IN VITRO CULTURE OF *IN VIVO*-PRODUCED

SHEEP, GOAT AND CATTLE EMBRYOS

by

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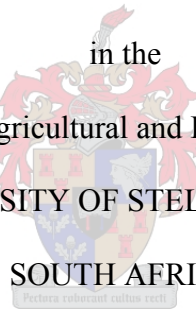Dissertation presented for the degree of

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work, and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Signature

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Date

ABSTRACT

As most researchers have foreseen, and many breeders have hoped, the *in vivo* and *in vitro* production of livestock embryos and the birth of subsequent offspring never really replaced artificial insemination during the past 30 years. This was, to a large extent, due to very variable and unreliable numbers of embryos produced using these two methods. The present study was therefore undertaken to investigate certain aspects of assisted reproductive techniques (ART) to try and solve some of these difficulties. Problems addressed were the management of follicular development on the ovary by controlling the dominant follicle, and investigating alternative and more cost-effective culture media and conditions for embryo culture.

A method to control the development of the dominant follicle in a cohort of follicles as well as the waves of follicular development in the ovaries of sheep, goats and cows with an estrogenic product was investigated. Estradiol cypionate (ECP) was used for this purpose, injected intramuscularly after the insertion of the progesterone or progestagen implant. ECP has a negative feedback effect on the secretion of pituitary FSH, and therefore follicular development. The animals of the three different species were randomly divided into two groups each, the ECP-group receiving the estradiol cypionate injection, and the control group receiving a saline injection. Five days after the ECP injection a program of follicular multi-stimulation with FSH hormone was initiated. The females of the different species were bred by either natural service (goats) or inseminated by laparoscopy (sheep) or trans-cervically (cows) to fertilize the ovulated ova. Embryos and unfertilized ova were collected surgically at the 8 to 16-cell stage 3 to 4 d after breeding in the sheep and goats, and trans-cervically in the cows. Significantly more CL formed, and a total number of ova and embryos, as well as transferable embryos, were collected from the ECP-group of sheep ewes and goat does compared to the control group that received no ECP ($p<0.01$). There was, however, no difference in the average

number of unfertilized ova that were collected in the two sheep or goat groups. In the cows the number of CL counted, the total number of embryos and ova and of transferable embryos collected, were significantly greater ($p<0.05$) in the group that were injected with ECP compared to the group that received no ECP. The control group also had a significantly larger number of unfertilized ova than the ECP-group ($p<0.05$). It could therefore be concluded that more reliable numbers of embryos can be produced *in vivo* if the development of the dominant follicle as well as the subordinate follicles is controlled with estradiol cypionate.

Since more than half a century ago, attempts have been made to culture cells and embryos outside the body (*in vitro* or *ex vivo*). This was done with different culture media and in various "incubators". Chapter 2 deals with two different culture media used: a standard TCM-199 culture medium and first trimester amniotic fluid (BAF) collected sterilely from pregnant cows after slaughter. Two different culture conditions were also investigated, the standard laboratory CO_2 incubator versus culturing bovine embryos in the vagina of a goat doe. Two experiments were done: Firstly the permeability of different receptacles to CO_2 gas was analyzed for possible culture in the vagina. Four-well plates and straws were used to incubate TCM-199 and BAF for a period of 120 h in the presence or absence of 5% CO_2 gas. The pH values were measured every 24 h and recorded. In the second experiment pre-compacted morula stage bovine embryos were incubated in the above culture media in sealed 0.25 mL straws in a standard laboratory incubator and in the vagina of a goat doe. Evaluation was done on (1) stage of development and (2) number of blastomeres after 96 h of culture. In experiment one it was shown that the CO_2 gas diffused out of the 4-well plate as well as the straws in the absence of CO_2 gas, while in the presence of CO_2, the pH of both media stabilized between 7.3 and 7.5. This meant that the semen straws were permeable to CO_2 gas and could therefore be used as receptacles for culturing early stage bovine embryos. In the second experiment no statistical differences ($p>0.05$) were found in the number of Grade 1 pre-compacted bovine embryos that

developed to the blastocyst stage, or the hatched blastocyst stage, neither for the culture medium used, or the method of culturing in the two incubators. Neither was there any difference (p>0.05) in the number of blastomeres that developed at the blastocyst stage between the two types of incubators used. Embryos tended to develop more blastomeres when cultured in BAF than when cultured in TCM-199 in both the standard laboratory incubator and when using the vagina of a goat doe as an incubator (p<0.05).

After the collection of *in vivo* produced livestock embryos, they are evaluated under high magnification (minimum of 80X) with the aid of an inverted or stereo microscope. The Grade 1 embryos will give the best conception results when transferred to synchronized recipient female animals, while the Grade 3 embryos will give the worst results. The aim of the next experiment was to culture all three quality grades of *in vivo* produced pre-compacted morula-stage embryos of sheep, goats and cows in two different culture media and then compare the development of the embryos by evaluating the number of embryos reaching the hatched blastocyst stage. The results have shown that there were no significant differences between the development of the Grade 1 and the Grade 2 embryos from any of the three species when either cultured in TCM-199 or heat inactivated early pregnancy-stage (<60 d) bovine amniotic fluid (BAF) were used as culture media. Significantly more *in vivo* produced Grade 3 pre-compacted morula-stage sheep, goat and cow embryos, however, developed to the hatched blastocyst stage when cultured in BAF with 10% FBS and antibiotics, compared to culture in TCM-199 with 10% FBS and antibiotics (p<0.05).

