EFFECTS OF CRYOPRESERVATION OF BOVINE GAMETES ON FERTILIZING POTENTIAL AND *IN VITRO* EMBRYO DEVELOPMENT

By

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Declaration

I the undersigned declare that this dissertation is original, my own and has never been presented in part or in full to any university for any award of a degree.

David Okello Owiny

Abstract

Cryopreservation of gametes has significant importance in the advancement of assisted reproductive technologies (ART) in the management of livestock and laboratory animal species, conservation of biodiversity, and treatment of human infertility. Cryopreservation also reduces the cost, genetic drift and diseases associated with maintaining live animals and cell lines. The increasing use of mammalian gametes obtained from the testis, and excurrent ducts, and the ovaries in ART has, therefore, been enhanced by the cryopreservation. There is need to maximize survival of cryopreserved gametes and the ability of cryopreserved gametes to produce embryos. Gametes from bovine ovaries and testes obtained from abattoirs, and culled buffalo testes were used to examine the effects of cryopreservation of gametes on embryo development, and the ability of frozen-thawed African buffalo (*Syncerus caffer caffer*) epididymal sperm in *in vitro* production of cattle x Buffalo hybrid embryos.

Effects of a commercial tris-egg yolk-based extender, Biladyl® (BIL) and modified Tyrode's lactate (MTL) on sperm fertilizing potential were compared by evaluating sperm motility, viability, and membrane and acrosome integrity. Tyrode's lactate medium was supplemented with 20 % foetal bovine serum (FBS) and 0.95 M glycerol (GLY), ethylene glycol (EG) or dimethyl sulfoxide (DMSO). Pre-freezing effects of the extenders and cryoprotectants were minimal in all treatments. Post-thaw parameters were lower ($P < 0.05$) than the pre-freezing parameters and the highest difference ($P < 0.0001$) was observed after 0 h of equilibration. Post-thaw motility and viability of treatments equilibrated for 2 h and 4 h in MTL, and frozen with GLY or EG were not different from the control ($P > 0.05$). Significant differences in post-thaw membrane and acrosomal status occurred between the control and treatments equilibrated for 2 h and 4 h. Using MTL with GLY or EG, and 2 h or 4 h of equilibration produced results comparable to the control. However, freezing bull epididymal spermatozoa without equilibration is not recommended, and DMSO should only be used in MTL in the absence of GLY and EG.

Bovine epididymal spermatozoa cryopreserved in BIL and MTL after equilibration for 2 h and 4 h was used to inseminate *in vitro* matured bovine oocytes. *In vitro* embryo development was assessed to compare the efficacy of BIL and MTL in cryopreserving the fertilizing potential of bovine epididymal spermatozoa. Cleavage rates varied between the treatments, and were lower ($P < 0.05$) for the treatments than that of the control (BIL). As embryo development progressed, differences between treatments decreased except for sperm cryopreserved in

DMSO that maintained a lower ($P < 0.0001$) development rate than other treatments and the control. Embryo development did not differ ($P > 0.05$) when sperm equilibrated for 2 h or 4 h was cryopreserved in BIL. However, embryo development was comparable when sperm equilibrated for 2 h or 4 h in MTL was cryopreserved with GLY or EG. There was no difference in blastomere numbers of embryos of all treatments equilibrated for 4 h. Spermatozoa cryopreserved in MTL containing 0.95 M GLY or EG, but not DMSO, produced embryos at rates comparable to BIL. Therefore, MTL supplemented with 20% FBS and 0.95 molar concentration of GLY or EG can be used as a substitute for commercial extender such as BIL for freezing bovine epididymal sperm, and possibly also of other species, for use in ARTs.

Frozen-thawed bovine cauda epididymal sperm were subjected to a second freeze-thaw cycle in BIL and MTL, and used for *in vitro* fertilization (IVF) of *in vitro* matured bovine oocytes. Cleavage, morula and blastocyst rates were lower ($P < 0.05$) for oocytes inseminated with sperm that underwent one freeze-thaw cycle in MTL and two freeze-thaw cycles in BIL and MTL (treatments), than the control (one freeze-thaw cycle in BIL). Embryo expansion and/or hatching on days 7-15, were not different ($P > 0.05$) among the treatments, but embryo expansion and/or hatching on the same days were lower ($P < 0.05$) when oocytes were inseminated with sperm refrozen in BIL. The blastomere numbers of embryos from sperm of freeze-thaw cycle one in BIL (119 ± 52.8) and MTL (130 ± 43) were not different ($P > 0.05$) from each other, but were higher ($P < 0.05$) than that of embryos from sperm refrozen in BIL (58 ± 29). It was concluded that bovine epididymal sperm can be refrozen in BIL, and MTL containing 20 % FBS, EG and used in ARTs.

Hybridization between cattle and its closest African wild relative, the African buffalo (*Syncerus caffer caffer*) was investigated. In an attempt to produce pre-implantation cattle x buffalo hybrid embryos *in vitro,* matured bovine oocytes were subjected to standard IVF procedure with either homologous bovine (n = 1166 oocytes) or heterologous buffalo (n = 1202 oocytes) frozen-thawed epididymal sperm. After IVF, 67.2% of the oocytes inseminated with homologous sperm cleaved. In contrast, insemination with buffalo sperm resulted in a 4.6% cleavage rate ($P < 0.0001$). Cleavage was also slower in hybrids than in cattle embryos. Up to 52.2% of the cleaved homologous embryos progressed to the morula stage compared with 12.7% for the hybrids ($P < 0.0001$). No hybrid embryos developed beyond the 16-cell stage, while 40.1% of cleaved bovine embryos developed to the blastocyst stage. Developmental anomalies such as polyspermy, uneven cleavage, vacuolization and absence of nuclei in some blastomeres were common in the hybrid embryos. It was concluded that interspecies fertilization of cattle oocytes with African buffalo epididymal sperm occurs *in vitro,* and that the

barrier to hybridization occurs in the early stages of embryonic development. Chromosomal disparity is likely the cause of the fertilization abnormalities, abnormal development and subsequent arrest impairing formation of pre-implantation hybrid embryos. Investigation into the developmental abnormalities including reciprocal hybridization and genetic studies of the hybrid embryos are recommended.

The effects of supplementing oocyte maturation medium with 100 μM cysteamine, and the use of a copper-wire cryoloop for vitrification of the *in vitro* matured bovine oocytes in the production bovine embryos *in vitro* was examined. Cysteamine did not improved the cleavage rate ($P > 0.05$), but improved morula and the blastocyst rates ($P < 0.05$) in one trial. In a second trial, cysteamine did not improve embryo development in the fresh oocytes and the cleavage rate of vitrified oocytes ($P > 0.05$), but improved the morula and the blastocyst rates ($P < 0.05$) of vitrified oocytes. Bovine oocytes can be successfully vitrified using a copper-wire cryoloop. Addition of cysteamine in maturation medium improves embryo development of vitrified-thawed mature oocytes. Cysteamine also improves embryo development in non-vitrified oocytes but this effect appears to be influenced by the pre-culture and culture conditions.

Opsomming

Kriopreservering van gamete speel 'n belangrike rol in die bevordering van ondersteunende reproduksietegnieke (ORT) tydens die bestuur van plaasdier- en laboratorium species, die bewaring van die biodiversiteit, en die behandeling van menslike infertiliteit. Kriopreservering verminder ook die koste, genetiese verskuiwing en siektetoestande wat met die bewaring van lewendige diere en sellyne geassosieer word. Die gebruik van soogdiergamete vanaf die testis en sy buise, en die ovaria tydens ORT word verder bevorder deur kriopreservering. Dit is dus aangewese om die oorlewing van gekriopreserveerde gamete en hul vermoë om embrio's te vorm, te optimaliseer Gamete vanaf beesovaria en –testis afkomstige vanaf die slagplaas, en uitgedunde buffels se testis is gebruik om die effek van kriopreservering op die ontwikkelingspotensiaal van gamete tot embrio's te ondersoek, sowel as die bevrugtingsvermoë van bevrore-ontdooide Afrika buffel (*Syncerus caffer caffer*) epididymale sperme tydens die *in vitro* produksie van bees met buffel hibried embrio's.

Die effek van 'n kommersieël beskikbare tris-eiergeel gebasseerde verdunner, Biladyl® (BIL) teenoor gemodifiseerde Tyrode's laktaat (MTL) op sperme se bevrugtingsvermoeë is vergelyk deur spermbeweging, -oorlewing, en spermmembraan- en -akrosoomintegriteit te evalueer. Tyrode's laktaat medium is verryk met 20% fetale bees serum (FBS) en 0.95 molaar gliserol (GLY), etileen glikol (EG) of demetielsulfoksied (DMSO). Die effek van die verdunners en kriopreserveermiddels in al die behandelings was minimaal. Na-ontdooiingswaardes was laer ($P < 0.05$) as die waardes voor kriopreservering. Die grootste verskille is na 0 h van ekwillibrasie waargeneem. Na-ontdooiingsbeweeglikheid en -oorlewing van behandelings met 2 h en 4 h ekwillibrasie in MTL, en gevries met GLY of EG, was nie verskillend van die kontrole nie ($P > 0.05$). Wesentlike verskille in membraan- en akrosoomstatus tussen die kontrole monsters en die behandelings ge-ekwillibreer vir 2 h en 4 h, het na ontdooiing voorgekom. Wanneer MTL met GLY of EG, en 2 h of 4 h ekwillibrasie gebruik is, is resultate gelykstaande aan die kontrolegroep verkry. Kriopreservering van bees epididymale sperme sonder ekwillibrasie is egter nie aan te beveel nie. DMSO behoort slegs met MTL in die afwesigheid van GLY en EG gebruik te word.

Bees epididymale spermatosoa bevries in BIL en MTL na ekwillibrasie vir 2 h en 4 h is gebruik om *in vitro* gematureerde bees oösiete te fertiliseer. *In vitro* embrionale ontwikkeling is daarna beöordeel om die doeltreffendheid van BIL en MTL as kriopreserveermiddels vir die bevrugtingsvermoeë van epididymale sperme te bepaal. Die tempo van seldeling het verskil tussen behandelings, en was laer ($P < 0.05$) vir die behandelings as vir die kontrole (BIL). Soos

embrionale ontwikkeling gevorder het, het verskille tussen die behandelingsgroepe afgeneem, behalwe vir sperme bevries in DMSO wat 'n stadiger ontwikkelingstempo ($P < 0.0001$) as die ander behandelings en kontrole getoon het. Embrionale ontwikkeling het nie verskil ($P > 0.05$) wanneer sperme wat vir 2 h en 4 h ge-ekwillibreer is, in BIL gekriopreserveer is nie. Wanneer sperme vir 2 h of 4 h in MTL ge-ekwillibreer is, en in GLY of EG bevries is, is soortgelyke resultate verkry. Daar was geen verskil in blastomeer aantal van alle groepe embrio's by sperme wat vir 4 h ge-ekwillibreer is nie. Sperme bevries in MTL met 0.95 molaar GLY of EG, maar nie DMSO nie, het embrio's geproduseer soortgelyk aan BIL. Daarom kan aanvaar word dat MTL met 20% FBS en 0.95 molaar GLY of EG as vervanging kan dien vir kommersiële verdunners soos BIL vir die bevriesing van bees epididymale sperme, en moontlik ook ander species, tydens ondersteunende reproduksie tegnieke.

Bevrore-ontdooide kouda epididymale sperme was blootgestel aan 'n tweede bevriesingssiklus in BIL en MTL, en daarna gebruik vir *in vitro* bevrugting van *in vitro* gematureerde bees oösiete. Kliewing, sowel as morula en blastosist ontwikkeling was laer ($P < 0.05$) vir oösiete wat ge-insemineer is met sperme wat eenmalig bevries is in MTL and tweemaal bevries is in BIL en MTL, as in die kontrole groep (eenmalig bevries in BIL). Embriovergroting en –ontkieming vanaf dag 7-15 was nie verskillend ($P > 0.05$) tussen die behandelingsgroepe nie, maar embriovergroting en –ontkieming op dieselfde dae was laer ($P <0.5$) wanneer oösiete ge-insemineer was met sperme herbevries in BIL. Die aantal blastomere van embrio's geproduseer met sperme van vriessiklus een in BIL (119 ± 52.8) en MTL (130 ± 43) was nie verskillend ($P < 0.05$) tussen behandelings nie, maar was hoër ($P < 0.05$) as in embrio's geproduseer deur sperme herbevries in BIL (58 ± 29). Hieruit kan afgelei word dat bees epididymale sperme hervries kan word in BIL en MTL wat 20% FBS, en EG bevat, en as sulks gebruik kan word in ORT.

Hibridisasie tussen beeste en sy naaste wilde Afrika familiegenoot, die Afrika buffel (*Syncerus caffer caffer*) is ondersoek. In 'n poging om bees x buffel hibried embrio's *in vitro* te produseer, is gematureerde bees oösiete blootgestel aan 'n standard IVF prosedure met òf homoloë bees (n = 1168 oösiete) òf heteroloë buffel (n = 1202 oösiete) bevrore-ontdooide epididymale sperme. Na IVF het 67.2% van die oösiete, ge-insemineer met die homoloë sperme, verdeel. In teenstelling daarmee het inseminasie met buffelsperme slegs 4.8% kliewing opgelewer ($P < 0.0001$). Verdeling was ook stadiger in die hibried- as in die beesembrio's. Tot 52.2% van die ontwikkelende homoloë beesembrio's het die morulastadium bereik, in vergelyking met 12.7% van die hibriedembrio's ($P < 0.0001$). Geen hibriedembrio het verder as die 16-sel stadium ontwikkel nie, terwyl 40.1% van die suiwer beesembrio's die blastosiststadium bereik het. Ontwikkelingsdefekte soos polispermie, oneweredige verdeling,

vakuolisasering en die afwesigheid van kerne in sekere blastomere het algemeen in die hibriedembrio's voorgekom. Daar is tot die gevolgtrekking gekom dat interspecie bevrugting van beesoösiete met Afrika buffel epididymale sperme wel *in vitro* plaasvind , en dat die buffer teen hibridisasie in die vroeë stadium van embrionale ontwikkeling plaasvind. Chromosomale ongelykheid is waarskynlik die oorsaak van bevrugtingsabnormaliteite, abnormale ontwikkeling en daaropvolgende staking in die ontwikkeling van pre-implantasie hibried embrio's. Ondersoeke na ontwikkelingsafwykings wat omgekeerde hibridisasie en die genetiese studie van die hibriedembrio's insluit, word aanbeveel.

Die effek van die byvoeging van 100 µM sisteamien by die maturasiemedium, en die gebruik van 'n koperdraad kriolus vir die vitrifikasie van *in vitro* gematureerde beesoösiete tydens die produksie van beesembrio's, is ondersoek. Sisteamien het nie kliewing verbeter nie ($P > 0.05$), maar het morula en blastosist getalle in een eksperiment met gevitrifiseerde oösiete verhoog ($P < 0.05$). Beesoösiete kan suksesvol gevitrifiseer word deur van 'n koperdraad kriolus gebruik te maak. Die byvoeging van sisteamien by die maturasiemedium verbeter embrionale ontwikkeling in gevitrifiseerde-ontdooide gematureerde beesoösiete. Sisteamien verbeter ook embrionale ontwikkeling in nie-gevitrifiseerde beesoösiete, maar hierdie aksie van sisteamien word klaarblyklik beïnvloed deur die prekultuur- en kultuurtoestande.

Dedication

Wholeheartedly dedicated to my wife Lillian Owiny, and our children Jonathan Omara and Marvin Ogwal who endured my absence during the years I toiled in a foreign land to get this manuscript done. May the Lord reward you immensely for your patience!

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Having been born the first boy-child in a semi-pastoral family, the job I had to first learn was herding the family cattle. Out of curiosity, I always wondered how conception would occur and the little "calf" would develop in the cow's womb to be born and grow into another cow or bull. Little did I know many years later my first choice for a university education would be veterinary medicine. No wonder, when I completed my bachelor course I was recruited to teach animal reproduction. Artificial insemination (AI) and embryo transfer (ET) became the topics I loved most. I did not only teach these topics but I widely practiced, especially AI. Because I did not do my masters in the field of assisted reproductive technology (ART) that I loved most, it was my dream that my doctoral study should be in this field. Then God sent Dr. Danie Barry to my department in Makerere University! My interest in ART climaxed. I wasted no time to travel to Stellenbosch University to initiate my doctoral program. Five years down the road my dream has come true. What a long journey!

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Lord God rest in peace the soul of Prof. Jude Ogaa who unfortunately passed away before he could read this manuscript, yet I would have loved him to do so. While to the University of Stellenbosch, I have no good words to express my appreciations any better. Thank you very much for giving me the admission and providing me with wonderful facilities such as the laboratory and transport, just to mention a few, that made it easy for me to study. All the staffs of the Department of Animal Sciences with whom I worked are wonderful people. They made me feel at home. Please keep it up!

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Abbreviations

AFGP	Anti-freeze glycoprotein
AI	Artificial insemination
ANOVA	Analysis of variance
ART(s)	Assisted reproductive technology/technologies
BIL	Biladyl
BSA	Bovine serum albumin
CASA	Computer assisted sperm analysis
CF-HCl	Ciprofloxacin hydrochloride antibiotic
CIDR	Controlled intravaginal drug release
COC(s)	Cumulus oocyte complex(es)
CO_2	Carbon dioxide gas
CR1	Charles Rosenkrans medium one
CR1aa	Charles Rosenkrans medium one with essential and non essential amino acids
DAPI	4',6-Diamidion-2-phenyindole, dilactate; DNA stain
DCI	Direct chilling injury
DMSO	Dimethyl sulfoxide
DNA	Dioxyribonucleic acid
DOPC	Dioleoylphosphatidylcholine
D-PBS	Dulbecco's phosphate buffered saline
dpi	Days post insemination
ECF	East Coast fever
EFAF	Essentially fatty acid free
EG	Ethylene glycol
EM	Equilibration medium for oocyte vitrification
EN	Eosin-Nigrosin
ET	Embryo transfer
ex situ	Outside normal habitat
F1	Filial one (first cross) of a hybrid
FBS	Fetal bovine serum
FMP	Forward motility proteins
GENMOD	Generalized linear module
GLY	Glycerol
GRB(s)	Genome resource bank(s)
GSH	Glutathione, a thiol compound
GV	Germinal vesicle stage of oocyte development
h	Hours
HM	Holding medium for oocytes during vitrification/thawing of oocytes
hMG	Human menopausal gonadotrophin
HOST	Hypo-osmotic swelling test
hpi	Hours post-insemination
ICM	Inner cell mass
ICSI	Intra-cytoplasmic sperm injection
IDCI	Indirect chilling injury
in vitro	Outside body
in vivo	Inside body
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation of oocytes in incubator
LDF	Low density lipoprotein fraction
LSMEANS	Least square means

MII	Metaphase plate two stage of oocyte development
MESA	Micro-surgical epididymal sperm aspiration method
min	Minutes
mRNA	Messenger ribonucleic acid
MTL	Modified Tyrode's lactose medium
NAA	Autosomal arm number
OPS	Open pulled straws
oFSH	Ovine follicular stimulating hormone
oLH	Ovine luteinizing hormone
PB	Polar body
2PB	Second polar body
PBS	Phosphate buffered saline
PESA	Percutenous epididymal sperm aspiration method
PG	Propylene glycol
PGD	Pre-implantation genetic diagnosis
pH	Hydrogen potential
PHE	Penicillamine, hypotaurine and epinephrine
PI	Propidium iodide
PN	Pronucleus
1PN, 2PN	One pronucleus, two pronuclei
PROC GLM	Procedure for general linear model
PVC	Polyvinyl chloride
PVP	Polyvinyl pyrrolidon
RNA	Ribonucleic acid
SAS	Statistical analysis system
sd	Standard deviation
SMQ	Sperm motility quantifier
SP	Seminal plasma
TALP	Tyrodes medium modified with albumin, lactose and pyruvate
TB	Trypan blue, a sperm stain
TCM-199	Tissue culture medium 199
TESE	Testicular sperm extraction
TM	Thawing medium for vitrified oocytes
TM1, 2 & 3	Thawing medium number one, two & three for serial dilution during thawing
TUGA	Trans-vaginal ultrasound guided aspiration of oocytes
UV light	Ultra-violet light
VM	Vitrification medium for oocytes
v/v	Volume for volume
w/v	Weight for volume
ZP3	Zona pellucida glycoprotein three

CHAPTER 1

General introduction

1.1 Spermatogenesis in mammals

Spermatogenesis is a process of cell division, differentiation and relocation by which spermatozoa are produced from germ cells. The germ cells form the epithelial lining of seminiferous tubules that composes the bulk of a testis (Elder and Dale, 2000). The germ cells undergo a cyclic process of mitosis, meiosis and terminal differentiation, as well as interaction over time and space within the seminiferous tubules (Zirkin, 1998). The activities of the three types of germ cells (i.e. spermatogonia, spermatocytes, and spermatids) partition spermatogenesis into spermatocytogenesis, meiosis and spermiogenesis (Johnson *et al.*, 1997; Johnson *et al.*, 2000). According to these authors, spermatocytogenesis involves mitotic division of the spermatogonia to produce stem cells and primary spermatocytes. Meiosis involves duplication of chromosomes, exchange of genetic material, and two cell divisions that reduce the chromosome number and yield four spermatids. During spermiogenesis, the spherical spermatids differentiate into elongated spermatids, which are released into the lumen of seminiferous tubules as spermatozoa. The differentiation consists of morphological transformations including condensation of nuclear chromatin, formation of the sperm tail and mid-piece containing the mitochondria, and development of the acrosome (Garner and Hafez, 1993).

Spermatogenesis starts at puberty and is regulated by endocrine and testicular paracrine/autocrine factors. Spermatogenesis occurs in waves, and at any location along the seminiferous tubules one or more generations of spermatogonia, spermatocytes or spermatids is present (Zirkin, 1998). Consequently, there are specific sets of cellular associations that succeed each other in place and over time, resulting into the continuous asynchronous production of the male gametes (Zirkin, 1998). The durations of the spermatogenic cycle and the entire process of spermatogenesis vary between species (Garner and Hafez, 1993). The cycle is 8.6-9 days in the boar (Franca *et al.*, 2005), 12.2 days in the stallion (Johnson *et al.*, 1997), 10 days in non-human primates (Millar *et al.*, 2000), and 10 and 14 days in ram and bull, respectively (Swierstra, 1968). The entire process of spermatogenesis lasts from 30 to 75 days in mammals (Franca *et al.*, 2005). In man, spermatogenesis requires approximately 70 days (Jeyendran, 2000), 37 days in non-human primates (Millar *et al.*, 2000), 45 days in bulls (Garner

and Hafez, 1993), and 40 days in boars (Franca *et al.*, 2005), 57 days in stallions (Johnson *et al.*, 1997), and 43.6-51.8 days in rabbits (Swierstra and Foote, 1965).

In studies where the duration of the seminiferous epithelium cycle and spermatogenesis were calculated using quantitative autoradiography after tritiated thymidine were incorporated into male germ cells of different animals, the following durations of spermatogenic cycles and spermatogeneisis shown in Table 1 were reported.

Table 1.1 Duration of seminiferous epithelium cycle and spermatogenesis in different mammals.

	Duration (Days)		
Species	Spermatogenic cycle	Spermatogenesis	
Boar	8.6	34.1	Clermont (1972)
Hamster	8.7	35.0	Clermont (1972)
Mouse	8.8	35.0	Clermont (1972)
Monkey	10.5	42.0	Fouquet and Dadoune (1986)
Ram	10.4	49.0	Clermont (1972)
Rabbit	10.5	51.8	Clermont (1972)
Rat	13.3	53.2	Clermont (1972)
Bull	13.5	54.0	Clermont (1972)
Dog	13.6	54.4	Foote *et al.* (1972)
Human	16.0	74.0	Clermont (1972)

(Adapted from Dadoune and Demoulin, 1993)

The efficiency of spermatogenesis is expressed as the estimated number of spermatozoa produced per day per gram of testicular parenchyma (Johnson *et al.*, 1997). Apparently the efficiency is dependent on the rate of cell division and differentiation that varies from species to species. According to Dadoune and Demoulin (1993), the efficiency of sperm production in mammals is 20-28 million sperm/g/day, except in the stallion and bull that produce 16 and 12 million sperm/g/day, respectively. In humans, the efficiency is much lower, about 20-40% of that in other mammals, attaining on average 6.5 million sperm/g/day at 20 years, which decreases to 3.8 million sperm/g/day at 50-60 years of age. This efficiency is, however, influenced in all mammals by the amount of germ cell degeneration, pubertal development, season, aging as well as other extrinsic factors (Johnson *et al.*, 1997). Table 1.2 below shows testicular efficiency in sperm production in some mammalian species according to Dadoune and Demoulin (1993).

Table 1.2 Testicular production and sperm cell reserves in mammalian species.

Species	Body wt (Kg)	Testis wt (pairs, g)	Prod/d/testis (pairs, x 10^9)	Prod/d/gm (testis, x 10^6)	Extragonadal reserve (x 10^9) Epididymis head	body	tail	v/deferens	Total	Ejaculated sperm (mean) (x 10^6)
Man	70	40	0.2	5	-	-	-	-	-	200
Bull	**1200**	**800**	**7.5**	**12**	**19**	**4.7**	**38**	**7.6**	**69**	**6000**
Stallion	1000	340	5.3	16	9/6	11	50	7.5	77	7000
Ram	100	500	9.5	21	23	11	126	-	>165	4000
Boar	200	720	16.2	23	36	51	104	-	>185	15000
Monkey	12	70	1.1	23	1.2	4.2	5.7	>2	>13	400
Rat	0.3	4	0.086	23	0.26		0.45	-	>0.7	58
Hamster	0.15	4	0.074	24	0.15		1.02	1.2	1.2	80
Rabbit	4	6	0.016	25	0.36	0.12	1.6	2.2	2.2	120

(Adapted from Dadoune and Demoulin, 1993)

1.2 Transport, maturation and storage of sperm in epididymis

Spermatozoa leave the testis in testicular fluid by way of numerous *tubulus recti* into a single *rete testis*, and a number of *vasa (ductuli) efferentia* (efferent ducts) that join to form a single duct of the epididymis (Jeyendran, 2000). Three anatomic parts of the epididymis that are important for the transportation, maturation and storage of spermatozoa (Ashdown and Hafez, 1993) are recognized. The *caput epididymis* (head) is a flattened part where the numerous *ductuli efferentia* join the ducts of epididymis. This continues into a narrow *corpus epididymis* (body), which terminates at the opposite pole in an expanded *cauda epididymis* (tail). The human epididymis has a larger head and a smaller tail when compared to other species (Tortora and Grabowski, 2003). The highly convoluted epididymis is very long in most mammals, for instance its approximately 36 m and 54 m in the bull and boar, respectively (Ashdown and Hafez, 1993). Consequently, the epididymal passage of spermatozoa is long (Table 1.3), about 7 days in the bull, 12 days in the boar (Swierstra, 1968), 16 days in ram (Amann, 1981), and 4-10 days in humans (Jeyendran, 2000).

Transport, storage and functional maturation of spermatozoa are the three main functions of the epididymis (Fournier-Delpech and Thibault, 1993). Despite the formation of the necessary morphological structures for motility, testicular spermatozoa are immotile (Elder and Dale, 2000). Testicular spermatozoa can develop rapid oscillations without progressive motility when extra-cellular pH is increased to 7.0-8.5 or undiluted samples are stored at 0 °C, however, they do not achieve a mature form of progressive motility (Voglmayr *et al.*, 1967). Consequently, transport of spermatozoa in the testis is accomplished by fluid flow (Jeyendran,

2000). In the ram, for instance, large volumes of fluid up to 60 mL leave the testis daily most of which is absorbed in the caput epididymis (Ashdown and Hafez, 1993).

Table 1.3 Duration of passage of spermatozoa (days) through the epididymis in different mammalian species.

Species	Caput	Corpus	Cauda	Total transit time
Man	1-2.5	0.5	5	1-12
Rhesus monkey	3		5	8-10
Stallion	1	1.5	6	7.5-10
Ram	1	3	8	13
Bull	**2**	**2**	**10**	**14**
Pig	3	2	4-9	9-14
Hamster	3	2	8	13
Guinea-pig	3	2	6-8	10-15
Rabbit	3	1	5-6	9-10
Rat	3	3	5	11
Mouse				3-5

(Adapted from Fournier-Delpech and Thibault, 1993)

The epididymis is functionally a complex organ which maintains a specific intra-luminal environment thought to be important for effecting sperm maturation in proximal regions, and sperm storage in distal regions of the duct (Brooks, 1983). Furthermore, the composition of the internal milieu is achieved both by transport between blood and lumen and *vice versa*, and by synthesis and secretion into the tubule lumen. Interactions between the spermatozoa with the luminal fluid micro-environment and with the epididymal epithelium are necessary for sperm maturation (Hinton and Palladino, 1995). Several low-molecular weight organic molecules achieve high concentrations in the epididymal lumen, though their functions in the events of sperm maturation and storage still remain unclear. A major role of the epididymis is to finely regulate the movement of these molecules into and out of the lumen to ensure that as spermatozoa progress along the duct, they are exposed to a continually changing, but optimal environment necessary for their maturation and survival (Hinton and Palladino 1995). Maturational process mostly occurs in the proximal epididymis where most of the proteins are secreted (Jones, 1999), commencing in the initial segment (Fawcett and Hoffer, 1979). This region of epididymis is lined by characteristic epithelium which secrets proteins at a greater rate than elsewhere in the epididymis, which is dependent for its function on the luminal fluid flowing from the testis (Jones, 1999).

During the epididymal passage, spermatozoa undergo functional changes leading to the acquisition of their fertilizing ability (Garner and Hafez, 1993; Tortora and Grabowski, 2003), and this is referred to as epididymal maturation (Elder and Dale, 2000). This maturational process involves modification of the sperm surface by epididymal proteins that are synthesized and secreted by epididymal epithelium under the control of androgens (Orgebin-Christ and Jahad, 1979). In addition, there is development of sustained progressive motility, alterations of metabolic patterns including the structural state of the mitochondria, changes in the nuclear chromatin and plasma membrane, movement and eventual loss of the cytoplasmic droplet, and acrosomal modification (Bedford, 1975). The immotile testicular spermatozoa become motile when they pass through the first part (*caput*) of the epididymis (Djakiew and Jones, 1982). Proteins secreted in the caput epididymis called forward motility proteins (FMP) seem necessary to induce progressive motility (Serres and Kann, 1984). Motility increases as spermatozoa enter the *corpus epididymis* (Garner and Hafez, 1993). The *cauda epididymis*' environment provides factors that enhance progressive forward motility and spermatozoa from this region give better fertility than those from *corpus epididymis* (Amann, 1981). The epididymal sperm, however, remain immotile while in the epididymis. This immotile state is thought to be due to the low pH, poor oxygen availability and, at least in rat and hamster, the presence of a viscous protein called immobilin (Turner and Reich, 1985) as well as the low cauda epididymal temperature (Fournier-Delpech and Thibault, 1993). Transport of epididymal sperm is achieved through peristaltic contractions of the smooth muscle in the wall of the epididymis (Tortora and Grabowski, 2003).

Other functions of the epididymis include the concentration of sperm through fluid absorption. Of the fluid that carries spermatozoa out of the testis through the efferent ducts, more than 95% is reabsorbed in the first part of the epididymis, leading to a sharp rise in spermatocrit (Hinton *et al.*, 1980; Setchell and Hinton, 1981). In addition, the epididymal epithelium continuously regulates the composition of the luminal milieu by transporting fluids and solutes, and secreting and absorbing specific proteins to ensure sperm survival (Jones, 1987; Clulow *et al.*, 1994). These proteins probably protect spermatozoa from reactive oxygen species, proteases and complements during sperm storage in the *cauda epididymis* (Hinton *et al.*, 1996). They may also play a role in sperm maturation by regulating the rate at which enzymes modify sperm proteins (Jones, 1999), or the amount of reactive oxygen species that modify the redox state of spermatozoa (Aitken and Vernet, 1998). Jones and Murdoch (1996) also suggested that the redox regulation may be involved in suppressing sperm motility and metabolism in the epididymis. Spermatozoa are intensely redox active cells and professional generators of reactive oxygen species. These activities are physiologically important to the

spermatozoa in regulating every aspect of sperm function examined, including their movement characteristics, capacitation, sperm-zona interaction, the acrosome reaction and sperm-oocyte fusion (Baker and Aitken, 2004).

In all mammals, sperm that is stored in the *cauda epididymis* accounts for about 75% of the total epididymal spermatozoa (Garner and Hafez, 1993), with spermatozoa remaining viable in the epididymis for several weeks (Bedford, 1975; Jeyendran, 2000). Minor storage sites include the *vas deferens* (Amann, 1981), and seminal vesicle and ampulla (Elder and Dale, 2000). During this storage the epididymal sperm remains immotile due to a low intracellular pH arising from the pH of cauda epididymal fluid. The pH of cauda epididymal fluid is approximately 5.8, and there appears to be a pH-dependant motility inhibitor in the cauda epididymis as elevation of the pH of this fluid to 7.0 stimulates cauda epididymal sperm motility (Carr and Acott, 1989). According to these authors, there is an increase in intracellular pH due to a lactate produced by sperm at ejaculation that stimulates sperm motility. The special ability of the cauda epididymis to store sperm depends on low scrotal temperature, and on the action of male sexual hormones (Foldesy and Bedford, 1982). The most extended sperm storage in mammals occurs in bats that hibernate (Gustafson, 1979) such as *Myotis lucifugus* and *M. velifer*. In this bat species, sperm is stored in the cauda epididymis for up to 10 months and their viability after 7 months was proven by successful artificial insemination into females (Racey, 1973).

Stored spermatozoa are released during ejaculation and spermatozoa not ejaculated are eliminated by excretion into urine or undergo senescence and eventually disintegrate (Garner and Hafez, 1993). Repeated ejaculation, however, does not affect the rate of sperm transport along the rest of the epididymis (Kirton *et al.*, 1967). In humans, Baumgarten *et al.* (1971) demonstrated that all the ducts proximal to the cauda epididymis has a neuro-musculature which ensures spontaneous, slow, rhythmical contractions while that of the cauda epididymis is quiescent, with the ability to produce nerve-mediated, brief, forceful contractions upon stimulation. This probably ensures that there are spermatozoa all along the epididymis at any given time. Prins and Zaneveld (1980) further proved the role of cauda epididymis in sperm storage by demonstrating that spermatozoa present in the vas deferens following ejaculation are returned to the cauda epididymis. These authors showed in rabbit that the distal vas deferens possesses greater contractile activity than the proximal duct after sexual activity, which explains the transport of vasal contents back to the epididymis following a sexual encounter.

The ability of the cauda epididymis to store spermatozoa and maintain their viability is extended after death of the male in most mammals. Viable epididymal sperm was recovered from post-mortem testicles of culled game animals transported at 4 °C (Bezuidenhout *et al.*, 1995; Shaw *et al.*, 1995; Kidson, 2000). Shaw *et al.* (1995) transported testicles of adult buffalo bulls in an insulated container containing ice and refrigerated for up to 24 h prior to use with development of blastocysts. Bezuidenhout *et al.* (1995) demonstrated that epididymal sperm of different game animal species survive at 4 °C for 2 to 5 days with buffalo epididymal sperm surviving longest. In mice, Songsasen *et al.* (1998) produced newborns after *in vitro* fertilization with spermatozoa retrieved from males euthanized and kept at 22 °C for 24 h. In that study, no differences in the percentages of spermatozoa with intact plasma and acrosomal membranes were observed regardless of the time after death. However, histological examination revealed that degenerative changes of the testes occurred within 6 h postpartum, whereas those of the epididymides were less obvious until 6 h later. This degenerative changes in the testis included pyknosis, release of intracellular contents, and disruption of intercellular bridges of the germ cells. In a later study on mouse sperm, sperm motility and intact membrane was lower when testicles were stored for 24 h and 48 h at 22 °C, than at 4 °C (Stilley, 2001). Stilley (2001) also showed that motility and membrane integrity of sperm from testicles stored at 4 °C did not decrease, when compared with the control until after 72 h of storage.

Martinez-Pastor *et al.* (2005) demonstrated that although epididymal sperm from Iberian red deer (*Cervus elaphus hispanicus*), and roe deer (*Capreolus capreolus linnaeus*), undergo a decrease of quality with post-mortem time, it could stay acceptable within many hours post-mortem. This, according to the authors, has implications for wildlife conservation programmes, as epididymal sperm is a good source of germ plasm. If valuable animals die and it is not possible to process their sperm immediately, it may still be possible to obtain viable spermatozoa many hours later. James (2004) demonstrated that bovine, caprine and equine cauda-epididymal sperm maintained acceptable levels of viability up to 96 h post-mortem. The author, however, noted that *in vitro* fertilization with cryopreserved bovine epididymal sperm was not efficient as there was limited embryo development.

1.3 Collection of epididymal sperm

Mammalian epididymal sperm is usually obtained from testicles collected at necropsy. Many techniques have been used, but retrograde flushing is the preferred method of retrieving epididymal sperm of many species at necropsy (Halangk *et al.*, 1990; Reyes-Moreno *et al.*, 2000; Cary *et al.*, 2004; Martinez-Pastor *et al.*, 2005). This involves isolation of the cauda

epididymis and vas deferens followed by catheterization of the vas deferens, and flushing of both the vas deferens and cauda epididymis with warm saline or semen extender. Halangk *et al.* (1990) obtained an average of 2 x 10^9 spermatozoa from one cauda epididymis of a slaughtered bull using retrograde flushing. Flushing can also be achieved using air (Howard *et al.*, 1986). Viable spermatozoa were collected from African elephant by flushing the excurrent ducts with air (Jones, 1973). Brackett *et al.* (1978) retrieved caput and corpus epididymal sperm from rabbits at necropsy by mincing, and cauda epididymis sperm by retrograde flushing. Mincing was also used to collect epididymal sperm of mice (Garner and Johnson, 1995). Meanwhile, epididymal sperm of goats was retrieved at post-mortem by applying pressure to the epididymis and squeezing out small droplets of sperm (Blash *et al.*, 2000).

A flotation technique and retrograde flushing was used to obtain sperm from terminal stallions with acceptable sperm quality (Cary *et al.*, 2004). The *cauda epididymis* and vas deferens were isolated and horizontal slashes made on them before they were covered with extender, then agitated and incubated at room temperature for 10 min. These authors showed that flotation method was the least cumbersome and showed a tendency to be superior to flushing in terms of sperm motility and percentage of cells passing through glass wool/sephadex filtration. In a study by Ellerman *et al.* (1998), the epididymis and vas deferens of rats were removed and carefully dissected. After extensive cleaning and rinsing, the epididymis was cut into the caput, corpus, and cauda. Cauda epididymal fluid was collected by making a single puncture in one tubule and allowing the release of the luminal content into a microtube. The content from the vas deferens was recovered by gently squeezing the tubule. For the caput and corpus regions, several incisions were made, and the luminal content was also recovered by squeezing the tissues.

Three methods are used for retrieving epididymal sperm from humans. These are the standard incisional, microsurgical epididymal sperm aspiration (MESA), the percutaneous epididymal sperm aspiration (PESA), and a modified mini-incision (mini-MESA) (Turek *et al.*, 1999). In MESA and mini-MESA, open surgical exploration of the epididymis is performed under general, regional or local anesthesia to isolate the epididymis and sperm are aspirated from a dilated tubule using micropipette or G23 hypodermic needle (Temple-Smith *et al.*, 1985; Collins *et al.*, 1996). Retrieval of epididymal sperm by PESA involves minimal invasion whereby under a local anesthesia, a G23 hypodermic needle is blindly inserted through the skin into the epididymis to aspirate the sperm (Collins *et al.*, 1996). *In vitro* fertilization with sperm retrieved by MESA was shown to be successful, though chances of fertilization were thought to be higher with intra-cytoplasmic sperm injection (Sperling *et al.*, 1995). The success rate of sperm

retrieval with MESA is higher than PESA, but the latter is simpler, cheaper and requires little experience (Elder and Dale, 2000). The overall fertilization rate of sperm retrieved using the two methods are comparable, though PESA cannot replace MESA because in some cases epididymal pathologies prevent the success of PESA (Lin *et al.*, 2000). In one method of PESA for collecting proximal epididymal sperm in obstructive azoospermic men (Steele *et al.*, 1999), the spermatic cord was injected with 10 mL of 0.5% bupivacaine without adrenalin. After 10 min, a G23 butterfly needle was introduced into the proximal epididymis on the upper pole of the testis and gentle suction applied with a 2 mL syringe. Epididymal sperm aspiration techniques similar to MESA and PESA may also be applied to mammalian species other than humans. A method similar to PESA was used by Morrell *et al.* (1997) who collected epididymal sperm from marmoset monkeys by piercing the lumen of *cauda epididymis* and vas deferens with a G20 hypodermic needle and gently washing the tissue with a sperm medium.

1.4 Oogenesis in mammals

Oogenesis is a much longer process than spermatogenesis. The formation of oocytes occurs during the early prenatal development when primordial germ cells migrate from the yolk sac to the gonadal ridges to colonize the indifferent gonads (Baker, 1971). Here the germ cells proliferate by mitosis (Gordon, 1994) and form associations with a small number of pre-granolosa cells to form primordial follicles (Telfer, 1998). These germ cells become the oogonia. According to Gordon (1994), oogonia undergo two meiotic divisions to reduce the chromosome number from the diploid (2n) to the haploid (n) stage. On entering prophase of the first meiotic division, as oogonium becomes a primary oocyte, and after completion of the first meiotic division and entry into the second, it becomes a secondary oocyte. The meiotic division of the secondary oocyte is, however, arrested at diplotene stage of prophase I and further oocyte development is resumed prior to ovulation. At birth several thousands to millions of the primordial follicles, depending on the species, constitute a store of oocytes in the ovarian cortex (Draincourt *et al.*, 1993).

The growth of the oocyte and its surrounding follicular cells is coordinated, with the oocyte progressing through a series of well-defined morphological stages that is regulated by unknown factors (Telfer, 1998). Follicle growth continues into an antral follicle and this is associated with marked structural changes, including an increased volume of the oocyte and multiplication and differentiation of the somatic cells forming different sizes of follicles. This marked follicle development is associated with the onset of puberty when development is observed to occur in three consecutive waves in every cycle (Sirois and Fortune, 1990). Similar

waves of follicular development have also been seen in pre-pubertal period (Hopper *et al.*, 1993) and pregnant animals (Gordon, 1994). Follicular development resulting into maturation and ovulation of the oocyte is a complex process, under the control of pituitary gonadotrophins with the interplay of ovarian hormones.

Follicles at various stages of development are present on the ovary at any time. According to Elder and Dale (2000), the vast majority of the numerous primordial follicles contained on the mammalian ovaries do not grow to maturity as over 99% of these follicles undergo atresia and die before ovulation. Consequently, only a few viable oocytes are produced during the reproductive lifespan of a female (Carroll *et al.*, 1990). Assisted reproductive technologies for *in vitro* maturation, fertilization and culture of oocytes (Eppig *et al.*, 1990), including intra-cytoplasmic sperm injection (ICSI) (Palermo *et al.*, 1992) and cryopreservation of oocytes (Fugger *et al.*, 1988) have been developed. These technologies are useful tools for the rescue of the would-be wasted large population of mammalian oocytes for conservation and future reproduction purposes. For these purposes, oocytes can be recovered from live, i.e. young, pregnant and incapacitated and from slaughtered or dead females (Gordon, 1994).

1.5 Oocyte recovery in mammals

In farm animals, ovaries collected from the abattoir provide the major source of oocytes. It has been shown that viable oocytes could be collected from abattoir ovaries of females of all ages including foetuses (Figueiredo *et al.*, 1993). Oocytes can also be retrieved from ovaries collected from animals that die of causes other than slaughter, making ART applicable to non-farmed mammals, especially endangered wildlife species. Proper handling of the ovaries is necessary to maintain the viability of the oocytes. It is important to consider the time interval between slaughter and death of the animal and oocyte recovery (Gibbons *et al.*, 1994). Temperature limits as well as the medium used for transportation and/or storage of ovaries before oocyte recovery are also important. Studies in cattle IVF recommended transportation of ovaries at about 33 °C in sterile Dulbecco's phosphate buffered saline (D-PBS) or normal saline (0.9% NaCl) and recovery of oocytes within 8 h from slaughter (Takagi *et al.*, 1992; Hamano and Kuwayama, 1993). In other studies, bovine ovaries were stored at 24-25 °C for 11 h (Yang *et al.*, 1990) and at 15-21 °C for 24 h (Schernthaner *et al.*, 1997) without reducing fertilization and blastocyst rates *in vitro*. Perhaps it is not necessary to collect ovaries in saline as long as the temperature remains within the normal range and desiccation is avoided, as was demonstrated with pig ovaries by Kidson (2004).

Oocytes are retrieved from the collected ovaries by puncture and aspiration of vesicular follicles that are 2-8 mm in diameter (Katska, 1984; Leibfried-Rutledge *et al.*, 1985) using a needle and a syringe or vacuum pressure. Methods of oocyte retrieval by follicular dissection and rupture (Lu *et al.*, 1987), and ovary slicing (Hamano and Kuwayana, 1993) have also been described. Furthermore, mass oocyte retrieval method by mechanical mincing of the ovary (Nuttinck *et al.*, 1993) and ovary digestion using enzymes (Strickland *et al.*, 1976) have also been used to recover oocytes from post-mortem ovaries.

Recovery of oocytes from live females involves methods such as endoscopy or laparoscopy. These may include methods such as percutaneous laparoscopic oocyte recovery (Lambert *et al.*, 1983) or trans-vaginal laparoscopic oocyte recovery (Reichenbach *et al.*, 1993). The modern methods of oocyte recovery from live animals, including humans, use ultrasound technology. Percutaneous ultrasound-guided oocyte recovery in farm animals has been reported by Hafez (1993). The technique of trans-vaginal ultrasound-guided oocyte retrieval is the most commonly used method in human IVF. This method was successfully used to recover oocytes from cows (Pieterse *et al.*, 1988), and is applicable for other animals.

1.6 Principles and techniques of gamete cryopreservation

Cryopreservation and storage of cells, including gametes, are significantly important in the advancement of ART by enabling better management of livestock and laboratory animal species, improved conservation of biodiversity, and improved modalities for the treatment of human infertility (Woods *et al.*, 2004). The use of ART reduces the cost, genetic drift and diseases that are usually associated with maintaining live animals and cell lines (Shaw *et al.*, 2000). However, attempts to cryopreserve mammalian cells have not been very successful as most of the cells die if exposed to low temperatures, unless they are placed in solutions that protect them, and cooled/warmed at specific rates (Shaw *et al.*, 2000).

A number of physical and biological changes occur as cells are cooled from body temperature to storage temperature and then warmed to body temperature again. Generally, the basic architecture of eukaryotic cells is a membrane bound cytoplasm containing supportive matrix of proteins (cytoskeleton) and numerous membrane bound organelles (Raven & Johnson, 1995). According to Cooper & Hausman (2004), cells are composed of water, organic ions and inorganic molecules, with water being the most abundant molecule forming more than 70% of the total cell mass. Consequently, the interactions of water with other molecules due to the bipolar nature of water molecule, the ability of water molecules to pass relatively freely

across the semi-permeable cell membrane, and the response of water molecules to cooling are very important in the cryobiology of cells.

Cooling cells to temperatures between 30 °C and 0 °C (chilling) compromises cell membrane integrity, cell metabolism, the cytoskeleton, and the capacity of the cell to control and repair damage e.g. by free oxygen radicals (Arav *et al.*, 1996; Ruffing *et al.*, 1993). Lowering temperature below 0 °C (freezing) introduces the risk of intracellular ice formation and possible rapid ice growth as the temperature falls (Ruffing *et al.*, 1993). When cooled to approximately −0.6 °C, biological water under isotonic conditions become thermodynamically unstable, and will favour the crystalline state (Karlsson and Toner, 1996). Ice formation is initiated by a nucleation process due to an aggregation of water molecules into thermodynamically stable clusters from which ice crystals grow (Turnbull and Fisher, 1949). The rate of crystallization, therefore, depends on the kinetics of nucleation and subsequent growth (Karlsson and Toner, 1996). As cooling progresses, a thermodynamic equilibrium is reached when crystallization ceases, and viscosity of the intracellular solution is high enough to effectively preclude molecular diffusion (Mazur, 1984). At this point, the intracellular solution undergoes a glass transition and solidifies into a non-crystalline glass state.

The rate of cooling to final storage temperature can profoundly affect the fate of the frozen biological samples (Karlsson and Toner, 1996). There are basically two cryopreservation protocols classified as "slow" and "rapid" depending on the cooling rate, and the type and concentration of additives used (Shaw *et al.*, 2000). To prevent intracellular ice formation, or minimize the damage it causes, all commonly known cryopreservation protocols are designed to dehydrate cells. In slow cooling protocols, dehydration is achieved by placing the cells in a solution containing low concentrations (10-11% for oocytes) of penetrating cryoprotectant solutes, then the temperature is lowered and extra-cellular ice crystal growth initiated (seeded) within the solution (Shaw *et al.*, 2000). In cryopreservation of sperm, for instance bull sperm, glycerol is added to a final concentration of 5-10% after cooling sperm in the non-cryoprotectant portion of the diluent to 5 °C for 4-6 h. Just after addition of glycerol, the semen is packed and frozen in liquid nitrogen vapour at −80 °C for 10 min and then stored in liquid nitrogen (Hafez, 1993).

Slow cooling protocols take advantage of the regulatory properties of extra-cellular ice by encouraging extra-cellular ice formation while minimizing the formation of intracellular ice. Extra-cellular ice formation occurs below the freezing point of water (0 °C), as the presence of cryoprotectant solutes and electrolytes depresses the freezing point of water, and thus allows

super-cooling of the extra-cellular solution (Karlsson and Toner, 1996). Because only pure water freezes, as more ice is formed and the ice crystals grow as the temperature decreases, the concentration of solutes in the unfrozen portion of cell solution increases and the viscosity of the solution increase as well. The increased solute concentration ("solution effect") causes water efflux from the cell and diffusion of solutes, especially the penetrating cryoprotectants, into the cell to reach equilibrium, though the permeability of cell membrane to water reduces as temperature falls (Karlsson *et al.*, 1994). The success of slow cooling, therefore, depends on achieving the optimal balance (equilibrium) between the rate at which water can leave a cell and rate at which it is converted into extra-cellular ice (Shaw *et al.*, 2000). Slow cooling has been extensively used in the cryopreservation of sperm and embryos (Mizukami *et al.*, 1999; Amann, 1999). To some extent, the procedure have been successful in the cryopreservation of oocytes of species that are not sensitive to chilling (e.g. mouse), but poor results were obtained with oocytes of species sensitive to chilling, for instance, pig, human and cow (Shaw *et al.*, 2000).

Most rapid cooling protocols use solutions with high concentrations of cryoprotectant solutes to cause rapid dehydration of the cells (Vajta *et al.*, 1998). In such solutions, the cells are sufficiently dehydrated and permeated by the cryoprotectants to the extent of tolerating direct immersion into liquid nitrogen or nitrogen vapour without equilibration. According to Shaw *et al.* (2000), there are two forms of non-equilibrium procedures of cryopreservation. When a measurable or visible amount of extra-cellular ice is formed during this type of cooling protocol, the procedure is termed rapid cooling or ultra-rapid cooling. However, when solutions used solidify into a glass state without ice crystal formation it is referred to as a vitrification procedure.

Despite the dehydration of cells caused by the high concentration of cryoprotectants during rapid and ultra-rapid cooling, intracellular ice formation and cryoprotectant toxicity are major problems in these procedures. Water exosmosis, in these methods, is slow compared with the rate at which intracellular solution becomes super-cooled, and intracellular ice formation is favoured (Karlsson and Toner, 1996). A factor implicated in the intracellular ice formation in these cooling procedures is the thermodynamic state of intracellular water that can be manipulated by dehydration only within an infinite temperature. At sufficiently low temperatures the cell membrane becomes effectively impermeable to water (Karlsson *et al.*, 1994). Therefore, for all cooling rates, cell dehydration stops at some characteristic temperature, for example −40 °C for cultured hepatocytes (Karlsson and Toner, 1996). Because the temperature at which the membrane becomes impermeable is quickly reached during rapid and ultra-rapid cooling, the remaining biological water is likely to form intracellular ice due to insufficient cell dehydration.

When cells are cooled at an extremely rapid rate in solutions that solidify without ice crystal formation at this cooling rate, the procedure is referred to as vitrification (Shaw *et al.*, 2000). According to Arav (1992), vitrification is a physical process whereby a liquid solution is transformed into a particular solid state called glass, when it is cooled to cryogenic temperatures. Cryopreservation by vitrification was first proposed by Luyet (1937), and later Luyet and Hodapp (1938) successfully vitrified frog sperm. Following the discovery of glycerol as the first cryoprotectant (Polge *et al.*, 1949), Rapatz and Luyet (1968) vitrified erythrocytes by cooling rapidly (10^4 K/min) in a solution containing 67% w/v glycerol. Boutron (1979) studied the stability of the amorphous state and potential of glass formation of commonly used cyroprotectants such as glycerol, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG). In 1985, Rall and Fahy obtained the first vitrification of embryos using PG, DMSO and acetamide.

Vitrification avoids the potentially damaging effects of ice by preventing both intracellular and extra-cellular ice crystal formation and growth. Usually high concentrations of permeating and non-permeating cryoprotectants are used in vitrification procedures. However, in 1941, Hoagland and Pincus, and recently Nawroth *et al.* (2002) and Isachenko *et al.* (2004a, 2004b) successfully vitrified human sperm without cryoprotectants. According to MacFarlane (1987), glass formation is usually observed when the cryoprotectant concentration is higher than 40%. This high concentration of cryoprotectants rapidly dehydrates the cells to minimize intracellular water that would be available for crystallization (Vajta *et al.*, 1998). The kinetic effects of dehydration depress the intracellular ice formation temperature of any remaining water molecules (Karlsson *et al.*, 1994). An extremely rapid cooling rate does not allow time for water molecules to arrange themselves into an orderly crystalline lattice structure, but instead form an amorphous network polymer. The resultant glass state has the ionic and molecular distribution of a liquid state, thus avoids the chemical and mechanical damage caused by ice crystals (Arav, 1992).

1.7 Cell cryo-injury and its control during cryopreservation

Stopping biological time and long-term cryopreservation should be achieved by procedures that allow the cells to reach the temperature of liquid nitrogen and, after an indefinite time, to return to physiological conditions without loss of viability (Arav, 1992). Cryopreservation processes, however, expose cells to thermal, chemical and mechanical constraints responsible for the profound effects of cryopreservation on the biological function of cells. The hostile environments the cells are subjected to during cryopreservation, i.e. chilling, high

electrolyte/solute concentrations and ice formation cause lethal damages (cryo-injury) to the cells (Karlsson and Toner, 1996).

Cryopreservation of cells, whether by slow, rapid, ultra-rapid or even vitrification procedures, usually expose the cells to chilling temperatures before the freezing temperature is reached. According to Levitt (1980), chilling injury is the damage caused by exposure of cells to low temperature without freezing. Exposure of cells to temperatures between +30 °C and 0 °C cause cell injury by compromising the membrane integrity, cell metabolism, the cytoskeleton and the cells' capacity to control and repair damage e.g. by free oxygen radicals (Ruffing *et al.*, 1993; Arav *et al.*, 1996). Two forms of chilling injury have been reported by Levitt (1980). Direct chilling injury (DCI) is expressed quickly upon cooling, and indirect chilling injury (IDCI) is expressed over a long period (days).

Direct chilling injury may result from lipid phase-transition on the cell membrane, as was demonstrated in pig sperm (Drobins *et al.*, 1993). This is likely to be the limiting factor for the successful, short-term, cold-storage or cryopreservation by freezing and vitrification of oocytes (Arav *et al.*, 1996). Chilling causes changes in the organelle and cytoskeletal components of the oocytes. The microtubule dynamics of oocytes are altered, and this may be responsible for the low fertilization rates of cryopreserved oocytes (Amann and Parks, 1994; Albertini, 1995). Cooling of metaphase II oocytes to non-physiological temperatures result in depolarization of meiotic spindle in mouse (Pickering and Johnson, 1987), cattle (Amann and Parks, 1994), human (Pickering *et al.*, 1990) and rhesus monkey oocytes (Songsasen *et al.*, 2002), although the degree of injury varies amongst the species. Cooling oocytes to low temperatures has also been observed to cause substantial disruption of cortical granules and plasma membranes that is thought to lead to poor fertilization and cell death, respectively (George & Johnson, 1993). Sensitivity to chilling attributable mainly to destruction of the plasma membrane was also demonstrated for pig oocytes (Didion *et al.*, 1990). Irreversible membrane lipid-phase changes of bovine oocytes at germinal vesicle (GV) stage has been demonstrated to occur at between 20 °C and 13 °C, while a very broad phase-transition, which centred around 10 °C, was observed for MII oocytes (Arav *et al.*, 1996).

Mazur *et al.* (1972) proposed the hypothesis of a two factor freezing injury. According to this theory, freezing damages cells either by formation of intracellular ice crystals during ultra-rapid cooling, or by chemical toxicity or osmotic stress, as well as mechanical damage due to "solution effect" and ice crystal growth during slow cooling. The cooling rate, i.e. the rate at which temperature of the freezing medium and that of the cell drops to −196 °C, is very

important during ice crystal formation (Arav, 1992). Because ice is formed from pure water (Mizukami *et al.*, 1999), slow cooling rates over a long duration is believed to cause "solution effect" injury resulting from extreme concentration of extra-cellular solutes (Critser *et al.*, 2002) and mechanical interactions between cells and the extra-cellular ice (Karlsson and Toner, 1996). Mazur (1984) and Schneider and Mazur (1987) demonstrated a significant inverse correlation between the unfrozen fraction and cell damage in freezing protocols. The "solution effect" causes osmotic stress and extreme dehydration that result in denaturation of lipoproteins, and induce haemolysis in red blood cells (Lovelock, 1957). Extreme dehydration and cell shrinkage are potentially damaging. There exists a critical minimum cell volume, shrinkage beyond which is deleterious to the cell (Meryman, 1970). According to Steponkus *et al.* (1983), osmotic dehydration can delete plasmalemma lipids from the membranes and it is likely that damage occurs during rehydration if there is insufficient membrane material for the cell to return to its isotonic volume.

Cells are usually sequestered into channels of unfrozen solution between ice crystals. The surrounding ice matrix acts as mechanical constraint and causes deformation as more extra-cellular ice forms (Karlsson and Toner, 1996). Mechanical contact between extra-cellular ice crystals and oocytes or embryos is believed to predispose these cells to damage at the site of contact (Ashwood-Smith *et al.*, 1988). Fujikawa and Miura (1986) and Fujikawa (1991) demonstrated ultra-structural changes in the plasma membranes caused by mechanical stress due to formation of extra-cellular ice. The presence of secondary branches on ice dendrites and possible mechanical shear forces due to the dendrites coarsening is also thought to be damaging to the cell (Hubel *et al.*, 1992).