The effect of co-culture on the survival of caprine embryos post transfer to a synchronized recipient female goat was also assessed. A total of 120 Kashmir embryos at the blastocyst stage were divided into three groups after thawing and reconstitution in four steps in glycerol and sucrose medium. The first group of embryos (G1, n=40) was individually transferred semi-

laparoscopically in D-PBS with 10% FBS and antibiotics to the ipsilateral horn of the CL over a period of 3 d. The second group of caprine blastocysts (G2, n=40) was similarly transferred in TCM-199 with FBS and antibiotics. The third group of frozen-thawed caprine blastocyst-stage embryos (G3, n=40) were first co-cultured for ~24 h in TCM-199 with serum and antibiotics in groups of up to five embryos inside a ~50 mm length of a semen straw in a cylindrical sponge in the anterior part of the vagina of a goat doe in her luteal phase. After the culture period these embryos were transferred in a similar way in TCM-199 without the co-culture as in G1 and G2. Ultrasound scanning showed that significantly more of the blastocyst embryos that were co-cultured in the vagina (G3) before transfer developed to a pregnancy compared with the embryos transferred in D-PBS (G1). The co-culture Group 3 blastocyst-stage caprine embryos produced significantly more offspring than the non-cultured embryos transferred in both D-PBS (G1) and TCM-199 (G2) ($p<0.05$).

The maturation of bovine oocytes to allow the oocyte to resume meiosis, is the first step in *in vitro* fertilization to produce IVMFC embryos. The composition of the maturation medium plays an important role in the success achieved with maturation. An investigation was therefore launched to evaluate the maturation ability of first trimester bovine amniotic fluid (BAF) to mature prophase I oocytes collected from abattoir ovaries to metaphase II oocytes, compared to a standard maturation medium such as TCM-199. In the first experiment three groups of ~100 oocytes each were matured in TCM-199 with estrus cow serum (ECS). The first group of oocytes was matured in a 50 µL micro-drop in an incubator, while the other two groups were matured in semen straws, one group in an incubator and the other group in the vagina of a goat doe in di-estrus. Six further groups of ~100 oocytes each, with BAF as maturation medium, three groups with ECS and three without ECS, were matured in the same receptacles and under the same conditions as with the TCM-199. No significant differences in number of oocytes reaching the metaphase II stage could be found for any of the nine treatment groups. In the

second experiment fresh and frozen-thawed BAF with or without ECS was tested as a maturation medium while the oocytes were matured in micro-drops inside a laboratory incubator. Although the fresh BAF with added 10% ECS was inclined to give better results as a maturation medium for bovine oocytes when compared to fresh BAF without serum, or frozen-thawed BAF with and without added serum, the differences between the four groups were not significant.

During the 33rd launch of the Soyuz capsule and Proton rocket by the Russian Rocket and Space Corporation (Energia) from the Baykonur launch pad in Kazachstan in April 2002, 1-cell ovine embryos were sent in a portable incubator to the International Space Station (ISS) for a culture period of 10 d to investigate embryonic development under micro-gravity conditions. The early-stage embryos were collected after follicular multi-stimulation from indigenous Tegeres breed ewes and artificial insemination with electro-ejaculated undiluted semen. Eight embryos were placed in two special, closed-system, plastic culture test tubes in a portable incubator, and eight embryos were cultured under similar conditions on earth. Pre-conditioned culture medium (TCM-199 with 10% fetal bovine serum, 1% antibiotics and 0.5% essential and non-essential amino acids) was injected into the special culture tubes to change the culture medium every second to third day by the cosmonaut, Mark Shuttleworth, the first African in space. A special 2 μ pore size filter inside the test tube prevented the embryos from being flushed from the tube during the medium changes. After the return of the samples from the ISS, the space and control embryos were frozen-stored in liquid nitrogen and transported to our laboratory in Stellenbosch, South Africa, where further evaluations and cell counts were done. There were no significant difference in developmental rate between the space and ground control samples in 1-cell embryos reaching the blastocyst or hatched blastocyst stage, but significantly more inner cell mass cells developed in the hatched blastocyst stage embryos cultured in space than in the

ground control embryos. Due to government animal health control regulations we were not allowed to transfer any of the embryos to recipient ewes.

The different research studies into the use of heat-inactivated first trimester bovine amniotic fluid (BAF) as a culture medium has allowed the following conclusions to be made:

1. BAF with 10% fetal bovine serum added will support the development of pre-compacted morula stage sheep, goat and cow embryos to the hatched blastocyst stage as well as or better than a standard embryo culture medium such as TCM-199 with added serum. This is probably due to the fact that BAF contains the necessary nutrients and growth factors needed for early embryonic development of these three species. Due to the inexpensive collection methods and possible supply of BAF, it is recommended that BAF can replace compound culture media in commercial and experimental setups where early-stage and later-stage embryos of the three species are cultured for various reasons. The transmission of possible micro-organisms such as viruses and pyrons should, however, be taken into account as a possible risk factor.

2. The earlier the stage of pregnancy, the more concentrated seems to be the constituents (growth factors and cytokines) present in amniotic fluid that is beneficial for embryonic development *ex vivo*. This can be seen in the developmental rate of bovine embryos to the hatched blastocyst stage in first trimester bovine amniotic fluid from different origins. In Chapter 3 BAF from a fetus of ~80 d of pregnancy supported 46% (35/75) of grade 1 pre-compacted morula stage bovine embryos to develop to the hatched blastocyst stage. This figure increased to 69% (67/97) when BAF from a ~60 d pregnancy was used as culture medium in Chapter 4 under the same conditions inside an incubator. It is therefore recommended that the amniotic fluid to be used as a culture medium for embryos should be

collected from the earliest possible stage of pregnancy. However, at <40 d of pregnancy the volume of amniotic fluid present is very small and not feasible to collect.

3. The concentration of growth or recuperating factors is higher in early-stage BAF than in serum. When TCM-199 with added 10% fetal bovine serum (FBS) was used as culture medium for grade 3 embryos in sheep, goat and cow embryos, 18%, 23% and 16% developed to hatched blastocyst, respectively. However, when BAF from a ~60 d pregnancy with 10% fetal bovine serum added was used as culture medium for similar grade 3 embryos, 43%, 49% and 53% of the pre-compacted stage embryos developed to the hatched blastocyst stage for sheep, goat and cows embryos, respectively. Because 10% fetal bovine serum was added to both culture media, it can be deducted that the recuperating proteins must have been only in the BAF, or in higher concentrations in the BAF than in the FBS. Further investigations into the effect of BAF from different stages of bovine and other livestock fetal development on the development of embryos should be investigated, to find the optimal time for amniotic fluid collection.

4. First trimester bovine amniotic fluid (BAF) will support bovine oocyte maturation. It was proven that pooled BAF collected from pregnancies of <90 d stimulated bovine prophase I oocytes in a 26 h culture period to develop to the metaphase II stage as well as TCM-199 with serum but without added hormones. No significant differences could be found between any of the groups even when other receptacles (wells with micro-drops vs straws) were used, or other incubators (laboratory incubator vs the vagina of a female goat) was used. Furthermore, freezing of the BAF after collection but before use did not seem to have been detrimental to the maturation capacity of BAF for bovine oocytes. When frozen-thawed BAF was compared to fresh BAF as culture media for the development of 2-cell murine embryos, there was a marked decrease in the number of murine embryos reaching

the blastocyst and hatched blastocyst stage when cultured in frozen-thawed BAF when compared to fresh BAF (unpublished data).

OPSOMMING

Soos 'n aantal navorsers in reproduksie dertig jaar gelede voorsien het, en telers visioene van gehad het, het *in vivo* en *in vitro* embrioproduksie en die daaropvolgende geboorte van nageslag, nooit werklik kunsmatige inseminasie as die mees populêre ondersteunende reproduksietegniek vervang nie. Dit was tot 'n groot mate toe te skryf aan onbetroubare en uiters varieërende embriogetalle geproduseer met beide hierdie metodes. Hierdie studie was dus onderneem om van die leemtes in hierdie embrioproduksietegnieke aan te spreek. Van die probleme wat ondersoek is, was die beheer van die golf van follikels deur die ontwikkeling van die dominante follikel te bestuur, sowel as om na alternatiewe en meer koste-effektiewe media en metodes van embriokweking te soek.

'n Metode om die ontwikkeling van die dominante follikel in 'n groep van follikels sowel as die golf van follikulêre ontwikkeling in skape, bokke en koeie te beheer met behulp van 'n estrogeniese produk, is ondersoek. Estradiolsipionaat (ECP)is vir hierdie doel gebruik, en binnespiers ingespuit na die toediening van 'n progesteroon- of progestageen implant. ECP het 'n negatiewe terugvoereffek op sekresie van FSH vanaf die adenohipofiese, wat gevolglik follikulêre ontwikkeling onderdruk. Vroulike diere van die drie species is ewekansig in twee verskillende groepe elk verdeel. Die een groep is ECP toegedien, en die ander groep 'n placebo inspuiting (steriele soutoplossing). Vyf dae na die ECP inspuiting is 'n follikulêre multi-stimulasieprogram geïnisieer. Die vroulike diere van die drie species is bevrug met dekking in die geval van die bokooie, en deur middle van inseminasies by die skaapooie (laparoskopies) en koeie (transservikaal). In die die skaap- en bokooie is die embrio's en onbevrugte ova chirurgies gekollekteer, teenoor transservikaal in die koeie, in die 8-sel tot 16-sel stadium 3 d tot 4 d na bevrugting. Beduidend meer CL het gevorm en beduidend meer totale aantal embrio's en ova, sowel as die embrio's van oorplaasbare kwaliteit, is by die ECP-groep skaap-

en bokooie gekollekteer, in vergelyking met die groepe wat die plasebo inspuiting ontvang het (p<0.01). Daar was egter geen betekenisvolle verskil in die aantal onbevrugte ova tussen die twee behandelingsgroepe vir skape of bokke nie. By die koeie is daar weereens beduidend meer CL en embrio's en ova sowel as oorplaasbare kwaliteit embrio's by die ECP groep gevind as by die placebo-groep. Hier het die plasebo (kontrole) groep egter beduidend meer onbevrugte ova as die ECP groep van koeie geproduseer.