Extensive chemical and physical damage to the extra-cellular and intracellular membranes of human sperm attributable to changes in the lipid phase-transition and/or increased lipid peroxidation has been reported to occur in conventional freezing or thawing (Alvarez and Storey, 1992; Mossad *et al.*, 1994). This damage to human sperm mainly occurs during thawing and is related to a reduced anti-oxidant defence activity during cooling and/or structural damage to cytoskeleton and/or anti-oxidant enzyme during cryopreservation (Alvarez and Storey, 1992).

During rapid cooling, the slow rate of exosmosis of cell water results in the cytoplasm becoming increasingly super-cooled with an associated increase in the probability of intracellular ice nucleation (Mazur, 1984). Although the mechanism by which intracellular ice damages cells is not well understood, it is generally believed that intracellular ice formation is an

inherently lethal event (Mazur, 1984; Acker *et al.*, 2001). There is significant evidence for a correlation between cell injury and intracellular ice formation during rapid cooling (Mazur, 1984). The general belief is that injury results from mechanical forces due to intracellular ice formation (Mazur, 1977), with plasma membrane (Fujikawa, 1980) and membranes of intracellular organelles (Mazur, 1984) as the possible sites of damage. Induction of gas bubble formation by intracellular ice (Morris and McGrath, 1981) and osmotic effects due to melting of intracellular ice during warming (Farrant, 1977) are the possible non-mechanical modes of injury by intracellular ice. However, Acker *et al.* (2001) have shown that conditions exist where intracellular ice may confer cryoprotection instead of cryo-injury to the cell. It was suggested that the innocuous intracellular ice formation results when the plasma membrane integrity is maintained by the intracellular propagation of intracellular ice between adjacent confluent monolayer (Acker *et al.*, 2001; Acker and McGann, 2002). Once ice forms inside the cell, there is no further cell shrinkage during slow cooling since osmotic equilibrium is maintained by growth of ice in the cell rather than by osmotic water transport across the plasma membrane.

Two major strategies are used to minimize cell damage during cryopreservation. Cell survival has been improved by cooling the cells at very high rate without causing intracellular ice formation, because the degree of cell injury is a function of time of exposure to the concentrated extra-cellular solutes (Mazur, 1984). Cryo-injury can also be minimized by the addition of low molecular weight compounds that function by colligatively reducing the salt concentration at subzero temperatures, delaying cell exposure to toxic extra-cellular salt concentrations, and/or minimizing damaging cell volume excursion (McGann, 1978; Mazur, 1984). The use of high concentrations of high molecular weight cryoprotective agents in addition to the low molecular weight cryoprotectants increases cell solution viscosity and favours formation of the glass state (vitrify) when cooled at very high rates. This prevents the extra-cellular ice formation and its cell-damaging osmotic and mechanical effects.

Low molecular weight permeating cryoprotectants e.g. glycerol, DMSO, EG, PG, acetamide and 1,2-propanediol, pass through cell membranes and act at both the intra- and extra-cellular levels. Non-permeating cryoprotectants such as proteins, sugars and polymers are large molecular compounds that are unable to pass through cell membranes and only act at the extra-cellular level. Cryoprotectants protect cells from cryo-injury by preventing ice formation, reducing injury due to "solution effects" (Karlsson & Toner, 1996), and stabilizing cell proteins (Arakawa *et al.*, 1990) and plasma membranes (Anchordoguy *et al.*, 1991). To maximize these protective properties, particularly during ultra-rapid cooling and vitrification,

cryoprotectants are used in high concentrations. It is known that the probability of vitrification increases relative to the increase in cryoprotectant concentration (Arav, 1992).

Cryoprotectant toxicity is a critical factor during vitrification as high cryoprotectant concentration enhances toxic damage to the cells (Arav, 1992), though the mechanism is not well understood. The high concentration of cyroprotectants, (30-50%) used in vitrification compared with 5-10% for slow cooling, cannot be applied to spermatozoa due to their lethal and osmotic effects (Mazur *et al.*, 2000). Excessive osmotic forces and cell volume changes occur during addition and removal of cryoprotectants due to the rate at which cryoprotectants and water molecules pass through the cell membrane. The high rate of cooling during vitrification and warming has been reported to cause cell fracture, for instance fracture of the zona pellucida (Rall and Mayer, 1989). Rapid cooling and warming, especially below the glass transition temperature (−110 °C) cause glass fractures with consequent damage to the zona pellucidae of oocytes (Arav, 1992).

Cryoprotectant toxicity depends on time and temperature of exposure and cells from different animal species display different levels of cryoprotectant tolerance (Arav, 1992). In freezing sperm, cryoprotectant toxicity is reduced by adding the cryoprotectant portion of the freezing medium at 5 °C just before freezing (Hafez, 1993). Reduction of the time and lowering the temperature of exposure to cryoprotectants improved vitrification of mouse embryos (Rall, 1987). Arav (1992) suggested reducing cryoprotectant toxicity by using small-volume samples, lowering the cryoprotectant concentration and adding other macromolecules e.g. anti-freeze glycoprotein (AFGP). Such macromolecules reduce cell damage by reducing the toxic effects of other cryoprotectants (Arav, 1992), and protect the cell membranes during cooling to cryogenic temperatures.

To reduce the osmotic changes caused by cryoprotectants during cooling and warming, Rall *et al.* (1987) suggested a step-wise exposure and dilution method, while Arav *et al.* (1988) used a titration method. Step-wise exposure appears to be preferred in most vitrification techniques, for instance cryoloop (Mavrides and Morroll, 2002) and open pulled straws (OPS) developed by Vajta *et al.* (1998). Cooling rate is a function of the heat transfer coefficient of the cooling liquid, the cell suspension solution and the vessel used, and their temperature differences (Arav, 1992). Cooling rate, therefore, vary with the volume of the sample and type of carrier vessel used (Shaw *et al.*, 1991). The probability of vitrification increases as the sample volume decreases (Arav, 1992). Therefore, methods such as OPS (Vajta *et al.*, 1998),

cryoloop (Mavrides & Morrol, 2002), and cryotop (Kelly *et al.*, 2004) that use small volumes, have been applied in vitrification of oocytes and embryos with variable success rates.

1.8 Importance of mammalian gamete cryopreservation in modern-day science

Cuada epididymis provides the major alternative to ejaculated sperm for use in assisted reproductive technologies (ARTs) and genome resource banks (GRBs). Successful use of epididymal sperm resulting in pregnancies have been reported in many species, including cattle (Watson, 1978), dog (Marks *et al.*, 1994), human (Patrizio *et al.*, 1995), goat (Blash *et al.*, 2000), rat (Nakatsukasa *et al.*, 2001), rabbit (Brackett *et al.*, 1978), pig (Rath and Niemann, 1997) and horse (Morris *et al.*, 2002). These studies indicate that when necessary, epididymal sperm can be used to substitute or supplement ejaculated sperm in propagation of a species through ARTs such as artificial insemination (AI), *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI), among others.

In domestic species, epididymal sperm could increase the breeding potential of genetically superior males, especially those that fail to mate naturally, cannot serve an artificial vagina or cannot be electro-ejaculated. Epididymal sperm is useful in extending the breeding life by rescuing germ cells from superior or valuable males, or males in danger of extinction when terminally ill or die suddenly. However, the cause of illness/death has to be investigated to avoid disease transmission (Howard *et al.*, 1986). Epididymal sperm of wildlife species collected at terminal illness or at necropsy can be used for similar purpose. In horses, Cary *et al.* (2004) showed that viable epididymal spermatozoa of acceptable quality can be collected from stallions with terminal illnesses such as traumatic injuries or colic, to extend their breeding carrier.

Cryopreservation of spermatozoa of mammals is a useful means of preserving genetic material. Genome resource banking (GRB) is a specific approach to preserving genetic material from threatened and endangered species (Gilmore *et al.*, 1998). Most GRB programmes use spermatozoa because they are easily accessible compared with other genetic resources (Johnson and Lacey, 1995). A sperm bank, say from epididymal origin, is useful for directly propagating a species or for studying its biology (Wildt, 1992; Johnson and Lacey, 1995). Epididymal sperm can be used in crossbreeding programmes and interspecies fertilization studies with possible production of new animal species. Several births in many mammals resulting from frozen-thawed epididymal spermatozoa through ART have already

been reported (Patrizio *et al.*, 1995; Rath and Niemann, 1997; Blash *et al.*, 2000; Nakatsukasa *et al.*, 2001).

Human epididymal sperm was believed to be incapable of fertilizing, as it was thought that a substantial maturation of sperm was required through the extremely long epididymis. However, Temple-Smith *et al.* (1985) and later Silber *et al.* (1988) reported aspiration of epididymal sperm from men with obstructive azoospermia resulting in pregnancies following IVF. Though the initial fertilization rate was low with IVF, the development of ICSI (Palermo *et al.*, 1992) markedly improved the rate of fertilization with epididymal sperm in the 90% range. The original indication for epididymal sperm aspiration in humans was congenital absence of the vas deferens. However, the indications now include failed vasectomy reversal, unreconstructable epididymitis, and other obstructive conditions of the vas deferens or ejaculatory ducts not amendable by vasal sperm aspiration (Turek *et al.*, 2000). The perfecting of freezing protocols, even for samples that would have been impossible to freeze, now allows routine cryopreservation of human epididymal sperm, regardless of their quality and quantity. Epididymal sperm retrieved by MESA and PESA can be successfully cryopreserved with fertilization, pregnancy and delivery rates similar to those of fresh sperm, a technique that helps prevent future retrievals, and cut costs of repeated sperm retrievals (Patrizio, 2000).

In the mammalian female the observation by Pincus and Enzmann (1935) that immature oocytes recovered from unstimulated rabbit ovaries could mature *in vitro* was a very important step in ART. This breakthrough evolved into the technology of IVF (Iritani and Niwa, 1977), ICSI (Palermo *et al.*, 1992) and cloning by nuclear transfer (Peura *et al.*, 1998; Vajta *et al.*, 2001), that are now applicable in farm animals and other mammalian species. Oocyte collection and their maturation *in vitro* enables production of embryos not only from live, but also from slaughtered or dead, young, pregnant and incapacitated females which is not possible with the conventional embryo transfer (ET) (Gordon, 1994). In farm animals, oocytes retrieved from post-mortem ovaries or from live animals can provide *in vitro*-produced embryos on a large scale for freezing and/or transfer as well as for research. In this way, extensive commercial application of ET in the normal farm practice as for artificial insemination (AI) can be made possible. Cryopreservation of oocytes also makes it possible to create female gamete genetic resource banks for *ex situ* conservation, especially for endangered mammalian species.

In humans, cryopreservation of oocytes has a wide range of applications. Storing of unfertilized oocytes preserves germ cells for individual women and avoids social, moral and legal complications which may arise if partners separate (Shaw *et al.*, 2000). Cryopreservation

of human oocytes also reduces costs of repeated ovum pickup in case of repeated IVF or ICSI. According to Porcu (2001), human oocyte cryopreservation is potentially an alternative solution to the ethical problems arising from embryo storage. In addition, oocyte freezing is the only method to preserve the reproductive capacity for women at risk of losing it because of premature ovarian failure, pelvic diseases, surgery, or anti-neoplastic treatments. Meanwhile, banks of frozen donated oocytes would facilitate the donation process, which is often complicated by a requirement for donor-recipient synchrony (Gook and Edgar, 1999). Furthermore, though controversial, oocyte storage would also open the door to the possibility of women with no medical indications, and no immediate plans to conceive, being able to store 'young eggs' for potential use at a later date.

1.9 Scope of the study

The study examined the effects of cryopreserving bovine gametes to promote the use of cryopreserved epididymal sperm and matured oocytes in ARTs; ease cryopreservation of epididymal sperm and oocytes; and maximise the utilization of cryopreserved sperm. The use of a saline extender suitable for laboratory and field conditions in the cryopreservation of mammalian spermatozoa using bovine cauda epididymal spermatozoa as a model was investigated. Epididymal spermatozoa extended and frozen in a commercial extender was compared with spermatozoa extended and frozen in a modified saline medium. The study also investigated the effects of spermatozoa equilibration time and permeating cryoprotectants, on cryopreserved epididymal spermatozoa. In addition, the study investigated the effects of repeated freeze-thawing of bovine epididymal sperm. Sperm fertilizing potential and the ability of sperm to fertilize oocytes and produce embryos *in vitro* were the measures for these effects. The ability of cryopreserved bovine and buffalo epididymal sperm in production of bovine and bovine x buffalo hybrid embryos *in vitro* was also studied. This was to understand whether fertilization occurs between the two species, and to what extent the hybrid embryos would develop. The hybridization was performed with the hope of providing information for exploring pathways for utilizing exotic alleles that could benefit livestock production and understand some biological mechanisms that control hybridization between the two species. Finally, in an attempt to overcome some of the difficulties associated with cryopreservation of oocytes, the study investigated the effects of cysteamine in an *in vitro* maturation medium and the use of a copper-wire cryoloop method in vitrification of bovine oocytes as a model for other animals. These effects were also assessed by *in vitro* embryo development.

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CHAPTER 2

Cryopreservation of bovine epididymal spermatozoa: Effects of extender, cryoprotectant and equilibration time on sperm fertilizing potential

2.1 Abstract

Cryopreservation of epididymal sperm is useful for conservation of biodiversity, animal production and management of human infertility. More efforts are necessary for improving sperm survival rates since up to 50% do not survive cryopreservation. This study compared the effects of a commercial tris-egg yolk-based extender, Biladyl® (BIL) and modified Tyrode's lactose (MTL) on sperm fertilizing potential. The MTL medium contained 20 % fetal bovine serum (FBS) and 0.95 M glycerol (GLY), ethylene glycol (EG) or dimethyl sulfoxide (DMSO). Epididymal sperm retrieved by retrograde flushing of bull epididymides were diluted with the cryoprotectant-free portion of BIL and MTL at room temperature and equilibrated at 5 °C for 0, 2 or 4 h. The sperm were frozen after addition of the cryoprotectant-containing portion of the respective extenders. Pre-freezing and post-thaw motility, viability, and membrane and acrosome integrity were subjectively evaluated. Pre-freezing effects of the extenders and cryoprotectants were minimal in all treatments, irrespective of duration of equilibration and temperature at which the cryoprotectants were added. Post-thaw parameters were significantly lower ($P < 0.05$) than the pre-freezing parameters with the most significant difference ($P < 0.0001$) observed after 0 h of equilibration. Post-thaw motility and viability of treatments equilibrated for 2 and 4 h in MTL containing cryoprotectants GLY or EG were not different from the control. However, differences in post-thaw membrane and acrosomal status were found for the control and treatments equilibrated for 2 and 4 h. Using MTL with GLY or EG, and 2 or 4 h of equilibration produced acceptable results comparable to the control. However, freezing bull epididymal spermatozoa without equilibration is not recommended, and DMSO should only be used in MTL in the absence of GLY and EG.

2.2 Introduction

The ultimate goal for cryopreservation of sperm is to maintain the fertilizing potential so that normal embryo development and pregnancy can be achieved using the cryopreserved sperm. In all species, sperm cryopreservation involves a precise process which suspends sperm in a mixture designed for adequate cell dehydration to prevent intracellular ice formation as the sperm is frozen. Sperm survival, therefore, depends on careful suspension, cooling, freezing and thawing in appropriate media. Semen is usually diluted with a suitable diluent and a cryoprotectant prior to cryopreservation at −196 °C in liquid nitrogen (Kundu *et al.*, 2000).

Although spermatozoa of many mammalian species have been successfully cryopreserved, some limitations still exist in the current methods. For this reason, survival of spermatozoa is too low for some applications (O'Dell *et al.*, 1958; Kennedy *et al.*, 1960; Berndtson and Foote, 1972; Mortimer *et al.*, 1976; Parkinson and Whitfield, 1987; Chen *et al.*,

1993a). In most mammalian species a high proportion of spermatozoa lose their motility or other functions after thawing and only about 50% of spermatozoa survive the freeze-thaw process (Watson, 2000). Such findings were observed in a cryopreservation study of African buffalo epididymal sperm (Lambrechts *et al.*, 1999). In other species, for instance chickens, survival is extremely low with up to 98-99% of spermatozoa lost during freeze-thawing (Etches, 1996). Meanwhile, pig spermatozoa are extremely sensitive to cryopreservation that severely reduces the fertilizing potential of such sperm (Almlid and Johnson, 1987; Parks and Graham, 1992).

Sperm damage during the freeze-thaw process is thought to be due to cold shock, osmotic stress, ice crystal formation (Mazur, 1984) and oxidative damage (Alvarez and Storey, 1992), though the mechanism is not well understood. However, it has been reported that cooling spermatozoa, even if the cells are not cooled below 0 °C, and warming cause metabolic and structural damages to the fertilizing integrity of the sperm (Amann, 1999). Amann (1999) further stated that cooling to 5 °C causes specific type of alteration that is related to membrane lipid phase-transitions different from those caused by freezing and thawing. Furthermore, cryopreservation causes changes in the sperm morphology, including damage to the sperm membrane, acrosome, tail and the organelles (Wooley and Richardson, 1978). Therefore, the proportion of fully functional sperm that retain their membranes, acrosomes, tails and organelles intact after freeze-thawing is low (Holt, 1997).

Sperm motility is particularly susceptible to cryopreservation damage (Henry *et al.*, 1993), and in many studies, for instance, Graham (1994) and Bezuidenhout *et al.* (1995) sperm motility was used as a measure of sperm viability. However, attributes other than motility are required for sperm to have a normal fertilizing potential, thus frozen-thawed sperm with a relatively high percentage of motility may not fertilize oocytes (Amann, 1999). A study by Graham (1994) on the effect of cryopreservation on motility of ejaculated and epididymal sperm showed no difference in the samples after cooling and thawing. The author, however, reported a higher post-thaw sperm motility for ram and bull epididymal sperm treated with seminal plasma than ejaculated sperm, though the reason was unclear. Other authors (Lasley and Bogart, 1944; Johnson *et al.*, 1980; Berger and Clegg, 1985) reported epididymal sperm to be more resistant to cold shock than ejaculated sperm.

Usually semen is diluted in a freezing medium (extender) that may be a simple buffered salts solution containing sugars such as fructose, glucose or lactose (Amann, 1999). To protect the spermatozoa from cryo-injury, complex macromolecules such as egg yolk, skimmed milk,

whole milk, and milk whey are added to the salts/sugars solution (Watson, 1981a; Chen *et al.*, 1993a; Ganguli *et al.*, 1997). Macromolecules of plant origin, for instance soybean extract (lecithin), are also used in the cryopreservation of sperm (Van Wagtendonk-de Leeuw *et al.*, 2000; Aires *et al.*, 2003). Further cryoprotection to the spermatozoa is provided by addition of permeating cryoprotectants such as glycerol (Polge *et al.*, 1949). Therefore, a basic sperm freezing medium is an osmotic supporting salts/sugars solution containing a suitable permeating cryoprotectant and protective macromolecular additives, mostly protein components of milk and egg yolk, or lipid components from vegetal origin (Bousseau *et al.*, 1998).

Protective macromolecular additives such as serum and serum albumin are used in the cryopreservation of oocytes and embryos in many studies, for instance Van Wagtendonk-de Leeuw *et al.* (1997) and Dinnyés *et al.* (2000). However, not much has been reported on the use of such macromolecules in the cryopreservation of mammalian spermatozoa. In one study, Chen *et al.* (1993b) examined the effects of adding sucrose and trehalose, known to have cryoprotective properties, blood serum, and the anti-oxidants taurine and hypotaurine on sperm motility after freezing and thawing at different rates. The authors found that up to 10% v/v of heat inactivated blood serum was generally beneficial for cryopreserving spermatozoa, but gave more variable responses with different bulls (Chen *et al.*, 1993b). In another study De Leeuw *et al.* (1993) used bovine serum albumin (BSA) and other substances including various cryoprotectants and membrane-stabilizing agents to freeze bovine sperm. The extender containing 6% w/v BSA yielded results which did not differ from those obtained with the standard extender. These authors recommended that further research on the cryopreservation of bovine spermatozoa should focus on membrane stabilization since the membrane-stabilizing compounds yield more promising results than the ice-preventing agents. In other species, like the fish, Aokil *et al.* (1997) successfully cryopreserved medaka (*Oryzias latipes*) spermatozoa in a medium containing fetal bovine serum (FBS) and 10% N, N-dimethylfromamide. Meanwhile, Beesley *et al.* (1998) demonstrated a high post-thaw viability in spermatozoa of the wood frog (*Rana sylvatica*) frozen in medium containing FBS.

Since the discovery of the cryoprotective property of glycerol against sperm cryo-injury (Polge *et al.*, 1949), glycerol has been the preferred cryoprotectant for freezing sperm of most mammalian species. However, glycerol is known to damage sperm cells at high concentrations and high temperatures. This could be related to the fact that glycerol induces more osmotic stress because it passes through cell membranes slower than other permeating cryoprotectants such as ethylene glycol (EG) and dimethyl sulfoxide (DMSO) (Amann, 1999). The extent of damage varies with species, for instance, marsupial spermatozoa can tolerate up to 20%

glycerol, while mouse spermatozoa are severely damaged if glycerol concentration exceeds 1.75% (Holt, 2000). In chickens, glycerol impairs fertility and should be diluted to 0.7% v/v (Etches, 1996) or removed by dilution, centrifugation and resuspension (Amann, 1999) before using the sperm for insemination.

Considerable attention has been given to other cryoprotectants, particularly EG and DMSO for freezing of genetic materials other than sperm of most mammals. However, in some species notably fish, amphibians and birds, these cryoprotectants are more commonly used for sperm cryopreservation than glycerol. Because glycerol impairs fertility, DMSO is preferred for crypreservation avian sperm (Etches, 1996). Dimethyl sulfoxide is the most common cryoprotectant for fish and amphibian sperm however, freezing of carp sperm in sugar-based extenders with methanol cryoprotectant yielded better post-thaw results than ionic extenders with DMSO cryoprotectant (Horváth *et al.*, 2003). Meanwhile, post-thaw motility similar to those of fresh semen was obtained when sea bass (*Dicetrarchus labrax*) semen was frozen in an extender containing EG as cryoprotectant (Sansone *et al.*, 2002). In mammals, EG has been used to freeze semen from dog (Vannucchi *et al.*, 1999); stallion (Alvarenga *et al.*, 2000; Mantovani *et al.*, 2002); cynomogus monkey (Li *et al.*, 2005) and alpaca (Santiani *et al.*, 2005) with very good results. Furthermore, cryopreservation of rabbit sperm in a sucrose-DMSO extender resulted to post-thaw motility and normal acrosome integrity comparable to those of fresh semen (Vicente and Viudes-des-Castro, 1996). However, reports on the use of these cryoprotectants for cryopreservation of bovine sperm, especially bovine epididymal sperm, are rare.

The amounts and methods of adding a permeating cryoprotectant, for instance glycerol, to the freezing medium vary with extenders, freezing methods and species (Foote, 1975). In freezing bovine sperm, glycerol is added after cooling spermatozoa to 5 °C or just before freezing to a final concentration of 5% (yolk-sugar extender) to 10% (in milk extender), though 7% is more preferred in most extenders (Hafez, 1993). The extended semen is usually held at 5 °C for 2 to 6 h, depending on the medium used, before freezing to allow sperm cells to equilibrate with the extender (Hafez, 1993; Herman *et al.*, 1994). Although it was thought to be necessary for sperm to equilibrate with glycerol, it is apparent that instead other changes take place at 5 °C because addition of glycerol just before freezing affords just as much cryoprotection (Hafez, 1993). In an earlier study of boar semen cryopreservation, no effect of equilibration time on post-thaw viability was demonstrated, suggesting that the primary cryoprotective effect of glycerol on boar semen may be extracellular (Almlid and Johnson, 1988). Furthermore, Colas (1975) showed in ram semen that addition of glycerol at +30 °C

resulted in a highly significant decrease in the mean proportion of motile spermatozoa immediately after thawing, compared with addition at +4 °C. In the same study, exposure time to glycerol had no significant effect on the survival of spermatozoa after thawing and incubation. Januskauskas *et al.* (1999) studied the effects of fast cooling (4.2 °C/min) and slow cooling (0.1 °C/min) rates during equilibration of dairy bull semen from room temperature to 4 °C, on *in vitro* post-thaw sperm viability and fertility after artificial insemination (AI). There was no significant difference in fertility rates (56-day non-return rates) between the two cooling rates. In addition, most of the *in vitro* post-thaw sperm viability parameters and the fertility results for the bulls after AI did not differ significantly between the two semen cooling procedures evaluated.

A sperm is a specialized cell morphologically and functionally designed to achieve its only function, which is fertilization. The morphological and functional characteristics of spermatozoa may be damaged during cryopreservation, which reduces their fertilizing potential (Holt, 1997). Therefore, to ascertain the fertilizing potential, many methods are usually used to evaluate the morphological and functional characteristics of spermatozoa's fertilization potential.

To accomplish fertilization, spermatozoa have to carry the male genome to the oocyte(s). In the female reproductive tract, sperm transport is by both inherent sperm motility, and muscle/cilliary and fluid movements of the oviduct (Scott, 2000). However, *in vitro* sperm transport is only achieved through the activity of the sperm flagellum or tail (Peters and Ball, 1987). Sperm motility is due to the propulsive apparatus (flagellum) equipped with contractile proteins strategically arranged in organelles, the coarse fibers, and associated subfilaments and microtubules (Hafez, 1993). The energy for this propulsion is generated in the mitochondria and transformed into a propulsive force by the microtubules (Amann, 1999). Sperm motility is, therefore, an important parameter for assessing sperm fertilizing potential. Strong, progressive motility is an important index of viability in a sperm population, usually seen as swirling, wave-like motion in the highly concentrated samples (Hunter, 1980). Traditionally, sperm motility evaluation is done subjectively, by visual assessment a microscope. The method is simple, easy and quick and widely used to determine the quality and extent of damage inflicted by, e.g. cryopreservation procedures (Rodriguez-Martinez, 2000). Furthermore, a significant relationship exists between subjectively assessed motility and fertility (Linford *et al.*, 1976; Kjaestad *et al.*, 1993). Objective methods of assessing sperm motility such as computer assisted sperm motility analysis (CASA) and sperm motility quantification (SMQ) are currently available (Van Der Horst *et al.*, 1999). However, in a comparative study of subjective and objective sperm motility evaluation, Walker *et al.* (1982) found that sperm motility could be subjectively rated with reasonable accuracy.

Sperm membrane integrity and proper function is essential for sperm motility, metabolism, capacitation, oocyte binding and the acrosome reaction (Jeyendran, 2000; Brito *et al.*, 2003). Both physical intactness and functional integrity of the sperm membranes are important indicators of the fertilizing potential of sperm. In the process of death, the sperm cell loses the physical integrity of its plasmalemma that becomes permeable to certain stains. A simple method of determining the physical status of the plasmalemma uses its permeability to vital stains such as eosin-nigrosin (EN) and trypan blue (TB). Vital stains have been widely used to evaluate plasmalemma physical integrity (Mayer *et al.*, 1951; Swanson and Bearden, 1951; Hackett and Macpherson, 1965; Dott and Foster, 1972). These stains differentially colour the cells such that live and dead sperm populations can be distinguished from each other and from the background (Dott and Foster, 1972). For instance, eosin in EN stains spermatozoa with physically broken or damaged membranes while it does not penetrate spermatozoa with physically intact membranes (Jeyendran, 2000). Recently, several stain combinations have been used to assess sperm viability. Amongst these are rhodamine 123 (R123) used to assess mitochondrial membrane potential and ethidium bromide to determine membrane integrity using flow cytometry (Evenson *et al.*, 1982). Other stain combinations include propidium iodide (PI), *Pisum sativum* agglutinin (PSA) and R123 (Garner *et al.*, 1990), Hoechst 33342 (Johnson *et al.*, 1989), Hoechst 33258 (De Leeuw *et al.*, 1991) and SYBR-14 and PI (Garner and Johnson, 1995). However, these staining procedures are slow, expensive and may not be performed in most laboratories. Therefore, simple but reliable techniques such as EN staining are still highly applicable in sperm analysis.

The integrity of the plasmalemma has also been evaluated using the hypo-osmotic swelling test (HOST) which evaluates the membrane's biochemical function (Jeyendran, 2000). Jeyendran *et al.* (1984) suggested that the ability of spermatozoa to swell in the presence of a hypo-osmotic diluent reflects normal water transport across the sperm membrane, which is a sign of normal membrane integrity and functional activity. Drevius and Erikson (1966) first demonstrated such an increase in cell volume of spermatozoa placed in hypo-osmotic solutions. This volume increase was associated with the spherical expansion of the cell membrane covering the tail, thus forcing the flagellum to coil inside the membrane. Coiling of the tail begins at the distal end of the tail and proceeds towards the midpiece and head as the osmotic pressure of the suspending medium is lowered (Drevius and Erikson, 1966; Jeyendran *et al.*, 1984). Therefore, the functional and active spermatozoa exposed to hypo-osmotic stress (HOST) swells due to water influx, and subsequently increase in volume to establish an equilibrium between the fluid compartment within the spermatozoa and the extracellular

environment. Spermatozoa with functionally damaged or inactive membranes are unable to support osmotic swelling as shown by those with normal membranes.

In a study by Correa and Zavos (1994) to assess frozen-thawed bovine sperm membrane integrity using HOST, a solution made of equal parts of fructose and sodium citrate at 100 mOsm/L produced the best swelling results. Revell and Mrode (1994) and Brito *et al.* (2003) prepared hypo-osmotic swelling test reagent by dissolving 0.9 g fructose and 0.49 g sodium citrate in 100 mL of double distilled water to give approximately 100 mOsm/L hypotonic solution. HOST has been reported to be very reliable (Zaneveld *et al.*, 1990), and very highly individual specific (Stark and Bernt, 1989). HOST is thus useful in evaluating epididymal sperm membrane functional integrity and the changes associated with freeze-thaw procedures.

Capacitation and the acrosome reaction are important processes the mammalian spermatozoa must undergo prior to penetrating the oocyte during fertilization. The acrosome reaction involves fusion of the sperm membrane with the outer acrosomal membrane, calcium influx (Bleil and Wassarman, 1983), vesiculation, and exocytosis of the acrosomal enzymes (Barros *et al.*, 1967; Yanagimachi and Noda, 1970). These processes are a prerequisite for sperm to penetrate the oocyte. A strong correlation exists between fertility and the percentage of sperm with normal acrosomes (Berndtson *et al.*, 1981). Normal or true acrosome reaction occurs only in live, intact spermatozoa (Thomas *et al.*, 1997). This process takes place at the surface of the zona pellucida, after the binding of sperm to the ZP3 receptor protein (Bleil and Wassarman, 1983; Rossiere and Wassarman, 1992) and precedes sperm penetration of the zona. Abnormal or false acrosome reactions may occur spontaneously without binding to the ZP3 receptor protein. Spontaneous acrosome reactions occur during sperm death (Thomas *et al.*, 1997), for instance during the process of freeze-thawing. Fertilization failure has been shown to be associated with absence of acrosomal enzymes (Dale *et al.*, 1994) or premature loss of acrosomal enzymes due to a spontaneous acrosome reaction (Chan *et al.*, 1999). Thus, evaluation of spermatozoa should not only focus on viability, but should determine sperm acrosomal status as well (Berndtson *et al.*, 1981).

Methods for evaluation of sperm acrosomal status include the triple stain procedure (Talbot and Chacon, 1980), the indirect immuno-fluorescence assay (Wolf *et al.*, 1985), chlortetracycline assay (Lee *et al.*, 1987), and *Pissum sativium* agglutinin-fluorescin isothiocyanate assay (Liu and Baker, 1988), among others. These methods may not be performed in a simple setting and the results are not readily available. Oettlé (1986a) reported the use of Spermac® stain for the evaluation of sperm morphology including the acrosome.

Since then, Spermac® stain has been used for staining fresh and extended semen from bull, ram, goat, dog, horse, boar, cheetah, and human (Oettlé and Soley, 1985; Oettlé, 1986a,b; Oettlé and Soley, 1986; Chan *et al.*, 1996; Hay *et al.*, 1996; Viggiano *et al.*, 1996; Watkins *et al.*, 1996). This metachromatic stain makes various degrees of acrosomal damage visible, and some of the damages that occur during the acrosomal reaction can be seen. It is a quick, reliable and easy procedure that can be performed in simple laboratories, and can also be used for diluted and frozen semen, because the smear's background does not take up the stain (Oettlé and Soley, 1985; Oettlé, 1986a).

The objective of this study was to use a non-commercial extender that could be easily applicable in a field condition in the cryopreservation of mammalian spermatozoa using bovine cauda epididymal spermatozoa as a model. The study, therefore, compared the efficacy of two extenders, a commercial extender Biladyl® (BIL) and modified Tyrode's lactose (MTL) medium; three cryoprotectants (glycerol, ethylene glycol and dimethyl sulfoxide); and three equilibration times of 0, 2 and 4 h in cryopreservation of the fertilizing potential of bovine epididymal sperm. Sperm evaluation done prior to freezing (i.e. after complete extension) and post-thaw was based on motility, live sperm, and membrane and acrosome integrity. The efficacy was tested using the null hypothesis "H_0: fertilizing potential of bovine epididymal sperm equilibrated at 5 °C for 0, 2 or 4 h in MTL containing glycerol, ethylene glycol or dimethyl sulfoxide, and in BIL for 0 and 2 h, and cryopreserved in liquid nitrogen is not different from equilibration in BIL for 4 h followed by cryopreservation".

2.3 Materials and methods

2.3.1 Preparation of extenders

Biladyl® (Minitüb, Germany) consists of a powder cocktail AB, solution A (49 mL) and solution B (250 mL). The preparation procedure was as described in the manufacturer's protocol. Briefly, at least 200 mL of fresh and clean chicken egg yolk (less than 48 h old) was obtained. The surfaces of the eggs were cleaned with 70% ethanol, the shell then broken to drain the albumen and the yolk gently placed onto a sterile tissue paper without breaking the yolk membrane. After rolling the yolk on the tissue paper to remove excess albumen, the yolk was aspirated using a sterile 20 mL syringe and pooled into a sterile beaker. The cocktail AB was then reconstituted in 12 mL of sterile water. Solutions A and B were each reconstituted by adding sterile water to the final volumes of 390 mL. To each of the 390 mL of the reconstituted solutions A and B, 100 mL of egg yolk and 10 mL of the reconstituted cocktail AB were added to final volumes of 500 mL of Biladyl A and B, respectively. These were mixed gently then

warmed to 30 °C in a water bath (Memmert®, Germany). Aliquots (10 mL) of the warmed Biladyl A and B were placed into sterile 15 mL test tubes and stored at −20 °C until used. All procedures were done under a laminar flow hood to prevent contamination.

Sperm Tyrode's lactose medium was prepared as described by Parrish *et al.* (1985) (see Appendix A2) under a laminar flow hood, sterile filtered then stored at 4 °C and used within one month. On the day of use, the sperm Tyrode's medium was supplemented with 0.2 mM sodium pyruvate, 20% FBS and 10 µg/mL ciprofloxacin hydrochloride (CF-HCl) to form modified Tyrode's lactose (MTL) extender that was divided into MTL A and B. To MTL B was added the permeating cryoprotectant glycerol (GLY), ethylene glycol (EG) or dimethyl sulfoxide (DMSO) each to a final concentration of 1.9 M.

2.3.2 Collection of testicles and sperm retrieval

Pairs of bull testicles were collected from nearby abattoir. The scrotal skin was removed leaving the *tunica vaginalis* covering the testicles. These were packed in clean polythene bags and transported to the laboratory in a polystyrene container containing an icepack. A cardboard sheet was placed between the icepack and testis to prevent direct contact, especially of the epididymis, with the icepack. The external surface of each testicle was sprayed with 70% ethanol and the surface dried with sterile tissue paper. To avoid cutting the epididymis, pressure was applied to the lateral and medial surfaces of the testis and a long incision made on the anterior border, opposite to where the epididymis is apposed. As the testis bulged out, the tunica vaginalis was detached from the epididymis and removed. The epididymis and *vas deferens* were then isolated from each testicle. The *cauda* and *corpus epididymis* were separated and *vas deferens* trimmed at the straight portion. All excess surrounding tissues were excised. Spermatozoa were retrieved by retrograde flushing of both the vas deferens and cauda epididymis (Halangk *et al.*, 1990; Reyes-Moreno *et al.*, 2000; Cary *et al.*, 2004; Martinez-Pastor *et al.*, 2005). Briefly, a blunted G21 hypodermic needle was inserted into the *vas deferens*. Using a 5 mL syringe, the sperm was flushed from the vas deferens and cauda epididymides of each pair of testicles with pre-warmed TCM-199 into a 50 mL centrifuge tube. The collected sample was incubated in a water bath at 37 °C for 10 min before evaluation for presence and motility of sperm. Preparations were made, stained and evaluated for viability, and membrane and acrosome integrity.

2.3.3 Washing and extension of sperm

The retrieved sperm samples were washed twice in TCM-199 by centrifugation at 700g for 6 min at 25 °C in a temperature controlled centrifuge (Allegra® X-22R, Beckman Coulter,

Germany) and the supernatant discarded. After the second washing, each sample was divided into two portions of approximately ¼ and ¾ in 15 mL test tubes. The ¼ portion was extended with pre-warmed Biladyl A to 4 mL and was called the **BIL A portion**. The ¾ portion was extended with modified Tyrode's lactose (MTL) A medium (without cryoprotectant) to 12 mL and was referred to as **MTL A portion**. These initial extensions were done at room temperature (24 °C). Aliquots of 1 mL of BIL A portion were made in three test tubes to be further extended, after equilibration, with an equal volume (1 mL) of **BIL B** (Minitüb, Germany) containing 14% (1.9 M) GLY (final concentration 7% GLY). Similar aliquots (1 mL) of MTL A portion were made in nine test tubes to be further extended with **MTL B** containing 14% GLY, 10.6% EG or 13.6% DMSO at the molar concentration of 1.9 M equivalent to that of GLY in Biladyl B. The final extensions were done either at room temperature for 0 h of equilibration or at 5 °C for samples equilibrated for 2 or 4 h. The respective treatments evaluated in this study are presented in the Experimental design (Table 2.1).

Table 2.1 Experimental design.

	Equilibration time (h)		
Medium	0	2	4
BIL	GLY 7% (0.95 M)	GLY 7% (0.95 M)	**GLY 7% (0.95 M)**
MTL	GLY 7% (0.95 M)	GLY 7% (0.95 M)	GLY 7% (0.95 M)
MTL	EG 5.3% (0.95 M)	EG 5.3% (0.95 M)	EG 5.3% (0.95 M)
MTL	DMSO 6.8% (0.95 M)	DMSO 6.8% (0.95 M)	DMSO 6.8% (0.95 M)
Initial extension	Room temperature	Room temperature	Room temperature
Cooling	5 °C for 0 h	5 °C for 2 h	5 °C for 4 h
Final extension	Room temperature	5 °C	5 °C
Sperm packing	0.25 mL straws	0.25 mL straws	0.25 mL straws
Freezing	Nitrogen vapour	Nitrogen vapour	Nitrogen vapour
Storage	Liquid nitrogen	Liquid nitrogen	Liquid nitrogen

2.3.4 Evaluation, freezing and thawing of semen

Sperm evaluation was performed before, at and after freezing. At the end of each equilibration period (0, 2 or 4 h), the samples were further diluted by slowly adding Biladyl B or MTL B followed by gentle mixing. Each sample was packed into four 0.25 mL Cassou straws (IMV®, France) and sealed with polyvinyl chloride (PVC) powder pre-cooled to 5 °C. Straws were frozen at minus 80 °C in nitrogen vapor (5 cm above liquid nitrogen) for 10 min before plunging into liquid nitrogen (−196 °C). The excess of the finally extended sperm samples were prepared for evaluation to obtain the pre-freezing values. The straws were stored in liquid

nitrogen for 1-4 weeks during which evaluation was performed. The frozen straws were thawed in a water bath at 37 °C for 1 min and the samples prepared for evaluation. Evaluation of sperm before, during and after freezing was done subjectively by one experienced person throughout the study. Spermatozoa were evaluated for progressive motility, viability, and membrane and acrosomal status.

2.3.4.1 Evaluation of progressive sperm motility

Aliquots (200 µL) of each sample were made in 3 mL test tubes, diluted with an equal volume of TCM-199 and kept in a water bath at 37 °C before motility assessment. A 10 µL drop of the sample was placed on a pre-warmed 35 mm Petri dish and subjectively assessed for progressive sperm motility on an inverted warm-stage (37 °C) Nikon microscope (Nikon®, Eclipse TE2000-E, Japan) at X200 magnification using a phase-contrast objective. Assessment was focused at the surface of the Petri dish. Progressive motility was defined as spermatozoa displaying forward linear movement and was scored as percentage of the total spermatozoa in the field being viewed to the nearest 5%, except for those with motility less than 5%.

2.3.4.2 Vital staining for live-dead sperm evaluation

Vital staining based upon differential staining of live and dead sperm using new improved dichromatic eosin-nigrosin (EN) stain (pH 8.14, osmolality 439, Taurus, South Africa) was used. Aliquots (200 µL) of each sample were placed in 3 mL test tubes, and diluted with an equal volume of 3% sodium citrate at room temperature (24 °C). A drop of 5 µL of the diluted sample was made at one end of a clean microscope slide and a 5 µL drop of EN stain placed on the sample drop. The two drops were mixed and incubated for 20 seconds at room temperature. A smear was made using a cover slip and dried at room temperature. The slide was examined with an objective bright field microscope (Nikon, 110096, Japan) at X400 magnification. Between 200 to 250 sperm cells were counted in different fields and scored as live or dead. Eosin stained dead sperm pink/red, while nigrosin coloured the background (slide) dark to provide the contrast needed for the live unstained sperm (Björndahl *et al.*, 2003).

2.3.4.3 Hypo-osmotic swelling test (HOST)

Hypo-osmotic swelling test reagent was prepared by dissolving 0.9 g fructose and 0.49 g sodium citrate in 100 mL of double distilled water to give approximately 100 mOsm/L hypotonic solution (Revell and Mrode, 1994; Brito *et al.*, 2003). A sperm sample (30 µL) was mixed and incubated with 300 µL of HOST reagent at 37 °C for 60 min (Brito *et al.*, 2003). A 7 µL drop of the incubated mixture and a 3 µL of EN stain were placed on a glass slide, the drops mixed and spread with wood stick applicator (Brito *et al.*, 2003). The smear was dried at room temperature

and examined under bright field microscopy (Nikon, 110096, Japan) at X400 magnification. Sperm with intact membranes resisted hypo-osmotic stress and demonstrated swelling and coiling of the tail, but not those with abnormal membranes. Between 200 to 250 sperm in different fields were evaluated and scored as having functional or abnormal membranes.

2.3.4.4 Evaluation of sperm acrosome integrity (Spermac® staining)

Spermac® stain (Stain Enterprises, South Africa) consists of the fixative (clear liquid), and stains A (red liquid), B (yellow/colourless liquid) and C (green liquid). Smear preparation, drying, fixing, staining and washing were done as described by Oettlé (1986a). Briefly, the sample was diluted 1:1 with 3% sodium citrate and a 10 µL drop of the diluted sample placed on a clean glass slide. A thin smear was made by spreading the drop with a cover slip and then allowed to air-dry for not more than 5 min. The smear was fixed for 10 min and then washed by dipping once in a cup of tap water. Excess water was drained with tissue paper and the slide dipped into stain A to stain for 2 min. Stain A was washed twice by dipping the slide in two separate cups of tap water. After draining the excess water, the slide was dipped in stain B for 1 min followed by washing once and draining excess water. The slide was finally dipped in stain C for 1 min, washed once and air-dried with the slide in a vertical position. The smears were evaluated using bright field microscopy (Nikon, 110096, Japan) under oil immersion objective magnification at X1000 for differential staining of normal and abnormal acrosomes. As described by Chan *et al.* (1999), sperm with normal intact acrosome showed green acrosomal and pink post-acrosomal regions with a well-defined thick green band forming a semicircle at the tip of the sperm with the mid-piece and tail colored green. Sperm with abnormal/reacted acrosomes showed a pink acrosomal region or green acrosomal region, with the semicircle band broken-up, discontinuous, vesiculated, botched, missing or fuzzy. Between 200 to 250 sperm cells in different fields were evaluated and classified as having a normal/intact or a reacted/damaged acrosome.

2.3.5 Statistical analysis

Data recorded for sperm retrieved from six pairs of epididymides were captured in Excel as numbers (viability, and membrane and acrosome integrity) or percentages (motility) then exported to a statistical analysis system (SAS) program (version 9.1) for analysis. The sperm parameters of the treatments were compared with that of 4 h equilibration in Biladyl as the control. The generalized linear module (GENMOD) procedure was used to compare treatments in terms of the differences in live sperm, and membrane and acrosome integrity. The least-squares means (LSMEANS) of the treatments were determined and the differences of LSMEANS used to test the differences between the individual treatments. The procedure for

general linear model (PROC GLM) was used to analyze sperm motility. An analysis of variance (ANOVA) test of the dependent variable (sperm motility) was performed and the effects of treatments determined, and the LSMEANS for the treatments were subsequently calculated. Differences between the LSMEANS were tested using a Bonferroni test adjusted for multiple comparisons of means to determine the significance of treatments. P-values less that 0.05 denoted significance.

2.4 Results

There was no difference in sperm parameters of sperm fertilizing ability between the control and treatments ($P > 0.05$) during the pre-freezing period. Equilibration time, extender and cryoprotectant used appeared not to have severely affected sperm motility, viability and acrosome integrity. However, the percentage of sperm with normal membrane integrity in the control was significantly different ($P < 0.05$) from that of the treatments, especially at 0 h of equilibration. Variable but not significant differences were also observed between few individual treatments in the pre-freezing sperm parameters. All post-thaw parameters were significantly lower ($P < 0.05$) when compared to pre-freezing parameters. Cryopreservation without equilibration was most damaging to spermatozoa, while an equilibration of 4 h produced the best results, and 2 h produced intermediate results.

It was noted during evaluation that sperm frozen in MTL was easier to examine than sperm frozen in BIL, because the latter medium is less transparent and sometimes reacted with other chemicals, for example stains, making it more difficult to examine. Secondly, spermatozoa frozen in extender containing DMSO was also difficult to examine, as DMSO reacted with the stains, resulting in poor staining.

2.4.1 Sperm motility

Figures 2.1, 2.2 and 2.3 show pre-freezing and post-thaw progressive sperm motility for spermatozoa equilibrated at 5 °C for 0, 2 and 4 h, respectively, and cryopreserved in extenders A (BIL), B (MTL + GLY), C (MTL + EG), and D (MTL + DMSO), with cryopreservation in BIL after 4 h of equilibration as the control. The series with different superscripts (a, b and c) are different ($P < 0.05$).

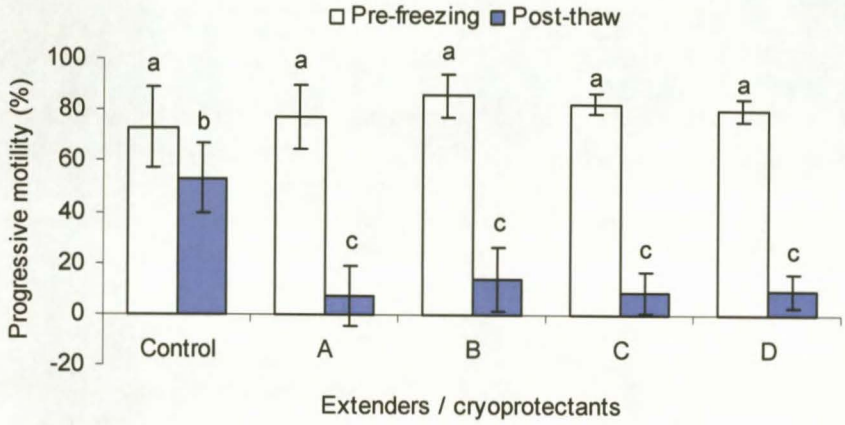

Fig. 2.1 Pre-freezing and post-thaw progressive sperm motility for spermatozoa equilibrated at 5 °C for 0 h.

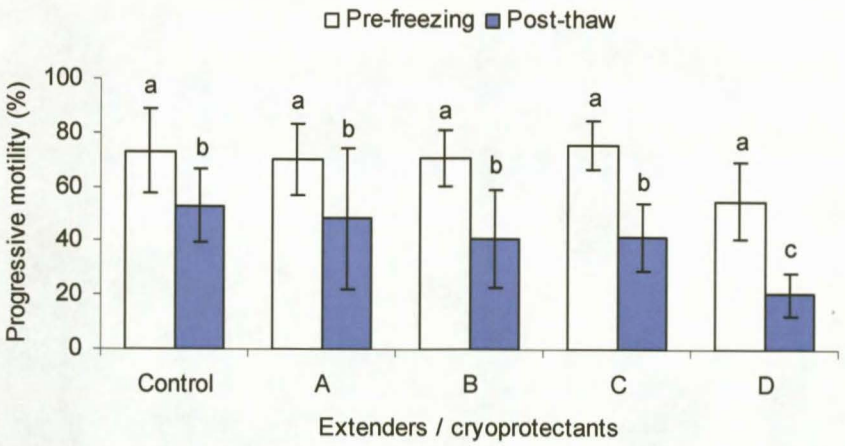

Fig. 2.2 Pre-freezing and post-thaw progressive sperm motility for spermatozoa equilibrated at 5 °C for 2 h.

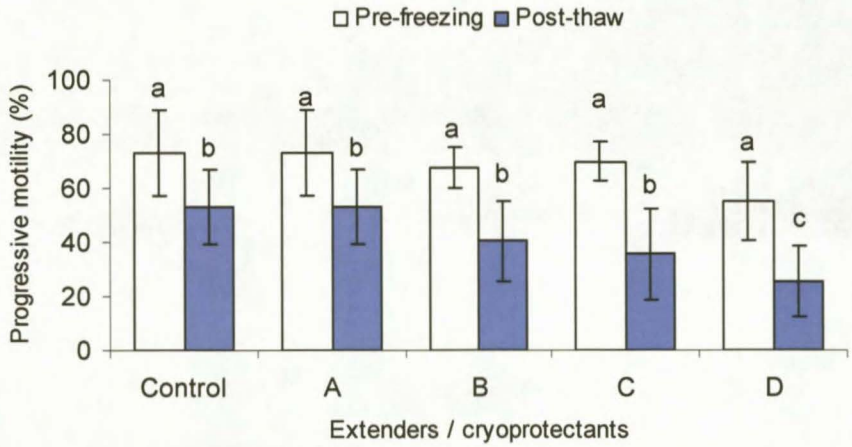

Fig. 2.3 Pre-freezing and post-thaw progressive sperm motility for spermatozoa equilibrated at 5 °C for 4 h.

Pre-freezing progressive motility for spermatozoa frozen after 0 h (Fig. 2.1), 2 h (Fig. 2.2) and 4 h (Fig. 2.3) of equilibration were not different ($P > 0.05$) from that of the control. The pre-freezing motility of spermatozoa equilibrated in MTL for 2 h and 4 h, and frozen with DMSO as cryoprotectant (Fig. 2.2 D and Fig. 2.3 D, respectively), tended to be lower than that of the rest of treatments, but these differences were not statistically significant ($P > 0.05$).

Addition of the cryoprotectant-containing portion of the respective extenders at room temperature without equilibration (Fig. 2.1) and at 5 °C after equilibration for 2 h (Fig. 2.2) and 4 h (Fig. 2.3) did not adversely affect the pre-freezing sperm motility. The mean pre-freezing sperm motility after equilibration for 0 h (81.5 ± 3.5%), 2 h (67.9 ± 9.0%) and 4 h (66.4 ± 7.9%) were no different from the overall mean motility for 0, 2 and 4 h (71.9 ± 9.6%). This indicated a minimal instant effect on sperm motility arising from addition of the cryoprotectants.

Post-thaw sperm motility was lower ($P < 0.001$) in all treatments frozen without equilibration (Fig. 2.1) than that of the control. Adverse effects of the freeze-thawing process on motility occurred irrespective of the extender and cryoprotectant used. The post-thaw progressive sperm motility in treatments equilibrated for 2 h (Fig. 2.2 A, B & C) and 4 h (Fig. 2.3 B & C) were lower, but not different ($P > 0.05$) from that of the control. However, equilibration for 2 h (Fig. 2.2 D) and 4 h (Fig. 2.3 D) in MTL followed by freezing in the presence of DMSO, exhibited a lower ($P < 0.05$) post-thaw progressive motility than the control and other treatments.

There was no difference ($P > 0.05$) in the mean post-thaw progressive motility between sperm equilibrated for 2 h (37.9 ± 11.9%), and 4 h (38.7 ± 11.4%). However, these were higher ($P < 0.0001$) than the mean post-thaw progressive motility for sperm equilibrated for 0 h (9.9 ± 2.9%). Post-thaw motility was best observed when sperm was equilibrated in BIL for 2 h (Fig. 2.2 A) or 4 h (Fig. 2.3 A). However, sperm equilibrated in MTL for 2 & 4 h and frozen in the presence of GLY (Fig. 2.2 B & Fig. 2.3 B) and EG (Fig. 2.2 C & Fig. 2.3 C), respectively, exhibited comparably good post-thaw progressive motility. Post-thaw motility after 2 and 4 h of equilibration was lower ($P < 0.05$) in MTL + DMSO (Fig. 2.2 D and Fig. 2.3 D) than the rest of treatments, although these were higher than the post-thaw motility of sperm frozen in the same extender without equilibration (Fig. 2.1 D).

2.4.2 Sperm viability

Figures 2.4, 2.5 and 2.6 show pre-freezing and post-thaw sperm viability for spermatozoa equilibrated at 5 °C for 0, 2 and 4 h, respectively, and cryopreserved in extenders A (BIL), B (MTL + GLY), C (MTL + EG), and D (MTL + DMSO), with cryopreservation in BIL after 4 h of equilibration as the control. The series with different superscripts (a - e) are different ($P < 0.05$).

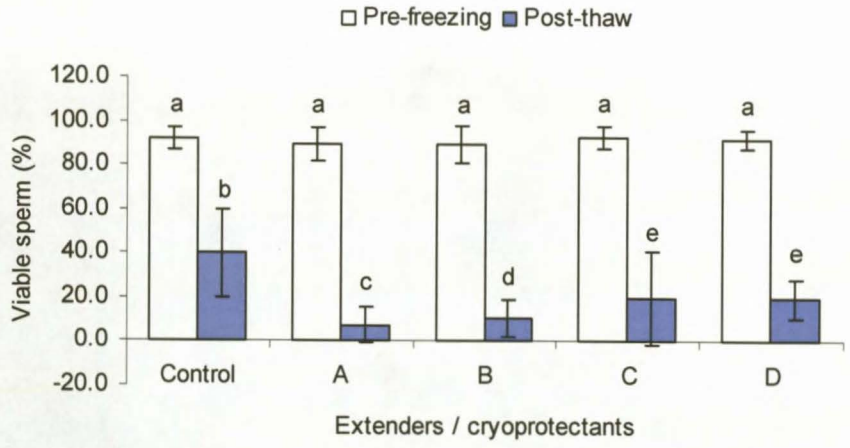

Fig. 2.4 Pre-freezing and post-thaw sperm viability for spermatozoa equilibrated at 5 °C for 0 h.

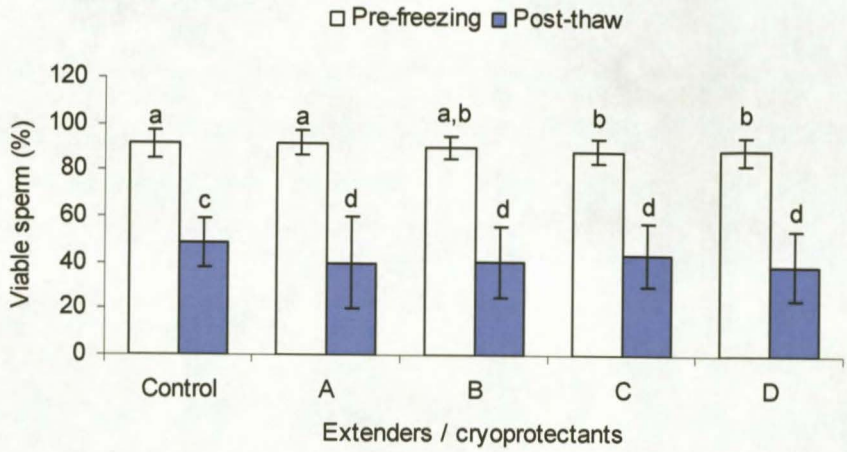

Fig. 2.5 Pre-freezing and post-thaw sperm viability for spermatozoa equilibrated at 5 °C for 2 h.

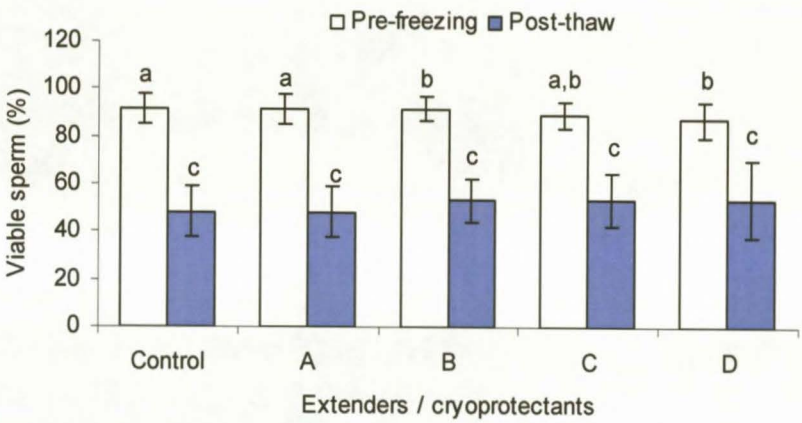

Fig. 2.6 Pre-freezing and post-thaw sperm viability for spermatozoa equilibrated at 5 °C for 4 h.

Pre-freezing percentage viable sperm for treatments frozen without equilibration did not differ ($P > 0.05$) from the control (Fig. 2.4). Similar findings were observed in spermatozoa equilibrated for 2 h (Fig. 2.5 A & B), while differences ($P < 0.05$) were observed in 2 h equilibration in treatments MTL + EG and MTL + DMSO (Fig. 2.5 C & D). Pre-freezing viability of sperm equilibrated for 4 h was lower in almost all the treatments (Fig. 2.6 B, C & D) compared to the control (Fig. 2.6 A = control). A difference ($P < 0.05$) was only demonstrated between MTL + DMSO (Fig. 2.6 D) and the control. The lower values of pre-freezing viability tended to occur after 2 h (Fig. 2.5 B, C & D) and 4 h (Fig. 2.6 B, C & D), but not 0 h (Fig. 2.4) of equilibration, and were associated with MTL extenders C & D containing EG and DMSO, respectively. The mean sperm viability for 0 h (90.0 ± 1.7%), 2 h (89.9 ± 1.5%) and 4 h (90.0 ±

2.0%) of equilibration, and an overall mean for 0, 2 and 4 h (90.2 ± 1.7%) showed a minimal variation in the pre-freezing sperm viability. This indicated that addition of the permeating cryoprotectants at room temperature or at 5 °C in BIL or MTL had minimal instant effects on the viability of bovine epididymal spermatozoa.