Sedert meer as 'n halfeeu gelede word pogings aangewend om selle en embrio's buite die liggaam te kweek (*in vitro* of *ex vivo*). Dit is gedoen met behulp van 'n verskeidenheid van mediums in verskillende tipes "inkubators". Hoofstuk 2 handel oor die vergelyking tussen twee verskillende kweekmediums: TCM-199 en eerste trimester amniotiese vloeistof (BAF) gekollekteer vanaf dragtige koeie na slagting. Twee verskillende kweekmetodes is ook ondersoek. Die standaard 5% CO_2 laboratorium-inkubator is vergelyk met kweking van gamete in die vagina van 'n bokooi. Two ondersoeke is gedoen, naamlik eerstens die deurlaatbaarheid van verskillende kweekhouers vir CO_2 gas vir moontlike vaginale kweking. Vierput bakkies en semenstrooitjies is gebruik om TCM-199 en BAF te inkubeer in die teenwoordigheid of afwesigheid van CO_2 vir 'n 120 h periode. Die pH waardes is elke 24 h bereken en aangeteken. Hier is bewys dat CO_2 gas uit die 4-put-bakkie sowel as deur die wand van die semenstrooitjie gediffundeer het in die afwesigheid van CO_2 in die inkubator, deur styging in die pH waarde na buite fisiologies normale vlakke. In die inkubator met CO_2 het die pH van beide mediums konstant tussen 7.3 en 7.5 gebly. In die tweede eksperiment is prekompakte morulastadium beesembrio's in die twee genoemde groeimedia binne-in 'n gedeelte van 'n afgeseelde 0.25 mL semenstrooitjie in die laboartoriuminkubator en die vagina van 'n bokooi gekweek. Evaluasie is gedoen ten opsigte van die (1) stadium van ontwikkeling en (2) aantal blastomere wat na 'n kweekperiode van 96 h ontwikkel het. Geen statistiese verskille kon tussen die graad 1 beesembrio's wat tot blastosiste of ontkiemde blastosiste ontwikkel, gevind word vir óf die

kweekmedium óf die inkubator gebruik nie. Daar was ook geen beduidende verskille in blastomeergetalle tussen die twee inkubators nie. In die BAF kweekmedium het daar egter beduidend meer blastomere in ontkiemde blastosiste ontwikkel in vergelyking waar TCM-199 as kweekmedium gebruik is (p<0.05).

Na die kolleksie van *in vivo*-geproduseerde lewende hawe embrio's, word die embrio's normaalweg ge-evalueer met behulp van 'n omgekeerde- of stereomikroskoop vergroting van minstens 80X. Die eerste graad embrio's lewer die beste dragtigheidssyfers wanneer die embrio's na gesinkroniseerde vroulike ontvangers oorgeplaas word, terwyl die derde graad embrio's die swakste konsepsiesyfers lewer. Die doel van die huidige eksperiment was om al drie kwaliteitsgrade van skaap-, bok- en bees prekompakte morula-stadium embrio's in die genoemde twee groeimedia te kweek en die aantal wat die ontkiemde blastosist stadium bereik, te evalueer. Die resultate het getoon dat daar geen beduidende verskil in die ontwikkeling van die eerste en tweede graad embrio's se onwikkeling in TCM-199 of hitte-geinaktiveerde vroeë dragtigeheid (<60 d) amniotiese vloeistof was nie. Statisties beduidend meer van die *in vivo*-geproduseerde derde graad skaap-, bok- en beesembrio's wat in BAF met 10% FBS en antibiotieka gekweek is, het egter die ontkiemde blastosist-stadium van ontwikkeling bereik in vergelyking met die wat in TCM-199 plus FBS en antibiotika ontwikkel het (p<0.05).

Die effek van ko-kultuur op die oorlewing van bokembrio's na oorplasing na gesinkroniseerde bokooie, is ook ondersoek. 'n Totaal van 120 Kashmir-embrio's in die blastosist stadium is in drie groepe verdeel na ontdooiing en rekonstuering deur vier stappe van verskillende konsentrasies gliserien en sukrose. Die eerste groep embrio's (G1, n=40) is individueel, semi-laparoskopies in D-PBS met 10% FBS en antibiotika, na die ipsilaterale uterushoring waar die CL voorgekom het oor 'n periode van 3 d oorgeplaas. Die tweede groep embrio's (G2, n=40) is op dieselfde wyse oorgeplaas, maar wel in TCM-199 met serum en antibiotika. Die derde groep

embrio's (G3, n=40) is eers vir ~24 h in TCM-199 met serum en antibiotika in groepe van vyf embrio's in 'n ~50 mm lengte van 'n semenstrootjie in die anterior gedeelte van die vagina van 'n bokooi in haar luteale fase saam met oviduktselle gekweek. Na die ~24 h periode is die 40 embrio's sonder die bykomstige selle op 'n soortgelyke wyse as in G1 en G2, oorgeplaas. Ultrasoniese skandering het aangetoon dat betekenisvol meer van die vooraf gekweekte embrio's (G3) dragtighede gevorm het teenoor die G1 embrio's (oorgeplaas in D-PBS, p<0.05), maar nie nie teenoor die G2 embrio's nie. Die G3 embrio's het ook beduidend meer lewendige lammers as die G1 en G2 embrio's (p<0.05) by geboorte gelewer.

Die maturasie van beesoösiete om die oösiet in staat te stel om meiose voort te sit, is die eerste stap in *in vitro*-fertilisasie om IVMFC embrio's te produseer. Die samestelling van die maturasiemedium speel 'n belangrike rol in die sukses wat met maturasie bereik word. 'n Ondersoek is geloods om die vermoeë van eerste trimester bees amniotiese vloeistof (BAF) om profase I oösiete, afkomstig vanaf slagplaas ovaria, na metafase II te matureer, in vergelyking met 'n kontrolemedium soos TCM-199. In die eerste eksperiment is drie groepe van ~100 oosiete in TCM-199 met 10% estrus koei serum (ECS) gematureer. Die eerste groep oosiete is in 50 µL druppels in 'n inkubator geplaas, en die tweede en derde groepe in 'n semenstrootjie, een groep in die inkubator en die ander groep in die vagina van bokooie in di-estrus. 'n Verdere ses groepe oösiete van ~100 elk is met BAF gematureer, drie groepe met ECS en drie groepe sonder serum en in soortgelyke houers en toestande as die eerste drie groepe. Geen beduidende verskille in maturasie kon tussen die nege groepe oösiete gevind word nie. In die tweede eksperiment is vars en bevrore BAF met of sonder ECS gebruik as maturasiemedia vir oosiete in 50 µL mikrodrupples in 'n laboratorium inkubator. Alhoewel die vars BAF met serum geneig was om beter maturasieresultate as die vars BAF sonder serum of bevrore-ontdooide BAF met of sonder serum te gee, was die verskille tussen die vier groepe nie statisties beduidend nie.

Gedurende die 33ste lansering van die Soyuz-kapsule en protonvuurpyl deur die Russiese Vuurpyl en Ruimte Organisasie (Energia) vanaf Baykonur lanseerbasis in Kazachstan (April 2002), het ons skaap 1-sel embrio's in 'n draagbare inkubator na die internasinale ruimtestasie (ISS) vir 'n kwekingsperiode van 10 d gestuur om die invloed van mikro-gravitasie op embrionale ontwikkeling te ondersoek. Hierdie vroeë stadium embrio's is gekollekteer na follikulêre multi-stimulasie van inheemse Tegeres skaapooie en bevrugting deur middle van kunsmatige inseminasie met elektro-ejakuleerde onverdunde semen. Ag 1-sel embrio's is in twee spesiale , geslote sisteem, plastiese kweekbuise in die draagbare inkubator na die ISS gestuur, en ag embrio's is onder soortgelyke omstandigehede op aarde gekweek. Vooraf pre-geïnkubeerde kultuurmedium (TCM-199 met 10% FBS, antibiotika en 0.5% essensiële en nie-essensiële aminosure elk) is elke tweede tot derde dag in die kweekbuise gespuit deur Afrika se eerste ruimtereisiger, Mark Shuttleworth, om die gebruikte kweekmedium te vervang. 'n Spesiale 2 μ porie-grootte membraan in die kweekbuise het voorkom dat die embrio's tydens mediumvervangings uit die buise gespoel word. Na die terugkeer van die monsters vanaf die ISS is die ruimte- en kontrole embrio's dadelik in vloeibare stikstof gevries en na ons laboartorium in Stellenbsosch, Suid-Afrika vervoer vir verdere evaluasie en seltellings. Daar was geen noemenswaardige verskil tussen die twee groepe 1-sel embrio's wat die blastosist en ontkiemde blastosist stadium bereik het nie, maar die ruimte-embrio's het beduidend meer binneste selmassa selle in die ontkiemde blastosist stadium gevorm as die kontrole embrio's. As gevolg van wetlike bepalings kon geen van hierdie embrio's na ontvanger ooie oorgeplaas word nie.

Die verskillende ondersoeke na die hitte-geïnaktiveerde eerste trimester bees amniotiese vloeistof (BAF) het op die volgende gevolgtrekkings gedui:

1. BAF met 10% bygevoegde fetale beesserum sal die ontwikkeling van pre-kompakte morula-stadium skaap-, bok- en beesembrio's tot die ontkiemde blastosiststadium ondersteun soortgelyk of selfs beter as 'n standaard embrio kweekmedium soos TCM-199 met bygevoegde serum. Dit is waarskynlik daaraan toe te skryf dat BAF die nodige voedingstowwe en groeifaktore benodig vir embrionale ontwikkeling in hierdie drie species bevat. Weens die eenvoudige kolleksiemetodes en voldoende voorsiening van 'n afvalproduk as kweekmedium, word dit aanbeveel dat BAf as alternatiewe kweekmedium gebruik kan word waar vroeë stadium embrio's van hierdie drie species gekweek moet word. Die moontlike oordraging van mikro-organismes soos virusse en prions moet egter as risikofaktor in ag geneem word.