Post-thaw sperm viability of spermatozoa frozen without equilibration was lower ($P < 0.0001$) when compared to the control (Fig. 2.4). Variations in post-thaw sperm viability associated with freezing medium and cryoprotectant used when freezing occurred without equilibration was also demonstrated in Fig. 2.4. Post-thaw viability was lower ($P < 0.0001$) when sperm was frozen without equilibration in BIL (Fig. 2.4 A) or MTL + GLY (Fig. 2.4 B) than in MTL + EG (Fig. 2.4 C) or MTL + DMSO (Fig. 2.4 D).

Post-thaw viability for spermatozoa frozen after 2 h of equilibration (Fig. 2.5) were lower ($P < 0.05$) than that of the control, but there were no differences ($P > 0.05$) between the treatments. Post-thaw viability of spermatozoa frozen after 4 h of equilibration (Fig. 2.6) did not differ between treatments and control. The post-thaw viability showed that the control provided better cryoprotection compared to the other extenders when cryopreservation occurred after 2 h (Fig. 2.5 A-D), but not 4 h (Fig. 2.6 B-D), of equilibration. Irrespective of the extender and cryoprotectant used, the average post-thaw viability was lowest when sperm was cryopreserved without equilibration (14.3 ± 6.4%), was intermediate when equilibrated for 2 h (40.7 ± 1.9%), and was highest when equilibrated for 4 h (52.4 ± 2.9%).

2.4.3 Sperm membrane integrity

Figures 2.7, 2.8 and 2.9 show pre-freezing and post-thaw sperm membrane integrity for spermatozoa equilibrated at 5 °C for 0, 2 and 4 h, respectively, and cryopreserved in extenders A (BIL), B (MTL + GLY), C (MTL + EG), and D (MTL + DMSO), with cryopreservation in BIL after 4 h of equilibration as the control. The series with different superscripts (a – i) are different ($P < 0.05$).

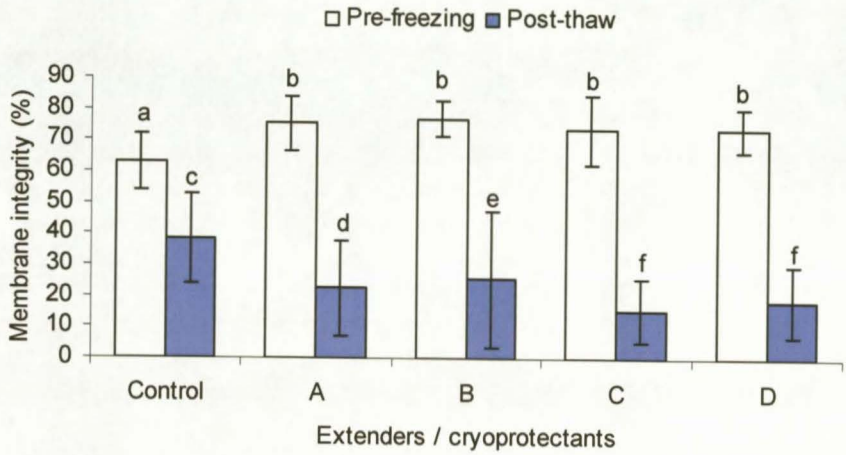

Fig. 2.7 Pre-freezing and post-thaw sperm membrane integrity of spermatozoa equilibrated at 5 °C for 0 h.

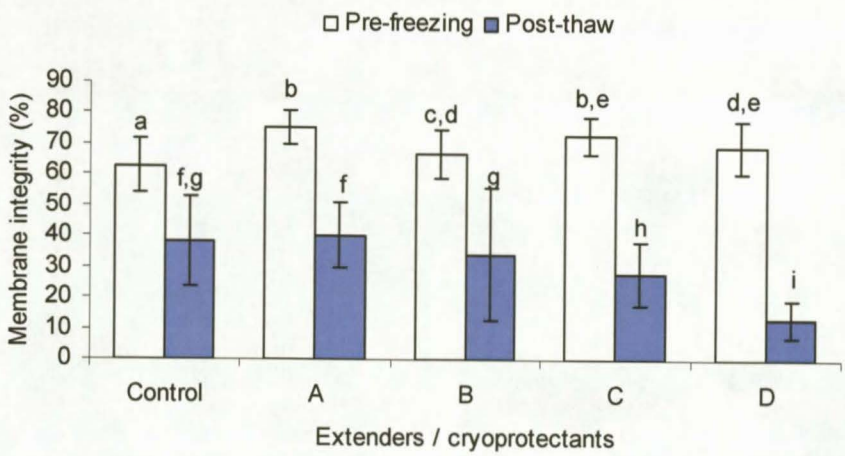

Fig. 2.8 Pre-freezing and post-thaw sperm membrane integrity for spermatozoa equilibrated at 5 °C for 2 h.

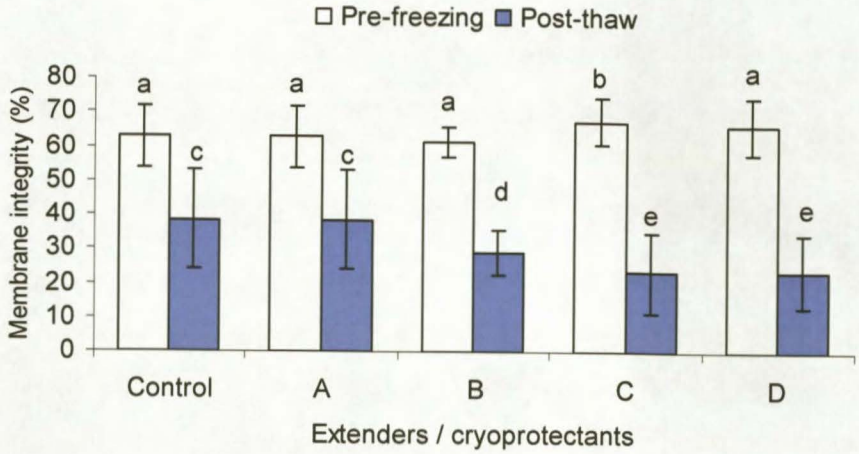

Fig. 2.9 Pre-freezing and post thaw sperm membrane integrity for spermatozoa equilibrated at 5 °C for 4 h.

Pre-freezing sperm membrane integrity for spermatozoa equilibrated for 0 h (Fig. 2.7) were higher ($P < 0.05$) than that of the control, but no differences ($P > 0.05$) were found between the treatments groups (Fig. 2.7 A-D). There were variations in the pre-freezing sperm membrane integrity for spermatozoa equilibrated for 2 h (Fig. 2.8) and 4 h (Fig. 2.9). The pre-freezing sperm membrane integrity for a 2 h equilibration (Fig. 2.8) was higher ($P < 0.05$) than for the control.

There was no difference ($P > 0.05$) in pre-freezing sperm membrane integrity did not differ between the control and equilibration period of 4 h for treatments MTL + GLY (Fig. 2.9 B), and MTL + DMSO (Fig. 2.9 D). Equilibration for 4 h for treatment MTL + EG (Fig. 2.9 C) showed a higher ($P < 0.05$) pre-freezing membrane integrity than the control (Fig. 2.9 C). The similarity in the mean pre-freezing normal sperm membrane integrity after equilibration for 0 h (74.9 ± 1.7%), 2 h (71.2 ± 3.7%), and 4 h (64.5 ± 2.9%) to the overall mean for 0, 2 and 4 h equilibration (70.2 ± 5.2%) demonstrated a minimal variation in membrane integrity.

Post-thaw sperm membrane integrity was highly variable for the three equilibration periods (Figs. 2.7-2.9). Equilibration for 0 h (Fig. 2.7) in all treatments showed a lower ($P < 0.0001$) post-thaw sperm membrane integrity than the control. However, post-thaw membrane integrity of spermatozoa frozen in MTL + GLY (Fig. 2.7 B) after 0 h of equilibration, was higher ($P < 0.05$) than that of spermatozoa frozen in BIL (Fig. A), and also higher ($P < 0.0001$) than that of spermatozoa frozen in MTL + EG (Fig. 2.7 C) and MTL + DMSO (Fig. 2.7 D).

Post-thaw membrane integrity of spermatozoa equilibrated for 2 h and frozen in BIL (Fig. 2.8 A) and in MTL + GLY (Fig. 2.8 B) did not differ ($P > 0.05$) from the control, but were different from each other ($P < 0.05$). Spermatozoa equilibration for 2 h and frozen in MTL + DMSO (Fig. 2.8 D) showed the lowest post-thaw sperm membrane integrity ($P < 0.0001$), when compared to the rest of the treatments and the control.

Post-thaw normal membrane integrity for 4 h equilibration (Fig. 2.9) were lower ($P < 0.0001$) than that of the control. There was no difference ($P > 0.05$) in post-thaw normal membrane integrity of spermatozoa equilibrated for 4 h and frozen in MTL + EG (Fig. 2.9 C) and MTL + DMSO (Fig. 2.9 D). However, equilibration for 4 h and freezing in these extenders (Fig. 2.9 C & D) resulted in a lower post-thaw membrane integrity ($P < 0.05$), than equilibration for 4 h and freezing in MTL + GLY (Fig. 2.9 B). Post-thaw sperm membrane integrity following equilibration for 2 h (29.0 ± 11.5%) and 4 h (28.7 ± 7.0%) were not different but higher than equilibration for 0 h (20.7 ± 4.4%).

2.4.4 Sperm acrosome integrity

Figures 2.10, 2.11 and 2.12 show pre-freezing and post-thaw sperm acrosome integrity for spermatozoa equilibrated at 5 °C for 0, 2 and 4 h, respectively, and cryopreserved in extenders A (BIL), B (MTL + GLY), C (MTL + EG), and D (MTL + DMSO), with cryopreservation in BIL after 4 h of equilibration as the control. The series with different superscripts (a – g) are different ($P < 0.05$).

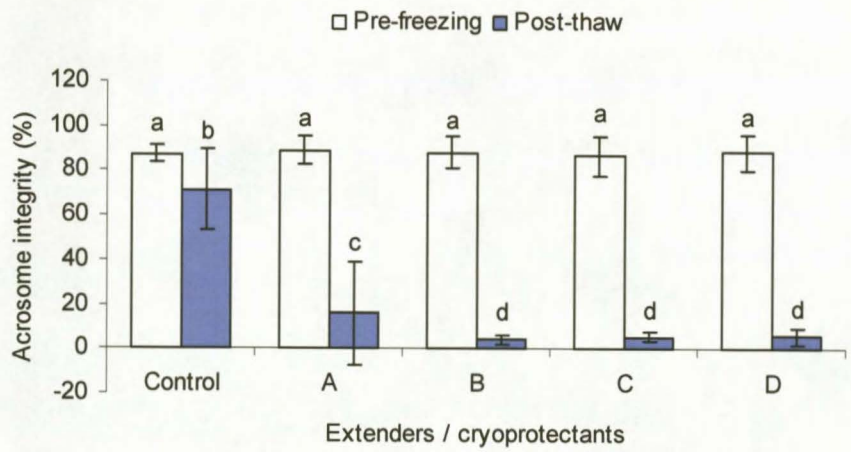

Fig. 2.10 Pre-freezing and post-thaw sperm acrosome integrity for spermatozoa equilibrated at 5 °C for 0 h.

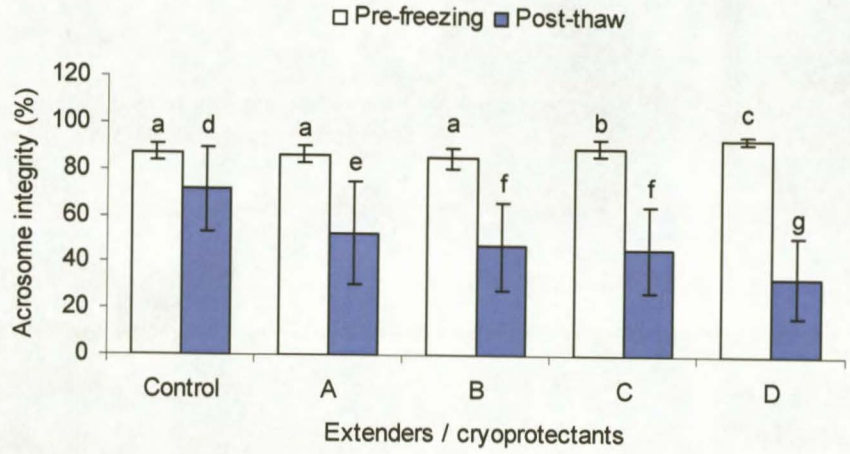

Fig. 2.11 Pre-freezing and post-thaw sperm acrosome integrity for spermatozoa equilibrated at 5 °C for 2 h.

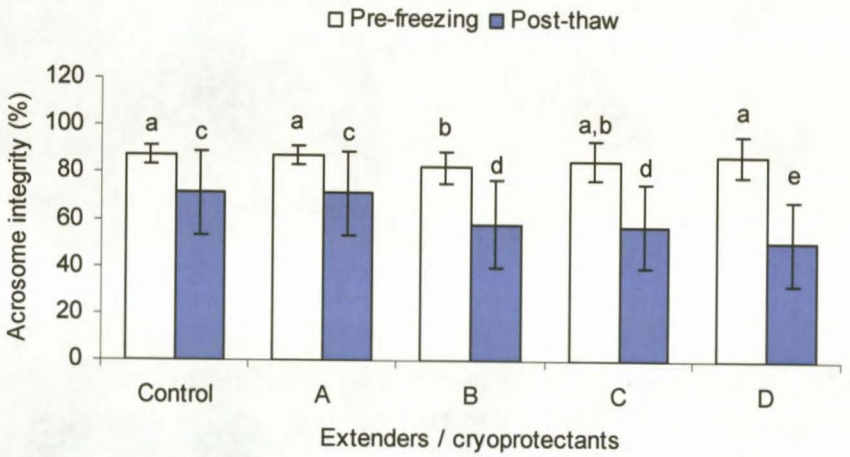

Fig. 2.12 Pre-freezing and post-thaw sperm acrosome integrity for spermatozoa equilibrated at 5 °C for 4 h.

The pre-freezing evaluation for acrosome integrity (Fig. 2.10) showed no differences ($P > 0.05$) between the control and all treatments equilibrated for 0 h. Equilibration of spermatozoa for 2 h in treatments BIL (Fig. 2.11 A) and MTL +GLY (Fig. 2.11 B) resulted in pre-freezing acrosome integrity not different ($P > 0.05$) from that of the control. Equilibration for 2 h in treatment MTL + DMSO (Fig. 2.11 D) showed a higher ($P < 0.0001$) pre-freezing sperm membrane integrity from the control and the rest of the treatments. Pre-freezing sperm membrane integrity of spermatozoa equilibrated for 4 h in treatments MTL + EG (Fig. 2.12 C) and MTL + DMSO (Fig. 2.12 D), did no differ ($P > 0.05$) from the control. However, equilibration for 4 h in treatment MTL + GLY (Fig. 2.12 B) resulted in a higher extent of acrosomal damage ($P < 0.05$) than for the control and other treatments. Average normal sperm acrosome integrity for equilibration for 0 h (88.1 ± 1.0%), 2 h (88.7 ± 3.8%) and 4 h (85.6 ± 2.3%), and overall

acrosome integrity for 0, 2 and 4 h equilibration (87.4 ± 2.8%) indicated a minimal variation in pre-freezing sperm acrosome integrity.

Irrespective of the duration of equilibration (0, 2 or 4 h), the post-thaw sperm acrosome integrity were lower ($P < 0.05$) than that of the control (Figs. 2.10-2.12). The lowest ($P < 0.0001$) post-thaw acrosome integrity was observed when sperm was frozen without equilibration (Fig. 2.10). Freezing sperm after equilibration for 2 h (Fig. 2.11) and 4 h (Fig. 2.12) did not differ in post-thaw acrosomal integrity. Freezing in MTL + DMSO after equilibration for 2 h (Fig. 2.11 D) and 4 h (Fig. 2.12 D) resulted in the lowest post-thaw sperm acrosome integrity ($P < 0.0001$), compared with the control and the rest of the treatments. There was no difference ($P > 0.05$) in post-thaw acrosomal integrity when sperm was equilibrated for 2 h and cryopreserved in MTL + GLY and MTL + EG (Fig. 2.11 B & C). Similarly, no difference ($P > 0.05$) was observed between treatments MTL + GLY and MTL + EG where sperm was equilibrated for 4 h (Fig. 2.12 B and Fig. 2.12 C). However, the post-thaw acrosomal integrity for these treatments were lower ($P < 0.05$) than that of the control. The highest post-thaw acrosome integrity was observed in spermatozoa equilibrated in BIL for 4 h. This was further shown by the mean post-thaw acrosome integrity for 4 h (59.4 ± 7.8%), 2 h (40.5 ± 7.8%) and 0 h (8.0 ± 5.4%) of equilibration.

2.5 Discussion

There were minimal variations between the treatment groups in the pre-freezing sperm parameters. Final dilution of spermatozoa at room temperature without equilibration showed no differences ($P > 0.05$) in sperm motility, sperm viability and acrosome integrity between the treatments and the control. Normal sperm membrane values following final dilution at room temperature did not differ ($P > 0.05$) between the treatments, but were higher ($P < 0.0001$) than that of the control (Fig. 2.7). These findings showed that MTL medium supplemented with 20% foetal bovine serum (FBS) supported the viability of spermatozoa at room temperature as much as Biladyl® (BIL). Sperm Tyrode's lactate medium prepared as described by Parrish *et al.* (1985) is widely used in processing spermatozoa in assisted reproductive biotechnologies such as *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI). The salt solutes in MTL are similar to the salt/sugar solutes in BIL thus provided a buffered medium for the spermatozoa. This finding is in agreement with Amann (1999) that a semen extender may be a simple buffered salts solution containing sugars such as fructose, glucose or lactose.

The egg yolk added to BIL is a complex protective macromolecular additive (Bousseau *et al.*, 1998) acting as a non-permeating cryoprotectant during freezing (Amann, 1999). Similar cryoprotection is probably provided by serum in MTL, with the latter being widely used in the cryopreservation of oocytes and embryos in many studies for example Van Wagtendonk-de Leeuw *et al.* (1997) and Dinnyés *et al.* (2000). The role of these compounds at room temperature is probably to stabilize the cell membranes, as it does during equilibration. Non-permeating cryoprotectants are known to stabilize cell proteins (Arakawa *et al.*, 1990; De Leeuw *et al.*, 1993) and reduce cell damage by reducing the toxic effects of other cryoprotectants (Arav, 1992). They may also provide metal-binding proteins that act as chelating agents (Goyer *et al.*, 1995) that can remove toxic metal ions from the media, thus providing a more suitable environment for the spermatozoa.

The current study also indicated that the concentration of 0.95 M for the permeating cryoprotectants used in this study is not instantly toxic to the sperm cells at room temperature. According to Bousseau *et al.* (1998), most extenders contain suitable permeating cryoprotectants at concentrations varying from 0.2-1.5 M, a concentration within these values was used in this study. The findings in the present study further agreed with a study by Silva *et al.* (2006) who observed no differences in progressive motility, morphology, acrosome integrity and membrane integrity in dog sperm when glycerol was added at room temperature (27 °C) and at 4 °C. The current study used three permeating cryoprotectants i.e. glycerol (GLY), ethylene glycol (EG) and dimethyl sulfoxide (DMSO). Cryoprotectants, especially permeating cryoprotectants, cause osmotic changes resulting in irreversible shifts in membrane structure, denaturation of proteins, disassembly of internal structures within the axoneme affecting sperm motility and may result in sperm death (Amann, 1999). The toxicity of permeating cryoprotectants to cells is known to depend on concentration, time and temperature of exposure (Arav, 1992). In the current study, freezing immediately after final extension must have prevented cryoprotectant toxicity that could have occurred at room temperature if exposure time was extended. This supports the findings of Rall (1987), who reported that a reduction in the time of exposure to cryoprotectants reduced cryoprotectant toxicity.

The final dilution of spermatozoa at room temperature did not affect ($P > 0.05$) sperm membrane integrity for the treatments, but resulted in higher ($P < 0.0001$) normal sperm membrane integrity in the treatments than the control (Fig. 2.7). This finding indicated that, like other sperm parameters studied, the sperm membrane integrity was not instantly affected by the addition of the cryoprotectants at room temperature. On the other hand, the finding showed that sperm membrane integrity is sensitive to low temperatures such as that of equilibration (5 °C).

The equilibration of the control at 5 °C for 4 h could have caused chilling injury (Levitt, 1980), thus compromising sperm's membrane integrity (Ruffing *et al.*, 1993; Arav *et al.*, 1996). The higher ($P < 0.0001$) pre-freezing sperm membrane integrity in the treatments than the control (Fig. 2.7) could not be attributed to cryoprotectant toxicity, because addition of cryoprotectants in the control occurred at low temperature (5 °C) and would unlikely result in toxicity (Arav, 1992). Paulenz *et al.* (2002) reported that sperm membrane damage in processed spermatozoa is mainly due to changes caused by handling, either during lowering of temperature (especially rapid cooling from 20 °C to 5 °C, resulting in cold shock injuries) and/or by aging of spermatozoa during storage. However, handling before final extension, for instance centrifugation, apparently did not affect the sperm membrane in this study.

Although there was no variation in pre-freezing sperm motility after 2 and 4 h of equilibration (Fig. 2.2 and Fig. 2.3), differences were noted in pre-freezing sperm viability, and normal membrane and normal acrosome integrity when equilibrated for the same duration at 5 °C. Chilling or cold shock injury could have been responsible for these pre-freezing variations, though duration of equilibration did not seem to influence these parameters. This agreed with the report that cooling from 20 °C to 5 °C results in cold shock injuries (Paulenz *et al.*, 2002) that affects cellular structures or alters cell functions (Amann, 1999). Exposure of cells to temperatures between plus 30 °C and 0 °C cause cell injury by compromising the membrane integrity, cell metabolism, the cytoskeleton and the capacity of the cell to control and repair damage e.g. by free oxygen radicals (Ruffing *et al.*, 1993; Arav *et al.*, 1996). The cryo-injury could have been due to direct chilling injury (DCI) which is expressed soon after cooling and may result from lipid phase-transition on the cell membrane, as was demonstrated in pig sperm (Drobins *et al.*, 1993). However, the effects due to cold shock were minimal for treatments equilibrated at 5 °C for 2 and 4 h because the cooling rate was slow. According to Amann (1999), slow cooling minimizes the damages caused by cold shock.

All post-thaw parameters were lower ($P < 0.0001$) than pre-freezing parameters. Freezing without equilibration (Figs. 2.1, 2.4, 2.7 & 2.10) was most damaging to spermatozoa, while equilibration for 4 h (Figs. 2.3, 2.6, 2.9 & 2.12) produced the best results and 2 h equilibration (Figs. 2.2, 2.5, 2.8 & 2.11) produced intermediate results. The low values of post-thaw sperm parameters following freezing without equilibration could have been due to rapid cooling. These treatments were rapidly cooled in liquid nitrogen vapor (5 cm above liquid nitrogen) from room temperature (24 °C) to −80 °C in 10 min. Rapid cooling is known to cause chilling or cold shock damage (Paulenz *et al.*, 2002). This injury, especially DCI, compromises the membrane integrity, cell metabolism, the cytoskeleton and the cell's capacity to control and

repair damage e.g. by free oxygen radicals (Ruffing *et al.*, 1993; Arav *et al.*, 1996). Extension at room temperature and freezing immediately probably did not give adequate time for the additive macromolecules to stabilize and cryoprotect the sperm membranes during freezing and thawing.

The extensive cryo-damage in spermatozoa frozen without equilibration (Figs. 2.1, 2.4, 2.7 and 2.10) could not have been due to cryoprotectant toxicity as has been explained above. It is known that the effects of cryoprotectants depend on concentration, time and temperature (Arav, 1992). However, in the treatments frozen without equilibration, despite exposure of spermatozoa to cryoprotectants at room temperature, exposure time was shortened by freezing immediately after adding the permeating cryoprotectant-containing portion of the extender. In addition, the cryoprotectant-containing portions of the extenders were added just before freezing in all other treatments as well, including treatments equilibrated for 2 h and 4 h. According to Hafez (1993), the cryoprotectant-containing portion of the extender can be added just before freezing to provide just as much cryoprotection to the spermatozoa. This further confirms that cell injuries that were seen in the post-thaw spermatozoa of treatments equilibrated for 0 h (Figs. 2.1, 2.4, 2.7 and 2.10) were due to cold shock during rapid cooling from room temperature to 0 °C in liquid nitrogen vapor, freezing in liquid nitrogen vapor to −80 °C and in liquid nitrogen to −196 ºC, and during thawing to 37 °C.

Post-thaw progressive motility, viability, and membrane and acrosome integrity in treatments equilibrated for 2h (Figs. 2.2, 2.5, 2.8 & 2.11, respectively) were lower ($P < 0.05$), when compared to the pre-freezing values of the same parameters in treatments equilibrated for 4 h (Figs. 2.3, 2.6, 2.9, & 2.12, respectively). These differences were also unlikely due to the cryoprotectant toxicity for two reasons. First, there were minimal pre-freezing variations in these parameters when extension was done at room temperature and the values were not different from those equilibrated for 2 or 4 h. Secondly, the cryoprotectant-containing portion of the extender was added at a low temperature (5 °C). The cryoprotectant injuries to sperm are minimal at low temperatures as these effects are temperature dependent (Arav, 1992). In the current study GLY, EG and DMSO were used as permeating cryoprotectants in MTL at the same molar concentration (0.95 M). The fact that cryoprotectant injuries to the sperm cells during the pre-freezing evaluation were minimal at both room temperature and 5 °C showed that these concentrations were acceptable for the freezing of bovine epididymal spermatozoa. This is because cell injuries due to cryoprotectants are also known to be concentration dependent (Arav, 1992).

The two extenders, tris-based (BIL) and HEPES-buffered saline (MTL) and the presence of the non-permeating macro-molecules from egg yolk and serum, respectively, seem to have had beneficial effects on bovine epididymal spermatozoa both at room temperature and cooling to 5 °C. The macro-molecules must have contributed to the minimal pre-freezing variations that occurred in treatments not equilibrated and those equilibrated for 2 and 4 h. In a study by De Leeuw *et al.* (1993), excluding egg yolk from the extender caused a serious decrease of sperm survival after both cooling and freezing. However, in the same study, the extender containing 6.5 mM dioleoylphosphatidylcholine (DOPC) vesicles in combination with 6% BSA yielded results which did not significantly differ from those obtained with the standard extender.

According to Bergeron *et al.* (2004), the beneficial effects of egg yolk during sperm preservation is derived from its low-density lipoprotein fraction (LDF). In ejaculated semen, the LDF binds with the seminal plasma (SP) protein that would otherwise bind to the sperm surface, causing cholesterol and phospholipids efflux from the sperm membrane, and eventual destabilization of the membrane (Manjunath *et al.*, 2002; Bergeron *et al.*, 2004). The binding of LDF to the SP proteins prevents lipid efflux from sperm membrane thereby stabilizing the membrane during equilibration, cryopreservation and post-thaw, which preserve sperm function. However, Cookson *et al.* (1984) demonstrated that lipoprotein bound firmly to spermatozoa and could not be removed by extensive washing, suggesting that the lipoprotein had become irreversibly associated with the sperm membranes. Possibly, the irreversible binding also prevents sperm membrane lipid efflux, and in this way the epididymal sperm that is not exposed to the SP protein is protected by the LDF during equilibration, cryopreservation and post-thaw. In a study by Chen *et al.* (1993b) it was found that up to 10% (v/v) of heat-inactivated blood serum was generally beneficial to bull sperm, but gave more variable responses with different bulls. The current study used 20% FBS v/v in MTL. The beneficial effects of the compounds known to protect sperm, including BSA, appear to result from protecting sperm from lipid peroxidation (Foote *et al.*, 1993). A similar beneficial effect to spermatozoa could be provided by FBS used in this study, as serum is reported to contain lipoproteins such as LDF (Havel *et al.*, 1955) as in the egg yolk. These lipoproteins in serum are, therefore, likely to cryoprotect sperm in a similar way as the LDF in egg yolk.

The MTL extender that contained serum also contained an energy substrate, sodium pyruvate (0.2 mM). In study of freezing sea bass (*Dicetrarchus labrax*) semen, Sansone *et al.* (2002) found that an extender consisting of a diluent, sodium pyruvate and 10% EG yielded the best post-thaw results when the extended semen was equilibrated for 6 h at 0-2 °C. In the current study, the role of sodium pyruvate in the performance of MTL in protecting sperm

fertilizing ability parameters before freezing could also be associated with its role as an energy substrate for sperm metabolism.

The post-thaw evaluation in all treatments showed a decrease in sperm motility (Figs. 2.1-2.3), sperm viability (Figs. 2.4-2.6), normal membrane integrity (Figs. 2.7-2.9), and normal acrosome integrity (Figs. 2.10-2.12) compared with pre-freezing evaluation. This agreed with a study by Lambrechts *et al.* (1999) in which cryopreservation significantly reduced the viability and motility of buffalo epididymal spermatozoa, an effect that was largely attributed to the thermal equilibration prior to cryopreservation. It further agreed with other studies by Henry *et al.* (1993), Willoughby *et al.* (1996) and Woods *et al.* (2004) that demonstrated the adverse effects of cryopreservation on various aspects of sperm structure.

The lower ($P < 0.0001$) progressive sperm motility in post-thaw than pre-freezing evaluation (Figs. 2.1-2.3) could be associated with the report by Henry *et al.* (1993) that sperm motility is particularly sensitive to cryopreservation. These authors suggested that motility, membrane integrity and mitochondrial function are similarly affected by freeze-thawing. The effects on motility seen in this study could have been mediated through damage to the mitochondrial activity (O'Connell *et al.*, 2002) and destruction of the axoneme (Amann, 1999). It is known that the energy for sperm propulsion is derived from metabolism that occurs in the mitochondria of sperm (Garner and Hafez, 1993), with the axoneme transforming this energy into the propulsive force (Amann, 1999) seen as sperm motility.

The most significant differences ($P < 0.0001$) between pre-freezing and post-thaw parameters were noted for all treatments equilibrated for 0 h (Figs. 2.1, 2.4, 2.7 and 2.10). A similar report was given by Vannucchi *et al.* (1999) from a study using dog semen extended in tris-fructose-citric acid extender with 7.5% EG and frozen without cooling. Similarly this produced the lowest progressive motility, forward velocity and the highest morphological abnormalities post-thaw, compared with the methods that involved stepwise equilibration. The extensive drop in sperm parameters occurred when equilibration time was 0 h, because rapid cooling is extremely dangerous to the sperm considering that cryopreservation affects all aspects of the sperm structure (Woods *et al.*, 2004). In earlier studies by Henry *et al.* (1993) and Willoughby *et al.* (1996), cytological evaluation revealed extensive damage to the plasma membrane, acrosomal region and tail configuration following such cooling. It's not known if stepwise freezing without equilibration, starting with higher temperature such as −20 °C down to −80 °C before plunging into liquid nitrogen could lead to improved post-thaw sperm survival. This could shorten the time spent in equilibration for 2 or 4 h.

Post-thaw sperm motility, and normal membrane and acrosome integrity of spermatozoa frozen in MTL + DMSO after equilibration for 2 h (Figs. 2.2 D, 2.8 D, 2.11 D) and 4 h (Figs. 2.3 D, 2.9 D, 2.12 D) were significantly lower ($P < 0.05$) than that of the other treatments and control. These showed that DMSO severely affected spermatozoa or failed to cryoprotect the sperm from cryo-injuries. Probably such effects are expressed post-thaw as it was insignificant in the pre-freezing evaluation. This could be because DMSO is more toxic than GLY as it crosses cell membrane faster (Amann, 1999). Amann (1999) also reported that permeating cryoprotectants cause irreversible changes in membrane structure, denaturation of proteins, and disassembly of internal structures within the axoneme, which affect sperm motility and may result in sperm death. One method through which permeating cryoprotectants protect cells from cryo-injury is by stabilizing cell proteins of organelles (Arakawa *et al.*, 1990) such as the mitochondrial membrane and the axoneme proteins. Apparently, DMSO did not adequately achieve this, resulting in cryo-damage to the mitochondria and the axoneme, thus reducing post-thaw sperm motility of spermatozoa frozen in MTL + DMSO after 2 and 4 h of equilibration. However, EG also crosses the sperm membrane faster than GLY, but showed post-thaw motility results for 2 h and 4 h of equilibration comparable to GLY. Similarly, in an earlier study by Alvarenga *et al.* (2000) GLY and EG were found to provide similar cryoprotective properties to stallion spermatozoa.

There were minimal variations in pre-freezing percentage viable spermatozoa following equilibration at 5 °C for 0 h (Fig. 2.4), 2 h (Fig. 2.5) and 4 h (Fig. 2.6). However, the post-thaw percentage viable sperm were significant lower ($P < 0.05$) than the pre-freezing values. The reduction was most significant ($P < 0.0001$) in treatments equilibrated for 0 h compared with 2 and 4 h. This finding agreed with the observations that though cryopreservation attempts to ensure sperm survival, it imposes irreversible damage to the sperm that may cause cell death (Hammersted *et al.*, 1990) or capacitation-like changes in the plasma membrane (Cormier *et al.*, 1997). This resulted from exposure of cells to thermal, chemical and mechanical constraints that negatively affected the biological function of cells, referred to as cryo-injury. The cryo-injury is explained by the thermodynamics and structural properties of the sperm membrane (Holt, 2000). It's known that the sperm membrane contains an unusual array of lipids (Parks *et al.*, 1987; Lin *et al.*, 1993) and that the plasma membrane is arranged into different domains (Holt and North, 1984). According to Amann (1999), each type of lipid in the membrane has a phase-transition temperature at which it changes from a liquid-crystalline state (liquid) to a crystalline-array state (gel). Proper function of the membrane requires a fluid membrane, and at body temperature all the lipid molecules in the membrane are in liquid-crystalline state. The sperm membrane lipids respond to temperature changes by alterations in their physical phase state

(Holt, 2000) which leads to damage of the membrane. Decreasing membrane temperature below the phase-transition of a particular lipid type leads to the molecules of that type of lipid (but not others) to aggregate into microdomains of lipid gel (Amann, 1999). This causes removal of molecules of this type of lipid from associations with proteins or other lipids, and formation of gaps between microdomains of lipid gel and fluid portions of the membrane, subsequently facilitating membrane rupture or fusion with other membranes.

Plasma membrane and acrosomal integrity are crucial to sperm fertilizing ability and the negative impact of cooling and cryopreservation on the fertilizing ability of sperm is mediated largely through damage of these structures (Parks and Graham, 1992). Sperm cells are protected from cryo-injury during the cryopreservation process (cooling, freezing and thawing) by cryoprotectants. In this study, the cryoprotectants used were permeating cryoprotectants (glycerol, ethylene glycol and dimethyl sulfoxide) and the non-permeating large molecules from egg yolk and serum. It is known that extracellular reagents (cryoprotectants) increase osmotic pressure of the medium, thereby inflicting damage to the cells (Curry and Watson, 1994; Liu and Foote, 1998). The increased intracellular and extra-cellular solute concentration known as "solution effect" irreversibly damages the membrane and internal structures causing loss of sperm function (Amann, 1999). "Solution effect" could have been responsible for the low post-thaw percentage of spermatozoa with normal membranes in both equilibrated and non-equilibrated treatments.

Cold shock must have contributed markedly to the poor post-thaw percentage of spermatozoa with normal membrane integrity in treatments equilibrated for 0 h (Fig. 2.7), and to some extent to the low post-thaw percentage in treatments equilibrated for 2 h (Fig. 2.8) and 4 h (Fig. 2.9). Cold shock, a damage due to rapid cooling to 0 °C, is also known to result from lipid phase-transition effects (Drobins *et al.*, 1993). As previously explained, cold shock affects the sperm membrane integrity that manifests during post-thaw period (Holt *et al.*, 1992). The rapid cooling from room temperature (24 °C) to −80 °C in liquid nitrogen vapour must have been responsible for the extremely low percentage of spermatozoa with normal membranes in treatments equilibrated for 0 h (Fig. 2.7). The slow cooling at 5 °C improved the percentage of spermatozoa with normal membrane integrity in treatments equilibrated for 2 h (Fig. 2.8) and 4 h (Fig. 2.9). This is in agreement with Amann (1999), who reported that slow cooling minimizes the damages caused by cold shock.

Another mechanism by which permeating cryoprotectants protect cells from cryo-injury is by the prevention of intracellular ice formation by permeating or dehydrating the cells (Karlsson

and Toner, 1996). The absence of an equilibration period and the rapid cooling in treatments equilibrated for 0 h (Fig. 2.4) might have resulted into formation of intracellular ice that is lethal to spermatozoa, explaining the very low post-thaw percentage of viable sperm. In these treatments, the cooling from room temperature (24 °C) to −80 °C in liquid nitrogen vapour was rapid enough to cause severe damage to the spermatozoa. According to Henry *et al.* (1993) the effects of cryo-injury on the overall sperm function including mitochondria are similar. The role of mitochondria in initiating cell death by apoptosis has been reported by Dinsdale *et al.* (1998) and Sun *et al.* (1999). This could have been responsible for the low ($P < 0.0001$) percentage of viable sperm in post-thaw spermatozoa frozen without equilibration (Fig. 2.4) as compared with 2 h (Fig. 2.5) and 4 h (Fig. 2.6) equilibration.

Cryoprotectants protect cells by reducing the "solution effect" (Karlsson and Toner, 1996), and stabilizing cell proteins (Arakawa *et al.*, 1990) and plasma membranes (Anchordoguy *et al.*, 1991), but these seem not to occur or is poor if freezing is done without equilibration (Figs. 2.1, 2.4, 2.7 & 2.10). Besides the osmotic activity, the toxic effects of cryoprotectants could have been responsible for the low levels of post-thaw survival following freezing without equilibration. This could be so if such effects were only expressed post-thaw as they were not demonstrated in the pre-freezing evaluation. Osmotic activities of cell membrane (Phelps *et al.*, 1999) and toxic effects of cryoprotectants (Arav, 1992) are pronounced at high temperature such as thawing temperature. Straws were thawed at 37 °C, a temperature higher than the room temperature (24 °C) at which complete extension of sperm frozen without equilibration was performed.

The ability of the cryoprotectants to stabilize the sperm membranes (Anchordoguy *et al.*, 1991) seem to have been compromised by the absence of equilibration. Consequently, sperm membrane integrity as evaluated by HOST was damaged ($P < 0.0001$) in post-thaw compared with pre-frozen spermatozoa without equilibration. Sperm membrane damage in processed spermatozoa is mainly due to changes caused by handling, either during lowering of temperature (especially rapid cooling from 20 to 5 °C, resulting in cold shock injuries) and/or by aging of spermatozoa during storage (Paulenz *et al.*, 2002). Cold shock is probably related to the phase-transition of membrane lipids, which result in phase separation and loss of selective permeability (Watson, 1981b). Compared with equilibration at 5 °C for 2 and 4 h, freezing at −80 °C in liquid nitrogen vapor without equilibration must have caused more severe cold shock and damage to sperm membrane. Consequently, normal membrane integrity was significantly lower ($P < 0.0001$) in post-thaw than pre-frozen, and in non-equilibrated than equilibrated spermatozoa.

At high temperatures, such as room temperature, and due to absence of equilibration, cryoprotectants are also likely to cause damage to and/or fail to cryoprotect the acrosome during freezing. In treatments where freezing was done without equilibration (Fig. 2.10), the percentage of sperm with normal acrosome was extremely low ($P < 0.0001$) in post-thaw as compared with pre-frozen spermatozoa. It is known that the negative impact of cooling and cryopreservation on fertility of sperm is mediated largely through damage of plasma membrane and acrosomal integrity (Parks and Graham, 1992). Cryopreservation causes irreversible damage to the sperm membranes that may cause cell death (Hammersted *et al.*, 1990) or capacitation-like changes in the plasmalemma (Cormier *et al.*, 1997). These would indicate that the fertilizing ability of spermatozoa frozen without equilibration irrespective of the extender used is greatly compromised unless biotechnology such as ICSI is employed.

Although all the post-thaw viability parameters examined were reduced ($P < 0.05$) for all the treatments equilibrated for 2 h (Figs. 2.2, 2.5, 2.8 & 2.11) and 4 h (Figs. 2.3, 2.6, 2.9 & 2.12) sperm survival was best achieved when BIL was used, especially, after 4 h of equilibration (control). In most mammalian species, a high proportion of spermatozoa lose their motility or other functions after thawing, with only about 50% of spermatozoa surviving the freeze-thaw process (Watson, 2000). During equilibration in these treatments, the temperature was reduced from room temperature (24 °C) to 5 °C, and according to Paulenz *et al.* (2002) this process could have caused some degree of cold shock damage to the spermatozoa. However, due to the presence of the non-permeating cryoprotectants during equilibration, and cooling for 2 and 4 h, the cold shock damage could have been minimal (Amann, 1999). Thus, the level of cryo-survival of spermatozoa was higher for both 2 and 4 h of equilibration compared with 0 h of equilibration that was cooled from 24 °C to −80 °C in 10 min before plunging into liquid nitrogen.

There were significant differences ($P < 0.05$) in the post-thaw normal membrane integrity and sperm acrosome integrity between the control and all treatments equilibrated for 2 h (Fig. 2.8; Fig. 2.11) and 4 h (Fig. 2.9; Fig. 2.12). However, post-thaw normal acrosome integrity of spermatozoa equilibrated for 2 h (Fig. 2.11 B & C) and 4 h (Fig. 2.12 B & C) and frozen in MTL + GLY and MTL + EG were not different ($P > 0.05$) from each other. Furthermore, the post-thaw sperm motility and viability of spermatozoa equilibrated for 2 h (Fig. 2.2 B & C; Fig. 2.5 B & C) and 4 h (Fig. 2.3 B & C; Fig. 2.6 B & C) and frozen in extenders containing GLY and EG were not significantly different ($P > 0.05$) from each other and the control. These findings showed that MTL + GLY and MTL + EG were comparable to each other and the control in cryoprotecting spermatozoa when freezing after 2 or 4 h of equilibration. The findings agreed with many reports that EG can be successfully used to freeze sperm from dog (Vannucchi *et al.*,

1999); stallion (Alvarenga *et al.*, 2000; Mantovani *et al.*, 2002); cynomogus monkey (Li *et al.*, 2005) and alpaca (Santiani *et al.*, 2005) with good results. The difference between extender A, and extenders B and C are that A is tris-based, and contains egg yolk and GLY, while B is MTL containing serum and GLY, and C is the same as B except that it has EG instead of GLY.

The performance of DMSO as a cryoprotectant after 2 and 4 h of equilibration was poorer than GLY and EG in all post-thaw parameters evaluated, except for percentage viable sperm (Fig. 2.5 D; Fig. 2.6 D) and normal membrane integrity (Fig. 2.9 D). This agreed with the findings of Li *et al.* (2005) who evaluated monkey (*Macaca fascicularis*) spermatozoa using sperm motility, plasma membrane and acrosomal integrity after freezing in different extenders. These authors concluded that the sperm cryopreservation properties of GLY and EG were similar and better than that of DMSO. However, in the same study it was reported that the mechanism of action of permeating cryoprotectants are not affected by extender composition which disagreed with the current study, because the control (tris-egg yolk-based extender) was better than MTL (HEPES-serum-based) extenders in cryoprotection. An earlier study by Alvarenga *et al.* (2000) concluded that EG at 10% v/v has similar cryoprotective properties as GLY as shown in the post-thaw examination of stallion spermatozoa for motility, and integrity of membrane and acrosome. The differences between the study by Alvarenga *et al.* (2000) and the current study are species, sources of spermatozoa, percentage of EG and the extenders used. Bovine sperm cryopreservation is mainly done in 7% GLY (0.95 M). This study used a similar concentration of GLY and the molar equivalent of EG (5.3%) and DMSO (6.8%). The results of the study showed that at a concentration of 0.95 M, GLY and EG can be used for cryopreservation of bovine epididymal sperm in MTL.

The extender MTL used in this study contained an energy substrate, sodium pyruvate (0.2 mM), while BIL contained the energy substrate, fructose (10 mg/mL). The cryoprotective effects of GLY and EG in MTL were similar. However, it was not known whether sodium pyruvate contributed to the similar post-thaw parameters in spermatozoa equilibrated for 2 and 4 h and frozen in extenders B and C that contained sodium pyruvate. A study by Sansone *et al.* (2002) on sea bass (*Dicetrarchus labrax*) semen found that the extender consisting of diluent, sodium pyruvate and 10% EG and equilibrated for 6 h at 0-2 °C gave the best post-thaw results. Chen *et al.* (1993b) reported that up to 10% v/v of heat treated blood serum was beneficial to sperm survival, but gave variable responses with different bulls after a freeze-thaw examination.

Extenders B, C and D basically consisted of MTL and 20% v/v FBS as a source of protein, while BIL contained 20% v/v egg yolk that is rich in lipoproteins and phospholipids.

Proteins and phospholipids are non-permeating cryoprotectants. Manjunath *et al.* (2002) and Bergeron *et al.* (2004) described the low-density lipoprotein fraction (LDF) as the non-permeating cryoprotectant in egg yolk. The LDF binds to and prevents lipid efflux from sperm membrane thereby stabilizing the membrane during equilibration, cryopreservation and post-thaw thus preserving sperm function. Though the non-permeating cryoprotectant in serum seems unidentified, serum contains lipoproteins including the LDF (Havel *et al.*, 1955). Because the cryopreservation efficiency of egg yolk and serum is not known and could be different, this could have contributed to the difference in post-thaw parameters shown between extender A and extenders B, C and D after 2 and 4 h of equilibration.

2.6 Conclusion

This study showed that bovine epididymal sperm can be cryopreserved in a serum-based, HEPES-buffered saline extender like modified Tyrode's lactose (MTL) with acceptable post-thaw sperm fertility parameters, but lower than that of Biladyl® (BIL). The post-thaw sperm fertilizing potential as measured by sperm motility, sperm viability, and sperm membrane and acrosome integrity, is dependent on the equilibration time and cryoprotectant used. Two and 4 h of equilibration are recommended, though 4 h is better, while 0 h of equilibration is not recommended. The cryoprotectants GLY and EG produced good results, while DMSO offers low cryoprotection at the concentration used. Although tris-egg yolk extenders with GLY such as BIL are very good in the cryopreservation of bull sperm, MTL (HEPES-buffered saline containing 20% v/v bovine serum) with GLY or EG produced promising results and the spermatozoa were easy to examine under the microscope because of the transparency. Further research on improving cryopreservation of sperm focusing on 0 h of equilibration using extenders that are easy to prepare and store at ambient temperature is recommended to ease sperm cryopreservation, especially for field conditions.

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CHAPTER 3

Cryopreservation of bovine epididymal spermatozoa: Effects of extender, cryoprotectant and equilibration time on *in vitro* embryo development

3.1 Abstract

Commonly mammalian sperm (usually ejaculate) cryopreserved in egg yolk- or milk-based extenders containing glycerol are evaluated based on morphological parameters that may not reflect the actual fertilizing ability of sperm. This study investigated the efficacy of modified Tyrode's lactose (MTL), a non-egg yolk-based extender, and a commercial egg yolk-based Biladyl® (BIL), cryoprotectants glycerol (GLY), ethylene glycol (EG) and dimethyl sulfoxide (DMSO), and 2 h and 4 h of equilibration in preserving the fertilizing ability of sperm, assessed by *in vitro* embryo production. Epididymal sperm collected by retrograde flushing was equilibrated at 5 °C for 2 or 4 h and cryopreserved in MTL supplemented with 20% foetal bovine serum (FBS) and 0.95 M GLY, EG or DMSO. Biladyl® was used as the control. *In vitro* matured bovine oocytes were subjected to a standard *in vitro* fertilization procedure with the frozen-thawed sperm. The presumptive embryos (n = 1632) and (n = 1754) for 2 and 4 h equilibration, respectively, were cultured in CR1aa supplemented with 3 mg/mL essentially fatty acid free bovine serum albumin (BSA-EFAF) and 5% FBS. Cleavage rates varied between the treatments and were significantly lower ($P < 0.05$) than that of the control. As embryo development progressed, differences between treatments decreased. However, embryos that resulted from sperm cryopreserved in DMSO maintained significantly lower ($P < 0.0001$) developmental rates than the other treatments and the control. Embryo development did not differ ($P > 0.05$) when sperm was equilibrated for 2 or 4 h and cryopreserved in BIL, while development was comparable when sperm equilibrated for 2 or 4 h in MTL was cryopreserved with GLY or EG and used for insemination. There was no difference in blastomere numbers of embryos of all treatments equilibrated for 4 h. Spermatozoa cryopreserved in MTL with 0.95 M GLY or EG, but not DMSO, produced embryos at rates comparable to Biladyl. Therefore, MTL with serum and GLY or EG successfully cryopreserved the fertilizing potential of bovine epididymal sperm, and the cryopreserved sperm can successfully be used for *in vitro* production of embryos.

3.2 Introduction

Cryopreservation of sperm has been most extensively practiced in cattle and most sperm cryopreservation protocols in other mammalian species use the cattle protocols with or without modifications. Since the first successful cryopreservation of sperm in liquid nitrogen using glycerol as cryoprotectant (Polge *et al.*, 1949), cryopreservation of spermatozoa has played a major role in animal production and conservation, as well as management of human infertility. To achieve this, cryopreservation is used in conjunction with assisted reproductive technologies (ARTs), notably artificial insemination (AI) and, recently, *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI). The major source of spermatozoa, particularly for

AI, has been ejaculated sperm. Of late, attention is given to epididymal sperm and its cryopreservation as one method of achieving the objectives of ART.

The ultimate goal in cryopreservation of a genetic material is preserving its viability after freeze-thawing. However, the process of cryopreservation and thawing has enormous effects on the viability of cells, including spermatozoa. Sperm damage during the freeze-thaw process is thought to be due to cold shock, osmotic stress, ice crystal formation (Mazur, 1984) and oxidative damage (Alvarez and Storey, 1992). Cooling spermatozoa to 0 °C and re-warming cause metabolic and structural changes that reduce the functional integrity of the sperm (Amann, 1999). In chickens, survival is extremely low with up to 98-99% of spermatozoa lost during freeze-thawing (Etches, 1996). Pig spermatozoa are extremely sensitive to cryopreservation as it severely reduces the fertilizing potential of pig sperm (Almlid and Johnson, 1987; Parks and Graham, 1992). In most mammalian species a high proportion of spermatozoa lose their motility and/or other functions after thawing, with only about 50% of spermatozoa surviving the freeze-thaw process (Watson, 2000). Generally, the proportion of fully functional sperm that retain intact membranes, acrosomes, tails and organelles after freeze-thawing is low (Holt, 1997).

Cryopreservation damages a sperm's membrane, acrosome, tail and organelles (Wooley and Richardson, 1978), and may stop the enzymatic and metabolic activity in a cell (Mazur, 1970). Perhaps, because sperm motility is particularly sensitive to cryopreservation damage (Henry *et al.*, 1993), sperm motility is frequently used as a measure of viability in many studies (Graham, 1994; Bezuidenhout *et al.*, 1995; Kundu *et al.*, 2000). However, attributes other than motility are required for sperm to have normal fertilizing potential. Thus frozen-thawed sperm with relatively high percentage of motility may not fertilize the oocytes (Amann, 1999). A major cause of reduced fertility of cryopreserved sperm, according to Watson (2000), is surface changes that affect recognition of oocyte receptors required during the sperm-zona binding. Theoretically, this causes clustering of membrane proteins that does not fully reverse upon thawing.

Apparently cryopreservation of undiluted semen with some success is possible only with human semen (Isachenko *et al.*, 2004). In other species, sperm is diluted in a freezing medium (extender) before cryopreservation. Freezing media widely vary between the classes (mammals, birds, and fish) and also between species within a class. An extender could be a simple buffered salts solution containing sugars such as fructose, glucose or lactose (0.5-2.0%), heated skim milk or a simple buffered salts solution containing 4-20% egg yolk (Amann, 1999).

Most extenders, therefore, feature a saline or saccharide bulk osmotic support, a suitable permeating cryoprotectant at concentrations varying from 0.2-1.5 M, and various protective macromolecular additives, mostly milk and egg yolk components, or lipid components from vegetal origin (Bousseau *et al.*, 1998). Kundu *et al.* (2000), for instance, froze goat cauda epididymal spermatozoa in modified Ringers's solution using glycerol (GLY), ethylene glycol (EG) and dimethyl sulfoxide (DMSO) as cryoprotectants. All these three permeating cryoprotectants showed a dose-dependent increase in motility recovery as well as a biphasic curve of cryoprotection. At optimal concentrations, DMSO (1.00 M) and EG (1.29 M) were effective in recovering sperm motility to the extent of 20% and 13%, respectively. These authors (Kundu *et al.*, 2000), however, concluded that these cryoprotectants have markedly lower cryoprotection potential than glycerol.

The type of cryoprotectant used varies widely between species, and sometimes within one species a cryoprotectant is successfully used in one study and is found to be unsuited in another study (Viveiros *et al.*, 2000). Glycerol is the preferred cryoprotectant for sperm of most mammalian species. Considerable attention has been given to other cryoprotectants, particularly EG and DMSO for cryopreservation of genetic materials of most mammals other than sperm, for instance cryopreservation of oocytes and embryos. However, in some species notably fish, amphibians and birds, these cryoprotectants are more commonly used for sperm cryopreservation than glycerol. The preference of DMSO for cryopreservation of bird sperm, though it is more toxic than glycerol (Etches, 1996), is because glycerol has a contraceptive property in birds (Hammerstedt and Graham, 1992). Meanwhile, DMSO is the most common cryoprotectant for fish and amphibian sperm; however, cryopreservation of carp sperm in sugar-based extenders with methanol cryoprotectant yielded better post-thaw results than ionic extenders with DMSO cryoprotectant (Horváth *et al.*, 2003). Post-thaw motility similar to that of fresh semen was obtained when sea bass (*Dicentrarchus labrax*) semen was cryopreserved in an extender containing EG as a cryoprotectant (Sansone *et al.*, 2002). So far in mammals, EG has been used to cryopreserve semen from dog (Vannucchi *et al.*, 1999); stallion (Alvarenga *et al.*, 2000; Mantovani *et al.*, 2002); cynomolgus monkey (Li *et al.*, 2005) and alpaca (Santiani *et al.*, 2005) with acceptable results. In a study by Vicente and Viudes-des-Castro (1996), cryopreservation of rabbit sperm in a sucrose-DMSO extender significantly improved the post-thaw motility and normal acrosome integrity compared to fresh semen.

The concentration of cryoprotectant and the type of extender used in cryopreservation of sperm also vary with animal species. In rabbits, Vicente and Viudes-des-Castro (1996) found that an extender with 0.5 M sucrose, 10% v/v egg yolk and 1.75 M DMSO significantly improved

the post-thaw motility of sperm. In goats Kundu *et al.* (2000) found that at their optimal concentrations, DMSO (1.00 M) and EG (1.29 M) recovered sperm motility of 20% and 13% post-thaw. However, DMSO and EG had markedly lower cryoprotection than 0.87 M GLY which resulted in a total motility recovery of 35%. Mantovani *et al.* (2002) recommended from their study that when cryopreserving stallion semen in skimmed milk, EG could substitute GLY if used at the same (3%) or lower concentration. A later study by Santiani *et al.* (2005) showed that skimmed milk- and egg yolk-based extenders containing EG or GLY for cryopreserving alpaca semen is superior to other extenders. Li *et al.* (2005) found that sperm-cryoprotective efficiencies of GLY and EG were similar and more effective than other cryoprotectants for cryopreserving cynomolgus monkey (*Macaca fascicularis*) spermatozoa in various extenders. Vannucchi *et al.* (1999) produced the best viability of canine spermatozoa when cryopreserved in tris-fructose-citric acid and 5% EG. These authors recommended that soybean lecithin-containing extender might be a better choice as a semen extender in the future.

Despite providing cryoprotection to cells during cryopreservation, cryoprotectants themselves cause osmotic and toxic stress to the cells including spermatozoa. If added too quickly before cryopreservation or removed too quickly upon thawing, cryoprotectants injure spermatozoa resulting in membrane damage due to shrinking and/or swelling (Gao *et al.*, 1993). The damage varies with species, for instance, marsupial spermatozoa can tolerate up to 20% glycerol, while mouse spermatozoa are severely damaged if glycerol concentration exceeds 1.75% (Holt, 2000). In birds glycerol impairs fertility; therefore glycerol in frozen-thawed avian sperm should be diluted to 0.7% v/v before using the sperm for insemination (Etches, 1996). Glycerol is used to cryopreserve stallion semen at 3%, and EG can be used at 3% or lower (Mantovani *et al.*, 2002). The volumes and methods of adding cryoprotectant, for instance glycerol, vary with extenders, cryopreservation methods and species (Foote, 1975). In cryopreserving bovine sperm, glycerol is added after cooling to 5 °C or just before freezing to a final concentration of 5% (yolk-sugar extender) to 10% in milk extender, though 7% is more preferred in most extenders (Hafez, 1993).

Freezing and thawing rates can also damage the fertilizing potential of sperm (Hammerstedt *et al.*, 1990). Semen is generally cryopreserved with 'slow cooling' methods. Optimal cooling rates for freezing semen are mostly found between 10 °C/min and 100 °C/min. To some extent, the reported differences may be related to the use of different types of cryoprotectants and different cryoprotectant concentrations. An extreme example is that fowl semen can be effectively cryopreserved at a cooling rate of approximately 600 °C/min when using dimethylacetamide as cryoprotectant, but not when using glycerol (Woelders *et al.*, 2006).

Cryoprotectants may differ widely in the cell membrane permeability, and also may affect the membrane permeability to water. These parameters greatly affect the velocity of dehydration, and therewith the optimal range of cooling rates. Usually bull sperm are frozen in liquid nitrogen vapour at −80°C for 10 min before plunging and storing in liquid nitrogen at −196 °C (Hafez, 1993). Epididymal sperm has been reported to be more resistant to cryo-injuries than ejaculated sperm (Lasley and Bogart, 1944; Johnson *et al.*, 1980; Berger and Clegg, 1985), though the reason is not well understood.