2. Hoe vroeër die stadium van dragtigheid, hoe hoër is die konsentrasie van bestanddele soos groeifaktore en sitikiene in amniotiese vloeistof wat voordelig is vir embrionale ontwikkeling. Dit kan gesien word in die ontwikkeling van beesembrio's *ex vivo* tot die ontkiemde blastosiststadium in BAF vanaf verskillende stadiums van dragtigheid. In Hoofstuk 3 het BAF afkomstig vanaf 'n beesfetus van ~80 d 46% (35/73) prekompakte morulastdium bees embrio's tot ontkiemde blastosiste ondersteun. Hierdie syfer het verhoog tot 69% 67/97) toe BAF vanaf 'n ~60 d fetus gebruik is vir soortgeluke embrio's en omstandighede in Hoofstuk 4. Dit word dus aanbeveel dat indien BAf as kweekmedium vir embrio's gebruik word, die BAF gekollekteer word van die vroegs moontlike stadium van dragtigheid. Op <40 d dragtigheid is die amniotiese vloeistof se volume egter so klein, dat kolleksie nie die moeite werd is nie.

3. Die konsentrasie van groei- en/of herstelfaktore in vroeë-stadium BAf is hoër as in serum. TCM-199 met addisionele 10% fetale beesserum (FBS) as kweekmedium vir graad 3 prekompakte skaap-, bok- en beesembrio's het 18%, 23% en 16% van die embrio's, respektiewelik, tot ontkiemde blastosiststadiums laat ontwikkel. Hierdie

syfers het verhoog tot 43%, 49% anend 53% toe ~60 d BAF met 10% begevoegde FBS gebruik is as kweekmdium vir soortgelyke graad 3 embrio's. Omrede 10% FBS teenwoordig was in beide hierdie kweekmediums, kan afgelei word dat die herstelfaktore slegs in BAF en nie in serum nie teenwoordig is, of in hoër konsentrasies in BAf as in FBS voorkom. Verdere ondersoeke na die effek van BAF op embrionale ontwikkeling gekollekteer vanaf verskillende stadiums van dragtigheid moet veder ondersoek word om die optimale stadium van BAF kolleksie te bepaal.

4. Eerste trimester beesamniotiese vloeistof (BAF) kan oösietmaturasie ondersteun. Die ondersoeke het bewys dat BAF gekollekteer vanaf <90 d dratighede die profase I beesoösiete na 26 h van inkubasie tot metafase II oösiete kon verander soortgelyk aan TCM-199 met serum maar sonder addisionele hormone. Geen wesentlike verskille is waargeneem in oösiet maturasie waar van ander kweekhouers (mikrodruppels vs semenstrooitjies) of alternatiewe inkubators gebruik gemaak is nie. Die bevriesing van die BAF het klaarblyk;lik ook nie die maturasiekapasiteit van BAF op beesoösiete nadelig beïnvloed nie. Waar bevrore-ontdooide BAF vergelyk is met vars BAF as kweekmedium vir die ontwikkeling van 2-sel muisembrio's, het statisties minder van die embrio's gekweek in bevrore-ontdooide BAF die ontkiemde blastosiststadium bereik as die embrio's gekweek in vars BAF (ongepubliseerde data).

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ABBREVIATIONS

AI	Artificial insemination
BAF	Bovine amniotic fluid
Blast	Blastocyst stage of the embryo
CL	Corpus luteum
CIDR	Controlled intra-vaginal release device (for estrus synchronization)
CO_2	Carbon dioxide gas
CSM	Complex serum-free medium
d	Days
DMEM	Dulbecco's minimal essential medium
D-PBS	Dulbecco's phosphate buffered saline
EBM	Eagle's basal medium
ECP	Estradiol cypionate
ECS	Estrus cow serum
EDTA	Ethylenediaminetetraacetic acid
et al.	And others
ex vivo	Outside the body
F12	Ham's F12 medium
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
h	Hours
HB	Hatched blastocyst stage of the embryo
hTF	Human tubal fluid
ICP	Immuno-Chemical Products, Auckland, New Zealand
IMV	IMV company, L'Aigle, France
In vitro	In an incubator (outside the body)
In vivo	Inside the body
IVF	In vitro fertilization
IVM	In vitro matured
IVMFC	In vitro matured, fertilized and cultured
IVP	In vitro produced
KSOM	Potassium simplex optimization medium
LH	Luteinizing hormone
mL	Milliliter
min	Minutes
MOET	Multi-ovulation and embryo transfer
Mor	Morula stage of the embryo
O_2	Oxygen gas
PBS	Phosphate buffered saline
SD	Standard deviation
SOF	Synthetic oviduct fluid
TCM	Tissue culture medium

CHAPTER 1

GENERAL INTRODUCTION

To develop and grow mammalian gametes outside the body similar to conditions that exist in the oviduct and uterus, has always been a major challenge to mankind. As far back as 1912 Brachet (1912) attempted to culture 5-7 day old rabbit embryos in clotted blood in glass dishes, while Lewis and Gregory (1929) did cinematographic studies on developing rabbit embryos. In the 1930s Pincus *et al.* (1938) experimented with mammalian eggs *in vitro* and *in vivo*. They showed that the release of the rabbit oocyte from its inhibitory influence of the follicle would allow the oocyte to mature and develop *in vitro*. It was in 1949 that Hammond (1949) showed that 8-cell mouse embryos could be cultured to the blastocyst stage in a simple salt culture medium enriched with hen egg white and yolk. This was followed by Whitten (1957), who showed that lactate is a critical constituent of a bicarbonate buffer solution supplemented with serum albumin to culture 2-cell mouse embryos to the blastocyst stage. It was McLaren and Biggers (1958) who first confirmed that mammalian embryos cultured *in vitro* could be transferred to surrogate mothers and will subsequently develop to live young.