Complex macromolecules found in egg yolk, skimmed milk, whole milk, and milk whey, as well as permeating cryoprotectants are added to provide adequate cryoprotection of sperm during cryopreservation (Watson, 1981; Chen *et al.*, 1993; Ganguli *et al.*, 1997). Plant products such as soybean extract (lecithin) are also used in the cryopreservation of sperm (Van Wagtendonk-de Leeuw *et al.*, 2000; Aires *et al.*, 2003) as macro-molecules. Milk or egg yolk is often used in freezing media for mammalian semen to protect the spermatozoa during cooling, freezing and thawing (Watson, 1976). Although serum and serum albumin are commonly used as macromolecules in the cryopreservation of mammalian oocytes and embryos, reports on their use in cryopreservation of bovine, especially epididymal spermatozoa, are rare. In other species, for instance fish, Aoki *et al.* (1997) successfully cryopreserved medaka (*Oryzias latipes*) spermatozoa in a medium containing FBS and 10% N, N-dimethylfromamide. In wood frog (*Rana sylvatica*) the post-thaw viability was found to be high for spermatozoa treated with both DMSO and FBS (Beesley *et al.*, 1998).

Cryopreservation of bovine sperm has been mainly in egg yolk- or milk-based extenders with glycerol as the permeating cryoprotectant. Reports on the use of other extenders and cryoprotectants for cryopreservation of bovine sperm, especially bovine epididymal sperm and their evaluation based on *in vitro* embryonic development, are not common. Evaluation of frozen-thawed spermatozoa based on the ability to produce embryos *in vitro* and/or resulting into normal term pregnancies would be more reliable in predicting conception than sperm viability. Van Wagtendonk-de Leeuw *et al.* (2000) compared Biociphos Plus, a lecithin-containing egg yolk-free extender with an egg yolk-based tris-standard extender and a tris-concentrate extender in cryopreserving bovine semen. Biociphos Plus resulted into a lower (77.1%) 56-day non-return rates compared with the egg yolk-based tris-standard (80.6%) and tris-concentrate (78.3%) extenders. However, in a later study Aires *et al.* (2003) showed that bovine semen cryopreserved in soy lecithin (egg yolk-free) extender had significantly superior quality and higher 56-day non-return rates (70.45%) compared with tris-egg yolk extenders (67.85%).

Successful use of epididymal sperm resulting into pregnancies have been reported in many mammalian species, including cattle (Watson, 1978), dog (Marks *et al.*, 1994), human (Patrizio *et al.*, 1995), goat (Blash *et al.*, 2000), rat (Nakatsukasa *et al.*, 2001) and horse (Morris *et al.*, 2002). Amann and Griel (1973) inseminated cows with testicular, cauda epididymal and ejaculated sperm. There was no significant difference in the fertilization rates for ejaculated (94%) and cauda epididymal (84%) sperm while no pregnancy resulted from testicular sperm. Goto *et al.* (1988) reported the birth of a healthy calf and several other pregnancies after *in vitro* fertilization with cauda epididymal sperm. In a study comparing individual variation amongst bulls using epididymal sperm (Goto *et al.*, 1989), normal rates of blastocyst development were obtained after *in vitro* maturation, fertilization and culture. Martins *et al.* (2000) found that the oocyte penetration ability of bovine epididymal sperm was higher than that of ejaculated sperm. In African buffalo *in vitro* embryo production, Shaw *et al.* (1995) used epididymal sperm retrieved from testicles transported at 4 °C for up to 24 h. A cleavage rate of 18% to 50% and embryo development to the morula stage of 33-65% was achieved, but no embryo developed to the blastocyst stage. Brackett *et al.* (1978) obtained a higher fertilization rate (73.1%) using cauda epididymal sperm, compared with 36.6% for ejaculated sperm in an *in vitro* fertilization study in rabbits.

Blash *et al.* (2000) used frozen-thawed goat epididymal sperm for IVF and accomplished a fertilization rate of 40% and a blastocyst development rate of 6%, a finding that was comparable to IVF with cryopreserved ejaculated semen. Later, Melican *et al.* (2001) conducted an *in vitro* fertilization study using epididymal and ejaculated sperm from a transgenic goat. These authors found that the overall cleavage rates of 67% (epididymal sperm) versus 57% (ejaculated sperm) and blastocyst development of 21% (epididymal sperm) versus 17% (ejaculated sperm) were comparable to each other. Pregnancy rates from transfer of day 2 embryos were equivalent (57% for either epididymal or ejaculated sperm) while, only the blastocysts produced with epididymal sperm resulted in pregnancies. These authors concluded that IVF utilizing cryopreserved epididymal transgenic sperm resulted in successful cleavage, blastocyst development and pregnancies. Melican *et al.* (2001) also concluded that this offers a potential alternative method for progeny development of production animals from transgenic founder male dairy goats.

In a pig *in vitro* fertilization study using frozen-thawed epididymal spermatozoa, Kikuchi *et al.* (1998) reported an oocyte penetration rate of 12% with 100% monospermic fertilization. In the same study, after transfer of the *in vitro* produced embryos, a recipient was pregnant and farrowed 5 normal piglets (3 males and 2 females). These authors concluded that

cryopreserved epididymal boar spermatozoa have the ability to fertilize *in vitro* and to produce piglets. A study conducted in rabbits (Brackett *et al.*, 1978) obtained higher fertilization rate (73.1%) with cauda epididymal sperm, compared with 36.6% for ejaculated sperm in an *in vitro* fertilization study. Seita *et al.* (2006) found that the percentage of two pronuclear (2PN) oocytes in rats were not significantly different following *in vitro* fertilization of *in vivo* matured oocytes with cryopreserved (59%) and fresh (62%) epididymal rat spermatozoa. Frozen-thawed cauda epididymal lion sperm was used for *in vitro* fertilization of *in vitro* matured lioness ova and fertilization rates of 12.7% and 11.5%, for 30 h and 36 h of maturation, respectively, were obtained (Bartels *et al.*, 2000).

In general, epididymal spermatozoa appear to be able to capacitate and fertilize oocytes *in vitro* much more easily than ejaculated spermatozoa (Yanagimachi, 1988). In their study where a higher *in vitro* fertilization rate using cauda epididymal sperm compared with ejaculated sperm was obtained in rabbits, Brackett *et al.* (1978) attributed it to the apparent ease of *in vitro* capacitation of cauda epididymal sperm, probably due to non-exposure to seminal secretions. Furthermore, a study by Martin *et al.* (1991) showed that the quality of human epididymal spermatozoa is comparable to ejaculated sperm, and are resistant to the deleterious effects of cryopreservation.

The current study investigated the efficacy of MTL, a non-egg yolk-based extender, and a commercial egg yolk-based Biladyl® (BIL) in preserving the fertilizing ability of sperm, assessed by means of *in vitro* embryo production. Furthermore, the study compared the effectiveness of permeating cryoprotectants GLY, EG and DMSO in cryoprotecting bovine epididymal spermatozoa frozen after 2 and 4 h of equilibration by assessing their effects on *in vitro* embryo development. The effects of the above mentioned factors were tested using the null hypothesis "H_0: development of embryos after fertilization with bovine epididymal spermatozoa equilibrated for 2 or 4 h and cryopreserved in MTL containing 20% FBS and 0.95 M GLY, EG or DMSO is not different from those after fertilization with spermatozoa equilibrated for 4 h and cryopreserved in Biladyl®".

3.3 Materials and methods

3.3.1 Collection, processing and cryopreservation of epididymal spermatozoa

Pairs of testicles from two sets of four mature bulls each marked 1 to 4 were collected and processed on two separate days. The testicles from a nearby abattoir were transported in an insulated (polystyrene) container containing an ice pack. A piece of paper cardboard was

placed between the ice pack and the testicles to prevent direct contact. The cauda epididymides were isolated and spermatozoa from each pair collected by retrograde flushing (Halangk *et al.*, 1990; Reyes-Moreno *et al.*, 2000; Cary *et al.*, 2004; Martinez-Pastor *et al.*, 2005) using pre-warmed (30 °C) tissue culture medium (TCM-199) into 50 mL plastic conical centrifuge tubes (Cellstar®, Greiner bio-one, Germany). The tubes with the samples were kept in a water bath (Memmert®, Germany) at 30 °C for at least 10 min before evaluation. A 20 µL drop of each sample was placed in a 35 x 10 mm Petri dish lid (Cellstar®, Greiner bio-one, Germany) and examined on an inverted microscope (Nikon®, EclipseTE2000-E, Japan) fitted with a warm stage for wave motion at X100, and mass motility at X400. Each sample was centrifuged at 700g for 6 min at 25 °C in a temperature controlled centrifuge (Allegra® X-22R, Beckman Coulter, Germany) and the supernatant discarded. While the tubes with spermatozoa remained in the water bath, the concentration of each sample was determined using the heamocytometer method (Hafez, 1993). Briefly, each sample was diluted at a ratio of 1:200 with distilled water coloured with 1% trypan blue. The spermatozoa in five diagonal squares of the 25 centre squares of the improved Neubauer counter (Spencer®, Germany) were counted to determine the concentration of sperm/mL.

Biladyl® (Minitüb, Germany) and sperm Tyrode's lactose medium (Parrish *et al.*, 1985) were prepared and used to process the spermatozoa as described previously (see Chapter 2). Briefly, Biladyl A and MTL A were prepared without the permeating cryoprotectant. Biladyl B contains GLY 14% v/v (1.9 M) as the cryoprotectant. Meanwhile, MTL B was prepared with GLY 14% (1.9 M), EG 10.6% (1.9 M) and DMSO 13.6% (1.9 M). Spermatozoa from bulls 1 and 2, and bulls 3 and 4 of Set one were pooled together in two sterile 15 mL plastic conical centrifuge tubes (Cellstar®, Greiner bio-one, Germany) with each bull contributing approximately 400 x 10^6 spermatozoa, based on the determined sperm concentration. The spermatozoa in the two test tubes were mixed into one sterile 15 mL tube then separated into 4 equal portions (approximately 400 x 10^6 spermatozoa) in 15 mL plastic centrifuge tubes. Each of these was initially diluted to 5 mL (approximately 80 x 10^6 spermatozoa/mL) with BIL A (one tube) and MTL A (three tubes), then cooled to 5 °C for 4 h. For final dilution of these, 5 mL of BIL B (one test tube) and MTL B (in three test tubes) were also cooled to the same temperature. Finally, the samples were diluted with the cooled 5 mL BIL B and MTL B after the 4 h of equilibration bringing the final concentration to approximately 40 x 10^6 spermatozoa/mL. The samples were gently mixed and designated as A1 (BIL, control), B1 (MTL + GLY), C1 (MTL + EG) and D1 (MTL + DMSO) each containing a final cryoprotectant concentration of 7% GLY, 7% GLY, 5.3% EG and 6.8% DMSO, respectively, equivalent to 0.95 M.

Spermatozoa from Set two's bulls were processed in the same way as that of Set one and concentration determined. Samples from bulls one and two and those from bulls three and four were pooled together into two tubes with each sample contributing 500 x 10^6 spermatozoa. The spermatozoa from the two tubes were further pooled into one sterile 15 mL tube, mixed and aliquoted into five equal portions (approximately 400 x 10^6 spermatozoa) in 15 mL tubes (Cellstar®, Greiner bio-one, Germany). These were each initially diluted to 5 mL (approximately 80 x 10^6 spermatozoa/mL) with BIL A (two test tubes) and MTL A (three test tubes) and cooled to 5 °C. For final dilution, 5 mL of BIL B (in two test tubes) and MTL B (in three test tubes) were prepared and cooled to 5 °C. After 2 h of equilibration, one tube initially diluted with BIL A and the three tubes diluted with MTL A were finally diluted with the corresponding cooled 5 mL BIL B and MTL B bringing the final volume to 10 mL and sperm concentration of approximately 40 x 10^6 spermatozoa/mL. The remaining one tube initially diluted in BIL A (control) was cooled for 4 h and finally diluted with BIL B. These sample were designated as A1 (BIL, control), A2 (BIL), B2 (MTL + GLY), C2 (MTL + EG) and D2 (MTL + DMSO) each containing a final cryoprotectant concentration of 7% GLY, 7% GLY, 7% GLY, 5.3% EG and 6.8% DMSO, respectively.

During the cooling of the samples, labelled sterile 0.25 mL Cassou straws (IMV®, France) and polyvinyl chloride (PVC) straw sealing powder were also cooled to the same temperature. After 2 or 4 h of equilibration, the samples were finally diluted and each sample packed into 30 colour-coded 0.25 mL semen straws using suction pressure. The straws were sealed with PVC powder and frozen in liquid nitrogen vapour at −80 °C (5 cm above liquid nitrogen) for 10 min before plunging into liquid nitrogen, and the straws were stored at −196 °C in liquid nitrogen until used for *in vitro* insemination.

Set one and Set two were used to fertilize bovine oocytes in two separate experiments. The first experiment compared embryo development *in vitro* after *in vitro* fertilization of *in vitro* matured bovine oocytes with sperm obtained from the first group of bulls i.e. A1 (control) compared with B1, C1 and D1. The second experiment compared embryo development *in vitro* after *in vitro* fertilization of *in vitro* matured bovine oocytes with set two spermatozoa i.e. A1 (control) compared with A2, B2, C2 and D2.

3.3.2 Ovary collection, oocyte retrieval and *in vitro* maturation

Bovine ovaries were collected from a local abattoir and transported in a plastic thermos container, without a saline carrier medium such as Dulbecco's phosphate buffered saline (D-PBS). The mean ovary transportation temperature was 31.0 ± 2.8 °C and 31.8 ± 3.2 °C for Experiments one and two, respectively, on arrival to the laboratory. Ovaries were washed three

times in pre-warmed (30 ºC) D-PBS supplemented with 10 µg/mL ciprofloxacin hydrochloride (CF-HCl) (ICN Biomedicals, Ohio, USA). Oocytes were aspirated from follicles 2-8 mm in diameter using a continuous flow suction pressure created through a series of plastic tubes connected to a vacuum water tap.

The aspirated follicular fluid was allowed to settle in a 50 mL centrifuge tube (Cellstar®, Greiner bio-one, Germany) for 10 min, the supernatant discarded and then re-suspended in pre-warmed modified D-PBS supplemented with 10 µg/mL CF-HCl and 1% v/v FBS. After re-suspending and discarding the supernatant twice, the sediment containing the cumulus-ocyte complexes (COCs) was transferred into 60 x 15 mm Petri dishes (Cellstar®, Greiner bio-one, Germany), examined and COCs (n = 1860, Experiment one) and (n = 1943, Experiment two), selected under a stereoscope (Nikon®, SMZ-2T, Japan). The selected COCs were washed twice in HEPES-TALP (see Appendix A2). The COCs were washed once in pre-incubated maturation medium (see Appendix A1). Groups of 15 COCs were transferred into pre-equilibrated 50 µL drops of maturation medium in 35 x 10 mm Petri dishes (Cellstar®, Greiner bio-one, Germany) under mineral oil (M8410-Sigma) and incubated at 39 ºC in a 5% CO_2 humidified air atmosphere for 24.2 ± 2.1 h and 25.1 ± 2.3 h for Experiments one and two, respectively. The duration from ovary collection to onset of maturation incubation was 4.1 ± 0.9 h and 4.3 ± 0.5 h for Experiments one and two, respectively.

3.3.3 Sperm preparation and *in vitro* fertilization

Motile epididymal spermatozoa were separated by swim-up in sperm-TALP (see Appendix A2) as described by Parrish *et al.* (1985). Briefly, 1 mL aliquots of pre-warmed (30 °C) sperm-TALP were made in labelled four x four (16 tubes for Experiment one) and four x five (20 tubes for Experiment two) sterile 5 mL test tubes. An extra four and five sterile 5 mL test tubes for Experiments one and two, respectively, were used to pool frozen-thawed spermatozoa of each treatment. Two straws from each sample were thawed in a water bath at 37 °C for 1 min and the contents emptied into the corresponding 5 mL extra test tubes. A diluted drop of each sample was placed on the surface of a 35 x 10 mm Petri dish lid and examined using a warm-staged inverted microscope (Nikon®, Eclipse TE2000-E, Japan). Approximately 90 µL of sperm from each treatment was layered under the 1 mL sperm-TALP in the four 5 mL test tubes for the corresponding treatment. The test tubes were placed slanting at about 45 degrees on a rack in a water bath at 37 ºC for sperm swim-up.

After 1 h of swim-up, the top 0.85 mL of the sperm-TALP medium in each of the four tubes of each treatment was aspirated and pooled into a 15 mL plastic centrifuge tube. These

samples were washed by centrifuging twice at 700g for 6 min at 25 °C in a temperature controlled centrifuge. After a second washing with sperm-TALP, the supernatant was aspirated and the pellet re-suspended in 0.2 mL fresh sperm-TALP. Sperm concentration was determined using a haemocytometer and the concentration adjusted to 10 x 10^6 sperm/mL. The matured COCs were washed twice in pre-warmed (37 °C) HEPES-TALP medium, and once in pre-incubated IVF-TALP (see Appendix A2). After washing, the oocytes were transferred into the pre-incubated 0.6 mL fertilization medium (IVF-TALP) in 4-well plates (Falcon®, USA) in groups of 30 per well. To each IVF well, 25 µL of PHE (2 mM penicillamine, 10 mM hypotaurine and 0.1 mM epinephrine) and 35 µL of sperm to a final concentration of 0.5 x 10^6 sperm/mL (Ward *et al.*, 2002) were added (about 12,000 sperm per oocyte). The IVF plates were incubated at 39 °C in a 5% CO_2 humidified air for 19.1 ± 3.1 and 19.5 ± 3.4 h in Experiments one and two, respectively.

3.3.4 *In vitro* culture and evaluation of embryo development

The presumptive embryos were washed twice in 2.5 mL of pre-warmed (37 °C) HEPES-TALP medium in 35 x 10 mm Petri dishes and once in pre-equilibrated culture medium, modified CR1aa (Rosenkrans *et al.*, 1990) supplemented with 3 mg/mL (0.3% w/v) BSA (EFAF), 5% v/v FBS and 10 µg/mL CF-HCL (see Appendix A3). The embryos were then transferred in groups of 10 into 30 µL drops of pre-equilibrated modified CR1aa medium under oil and incubated at 39 °C in a 5% CO_2 in humidified air atmosphere for 14 days. The presumptive embryos were cultured without stripping the cumulus cells. The cumulus cells attached to the surface of the Petri dish and formed a monolayer onto which the embryos were co-cultured. Half of the culture medium in the droplets was replaced with fresh pre-incubated medium every second day. Embryonic development was evaluated on day 3 for cleavage, day 5 for morula formation and day 7 for blastocyst formation. Embryos were also evaluated for blastocyst expansion on days 7, 9 and 11, and for blastocyst hatching on days 9, 11, 13 and 15, after which they were discarded. Some 8-day old unhatched blastocysts were selected and cryopreserved in 1.5 M ethylene glycol in modified PBS supplemented with 0.4% BSA (American Embryo Systems, Grand Prairie, Texas) using a programmable freezer (Freeze Control® CL863, CryoLogic, Australia). The blastocysts were stored in liquid nitrogen until they were thawed and stained for blastomere count.

3.3.5 Evaluation of embryonic cell number

Day 8 blastocysts were stained with DAPI (D9564-Sigma), a DNA-binding fluorescent stain, and evaluated as described by Moreira *et al.* (2001) with slight modifications. Briefly, a 0.01 mg/mL DAPI was prepared in 1 mg/mL solution of polyvinyl pyrrolidon (PVP) in PBS (PBS-

PVP), stored at 4 °C wrapped in aluminium foil and used within 6 months. Prior to use, the 1 mg/mL DAPI solution was diluted to 1 µg/mL with PBS-PVP and kept away from bright light. A fixative, 4% (40 mg/mL) solution of paraformaldehyde in PBS (pH 7.4), was also prepared. The straws were thawed in a water bath at 37 °C for 1 min, and the contents emptied into 2.5 mL of HEPES-TALP in a 35 x 10 mm Petri dish. The embryos were then cultured for 1 to 2 h in modified CR1aa before staining.

A 4-well plate was used for the staining process. The two right wells were each filled with 0.5 mL of PBS-PVP, the top left with the fixative (0.5 mL) and the bottom left to be filled with 0.5 mL DAPI staining solution. After culture, the embryos were transferred into the top right well containing PBS-PVP and washed once, and then transferred into the fixative to fix for 30 to 60 min. The staining well was filled with 0.5 mL DAPI preparation at the end of fixing and the embryos transferred into this well where they were stained for 10 min. This was followed by washing once in PBS-PVP contained in the right lower well. Stained embryos were kept in this well and protected from direct light as they were examined one at a time.

Each embryo was transferred onto a clean microscope slide with a small drop (10 µL) of the PBS-PVP medium then examined first with bright light and then UV light on an inverted microscope (Olympus® 1x70, Tokyo, Japan). A cover slip was very gently placed over the embryo to spread and make it possible to focus the blastomeres concurrently. The image was picked by a camera (Colorview®, Soft Imaging System, GmbH, Germany). The image was displayed on a computer monitor by software (AnalySIS®, Soft Imaging System, GmbH, Germany) and snapshots were recorded. At X100 or X200 magnification, the bright-blue nuclei of blastomere were counted using the Touch-count program of the AnalySIS software. In cases where the blastomeres spread beyond the view of the camera and were not fully displayed on the computer monitor for touch-counting, direct counting of the nuclei was performed.

3.3.6 Statistical analysis

Data from nine and eight replicates of bovine embryo cultures for Experiments one and two, respectively, were analysed. Data captured in Microsoft Excel and exported to SAS (version 9.1, 2006). This was analyzed by running the logistic regression of the generalized linear module (GENMOD) procedure and P-values less than 0.05 were taken as indicating significant difference. The correlation of pre-culture conditions with embryo development was also analyzed by logistic regression. Differences of least square means were used to determine the correlations between embryo development and the respective sperm treatments. The data on blastomere count for 4 h equilibrated sperm was analyzed by one way analysis of variance

(ANOVA). A critical number of observations for blastomere count for 2 h sperm equilibration was not attained, therefore, analysis was by MEANS procedure.

3.4 Results

The average concentration of spermatozoa collected from the 4 bulls was 1800 x 10^6 and 828 x 10^6 sperm per mL for sets one and two, respectively. All the collected samples showed strong to very strong wave motion and mass motion of ≥ 80%. Frozen-thawed bovine epididymal spermatozoa extended in Biladyl® (BIL) and modified Tyrode's lactose (MTL) containing glycerol (GLY), ethylene glycol (EG) or dimethyl sulfoxide (DMSO) cryoprotectants and equilibrated for 2 and 4 h produced embryos *in vitro*. Tables 3.1 and 3.2 show embryo development in Experiment one where sperm cryopreservation was done after 4 h of equilibration.

Table 3.1 Development of embryos from epididymal sperm equilibrated for 4 h and cryopreserved in Biladyl (A1), and in MTL containing glycerol (B1), ethylene glycol (C1) and dimethyl sulfoxide (D1). Embryo development was evaluated by cleavage rate (CR), morula (MOR), blastocyst (B), expanded blastocyst (XB) and hatched blastocyst (HB) on the corresponding days in brackets. Day 0 was insemination day.

		Embryo development (%)						
Treatments	No. of ocytes cultured (Day 1)	CR (Day 3)	MOR (Day 5)	B (Day 7)	XB/HB (Day 9)	HB (Day 11)	HB (Day 13)	HB (Day 15)
A1	432	85.6[a]	63.9[a]	62.0[a]	23.4[a]	10.6[a]	13.9[a]	15.3[a]
B1	441	82.8[b]	65.1[a]	65.8[a]	21.3[a]	13.6[a]	15.9[a]	16.6[a]
C1	447	78.3[c]	57.0[b]	55.5[b]	20.1[a]	10.1[a]	12.1[a]	12.5[a]
D1	443	47.9[d]	23.3[c]	19.6[c]	4.5[b]	0.9[b]	1.4[b]	1.6[b]

[a,b,c,d]Values within columns with different superscripts differ significantly ($P < 0.05$).

Table 3.2 Embryo development beyond day 7 for embryos from sperm equilibrated for 4 h as evaluated by blastocyst expansion (XB) and blastocyst hatching (HB) on the corresponding days in brackets.

		Embryo development (%)						
Treatments	No. of blastocysts (Day 7)	XB (Day 7)	XB (Day 9)	HB (Day 9)	XB (Day 11)	HB (Day 11)	HB (Day 13)	HB (Day 15)
A1	175	14.3[a]	46.3[a]	11.4[a]	29.7[a]	26.3[a]	34.3[a]	37.7[a]
B1	183	14.8[a]	38.8[a,b]	12.6[a]	21.9[a]	32.8[a]	38.3[a]	39.9[a]
C1	163	13.5[a]	44.2[a,b]	11.0[a]	25.8[a]	27.6[a]	33.1[a]	34.4[a]
D1	63	7.9[a]	30.2[b]	1.6[b]	28.6[a]	6.3[b]	9.5[b]	11.1[b]

[a,b]Values within columns with different superscripts differ significantly ($P < 0.05$).

Table 3.3, the correlations of embryo development with some pre-culture conditions namely ovary transport temperature and duration, and oocyte maturation and insemination durations are shown. The underlined P-values showed significant correlations of pre-culture conditions with embryo development.

Table 3.3 Correlations of rates of cleavage (CR), morula (MOR), blastocyst (B), blastocyst expansion (XB) and blastocyst hatching (HB) with some pre-culture conditions in development of embryos after insemination with sperm equilibrated for 4 h before freezing.

	Embryo development (%)					
Pre-culture conditions	CR (Day 3)	MOR (Day 5)	B (Day 7)	XB/HB (Day 9)	HB (Day 11)	HB (Day 13)
Transport temp	P = 0.0060	P = 0.2166	P = 0.0502	P = 0.3388	P = 0.9627	P = 0.6056
Transport time	P = 0.8412	P = 0.9123	P = 0.1809	P = 0.8830	P = 0.5064	P = 0.4521
IVM duration	P = 0.1356	P = 0.4730	P = 0.0166	P = 0.0074	P = 0.0193	P = 0.0044
IVF duration	P = 0.2461	P = 0.5401	P = 0.0007	P = 0.0002	P = 0.0409	P = 0.0931

A total of 1754 and 1632 presumptive embryos were cultured in Experiments one and two, respectively. The cleavage rates of embryos from all sperm treatments were significantly lower ($P < 0.05$) than that of the control. Sperm cryopreserved after equilibration for 4 h in MTL + DMSO (treatment D1) resulted in the lowest ($P < 0.0001$) cleavage rate compared to the control. All the treatments were significantly different ($P < 0.05$) from each other. Cleavage rate correlated ($P < 0.05$) with transport temperature, but not the other statistical factors. Morula formation in treatment B1 (4 h equilibration in MTL + GLY) was not different ($P > 0.05$) from that of the control, while treatments C1 (4 h equilibration in MTL + EG) and D1 were different ($P > 0.05$) from that of the control. All comparisons between individual treatments showed significant

differences ($P < 0.05$). Morula formation was not correlated ($P > 0.05$) with any of the pre-culture conditions (Table 3.3). Blastocyst formation on day 7 in treatments C1 and D1 were different, ($P < 0.05$ and $P < 0.0001$, respectively), from the control and B1 ($P < 0.05$ and $P < 0.0001$, respectively). Treatments C1 and D1 were also different $P < 0.0001$ from each other, while B1 was not different ($P > 0.05$) from the control. Blastocyst formation on day 7 correlated with the durations of maturation and insemination ($P < 0.05$).

Embryo expansion/hatching on days 9 and 11, and hatching on day 13 in treatment D1 were different ($P < 0.0001$) from that of the control and the rest of the treatments. Expansion/ hatching of embryos on days 9 and 11, and hatching on day 13 for treatments B1 and C1 were not different ($P > 0.05$) from the control and each other. Embryo expansion and/or hatching on days 9 and 11 correlated with durations of maturation and insemination incubation ($P < 0.05$). Embryo hatching on day 13 correlated only with duration of maturation ($P < 0.05$).

Evaluation of embryo development in Experiment one based on number of blastocysts on day 7 showed further differences between treatment D1 and the rest of the treatments as well as the control. Embryo expansion on days 7, 9 and 11; and embryo hatching on days 9, 11, 13 and 15 in treatment D1 were different ($P < 0.05$) from those of the control (A1), and treatments B1 and C1 (Table 3.1). However, when the blastocyst development was evaluated based on the number of blastocysts in culture on day 7 (Table 3.2) no differences ($P > 0.05$) in blastocyst expansion between the treatments as well as the control were noted. Embryo expansion on day 9 for treatment D1 was different ($P < 0.05$) from the control but not treatments B1 and C1 ($P > 0.05$), while treatments B1 and C1 were not different from each other as well as the control. Hatching of the blastocysts of treatment D1 on day 9 was, however, different from those of the control, B1 and C1 ($P < 0.05$), while B1 and C1 did not differ from each other and from the control. There were no differences ($P > 0.05$) in expanded blastocysts between the treatments and the control on day 11. Hatching of the blastocysts on days 11, 13 and 15 showed similar trend in which treatment D1 was different ($P < 0.05$) from the control as well as B1 and C1, while treatments B1 and C1 did not differ from each other and the control.

Embryo development in Experiment two where sperm equilibration was for 2 h, except for the control (4 h), and the correlations of embryo development with pre-culture conditions, are shown in Tables 3.4, 3.5 and 3.6. There were no differences in cleavage rates between the control (BIL-4 h) and A2 (BIL-2 h) and control and B2 (MTL + GLY) ($P > 0.05$). However, the cleavage rate of treatment D2 (MTL + DMSO) was significantly different ($P < 0.0001$) from the rest of treatments. The cleavage rate of treatment C2 (MTL + EG) was also different from other

treatments ($P < 0.05$) except treatment B2 (MTL + GLY). Ovary transport temperature correlated with cleavage rate ($P < 0.05$).

Embryo development to the morula stage was similar to that of cleavage. There were no differences in morula rates between the control and A2, and control and B2 ($P > 0.05$). Treatments B2 and C2 were also not different ($P > 0.05$) in morula rates. However, the morula rate of treatment D2 differed ($P < 0.0001$) from the rest of treatments. Ovary transport temperature, but not other pre-culture conditions, correlated with cleavage rate ($P = 0.05$).

Treatment D2 was different ($P < 0.0001$) from all other treatments in blastocyst formation by day 7, while the rest of treatments were not different ($P > 0.05$) from the control. However, there was a difference ($P < 0.05$) between A2 and C2, while no difference ($P > 0.05$) existed between the rest of the treatments. Duration from ovary collection to maturation correlated ($P < 0.05$) with blastocyst formation.

Tables 3.4 and 3.5 show embryo development in Experiment two where sperm cryopreservation was performed after 2 h of equilibration. In Table 3.6, the correlations of embryo development with pre-culture conditions namely ovary transport temperature and duration, and oocyte maturation and insemination durations are presented. The underlined P-values denoted correlations of the pre-culture conditions with embryo development.

Table 3.4 Development of embryos from epididymal sperm cryopreserved in Biladyl after equilibration for 4 h (A1) and 2 h (A2); and equilibration for 2 h followed by cryopreservation in MTL containing glycerol (B2), ethylene glycol (C2) and dimethyl sulfoxide (D2). Embryo development was evaluated by cleavage rate (CR), morula (MOR), blastocyst (B), expanded blastocyst (XB) and hatched blastocyst (HB) on the corresponding days in brackets. Day 0 was insemination day.

		Embryo development (%)						
Treatments	No. of oocytes cultured (Day 1)	CR (Day 3)	MOR (Day 5)	B (Day 7)	XB/HB (Day 9)	XB/HB (Day 11)	HB (Day 13)	HB (Day 15)
A1	330	80.9^{a}	59.4^{a}	47.6^{a}	17.3^{a}	19.4^{a}	14.5^{a}	14.8^{a}
A2	306	80.3^{a}	63.1^{a}	$55.9^{a,c}$	21.6^{a}	21.9^{a}	14.1^{a}	15.4^{a}
B2	320	$75.9^{a,b}$	$58.8^{a,b}$	50.0^{a}	$22.5^{a,c}$	21.9^{a}	16.3^{a}	18.1^{a}
C2	339	72.3^{b}	51.9^{b}	$44.5^{a,d}$	$15.9^{a,d}$	17.7^{a}	14.2^{a}	16.2^{a}
D2	337	32.3^{c}	16.6^{c}	11.9^{b}	0.3^{b}	1.5^{b}	0.3^{b}	0.3^{b}

^{a,b,c,d} Values within columns with different superscripts differ significantly ($P < 0.05$).

Table 3.5 Embryo development beyond day 7 for embryos from sperm equilibrated for 2 h as evaluated by blastocyst expansion (XB) and blastocyst hatching (HB) on the corresponding days in bracket.

		Embryo development (%)						
Treatments	No. of blastocysts (Day 7)	XB (Day 7)	XB (Day 9)	HB (Day 9)	XB (Day 11)	HB (Day 11)	HB (Day 13)	HB (Day 15)
A1	157	17.2[a]	22.3[a]	14.0[a]	15.9[a]	24.8[a]	30.6[a]	31.2[a]
A2	171	13.5[a]	29.2[a]	9.4[b]	19.9[a]	19.3[a,b]	25.1[a]	27.5[a]
B2	160	20.0[a]	28.1[a]	16.9[a]	12.5[a]	28.8[a,c]	32.5[a]	36.3[a]
C2	151	14.6[a]	25.2[a]	11.3[a]	17.2[a]	22.5[a]	31.8[a]	36.4[a]
D2	40	0	2.5[b]	2.5[c]	10.0[a]	2.5[d]	2.5[b]	2.5[b]

[a,b,c,d]Values within columns with different superscripts differ significantly ($p < 0.05$).

Table 3.6 Correlations of rates of cleavage (CR), morula (MOR), blastocyst (B), blastocyst expansion (XB) and blastocyst hatching (HB) with some pre-culture conditions in development of embryos after insemination with sperm equilibrated for 2 h before freezing.

	Embryo development (%)						
Treatments	CR (Day 3)	MOR (Day 5)	B (Day 7)	XB/HB (Day 9)	XB/HB (Day 11)	HB (Day 13)	HB (Day 15)
Transport temp	P = 0.0098	P = 0.0431	P = 0.7647	P = 0.6070	P = 0.9121	P = 0.7189	P = 0.7618
Transport time	P = 0.4382	P = 0.4382	P = 0.0249	P = 0.0010	P < 0.0001	P = 0.0015	P = 0.0013
IVM duration	P = 0.4631	P = 0.4631	P = 0.2111	P < 0.0001	P < 0.0001	P = 0.0001	P < 0.0001
IVF duration	P = 0.7463	P = 0.7048	P = 0.9099	P = 0.1388	P = 0.0121	P = 0.1076	P = 0.0231

The rate of embryo expansion/hatching on day 9 for treatment D2 was different ($P < 0.0001$) from the rest of the treatments. Treatments B2 and C2 were also different ($P < 0.05$) from each other. Furthermore, treatment D2 was significantly different ($P < 0.0001$) from the rest of treatments in embryo expansion and/or hatching on day 11 as well as embryo hatching on days 13 and 15. No differences ($P > 0.05$) were found between in embryo expansion and/or hatching on days 11, 13 and 15 and the rest of the treatments. Durations of ovary transportation and maturation significantly correlated ($P < 0.05$) with expansion and/or hatching on day 11, 13 and 15, and duration of insemination on days 11 and 15.

Embryo development beyond day 7 was also based on the number of blastocysts formed by day 7. No embryo of treatment D2 had expanded by day 7. There were no differences ($P > 0.05$) in blastocyst expansion between the other treatments and control. Blastocyst expansion was different ($P < 0.05$) between D2 and the control, and between D2 and

rest of treatments on day 9. However, D2 and A2 were different ($P < 0.05$) from each other, the control and other treatments in embryo hatching on day 9. The rest of the treatments were not different from each other and from the control in embryo expansion and hatching on day 9. There were no differences ($P > 0.05$) between all treatments as well as the control in embryos that had expanded, but not hatched on day 11. Furthermore, blastocyst hatching on day 11 was not different ($P > 0.05$) in treatments A2, B2 and C2 as well as the control. Hatching of the blastocysts on days 13 and 15 had similar trends in which treatment D2 significantly differed ($P < 0.05$) from the control and the other treatments, while the rest of the treatments did not differ from each other and the control.

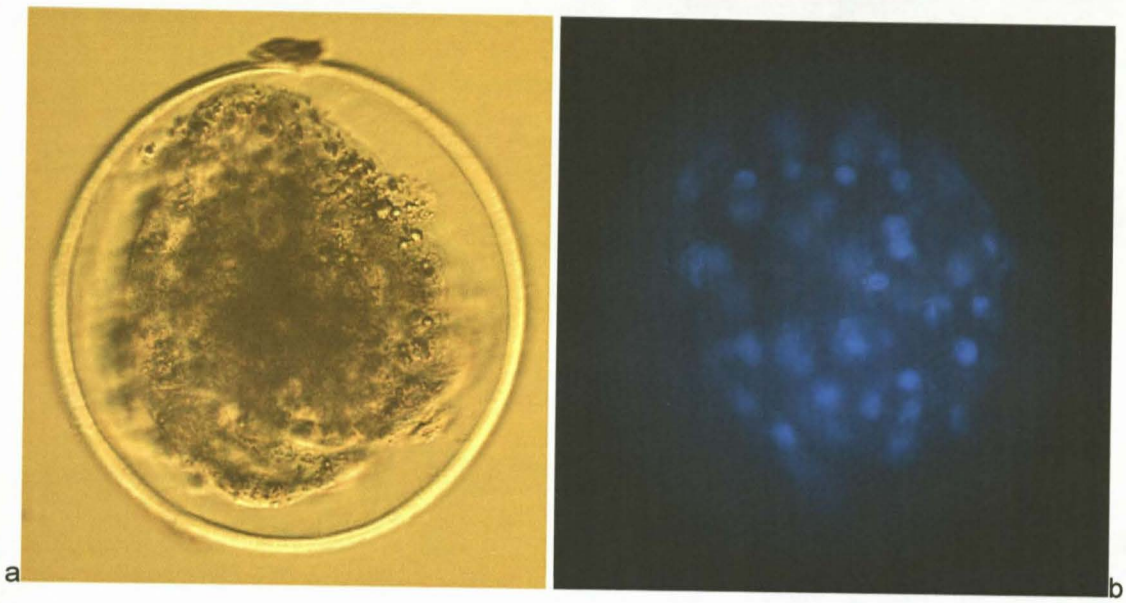

Fig. 3.1 A DAPI-stained frozen-thawed day 8 blastocyst (day 0 = insemination day) (a) bright field microscopy; and (b) the same embryo under UV light microscopy showing bright-blue stained nuclei of blastomeres at X200 magnification.

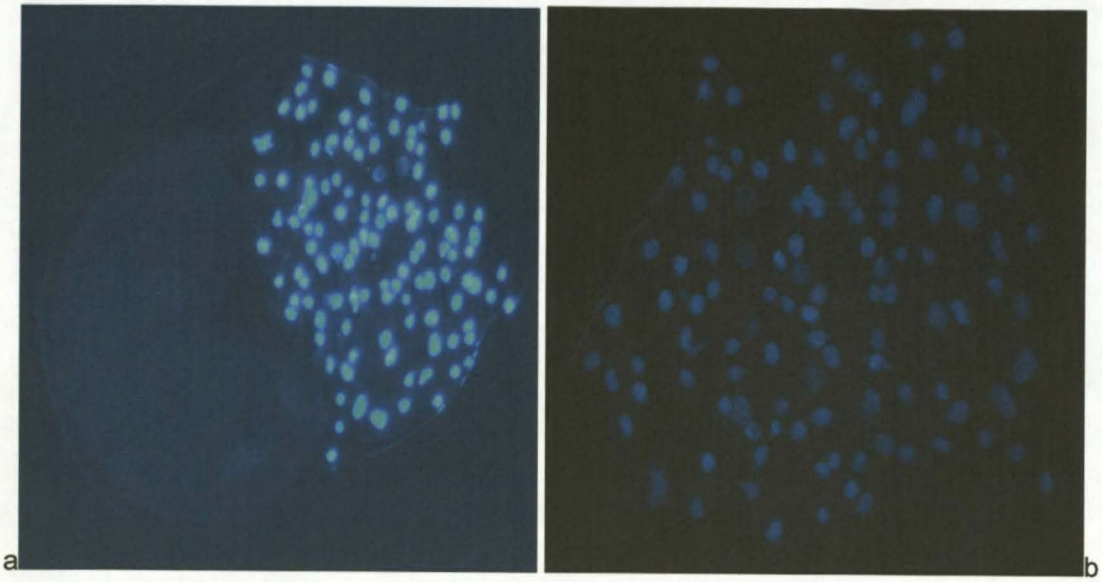

Fig. 3.2 A DAPI-stained frozen-thawed day 8 blastocyst (day 0 = insemination day) under UV microscopy after placing a cover slip on the embryo to spread the blastomeres (a) X100 (b) X200 magnification.

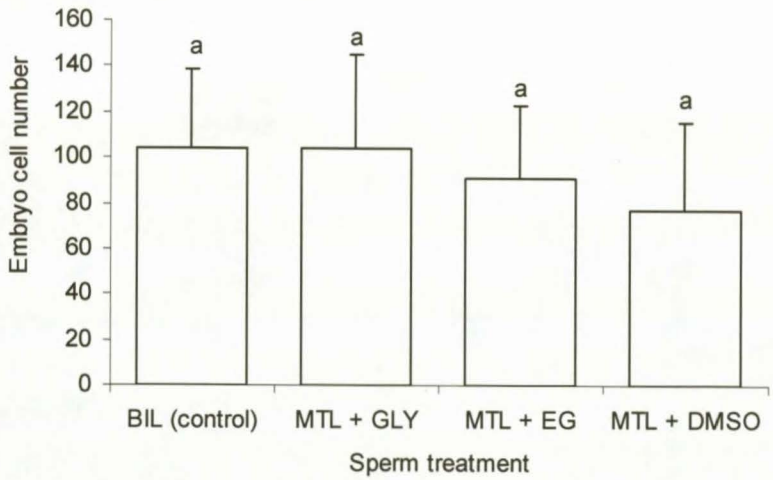

Fig. 3.3 Blastomere numbers of day 8 blastocysts (day 0 = insemination day) from epididymal sperm equilibrated for 4 h and cryopreserved in BIL (control), and MTL containing glycerol, ethylene glycol or dimethyl sulfoxide.

Blastomeres were counted from day 8 blastocysts (n = 51) of Experiment one. A range of 32-174 and mean blastomere count of 95.6 ± 36.7 were recorded in all the treatments. Despite the wide range, there were no differences ($P > 0.05$) in blastomere number between the

control and treatments. The observations for embryo cell count from sperm equilibrated for 2 h (Experiment two) were below the critical number of observation for ANOVA. However, the mean blastomere counts were 105 ± 48.8, 84 ± 24.9, 117 ± 30.1 and 66 ± 0.7 blastomeres for treatments A1, A2, B2 and C2, respectively. Only one embryo of treatment D2 was recovered and blastomeres counted, but could not be included in the results.

3.5 Discussion

This study has shown that bovine epididymal spermatozoa cryopreserved in modified Tyrode's lactose (MTL) with 20% bovine serum and 0.95 M glycerol (GLY), ethylene glycol (EG) or dimethyl sulfoxide (DMSO) as cryoprotectants produced embryos *in vitro* to the hatched blastocyst stage. Similarly, Kundu *et al.* (2000) successfully froze goat cauda epididymal spermatozoa in modified Ringer's solution using GLY, EG and DMSO as permeating cryoprotectants, although evaluation was based on sperm motility and not embryo development, as in this study. Furthermore, Kundu *et al.* (2000) used 0.87 M GLY, 1.29 M EG and 1.00 M DMSO as the optimal concentration, and they did not use macromolecules such as bovine serum, as was used in this study. In similar studies, frozen-thawed epididymal sperm have been used to produce embryos in goats (Blash *et al.*, 2000; Melican *et al.*, 2001), pigs (Kikuchi *et al.*, 1998), lions (Bartels *et al.*, 2000) and rats (Seita *et al.*, 2006), though using variable cryopreservation protocols.

Reports on the use of extenders other than egg yolk-based and cryoprotectants other than glycerol for cryopreservation of bovine sperm, especially bovine epididymal sperm, and their evaluation based on *in vitro* embryonic development, are rare. Evaluation of frozen-thawed spermatozoa based on the ability to produce embryos *in vitro* and/or resulting in term pregnancies are more reliable than sperm viability parameters. Amann (1999) stated that attributes other than motility are required for sperm to have a normal fertilizing potential, thus implying that frozen-thawed sperm with relatively high percentage of motility may not fertilize the oocytes. Furthermore, Brito *et al.* (2003) compared plasmalemma evaluation methods with IVF rates using bovine spermatozoa. These authors found that determination of plasmalemma functional integrity by the hypo-osmotic swelling test (HOST) was the only test that correlated with IVF rates. A study on the use of Biociphos Plus, a soybean extract- (lecithin-) containing egg yolk-free extender for cryopreservation of bovine semen, resulted in a lower (77.1%) 56-day non-return rates compared with the egg yolk-based tris-standard (80.6%) and tris-concentrate (78.3%) extenders (Van Wagtendonk-de Leeuw *et al.*, 2000). In a later study, however, Aires *et al.* (2003) showed that bovine semen cryopreserved in soy lecithin (egg yolk-free) extender had

a significantly superior quality and higher 56-day non-return rates (70.45%) compared to bovine semen cryopreserved with tris-egg yolk extender (67.85%). *In vivo* embryo production and pregnancy are credible means of spermatozoa evaluation. In the current study *in vitro* embryo development to the hatched blastocyst stage was used to evaluate the fertilizing potential of frozen-thawed bovine epididymal spermatozoa.

Cleavage and subsequent embryo development that occurred in all treatments showed that although dilution, cryopreservation and thawing adversely affect sperm fertilizing potential, some bovine epididymal spermatozoa were capable of withstanding these effects and produced normal embryos. The extenders and cryoprotectants used in the current study and the subsequent cryopreservation resulted to viable spermatozoa. This showed that the method used could be applicable for genome resource banks and/or production of embryos using ART such as IVF and ICSI as reported by Anel *et al.* (1999, 2000). The cryopreservation of bovine sperm has been mainly in egg yolk- or milk-based extenders with glycerol as the preferred permeating cryoprotectant. This study showed that MTL, serum and other permeating cryoprotectants can also be used in cryopreservation of bovine sperm. Under field conditions for the purpose of conservation of endangered species, serum can be obtained from the male from which sperm is collected or a related species. The problem of using serum is the risk of disease transmission. However, Baker *et al.* (2003) has shown in humans that washing of sperm before use in ART can minimize such risks in HIV patients. In ART, with the exception of AI, sperm is usually washed before use.

The amounts and methods of adding a cryoprotectant, for instance glycerol, vary with extenders, cryopreservation methods and species (Foote, 1975). In cryopreservation of bovine sperm, glycerol is added after cooling to 5 °C or just before freezing to a final concentration of 5% (yolk-sugar extender) to 10% (in milk extender), though 7% is more preferred in most extenders (Hafez, 1993). In the current study the cryoprotectants to a concentration of 0.95 M were added after cooling the spermatozoa to 5 °C for 2 or 4 h. Furthermore, the 0.95 M concentration of all permeating cryoprotectants (equivalent of 7% glycerol) used in this study seem to have provided cryoprotection to fertilizing potential of bovine epididymal spermatozoa, as was shown by the cleavage and subsequent embryo development.

One of the measures of *in vitro* fertilization efficiency is the cleavage rate (Gordon, 1994). The penetration of the oocyte by the sperm is followed by a series of events such as sperm head decondensation, formation of two pronuclei and a second polar body and syngamy culminating in cleavage. Post-thaw sperm viability and hence the effect of cryopreservation on

sperm fertilizing potential can be based on cleavage rates because only normal spermatozoa are capable of fertilizing. In a study to assess sperm quality parameters of bulls showing different abilities to produce embryos *in vitro*, fertilization rates ranged from 59.9% to 79.3% (Alomar *et al.*, 2006). These authors concluded that fertilization rate was the only parameter to show some correlation with blastocyst rate for all the bulls. Based on cleavage rate, fertilization rates in this study are comparable to or higher than those reported in other studies using frozen-thawed or fresh epididymal sperm. Blash *et al.* (2000) used frozen-thawed goat epididymal sperm for IVF and attained a fertilization rate of 40%. In pigs, Kikuchi *et al.* (1998) reported an oocyte penetration rate of 12% with frozen-thawed epididymal spermatozoa. A study using rabbit epididymal sperm (Brackett *et al.*, 1978) obtained a fertilization rate of 73.1%. Seita *et al.* (2006) recorded an *in vitro* fertilization rate of *in vivo* matured oocytes of 59% using cryopreserved rat epididymal spermatozoa. In lions the *in vitro* fertilization rate of *in vitro* matured lioness ova with frozen-thawed cauda epididymal lion sperm was 12.7% and 11.5%, for 30 h and 36 h of maturation, respectively (Bartels *et al.*, 2000).

Overall average cleavage, morula and blastocyst rates obtained in this study i.e. 73.7%, 52.3% and 50.7%, (4 h equilibration) and 68.3%, 50.0%, and 42.0% (2 h equilibration), respectively, were similar to that reported elsewhere for the bovine, caprine and rabbits. Parks *et al.* (1999) obtained an average cleavage rate (≥ two-cell) of 82.3% and a day 7 blastocyst rate of 32.5% in a bovine IVF study. Melican *et al.* (2001) conducted an *in vitro* fertilization study using epididymal and ejaculated sperm from a transgenic goat. The overall cleavage and blastocyst rates of 67% and 21% respectively, for epididymal sperm, and cleavage and blastocyst rates of 57% and 17% respectively, for ejaculated sperm were reported (Melican *et al.*, 2001). In rabbits, Brackett *et al.* (1978) obtained fertilization rate of 73.1% using cauda epididymal sperm in an *in vitro* fertilization study.

The fact that inseminated oocytes cleaved and embryos developed to the hatched blastocyst stage (Tables 3.1, 3.2, 3.4 and 3.5) indicated that MTL containing 20% bovine serum and 0.95 M GLY, EG or DMSO can be used to cryopreserve bovine epididymal spermatozoa, and embryo production using ART. This could have been because the cryopreservation method used in the current study was efficient for cryopreservation of epididymal sperm. Epididymal sperm has also been reported to be more resistant to cryo-injuries than ejaculated sperm (Lasley and Bogart, 1944; Johnson *et al.*, 1980; Berger and Clegg, 1985), although a comparative study was not performed.

Cleavage rates of treatments B1 (82.8%) and C1 (78.3%) in Experiment one (4 h equilibration) (Table 3.1) and A2 (80.3%), B2 (75.9%) and C2 (72.3%) in Experiment two (2 h equilibration) (Table 3.4) were comparable to the respective controls, 85.6% and 80.9%. This finding indicated that cryopreservation of bovine epididymal sperm in MTL with 20% bovine serum and 0.95 M GLY or EG is comparable to cryopreservation in the commercially prepared Biladyl. Furthermore, the findings showed that there is little difference in cryopreserving bovine epididymal spermatozoa in these extenders and cryoprotectants after 4 or 2 h of equilibration. These findings were comparable to the bovine IVF cleavage rates reported by Shi (1991) and Zhang *et al.* (1992), though these authors used ejaculated sperm. These cleavage rates were above those reported in other species using epididymal sperm, for instance 67% in goats (Melican *et al.*, 2001), 18 to 50% in African buffalo (Shaw *et al.*, 1995) and in lions 12.7% and 11.5%, for 30 h and 36 h of maturation, respectively (Bartels *et al.*, 2000). Cleavage rate is determined 48 hpi (Gordon, 1994); however, in this study cleavage rates was evaluated on day 3 post insemination, which coincided with the first changing of the culture medium. The cumulus cells that were used to co-culture the embryos had attached by day 3 (but not day 2) and it was easy to detached the embryos from the cumulus cells to allow for the proper evaluation of cleavage.

Unlike in other treatments, cleavage rates for treatments D1 (47.9%) and D2 (32.3%), were significantly different from their controls and other treatments. Since all the MTL extenders had a similar composition except for the permeating cryoprotectant, the low cleavage rates exhibited by these treatments must have arisen from the effect of DMSO on the fertilizing ability of the spermatozoa. Cryoprotectants are known for their osmotic and toxic effects on cells. Dimethyl sulfoxide crosses the cell membrane faster and is more toxic than GLY and EG (Amann, 1999), thus it's likely to be toxic to the spermatozoa even when added at low temperatures. Cryoprotectants damage spermatozoa membranes due to shrinking and/or swelling during freezing and thawing (Gao *et al.*, 1993). Permeating cryoprotectants cause cell injury by denaturing enzymes and can also increase retention of intracellular water, with subsequent intracellular ice formation and cell injury (Diller & Lynch, 1983; Karlsson *et al.*, 1994). They also alter microtubule organizations (Vincent and Johnson, 1992) therefore, affecting the cytoskeletal component of the sperm necessary for cleavage after fertilization.

Preparation of sperm for *in vitro* fertilization using the swim-up method improves sperm motility (Gordon, 1994). However, even the motile spermatozoa could have had injuries affecting other sperm structures vital for fertilization, for instance the sperm membrane and acrosome. An intact sperm membrane is necessary for sperm-zona binding, a prerequisite for

zona penetration (Saling *et al.*, 1979; Saling and Storey, 1979). In addition, a study comparing bovine sperm evaluation methods with IVF rates found that determination of sperm membrane functional integrity by HOST was the only test that correlated with IVF rates (Brito *et al.*, 2003). Therefore, motile spermatozoa with damaged membranes may fail to fertilize an oocyte due to their inability to bind to the zona. Cormier *et al.* (1997) showed that premature capacitation occurs in partially (extended and cooled) and fully cryopreserved bovine spermatozoa. This would cause premature release of the acrosomal enzymes necessary for fertilization, hence a poor fertilization rate by an otherwise motile sperm sample. The low fertilization rate from spermatozoa cryopreserved in DMSO could also have been due to damage to the DNA. Furthermore, it's possible that the DMSO at 0.95 M was not toxic to the spermatozoa since it was added at 5 °C, but rather did not provide adequate cryoprotection to the spermatozoa. Kundu *et al.* (2000) used an optimal concentration of 1.00 M DMSO and 1.29M EG to cryopreserve goat sperm, but found that DMSO and EG had markedly lower cryoprotection potential than GLY at 0.87 M. The concentration of DMSO used in this study (0.95 M) was similar to the 1.00 M used by Kundu *et al.* (2000).

Development of the embryos to morula (day 5) and blastocyst (day 7) stage were similar in Experiments one (4 h equilibration) and two (2 h equilibration). In the former, morula and blastocyst rates of treatment B1 (65.1% and 65.8%) were not different from, and even higher than that of the control, i.e. 63.9% and 62.0%, respectively. Apparently, all the embryos that had reached the morula stage by day 5 and some of those that had not, developed to the blastocyst stage by day 7. This could be because both B1 and the control contained 0.95 M GLY that has been the preferred cryoprotectant and its concentration for cryopreservation of bovine sperm (Hafez, 1993). Probably this was due to the excellent cryoprotection properties of GLY compared with EG and DMSO as reported by Kundu *et al.* (2000) in the cryopreservation of goat epididymal sperm. In a study investigating caprine blastocyst development after *in vitro* fertilization with spermatozoa cryopreserved in different extenders, Keskintepe *et al.* (1998), however, found the highest proportion of blastocysts from the use of spermatozoa diluted in a skimmed milk extender, heparin capacitated, and inseminated in medium containing lamb serum. The current study used 2 µg/mL heparin for capacitation and inseminated oocytes in IVF-TALP containing 6 mg/mL bovine serum albumin.

Morula and blastocyst rates of treatment C1 were lower than B1 and the control ($P < 0.05$), while those of D1 were significantly lower ($P < 0.0001$) than those of the rest of the treatments and the control. Probably, the effect of EG is exhibited later in embryo development while the marked effect of DMSO continues after cleavage. The effects of these treatments,

especially D1, could be associated with damage to the sperm DNA and inability to synthesize adequate mRNA leading to embryonic developmental arrest. Maxwell and Salamon (1993) reported a decreased embryonic development in cryopreserved compared to fresh ovine spermatozoa. Meanwhile in an earlier study, Bladou *et al.* (1991) reported a high incidence of degeneration of human embryos produced by IVF with epididymal sperm of infertile men.

The rates of morula and blastocyst formation after 2 h of equilibration were similar to, but lower than that after 4 h of equilibration. This indicated that equilibration for 4 h is better than 2 h, though the morula and blastocyst rates are comparable to those of other studies. Spermatozoa cryopreservation studies with evaluation based on sperm viability parameters have variable reports on the effects of equilibration time, while those based on embryo development are hard to come by. No differences in the percentage of intact acrosome and motile bovine spermatozoa were seen after equilibration for 0.5 to 2 h (Wiggin and Almquist, 1975a). The same authors (Wiggin and Almquist, 1975b) reported that equilibration times longer than 4 h are detrimental for sperm and recommended 2 h. However, Herold *et al.* (2004) observed no detrimental effect after equilibration time exceeding 4 h in cryopreserving buffalo epididymal sperm, but equilibration times of 2 and 3 h were inferior to longer equilibrations. These studies, however, equilibrated sperm in extenders containing permeating cryoprotectants and evaluated sperm cryopreservation by sperm viability parameters other than embryo development. In the current study, the permeating cryoprotectants were added just before cryopreservation after cooling to 5 °C, a method commonly used for cryopreserving bovine sperm (Hafez, 1993). Treatments A2 and B2 were not different from, and to some extent better than, the control in morula and blastocyst rates. The common factor in these treatments and the control was glycerol. Perhaps this further confirms the effectiveness of glycerol in cryoprotecting bovine epididymal sperm after 2 h of equilibration in extenders containing glycerol. Like in Experiment one and possibly for the same reasons, morula and blastocyst rates of C2 and D2 in Experiment two were lower than that of the other treatments and the control, and also different from each other, with the lowest rates exhibited by D2.

Embryo development was evaluated beyond day 7 and the number of embryos that had expanded and/or hatched by days 9, 11, 13 and 15, recorded. The trend of development beyond day 7 was similar in Experiments one and two. Treatments B1 and C1 in Experiment one, and treatments A2, B2 and C2 in Experiment two were not different from their respective controls, and were also not different from each other ($P > 0.05$). Similar embryo developmental trends were observed when development on and beyond day 7 were analyzed based on the total number of blastocysts of each treatment on day 7 (Tables 3.2 and 3.5). These results

indicated that spermatozoa cryopreserved in freezing media A, B and C after 4 or 2 h of equilibration produce embryos that are capable of undergoing implantation, and thus causing normal pregnancy at similar rates as shown by embryo expansion and hatching. Brandao *et al.* (2004) reported that prolonged *in vitro* embryo culture resulting into expansion, hatching and elongation more precisely predict the potential development of a bovine conceptus other than blastocyst quality or parameters related to the embryonic-foetal development in a foster mother. Although most of the day 5 morulae appeared to have developed into blastocysts by day 7, less than 50% of the day 7 blastocysts expanded and hatched in their subsequent development in Experiments one and two (Tables 3.2 and 3.4). This was lower than expansion and hatching rates reported by Wang *et al.* (1992) for bovine IVF. The decreased embryonic development could be the result of DNA damage caused by sperm cryo-damage. A decreased rate of embryonic development has also been reported in cryopreserved or cooled ovine sperm compared with fresh sperm (Maxwell and Salamon, 1993). In humans, Bladou *et al.* (1991) reported a high incidence of embryo degeneration (> 50%) for embryos produced by IVF with epididymal sperm from infertile men.

Embryo development beyond day 7 was lower in D1 and D2 ($P < 0.0001$), compared to that of the control and the other treatments. Evaluation of embryo development on and beyond day 7 based on the number of blastocysts on day 7 showed similar trends. This evaluation indicated poorer development rates in D2 than D1 ($P < 0.05$) (Tables 3.2 and 3.4). The continued drop in the rates of embryo development in these treatments was an indication of severe damage to the spermatozoa cryopreserved in D1 and D2. Spermatozoa of these treatments possibly suffered irreversible DNA damage that could have resulted into improper synthesis of mRNA and thus proteins, resulting in the arrest of embryo development. However, the mechanism of this damage cannot be explained and further research is recommended.

It was noted that the duration of transport of ovaries, maturation of oocytes and oocyte insemination individually or in combination, significantly correlated with embryo development on day 7 and beyond. The mean duration from ovary collection to onset of maturation incubation was 4.1 ± 0.9 h and 4.3 ± 0.5 h, and mean temperature was 31.0 ± 2.8 °C and 31.8 ± 3.2 °C for Experiments one and two, respectively. The ovaries were transported in insulated containers without a saline carrier medium. Studies in cattle IVF recommended transportation of ovaries at about 33 °C in sterile PBS or normal saline (0.9% NaCl), and recovery of oocytes within 8 h from slaughter (Sato *et al.*, 1990; Takagi *et al.*, 1992; Hamano and Kuwayama, 1993). However, bovine ovaries were stored at 24-25 °C for 11 h (Yang *et al.*, 1990) and at 15-21 °C for 24 h (Schernthaner *et al.*, 1997) without reducing fertilization and blastocyst rates *in vitro*.

The ovary transport time and temperature in this study were within these limits and thus could not have caused the low embryo expansion and hatching. Apparently it is not necessary to transport ovaries in saline as long as the temperature remains within the normal range and desiccation is avoided. Like in this study, Kidson (2004) collected pig ovaries in insulated containers without saline and there was no effect on *in vitro* embryo development.

The mean maturation duration was 24.2 ± 2.1 and 25.1 ± 2.3 h for Experiments one and two, respectively. Maturation duration correlated ($P < 0.05$) with embryo development beyond day 7 in both Experiments. However, this correlation could not be the cause of low blastocyst expansion and hatching observed in our study. A study by Prokofiev *et al.* (1992) concluded, on the basis of embryo yields, that there is a wide range of 18-24 h in the duration of oocyte maturation. In a later study, Monaghan *et al.* (1993) found that 24 h was superior to an 18 h maturation period. Furthermore, Ward *et al.* (2002) confirmed that the optimal duration of maturation of bovine oocytes *in vitro* to maximize blastocyst yield is 24 h.

The mean insemination time was 19.1 ± 3.1 h and 19.5 ± 3.4 h for Experiments one and two, respectively. Mean insemination time correlated with the rates of most of the embryo development stages beyond day 7 in both Experiments one and two. A study by Ward *et al.* (2002) in which matured bovine oocytes were co-incubated with sperm for 1, 5, 10, 15 or 20 h, showed that sperm-oocyte co-incubation for 10 h is sufficient to ensure maximal blastocyst yields. Perhaps the discrepancy in insemination time reported by Ward *et al.* (2002) and that used in this study partly explains the low rates of embryo expansion and hatching seen in the current study. A study by Sumantri *et al.* (1997) showed that the duration of sperm-oocyte incubation has effects on *in vitro* embryo development. A sperm concentration of 0.5×10^6 spermatozoa/mL in the fertilization medium was used in the current study. Ward *et al.* (2002) found that sperm concentrations of 0.25×10^6 spermatozoa/mL and 0.5×10^6 spermatozoa/mL yielded significantly more blastocysts than any other concentration within the range of 0.01 to 1.0×10^6 spermatozoa/mL.

Determination of the total cell number, as well as the number of the inner cell mass (ICM) cells, provides a useful standard against which IVC treatments can be compared (Gordon, 1994). Because this study was designed to assess the effects of sperm treatments using embryo development, cells of the embryos were counted to assess the sperm treatments. Eight-day old embryos were stained with DAPI, a DNA-binding fluorescent stain and the total embryonic cells were counted. The mean blastomere counts were 104 ± 35.0, 104 ± 40.5, 91 ± 32.4 and 77 ± 38.6 for A1 (control), B1, C1 and D1, respectively, in Experiment one (Fig. 3.3).

However, there was no significant difference ($P > 0.05$) in embryo cell numbers between the treatments as well as the control. In Experiment two, the mean blastomere counts were 105 ± 48.7, 84 ± 24.8, 117 ± 30.0 and 66 ± 0.7 for A1 (control), A2, B2 and C2, respectively. The embryos of treatment D2 for cell count were too few to be counted. The results of blastomere count in the current study were comparably lower than those of Jiang *et al.* (1992). However, Mori *et al.* (2002) showed that although the day 7 and day 8 embryos are smaller than the day 9 expanded blastocysts, the cell number in both types of embryos are similar. These authors further found that cell numbers of blastocysts and expanded blastocysts decreased with an increase embryo age. Perhaps this explains the apparently no differences that existed between treatments B1 and C1, and A2 and B2, and their respective controls.

Many *in vitro* embryo culture systems have been used for the culture of bovine embryos. In the current study embryos were cultured in CR1aa (Rosenkrans *et al.*, 1990) supplemented with 3 mg/mL BSA (EFAF) and 5 % v/v FBS. Furthermore, presumptive embryos were not stripped off the cumulus cells, and these cells attached to the Petri dish surface to form a monolayer with which the embryos were co-cultured. Most of the day 5 morulae developed to the blastocyst stage in all treatments, except D1 and D2. Furthermore, some of the blastocysts that developed early had expanded by day 7, an indication of an efficient embryo culture system. Rosenkrans *et al.* (1990) and Rosenkrans and First (1991) concluded from their studies that a simple medium, Charles Rosenkrans 1 (CR1), with essential and non-essential amino acids, can support the development of bovine embryo *in vitro* even in the absence of feeder cells. This was supported by the findings of the current study.

A study by Mastromonaco *et al.* (2004) indicated that both serum and BSA (fatty acid free) are comparable in embryo development of IVF bovine embryos. Sung *et al.* (2004) showed that differential embryotrophic factor(s) contained in BSA and serum is/are necessary for promoting successful morula and blastocyst development in cattle embryos. Perhaps, the combination of BSA, FBS and cumulus cells provided an efficient embryo culture system to achieve the good embryo development reported in this study. Kane *et al.* (1992) and Bavister (1995) reported that somatic cells could provide embryonic growth factors or remove embryotoxic substances from the culture medium, or both. Here the use of cumulus cell co-culture was seen to save time and resources, and controls the risks of cross contaminations arising from the use of cells from other animals or species. Similarly it was shown by Zhang *et al.* (1995) that cumulus cell co-culture significantly improves rates of embryo development to morula or blastocyst stages.

3.6 Conclusion

It was concluded from the findings of this study that bovine embryos can be produced *in vitro* using epididymal spermatozoa cryopreserved in a serum-based saline medium, modified Tyrode's lactose (MTL), containing permeating cryoprotectants. When cryopreserving bovine epididymal spermatozoa in MTL for *in vitro* fertilization, glycerol or ethylene glycol, but not dimethyl sulfoxide, should be the preferred permeating cryoprotectants. An equilibration time of 2 and 4 h in the non-glycerol or non-ethylene glycol portion of the extender produced comparable embryo development *in vitro*. Embryo development *in vitro* to the hatched blastocyst stage from bovine epididymal spermatozoa cryopreserved in MTL supplemented with 20% bovine serum, 0.95 M glycerol or ethylene glycol are comparable to those cryopreserved in the commercial extender Biladyl® (Minitüb, Germany). A 2 h equilibration period in the non-cryoprotectant portion of MTL is recommended to reduce the equilibration time. An *in vivo* fertilization study through artificial insemination (AI) using bovine epididymal sperm cryopreserved in MTL supplemented with 20% bovine serum, 0.95 M glycerol or ethylene glycol is recommended. Further research in the cause of the low *in vitro* embryo development following insemination with bovine epididymal spermatozoa cryopreserved in DMSO is also recommended.

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CHAPTER 4

The effects of repeated freeze-thawing of bovine cauda epididymal spermatozoa on *in vitro* embryo development

4.1 Abstract

Assisted reproductive technologies (ARTs) have shown that fertilization requires only a few spermatozoa, even from sources other than ejaculates. This has refocused recent efforts in sperm cryopreservation on maximizing the use of spermatozoa by single sperm freezing, refreezing and freeze-drying. Refreezing is important when sperm is cryopreserved in bulk, in limited supply, highly valuable or mistakenly thawed. This study examined the use of Biladyl® (BIL) and modified Tyrode's lactose medium (MTL) containing 20% foetal bovine serum (FBS) and ethylene glycol (EG), in refreezing bovine cauda epididymal sperm for production of embryos by *in vitro* fertilization (IVF) procedures. Sperm collected by retrograde flushing were subjected to freeze-thaw cycle one (BIL 1 and MTL 1). Thirty straws of each of the cryopreserved sperm samples were thawed and motile sperm selected by swim-up before subjecting the sperm to freezing cycle two in the corresponding medium (BIL 2 and MTL 2). *In vitro* matured bovine oocytes were subjected to standard IVF with the spermatozoa of freeze-thaw cycles one and two. The presumptive embryos (n = 1301) were cultured in CR1aa supplemented with 3 mg/mL BSA and 5% v/v FBS for 14 days. Embryo development was assessed for cleavage, morula and blastocyst formation, blastocyst expansion and hatching, and blastomere numbers. Cleavage, morula and blastocyst rates were significantly higher ($P < 0.05$) in the control (BIL 1) than the treatments (BIL 2, MTL 1 and MTL 2). Embryo expansion and/or hatching (days 7-15), however, were not different ($P > 0.05$) in BIL 1, MTL 1 and MTL 2. Meanwhile, expansion and/or hatching on the same days were significantly lower in BIL 2. The blastomere numbers for treatment BIL 1 (119 ± 52.8) and MTL 1 (130 ± 43) were not different ($P > 0.05$). However, the blastomere number for treatment BIL 2 (58 ± 29) was lower than those for BIL 1 and MTL 1 ($P < 0.05$). It was concluded that bovine epididymal sperm can undergo two freeze-thaw cycles in Biladyl®, and in modified Tyrode's lactose with foetal bovine serum and ethylene glycol for use in embryo production using ARTs.

4.2 Introduction

Epididymal sperm is the major alternative source of sperm to ejaculates. Cryopreservation of epididymal sperm is therefore important for animal production, conservation science and management of human infertility through assisted reproductive technologies (ARTs). Cryopreservation is presently widely used as a method of storing different cell types and tissues, including male and female gametes as well as embryos. It has been possible to cryopreserve spermatozoa of several mammalian species effectively, particularly bovine and human sperm since the late 1930's-1940's (Bernschtein and Petropavlovski, 1937; Polge *et al.*, 1949; Smirnov, 1949). However, cryopreservation is known to cause changes in a sperm's morphology that impair its fertilizing potential (Wooley and Richardson, 1978). In most mammalian species a high proportion of spermatozoa lose their motility and/or other functions

after thawing, with only about 50% of spermatozoa surviving the freeze-thaw process (Watson, 2000). In the past five decades research efforts on sperm cryopreservation has been to improve the technique to ensure better post-thaw results in terms of quality and quantity of spermatozoa, that can be used in assisted reproductive technologies (ART). Application of assisted reproductive techniques (ART) such as *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI) has, however, shown that fertilization requires a few spermatozoa, even from sources other than ejaculate. Further efforts have, therefore, been on maximizing the use of spermatozoa by single sperm freezing (Cohen *et al.*, 1997), refreezing (Arav *et al.*, 2002; McCue *et al.*, 2004) as well as easing storage and transportation by freeze-drying (Keskintepe *et al.*, 2002; Liu *et al.*, 2004). Amongst these, refreezing has so far been the most promising.

There is no doubt that the process of freeze-thawing is damaging to sperm. The major effect of repeated freeze-thaw cycles is, therefore, likely to be the increased intensity of this damage. Most cryopreservation damage to cells is caused by high solute concentration (solution effects) and intracellular ice formation (Stachecki *et al.*, 1998). In a study on human erythrocytes, Pegg and Diaper (1988) demonstrated that cryo-injury of cells is due solely to changes that occur in the composition of their surrounding milieu. According to these authors, this is most probably mediated by a temporary leak in the plasma membrane that occurs during the thawing (re-expansion) phase. Extensive chemical and physical damage to the extra-cellular and intracellular membranes of human sperm attributable to changes in the lipid phase-transition and/or increased lipid peroxidation has been reported to occur during conventional freezing or thawing (Alvarez and Storey, 1992, 1993; Mossad *et al.*, 1994). This damage to human sperm mainly occurs during thawing and is related to a reduced antioxidant defence activity during cooling and/or structural damage to the cytoskeleton and/or antioxidant enzymes during cryopreservation (Alvarez and Storey, 1992, 1993). The general belief is that cryo-injury results from mechanical forces due to intracellular ice formation (Mazur, 1977), with plasma membrane (Fujikawa, 1980) and possibly the membranes of intracellular organelles as the possible sites where damage occur.

Sperm morphology, motility, mitochondrial activities and viability are equally susceptible to cryopreservation-induced damage. O'Connell *et al.* (2002) demonstrated a 30-50% reduction in these parameters after one freeze-thaw cycle of human sperm. Watson (2000) reported that only about 50% of spermatozoa survive the freeze-thaw process in most mammalian species, as a high proportion of spermatozoa lose their motility or other functions after thawing. The freeze-thawing procedure has a detrimental effect on chromatin, morphology, membrane integrity, and vitality of human spermatozoa either by freezing above static liquid nitrogen

vapour or by using a computerized biological freezer (Hammadeh *et al.*, 2001). The proper condensation of chromatin is related to fertility (Hammadeh *et al.*, 1999) and there is some evidence that cytoskeletal elements of spermatozoa may be sensitive to cooling (Holt and North, 1991). Human sperm nuclear DNA, assessed by the alkaline Comet assay, was significantly damaged by slow freezing followed by fast thawing (Thompson-Cree *et al.*, 2003). Though this did not affect cleavage and blastocyst rates, pregnancies tended to be lower in cryopreserved (26%) than fresh (30%) testicular sperm extraction (TESE) cycles. Fertility of frozen-thawed spermatozoa is also known to be reduced due to precocious capacitation-like changes that are known to occur during cryopreservation. Cormier and Bailey (2003) used a chlortetracycline assay and the ability of the sperm to undergo the lysophosphatidylcholine-induced acrosome reaction to confirm that a sub-population of cryopreserved sperm is capacitated at thawing. The authors, however, failed to prevent cryo-capacitation, according to the chlortetracycline assay and profile of phosphotyrosine-containing sperm proteins. The present study explored the effects of these sperm cryo-damages on embryo development after one and two freeze-thaw cycles.

Sperm cells are protected from cryo-injury by permeating and non-permeating cryoprotectants included in the freezing medium. A sperm freezing medium (extender) could be a simple buffered salts solution containing sugars such as fructose, glucose or lactose (0.5-2.0%), heated skim milk or a simple buffered salts solution containing 4-20% egg yolk (Amann, 1999). Generally, most freezing media feature a saline or saccharide bulk osmotic support, a suitable permeating cryoprotectant at concentrations varying from 0.2-1.5 M, and various protective macromolecular additives, mostly milk and egg yolk, or lipids from vegetal origin (Bousseau *et al.*, 1998). The sugars and proteins in milk, lipoproteins in egg yolk and plant lipids serve as non-permeating cryoprotectants (Amann, 1999). They, together with permeating cryoprotectants osmotically remove intracellular water preventing intracellular ice formation. In addition, they remove the permeating cryoprotectants from intracellular space by creating a hyper-osmotic extra-cellular environment (Mizukami *et al.*, 1999). Basically, cryoprotectants protect cells from cryo-injury by preventing ice formation, thus reducing injury due to "solution effects" (Karlsson and Toner, 1996), and by stabilizing cell proteins (Arakawa *et al.*, 1990) and plasma membranes (Anchordoguy *et al.*, 1991). Although sperm from some species, for instance, medaka fish (*Oryzias latipes*) (Aoki *et al.*, 1997) and wood frog (*Rana sylvatica*) (Beesley *et al.*, 1998) have been successfully cryopreserved in serum, little is known about refreezing bovine epididymal spermatozoa in media containing serum. Likewise, the cryoprotective property of ethylene glycol in refreezing bovine epididymal sperm is not known.

When using frozen-thawed spermatozoa in ART, usually the motile sperm is selected as a high proportion of spermatozoa do not survive cryopreservation. A simple swim-up or density gradient centrifugation procedure allows for the selection of spermatozoa with progressive motility, normal morphology and sperm with non-damaged DNA (Isachenko *et al.*, 2004). Other workers (O'Connell *et al.*, 2003) have also shown that this pre-selection improved post-thaw sperm quality including DNA integrity. Nawroth *et al.* (2002) also reported an improvement in post-thaw sperm viability following vitrification of swim-up-prepared human spermatozoa. In a later study, Isachenko *et al.* (2004) demonstrated that the swim-up method of preparing the sperm result in a significant improvement in the quality of spermatozoa. Despite the effects of repeated freeze-thaw cycles on spermatozoa, such spermatozoa prepared by the swim-up method should achieve fertilization and blastocyst rates similar to those of spermatozoa that underwent a single freeze-thaw cycle. In humans, however, low fertilization and pregnancy rates are achieved when frozen-thawed spermatozoa are used for intra-uterine insemination (Mack and Zaneveld, 1987) and conventional IVF (Szczygiel *et al.*, 2002). Furthermore, the fertilizing ability of frozen-thawed spermatozoa of most mammalian males has been reported to be lower than that of fresh sperm (Amann, 1999).

A prospective analysis of motility and viability of human semen after repetitive freeze-thaw cycles demonstrated that multiple freeze-thaw cycles are possible even with markedly impaired semen (Gilbert and Brown, 1999). Rofeim *et al.* (2001) demonstrated that human sperm can undergo up to seven freeze-thaw cycles, though with markedly impaired fertilizing potential. In another study on human sperm by Bandularatne and Bongso (2002), it was reported that the number of normal vital sperm after three serial freeze-thaw cycles is adequate for normal fertilization by ICSI. These authors recommended that leftover washed sperm in IVF laboratories can be refrozen, thawed and refrozen several times for future use. In a study by Verza and Esteves (2004), a significant reduction in motility, vitality and morphology between first and second freeze-thaw cycles was reported. The authors, however, concluded that refreezing human semen by the technique of liquid nitrogen vapour allows the retrieval of viable spermatozoa after thawing. A comparative investigation of motility recovery among methods of thawing human semen in serial freeze-thaw processes was conducted by Leffler and Walters (1996). They demonstrated that sperm frozen rapidly in liquid nitrogen and for sperm subjected to multiple freeze-thaw processes, motility recovery is improved if thawed at 37 °C rather than at room temperature. It was, however, noted that cryopreserved human semen that is intended to be re-used in future assisted reproduction treatments should be thawed only once and aliquoted in the original freezing medium before refreezing (Polcz *et al.*, 1998).

In the horse, Choi *et al.* (2006) produced blastocysts from ICSI with motile and non-motile stallion sperm that are subjected to two freeze-thaw cycles. They concluded that cryopreserved stallion semen may be thawed, diluted, and refrozen without an effect on the ability of spermatozoa to initiate embryo development after ICSI. An earlier study on stallion spermatozoa by McCue *et al.* (2004) demonstrated that refrozen equine sperm retained approximately 70% of their initial post-thaw motility. Dilution prior to refreezing would allow for the re-allocation of a single 0.5 mL straw into hundreds or thousands of smaller straws for future judicious use of valuable stallion semen in assisted reproduction. Frozen-thawed sheep semen has been sorted, refrozen and subsequently used to produce offspring (Evans *et al.*, 2004). In cattle, Filseth and Vatn (1979) used repeated freeze-thaw cycles to predict the viability and fertility of bull semen. Although motility after refreezing had decreased, there was no difference in 60-days non-return rates between single frozen (66.86%) and refrozen (65.30%) semen. Furthermore, in a study of double freezing of large volumes of bull semen, Arav *et al.* (2002) successfully produced pregnancies without difference between double frozen (44.0%) and the control (45.5%). This would be beneficial for cryo-banking bull semen in large volumes and later refrozen in regular insemination volumes. Horiuch *et al.* (2002) reported birth of normal calves after ICSI of bovine oocytes with non-motile spermatozoa "killed" by repeated freeze-thawing, though blastocyst rate was higher in immobilized (20%) than "killed" (1%) sperm. A marked difference in cryo-survival was observed in bovine ejaculated and epididymal spermatozoa in serial freeze-thaw cycles (James, 2004). In that study, James found that although the ejaculated sperm survived more than one freeze-thaw cycle, the epididymal sperm harvested after 24 h of cool storage did not, and therefore a single freeze-thaw cycle in smaller volumes for epididymal sperm was recommended.

The potential for maximizing spermatozoa use by refreezing are promising. Although serial freezing of spermatozoa would result in more severe impairment of sperm fertilizing ability, research has shown that some spermatozoa do survive and can be used for normal fertilization. In humans, this would be applicable in cryopreserving excess sperm in IVF laboratories, especially for men with difficulty in producing sperm or with declining sperm quality (Bandularatne and Bongso, 2002). Furthermore, this procedure provides men with severely impaired semen quality or those about to undergo treatment that will adversely affect spermatogenesis, the means to preserve their gametes (Gilbert and Brown, 1999). In farmed and game animals, refreezing allows judicious use of highly valuable semen in limited supply (McCue *et al.*, 2004). Sperm sexing (sorting) by flow cytometry, for instance, results in highly valuable spermatozoa in limited number (Evans *et al.*, 2004) therefore, any excess should be refrozen. In case of large volume cryo-banking, the semen can be thawed, re-extended and

refrozen in smaller volumes to maximize sperm utilization (Arav *et al.*, 2002). Therefore, sperm could be cryopreserved in high concentrations or large volumes to ease storage and transportation and later thawed, aliquoted in working volumes or concentrations and refrozen. Meanwhile, when there is a mistaken thawing, the straw can be refrozen to save the economic and genetic loses that could be incurred (James, 2004).

The current study compared sperm cryoprotective properties of a commercial extender, Biladyl® (BIL), and modified Tyrode's lactose (MTL) containing foetal bovine serum and ethylene glycol after one and two freeze-thaw cycles of bovine epididymal sperm, as evaluated by *in vitro* embryo development. This was tested using the null hypothesis "H_0: development of embryos produced by fertilizing bovine oocytes with bovine epididymal sperm that underwent a single freeze-thaw cycle in MTL and two freeze-thaw cycles in MTL and BIL is not different from those fertilized with spermatozoa after one freeze-thaw cycle in BIL".

4.3 Materials and methods

4.3.1 Collection, processing and freeze-thaw cycle one of epididymal spermatozoa

Pairs of testicles were collected from four mature bulls from a nearby abattoir and transported in an insulated container (polystyrene) containing an ice pack. A piece of paper cardboard was placed between the icepack and the testicles to prevent direct contact. The cauda epididymides were isolated on the same day and spermatozoa from each pair retrieved by retrograde flushing (Halangk *et al.*, 1990; Reyes-Moreno *et al.*, 2000; Cary *et al.*, 2004; Martinez-Pastor *et al.*, 2005). Pre-warmed (30 °C) tissue culture medium (TCM-199) was used to flush the spermatozoa into a 50 mL plastic centrifuge tube (Cellstar®, Greiner bio-one, Germany). After flushing, the tubes with the respective samples were kept in a water bath (Memmert®, Germany) at 30 °C for at least 10 min before evaluation.

The spermatozoa from all the 4 bulls were evaluated for mass motility, concentration, viability, and membrane and acrosome integrity (Table 4.1) as earlier described (see Chapter 2). Briefly, a drop of each sample was placed in a 35 x 10 mm Petri dish lid (Cellstar®, Greiner bio-one, Germany) and examined for wave motion at X100 and mass motility at X400 magnification on an inverted microscope (Nikon®, EclipseTE2000-E, Japan) fitted with a warm stage. Smears were stained with eosin-nigrosin and Spermac® stains for viability and acrosome integrity, respectively. The functional integrity of the spermatozoa was determined by the hypo-osmotic swelling test (HOST), (see Chapter 2). Each sample was centrifuged at 700g for 6 min at 25 °C in a temperature controlled centrifuge (Allegra® X-22R, Beckman Coulter, Germany)

and the supernatant discarded, thus leaving about 3 mL of the medium with spermatozoa. While the tubes with spermatozoa remained in the water bath, the concentration of each sample was determined using the haemocytometer method (Hafez, 1993). Briefly, each sample was diluted at ratio of 1:200 with distilled water coloured with trypan blue (1%) and spermatozoa in five diagonal squares of the 25 centre squares of the improved Neubauer counter (Spencer®, Germany) counted. The concentration was determined using the formula N x D x 50,000 to give the number of spermatozoa x 10^6/mL (Hafez, 1993). In this formula, N is the average number of spermatozoa counted in the two counting chambers of the haemocytometer, and D is the dilution factor (200).

Biladyl® (Minitüb, Germany) and sperm Tyrode's lactose medium (Parrish *et al.*, 1985) were prepared and used to process the spermatozoa as described earlier (see Chapter 2 and Chapter 3). Briefly, BIL A and MTL A were prepared without permeating cryoprotectant. Only ethylene glycol was used in this study as the permeating cryoprotectant in MTL. Biladyl B contains GLY 14% v/v (1.9 M) as the permeating cryoprotectant therefore, MTL B was prepared with same molar concentration (1.9 M) of ethylene glycol equivalent to 10.6% v/v. Sperm from the four bulls was aliquoted into eight tubes (two per bull) each containing approximately 400 x 10^6 spermatozoa based on the concentration determined. Spermatozoa from bulls one and two were pooled into two 50 mL test tubes labelled as BIL (1 + 2) and MTL (1 + 2); while those from bulls three and four were pooled into two other 50 mL test tubes and labelled BIL (3 + 4) and MTL (3 + 4). Each 50 mL test tube, therefore, contained about 800 x 10^6 spermatozoa. Correspondingly, these were extended with BIL A and MTL A to 10 mL (80 x 10^6 spermatozoa/mL) then cooled to 5 °C for 4 h. Twenty (20) mL of BIL B and MTL B, labelled 0.25 mL sterile semen straws (Cassou straws, IMV®, France) and PVC straw sealing powder were also cooled to the same temperature. After the 4 h of cooling, the spermatozoa in tubes BIL (1 + 2) and BIL (3 + 4) were each extended further with 10 mL of BIL B, while the spermatozoa in tubes MTL (1 + 2) and MTL (3 + 4) were extended further with 10 mL of MTL B, bringing the final concentration to about 40 x 10^6 sperm/mL. These were each loaded into 50 colour-coded 0.25 mL semen straws, sealed with PVC powder and frozen in liquid nitrogen vapour at −80 °C for 10 min, before they were plunged and stored in liquid nitrogen.

4.3.2 Thawing of cryopreserved epididymal sperm, processing and freeze cycle two

Sperm-TALP was prepared as described by Parrish *et al.* (1985) (see Appendix A2). Aliquots of 1 mL were made in thirty-five (35) 5 mL test tubes (per set) and kept in a water bath at 37 °C. Thirty (30) straws of each cryopreserved set i.e. BIL (1 + 2), BIL (3 + 4), MTL (1 + 2) and MTL (3 + 4) were thawed in a water bath at 37 °C for 1 min and the contents of each set

pooled in a test tube. The pooled samples were evaluated for motility, viability, and sperm membrane and acrosome integrity. About 200 µL of the pooled samples was under-layered at the bottom of each of the thirty-five 1 mL aliquots of sperm-TALP and the test tubes were placed slanting at about 45 degrees on a rack in a water bath at 37 °C. After 1 h of swim-up, 0.85 mL of the top of the medium was aspirated from each tube and pooled into three 15 mL centrifuge tubes per set. The tubes were centrifuged twice at 700g for 6 min and the supernatant discarded. The final pellet of each set was re-suspended in 0.5 mL of the corresponding portion A of freezing medium and pooled into one 15 mL centrifuge tube. The tubes were kept in a water bath as the samples were re-evaluated for motility, concentration, viability and integrity of sperm membrane and acrosome. There was difficulty in evaluating spermatozoa cryopreserved in BIL as it reacted with the stains, while concentration could not be determined as the reaction obstructed vision of sperm cells. Spermatozoa from each set was diluted with the corresponding BIL A or MTL A to 5 mL and cooled to 5 °C for 4 h. These were further diluted with an equal volume of BIL B and MTL B that has been cooled to the same temperature, to 10 mL. The finally extended samples from each set were packed in 20 colour-coded 0.25 mL Cassou straws (IMV®, France) and sealed with PVC powder. The straws were frozen in liquid nitrogen vapour at −80 °C for 10 min before plunging and storing in liquid nitrogen.

4.3.3 Ovary collection, oocyte retrieval and *in vitro* maturation

Bovine ovaries were collected from a local abattoir and transported in a plastic thermos container without a saline carrier at a mean temperature of 32.9 ± 2.6 °C. Ovaries were washed three times in warm sterile (30 °C) normal saline (0.9% NaCl) supplemented with 10 µg/mL ciprofloxacin hydrochloride (CF-HCl) (ICN Biomedicals, Ohio, USA). Oocytes were aspirated from follicles 2-8 mm in diameter using a continuous flow suction pressure as described in Chapter 3. The collected follicular fluid was allowed to settle in a 50 mL conical centrifuge tube (Cellstar®, Greiner bio-one, Germany) for 10 min, the supernatant discarded and re-suspended in pre-warmed modified D-PBS supplemented with 10 µg/mL CF-HCl and 1% v/v FBS. After re-suspending and discarding the supernatant twice, the sediment containing the cumulus oocyte complexes (COCs) was transferred into 60 x 15 mm Petri dishes (Cellstar®, Greiner bio-one, Germany). The COCs were examined under a stereoscope (Nikon®, SMZ-2T, Japan), and those with three or more layers of cumulus cells and homogeneously granulated cytoplasm (n = 1425) were selected for further processing. The selected COCs were washed twice in HEPES-TALP (see Appendix A2) and then washed once in pre-incubated maturation medium (see Appendix A1). Groups of 15 COCs were transferred into pre-equilibrated 50 µL drops of maturation medium in 35 x 10 mm Petri dishes (Cellstar®, Greiner bio-one, Germany) under mineral oil (M8410-Sigma) and incubated at 38.5 °C in a 5% CO_2 humidified air atmosphere for

24.1 ± 1.3 h. The duration from ovary collection to onset of maturation incubation was on average 4.9 ± 0.4 h.

4.3.4 Sperm preparation and *in vitro* fertilization

Spermatozoa from freeze-thaw cycles one (BIL 1 and MTL 1) and two (BIL 2 and MTL 2) each consisting of sperm from all the four bulls, were used in four fertilization treatments with freeze-thaw cycle one in BIL (BIL 1) as the control. Motile spermatozoa were separated by swim-up in sperm-TALP as described in 4.3.2, and then further processed for IVF (see Chapter 3). Sperm from the respective treatments were also evaluated before and after swim-up for motility, viability and integrity of sperm membranes and acrosomes (see Chapter 2).

The matured COCs were washed twice in HEPES-TALP medium and once in pre-incubated IVF-TALP (modified Tyrode's medium supplemented with 25 mM sodium bicarbonate, 10 mM sodium lactate, 0.5 mM sodium pyruvate, 0.6% BSA essentially fatty acid free (EFAF), 10 µg mL^{-1} CF-HCl and 2 µg mL^{-1} heparin sodium salt). After washing, the oocytes were transferred into the pre-incubated 0.6 mL fertilization medium (IVF-TALP) in 4-well plates (Falcon®, USA) in groups of 30 per well. To each IVF well, 25 µL of PHE (penicillamine 2mM, hypotaurine 10 mM and epinephrine 0.1 mM) and approximately 35 µL of sperm to a final concentration of 0.5 x 10^6 sperm/mL (Ward *et al.*, 2002) was added. The IVF plates were incubated at 39 °C in a 5% CO_2 humidified air for an average of 20.6 ± 3.1 h. The remaining sperm samples were evaluated for motility, percent viability and normal membrane and acrosome integrity.

4.3.5 *In vitro* culture and evaluation of embryo development

The presumptive embryos were washed twice in 2.5 mL of HEPES-TALP medium in 35 x 10 mm Petri dishes and once in pre-equilibrated culture medium, modified CR1aa (Rosenkrans *et al.*, 1990) (see Appendix A3). They were then transferred in groups of 10 into 30 µL drops of pre-equilibrated modified CR1aa medium under oil and incubated at 39 °C in a 5% CO_2 in humidified air atmosphere for 14 days. The presumptive embryos were cultured without stripping the cumulus cells. These cells attached to the surface of the Petri dish to form a monolayer with which the embryos were co-cultured. Half of the culture medium in the droplets was replaced with fresh pre-incubated medium every second day. A total of 1301 presumptive embryos were cultured. Embryonic development was evaluated on days 3, 5 and 7 for cleavage, morula and blastocyst formation, respectively. Embryos were also evaluated for blastocyst expansion on days 7, 9 and 11, and for blastocyst hatching on days 9, 11, 13 and 15, after which they were discarded. Fifteen day 8 pre-hatched blastocysts were selected and

cryopreserved in 1.5 M ethylene glycol in modified PBS supplemented with 0.4% BSA (American Embryo Systems, Grand Prairie, Texas) using a programmable freezer (Freeze Control® CL863, CryoLogic, Australia), and stored in liquid nitrogen until they were thawed and stained for blastomere counting.

4.3.6 Evaluation of embryonic cell number

Day 8 blastocysts were stained with DAPI (D9564-Sigma), a DNA-binding fluorescent stain, and evaluated as described by Moreira *et al.* (2001) with slight modifications. Briefly, a 0.01 mg/mL DAPI was prepared in 1 mg/mL solution of polyvinyl pyrrolidon (PVP) in PBS (PBS-PVP) and stored wrapped in aluminium foil at 4 °C. This was used within 6 months of storage. Prior to use, this was diluted to 1 µg/mL with PBS-PVP and kept away from direct light (wrapped in aluminium foil). A fixative, 4% (40 mg/mL) solution of paraformaldehyde in PBS (pH 7.4) was also prepared. The straws were thawed in a water bath at 37 °C for one min, and the contents emptied into 2.5 mL of HEPES-TALP in a 35 x 10 mm Petri dish. The embryos were then cultured for 1 to 2 h in modified CR1aa before staining.

A 4-well plate was used in the staining process. The two right wells were filled with 0.5 mL PBS-PVP, the top left with the fixative (0.5 mL) and the bottom left to be filled with 0.5 mL DAPI staining solution. After culture, the embryos were transferred into the top right well containing PBS-PVP and washed once then transferred into the fixative to fix for 30 to 60 min. The staining well was filled with 0.5 mL DAPI at the end of fixing and the embryos transferred into this well where they were stained for 10 min. Staining was followed by washing once in PBS-PVP contained in the right lower well. Stained embryos were kept in this well as they were examined one at a time.

Each embryo was transferred onto a clean microscope slide with a small drop (about 10 µL) of the PBS-PVP medium then examined first using bright field, and then UV light on an inverted microscope (Olympus® 1x70, Tokyo, Japan). A cover slip was very gently placed over the embryo to spread and make it possible to focus on all of the blastomeres concurrently. The image was captured by a camera (Colorview®, Soft Imaging System, GmbH, Germany) attached to the microscope, and displayed on a computer monitor by a software system (AnalySIS®, Soft Imaging System, GmbH, Germany). The DNA in the nuclei of blastomeres appeared bright-blue under UV light. At X100 or X200 magnification, the cells were counted using the Touch-count program of the AnalySIS software and snapshots were also recorded. In cases where blastomeres spread beyond the focus of the camera, and thus could not be displayed on the computer monitor, the blastomeres were counted directly.

4.3.7 Statistical analysis

The data on embryo development were collected from six replicates of bovine embryo cultures arising from fertilization with sperm treatments BIL 1, BIL 2, MTL 1 and MTL 2. The data captured in Microsoft Excel were exported to SAS (version 9.1, 2006) and analyzed using the generalized linear module (GENMOD) procedure. The influence of pre-culture conditions (ovary transport temperature and duration, and oocyte maturation and insemination duration) on embryo development was also analyzed using GENMOD. Differences of least square means were used to determine the correlation between embryo development and the respective sperm treatments. The data on blastomere counts were analyzed by a general linear model (GLM) procedure and the Benferroni test used for least square means adjustment for multiple comparisons.

4.4 Results

Table 4.1 shows the concentration and sperm fertilizing potential parameters of epididymal spermatozoa from the four bulls before the spermatozoa were subjected to freeze-thaw cycles one and two. All the samples showed strong to very strong wave motion and mass motion of 85% and above.

Table 4.1 Evaluation of freshly collected epididymal sperm from four bulls.

Bull	Concentration $x10^6$	% mass motility	% viable sperm	% normal membrane	% normal acrosome
1	980	90.0	88.2	79.1	95.0
2	990	95.0	93.6	85.5	96.6
3	1270	85.0	86.4	89.3	97.7
4	1890	95.0	87.8	79.7	97.1
Mean	1283.0 ± 426.7	91.3 ± 4.8	89.0 ± 3.2	83.4 ± 4.9	96.6 ± 1.2

Figure 4.1 shows the effects of freezing and refreezing bovine epididymal sperm on sperm motility, viability, and membrane and acrosome integrity, based on subjective evaluation. The post-thaw values of freeze-thaw cycle one for both Biladyl (BIL 1) and modified Tyrode's lactose medium (MTL 1) before swim-up (BS) and after swim-up (AS) were lower ($P < 0.05$) than those of the fresh spermatozoa. These were, however, higher ($P < 0.05$) than the values after freeze-thaw cycle two. The post-thaw sperm values before swim-up were low but improved after swim-up in both freeze-thaw cycle one and two. However, apparently the

percentages never reach that of fresh spermatozoa, except motility after swim-up of spermatozoa cryopreserved in Biladyl in freeze-thaw cycle one. Of all the post-thaw sperm parameters examined, the motility seemed to improved best following swim-up.

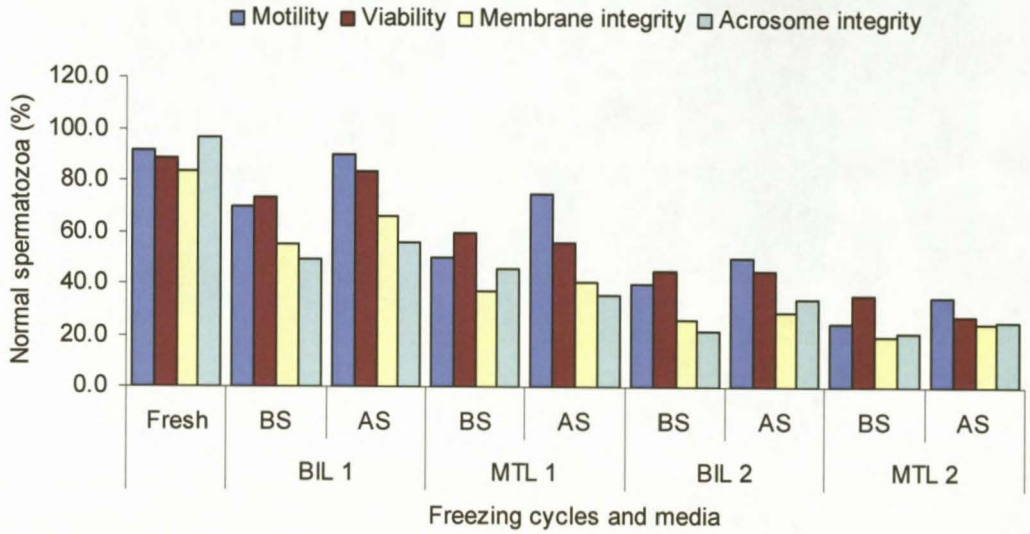

Fig. 4.1 Quality of fresh and frozen-thawed epididymal sperm of freeze-thaw cycles one and two. The spermatozoa were evaluated before swim-up (BS) and after swim-up (AS) following cryopreservation in BIL and MTL in freeze-thaw cycle one (BIL 1 and MTL 1), and two (BIL 2 and MTL 2), respectively.

Table 4.2 Embryo development from epididymal sperm of freeze-thaw cycles one (BIL 1 and MTL 1), and two (BIL 2 and MTL 2) evaluated based on cleavage rate (CR), morula (MOR), blastocyst (B), and expanded (XB) and hatched (HB) blastocyst on the corresponding days in brackets. Day 0 was oocyte insemination day.

		Embryo development (%)						
Sperm treatments	No. of oocytes cultured (day 1)	CR (Day 3)	MOR (Day 5)	B (Day 7)	XB/HB (Day 9)	XB/HB (Day 11)	HB (Day 13)	HB (Day 15)
BIL 1	302	87.1[a]	69.2[a]	63.6[a]	26.2[a]	28.5[a]	20.2[a]	20.5[a]
MTL 1	332	73.2[b]	54.5[b]	50.9[b]	24.1[a]	25.3[a]	18.1[a]	19.3[a]
BIL 2	329	54.4[c]	31.9[c]	29.2[c]	9.4[b]	10.0[b]	5.5[b]	6.1[b]
MTL 2	338	57.7[c]	41.1[d]	37.0[d]	16.9[c]	15.7[c]	12.4[a]	13.3[a]

[a,b,c,d]Values within columns with different superscripts differ significantly ($P < 0.05$).

Table 4.3 Embryo development from epididymal spermatozoa of freeze-thaw cycles one (BIL 1 and MTL 1), and two (BIL 2 and MTL 2) beyond day 7 post-insemination evaluated for blastocyst expansion (XB) and hatching (HB) based on number of blastocysts on day 7.

		Embryo development (%)						
Sperm treatments	No. of blastocysts (Day 7)	XB (Day 7)	XB/HB (Day 9)	HB (Day 9)	XB/HB (Day 11)	HB (Day 11)	HB (Day 13)	HB (Day 15)
BIL 1	176	15.3[a]	44.9[a]	13.1[a]	48.9[a]	29.5[a]	34.7[a]	35.2[a]
MTL 1	155	15.5[a]	51.6[a]	11.0[a,b]	54.2[a]	31.6[a]	38.7[a]	41.3[a]
BIL 2	88	6.8[b]	35.2[b]	6.8[b]	37.5[b]	13.6[b]	20.5[b]	22.7[b]
MTL 2	118	16.1[a]	48.3[a]	10.2[a,b]	44.9[a,b]	19.7[a]	35.6[a]	38.1[a]

[a,b]Values within columns with different superscripts differ significantly ($P < 0.05$).

Table 4.4 Correlations of rates of cleavage (CR), morula (MOR), blastocyst (B), blastocyst expansion (XB) and blastocyst hatching (HB) with pre-culture conditions in development of embryos after insemination with sperm that underwent one and two freeze-thaw cycles in BIL and MTL.

		Embryo development (%)					
Pre-culture conditions	CR (Day 3)	MOR (Day 5)	B (Day 7)	XB/HB (Day 9)	XB/HB (Day 11)	HB (Day 13)	HB (Day 15)
Transport temp	P = 0.4982	P = <u>0.0082</u>	P = <u>0.0355</u>	P < <u>0.0001</u>	P = <u>0.0017</u>	P = <u>0.0063</u>	p=9492
Transport time	P = 0.0732	P = 0.3897	P = 0.5506	P = 0.1145	P = 0.7119	P = 0.0609	p=8537
IVM time	P = 0.2329	P = 0.2073	P = 0.1070	P = 0.9439	P = 0.2181	P = 0.9970	p=6910
IVF time	P = 0.3423	P = <u>0.0062</u>	P = <u>0.0029</u>	P = 0.4010	P = 0.0760	P = 0.3169	p=2939

The underlined p-values showed significant correlations of pre-culture conditions with embryo development.

Bovine epididymal spermatozoa refrozen in egg yolk-based BIL and MTL supplemented with 20% foetal bovine serum and 0.95 M ethylene glycol resulted in *in vitro* embryo development. However, the rates of embryo development were lower than that from sperm that underwent single freeze-thaw cycle (Table 4.2 and Table 4.3). The cleavage rates of all treatments were lower ($P < 0.0001$) than that of the control (BIL 1). There were also differences ($P < 0.0001$) in cleavage rates between treatments, except BIL 2 and MTL 2 that were not different ($P > 0.05$) (Table 4.2). No pre-culture condition correlated ($P > 0.05$) with cleavage rates (Table 4.4). Morula and blastocyst rates of all treatments were different ($P < 0.05$) from each other and from that of the control. Ovary transport temperature and the duration of insemination were correlated ($P < 0.05$) with morula and blastocyst rates.

Spermatozoa of freezing cycle two (BIL 2 and MTL 2) were different ($P < 0.05$) from spermatozoa of freezing cycle one (BIL 1 and MTL 1) in blastocyst expansion/hatching on days 9 and 11 (Table 4.2). Spermatozoa of freeze cycle two were also different from each other, while those of freeze cycle one were not different from each other in blastocyst expansion/hatching. Ovary transport temperature were correlated with embryo expansion and/or hatching on days 9, 11 and 13 ($P < 0.05$), but not day 15 ($P > 0.05$).

A closer evaluation of embryo development on and after day 7 was based on the number of blastocysts on day 7 (Table 4.3). Some blastocysts had expanded by day 7 (Table 4.3) and there were no differences ($P > 0.05$) between treatments MTL 1, MTL 2 and the control (BIL 1) in blastocyst expansion on day 7. However, blastocyst expansion on day 7 was lower ($P < 0.05$) in treatment BIL 2 than those of the rest treatments and the control. Similarly, there were no differences between treatments in blastocyst expansion/hatching on day 9, except those of treatment MTL 2 that were lower ($P < 0.05$) than those of other treatments and the control. The number of embryos that had hatched on day 9 differed ($P < 0.05$) between treatment BIL 2 and the control, but not between the rest of treatments. The number of embryos that had expanded/hatched by day 11 was lower in BIL 2 than MTL and the control ($P < 0.05$). Similarly, embryo hatching on days 11, 13 and 15 were lower in BIL 2 ($P < 0.05$) than the control and other treatments, which were otherwise not different from each other.

Figure 4.2 shows embryo development rates from cleavage on day 3 to the hatched blastocysts on day 15 based on the number of presumptive embryos cultured on day 1. There was a progressive decrease in development up to the blastocyst stage on day 7, after which development rate appeared not to change up to the hatched blastocyst on day 15. However, when embryo development on and after day 7 was assessed based on the number of blastocysts on day 7 (Fig. 4.3), there was a progressive increase in blastocyst expansion and hatching rates up to day 15. It was noted that most blastocysts expanded/hatched between days 9 and 13, and there was no difference ($P > 0.05$) in blastocyst hatching on days 13 and 15.

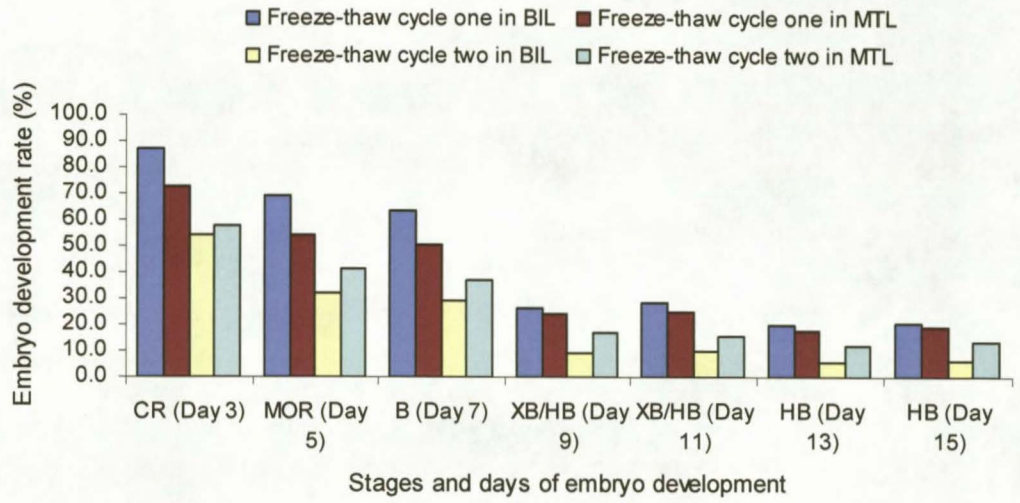

Fig. 4.2 Development of embryos from epididymal sperm of freeze-thaw cycles one and two.

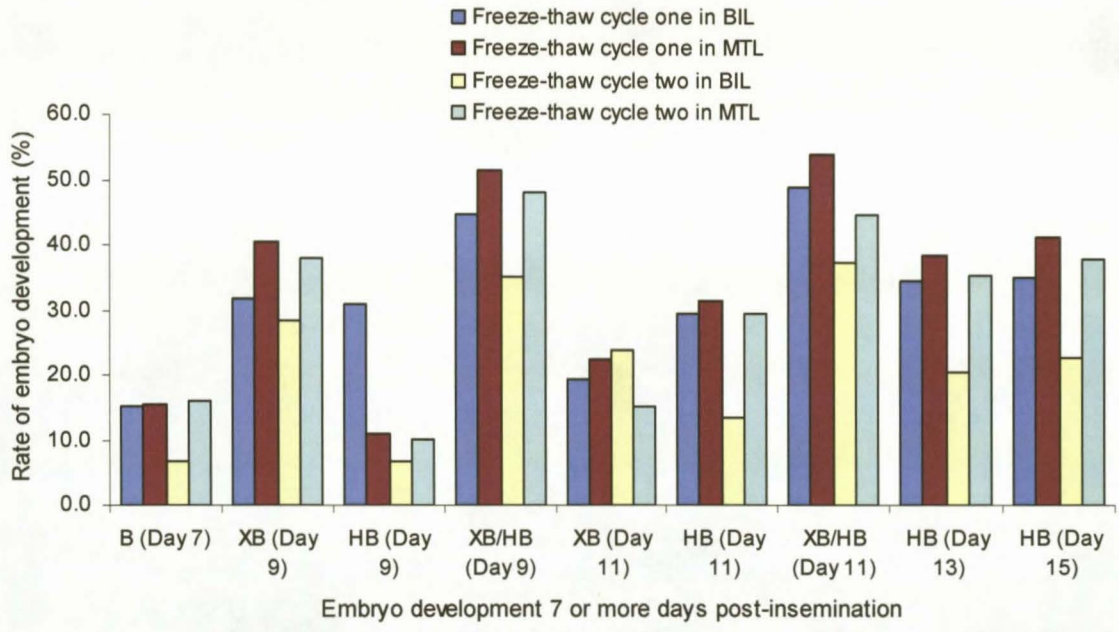

Fig. 4.3 Development of embryos resulting from epididymal spermatozoa of freeze-thaw cycles one and two beyond day 7 post-insemination.

Day 0	=	Insemination day
CR	=	Cleavage rate
MOR	=	Morula rate
B	=	Blastocysts
XB	=	Expanded blastocysts
HB	=	Hatched blastocysts
XB/HB	=	Expanded and hatched blastocysts

Figs. 4.4 and 4.5 show a DAPI-stained blastocyst from spermatozoa that underwent one freeze-thaw cycle in BIL. As the embryo was examined using UV light for blastomere count, the nuclei of the blastomeres appeared bright blue.

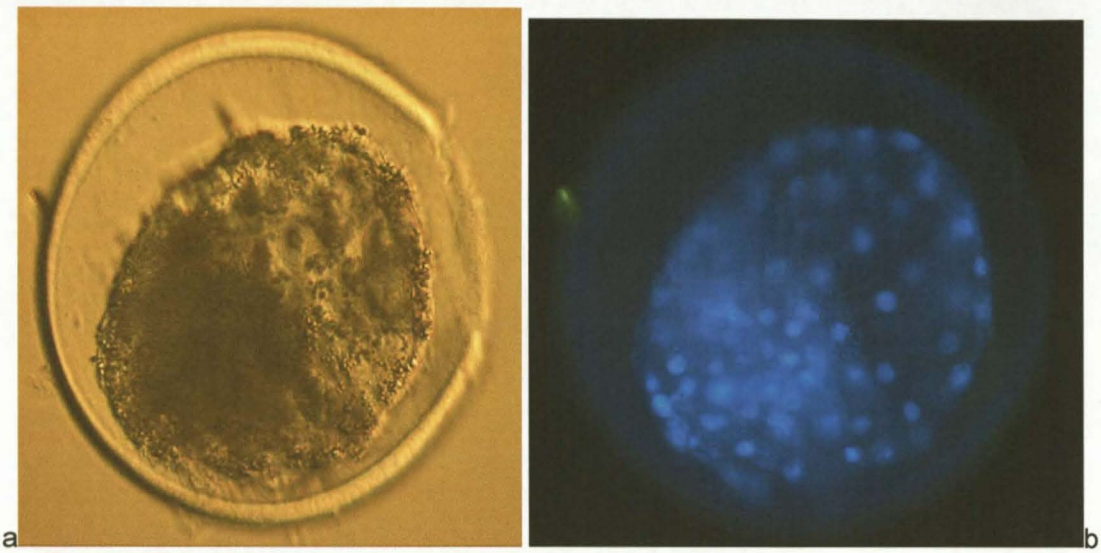

Fig. 4.4 A DAPI-stained day 8 embryo from spermatozoa cryopreserved in BIL (a) under bright field and (b) UV light before placing cover slip on the embryo to spread the blastomeres for counting (X200 magnification).

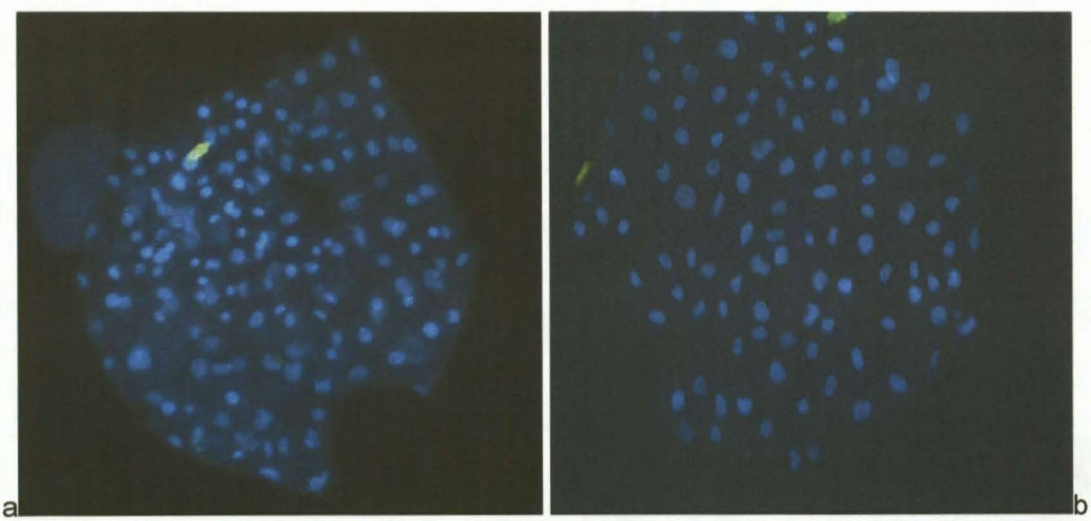

Fig 4.5 A DAPI-stained day 8 embryo after placing a cover slip on the embryo to spread the blastomeres for cell count (a) at X100 (b) at X200 magnification.

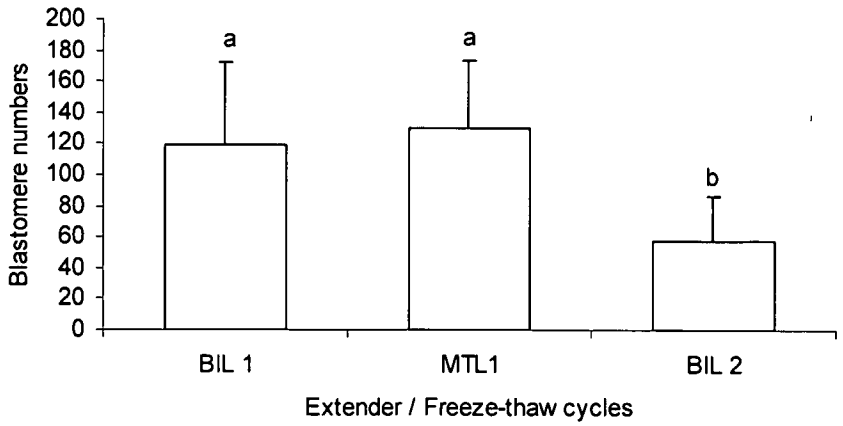

Fig. 4.6 Blastomere numbers of day 8 embryos from freeze-thaw cycles one (BIL 1 and MTL 1) and two (BIL 2).

Figure 4.6 shows the blastomere count of day 8 embryos. Embryos from treatments BIL 1, BIL 2 and MTL 1 had an average blastomere number of 109 ± 51.3, and a range of 32 – 182 (Fig. 4.4). The blastomere number for treatments BIL 1 (119 ± 52.8, n = 7) and MTL1 (130 ± 43.3, n = 9) were not different ($P > 0.05$). However, blastomere number for treatment BIL 2 (58 ± 28.9, n = 5) was lower than that for treatments BIL 1 and MTL 1 ($P < 0.05$). Treatment MTL 2 was not evaluated as very few blastocysts were recovered after cryopreservation.

4.5 Discussion

The study demonstrated that bovine epididymal spermatozoa quality in terms of motility and percent viability, normal membrane and acrosome integrity, markedly decreased when refrozen (Fig. 4.1). There was a progressive decline in all these parameters through the first to second freeze-thaw cycles. This agrees with James (2004) who found that bovine epididymal sperm stored within the epididymides of the testis for 24 h at 4 °C did not survive freeze-thaw cycles well. In the current study the testicles were transported in an insulated container with an icepack, the spermatozoa were collected, processed and cryopreserved on the same day. The poor survival of bovine epididymal spermatozoa is, therefore, likely to be inherent and not due to storage at 4 °C. Furthermore, the reports that epididymal sperm is more resistant to cryo-injuries than ejaculated sperm (Lasley and Bogart, 1944; Johnson *et al.*, 1980; Berger and Clegg, 1985), may only apply to one freeze-thaw cycle. This study, however, did not compare epididymal sperm with ejaculated sperm.