The *in vitro* culture of *in vivo* and *in vitro*-produced livestock embryos is today widely used for a number of reasons. Providing conditions that will allow the embryos to survive freezing, thawing and transplantation, and to create the best culture conditions for the development of livestock embryos, has been an irresistible challenge to many animal biotechnologists worldwide for many years. More than a thousand scientific papers dealing with livestock embryo culture published during the past decade gives one some idea of the challenges and interest in the subject.

But why this much effort to optimize *in vitro* embryo culture conditions? The benefits of this biotechnology procedure to both human and animal reproduction are numerous. Not only does it allow the production of viable embryos from ova and sperm in an incubator, but it also optimize the survival of partially damaged embryos (Hansel 2003).

The fact that only about 20 to 30% of selected ova collected from slaughterhouse ovaries will ever reach the transferable blastocyst stage (Thompson and Duganzich 1996), however, shows that our culture media, conditions and knowledge of the subject is still far from optimal or complete. This study therefore concentrated on testing different media and culture conditions to increase the survival of *in vivo*-produced early-stage mammalian embryos.

The successful culturing of livestock embryos also stimulated much interest in programmed embryonic death, called apoptosis. Much research is focused on determining why only about 60% of *in vivo* produced embryos in animals develop into pregnancies that are carried to term (Pieterse *et al.* 1990, Carnevale *et al.* 2000).

Another useful tool during the culturing of livestock embryos is the investigation of the effect of different additives like hormones, growth factors, cytokines, sera and other proteins, anti-oxidants, amino-acids, micro-minerals and many other substances that might play a major or minor role in embryonic development. In this study a comprehensive investigation into the effect of first trimester bovine amniotic fluid on embryonic development was done.

The incidence of the so-called large calf syndrome leading to dystocia, increased birth weight and even a higher incidence of post-natal deaths or very weak calves, have been attributed to the long-term culture of livestock, especially bovine, embryos. The incidence of this phenomenon seems to be higher in *in vitro* produced embryos and where serum additives of 10% or above and/or co-culture have been used during the culture period (Jocobsen *et al.* 2000).

Embryo culture is one aspect of embryo transfer that must constantly be maintained in order to obtain optimal results under highly adverse and variable conditions. A survey of the media used in 26 embryo transfer companies in the USA has shown that as many as 14 different combinations of four culture media were used, and only 11 of the 26 companies consistently used the same medium for flushing of the *in vivo* produced embryos, culture of the embryos and freezing of the embryos Nelson and Nelson 2001). As all of the 26 companies have been in business for many years, and therefore must have obtained pregnancy results that are very acceptable, it shows that no specific culture medium will dramatically improve pregnancy rates in a multi-ovulation and embryo transfer (MOET) program if the media used for holding and culturing the embryos up to that stage were already acceptable prior to the change (Stringfellow 1998). During the past three decades culture media used in embryo transfer programs slowly changed from bicarbonate buffered solutions (Ham's F-10 and TCM-199) to phosphate buffered saline (PBS), or modified and enriched PBS, due to pH changes of the bicarbonate buffered culture media outside the CO_2 incubator. Since the 1990's, PBS was then again left aside for zwitterion-buffered (Emcare) and HEPES-buffered (ViGro) cultured media in MOET programs.

Many attempts have been made to use protein-free media for human and animal embryo culture conditions. A protein-free culture medium will allow investigations into nutrient requirements, metabolism, hormone, and steroid and protein production of cultured embryos. The addition of serum or protein always has the potential of transmitting disease to the recipient of the *in vitro* produced or *in vivo* collected embryo. The generation of viable human embryos in a chemically defined culture medium devoid of any protein additives beginning from oocyte collection to the cleaved embryo stage, have not been described. This shows that chemical media without protein additives are not optimally defined (Ali *et al.* 2000).

The enhancement of "helper" cells, or co-culture monolayers, on the growth and development of cultured embryos have also been investigated in the quest to find optimal conditions for the culturing of early-stage embryos (Thibodeaux and Godke 1992). Trophoblastic vesicles prepared from sectioned trophectoderm of 12 to 14 day bovine conceptuses were used as a co-culture system for their embryotropic effect. More than double the number of one- to eight-cell bovine embryos reached the morula stage (past the eight to 16-cell block stage) when compared to similar grade and stage embryos cultured in Menezo's culture medium alone (46% vs <20%) (Camous *et al.* 1984) It appears that trophoblastic vesicles should be prepared after day 10 and before day 14 of development in the cow, sheep and goat for maximum embryotropic activity during *in vitro* embryo culture (Thibodeaux and Godke 1992).

Fibroblasts from adult cow or fetal uterine tissue, when used as co-culture, had a beneficial effect on developing bovine, equine, and caprine embryos (Wiemer *et al.* 1987). Even bovine demi-embryos mechanically split in two benefited from the embryotropic effect of the uterine-cell

monolayer either by the factors secreted by the co-culture cells, by the removal of toxic metabolic waste products by the co-culture, or by lowering the oxygen tension in the immediate vicinity of the developing embryo (Voelkel *et al.* 1985). Caprine endometrial cells gave a 63% hatching rate when used as co-culture cells for 2- to 4-cell caprine embryos. Even better hatching results (87%) were obtained when caprine oviduct epithelial cells were used with similar caprine embryos (Pritchard *et al.* 1990).