Swim-up is a procedure widely used to select spermatozoa for use in ART. The swim-up procedure has been reported to allow for the selection of spermatozoa with progressive motility, normal morphology or even those with undamaged DNA (Isachenko *et al.*, 2004; O'Connell *et al.*, 2003). However as shown in Fig. 4.1, swim-up improved ($P < 0.05$) post-thaw sperm motility, but viability, and membrane and acrosome integrity improved to a lesser extent. Perhaps some of the motile spermatozoa selected by swim-up were morphologically damaged by cryo-injury rendering parameters such as membrane and acrosome integrity less improved after swim-up than motility. Berndtson *et al.* (1981) showed for ejaculated bull sperm that post-thaw motility and acrosomal integrity constituted distinct and separate features of spermatozoal integrity that varied independently of each other. The current study has shown that the sperm parameters, including motility, never improved after swim-up to pre-freezing level, showing that refreezing results in an increased sperm damage.

The study also demonstrated that despite low survival after the second freeze-thaw cycle, spermatozoa from one and two freeze-thaw cycles produced embryos *in vitro*. The cleavage rates for MTL 1 (73.2%), MTL 2 (57.7%) and BIL 2 (54.4%) were lower ($P < 0.0001$) than that of the control, BIL 1 (87.1%). In addition, the cleavage rates for BIL 2 and MTL 2 (freeze-cycle two) did not differ ($P > 0.05$) from each other. Evaluation of *in vitro* fertilization efficiency can be based on the cleavage rate (Gordon, 1994). It's only the morphologically normal spermatozoa that, in the current study, survived the process of freeze-thawing, would fertilize an oocyte and result into cleavage. A normal sperm membrane is necessary for sperm-zona binding that triggers the acrosome reaction, which result in penetration of the zona (Gordon, 1994). In human sperm, it has been shown that freeze-thawing significantly damages not only the sperm's morphology and membrane integrity, but the chromatin as well (Royere *et al.*, 1988; Hammadeh *et al.*, 1999). The fertilizing sperm contributes three critical components to the oocyte, namely oocyte activation, the haploid chromatins and the centrosome which are vital for cleavage of the fertilized oocyte (Hewitson and Schatten, 2003). Sperm chromatin cryo-damage may lead to DNA fragmentation that has been reported to correlate negatively with fertilization rates in IVF (Sun *et al.*, 1997).

The findings of this study also showed that the egg yolk-based glycerol-containing Biladyl offered better protection to preserve the fertilizing potential of spermatozoa than serum-based ethylene glycol-containing MTL during freeze-thaw cycle one. However, the cryoprotection by both extenders seem to be poor in freeze-thaw cycle two, with BIL showing poorer cryoprotection than MTL as noted from the cleavage rates. The role of serum components in cryopreserving sperm fertilizing potential is not clear. However, egg yolk is

thought to cryoprotect membrane structures as the low-density lipoprotein fraction, glycolipids and cholesterol may be incorporated into the membranes, reducing the tendency to form a gel during cooling (Watson, 1976; Parks and Lynch, 1992). Furthermore, the egg yolk-glycerol medium binds the nitrite (NO^-), oxygen (O_2^-) and hydrogen peroxide (H_2O_2) radicals (Chatterjee and Gagnon, 2001). There could, therefore, be a dual action whereby the membranes are coated and isolated from the cryoprotective agents while preserving their fluidity and flexibility at low temperature, thereby reducing the cytoskeletal damage (Isachenko *et al.*, 2004). The bovine serum in MTL probably acts in the same way, but the sperm response to these cryoprotective roles seem to be markedly reduced in subsequent freeze-thaw cycles in both media.

Cleavage rates of 87.1% and 73.2% were obtained for embryos resulting from spermatozoa of freeze-thaw cycles one in BIL and MTL, respectively. The cleavage rates for freeze-thaw cycle one were comparable to that obtained from epididymal spermatozoa reported elsewhere in bovine, caprine and rabbit species. Park *et al.* (1999) found an average cleavage rate (≥ two-cell) of 82.3% in a bovine IVF study. Using cauda epididymal sperm Melican *et al.* (2001) obtained an overall cleavage rate of 67% from transgenic goats, while Brackett *et al.* (1978) reported a fertilization rate of 73.1% in a rabbit IVF study.

Although the cleavage rates of embryos from spermatozoa of freeze-thaw cycles two in both extenders in this study were low, 54.4 % and 57.7% for BIL and MTL, respectively, these were higher than the 28% reported by Horiuch *et al.* (2002) for ICSI using cryo-damaged bovine sperm. This low cleavage rates for freeze-thaw cycle two could be associated with DNA damage during repeated cryopreservation. According to Lopes *et al.* (1998), the proportion of spermatozoa with fragmented DNA negatively correlated with fertilization rates in ICSI. A fertilization rate of 90%, i.e. two pronuclei and two polar bodies, was attained in a study by Horiuch *et al.* (2002). However, in that study many fertilized oocytes did not cleave (Horiuch *et al.*, 2002). Similarly, cleavage failure of some otherwise fertilized oocytes could have occurred in the current study because assessment of fertilization by pronuclei and polar body formation was not performed, as assessment of fertilization was based on cleavage rate.

The comparably low cleavage rates for the second freeze-thaw cycles were also attributed to sperm refreezing cryo-injury that could have caused extensive morphological and/or chromatin damage. In human sperm, Hammadeh *et al.* (2001) reported the freeze–thawing procedure has a detrimental effect on chromatin, morphology, membrane integrity, and vitality of sperm. Furthermore, the cytoskeletal elements of spermatozoa are reported to be

sensitive to cooling (Holt and North, 1991). Because of their importance in fertilization, the detrimental effects of freeze-thawing on these structures would result in low fertilization rates, hence low cleavage rates as some oocytes would not cleave due to the damaged chromatins and centrosomes. In human ART, up to 25% of the otherwise unfertilized oocytes 24 hpi were actually penetrated by one or more sperm but embryonic development had arrested at some point (Asch *et al.*, 1995). Epifluorescent, laser-scanning confocal microscopy of these arrested oocytes suggested defects in the sperm centrosome as one cause of fertilization failure (Hewitson and Schatten, 2003).

The current study used the swim-up method to prepare sperm for refreezing and for *in vitro* fertilization. The cleavage rates of freeze-thaw cycle one were comparable to that of 71% obtained form frozen-thawed ejaculated semen prepared by swim-up method in a study by Seidel *et al.* (1995). Sperm preparation by swim-up results in a higher percentage of motile sperm than other methods of sperm preparation for ART (Gordon, 1994; Seidel *et al.*, 1995). Sperm swim-up was also reported by Isachenko *et al.* (2004) and O'Connell *et al.* (2003) to allow the selection of spermatozoa with progressive motility, normal morphology or even those with undamaged DNA and centrosomes. In this study, however, spermatozoa membrane and acrosome integrity did not improve after swim-up as much as motility (Fig. 4.1). Some of the motile selected sperm could have had damaged membranes and acrosome. Probably some of the fertilizing sperm had a damaged cytoskeleton or DNA, especially after the second freeze-thaw cycle, which could have contributed to the reduced cleavage rates from spermatozoa of two freeze-thaw cycles.

There was a decline in embryo development from day 3 (cleaved oocytes) to day 5 (morula) in all treatments ranging from 16.6 % (MTL 2) to 22.5% (BIL 2). However, little difference was seen between days 5 (morula) and 7 (blastocysts). In humans, according to Gardner and Schoolcraft (1998), the developmental potential of an embryo is basically determined by the normalcy of the gametes from which it is derived. In the same context, these authors further reported that a significant percentage of gametes lack normal developmental competence because they are genetically or chromosomally abnormal. Furthermore, Rizos *et al.* (2002) demonstrated that the intrinsic quality of the oocyte is the main factor affecting blastocyst yields in bovine IVF. The highest decline between cleaved oocytes and morula was seen in BIL 2, which could be associated with cryo-damage on some fertilizing-spermatozoa DNA as was earlier reported by Gardner and Schoolcraft (1998). Fertilization with abnormal cryo-damaged spermatozoa could results in the absence or inadequate activation of oocytes.

Failure in genomic activation in humans, and probably cattle, may result in the arrest of embryos at 8-cell to morula stages (Bongso and Gardner, 2000).

Ovary transport temperature and insemination duration correlated ($P < 0.05$) with morula rates. It is unlikely that these pre-culture conditions temperature contributed to the arrest of development of some embryos to morula, because the ovaries were transported at 32.9 ± 2.6 °C, and insemination time was on average 20.6 ± 3.1 h. Studies in cattle IVF recommended transportation of ovaries at about 33 °C in sterile PBS or normal saline (0.9% NaCl), and recovery of oocytes within 8 h from slaughter (Sato *et al.*, 1990; Takagi *et al.*, 1992; Hamano and Kuwayama, 1993). Probably, insemination duration could have been responsible for the decline in embryo development, for Ward *et al.* (2002) demonstrated that sperm-oocyte co-incubation for 10 h is sufficient to ensure maximal embryo yields.

A morula rate of 69.2% and blastocyst rate of 63.6% for the control were significantly higher than those of the other treatments, and differences also existed between the treatments (Table 4.2). The lowest morula and blastocysts rates (31.9% and 29.2%) were recorded for BIL 2, followed by MTL 2 (41.1% and 37.0%), while those of MTL 1 were 54.5% and 50.9%, respectively. The blastocyst rates of freeze-thaw cycles one were similar to those obtained in Chapter 3. The blastocyst rates of freeze-thaw cycle two were comparable to, and those of cycle one were higher than, those obtained by Jiang *et al.* (1991) and Maeda *et al.* (1996). The high rate of embryo development in freeze-thaw cycle one was probably due to the excellent cryoprotection of sperm fertilizing ability by BIL and MTL during single freeze-thaw cycle. Furthermore, in all treatments most of the day 5 morulae developed into blastocysts, and some had expanded by day 7. This could be attributed to the excellent culture system used. This study used CR1aa (Rosenkrans *et al.*, 1990) supplemented with 3 mg/mL (0.3% w/v) BSA essentially fatty acid free (EFAF) fraction and 5% v/v FBS. The presence of differential embryotrophic factor(s) in BSA and serum necessary for promoting competent morula and blastocyst development in cattle embryos was demonstrated by Sung *et al.* (2004). Perhaps the combination of BSA and FBS provided an efficient embryo culture system to achieve the high rate of embryo development from the morula to the blastocyst stage. Furthermore, the embryos were co-cultured with cumulus cells. The cumulus cells surrounding the oocytes attached to the surface of the Petri dish during the embryo culture to form the co-culture system. The intact state of surrounding cumulus cells of oocytes or embryos is beneficial for maturation, fertilization and possibly early embryo development shortly after insemination (Zhang *et al.*, 1995). Fukuda *et al.* (1990) and Nakao and Nakatsuji (1990) successfully demonstrated the use of cumulus cells surrounding the oocytes as co-culture in bovine IVF. Kane *et al.* (1992)

and later Bavister (1995) reported that somatic cells could provide embryonic growth factors and/or remove embryotoxic substances from the culture medium. In the current study the cumulus cells were easier to use because they were readily available as part of the routine *in vitro* embryo culture procedure as reported by Gordon (1994).

The low morula and blastocyst rates after freeze-thaw cycle two, especially for treatment BIL 2, were probably due to the influence of fertilization with cryo-damaged spermatozoa on embryo development. Although swim-up recovers highly motile spermatozoa, other structures such as the organelles, particularly the chromatin content and centrosome could have been severely damaged after freeze-thaw cycle two. Chromosomally abnormal gametes lack normal developmental competence (Gardner and Schoolcraft, 1998). Cryo-damage to the sperm DNA could have been extensive in the freeze-thaw cycle two, and this could be responsible for development arrest in treatment BIL 2 and MTL 2. Such development arrest could have occurred during the genomic activation due to epigenetic effects of defective sperm (Elder and Dale, 2000). The defective sperm in the current study could have been caused by damage during the cryopreservation procedure.

Embryo development beyond day 7 continued to be best in the control (BIL 1), but there were no significant differences ($P > 0.05$) between MTL 1, MTL 2 and the control, except for the embryos that expanded/hatched on days 9 and 11 (Table 4.2, Table 4.3, and Fig. 4.2). A similar embryo development trend was also observed on the day 7 blastocysts that expanded and/or hatched thereafter. In all these, development of embryos of treatment BIL 2 was significantly lower ($P < 0.05$) than other treatments. Considering that all other factors were constant in all treatments, except sperm freezing and the freezing media used, sperm cryo-damage could be the cause of the poorer embryo expansion and hatching in BIL 2 than BIL 1, MTL 1 and MTL 2. According to Elder and Dale (2000), sperm quality may have an influence on embryogenesis and implantation potential. Evaluation of implantation potential can be based on embryo expansion and hatching. Brandao *et al.* (2004) reported that the *in vitro* culture of embryos to expansion and hatching can precisely predict the potential development of a bovine conceptus.

There was a marked decline in embryo development rates between the blastocyst stage (day 7) and the expanded/hatched blastocyst stage (day 9) ranging from 19.8% for BIL 2 to 37.4% for BIL 1. Though there was little variation in embryo development after day 9, generally, the rates of embryo expansion and hatching were lower than reported for bovine IVF by Wang *et al.* (1992). The lower rate of embryo development recorded in this study could be attributed

to the epigenetic effects of defective spermatozoa on embryo development, similarly reported by Elder and Dale (2000). There is no doubt that the freeze-thaw cycle, particularly when repeated, caused extensive cryo-injury to the spermatozoa such as the sperm's DNA damage. Probably, some effects of sperm cryo-injury are expressed later in embryonic development, perhaps, in the form of developmental retardation or arrest. It was earlier stated by Gardner and Schoolcraft (1998) that the developmental potential of an embryo is basically determined by the normalcy of the gametes from which it is derived. Although Rizos *et al.* (2002) demonstrated the intrinsic quality of the oocyte as the main factor affecting blastocyst yields in bovine IVF, in this study the ovary and thus the oocyte was a constant factor as they were derived from the same source.

In the current study the embryo culture medium was CR1aa which contained energy substrates such as pyruvate, lactate and glutamine in addition to a protein source (3 mg/mL BSA and 5% v/v FBS). The low expansion and hatching rates recorded in this study could be attributed to the culture medium. In human IVF, it has been observed that the mean mitotic index gradually decreased, while the dead cell index increased, when embryos cleaved from days 5 to 7 (Bongso and Gardner, 2000). Such observations on increased cell death with reduced mitotic index by day 7 were suggestive of inadequate culture conditions for blastocyst development (Hardy, 1993). The low rate of blastocyst development in the current study could further be explained by the fact that the culture medium (CR1aa) lacked glucose. A report by Javed and Wright (1991) showed that glucose utilization by bovine embryos is low until 16-cell stage, after which utilization significantly increase. Renard *et al.* (1978) found that day 10 cattle embryos that had a high rate of glucose utilization, developed more readily *in vitro* and *in vivo* than did embryos utilizing little or no glucose, regardless of their morphological appearance. Renard *et al.* (1980) suggested that glucose may be essential for the hatching of bovine embryos. Lonergan *et al.* (2001) reported that events further back along the developmental axis determine the proportion of immature oocytes reaching the blastocyst stage, including expansion and hatching. Such factors, for instance the age of the cows/heifers from which the ovaries were collected, were not considered in the current study. Looney *et al.* (1995) demonstrated a significant difference in embryo development from oocytes collected from pre-pubertal heifers and mature cows.

The average number of blastomere counted for day 8 embryos was 109 ± 51.3 and ranged from 32 to 182. The blastomere number for BIL 1 (119 ± 52.8, n = 7) and MTL1 (130 ± 43.3, n = 9) did not differ ($P > 0.05$). However, blastomere number for treatments BIL 1 and MTL 1 were significantly higher ($P < 0.05$) than the blastomere number for treatment BIL 2 (58 ±

28.9, n = 5). The blastomere numbers of freeze-thaw cycle one (BIL 1 and MTL 1), but not freeze-thaw cycle two (BIL 2), were comparable to those reported by Jiang *et al.* (1991, 1992) and Stojkovic *et al.* (2002) for bovine IVF. Embryos of treatment BIL 2 could have had retarded development due to the epigenetic effects of sperm defects that was associated with cryo-injury due to repeated freeze-thawing as reported by Elder and Dale (2000). However, MTL 2 embryo cell count could not be included in the analysis as the embryos recovered were too few, therefore, the effects of repeated freeze-thawing in this medium could not be confirmed. The high blastomere numbers in BIL 1 and MTL 2 indicated that although CR1aa contains no glucose, an important energy substrate in late embryonic development (Javed and Wright, 1991; Renard *et al.*, 1978, 1980), some embryos are competent enough to develop to the hatched blastocyst stage. Donnay *et al.* (2002) observed a slight decrease in embryonic cell number at day 7 in the presence of glucose, while neither the inner cell mass : trophoblastic cell ratio nor the apoptotic index were affected.

4.6 Conclusion

This study demonstrated that bovine epididymal spermatozoa cryopreserved in Biladyl® and modified Tyrode's lactose supplemented with bovine serum and ethylene glycol can be refrozen in the same media after swim-up and can be used to produce embryos *in vitro*. Survival of spermatozoa is, however, markedly reduced after freeze-thaw cycle one and the rate of embryo production is consequently lower for spermatozoa refrozen in the respective media. Bovine epididymal spermatozoa can undergo two freeze-thaw cycles in Biladyl®, but preferably modified Tyrode's lactose and the sperm can be used in assisted reproductive technology. Further research on *in vivo* embryo production and pregnancy rates using bovine epididymal sperm subjected to two or more freeze-thaw cycles is recommended.

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CHAPTER 5

In vitro production of bovine x buffalo hybrid embryos using bovine oocytes and African buffalo (*Syncerus caffer caffer*) epididymal sperm

5.1 Abstract

Interspecies hybridization of bovids occurs between domestic cattle and at least three other species; American bison (*Bison bison*), yak (*Bos grunniens*) and banteng (*Bos banteng*). Birth of a cattle x buffalo (*Bubalus bubalis)* hybrid was reported in Russia and China, but these reports were never authenticated. Such hybrids could be important in improving livestock production and management of diseases that impede production in tropical Africa. This study investigated hybridization between cattle and its closest African wild relative, the African buffalo (*Syncerus caffer caffer*). In an attempt to produce pre-implantation cattle x buffalo hybrid embryos *in vitro,* matured bovine oocytes were subjected to a standard *in vitro* fertilization (IVF) procedure with either homologous bovine (n = 1166 oocytes) or heterologous buffalo (n = 1202 oocytes) frozen-thawed epididymal sperm. After IVF, 67.2% of the oocytes inseminated with the homologous sperm cleaved. In contrast, insemination with buffalo sperm resulted in a 4.6% cleavage rate ($P < 0.0001$). Cleavage rate was also slower in hybrids than in cattle embryos. Up to 52.2% of the cleaved homologous embryos progressed to the morula stage, compared with 12.7% for the hybrid embryos. No hybrid embryos developed beyond the 16-cell stage, while 40.1% of the cleaved bovine embryos developed to the blastocyst stage ($P < 0.0001$). Transfer of the hybrid morula stage embryos to cattle surrogates caused no pregnancy. Developmental anomalies such as polyspermy, uneven cleavage, vacuolization and absence of nuclei in some blastomeres were common in the hybrid embryos. It was concluded that interspecies fertilization of cattle oocytes with African buffalo epididymal sperm occurs *in vitro,* and that the barrier to hybridization occurs in the early stages of embryonic development. Chromosomal disparity is possibly the cause of the fertilization abnormalities, abnormal development and the subsequent arrest impairing the formation of pre-implantation hybrid embryos. Transfer of the hybrid embryos did not rescue the embryos from development arrest. Investigation into the developmental abnormalities including reciprocal hybridization and genetic studies of the hybrid embryos are recommended.

5.2 Introduction

In view of the ever increasing global human population and the demand for food, human being must utilize all the possible resources for survival, for instance improving animal production. In tropical Africa, however, diseases such as trypanosomiasis and theileriosis and the control thereof, pose major constraints in areas which have the greatest potential for significant increases in domestic livestock populations and livestock productivity. While programmes for eradication of these diseases from the entire continent are unrealistic, considerable effort has been invested in the control of these diseases through treatment, vector management and the use of genetically resistant indigenous breeds (D'Ieteren *et al.*, 1998). Attempts towards the development of vaccines against such diseases (Mkunza *et al.*, 1995;

Beniwal *et al.*, 1997) may not succeed in the near future. Furthermore, drug resistance to these diseases is developing faster while the environmental impact of the chemical vector control is enormous.

Selection, crossbreeding and use of disease-tolerant breeds are some of the options for improving livestock production in disease endemic areas in Africa. Some indigenous breeds and their crosses are known to have some degree of resistance to theileriosis (Preston *et al.*, 1992), while other breeds of cattle, particularly the N'Dama, are trypano-tolerant. However, according to Benjamin (1996), crossbreeding of domestic cattle without proper health cover and nutritional support may lead to a senseless waste of both the precious germ plasm assets and liabilities. Innate disease resistance, heat tolerance, cold tolerance and performance capabilities do differ with breeds/species performing under various environmental stresses. Sensible selection and hybridization have been attempted to increase production in tropical Africa while maintaining disease resistance (Syrstad, 1996). Unfortunately, some of the crosses are as susceptible to these diseases as their exotic parents (Paling *et al.*, 1991). The wildlife ungulate relatives of cattle such as the African buffalo (*Syncerus caffer caffer*) and eland (*Tragelaphus oryx*) have for centuries co-existed with the vectors of these tropical African diseases. However, attempts to utilize the gene pool of these cattle-cousins in enhancing cattle disease resistance thus improving production, has been minimal, perhaps, due to interspecies and ethical barriers. Application of the available technologies such as *in vitro* fertilization and gene transfer could help understand and possibly overcome some of the bottlenecks in exploiting such gene pools for improving domestic livestock productivity.

Quantification of traits, selection, heritability estimates, and planned mating are animal breeding biotechnologies that have played and still continue to play an important role in selection and propagation of desirable and economically important characteristics in livestock. Selection, according to Wheeler and Campion (1993), does not create new genes but increase the frequency of desirable genes in the population, and decrease the frequency of less-desirable genes. These authors further argued that genetic improvement will plateau using selection alone, but if combined with molecular genetics, greater genetic progress will be achieved. The ability to select, breed, and genetically engineer livestock for disease resistance will have a tremendous effect on the efficiency of milk and meat production. In a variety of animal species, the major genes that control humoral and cellular immune responses to a variety of antigens have been mapped to a chromosomal region known as the multi-histocompatibility-complex (MHC) (Lewin, 1989). Hanotte *et al.* (2003) mapped the genes probably responsible for trypano-tolerance in crosses between trypano-tolerant West African

N'Dama and trypano-susceptible improved Kenya Boran cattle. Such genes can be propagated through technologies such as production of transgenic animals with an altered genetic makeup for improved disease resistance, thereby improving livestock productivity. The attainment of such improvements will, therefore, depend heavily on the ability to quantify desirable traits, to identify markers linked to gene(s) responsible for those traits, to select or redesign populations of superior individuals, and to propagate those animals.

The production potential of livestock is, perhaps, based on the repertoire of alleles or genes available for recombination. Within a species, the repertoire of alleles can be changed because of extinction of some alleles, or emergence of new alleles through mutation. New alleles can be introduced by hybridization which may even give rise to a new species. Livestock species such as cattle have been intensively selected for traditional production alleles, with consequent stochastic extinction of some potentially beneficial alleles. In the tropics, selection has not been so intense but the introduction of exotic breeds is progressively eroding the genetic base of indigenous cattle (Syrstad and Ruane, 1998). Moreover exotic, and to some extent indigenous, breeds lack desirable attributes such as adaptation to environment in which production is expected to occur. One of the key attributes is resistance to diseases, notably trypanosomiasis, theileriosis, especially East Coast fever (ECF), and helminthiasis. The cost of losses due to these diseases is enormous and constitutes a major constraint to optimal production.

Wild ruminants such as the African buffalo (*Syncerus caffer caffer*) and eland (*Tragelaphus oryx*) have roamed the African savannah for centuries, and are extremely resistant to trypanosomiasis, helminthiasis and ECF (Brocklesby, 1964). African buffalo (*Syncerus caffer caffer*) is the closest wild African relative of the domesticated cattle (*Bos taurus* and *Bos indicus*). However, the diploid number of chromosomes in African buffalo is 52 (Wurster and Benirschke, 1968; Gallagher Jr. and Womack, 1992) compared with 60 in cattle (Hafez, 1993), with extensive band homologies (Gallagher Jr. and Womak, 1992). The African buffalo is well adapted to survive the hordes of parasites that afflict Africa. However, it is reputed to have a mean temperament that, perhaps, prevents its domestication, yet it does not interbreed with cattle. Application of reproductive technologies such as *in vitro* fertilization have been used to overcome some of the problematic events associated with ovulation, fertilization and embryo development, which are prerequisites in the production of interspecies hybrids. In addition, such technologies provide a starting point for the generation of reproductive materials for a number of advanced reproductive techniques such as nuclear transfer in production of interspecies hybrids. The hybrid embryos could also provide materials for gene mapping and

understanding the differences between bovine and buffalo genes that could be responsible for the survival of buffaloes in tsetse- and tick-infested tropical Africa.

Cattle and the African buffalo both belong to family Bovidae but are members of two different species. A species can be defined as a reproductively isolated group of organisms or population with a similar gene pool that interbreed in nature to produce offspring (Strickberger, 1996). Cross-species hybridization has been reported to occur in nature between certain species, but not between cattle and African buffalo. Cross fertilization of domestic cattle (2n = 60) and water buffalo (2n = 50) gametes *in vitro* has been reported, but development failure of the hybrid embryos followed after the first few cell divisions (Kochhar *et al.*, 2002; Patil and Totey, 2003). In Australia, a few cattle and riverine buffalo (2n = 50) hybrid embryos developed to the blastocyst stage (Tatham, 2000). These observations implied that the disparity in chromosome numbers is not the constraint to hybridization of domestic cattle and water buffalo. It is not known whether interspecies fertilization occurs between African buffalo and cattle, and the extent of embryonic development has never been reported.

The potential for the hybridization of African buffalo and cattle could be enormous. A new species more adaptable to, and with better production in the harsh conditions of the African savannah could result from such an interspecies hybridization. For instance, hybrids of cattle and American bison (Solti *et al.,* 2000) and cattle and yak (Gray, 1972) have been reported to perform between, and in some respects better than their parents. In the game and tourism industry, the African buffalo is one of the "Big 5". Although the wild population of African buffalo is stable, many of them are in regions endemic to diseases like tuberculosis, foot-and-mouth disease, corridor disease; thus they cannot be introduced to agricultural areas (Solti *et al.*, 2000). There has, therefore, been a considerable effort for the production of disease-free buffaloes, particularly in South Africa. One suggested method was the application of reproductive biotechnologies to produce buffalo embryos *in vitro* and transfer into cattle surrogates. However, the problem of immunological incompatibility as occurred in interspecies transfer of gaur embryos to cattle surrogates (Hradecky *et al.*, 1988) would make term gestation impossible. If a hybrid of cattle and African buffalo could be produced, the genetic make-up of the hybrid would be between that of the parents and, therefore, is likely to carry term pregnancy of either species. This would make it possible to apply *in vitro* embryo production and transfer technology in wildlife conservation.

Hybrids are the offspring of two closely related breeds, species or genera (Jainudeen and Hafez, 1993). Hybridization between related genera is very rare, while many reports

document hybridization between closely related species occurring in nature. Among the bovidae, a wide variety of antelope hybrids have been recorded in zoos. Mating between a male eland and a female kudu produced a sterile male hybrid that resembled the eland (Gray, 1972; Jorge *et al.*, 1976). Hybrids between Kirk's and Guenther's dik-dik exists, but are infertile (Ryder *et al.*, 1989). Waterbucks, *Kobus ellipsiprymnus* (2n = 50-54), *K. kob* (2n = 48) and *K. megaceros* (2n = 52), according to a comprehensive study by Kingswood *et al.* (1998), appear to have incomplete reproductive isolation because hybridization among them is common. Griner (1983) autopsied two hybrid waterbucks in San Diego, and Gray (1972) also referred to numerous hybrids among these different kob species. Meanwhile, the blue wildebeest and the smaller black wildebeest, both with 2n = 58 (Wurster and Benirschke, 1968; Corbet and Robinson 1991), produce fertile hybrids (Fabricius *et al.*, 1988).

Among the domestic bovidae, sheep (2n = 54) and goats (2n = 60) seem similar and can be mated, but offspring are generally stillborn due to a chromosome mismatch. Recently, the Botswana Ministry of Agriculture reported a nanny goat impregnated by a ram housed with the goat, and a live offspring was produced with 57 chromosomes (Letshwenyo and Kedikilwe, 2000; Mine *et al.*, 2000). This "chimera" was called "The Toast of Botswana" (Schaub, 2006). Although infertile, the hybrid had to be castrated at 10 months of age because it continually mounted sheep and goats in the enclosure. The hybrid at 5 years of age weighed 93 kg compared with 53.73 ± 13.83 kg, the average weight of castrated Tswana male goats of the same age (Mine *et al.*, 2000). These authors raised a question as to whether the animal could be of commercial interest in meat production in Botswana.

Moore *et al.* (1981), produced hybrid embryos between Barbary ram (*Ammotragus lervia)* (2n = 58) and nanny goats (*Capra hircus*) and the domestic ewes (*Ovis aries*), but only one Barbary ram x nanny goat male kid was born with the karyotype of 2n = 59XY. However, a study by Flint *et al.* (1983) based on the concentration of plasma progesterone, ovarian histology and corpus luteum regression showed that the Barbary sheep resembles the domestic sheep rather than the goat.

Among bovids, American Bison bulls (American "buffalo") have been crossed with domestic cattle to produce Beefalo and Cattalo. Domestic cattle and bison have a chromosomal number 2n = 60 with the only difference being that the Y chromosome is acrocentric in bison and submetacentric in cattle (Jainudeen and Hafez, 1993). The Zubron is a hybrid between a domestic cow and a Wisent (European bison, *Bison bonasus*). As such it is analogous to the American Beefalo or Cattalo. First generation Zubron males are infertile and

cannot be used for breeding, but the females are fertile and may be bred back (back-crossed) to either Wisent or to domestic bulls. Males from these back-crosses are fertile. The Bison (American "buffalo") has also been bred with the domestic Tibetan yak to create the Yakalo. The Yakalo females are fertile, the males are sterile and the meat is considered superior to beef. In the Yunnan Province of China, the mithan is a livestock breed created by hybridizing gaur with domestic cattle (Cheng, 1984). The chromosomal number of the mithan (*Bos g. frontalis*; 2n = 58) is midway between that of the gaur (*Bos gaurus*; 2n = 56) and *Bos taurus* (2n = 60) (Solti *et al.*, 2000).

Crossing the river buffalo (*Bubalus bubalis bubalis*, 2n = 50) and the swamp buffalo (*Bubalus bubalis carabanesis*, 2n = 48) results in F1 hybrids with intermediate chromosomal number 2n = 49 between these types of water buffaloes (Bongso and Jainudeen, 1979). Unlike in other hybrids, both male and female hybrid buffaloes are fertile, despite the males having a high percentage of degenerating spermatocytes and abnormal spermatids in their testes (Bongso *et al.*, 1983). A reputed hybrid between domestic buffalo (*Bubalus bubalis*) and cattle was reportedly born in the old USSR, with similar hybrids including fertile females have been reported in China (Mason, 1974). These hybrids were reportedly very similar to their buffalo dams. However, both reports seem doubtful because despite many attempts, no other proven hybrids have ever been produced.

Sterile matings between buffalo (*Bubalus bubalis*) and cattle were reported to be common. However, insemination of 7 cows with buffalo (*Bubalus bubalis*) semen did not result in pregnancy, and domestic bulls failed to fertilize 4 buffalo cows either by natural mating or by artificial insemination (Tatham, 2000). Other studies also failed to obtain hybrids between *Bos taurus* and *Bubalus bubalis* (Gray, 1953). According to Tatham (2000), no case of hybridization between the species has been recorded. Zebu bulls frequently and European bulls not uncommonly, have been seen to mate with buffalo females, but no hybrid offspring have been produced. Buffalo males will occasionally mate with zebu and European cows. No offspring result either from natural mating or from artificial insemination.

Perhaps, it should be noted that the Bovidae family is the most diverse of the nine artiodactyla families, with 45 extant genera and 124 extant species (Vaughan, 1986). The diploid chromosome number (2n) of the bovidae ranges from 30 to 60, but the autosomal arm number (NAA) is relatively constant at 56-58 for most karyotyped bovids (Othman, 2004). This is probably why hybrids between cattle (2n = 60) and water buffalo (2n = 50) or African buffalo

(2n = 52) does not exist. Understanding the barriers to such hybridization requires the application of reproductive technologies in an attempt to produce such hybrids.

Morphological, genetic and behavioural attributes found in hybrid offspring are often shown to be a mix of both parents' features, as well as a few not found in either (Moore *et al.*, 1999). The purpose of crossbreeding or hybridization is normally to combine desirable attributes of breeds or species with a possibility that the performance of the hybrids will exceed the average of the parents (hybrid vigour). Cattle interspecies hybrids of interest are those between cattle and American bison, and cattle and yak. Beefalos and cattalos are fertile hybrids of domestic cattle (*Bos taurus*) and the American bison (*Bison bison*), both with a diploid number of chromosomes being 60 (Jainudeen and Hafez, 1993). The beefalos are very variable in type and colour depending on the breed of cattle used e.g. Herefords and Charolais (beef cattle), Holsteins (dairy) or Brahman (humped cattle). The aim of this hybridisation between domestic cattle and American bison is to produce high protein, low fat and low cholesterol beef. Beefalos combine the superior hardiness, forage ability, calving ease and meat quality of bison with the fertility, milking ability and ease of handling of cattle (Solti *et al.*, 2000). The cattle and yak hybrid surpass their parents in strength and vigour (Clutton-Brock, 1999). Cattle-yak F1 hybrids have higher birth-weights (Zhang, 1984; Zhao, 1984), attain sexual maturity earlier, grow faster and are larger in size, have a better milk yield, (Zhang, 2000), especially if crossed with improved breeds of cattle, than pure yak. Although the hybrid males are sterile, they are very suitable for use as draught animals, for they are easily tamed and have a better meat compared to that of pure yaks (Zhang, 2000).

Despite the successes in hybridization of cattle and bison as well as cattle and yak, it's not known as to what would be the result of a cattle and African buffalo hybrid, if such a hybrid could be produced. Cattle and African buffalo belong to different species. The biological concept according to Strickberger (1996) defined a species as a group of interbreeding individuals normally separated from other species by the absence of genetic exchange, that's by reproductive isolation. Through this, nature imposes interspecies breeding barriers that safeguard the purity of individual species. These barriers or isolating mechanisms may be geographic (allopatric) or may occur within the same geographic locality (sympatric). Mayr (1963) categorized sympatric isolation as pre-mating e.g. seasonal, behavioural or mechanical isolation; and post-mating such as gamete mortality, zygote mortality and hybrid sterility.

The mammalian oocyte is believed to be protected against foreign sperm penetration by a barrier, which operates at the level of the zona pellucida (ZP) and/or the vitelline membrane

(Gordon, 1994). Kouba *et al.* (2001) studied gamete interaction between bovine oocytes and fringe-eared (FE) oryx (*Oryx gazella callotis*) spermatozoa and concluded that species-specific differences in gamete interaction may exist even between very closely related non-domestic bovids. Slavik and Fulka (1999) studied the contribution of oviductal fluid to the species-specific barrier by fertilizing *in vitro* matured cattle and sheep oocytes with ram and bull spermatozoa, respectively. From the study, Slavik and Fulka (1999) suggested that the properties of zona pellucidae of ovulated and *in vitro* matured oocytes are not identical, and may be modified by contact with oestrous oviductal fluid. In another study on sperm-zona binding using bovine oocytes and bovine, porcine and equine spermatozoa, it was recommended that the dogma of strict species-specific sperm-zona interactions under *in vitro* conditions has to be reconsidered (Sinowatz *et al.*, 2003).

Modern biotechnology methods such as manipulation of reproductive cycles, artificial insemination (AI), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) provide means of understanding and overcoming some of the interspecies hybridization isolation mechanisms. Moore *et al.* (1981) inseminated nanny goats and ewes with Barbary ram semen and embryos were produced, though only one nanny goat x Barbary ram embryo developed to a full-term foetus. Tatham (2000) attempted to overcome the hybrid isolation mechanism between water buffalo and cattle by artificially inseminating seven cows with buffalo semen and four buffalo cows with domestic bull semen, but no pregnancy resulted. Frozen-thawed semen of improved cattle breeds have successfully been used in hybridization programmes of cattle and yak through AI (Zhang, 2000), though natural mating is possible.

A study on using bovine oocytes for a heterologous fertility test by ICSI and comparison of pronuclear formation of ram, bull and minke whale *(Balaenoptera acutorostrata)* spermatozoa after injection into bovine oocytes was done by Wei and Fukui (2000). Though blastocyst formation was only observed when bull spermatozoa were used, the results indicated that dead foreign spermatozoa can fertilize bovine oocytes after ICSI. In an earlier study, Kim *et al.* (1999) determined the incidence of activation, male pronuclear formation, and apposition of pronuclei in porcine oocytes, following intracytoplasmic injection of various porcine sperm components and spermatozoa of cattle, mouse or human. Mouse, bovine, or human spermatozoon all activated porcine oocytes, a result suggesting that the sperm-borne activation factor(s) is not strictly species-specific.

The interspecies fertilization barrier is believed to exist at the level of the zona pellucida (ZP) and/or vitelline membrane (Gordon, 1994). For fertilization to take place, the oocyte must

recognize the sperm. In the mouse, for instance, a glycoprotein ZP3 provides the receptor sites on the oocyte for sperm recognition and binding, and eventual acrosome reaction, and sperm penetration of the oocyte (Wassarman, 1994). This author further stated that apparently homologous molecules regulate fertilization in other mammals, including man. However, Slavik *et al.* (1990) demonstrated that ram sperm can penetrate *in vitro* matured bovine oocytes as readily as bull sperm. A subsequent report by Slavik and Fulka (1992) showed that the ZP of the bovine oocyte does not represent a barrier against penetration by goat sperm. Later, Slavik *et al.* (1997) produced hybrid zygotes by *in vitro* fertilization of matured bovine oocytes with ram spermatozoa. However, there was development failure in the hybrid embryos, a phenomenon that was attributed to delay or inefficient reactivation of the embryonic genome in the hybrid embryos. The penetration of bovine oocytes by ram sperm *in vitro* was also reported by Kelk *et al.* (1991); however, when cows were inseminated with ram semen, no evidence of fertilization was obtained. The author concluded that fertilization of bovine oocytes by ram sperm was an *in vitro* phenomenon.

In deer, Fukui *et al.* (1991) reported that development of effective IVF techniques could be valuable in the *in vitro* hybridization of different deer species which do not hybridize naturally. Meanwhile, Moreno *et al.* (1994) used fresh extended and frozen-thawed American bison (*Bison bison*) sperm to fertilize bovine oocytes, with the highest percentage of F1 morulae/blastocysts produced with the fresh extended semen. Roth *et al.* (1998) were successful in the production and development of hybrid embryos after co-incubation of scimitar-horned oryx (*Oryx dammah*) sperm with cow oocytes, suggesting that the two bovid species have similar fertilization mechanisms. Patil and Totey (2003) attempted to hybridize water buffalo and cattle using the IVF procedure, but *in vitro* embryo development of only a few embryos progressed beyond the 8-cell stage. Gordon (1994) recommended that such apparent *in vitro* block to fertilization may be useful model for studying certain forms of fertilization failure.

This study attempted to produce cattle and African buffalo hybrid embryos using IVF procedures to understand whether fertilization occurs, and to what extent the hybrid embryos would develop. It was hoped that this would provide information for exploring pathways for utilizing exotic alleles that could benefit livestock production and understand some biological mechanisms that control hybridization between the two species. The null hypothesis "H_o: *in vitro* development of embryos from *in vitro* matured bovine oocytes *in vitro* fertilized using the homologous bovine and heterologous buffalo epididymal sperm are not different" was tested at a 95% confidence level.

5.3 Materials and methods

5.3.1 *In vitro* maturation

Bovine ovaries were collected from a local abattoir and transported in an insulated container in D-PBS supplemented with 10 µg/mL ciprofloxacin hydrochloride (CF-HCl) (ICN Biomedicals, Ohio, USA) at 32 ± 2.2 °C. Ovaries were washed three times in pre-warmed (30 °C) D-PBS, and oocytes aspirated from follicles 2-8 mm in diameter using suction pressure as described in Chapter 3. The collected follicular fluid was allowed to settle in a 50 mL centrifuge tube (Cellstar®, Greiner bio-one, Germany) for 10 min, the supernatant discarded and re-suspended in pre-warmed modified D-PBS (D-PBS supplemented with 10 µg/mL CF-HCl and 1% v/v FBS). After re-suspending and discarding the supernatant twice, the sediment containing cumulus oocytes complexes (COCs) was transferred into 60 x 15 mm Petri dishes (Cellstar®, Greiner bio-one, Germany), examined and COCs selected under a stereoscope (Nikon®, SMZ-2T, Japan). The selected COCs (n = 3371) were washed twice in HEPES-TALP (see Appendix A2) and then washed once in a pre-incubated maturation medium (see Appendix A). Groups of 15 COCs were then transferred into pre-equilibrated 50 µL drops of maturation medium in 35 mm Petri dishes under mineral oil, and incubated at 38.5 °C in a 5% CO_2 humidified atmosphere for an average of 24 h. The mean duration from ovary collection to onset of maturation incubation was approximately 5 h.

5.3.2 *In vitro* fertilization

Frozen epididymal sperm from culled African buffalo bulls (n = 2), and bovine (n = 2) epididymal sperm from abattoir materials were used for *in vitro* fertilization of the matured oocytes. Bovine and buffalo epididymal sperm were retrieved by retrograde flushing (Halangk *et al.*, 1990; Reyes-Moreno *et al.*, 2000; Cary *et al.*, 2004; Martinez-Pastor *et al.*, 2005) and frozen in Biladyl® (Minitüb, Germany), as described previously (Chapter 2). Two 0.25 mL straws of sperm from the different buffalo and bovine bulls were thawed in a water bath at 37 °C for 1 min. Motile sperm were separated by swim-up in sperm-TALP (see appendix A2) as described by Parrish *et al.* (1985).

After 1 h of swim-up, the top 0.85 mL of sperm-TALP medium in each test tube was aspirated, and pooled into a 15 mL plastic centrifuge tube, and washed by centrifuging (Allegra® X-22R, Beckman Coulter, Germany) twice at 700g for 6 min. After the second wash, the medium was aspirated and the pellet re-suspended in 0.2 mL sperm-TALP. Sperm concentration was determined using a haemocytometer, and the concentration adjusted to 10-20 x 10^6 sperm/mL. The matured COCs were washed twice in HEPES-TALP medium, and once in IVF-TALP (see Appendix A2). The oocytes were then transferred in groups of 30

oocytes per well of a 4-well plate (Falcon®, USA) each containing 0.6 mL of a pre-incubated fertilization medium (IVF-TALP). Twenty four micro-litre of PHE (2 mM penicillamine, 10 mM hypotaurine and 0.1 mM epinephrine) and approximately 30 µL of sperm (final concentration of 0.5 to 1.0 x 10^6 sperm/mL) (Ward *et al.*, 2003) was added to each well, and the IVF plates were incubated at 38.5 °C in a 5% CO_2 humidified air atmosphere for 19.7 ± 2.9 h.

5.3.3 *In vitro* culture

After fertilization, the presumptive embryos were washed through four 200 µL drops of pre-incubated *in vitro* culture (IVC) medium (TCM-199 supplemented with 10% FBS, 0.2 mM sodium pyruvate, 1 mM L-glutamine and 10 µg/mL CF-HCl) under mineral oil. Bovine (n = 1166) and hybrid (n = 1202) presumptive embryos were selected for culture. The presumptive embryos were co-cultured in groups of 10 to 15 with bovine oviductal epithelial cell monolayer in 100 µL drops of IVC medium under mineral oil at 38.5 °C in a 5% CO_2 humidified air atmosphere for 8 days. On every second day, half of the IVC medium was replaced with fresh medium. Embryonic development was assessed at 72 h post insemination (hpi, time zero was the insemination time) for cleavage, morula formation (day 5), and blastulation (day 7 and 9).

5.3.4 Transfer of hybrid embryos to bovine surrogates

Cycling recipient beef cows with moderate body conditions were selected for recipients. The recipients were synchronized using controlled intra-vaginal drug release devices implants (CIDR®, Pfizer Animal Health, USA). The implants were withdrawn 12 days after implantation and an injection of $PGF_2\alpha$ was given at time of CIDR removal. The cows came on heat 2-3 days after CIDR withdrawal. Morula-stage hybrid (n = 4) and blastocyst-stage bovine (n = 2) embryos were non-surgically transferred to six surrogate bovine cows. During the embryo transfer procedure on day 7, the cows were rectally examined for presence, location and quality of the corpus luteum (CL). Upon the presence of a good quality CL, cows were given 2 mL of a 2% lignocaine through the sacro-coccygeal joint into the epidural space to reduce straining. Each recipient cow received one embryo deposited towards the tip of the uterine horn, ipsilateral to the corpus luteum. Two months after the transfer, the cows were examined for pregnancy by rectal palpation by an experienced veterinarian.

5.3.5 Staining of embryos with orcein and DAPI stains

Preliminary staining of the presumptive embryos was done to ascertain if fertilization took place. Ten hybrid and bovine presumptive embryos 24 hpi were removed from those to be cultured, and denuded by vortexing. The presumptive embryos were washed once in HEPES-TALP then fixed in 1 mL of acid alcohol (acetic acid:ethanol, 1:3) for 24 h in a 24-well plate.

After fixing, the presumptive embryos were stained by adding approximately 10 µL of aceto-orcein stain (prepared as 1% w/v orcein stain in acid alcohol) into the wells of the 24-well plates. The stained presumptive embryos were examined using an inverted microscope (Olympus®1x70, Tokyo, Japan) for the presence of a second polar body and formation of a male pronucleus. The images were captured by a camera (Colourview®, Soft Imaging System (SIS), Germany) attached to the microscope and displayed on a computer monitor by a software (AnalySIS®, SIS, Germany). Snapshots of the images were recorded and stored for later evaluation.

The embryos that were cultured and underwent cleavage were stained to determine whether the blastomeres possessed nuclei. A protocol of preparing DAPI stain described by Moreira *et al.* (2001) was followed. Briefly, step one involved preparation of 1 mg/mL solution of polyvinyl pyrrolidon (PVP) in PBS and 4% (40 mg/mL) solution of paraformaldehyde in PBS (pH 7.4). In step two, DAPI (D9564-Sigma), a DNA-binding stain was prepared by dissolving 10 mg in 10 mL of PBS to make a 0.1 mg/mL solution that was stored wrapped in aluminium foil at −20 °C for later use. It was noted that DAPI dissolves slowly therefore, adequate time was given to allow it dissolve completely. Thirdly, 1 mL of the 0.1 mg/mL of step two solution was diluted to 10 mL to make a 0.01 mg/mL solution that was stored wrapped in aluminium foil at 4 °C for use within 6 months. In the fourth step, a working solution was prepared at the time of use by adding 100 µL of the 0.01 mg/mL (step two) solution to 900 µL PBS/PVP. This was used once and the excess discarded.

The DAPI staining procedure was done in a 4 well plate. The embryos were washed twice in 500 µL of PBS/PVP contained in the two right wells. They were then fixed in 500 µL of PBS-paraformaldehyde solution (4% w/v in PBS, pH 7.4) in the left upper well for 1 h at room temperature. Embryos were stained by transferring into the left lower well containing 500 µL of DAPI working solution and left to stain for 10 min. Two wells of a second 4-well plate were filled with 500 µL of PBS-PVP solution and embryos washed by transferring them from well one to well two. The embryos were kept in well two while each embryo was transferred onto a clean microscope slide with a small droplet (approximately 10 µL) of the PBS-PVP solution and examined using an inverted fluorescent microscope (Olympus®, 1x70 Tokyo, Japan) at X200 magnification. The colorview® camera attached to the microscope captured the images and the embryo evaluated with particular attention to the bright-blue-stained DNA of the blastomere nuclei.

5.3.6 Statistical analysis

Data from 15 replicates of embryo culture were captured in Microsoft Excel and exported to SAS (version 9.1, 2006) for statistical analysis. Data were analyzed by running the generalized linear model (GENMOD) procedure. Correlations of statistical factors (i.e. temperature during embryo transportation, duration of ovary collection to onset of maturation, and duration of maturation and fertilization periods) with embryo development were also calculated. P-values less than 0.05 were denoted as indicating significance.

5.4 Results

Fertilization occurred when *in vitro* matured bovine oocytes were *in vitro* inseminated with both the homologous bull and heterologous buffalo epididymal spermatozoa. Although the bovine embryos developed to the blastocyst stage, the hybrid embryos did not develop beyond the morula stage (Table 5.1). No pregnancy resulted from the four hybrid and two bovine embryos transferred to bovine cow surrogates.

Table 5.1 Development of embryos after fertilization of *in vitro* matured bovine oocytes with bovine and buffalo epididymal sperm. Values in brackets are numbers and those not in brackets are percentages. Insemination of oocytes was on day 0.

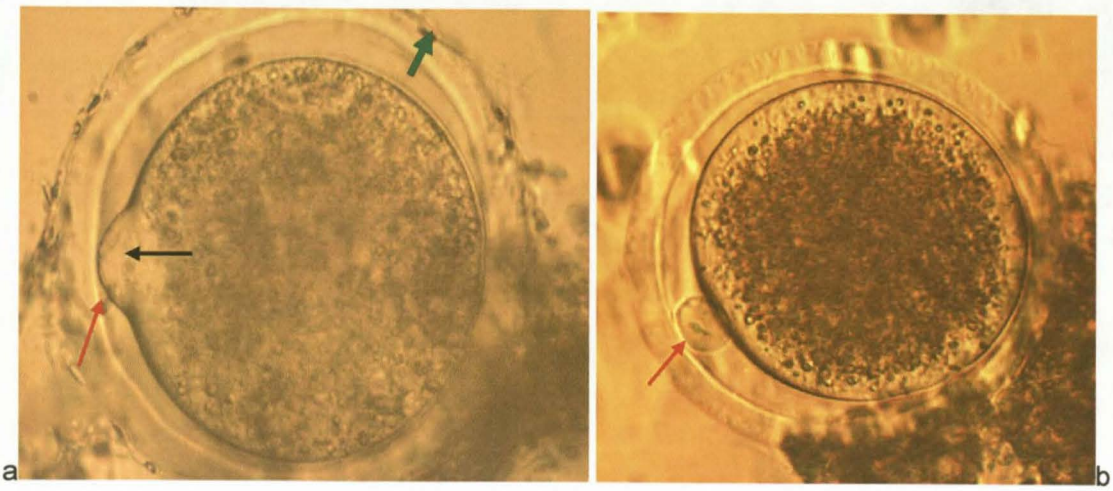
Sperm source	No. of presumptive embryos cultured (Day 1)	Cleaved oocytes (Day 3)	Morula (Day 5)	Blastocyst (Day 7)	Expanded blastocyst (Day 9)
Bovine	1166	67.2[a] (784/1166)	52.2[a] (409/784)	40.1[a] (314/784)	6.0[a] (47/784)
Buffalo	1202	4.6[b] (55/1202)	12.7[b] (7/55)	0[b] (0/55)	0[b] (0/55)

[a,b] Columns with different subscripts differ significantly ($P < 0.0001$)

Of the 784 (67.2%) and 55 (4.6%) presumptive bovine and hybrid embryos that cleaved, 409 (52.2%) and 7 (12.7%) progressed to the morula stage, respectively. None of the hybrid embryos developed beyond the 16-cell stage. However, 314 (40.1%) of the cleaved oocytes, or 76.8% (314/409) of the morula stage bovine embryos, developed to the blastocyst stage by day 7 post-insemination. Cleavage rate and morula formation in the hybrid and bovine embryos were different ($P < 0.0001$). There was also a difference ($P < 0.0001$) between the buffalo and bovine sperm in embryo production. The duration from ovary collection to onset of maturation correlated with cleavage rate ($P < 0.05$) and morula formation ($P < 0.0001$), and temperature of ovary transportation also correlated with cleavage rate ($P < 0.05$).

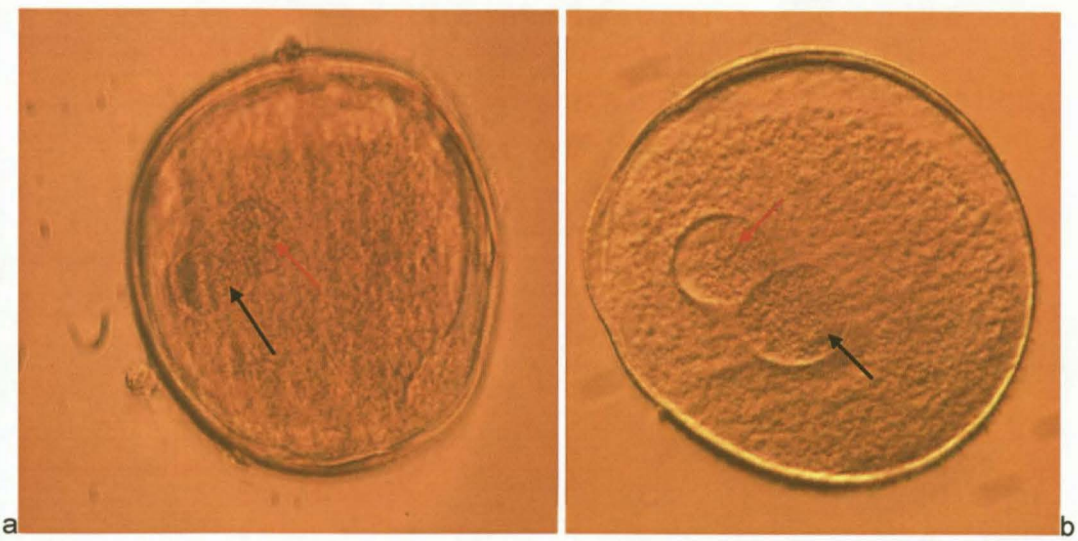
5.4.1 Different stages of fertilization and development of hybrid and bovine embryos

Initial examination showed evidence of fertilization with formation of a second polar body (Fig. 5.1a), development of two pronuclei (Fig. 5.2a) and cleavage beyond the two-cell embryo stage (Figs. 5a and 5.4a). Fertilization and cleavage was also confirmed by presence of more than two nuclei in blastomeres of DAPI-stained hybrid embryos. The development of some hybrid and bovine embryos (Figs. 5.3 and 5.4) were similar. Generally, the hybrid embryos developed at a slower rate than their bovine age-mates.



a b

Fig. 5.1 Unstained presumptive hybrid embryos 7 hpi showing (a) an extruded first polar body (red arrow), extrusion of second polar body (black arrow) and accessory sperm (green arrow); and (b) an extruded first polar body before or just after fertilization of the oocyte (X200 magnification).



a b

Fig. 5.2 Presumptive embryos 24 hpi stained with orcein stain showing male and female pronuclei in (a) hybrid embryo and (b) bovine embryo just before syngamy (X200 magnification).

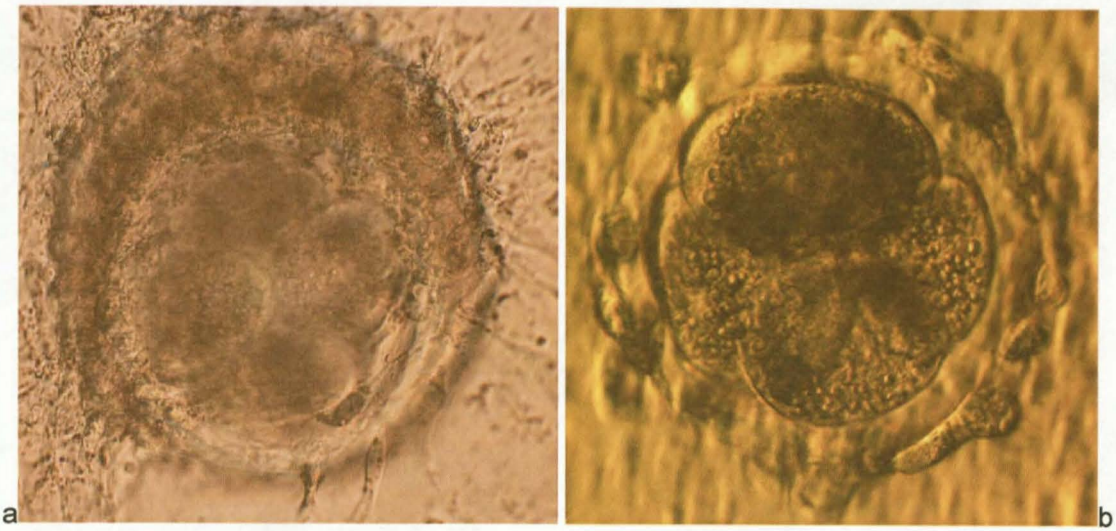


Fig. 5.3 Unstained 4-cell embryos 48 hpi: (a) a hybrid embryo and (b) a bovine embryo showing even cleavage. The embryos are attached to the cumulus cells and the co-culture can be seen in the background (X200 magnification).

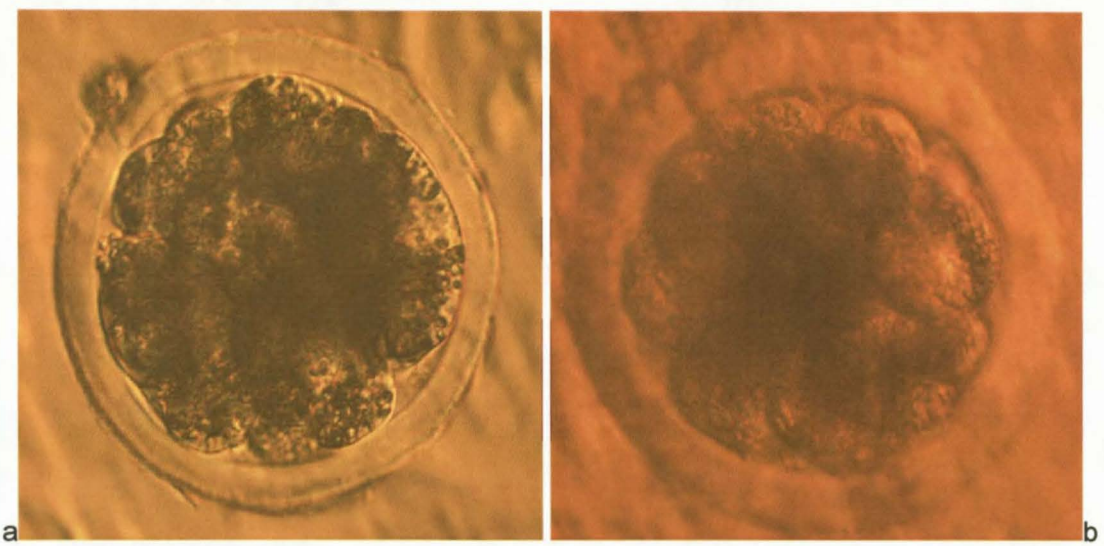


Fig. 5.4 Morula stage of (a) a hybrid embryo and (b) a bovine embryo 5 days post-insemination. There is even granulation of both embryos, showing normal embryo development (X200 magnification).

5.4.2 Abnormal fertilization and development in hybrid embryos

Embryo developmental anomalies such as uneven cleavage (Fig. 5.5b) and degeneration of blastomeres (Fig. 5.6a) were more common in hybrid embryos than the bovine embryos. Presumptive hybrid embryos developed vacuoles within the blastomeres (Fig. 5.6a) or apparently without cleavage but appeared as cleaved embryos (Fig. 5.6b). Polyspermy in hybrid embryos indicated by presence of 3 pronuclei (Fig. 5.5a) and was more common in the

hybrid embryos. Examination of hybrid and bovine embryos stained with DAPI showed absence of nuclei in some of the blastomeres of the hybrid embryos.

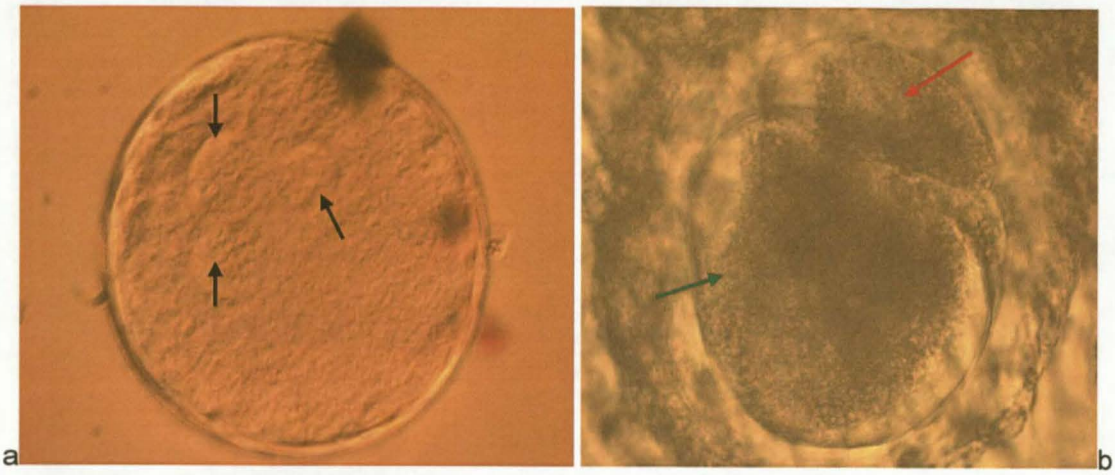


Fig. 5.5 Hybrid embryos: (a) an orcein-stained presumptive embryo 24 hpi showing polyspermy i.e. 3 pronuclei shown with black arrows, and (b) a 2-cell embryo 48 hpi showing uneven cleavage, i.e. the blastomere indicated with the green arrow is bigger than the one indicated with the red arrow (X200 magnification).

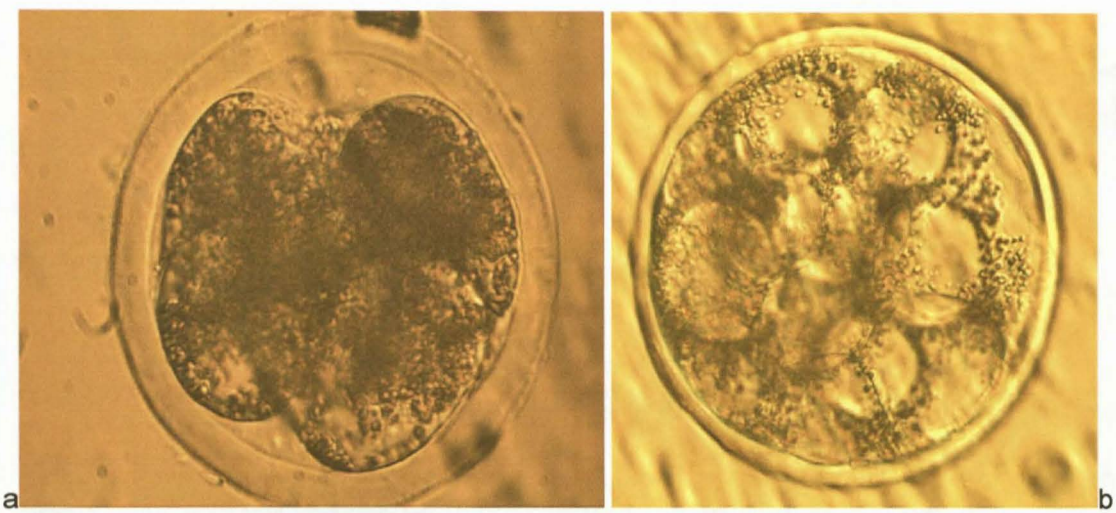


Fig. 5.6 Unstained hybrid embryos (a) 4-cell embryo 48 hpi showing signs of blastomere degeneration, blastomeres with a poor outline, uneven granulation and vacuoles in the blastomeres, and (b) a seemingly unfertilized oocyte with vacuoles (4 dpi) that could be confused with dividing blastomeres of a normal embryo (X200 magnification).

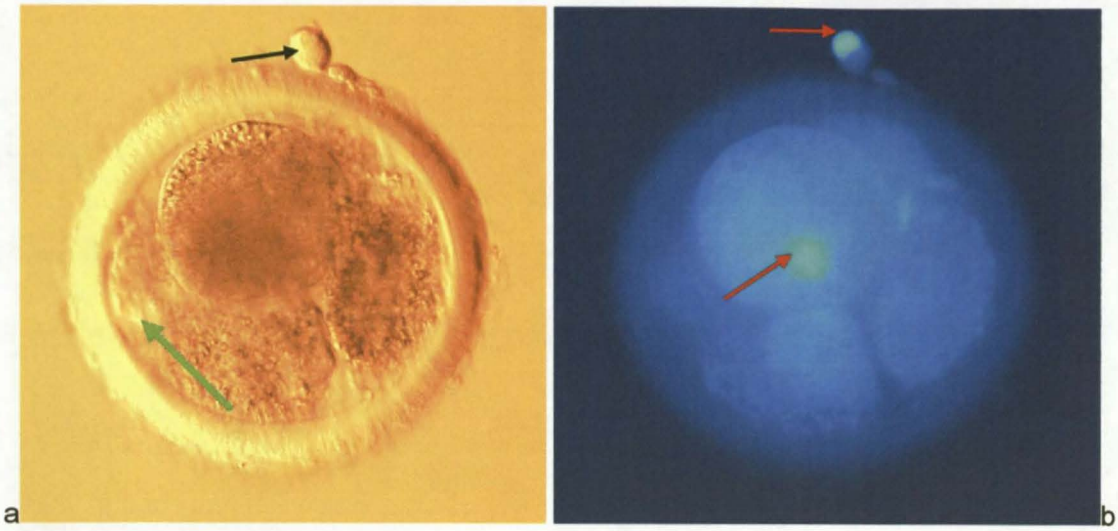


Fig. 5.7 DAPI-stained 4-cell hybrid embryo 6 dpi: (a) bright field microscopy showing 3 blastomeres, a degenerated fourth blastomere (green arrow) and an attached cumulus cell (black arrow), and (b) the same embryo under fluorescent microscopy showing only one nucleus and the cumulus cell with a nucleus (red arrows) (X200 magnification).

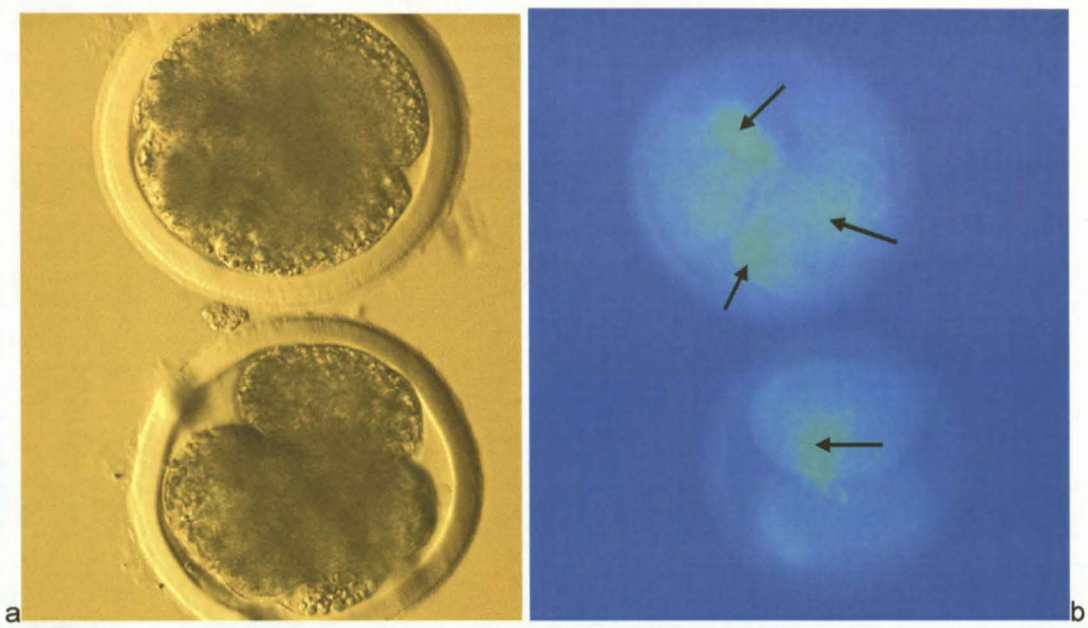


Fig 5.8 DAPI-stained hybrid embryos 6 days post-insemination: (a) an 8-cell (top) and 4-cell (bottom) embryos under bright field microscopy; and (b) the same embryos under fluorescent microscopy showing only three and one nuclei (black arrows) of the 8-cell and 4-cell embryos, respectively. The DNA is not condensed but rather dispersed within the blastomeres (X100 magnification).

5.5 Discussion

The study demonstrated that interspecies *in vitro* fertilization of *in vitro* matured bovine oocytes by African buffalo epididymal spermatozoa occurs, but that the embryo development rate is low and slow, progressing only up to the pre-compacted morula (16-cell) stage. Fertilization was indicated by the presence of accessory sperm, formation of a second polar body (2PB) and two pronuclei (2PN), and cleavage. These indications of successful fertilization conformed to the description of fertilization by Elder and Dale (2000). Elder and Dale (2000) described fertilization as a complex process of cell-cell interaction which starts with specific sperm-oocyte recognition and binding, oocyte penetration, sperm nuclear decondensation and formation and fusion of the pronuclei. However, according to Gordon (1994), it's generally believed that a barrier operating at the level of the ZP and vitelline membrane protects mammalian oocytes against penetration by foreign sperm. In a study by Bongso and Trounson (2000), no recognition of the zonae of other species by human sperm was noted, leading to the conclusion that the interactions between sperm, and the ZP and the oocyte's membrane are species-specific. The current study, however, did not agree with these reports and further disagreed with a report by Kouba *et al.* (2001) that species-specific differences in gamete interactions may exist even between closely related bovids. There is genetic disparity between the African buffalo (2n = 52) and cattle (2n = 60) yet interspecies fertilization occurred between these bovid species in the current study. These findings also showed that sperm-oocyte recognition which in mammals is mediated, for instance the mouse, by ZP3, a glycoprotein receptor on the ZP surface (Gordon, 1994), is not a species-specific event. Perhaps, in agreement with this study, Sinowatz *et al.* (2003) recommended, from their study, that the dogma of strict species-specificity of sperm-zona interactions under *in vitro* conditions has to be reconsidered.

The presumptive hybrid embryos formed a 2PB (Fig. 5.1a) and 2PN (Fig. 5.2a) and subsequently cleaved (Fig. 5.3a and Fig. 5.4a). Activation of the oocyte by sperm results in completion of the second meiotic division as indicated by extrusion of the second PB and formation of the male and female pronuclei (Bongso and Trounson, 2000). The formation of the second PB and 2PN in the presumptive hybrid embryos indicated that buffalo sperm activated bovine oocytes *in vitro*, and that oocyte activation by sperm is not, at least in these two species, species-specific. This agreed with a report by Elder and Dale (2000) that the factors responsible for sperm nuclear decondensation and pronuclear formation are found in the oocyte cytoplasm and not in the sperm, and these factors are not species-specific.

Fertilization was assessed by formation of the 2PB, 2PN and cleavage rate. Under normal circumstances, fertilization rates of up to 80.0% can be achieved with bovine IVF (Chohan and Hunter, 2004). The cleavage rates in this study were 62.7% and 4.6% for insemination with the bovine and buffalo spermatozoa, respectively. The low fertilization rates in this study could be due to the sperm preparation medium, capacitation agent used, as well as the IVF conditions during fertilization with the bovine sperm. In addition to these, the species difference could have been the main cause of the low fertilization rate seen in fertilization with heterologous buffalo sperm. In this study, both bovine and buffalo sperm were prepared in sperm-TALP (Parrish *et al.*, 1985), and heparin was used as the capacitating agent (Parrish *et al.*, 1989a,b) at a concentration of 2 µg/mL in the fertilization medium (IVF-TALP). In water buffalo (*Bubalus bubalis*), Totey *et al.* (1992) found that frozen-thawed spermatozoa prepared in Bracket-Oliphant (BO) medium and treated with 5 mM caffeine + 10 µg heparin had a higher fertilization rate (29.8%) than those prepared in HEPES-TALP and treated with 10 µg/mL heparin (19.6%). However, fertilization rate was significantly improved (50%) when fresh ejaculated spermatozoa treated with 5 mM caffeine and 10 µg/mL heparin in BO medium was used. The current study used frozen-thawed African buffalo (*Syncerus caffer caffer*) epididymal sperm prepared in sperm-TALP and treated with 2 µg/mL heparin. Patil and Totey (2003), in their hybridization study between *Bubalus bubalis* and bovine found no differences in fertilization rates. The overall fertilization rate of *Bubalus bubalis* oocytes obtained with cattle spermatozoa was 78.4%, which did not differ from that obtained with buffalo spermatozoa (80.2%). Similarly, Tatham *et al.* (2003) found no significant difference in fertilization rates when buffalo and bovine spermatozoa were used to fertilized bovine oocytes. In another hybridization study by Roth *et al.* (1998), scimitar-horned oryx (*Oryx dammah*) sperm from different males were capable of fertilizing cow oocytes at rates ranging from 65.4% to 96.2%.

Culture of presumptive hybrid embryos resulted to cleavage beyond the 2-cell stage, a further evidence that buffalo sperm fertilized bovine oocytes *in vitro*. Gordon (1994) stated that one of the standard procedures for evaluating *in vitro* fertilization is the percentage of inseminated oocytes that cleaved 2 days post-insemination, a measure referred to as cleavage rate. In this study cleavage rate was evaluated 3 days post insemination because of the slow cleavage rate of the hybrid embryos. There was a difference ($P < 0.0001$) in cleavage rate between the hybrid embryos (4.6%), compared to the bovine embryos (67.2%). In an interspecies hybridization study by Kouba *et al.* (2001) a similarly low cleavage rate (8.0%) for *in vitro* matured bovine ($2n = 60$) oocytes *in vitro* inseminated with fringe-eared oryx (*Oryx gazelle callotis*) with chromosomal number $2n = 58$. However, these authors reported that the low cleavage rate in hybrid embryos was not very different from parthenogenic controls (4.2%).

The current study did not do a parthenogenic control. Embryo development ability similar to that of the current study was observed by Tatham *et al.* (2003) in water buffalo x cattle hybrid embryos compared with a cattle control. The similarity of the study by Tatham *et al.* (2003), and the current study was the insemination of bovine oocytes with bovine and buffalo sperm. However, in a study on hybridization in which water buffalo (*Bubalus bubalis*, 2n = 50) oocytes were fertilized with water buffalo and bovine sperm, the initial cleavage rate between buffalo and hybrid embryos was similar, 26.0 ± 4.1% versus 24.3 ± 4.8% (Patil and Totey, 2003) which disagreed with the findings of the current study. This could be because the current study used buffalo epididymal sperm to fertilize bovine oocytes, while Patil and Totey (2003) used ejaculated bovine sperm to fertilize buffalo oocytes. The buffalo breeds involved in the two studies were also different. A further possible explanation is that when successful interspecies hybridization has been demonstrated, there are marked differences in the success of reciprocal crosses such that a high rate of fertilization is possible in one direction but not the other (Bazer *et al.*, 1993). Reciprocal fertilization could not be done in this study because of the difficulties in obtaining the African buffalo oocytes.

The low cleavage rate could also be explained by improper zygotic gene activation. According to Elder and Dale (2000), successful development of a fertilized oocyte beyond the early cleavage stage requires initiation and regulation of new embryonic genome transcription. The oocyte cytoplasm provides a specialized environment for the newly formed zygotic nucleus. During the transition from maternal to zygotic gene activation the embryo begins to synthesize its own RNA and protein rather than relying on that inherited from the mother. In the absence of appropriate activation and maintenance of embryonic gene expression, the embryo will simply fail to develop beyond the cleavage stage.

The Rate of development of the hybrid embryos was lower and progressed at a slower rate than in the bovine embryos. From the 4.6% of the hybrid embryos that cleaved, only 12.7% (7 of 55) developed to the morula (16-cell) stage, and development was arrested at this stage. There was a significant difference ($P < 0.0001$) in morula formation between the hybrid and bovine embryos. Out of the 62.7% bovine oocytes that cleaved, 52.2% developed to morula stage and 76.8% of the morulae developed to blastocysts. Meanwhile, only 4.6% of the presumptive hybrid embryos cleaved and 12.7 % of the cleaved embryos developed to the morula stage, but none developed to the blastocyst stage. Similarly, Tatham *et al.* (2003) observed a difference in the developmental ability of water buffalo x cattle hybrid embryos compared to that of the cattle control. However, in the same study (Tatham *et al.*, 2003) fertilization of cattle oocytes with buffalo sperm resulted into 7.8% of the oocytes developing into

hybrid embryos, though the actual stage was not reported. In this study no development beyond the 16-cell stage was observed.

A study by Patil and Totey (2003), to a large extent, disagrees with the findings of this study. These authors reported that there was no difference in embryo developmental rate until the 8-cell stage, and 5.3% that of the water buffalo x cattle hybrid embryos developed into blastocysts compared to that of the buffalo control (21.7%). Likewise, buffalo breed difference, source of sperm and the possibility of lack of success in reciprocal crossing as explained for cleavage rate, could have contributed to the disagreement between this study and that of Patil and Totey (2003).

In a hybridization study in which bovine (2n = 60) oocytes were fertilized with ram (2n = 54) spermatozoa, it was reported that the ability of hybrid embryos to reach 8-cell stage was similar to that of the control embryos, but not thereafter (Slavik *et al.*, 1997). The current study however, did not agree with the report by Slavik *et al.* (1997) possibly because the chromosomal disparity of the sheep (2n = 54) and cattle (2n = 60) are closer to each other compared to that of cattle (2n = 60) and African buffalo (2n = 52). In a study comparing cattle hybrid and pure-breed *in vitro*-derived embryos, Boediono *et al.* (2003) found a difference in blastocyst production rate and development of blastocyst stage between hybrid and pure-breed embryos. Furthermore, they found that almost all the *in vitro*-produced blastocysts were obtained from zygotes that had developed to the 8-cell stage by 48 hpi. The implications of these to the current study are that the rate and the extent of development of hybrid embryos are related to the genetic similarity or diversity of the parents.

The transition from maternal genome (cytoplasmic and mitochondrial DNA) to embryonic genome (nuclear DNA) takes place at the 8 to 16-cell stage of embryo development. This could have played a role in failure of development of the hybrid embryos beyond the 16-cell stage, (Elder and Dale, 2000) for genome activation is considered critical for the morula/blastocyst transition. During this critical stage, the embryonic gene activated during the zygotic stage should be able to synthesize the embryo's own and adequate RNA and proteins required to drive further development. According to Ménézo and Janny (1997), a complex remodeling takes place, and poor sperm quality can compromise this transition. In this study the use of buffalo sperm (n = 26) to fertilize bovine oocytes (n = 30) could have been recognized by the oocytes as poor quality sperm resulting into developmental arrest. This is because the gene regulating properties of sperm assist in the preparation of the paternal genome to contribute to the embryogenesis and the genome of the zygote (Emery and Carrell, 2006). The

developmental arrest could have also been caused by abnormal reprogramming that occurs in some hybrids after fertilization and during pre-implantation embryo development which is, in part, responsible for hybrid dysgenesis (Shi *et al.*, 2005). Furthermore, assessment of transition from maternal to embryonic genome control in cattle x goat hybrid embryos, according to the onset of RNA synthesis, indicated the differences in the frequency of labeled nuclei and the intensity of their labelling (Slavik *et al.*, 1997). Because the frequency of the labelled nuclei and the intensity of their labelling were remarkably lower in hybrid embryos, reflecting their developmental failure, the authors (Slavik *et al.*, 1997) concluded that these observations are consistent with delay or inefficient reactivation of embryonic genome in the hybrid embryos.

Abnormal fertilization such as multi-pronuclear, especially three pronuclear (3PN), and uni-pronuclear (1PN) oocytes was observed in some hybrid embryos, which possibly contributed to the poor/abnormal cleavage and developmental arrest that was later observed in the hybrid embryos. In human IVF, Martin-Pont *et al.* (1991) confirmed that most multi-pronuclear one-cell zygotes are polyploids. Triploidy is reported to occur when an oocyte is fertilized by two sperm and is referred to as polyandry (Jainudeen and Hafez, 1993), or may arise due to polygyny, when there is failure of the oocyte to extrude the first or second PB (Xu and Greve, 1987, 1988). In an ICSI study of abnormal fertilization, Sean *et al.* (1995) demonstrated 3PN and a single PB indicating that the second PB developed into the third PN. These authors concluded that the principal cause of fertilization failure after ICSI is failure of oocyte activation by sperm penetration, implying that sperm penetration may occur but oocyte activation may be inefficient. This phenomenon may be possible in interspecies fertilization where oocyte penetration could occur but due to failure of oocyte activation, the second PB develops into a third PN; or polyspermy occurs due to failure of the cortical reaction. Such oocytes with abnormal fertilization may not cleave, leading to reduction in cleavage rate, or may cleave but development arrested at some stage. The low cleavage rate could have also arisen from polyspermy. This would be explained further by a similar hybridization study by Roth *et al.* (1998) in which they fertilized bovine oocytes with scimitar-horned oryx (*Oryx dammah*) sperm. From the 24 uncleaved oocytes, 7 (29.2%) were as a result of polyspermy. Development of 1PN could have been due to asynchronous PN development that could have also arisen due to inadequate oocyte activation. Asynchronous development of pronuclei is a phenomenon responsible for early developmental arrest in IVF embryos (Schmiady *et al.*, 1987).

Polyspermy has been reported to occur in cattle IVF at a rate of 8% (Chikamatsu *et al.*, 1989) to 55% (Long *et al.*, 1993) depending on many factors such as the bull, sperm concentration, and fertilization and capacitation media. Polyspermy was, however, not

observed in the presumptive bovine embryos examined in this study, but it was more common in hybrid embryos possibly due to the inability of buffalo sperm to stimulate an effective block to polyspermy. According to Bazer *et al.* (1993), the block to polyspermy that occurs at the ZP and vitelline membrane is initiated by sperm penetration resulting into the release of cortical granules and reorganization of ZP and/or vitelline surface. This 'cortical reaction' results in hardening of the ZP and inactivation of the ZP3 receptors. Iwasaki *et al.* (1989) found that polyspermy is the most common form of abnormal fertilization causing chromosomal anomalies in bovine *in vitro* fertilization. Iwasaki *et al.* (1992) reported that developmental anomalies at early stages of *in vitro* fertilization-derived embryos were caused by such abnormal fertilization. In human IVF, Boyers *et al.* (1987) found that cleavage rate was higher (92.7%) in the diploid than polyploidy oocytes (65.5%).

It was not known whether the tri-pronuclear oocytes actually arose out of fertilization by more than one sperm, which may be associated with an abnormally high concentration of sperm in the fertilization medium. This study used 0.5-1.0 x 10^6 sperm/mL, which is the concentration normally used in bovine IVF. According to Handyside (1992), the main abnormality arising at fertilization *in vitro* is fertilization by more than one sperm which may partly be caused by the abnormally high sperm concentration used to inseminate oocytes. In human IVF, tri-pronuclear oocytes have been seen to have altered first cleavage pattern in which they cleave directly to 3-cell at first cleavage (Kola *et al.*, 1987). Tri-pronuclear oocytes are usually formed by dispermic fertilization and, as a result of the formation of a tripolar spindle, the majority (62%) divide into 3 cells with severe abnormal chromosome numbers (Garello *et al.*, 1999). Recently Lundin *et al.* (2001) showed in human IVF that early cleavage rate in three pronuclear (3PN) zygotes was only 12%, compared to 26.9% in 2PN zygotes.

Uneven cleavage was another form of anomaly in embryo development seen among the hybrids embryos in this study. This could have been related to uncoordinated regulation of cleavage caused by the unequal number of chromosomes from the buffalo sperm (n = 26) and the bovine oocytes (n = 30). This argument is supported by a report by Emery and Carrell (2006) that the epigenetic (gene regulatory) properties and mechanisms of sperm assist in the preparation of the paternal genome to contribute to embryogenesis and the genome of the zygote. Edwards and Beard (1997) postulated that the pronuclei undergo arrangement governed by cytoplasmic contraction waves organized by the sperm centrosome, and this may establish polarity in the oocyte, thus re-setting a new axis after fertilization. Embryos unable to achieve optimal pronuclear orientation, possibly due to shorter cytoplasmic waves (Payne *et al.*, 1997), may exhibit poor morphology, for example uneven cleavage or fragmentation (Garello *et*

al., 1999). Unevenly sized blastomeres may be the manifestation of an uneven distribution of genetic material (Ebner *et al.*, 2003) that could be expected in hybridization between species with a wide difference in chromosomal numbers, as the case in this study. A significantly higher number of blastomeres affected by numeric chromosomal aberrations has been reported in unevenly cleaved embryos (29.4%) as compared with evenly cleaved ones (8.5%) (Hardarson *et al.*, 2001). Hardarson *et al.* (2001) found that uneven blastomere cleavage was highly correlated with aneuploidy and multinuclear rates in human embryos. Perhaps, the development of unevenly cleaved hybrid embryos was bound to be arrested at some stage. For instance, Almeida and Bolton (1998) showed that 63.4% of embryos that arrest between the pronucleate and the eight-cell stage are chromosomally abnormal, and abnormal embryo morphology is associated with chromosomal abnormalities. In addition, Hardarson *et al.* (2001) concluded from their study that uneven blastomere cleavage has a negative effect on both implantation and pregnancy rates in human IVF.

Abnormal development of the hybrid embryos also occurred in the form of vacuoles. Vacuoles have been described by Guelman and Patrizio (2003) as one or more fluid-filled pockets inside the cytoplasm, and can differ in terms of sizes and shapes. According to the same authors, vacuoles may occur in an oocyte or blastomeres at any time, although little is known about vacuolization or why it takes place. In this study, the presence of vacuolated oocytes could have arisen from abnormal fertilization of the bovine oocytes (n = 30) by the buffalo sperm (n = 26), resulting into an endocytotic process of cytoplasm repair or atresia. According to Van Blerkom (1989) and Van Wissen *et al.* (1992), vacuoles may be the result of an endocytotic process of cytoplasm repair, or the result of abnormal endocytosis due to oolema abnormality or atrasia.

When the hybrid embryos were stained with DAPI to investigate whether the blastomeres had nuclei, it was noticed that some blastomeres were anucleate, a finding that could have arisen from abnormal fertilization of the cattle oocytes (n = 30) by the buffalo sperm (n = 26). In human IVF, Handyside (1992) reported an incidence of about 1% of embryos with one or no pronuclei, but with a second polar body, indicating a parthenogenetic activation. Multi-nucleation or absence of a nucleus (anucleate blastomere) indicates gross abnormalities in the timing between cytokinesis (cell division) and karyokinesis (nuclear division), that result in embryo developmental incompetence (Kligman *et al.*, 1996). This could have occurred in the hybrid embryos due to the difference in chromosomal numbers of the sperm and the oocytes. In human IVF, anucleate blastomeres are also considered as an extreme example of embryo fragmentation. The presence of anucleate blastomeres lowers the embryo viability, and if

multiple anucleate blastomeres appear the embryo may be considered 'not viable' due to gross errors in embryo cleavage.

5.6 Conclusion

It was concluded that interspecies fertilization of *in vitro* matured cattle oocytes with African buffalo epididymal sperm occurs *in vitro,* and that the barrier to hybridization is in the early stages of embryonic development. Chromosomal disparity is likely the cause of the fertilization abnormalities, abnormal development and subsequent arrest impairing formation of pre-implantation hybrid embryos. Transfer of the hybrid embryos did not prevent development arrest of the embryos. Investigation into the developmental abnormalities including reciprocal hybridization and genetic studies of the hybrid embryos are recommended, because hybridization may occur in one direction but not *vice versa*.

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CHAPTER 6

The influence of cysteamine and vitrification with a copper-wire cryoloop on *in vitro* bovine embryo development

6.1 Abstract

This study examined the effects of supplementing oocyte maturation medium with cysteamine, and the use of copper-wire cryoloop vitrification of mature bovine oocytes on *in vitro* embryo development. Bovine oocytes were *in vitro* matured in TCM-199 with or without supplementation with 100 µM cysteamine. In the first trial, all matured oocytes were fertilized fresh, while in the second trial the oocytes were either fertilized fresh or after vitrification in 20% ethylene glycol and 20% dimethyl sulfoxide using a copper-wire cryoloop. Fresh and frozen-thawed oocytes were fertilized with frozen-thawed bovine epididymal sperm and cultured for 8 days. Cysteamine did not improve the cleavage rate ($P > 0.05$), but improved the morula and the blastocyst rates ($P < 0.05$) in the first trial. In the second trial, cysteamine did not improve embryo development in the fresh oocytes and the cleavage rate of vitrified oocytes ($P > 0.05$). However, cysteamine improved the morula and the blastocyst rates ($P < 0.05$) of vitrified oocytes. Bovine oocytes can be successfully vitrified using a copper-wire cryoloop though the method requires improvement, and addition of cysteamine in the oocyte maturation medium improves embryo development after vitrification with a copper-wire cryoloop. Cysteamine also improved embryo development in non-vitrified oocytes, but this effect appears to be influenced by pre-culture and culture conditions.

6.2 Introduction

Cryopreservation and storage of gametes, somatic cells and tissues is significantly important in the advancement of assisted reproductive technology (ART) by enabling improved management of livestock and laboratory animal species, improved conservation of biodiversity, and improved modalities for the treatment of human infertility (Woods *et al.*, 2004). The application of ART reduces the cost, genetic drift and diseases that are usually associated with maintaining live animals and cell lines (Shaw *et al.*, 2000). Cryo-banking of female gametes will contribute to improving genetic breeding, reproductive technologies and biotechnology (Ledda *et al.*, 2001). This also makes it possible to create female gamete genetic resource banks for *ex situ* conservation, especially of endangered mammalian species.

In humans, cryopreservation of oocytes has a wide range of applications. Storing of unfertilized oocytes preserves germ cells for individual women and avoids social, moral and legal complications which may arise if partners separate (Shaw *et al.*, 2000). According to Porcu (2001), human oocyte cryopreservation is potentially an alternative solution to the ethical problems arising from embryo storage. In addition, oocyte cryopreservation preserves the reproductive capacity for women at risk of losing it because of premature ovarian failure, pelvic

diseases, surgery, or anti-neoplastic treatments. Banks of frozen donated oocytes would facilitate the donation process, which is often complicated by a requirement for donor-recipient synchrony (Gook and Edgar, 1999). Though controversial, oocyte storage would also open the door to the possibility of women, with no medical indications and no immediate plans to conceive, being able to store 'young eggs' for potential use at a later stage. This technology also reduces the costs of repeated trans-vaginal ultrasound guided aspiration (TUGA) in the case of repeated *in vitro* fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI) in humans and other animal species.

Despite the successes made in cryopreservation of the male gamete and embryos of most mammals, cryopreservation of the female gamete has faced technical difficulties because of the specific features of the oocyte. The oocyte is a large cell with a low surface to volume ratio that limits the rates of movement of cryoprotectants and water into and out of the oocyte (Vajta, 2000). In addition, the low temperature sensitivity of oocytes (Martino *et al.*, 1996; Lane *et al.*, 1999), alterations that occur in the zona pellucida (Fuku *et al.*, 1995), and the requirement for removal of cumulus cells (Vajta, 2000; Le Gal and Massip, 1999) pose further difficulties in the cryopreservation of oocytes.

A major reason why most cryopreservation protocols successful in cryopreserving sperm and embryos have not been successful in cryopreserving oocytes is the sensitivity of the oocyte to cooling and freezing (Lane *et al.*, 1999). Cooling exposes oocytes to chilling injury that occurs at low temperatures above freezing point (Levitt, 1980). Temperatures between +30 °C and 0 °C compromises the membrane integrity, cell metabolism, the cytoskeleton and a cell's capacity to control and repair damage e.g. by free oxygen radicals (Ruffing *et al.*, 1993; Arav *et al.*, 1996). Mammalian oocytes contain the actin-based microfilamentous cytoskeleton that controls the cytoplasmic movements including oocyte maturation, fertilization and cell division, but the cytoskeleton is sensitive to physical changes (Bernard and Fuller, 1996). In the mouse oocyte, chilling affects the second meiotic spindle by disrupting the microtubules due to tubulin depolimerization (Rall and Fahy (1985). Cooling of human oocytes to room temperature significantly reduced the spindle assembly (Pickering *et al.*, 1990). The immature germinal vesicle (GV) stage oocytes have chromatin at the diplotene stage of prophase I and do not possess a spindle apparatus. This state would make the GV stage oocytes less sensitive to chilling, and therefore, a suitable alternative approach to oocyte cryopreservation, than the metaphase two (MII) stage that are sensitive to cryopreservation due to the spindle formation (Picton *et al.*, 2002). However, for some reason, the GV stage oocytes are more sensitive to freezing than the mature MII stage oocytes (Fuku *et al.*, 1995).

The phase behaviour and physical properties of membrane lipids are sensitive to acute changes in temperature (Hazel, 1995). Chilling oocytes has been observed to cause substantial disruption of cortical granules and plasma membranes that is thought to lead to poor fertilization and cell death, respectively (George and Johnson, 1993). The poor fertilization is associated with zona hardening due to premature cortical granule exocytosis observed in chilled bovine oocytes (Vajta *et al.*, 1998). Sensitivity to chilling attributable mainly to destruction of the plasma membrane was also demonstrated in pig oocytes (Didion *et al.*, 1990). Irreversible membrane lipid-phase changes of bovine oocytes at GV stage have been demonstrated to occur at between 20 °C and 13 °C, while a very broad phase-transition, which centred around 10 °C, was observed for MII oocytes (Arav *et al.*, 1996). In a study by Saunders and Parks (1999), actin staining revealed craters and apparent holes in the plasma membrane of chilled bovine oocytes. Because cortical actin is closely associated with the plasma membranes, Saunders and Parks (1999) suggested that alterations in microfilament distribution after cooling may be a result of damage to the plasma membrane rather than a direct result of cooling.

The cryopreservation sensitivity of oocytes is probably based on their large size and the amount of intracellular water. According to Mazur *et al.* (1972), freezing damages cells either by formation of intracellular ice crystals during ultra-rapid cooling, or by chemical toxicity, osmotic stress and mechanical damage due to a "solution effect" and ice crystal growth during slow cooling. Freezing rate is very important in ice crystal formation (Arav, 1992). Because ice is formed from pure water (Mizukami *et al.*, 1999), slow freezing rates over a long duration are believed to cause a "solution effect" injury resulting from the extreme concentration of extra-cellular solutes (Critser *et al.*, 2002), and mechanical interactions between cells and the extra-cellular ice (Karlsson and Toner, 1996). Using mouse embryos, Schneider and Mazur (1987) demonstrated a significant inverse correlation between the unfrozen fraction and cell damage in freezing protocols. The "solution effect" causes osmotic stress and extreme dehydration that result in denaturation of lipoproteins, and induce haemolysis in erythrocytes (Lovelock, 1957). The extreme dehydration and cell shrinkage are potentially damaging to the cell. Apparently, there is a critical minimum cell volume beyond which further shrinkage is deleterious to the cell (Meryman, 1970). According to Steponkus *et al.* (1983), osmotic dehydration can delete plasmalemma lipids from the membranes, and it is likely that damage occurs during rehydration if there is insufficient membrane material for the cell to return to its isotonic volume.

Cryopreservation also causes drastic changes in the organization of the meiotic spindle and chromosomes in human (Sathananthan *et al.*, 1987) and mouse (Sathananthan *et al.*, 1988) oocytes. In a study by Saunders and Parks (1999), freezing bovine oocytes led to

complete disappearance of spindle in over 70% of the oocytes, and the normal spindle structure was not recovered after 3 h of incubation. Saunders and Parks (1999) observed a dramatic change in the distribution of actin in bovine oocytes after freeze-thawing, and many oocytes with disrupted microfilament organization also had discontinuous or lysed plasma membranes. Such changes in the organization of the cytoskeletal actin were earlier observed in mouse and rabbit oocytes, but were less dramatic and irreversible after thawing (Vincent *et al.*, 1989; George and Johnson, 1993). Frozen-thawed human (Sathananthan *et al.*, 1987) and bovine (Schmidt *et al.*, 1995) oocytes have been observed to have disrupted the plasma membrane and mitochondria. Increased permeability to calcium ions (Ca^{2+}) and loss of mitochondria would be expected to alter the dynamic assembly and cause the disassembly of both microtubules and microfilaments (Saunders and Parks, 1999). Osmotic stress, solute effects, or intracellular ice formation that occur during freezing process may irreversibly change the structure of plasma membrane or cytoskeleton so that it can no longer adjust to changes, including those cause by sperm penetration.

The effect of intracellular ice formation during cryopreservation is reduced by the addition of cryoprotectants in the freezing medium. Cryoprotectants dehydrate the cells and prevent intracellular ice formation during freezing (Leibo *et al.*, 1974; Rall, 1987). However, according to Gosden (2005), exposure of oocytes to cryoprotectants may increase intracellular Ca^{2+} and trigger premature exocytosis of cortical granule material, which modifies the zona pellucida enzymatically and renders it harder to bind spermatozoa ("zona hardening"). This problem, however, has been circumvented by the technique of ICSI (Palermo *et al.*, 1992), which has become a routine in all protocols for cryopreserved human oocytes. In mouse and rabbit oocytes, cryoprotectants alter microtubule organization (Vincent and Johnson, 1992); therefore, affect the cytoskeletal component of the cell that modulates chromosomal movement during meiosis and mitosis. Permeating cryoprotectants also increase retention of intracellular water, with subsequent intracellular ice formation and cell injury (Diller and Lynch, 1983; Karlsson *et al.*, 1994). During the addition and removal of cryoprotectants, excessive osmotic forces occur due to the rate at which cryoprotectants and water molecules pass through the cell membrane. Since water crosses faster, there is excessive exosmosis and consequent shrinkage during initial exposure to, and excessive osmosis and consequent swelling during initial removal of cryoprotectants. These volume changes, if excessive, may be harmful to the cell (Arav, 1992).

Cryopreservation of oocytes using the equilibration method has generally been unsuccessful because of the sensitivity of the oocytes to the chilling and freezing processes

associated with equilibration and slow freezing. However, the ultra rapid non-equilibration method of cryopreservation referred to as vitrification has, to some extent, been successful in cryopreserving the mammalian oocytes. Vitrification, or achieving a glass-like state without ice formation, was first proposed by Luyet (1937). Luyet and Hodapp (1938) vitrified frog sperm, while Rall and Fahy (1985) accomplish the first vitrification of mouse embryos. Since chilling injury is time dependent, the rationale is to prevent ice formation and injury by freezing at a rate fast enough to solidify the intracellular water before it can crystallize (Martino *et al.*, 1996). This is accomplished by exposing the cell to high concentrations of cryoprotectants for a very short equilibration period followed by very rapid cooling by plunging the cell into liquid nitrogen. The high osmolarity of the vitrification solution rapidly dehydrates the cell and immersion into liquid nitrogen quickly solidifies the cell before the remaining intracellular water has time to form the damaging ice crystals. The high concentrations of low and high molecular weight cryoprotective agents increases cell solution viscosity and favours formation of glass state (vitrify) when cooled at ultra-high rates. Vitrification prevents the extra-cellular ice formation and its cell-damaging osmotic and mechanical effects.

Two important concerns with vitrification are the increased toxicity of high levels of cryoprotectants at room temperature (Shaw *et al.*, 1992), and the ability to freeze and thaw fast enough to avoid ice crystal formation and devitrification (Vajta *et al.*, 1998). Cryoprotectant toxicity depends on duration and temperature of exposure, with cells from different animal species having different levels of cryoprotectant tolerance (Arav, 1992). Toxicity is controlled by exposing the cell to the high concentrations of cryoprotectants for a very short equilibration period, followed by very rapid cooling by plunging into liquid nitrogen (Picton *et al.*, 2002). To reduce the toxicity and osmotic changes caused by cryoprotectants during cooling and warming, Rall *et al.* (1987) suggested a stepwise exposure and a dilution method. Step-wise exposure appears to be the preferred method in most vitrification techniques. The open pulled straws (OPS) developed by Vajta *et al.* (1998), cryoloop (Mavrides and Morroll, 2002) and cryotop (Kelly *et al.*, 2004), used step-wise exposure of oocytes to cryoprotectants.

Premature cortical reaction and subsequent zona hardening caused by chilling is reduced by direct plunging into liquid nitrogen to achieve a rapid cooling rate without allowing time for chilling. Premature release of the cortical granules with subsequent zona hardening is also associated with high concentration of cryoprotectants (Gosden, 2005). This form of premature release of cortical granules is reduced by use of foetal bovine serum (FBS) in the vitrification medium (Vincent and Johnson, 1992; Carroll *et al.*, 1993). The FBS possibly binds the active components in the cortical granules exudates and prevent modifications of the

structural proteins in the zona (Bernard and Fuller, 1996). The failure of fertilization commonly seen after freezing mouse oocytes (Glenister *et al.*, 1987; Carroll *et al.*, 1989) is associated with changes occurring in the zona (Carroll *et al.*, 1990), and can be avoided by addition of FBS to the freezing and/or dilution media (Carroll *et al.*, 1993). In humans, a high level of fertilization was recorded when oocytes were cooled and frozen by a slow technique in media containing bovine serum albumin (Hunter *et al.*, 1991; Bernard *et al.*, 1992).

Vitrification requires an extremely high cooling rate. Cooling rate is a function of the heat transfer coefficient of the cooling liquid, the cell suspension solution and the vessel used, and their temperature differences (Arav, 1992). The cooling rate therefore, vary with the volume of the sample and type of carrier vessel used (Shaw *et al.*, 1991). The probability of vitrification increases as the sample volume decreases (Arav, 1992). Therefore, methods such as OPS (Vajta *et al.*, 1998), cryoloop (Lane *et al.*, 1999; Mavrides & Morroll, 2002), and cryotop (Kelly *et al.*, 2004) that use small volumes have been applied in the vitrification of oocytes and embryos, with variable success rates. A high cooling rate required for vitrification is also enhanced by use vessels such as metals that enhance heat conduction to the oocyte and cytoplasm. This, in combination with small volume, was applied in vitrification of bovine oocytes using an electron microscope grid, (Yoon *et al.*, 2003).

A study by De Matos *et al.* (1995) demonstrated that the percentage of embryos that developed to the blastocyst stage was significantly higher ($P < 0.01$) for oocytes matured in medium containing 100 µM of cysteamine than for those matured in the control medium. In the same study these authors further showed that addition of cysteamine, a thiol compound, to an *in vitro* maturation medium increased ($P < 0.05$) the intracellular glutathione (GSH) levels of the oocytes. Later, De Matos *et al.* (1997) demonstrated the role of cumulus cells in GSH synthesis during *in vitro* maturation (IVM) and importance of GSH in embryo development of the bovine oocytes. In pigs, Grupen *et al.* (1995) demonstrated that addition of 500 µM cysteamine to the maturation medium increased synchronous pronuclear formation and normal embryonic development in porcine oocytes matured and fertilized *in vitro*. However, Yamauchi and Nagai (1999) observed that the addition of 150 µM cysteamine to porcine oocyte culture medium increased oocyte GSH content and promoted male pronuclear formation after sperm penetration of denuded oocytes, but had no effects on the maturation rate or kinase activity. Addition of cysteamine to the IVM medium showed no beneficial effect on maturation and cleavage rate of buffalo (*Bubalus bubalis*) oocytes, however, *in vitro* embryo production efficiency improved, but not satisfactorily (Gasparrini *et al.*, 2000).

A study by Kelly *et al.* (2005) examined the effect of adding cysteamine to maturation media on survival of bovine and ovine oocytes following vitrification. Blastocyst development was influenced by a significant ($P < 0.001$) interaction between species and whether or not oocytes were vitrified. This interaction occurred because cysteamine improved the blastocyst rate in fresh ovine and vitrified bovine oocytes, but not in other treatments. These authors concluded that bovine oocytes with a 38.3% blastocyst rate can be vitrified successfully when maturation occurs in the presence of cysteamine; however, a comparable result did not occur in ovine oocytes (10.6% blastocyst rate) despite a 70% cleavage rate. In addition, Oyamada and Fukui (2004) observed that supplementation of bovine IVM medium with cysteamine improved the developmental competence and cryo-resistance of oocytes following vitrification, probably due to an increased GSH synthesis during the IVM process.

Methods such as OPS (Vajta *et al.*, 1998), cryoloop (Lane *et al.*, 1999; Mavrides and Morroll, 2002), and cryotop (Kelly *et al.*, 2004) that have demonstrated variable successes in vitrification of bovine oocytes utilize plastic and nylon in the vitrification of oocytes. The use of metals such as the electron microscope grid enhances heat conduction to the oocyte and cytoplasm, thereby achieving the high rate of cooling required for vitrification to occur (Yoon *et al.*, 2003). The current study examined the use of copper-wire cryoloop in vitrification of *in vitro* matured bovine oocytes. The study also examined the effects of cysteamine in *in vitro* maturation medium on subsequent embryo development from the fresh *in vitro* matured oocytes and copper-wire cryoloop-vitrified bovine oocytes.

6.3 Materials and methods

6.3.1 Ovary collection, oocyte retrieval and *in vitro* maturation

Bovine ovaries were collected from a local abattoir for four replicates. Ovaries were transported without saline in a polythene bag placed in an insulated container. Ovaries were washed three times in pre-warmed (30 °C) normal saline supplemented with 10 µg/mL ciprofloxacin hydrochloride (CF-HCl) and oocytes aspirated from follicles 2-8 mm in diameter using suction pressure. The collected follicular fluid was allowed to settle for 10 min in 50 mL centrifuge tubes (Cellstar®, Greiner bio-one, Germany), the supernatant discarded and re-suspended in pre-warmed modified D-PBS (D-PBS supplemented with 10 µg/mL CF-HCl and 1% FBS). After re-suspension and discarding the supernatant twice, the remaining 2 mL of the medium was transferred to 60 x 15 mm Petri dishes (Cellstar®, Greiner bio-one, Germany). The medium was then examined under a stereoscope (Nikon®, SMZ-2T, Japan), and cumulus

oocyte complexes (COCs) with compact multi-layered cumulus investment and homogenous ooplasm selected.

The selected COCs were washed twice in HEPES-TALP (see Appendix A2) and for each trial the oocytes were randomly divided into two groups. Group one (control/not treated with cysteamine) was washed and cultured in a pre-equilibrated maturation medium (see Appendix A1). The oocytes to be matured with cysteamine (group two) were washed and cultured in the maturation medium same as in group one, but with 100 µM cysteamine added. Oocytes were cultured in groups of 10-15 in 50 µL drops of the maturation medium under mineral oil, and incubated at 39 °C in a 5% CO_2 in humidified air atmosphere for 24 h. The duration from ovary collection to beginning of maturation was approximately 5 h.

In Experiment one, the oocytes matured with and without cysteamine were subjected to standard *in vitro* fertilization procedure without vitrification. However, in Experiment two the oocytes matured with/without cysteamine were *in vitro* fertilized fresh (without vitrification) or after vitrification.

6.3.2 Preparation of vitrification and thawing media

Fresh vitrification and thawing media were prepared on the day of use and the media preparations were performed under a laminar flow cabinet. Oocyte holding medium (HM), equilibration medium (EM) and vitrification medium (VM) were prepared for the oocyte vitrification procedure. Oocyte HM was HEPES-buffered Tyrode's lactose medium supplemented with 20% FBS, 0.2 mM sodium pyruvate and 10 mg/mL CF-HCl. Oocyte EM consisted of 5% ethylene glycol (EG) and 5% dimethyl sulfoxide (DMSO) in HM, while oocyte VM was made of 20% EG, 20% DMSO and 1 M sucrose in HM. In every vitrification cycle, 4 mL each of HM and EM, and 2 mL of VM were prepared.

The thawing procedure consisted of three thawing media (TM) and the HM. Thawing medium one (TM1) consisted of a 1 M sucrose in HM, thawing medium two (TM2) was 0.5 M sucrose in HM, and thawing medium three (TM3) was 0.25 M sucrose in HM. A total of 4 mL of each medium was prepared for each thawing cycle.

6.3.3 Preparation of copper-wire cryoloops

Cryoloops with an internal diameter of approximately 1 mm were made using a thin copper wire 71.0 ± 0.3 µm in diameter. The loose ends of the loop were trimmed and glued into

a 20 µL pipette tip. The cryoloops were sterilized by treating with absolute ethanol before they were used.

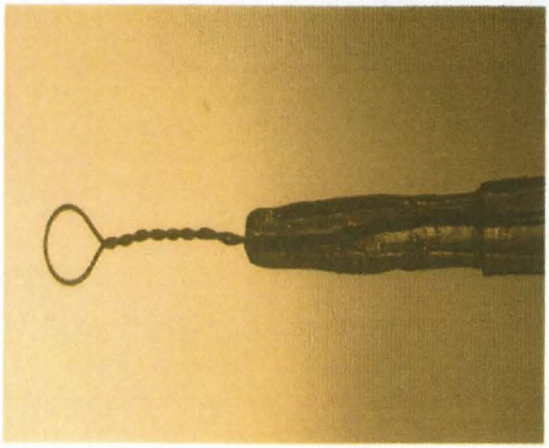


Fig. 6.1 The vitrification copper-wire loop fixed to the end of 20 µL micro-pipette tip.

6.3.4 Oocyte vitrification

Oocyte holding, equilibration and vitrification were performed at room temperature (24 °C) under a laminar flow cabinet. Two experiments were conducted. Experiment one compared the vitrification of *in vitro* matured oocytes of the control group with oocyte matured in a medium containing 100 µM cysteamine, using the copper-wire cryoloop method of vitrification. After maturation, the cysteamine-treated and the non-treated oocytes were each randomly divided into the non-vitrification and vitrification groups. Those in the non-vitrification group were subjected to a standard *in vitro* fertilization (IVF) procedure without vitrification. The oocytes of vitrification group were vitrified before subjecting them to a standard IVF procedure.

Before vitrification, the oocytes were subjected to a solution of 300 µg/mL hyaluronidase in M2 medium for 1 min, while they were agitated through aspiration and squirting to partially denude them. The oocytes were washed twice in HM and then kept for 5-20 min in the same medium in a 35 x 10 mm Petri dish before equilibration. Groups of 5-10 oocytes were transferred into 2 mL of EM in a 35 x 10 mm Petri dish where they were kept for 1 min during the vitrification process. A 100 µL droplet and five to seven 10 µL droplets of VM were made on the inner surface of a 35 x 10 mm Petri dish lid for vitrification (Fig. 6.2). Oocytes were transferred using a fine fire-pulled Pasture pipette from the EM to the 100 µL VM drop, then immediately to one of the 10 µL VM drops, and finally loaded onto a thin film of VM on the copper-wire cryoloop with a minimum volume of the VM. The procedures were all performed within 30 seconds. The cryoloop loaded with the oocytes was directly immersed into liquid

nitrogen and fitted into a trimmed colour-coded 0.5 ml plastic straw to protect the vitrified oocytes. The cryoloops were packed in goblets and stored in liquid nitrogen at −196 °C.

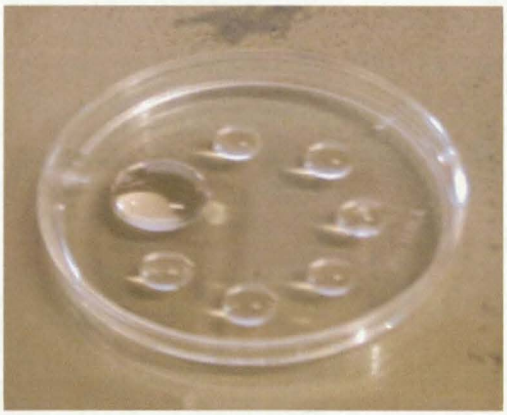


Fig. 6.2 Drops of vitrification medium (VM) in the lid of a 35 mm Petri dish.

6.3.5 Thawing of vitrified oocytes

The vitrified oocytes were thawed after 24 to 48 h of storage in liquid nitrogen using a stepwise thawing procedure (Rall *et al.*, 1987). Briefly, 2 mL each of TM1, TM2, TM3 and HM were prepared in 35 x 10 mm Petri dishes for the thawing of the oocytes. The TM1 was pre-warmed to 37 ºC, while the rest of the media were kept under a laminar flow hood on a warm stage at approximately 30 °C. The cryoloops were removed from liquid nitrogen, the 0.5 ml straw covering the loop immediately removed and the loop immersed in TM1, in which the oocytes dropped due to the change in surface tension forces. The oocytes were held for 1 min in TM1 then transferred to TM2, and then to TM3 where they were held for 3 min in each medium. Thereafter, the ooctyes were transferred to HM where they were held for 5-20 min and then incubated in a post-thaw incubation medium (TCM-199, 20% FBS, 10 µg/mL CF-HCl) for 30 min to 1 h before IVF. During their preparation for IVF, the oocytes were evaluated and selected based on the morphological appearance of the zona, cytoplasm and plasma membrane, and perivitelline space, as described by Mavrides and Morroll (2002). Oocytes with fragmented cytoplasm, indistinct plasma membranes, increased perivitelline space and fractured zona pellucidae were considered not to have survived vitrification and were not used for IVF. The number of oocytes that degenerated during IVF and *in vitro* culture (IVC) were also recorded.

6.3.6 *In vitro* fertilization

In vitro fertilization of non-vitrified and vitrified oocytes was performed on different days. Two 0.25 ml straws of frozen bovine epididymal sperm from three bulls were thawed in a water bath at 37 ºC for 1 min. Motile sperm were separated by swim-up in sperm-TALP (see

Appendix A2) as described by Parrish *et al.* (1985). Sperm concentration was determined using haemocytometer and sperm concentration adjusted to 10 x 10^6 sperm/mL. The thawed selected oocytes were washed twice in HEPES-TALP medium and once in IVF-TALP medium (see Appendix A2). Groups of 15-20 oocytes were transferred into pre-equilibrated 100µL IVF-TALP drops under oil in 35 x 10 mm Petri dishes. To each IVF drop, 4 µL of PHE (penicillamine 2 mM, hypotaurine 10 mM, epinephrine 0.1 mM) and 10 µL of sperm (1 x 10^6 sperm/mL final concentration) was added and the plates incubated at 39 °C in 5% CO_2 in humidified air.

6.3.7 *In vitro* culture and embryo evaluation

After *in vitro* fertilization, presumptive embryos were washed twice in HEPES-TALP and once in modified CR1aa medium (see Appendix A3). The presumptive embryos were cultured in groups of 15-20, with the cumulus cells intact. Embryo culture was performed in 30 µL drops of modified CR1aa under oil at 39 °C in 5% CO_2 in humidified air. Evaluation of embryos was done on days 3 for cleavage (day zero was the day of insemination), on day 5 for morula formation, on days 7 and 8 for blastulation, and on day 8 for blastocyst expansion.

6.3.8 Statistical analysis

Data from four replicates for each trial were captured in Microsoft Excel and exported to statistical analysis system (SAS, version 9.1, 2006) for analysis. The data on oocyte recovery, oocyte survival (morphological appearance), and embryo development were analyzed using the generalized linear model (GENMOD) procedure, while blastomere number of the day 8 embryos was compared using an analysis of variance (ANOVA) procedure.

6.4 Results

Table 6.1 shows the effect of cysteamine on development of embryo from bovine oocytes (n = 276) *in vitro* matured in a medium without cysteamine (n = 133) and with 100 µM cysteamine (n = 143). Cleavage rate for oocytes *in vitro* matured without cysteamine (60.9 ± 6.4%) did not differ ($P > 0.05$) from those *in vitro* matured in a medium containing cysteamine (66.4 ± 19.0). However, morula and blastocyst formation on days 5 and 7 were at higher rates ($P < 0.05$) for oocytes matured with cysteamine than those matured without cysteamine. Similarly, blastocyst formation on day 8 was lower ($P < 0.05$), in the control than the treated group. Blastocyst expansion on day 8 in oocytes matured without cysteamine was also lower ($P < 0.05$) than that of oocytes matured with cysteamine.

Table 6.1 Embryo development after *in vitro* maturation without and with cysteamine.

		Embryo development (%)				
	No. of oocytes	Cleaved (Day 3)	Morula (Day 5)	Blastocyst (Day 7)	Blastocyst (Day 8)	Expanded blastocyst (Day 8)
Cysteamine - (control)	133	60.1±6.4[a]	36.1±12.8[b]	26.3±11.3[b]	26.3±11.3[b]	12.0±5.4[b]
Cysteamine + (treatment)	143	66.4±19.0[a]	50.3±13.5[a]	42.0±12.9[a]	42.0±12.9[a]	23.8±5.4[a]

Columns with different superscripts (a, b) are significantly different ($P < 0.05$).

There was no difference ($P > 0.05$) in blastomere number between day 8 embryos from oocytes matured without cysteamine (99.3 ± 28.5) and with cysteamine (103 ± 30.5) (Fig. 6.2).

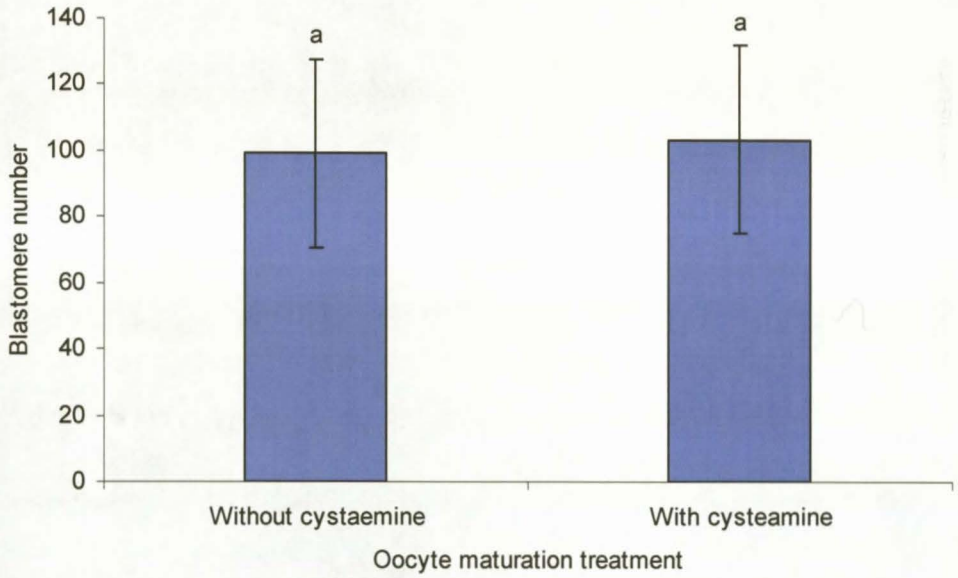


Fig. 6.3 Blastomere number of day 8 embryos from oocytes *in vitro* matured without and with cysteamine.

Exposure of oocytes to hyaluronidase (300 µg/mL in M2 medium) for 1 minute was sufficient to cause partial denudation of the oocytes for vitrification (Fig. 6.4). Extension of exposure resulted into complete denudation.

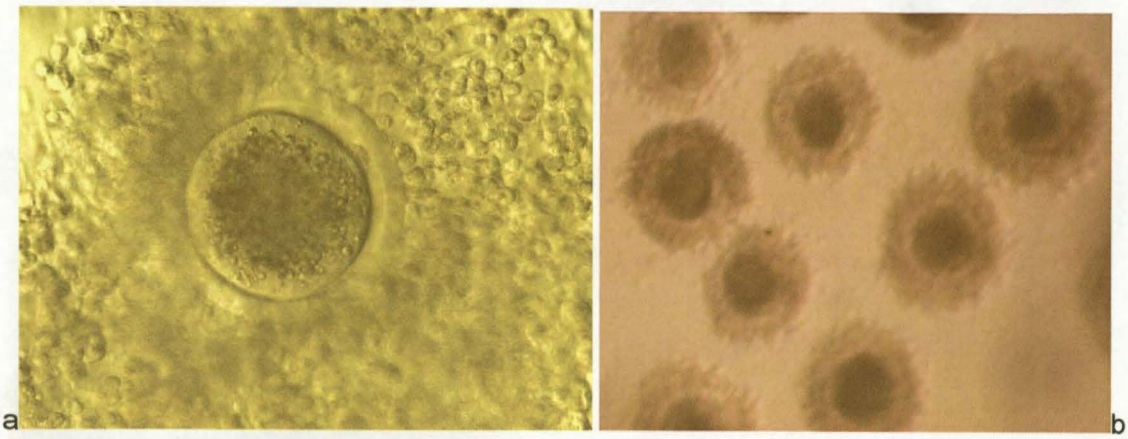


Fig. 6.4 *In vitro* matured bovine oocytes: (a) an oocyte before and (b) oocytes after treatment with hyaluronidase for 1 min (X40 magnification).

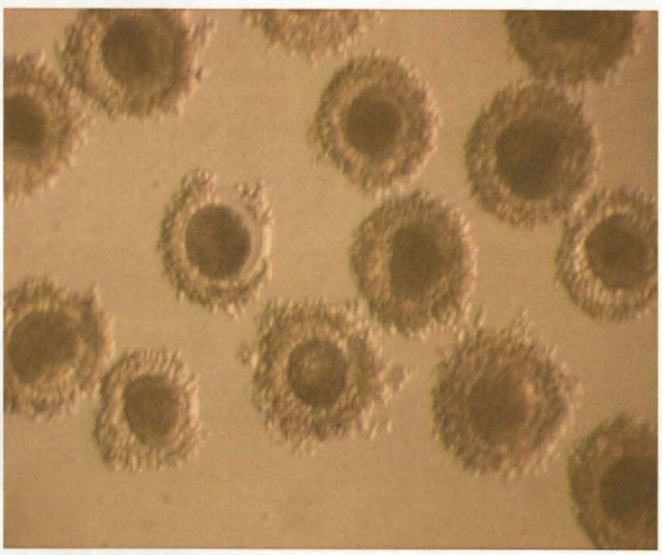


Fig. 6.5 *In vitro* matured bovine oocytes in equilibration medium for 1 min (X40 magnification).

Exposure of oocytes to the equilibration medium (EM) caused initial shrinkage of the cytoplasm (Fig. 6.5), but subsequently the oocytes regained their cytoplasmic volume as equilibration occurred in the medium. Transferring the oocytes from equilibration medium into vitrification medium (VM) drops caused further shrinkage of the cytoplasm. The oocytes were immediately immersed into liquid nitrogen to vitrify.

After thawing and exposure of the oocytes through the serial dilutions of the thawing medium and holding medium, the post-thaw morphology of the oocytes that survived vitrification appeared normal (Fig. 6.6a). The oocytes that survived the process of vitrification cleaved after fertilization (Fig. 6.6b).

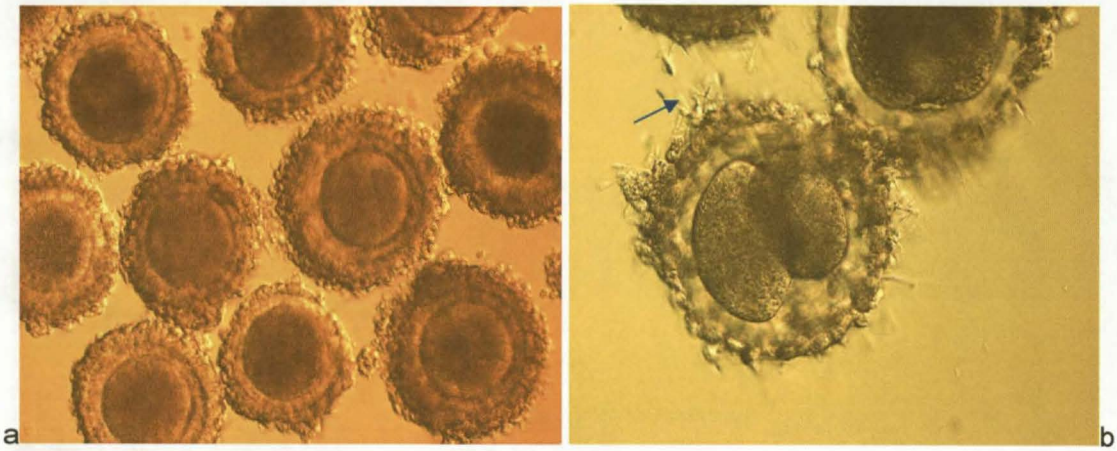
a  b

Fig. 6.6 Post-thaw vitrified *in vitro* matured bovine oocytes (a) before *in vitro* fertilization and (b) 21 hpi in fertilization medium showing 2-cell embryo and accessory sperm (arrow) attached to the zona (X40 magnification).

Table 6.2 Post-thaw recovery and survival rates of oocytes matured with or without cysteamine and vitrified using a copper-wire cryoloop method.

	No. of oocytes vitrified	No. of oocytes recovered	Normal oocytes post-thaw	Normal oocytes fertilized	Normal oocytes cultured
Cysteamine - (control)	166	163 (98.0±3.3)	154 (95.3±6.0)	108 (68.1±15.6)	64 (41.0±13.3)
Cysteamine + (treatment)	181	164 (91.3±7.7)	145 (89.0±13.6)	106 (65.4±18.0)	70 (43.3±6.5)

Table 6.2 shows an oocyte recovery rate of more than 90% and a decreased survival rate after thawing. The percentage of normal oocytes decreased from 95.3 ± 6.0 and 89.0 ± 13.6 during the post-thaw culture, and 68.1 ± 15.6 and 65.4 ± 18.0 in IVF culture to 41.0 ± 13.3 and 43.3 ± 6.5 during the IVC of oocytes matured without and with cysteamine, respectively. There were no significant differences ($P > 0.05$) in the percentage of normal oocytes during the post-thaw, IVF and IVC culture between the control and the treatment group.

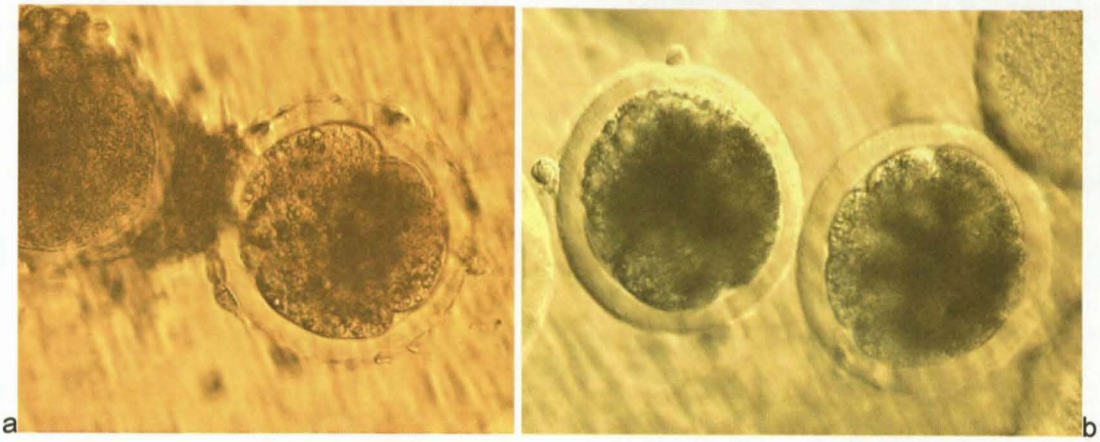


Fig. 6.7 Embryos from oocytes *in vitro* matured in a medium without cysteamine and vitrified using the copper-wire cryoloop method: (a) 4-cell embryo 48 hpi, and (b) morulae 5 days post-insemination (X100 magnification).

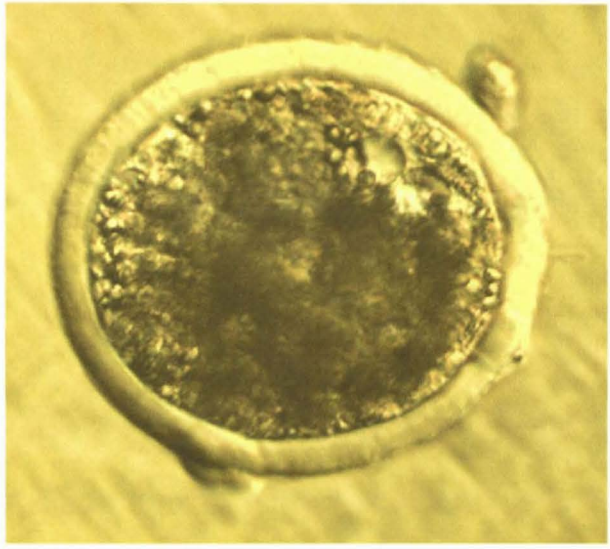


Fig. 6.8 Day 7 early blastocyst from an oocyte *in vitro* matured in a medium containing cysteamine and vitrified using a copper-wire cryoloop method (X200 magnification).

Figure 6.7 and Figure 6.8 show normal development of embryos from oocytes vitrified after maturation in medium without and with cysteamine, respectively. Cysteamine did not improve ($P > 0.05$) embryo development in the non-vitrified oocytes, but embryo development in general was low (Table 6.3). Similarly, there was no difference ($P > 0.05$) in day 3 cleavage rate of vitrified oocytes following maturation without and with cysteamine. However, the morula and blastocyst rates were higher ($P < 0.05$) for oocytes matured with cysteamine than without cysteamine, and vitrified using the wire-loop. The day 5 morula rate and days 7 and 8

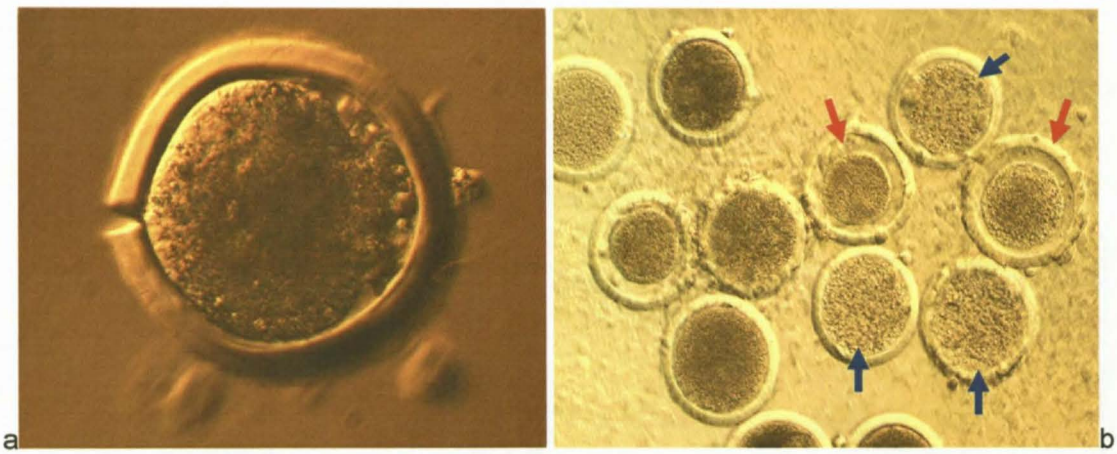
blastocyst rate of oocytes vitrified after maturation in cysteamine tended to be similar ($P > 0.05$) to that of oocytes matured without and with cysteamine, but not vitrified (Table 6.3).

Table 6.3 Embryo development from oocytes matured without and with cysteamine and vitrified using a copper-wire cryoloop. Values in brackets represent percentages.

Fresh/ Vitrified	Cystea- mine	No. of oocytes	No. of oocytes cleaved (Day 3)	Morula (Day 5)	Blastocyst (Day 7)	Blastocyst (Day 8)
Fresh	-	130	89 (68.5)^{a}	56 (43.1)^{a}	36 (27.7)^{ac}	36 (27.7)^{ac}
Fresh	+	136	87 (64.0)^{a}	55 (40.4)^{ac}	39 (28.7)^{ac}	39 (28.7)^{ac}
Vitrified	-	51	18 (35.3)^{b}	3 (5.9)^{b}	2 (3.9)^{b}	2 (3.9)^{b}
Vitrified	+	54	25 (46.3)^{b}	15 (27.8)^{c}	10 (18.5)^{c}	10 (18.5)^{c}

^{a,b,c}Values in columns with different superscripts are significantly different ($P < 0.05$)

Oocytes with abnormal post-thaw morphology were not selected for fertilization or failed to cleave after IVF and IVC incubation. The morphological abnormalities appeared mainly in the form of fractured zona and fragmented cytoplasm (Fig. 6.9a), and enlarged perivitelline spaces, and fragmented cytoplasm (Fig. 6.9b).



a b

Fig. 6.9 Uncleaved vitrified and cryo-damaged *in vitro* matured bovine oocytes showing (a) a fractured zona pellucida and fragmented cytoplasm (X100 magnification), and (b) degenerated oocytes with enlarged perivitelline spaces (red arrows) and fragmented cytoplasm (blue arrows) (X40 magnification).

6.5 Discussion

In vitro development of embryos from oocytes matured with/without adding cysteamine in the maturation medium is shown in Fig. 6.1. Morula formation on day 5, blastocyst formation on days 7 and 8, and blastocyst expansion on day 8 were higher ($P < 0.05$) for oocytes matured with cysteamine than for those matured without cysteamine. However, there were no differences ($P > 0.05$) in cleavage rate on day 3 (Table 6.1) and in the day 8 blastomere number (Fig. 6.2) for oocytes matured in cysteamine or without cysteamine. These findings agreed with a report on bovine IVF by De Matos *et al.* (1995), where no differences ($P > 0.05$) in maturation, cleavage rates and in the mean cell numbers per blastocyst among treatments were observed. Another study by Gasparrini *et al.* (2000) also observed no beneficial effect of cysteamine on maturation and cleavage rate of buffalo (*Bubalus bubalis*) oocytes. These authors further reported that the percentage of embryos that developed to the compact morula and the blastocyst stages, and the percentage of transferable embryos, were significantly higher ($P < 0.01$) for oocytes matured in medium containing 50 µM of cysteamine, than for oocytes matured in medium containing 0, 100 and 200 µM cysteamine. In porcine IVF, Grupen *et al.* (1995) also showed that the addition of 500 µM cysteamine to maturation medium increased normal embryonic development.

Studies in bovine IVM (De Matos *et al.*, 1995) and in porcine IVM (Yamauchi and Nagai, 1999) demonstrated that addition of cysteamine in IVM medium increased the intracellular glutathione (GSH) levels of the oocytes. A further study by De Matos *et al.* (1997) showed the role cumulus cells play in GSH synthesis during IVM, and the GSH importance in embryo development. Glutathione is a thiol compound. According to De Matos and Furnus (2000), the high intracellular GSH levels after induction of GSH synthesis in bovine IVM by thiol compounds are maintained during IVF and are still present at the beginning of IVC, which improve developmental rates. In addition, these authors reported that this metabolic pathway is an important component of the cytoplasmic maturation process that affects the subsequent steps of *in vitro* embryo production. In the pig, the improvement in normal embryo development following addition of cysteamine in oocyte maturation medium is associated with synchronization of pronuclear formation (Grupen *et al.*, 1995; Yamauchi and Nagai, 1999).

There was no difference ($P > 0.05$) in embryo development in the non-vitrified oocytes that were matured without and with cysteamine (Table 6.3). This supports findings of Kelly *et al.* (2005) who obtained no differences in cleavage and blastocyst rates for fresh bovine oocytes matured without and with cysteamine. However, in the study by Kelly *et al.* (2005) a significant difference for ovine oocytes with the same treatment was reported. In a study by Gasparrini *et*

al. (2000), no beneficial effect cysteamine on maturation and cleavage rate was observed in buffalo oocytes, however, *in vitro* embryo production efficiency improved, but not satisfactorily. In studies in the cow (De Matos *et al.*, 1995), pig (Grupen *et al.*, 1995; Yamauchi and Nagai, 1999), and goat (Kelly *et al.*, 2005), embryo development improved with the addition of cysteamine in the maturation medium. A similar observation was made in the current study in an earlier trial (Table 6.1).

In pigs the improvement in embryo development has been shown to be associated with synchronization of pronuclear formation. Inhibition of protein synthesis during certain critical stages of maturation in porcine oocytes dramatically impairs the transformation of the sperm nucleus to a male pronucleus (Ding *et al.*, 1992). A critical step in the formation of a male pronucleus is the decondensation of the sperm nucleus (Eppig, 1996). A male pronucleus does not form after sperm penetration of immature oocytes (Perreault, 1990). Formation of the male pronucleus probably requires the reduction of the disulfide bonds of protamine so that factors within the oocyte can extract or degrade the protamines in the sperm nucleus and replace them with histones (Perreault, 1990). The reducing agent glutathione plays a key role in this process (Perreault *et al.*, 1988; Yoshida, 1993). The concentration of glutathione increases during oocyte maturation, and inhibition of the synthesis of this reducing agent suppresses the formation of the male pronucleus (Perreault *et al.*, 1988; Yoshida, 1993). Thus, the production of glutathione is a critical part of cytoplasmic maturation and subsequent embryo development (Eppig, 1996).

In the cow, the mechanism of improvement in embryo development associated with supplementation of IVM medium with cysteamine involves GSH metabolic pathways, that is maintained during IVM, IVF and IVC (De Matos and Furnus, 2000). However, in the current study (Table 6.1 and Table 6.3), and in studies by De Matos *et al.* (1995), Kelly *et al.* (2005), there were no significant differences in cleavage rates of oocytes that were matured without cysteamine and not vitrified. The reason for these findings was not known. This can possibly be ascribed to different factors such as ovary and oocyte handling before *in vitro* maturation, which can affect subsequent embryo development. In the current study the results in Table 6.1 and Table 6.3 were from two different trials that could have been influenced by such factors during the *in vitro* procedures.

Table 6.3 also shows a lower ($P < 0.05$) day 5 morula rate and days 7 and 8 blastocyst rates for oocytes matured without cysteamine than those matured with cysteamine followed by vitrification. A similar observation was made by Oyamada and Fukui (2004) who reported that

the addition of cysteamine to bovine IVM medium improved the developmental competence and cryo-resistance of oocytes following vitrification, probably due to an increased GSH synthesis during the IVM process. Kelly *et al.* (2005) reported a blastocyst rate of 38% compared to 5% from vitrified bovine oocytes *in vitro* matured with and without cysteamine, respectively. In the current study, the blastocyst rates were 18% and 3.9% for vitrified oocytes following IVM in medium with and without cysteamine, respectively. This difference could be due to the factors such as ovary transportation temperature and duration, associated with handling of ovaries and oocytes before and during culture.

The development of vitrified oocytes, particularly those matured without cysteamine was significantly lower ($P < 0.05$) than that of non-vitrified oocytes matured with and without cysteamine (Table 6.3). However, as the embryos developed to the blastocyst stage, the development of embryos from oocytes vitrified after maturation in medium with cysteamine tended to be similar to that of oocytes matured without and with cysteamine but not vitrified ($P > 0.05$). The study by Kelly *et al.* (2005) made similar findings in bovine, but not ovine oocytes. Generally, the process of vitrification is detrimental to oocytes. Vitrification subjects the oocytes to extremely toxic level of cryoprotectants, and the associated osmotic changes (Shaw *et al.*, 1992), and if freeze-thawing is not fast enough, there is a lethal ice crystal formation and devitrification (Vajta *et al.*, 1998). Therefore, the development rate of vitrified oocytes would be lower than the non-vitrified oocytes as found in the current study. Addition of thiol compounds such as cysteamine appear to improve the development to blastocysts of vitrified oocytes towards the level attained by the non-vitrified oocytes, as was observed in bovine oocytes in the study by Kelly *et al.* (2005) and in the current study. In the cow, the addition of cysteamine to the IVM medium improved the developmental competence and cryo-resistance of oocytes following vitrification, probably due to an increased GSH synthesis during the IVM process (Oyamada and Fukui, 2004). Such high levels of intra-cellular GSH are maintained up to the beginning of IVC, resulting to an improved embryo development *in vitro* (De Matos and Furnus, 2000).

The recovery rate of 98.0 ± 3.3% and 91.3 ± 7.7% for oocytes matured without and with cysteamine, respectively, was attained using the copper-wire cryoloop vitrification (Table 6.2). This was comparable to the 95.2 ± 1.5% and 97.5 ± 1.2% reported for slow freezing in 0.25 ml straws and nylon cryoloop vitrification (Mavrides and Morroll, 2002). These authors attributed the 'lost' oocytes to dropping off the cryoloop or to operator error. The un-recovered oocytes in the current study could also be attributed to similar factors.

The percentage of normal oocytes was 95.3 ± 6.0 and 89.0 ± 13.6 during the post-thaw culture of oocytes matured without and with cysteamine, respectively (Table 6.2). The survival rate decreased to 68.1 ± 15.6 and 65.4 ± 18.0, in IVF culture, and to 41.0 ± 13.3 and 43.3 ± 6.5, during the IVC of oocytes matured without and with cysteamine, respectively (Table 6.2). Oocyte survival in the post-thaw culture was similar to the 90.5 ± 2.3% obtained by Mavrides and Morroll (2002) using nylon cryoloop vitrification of bovine oocytes. The findings of the current study was also comparable to the survival rates obtained in a study by Chen *et al.* (2001), who recorded the post-thaw survival rates of 79% for closed pulled straws (CPS), 77% for straw, 63% for opened pulled straws (OPS) and 39% for the grid methods of vitrification of mouse oocytes.

There were no differences ($P > 0.05$) in the percentage of normal oocytes during the post-thaw, IVF and day three IVC culture between the cysteamine-treated and the non-treated group. Inability of the oocytes to survive vitrification was indicated by an enlarged perivitelline space, fragmented cytoplasm and the absence of cleavage 3 days post insemination (Fig. 6.9b). A likely cause of oocyte cryo-damage seen in this study may be a high concentration of cryoprotectants (20% EG and 20% DMSO) used in vitrification. Although cryoprotectant concentration of 40% seems to be the optimal for vitrification of bovine oocytes (Otoi *et al.*, 1998), the increased toxicity of this high level of cryoprotectants at room temperature is a concern (Shaw *et al.*, 1992). Cryoprotectant toxicity depends on duration and temperature of exposure, with cells from different animal species tolerating different levels of cryoprotectants (Arav, 1992). Bovine oocytes are very sensitive to exposure to high concentrations of cryoprotectant solutions (Parks and Ruffing, 1992), and the latter can produce irreversible damage to the cytoskeletal organization of the oocytes (Fuku *et al.*, 1995). The sensitivity of bovine oocytes to high concentration of cryoprotectants could have resulted in the low cleavage rates of vitrified-thawed oocytes.

Another concern with vitrification is the ability to cool and thaw fast enough to avoid crystal formation and devitrification (Vajta *et al.*, 1998). The cooling rate vary with the volume of the sample and type of carrier vessel used (Shaw *et al.*, 1991), and the probability of vitrification increases as the sample volume decreases (Arav, 1992). The current study used a copper-wire cryoloop (1 mm in diameter) and a small volume (<1 µL) of vitrification solution in a thin film. Similar methods but with a nylon cryoloop have been used by Lane *et al.* (1999) and Mavrides and Morroll (2002). The small volume and heat conduction of copper permits rapid and uniform heat transfer during cooling required for vitrification as reported by Lane *et al.* (1999). However, it was not known whether the ultra rapid heat changes during vitrification and thawing resulted

in zona fractures seen in some oocytes post-thaw (Fig. 6.9a). It was also not known whether copper is toxic to the oocytes that could have contributed to the low rates of cleavage and subsequent embryo development.

Partial removal of the cumulus-oocyte complex (COC) is a requirement for vitrification of matured oocytes (Le Gal and Massip, 1999; Vajta, 2000). A study by Varga *et al.* (2006) in pigs demonstrated the importance of cumulus cells in protecting the viability of oocytes during vitrification/warming procedure. In the current study, the oocytes were partially denuded using 300 µg/mL porcine hyaluronidase (Fig. 6.3b). A major constituent of the COC is hyaluronan, which is involved in maintaining the structure and shape of the COC that is necessary for maturation and fertilization (Richards, 2005). Hyaluronidase digests the hyaluronan interspaced between the cumulus cells, a process that removes the COC (Sabeur *et al.*, 1997). Apart from this artificial disruption of the COC, the other direct or indirect effects of hyaluronidase in the development of bovine embryos are not known. A study by Fatehi *et al.* (2002) showed that removal of cumulus cells prior to IVF significantly reduced the cleavage rate of bovine oocytes (25% for denuded oocytes versus 56% for COCs). An earlier study by Zhang *et al.* (1995) also demonstrated a significantly reduced rate of fertilization, cleavage, morula and blastocyst in denuded oocytes when compared to COCs. Perhaps, partial denudation could have been one of the causes for the low cleavage rate and subsequent embryo development in the vitrified oocytes in the current study.

6.6 Conclusion

In vitro matured bovine oocytes can successfully be vitrified using a copper-wire cryoloop and used for ARTs. Maturation of bovine oocytes in cysteamine improved embryo development in fresh and vitrified oocytes. It was concluded from the findings of this part of the study that cysteamine improves embryo development in fresh and vitrified oocytes, but under certain circumstances of *in vitro* embryo culture, this effect is not demonstrated. A further study towards improvement of the copper-wire cryoloop method of oocyte vitrification is recommended to improve oocyte survival and embryo development rates.

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CHAPTER 7

Conclusions and recommendations

The development of effective methods of cryopreserving mammalian gametes is important in the application and promotion of assisted reproductive technologies (ARTs). For instance, the application of artificial insemination particularly in cattle was greatly facilitated by development of effective sperm freezing procedures. It is known that other than ejaculated sperm, sperm from the excurrent ducts, namely the vas deferens and epididymis, as well as testicular sperm can fertilize oocytes through the application of ART. Likewise, oocytes obtained from the ovaries are capable of being fertilized using these technologies to produce viable embryos. Although sperm freezing has been largely successful, there is need to study the effects of crypreservation on sperm other than ejaculated sperm because of their increasing importance in ARTs. Oocyte cryopreservation, however, has variable successes and the search for improved methods of cryopreservation has been ongoing.

This study examined the efficacy of a commercial and a saline extender and cryoprotectants in cryopreserving the fertilizing potential and *in vitro* embryo production by bovine epididymal sperm. The study also compared cryopreserved bovine and buffalo epididymal sperm for the *in vitro* production of bovine and hybrid embryos, respectively. Effects of adding cysteamine to the maturation medium of bovine oocytes, followed by *in vitro* fertilization and culture of the *in vitro* matured, fresh or vitrified-thawed oocytes, were also investigated. The following conclusions and recommendations were made:

1. Bovine epididymal sperm can be cryopreserved in a serum-based, HEPES-buffered saline extender like modified Tyrode's lactose (MTL) with acceptable post-thaw sperm fertility parameters, but lower than that of Biladyl (BIL), a commercial extender. The post-thaw sperm fertilizing potential as measured by sperm motility, sperm viability, and sperm with normal membranes, and acrosome integrity, is dependent on the equilibration time and cryoprotectant used. Equilibration for 2 h and 4 h is recommended, though 4 h yielded better results, while 0 h of equilibration is not recommended. The cryoprotectants glycerol (GLY) and ethylene glycol (EG) produced good results, while dimethyl sulfoxide (DMSO) offered little cryoprotection at the concentration used in this study. Although tris-egg yolk extenders with GLY such as BIL are very useful for the cryopreservation of bull epididymal sperm, MTL (HEPES-buffered saline containing 20% v/v bovine serum) with GLY or EG, and 2 h or 4 h of equilibration produced promising results. The spermatozoa in MTL are also easy to examine under the microscope

because of their transparency. Further research on improving cryopreservation of sperm focusing on reducing the equilibration time to 0 h, and using extenders that are simple to prepare and store at ambient temperature are recommended to ease sperm cryopreservation, especially for field conditions.

2. Bovine embryos can be produced *in vitro* using epididymal spermatozoa frozen in a serum-based saline medium (MTL) containing permeating cryoprotectants. When freezing bovine epididymal spermatozoa in MTL for *in vitro* fertilization, GLY or EG, but not DMSO, are the preferred permeating cryoprotectants. An equilibration time of 2 h and 4 h in the non-glycerol or non-ethylene glycol portion of the extender produced comparable results of embryo development *in vitro*. Embryo development *in vitro* i.e. to the hatched blastocyst stage, from bovine epididymal spermatozoa frozen in MTL supplemented with 20% bovine serum, 0.95 M GLY or EG, are comparable to that obtained with sperm cryopreserved in a commercial extender such as BIL. A basic saline solution such as MTL supplemented with 20% serum in the presence of the appropriate molar concentration of GLY or EG can be used as a substitute for commercial extender such as BIL for freezing bovine epididymal sperm, and possibly also of other species. A 2 h equilibration period in the non-cryoprotectant portion of MTL is recommended to reduce the equilibration time. An *in vivo* fertilization study through artificial insemination (AI) using bovine epididymal sperm cryopreserved in MTL supplemented with 20% bovine serum, 0.95 M glycerol or ethylene glycol is recommended. Further research in the cause of the low *in vitro* embryo development following insemination with bovine epididymal spermatozoa cryopreserved in DMSO is also recommended.

3. Bovine epididymal spermatozoa cryopreserved in Biladyl® and modified Tyrode's lactate supplemented with bovine serum and ethylene glycol can be refrozen in the same media after swim-up and can be used to produce embryos *in vitro*. Survival of spermatozoa is, however, markedly reduced after freeze-thaw cycle one and the rate of embryo production is consequently lower for spermatozoa refrozen in the respective media. Bovine epididymal spermatozoa can undergo two freeze-thaw cycles in Biladyl®, but preferably modified Tyrode's lactose and the sperm can be used in ARTs. Further research on *in vivo* embryo production and pregnancy rates using bovine epididymal sperm subjected to two or more freeze-thaw cycles is recommended.

4. Cryopreserved epididymal sperm of the African buffalo can *in vitro* fertilize *in vitro* matured bovine oocytes. However, the low and slow rate of development of the hybrid embryos, abnormal embryo development, and inability to progress beyond the pre-compacted

morula (16-cell) stage, are indications of a barrier to hybridization in the early stages of embryonic development. Chromosomal disparity is likely the cause of the fertilization abnormalities, abnormal development and subsequent arrest impairing formation of pre-implantation hybrid embryos. Transfer of the hybrid embryos does not prevent the development arrest of the embryos. Investigation into the developmental abnormalities including reciprocal hybridization and genetic studies of the hybrid embryos are recommended, because hybridization may occur in one direction but not *vice versa*.

5. *In vitro* matured bovine oocytes can successfully be vitrified using a copper-wire cryoloop and used for ARTs. Maturation of bovine oocytes in cysteamine improved embryo development in fresh and vitrified oocytes. It was concluded from the findings of this part of the study that cysteamine improves embryo development in fresh and vitrified oocytes, but under certain circumstances of *in vitro* embryo culture, this effect is not demonstrated. A further study towards improvement of the copper-wire cryoloop method of oocyte vitrification is recommended to improve oocyte survival and embryo development rates.

Appendices

Appendix A
Preparation of media and media additives

1. Preparation of oocyte maturation medium

a) Maturation medium with hMG

	Stock	Final	Vol (mL)
TCM-199	-	-	3432
FBS	-	10% v/v	0.400
L-glutamine	15.0 mg/mL	1.0 mM	0.040
Na-pyruvate	2.2 mg/mL	0.2 mM	0.040
Ciprofloxacin HCl	10 mg/mL	10 µg/mL	0.004
hMG	0.1 mg/mL	2.0 µg/mL	0.080
Estradiol	1.0 mg/mL	1.0 µg/mL	0.004
Final volume			**4.0 mL**

b) Maturation medium with ovine follicle stimulating hormone (oFSH) and luteinizing hormone (oLH)

	Stock	Final	Vol (mL)
TCM-199	-	-	3.482
FBS (10%)	-	10% v/v	0.400
L-glutamine	15.0 mg/mL	1.0 mM	0.040
Na-pyruvate	2.2 mg/mL	0.2 mM	0.040
Ciprofloxacin HCl	10 mg/mL	10 µg/mL	0.004
Estradiol benzoate 17β (E_2)	1.0 mg/mL	1.0 µg/mL	0.004
Luteinizing hormone (oLH)	2.5 units/mL	5 µg/mL	0.020
Follicle stimulating hormone (oFSH)	5 units/mL	0.5 µg/mL	0.010
			4.0 mL

2. Preparation of Tyrode's-albumin-lactate-pyruvate (TALP) medium

Step 1: Prepare stock salt solutions for making Tyrode's-Lactate medium.

Chemical	mg/mL of water	Vol. (mL)	Storage
NaCl	133.3	50	4°C indefinitely
KCl	11.76	50	4°C indefinitely
$NaHCO_3$	21.04	10	4°C for 1 week
$NaH_2PO_4 .H_2O$	4.7	50	4°C indefinitely
$CaCl_2 .2H_2O$	29.4	50	4°C indefinitely
$MgCl_2 .6H_2O$	20.34	50	4°C indefinitely
Phenol red	1.0	10	4°C indefinitely
Na-pyruvate	2.2	25	4°C 1 month, aluminium foil
L-glutamine	15.0	50	-20°C indefinitely, aluminium foil
HEPES*	238	50	4°C indefinitely, aluminium foil

Other stocks

Chemical	mg/mL	Storage
BSA (Frac V)	120	-20°C
BSA (EFAF)	120	-20°C
Heparin	1	-20°C
Ciprofloxacin HCl	10	-20°C
Na-lactate	60% syrup	4°C

**Adjust pH to 7.0 before bringing to the final volume.*

Step 2: Prepare Tyrode's-Lactate (TL) medium.

Stock solution (mL)	Sperm-TL	HEPES-TL	IVF-TL
NaCl	2.17	2.5	1.25
KCl	0.98	1.0	0.5
$NaHCO_3$	5.0	0.4	2.5
$NaH_2PO_4 .H_2O$	0.5	0.5	0.25
Na-lactate (60% syrup)	0.184	0.093	0.048
HEPES	0.5	0.5	-
$CaCl_2 .2H_2O$	0.5	0.5	0.25
$MgCl_2 .6H_2O$	0.55	0.25	0.125
Phenol red	0.5	0.5	0.25
Sterile water	39.116	43.757	19.828
Final volume	**50.00 mL**	**50.00 mL**	**25.00 mL**
Adjust pH to	**7.4**	**7.3**	**7.4**

Sterile filter, store at 4°C and use within 1 month.

Step 3: Prepare Tyrode's-Lactate-Albumin-Pyruvate (TALP) medium.

Chemical (ml)	Sperm-TALP	HEPES-TALP	IVF-TALP
TL-Stock	8.990	9.490	4.615
BSA (Fraction V)	0.500	0.250	--
BSA (EFAF)	--	--	0.250
Na-Pyruvate	0.500	0.250	0.125
Ciprofloxacin HCl	0.010	0.010	0.005
Heparin	--	--	0.005
Final volume	**10 mL**	**10 mL**	**5 mL**

Make fresh preparations of TALP and use immediately. DO NOT store the unused portion.

3. Preparation of oocyte culture (CR1aa) medium

Step 1: Prepare CR1 stock.

CR1 stock can be prepared using the salt solutions for making TL medium.

	Stock conc. Mg/mL	Per 50 mL		Per 100 mL	
		mg	mL	mg	mL
NaCl	133.3	335.0	2.513	670	5.026
KCl	11.76	11.5	0.998	23	1.996
$NaHCO_3$	21.04	110.0	5.228	220	10.456
Na-pyruvate	2.20	2.0	0.909	4	1.818
L-glutamine	15.00	7.5	0.500	15	1.000
Hemi-calcium lactate	55.00	27.5	0.500	55	1.000
Phenol red*	1.00	0.5	0.500	1	1.000
Sterile water to			**50 mL**		**100 mL**

**Phenol red is optional.*
- Adjust pH to 7.4 before bringing to the final volume.
- Filter CR1 using 0.22 µM milli-pore syringe filter then aliquot 5 mL into sterile test tubes. Store frozen and only use once when thawed, do not refreeze unused portion.

Step 2: Preparation of CR1aa medium.

	Cat #	Stock conc.	Per 3 mL	Per 5 mL
CR1 stock		-	2682 µL	4470µL
BSA (EFAF) (0.3%)	A6003 Sigma	120 mg/mL	75 µL	125µL
FBS (5%)	F4135 Sigma	-	150 µL	250µL
Essential amino acids	B6766 Sigma	50x	60µL	100µL
Non-essential amino acids	M7145 Sigma	100x	30µL	50µL
Ciprofloxacin HCl	199020 ICN Biomed	10 mg/mL	3µL	5µL

Use CR1aa soon after preparing and do not store unused portion.

Direction for using CR1aa medium

Make 50 µL drops of CR1aa medium; equilibrate for 2 hours and culture 10-15 fertilized oocytes per drop. Oocytes may be denuded before culture or culture with cumulus cells that attach to the surface of the Petri dish to form a co-culture. Evaluate the oocytes for cleavage on 3rd day post-insemination, and morula and blastocyt on days 5 and 7, respectively. Change half of the culture medium with fresh pre-equilibrated medium every second day.

4. Preparation of penicillamine, hypotaurine & epinephrine (PHE)

PHE is used for enhancing sperm motility and penetration of oocytes. The following steps are for the preparation of 44 ml of PHE (Must be prepared under a low density light).

Step 1: Preparation of solution A (Lactate metabisulphite).

i) Make 250 mL of normal saline (0.9% NaCl).
ii) Measure 100 mL of the saline in a beaker.
iii) Add 100 mg Na-Metabisulphite ($Na_2S_2O_5$) to the saline.
iv) Add 328.1 mg Sodium lactate (= 252 µl of 60% Na-Lactate syrup) to the saline.
v) Adjust the pH to 4.004 at room temperature.

Step 2: Preparation of solution B (stock solutions).

i) Dissolve 15 mg of Penicillamine in 50 mL of saline in a separate beaker. Call this PENICILLAMINE STOCK SOLUTION.
ii) Dissolve 5 mg of Hypotaurine in 50 mL saline in a separate beaker. Call this HYPOTAURINE STOCK SOLUTION.
iii) Dissolve 18.2 mg of epinephrine into 5 mL of solution A (Lactate metabisulphite) - use low light intensity as epinephrine is sensitive to light.
iv) Get 19.5 mL of solution A (sodium metabisulphite) into a test tube and to this add 0.5 mL of step 2(iii) solution above and call this EPINEPHRINE STOCK SOLUTION.

Step 3: Preparation of PHE

Use a fresh 50 ml test tube (low light density room) to add the following to make PHE:

i)	Saline (fresh)	16.0 mL
ii)	Penicillamine stock	10.0 mL
iii)	Hypotaurine stock	10.0 mL
iv)	Epinephrine stock	8.0 mL
	Total	**44.0 mL**

- *Sterile filter and store in 0.5 ml Eppendorf vials at – 20°C in darkness indefinitely.*
- PHE is used at a concentration of 40 µL/mL of *in vitro* fertilization-TALP medium.

5. Preparation of Cysteamine

Cysteamine (M9768 – Sigma), molecular weight = 77.15, packing – 5 g bottle, sealed in an airtight polythene bag and then in a tin.

A working solution was prepared by dissolving 50 mg in 32 mL of sterile water to make a 1.543 mg/mL solution equivalent to 20 mM.

This was sterile filtered and aliquots of 1.5 ml stored frozen at -20 °C.

Five micro-litres (5 µL) were used in every ml of medium to give a final concentration of 100 µM (7.715 µg/mL) in the medium.

6. Preparation of phosphate buffered saline (PBS)

Prepare two stock solutions (A and B) for making PBS as below.

Stock solution A (10x)		**Stock solution B (10x)**	
	g/L		g/100 mL
NaCl	80.0	$MgCL_2$ .6H2O	1.0
KCl	2.0	$CaCl_2$.$2H_2O$	0.67
KH_2PO_4	2.0	or	
Na_2HPO_4 .$12H_2O$	29.0	$CaCl_2$.$6H_2O$	1.0
or			
Na_2HPO_4 (anhydrous)	11.5		

Make 1 L of solution A (10X) and 100 mL of solution B (10X) in sterile deionised water and store both solutions separately at room temperature. To make 1 L (1000 mL) of PBS, take 100 mL of solution A and 10 mL of solution B and add 890 mL of sterile deionised water. Adjust the pH to 7.4, sterile filter and store at room temperature.

Appendix B
Sperm and oocyte evaluation; freezing and vitrification

1. Preparation of Biladyl medium (Sperm extender/freezing medium)

(Manufacturer's protocol)

Biladyl (Minitüb, Germany) comes in three different containers consisting of:

1. Biladyl Cocktail AB (powder)

a. Tyrosine	60 mg
b. Gentamycin	300 mg
c. Spectinomycin	360 mg
d. Lincomycin	180 mg

2. Biladyl solution A (50 mL)

a. Tris	12.1g
b. Citric acid	6.9 g
c. Fructose	5.0 g

3. Biladyl solution B (140 mL)

a. Glycerol	86.0 g
b. Tris	12.1 g
c. Citric acid	6.9 g
d. Fructose	5.0 g
e. Tylosin	2.5 mg
f. Gentamycin	12.5 mg
g. Lincomycin	7.5 mg
h. Spectinomycin	15.0 mg

Step 1: Prepare egg yolk

- Prepare 200 mL of egg yolk from freshly laid (within 24 hours) chicken eggs.
- Clean the egg yolk by rolling the yolk between two sheets of sterile filter/tissue paper to remove the membranes and excess egg white.
- Use a syringe to aspirate the egg yolk out of the yolk sac.

Step 2: Prepare cocktail AB

- Reconstitute cocktail AB by adding 12 mL of double distilled/sterile water using a syringe. The final composition ingredients in the reconstituted cocktail AB expressed as active units of antibiotics per 0.02 mL are: Tylosin 100 µg, Gentamycin 500 µg, Lincomycin 300 µg and Spectinomycin 600 µg.

Step 3: Prepare Biladyl A

- Reconstitute 49 mL (49 g) of solution A with sterile water to a combined volume of 390 mL.
- Add 100 mL of the freshly prepared egg yolk to bring the volume to 490 mL.
- Add 10 mL of the reconstituted cocktail AB using a sterile syringe (final volume 500 mL).
- Mix gently and warm the mixture to 30°C. Call this BILADYL A.
- Filter through sterile a filter, aliquot 5 mL volumes into 15 mL tubes and store at -40°C.

Step 4: Prepare Biladyl B

- Reconstitute 250 mL (250 g) of solution B with 140 mL of sterile water to make 390 mL.
- Add 100 mL of the freshly prepared egg yolk to bring the volume to 490 mL.
- Add 10 mL of the reconstituted cocktail AB using a sterile syringe (final volume 500 mL).
- Mix gently and warm the mixture to 30°C. Call this BILADYL B.
- Filter through a sterile filter, aliquot 5 mL volumes into 15 mL tubes and store at -40°C.

Step 5: Using Biladyl
- Dilute semen/sperm in a pre-warmed Biladyl A and equilibrate for an appropriate time at 5°C.
- Dilute equilibrated sperm with an equal volume of Biladyl B that had been cooled to the same temperature.
- Pack semen in straws and freeze using an appropriate freezing method.

Final composition of Biladyl freezing medium containing solutions A and B per 100 mL
Yolk 20%, Glycerol 7%, Tris 2.42 g, Citric acid 1.38 g, Fructose 1.0 g, and active units of antibiotics: Tylosin 5.25 mg, Gentamycin 26.25 mg, Lincomycin 15.75 mg, Spectinomycin 31.5 mg, and double distilled water.

2. **Spermac staining procedure** (Manufacturer's protocol)

Staining method for human spermatozoa
For *in vitro* diagnostic use only

Intended use
Spermac is a diagnostic kit for staining human spermatozoa. The purpose of staining spermatozoa is to be able to differentiate morphologically normal from abnormal sperm cells.

General information
The definition and criteria for normality have been largely based on studies done on sperm recovered from the female reproductive tract (especially in post-coital cervical mucus) which is considered to be normal. Still different criteria have been proposed, the main ones being the WHO criteria[1] and the Tygerberg (or strict) criteria[2,3]. These methods differ mainly in the fact that so called borderline normal spermatozoa, according to WHO, is classified as abnormal by strict criteria[3].
Spermac stain aids in evaluating morphology in a way that it helps to distinguish the different parts of the sperm cell (head, acrosome[4], equatorial region, mid-piece, and tail), making it easier to differentiate between a normal and an abnormal spermatozoon.

Material included with the test
Reagent A: red stain – 50 mL or 200 mL
Reagent B: pale green – 50 mL or 200 mL
Reagent C: dark green – 50 mL or 200 mL
Fix: fixative – 50 mL or 200 mL

Material not included with the test
Glassware
Coplin jars
Microscope
Immersion oil
Tap water

Storage and stability
Spermac stain should be stored in closed Coplin jars or the original bottles, at 15-25°C. The reagents are stable for 36 months after date of manufacture, if unused. However, staining removes constituents and introduces contaminants, and thus stains should be replaced when adequate staining is no longer achieved. Filter stains if deposit is noted.

Preparation
Pour the reagents in Coplin jars, make sure the fluid level is high enough to cover the area that is to be stained.
Fill a fifth Coplin jar, or any other recipient that can contain a complete object glass, with tap water (for washing the slides between the different dyes).
Clean, wash in alcohol and dry slides before use.

Method
1. Allow a thin feathered-edge smear of fresh, liquefied, undiluted semen to air dry for a maximum of 5

minutes.
2. Fix the smear by immersing the slide for a minimum of 5 minutes in a Coplin jar containing the fixative. Remove slide from fixative, place vertically on absorbent paper to drain excess fixative, and then allow to dry completely, either on a warm plate at 37 degree Celsius for 15 minutes or overnight
3. Wash by gently dipping 6-7 times in tap or distilled water. Briefly drain excess water off by touching end of slide onto absorbent paper. Do not touch the specimen with the paper.
4. Stain 1-2 minutes in stain A. Wash as above.
5. Repeat the washing in fresh water. This double washing step after Stain A is important.
6. Stain 1 minute in stain B. Wash as above.
7. Stain 1 minute in stain C. Wash as above.
8. Allow smear to air dry.
9. Observe staining under a light microscope (X100) using oil immersion:
   - acrosome = dark green
   - nucleus = stained red
   - equatorial region = pale green
   - mid-piece and tail = green

Interpretation of the results
- Count at least 100 and preferably 200 spermatozoa and classify them as either normal or abnormal, specifying which defects are most common.
- Only include identifiable sperm cells in the count.
- The criteria for classifying sperm cells as either normal or abnormal depend on the classification method used in the lab.
- According to WHO, using WHO criteria, a sample is considered normal if at least 30 % of spermatozoa show normal forms[1].
- According to strict criteria the rate of fertilization per cycle (in IVF) drops dramatically when the percentage of normal forms is below 15 %[2,3].

Storage
Spermac stain should be stored at room temperature (15-25°C).

Mounting slides
If slides are mounted staining will fade under mounting medium (after weeks). So do not mount slides if you want to refer back later. Gently blot off immersion oil, which also fades the staining. It is preferable to make duplicate slides for future reference if necessary, or photographic or video records.

Warnings and Precautions
- All semen samples should be considered potentially infectious. Handle all specimens as if capable of transmitting HIV or Hepatitis.
- Fix contains Formaldehyde: Toxic by inhalation, in contact with skin and if swallowed. May cause irritation to mucus membranes. Listed as a carcinogen. Possible risks of irreversible effect. May cause sensitization by skin contact.
- All other ingredients have not been established as toxic. Full Material Safety Data Sheet is available on request from the manufacturer.

Remarks on use
- Pertinacious or gelatinous samples and frozen samples must be diluted 1:1 with 3% sodium citrate prior to smearing. However, if the smear is immersed in the fixative as soon as the smear is dry, this citrate dilution step is unnecessary.
- A stained slide should be transparent with only a very slight hint of green hue. If the slide is dark green, then the slide was dried too long prior to fixing.

References

[1] WHO (1992). Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 3ed, Cambridge University Press, Avon, pp.107.

[2] Menkveld R, Kruger TF *et al.* (1991). Atlas of human morphology. Williams and Wilkins, Baltimore.

[3] Menkveld R, Stander FSH *et al.* (1990). The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Human Reproduction* 5(5): 286-92.

[4] Oettlé EE (1986). An improved staining technique which facilitates sequential monitoring of the

acrosome state. *Development, Growth and Differentiation* (Suppl.), p28.
Spermac is produced by Stain Enterprises Inc., South Africa.

3. Orcein staining of oocytes

Step 1: Preparation of fixative
- Mix one part of glacial acetic acid into three parts ethanol (1:3 v/v mixture).

Step 2: Preparation of stain
- Make a 1% w/v (10 mg/mL) solution orcein stain in the fixative.
- Filter with filter paper to remove the un-dissolved granules of stain.
- Keep the stain in a dark place or wrap with aluminium foil.

Step 3: Preparation of oocyte for staining
- Denude the oocytes at appropriate time of maturation using hyaluronidase and/or vortexing.
- Place 1 mL of the fixative in 24-well plate or fill the wells because the fixative evaporates readily.
- Transfer the oocytes into fixative in small groups (e.g. 5 per well) and fix for 24 hours.
- Add 10 to 15 µL of stain into each well and give about 10 minutes to stain.
- Examine with an inverted microscope at X100 or X200 magnification. Examination can be done on the same day or at a later time as long as you keep adding the fixative to avoid drying.

4. Preparation of DAPI and blastomere staining

The following steps were used in the preparation DAPI and staining the embryos for blastomere enumeration:

Step 1:
Prepare a 1 mg/mL solution of polyvinyl pyrrolidon (PVP) in phosphate buffered saline (PBS) and call it PBS-PVP solution.

Prepare a 4% (40 mg/mL) solution of paraformaldehyde in PBS (pH 7.4) call this the fixative.

Step 2:
Dissolve 10 mg DAPI (D9564-Sigma) in 10 mL PBS to make a 0.1 mg/mL solution of DAPI. *(Note: DAPI dissolves slowly, give it time to dissolve). Store DAPI at -20°C.*

Dilute 1 mL (0.1 mg) of step 1 solution to 10 mL to make a 0.01 mg/mL solution. Store at 4°C for 6 months.

Step 3:
Make a working solution on day of use by adding 100 µL of step 2 solution to 900 µL PBS/PVP to a final concentration of 0.001 mg/mL (1 µg/mL). Use once and discard excess.

Step 4: Blastomere staining
A 4-well plate is convenient for staining.
- Fill the two right wells with 0.5 mL PBS-PVP medium and the top left well with 0.5 mL fixative.
- Leave the bottom left well empty (to be filled with 0.5 mL DAPI staining solution).
- Transfer the embryos from culture medium to the top right well containing PBS-PVP to wash once then transferred into the fixative to fix for 30 to 60 minutes.
- Fill the lower left well with 0.5 ml DAPI at the end of fixing. Transfer the embryos into this well and stain for 10 minutes.
- Transfer the embryos into the lower right well containing PBS-PVP to wash once.
- Keep the stained embryos in this well as you examine them one at a time.
- Transfer each embryo onto a clean microscope slide with a small drop (about 10 µL) of the PBS-PVP and examine under an inverted microscope first with bright light then with UV light. Blastomere nuclei appear bright blue against a dark background.
- Gently place a cover-slip over the drop. The zona pellucida will break and the blastomeres will spread. Examine with the UV light at X100 or X200 magnification and count the bright-blue nuclei of blastomeres.

5. Determination of cryoprotectant concentration in sperm freezing media

Cryoprotectants can be in liquid or solid form and their concentrations may be given as a molar value, or as a percent (volume/volume or weight/volume). The formula below was used to convert from molar concentration to percentage concentrations and vice versa. This conversion requires the knowledge of molecular weight and specific gravity of the substances. When using liquids, it's convenient to work with liquid volume percentages.

	Chemical formula	Molecular weight	Specific gravity ($g\ cm^{-3}$) at 20 °C
Glycerol	$C_3H_5(OH)_3$	92.10	1.25
Ethylene glycol	$(CH_2)_2(OH)_2$	62.07	1.11
DMSO	$(CH_3)_2SO_4$	78.13	1.10
Sucrose	$C_{12}H_{22}O_{11}$	342.30	----

$$\frac{\mathbf{M \times W}\ (g)}{\mathbf{SG}\ (g\ cm^{-3})} = \mathbf{V}\ (cm^3)$$

M = molarity of solution
W = molecular weight of the substance
SG = specific gravity of the substance
V = volume of substance; added to 1 litre without solvent

Definitions:
a) A 1 molar solution equals the molecular weight of a substance in grams dissolved in 1 litre
b) A 1% (w/v) solution equals 10 g of a substance dissolved in 1 L (or 10 mg/mL solution)
c) A 1% (v/v) solution equals to the volume of a substance made to 1 L (or mL/L solution)
d) Specific gravity (SG) is the ratio of the weight of a given volume of the substance compared to the weight of the same volume of water at 0 °C.

a) To convert 7% glycerol to molar concentration

A 7% v/v solution of glycerol is 70 mL of glycerol qs to 1000 mL

$$\frac{M \times 92.10}{1.25} = 70$$

$$M = \frac{70 \times 1.25}{92.10} = 0.95\ M$$

Therefore a 7% glycerol is equivalent to 0.95 molar concentration of glycerol in the solution.

b) To convert 0.95 M ethylene glycol to percentage

$$\frac{M \times W}{SG} = V$$

$$\frac{0.95 \times 62.07}{1.11} = 53\ mL$$

$$53\ mL\ qs\ to\ 1000\ mL = \frac{53 \times 100}{1000} = 5.3\%$$

Therefore, a 0.95 M solution of ethylene glycol is equivalent to 5.3% v/v.

Appendix C
Chemicals, media, media additives and stains used and their sources

1. Salts/sugars

Chemicals	Mol wt	Source/company
Calcium chloride ($CaCl_2.2H_2O$)	110.99	MERCK
Choline chloride ($C_5H_{14}NO.Cl$)	139.63	SIGMA
D-fructose ($C_6H_{12}O_6$)	180.16	MERCK
D-glucose anhydrous (dextrose) $C_6H_{12}O_6$	180.16	BHD
di-Potassium hydrogen phosphate ti-hydrate ($K_2HPO_4.3H_2O$)	228.23	MERCK
Magnesium chloride ($MgCl_2.6H_2O$)	203.30	MERCK
Potassium chloride (KCl)	74.56	MERCK
Potassium dihydrogen phosphate (KH_2PO_4)	136.09	MERCK
Sodium bicarbonate ($NaHCO_3$)	84.01	MERCK
Sodium chloride (NaCl)	58.44	MERCK
Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$)	137.99	MERCK
Tri-sodium citrate ($C_6H_5Na_3O_7.2H_2O$)	294.10	MERCK

2. Media and supplements

Supplement	Cat #	Source/company
Bovine serum albumin (BSA) fraction V	A9418	Sigma
Bovine serum albumin (BSA) fatty acid free	A6003	Sigma
Calcium L-lactate	L4388	Sigma
Ciprofloxacin HCl	199020	ICN Biomedicals
Cysteamine	M9768	Sigma
Epinephrine	E4250	Sigma
Essential amino acids (BME) x50	B6766	Sigma
Estradiol benzoate-17$_\beta$ (water soluble)	E4389	Sigma
Foetal bovine serum (FBS)	F4135	Sigma
Heparin sodium salt	H3149	Sigma
HEPES sodium salt	H7006	Sigma
Human menopausal gonadotrophin (hMG)	G5270	Sigma
Hyaluronidase	H4272	Sigma
Hypotaurine	H1384	Sigma
L-glutamine	G8540	Sigma
Non-essential amino acids (MEM) x100	M7145	Sigma
Ovine follicular stimulating hormone (oFSH)	F8174	Sigma
Ovine luteinizing hormone (oLH)	L5269	Sigma
Penicillamine	P4875	Sigma
Phenol red	P3532	Sigma
Sodium lactate syrup (60%)	L1375	Sigma
Sodium-metabisulfite	S1516	Sigma
Sodium pyruvate	P4562	Sigma
Tissue culture medium (TCM-199)	M7528	Sigma

3. Other chemicals

Chemical	Cat #	Source/company
Acetic acid 100% (glacial)	27225	Sigma
Dimethyl sulfoxide (DMSO)	D2650	Sigma
Ethanol 96.4%	-	KIMIX
Ethylene glycol (EG)	E9129	Sigma
Glycerol (GLY)	G5516	Sigma
Mineral oil	M8410	Sigma
Paraformaldehyde	P6148	Sigma
Polyvinyl pyrrolidon (PVP)	PVP-360	Sigma

4. Stains

Stain	Company	Country
Eosin-nigrosin	Taurus	South Africa
Spermac stain	Stain enterprises	South Africa
Orcein stain	Merck	Germany
Trypan blue	Merck	Germany
DAPI (D9564)	Sigma	USA