Oviduct epithelial cells of various mammalian species are able to stimulate embryonic development of a number of animal species. It was already reported by Boland in 1984 that the oviduct of the rabbit would maintain or enhance *in vivo* development of mouse, sheep, cow, pig, goat and horse embryos. An oviduct epithelial co-culture system supports embryo development *in vitro* to obtain an adequate pregnancy rate, even when a simple culture medium without any protein supplementation is used for *in vitro* culture of bovine embryos (Ellington *et al.* 1990). It is therefore suggested that oviduct cell monolayers are the most effective co-culture system for early-stage farm animal embryos, while fibroblast monolayers are adequate for the development of later-stage embryos (morula to blastocyst), but less effective for the development of earlier-stage embryos through the hatched blastocyst stage (Rexroad 1989).

Granulosa cells from mural and cumulus origin play an important role in oocyte maturation, fertilization and further development (Critser and First 1986; Faundez *et al.* 1988). Cumulus cell monolayers have shown the ability to support embryo development that culminated in live offspring when both fresh and frozen-thawed bovine embryos cultured on this monolayer system were transferred to recipient cows (Fukuda *et al.* 1990). A total of 47% of the *in vitro* matured,

fertilized and cultured (IVMFC) bovine oocytes grown by Zhang *et al.* (1992) in cumulus cell co-culture reached the morula stage of development. Even cross-species embryo enhancement was demonstrated when bovine cumulus granulosa cell co-culture was used to grow porcine IVF-derived embryos (Zang *et al.* 1991), and bisected equine embryos (Rodriguez *et al.* 1991).

Growing cell cultures produce certain growth factors that are beneficial to embryo development. This is the principle behind conditioned media. Blakewood *et al.* (1989) cultured murine embryos in the amniotic sac of a developing chick embryo (Blakewood and Godke 1989). The growing chick embryo and the wall of the amniotic sac secrets certain growth factors into the amniotic fluid that are beneficial to the development of murine embryos.

The new century has set a revolution in the application of biotechnological procedures to farm animals. This revolution was started by the production of transgenic mice by Brinster *et al.* (1982), followed by the production of transgenic cattle, sheep and pigs (Wall *et al.* 1992 and Ebert and Schindler 1993). Then followed the development of a practicable sperm sexing procedure in cattle with its enormous potential to produce offspring of a desired gender (Seidel *et al.* 1997, 1999; Seidel and Garner 2002). Biotechnology reached an unprecedented level with the production of the sheep, Dolly, cloned from a somatic mammary gland cell by Campbell *et al.* (1996) and Wilmut *et al.* (1997). This new technique offered almost unlimited possibilities for the production of genetically modified animals (Cibelli *et al.* 1998; Park *et al.* 2002). However, the successful development and application of these and related technologies is critically dependent on a whole range of basic reproductive technologies such as *in vitro* maturation of oocytes, *in vitro* fertilization, *in vitro* culture of embryos and *in vitro* culture of sperm precursors. Without

significant improvements in these reproductive technologies, application of developments in cloning and the production of transgenic farm animals will remain limited and extremely costly.

The application of a reliable system for *in vitro* produced (IVP) livestock embryos can be manifold. The first advantage that comes to mind is the increased supply of embryos from high genetic merit cows and bulls using oocytes obtained by ultrasound ovum pick-up procedures, or from abattoir material. To optimize the production of such embryos, the *in vitro* culture conditions to grow such embryos have to be improved (Kane 2003).

Secondly, IVP embryos will facilitate the production of twins in beef cattle. Either two embryos could be transferred non-surgically to the uterus of a synchronized recipient, or the recipient could be bred to AI when in estrus and one week later receive an IVP embryo by embryo transfer (Gordon and Boland 1978; Gordon and Lu 1990). However, interest in this approach has declined mostly due to varying results, and a low blastocyst rate. If the techniques for *in vitro* grown (IVG) oocytes developed by Eppig and O'Brien (1996) in the mouse could be adapted and improved for cattle oocytes, to allow the production of thousands of fertilizable oocytes from fetal calf ovaries, this would enormously improve the IVP embryo supply situation and could change the attitude to twinning in beef cattle. Again maturation and culture conditions will have to be optimized before this would become a reality.

Thirdly, a spin off advantage of the production of IVP embryos could be an ample supply of suitable oocytes for use in embryo cloning procedures, if the *in vitro* maturation procedure could be improved (Kane 2003).

Progeny testing of female livestock animals as compared with male animals has always been drastically forestalled by the small number of offspring produced by one female. The development of successful procedures for the production of thousands of viable IVG oocytes from a single ovary would lead to a fourth advantage of IVP embryos which could dramatically change the whole area of female progeny testing in cattle. IVP embryos produced from IVG oocytes could be stored frozen, while embryos from the same cohort are transferred to recipients for progeny testing.

Extension of successful IVP embryo production techniques could, in the fifth place, have major importance in the preservation of endangered species. The use of the endangered individuals for oocyte or embryo production should, however, not be a threat to the life of such an animal, or else the techniques itself could increase the danger of species extinction before the techniques could be put to beneficial effect. Reproductive techniques used *in vivo* as well as *in vitro* should, therefore, still be further improved before these procedures could be used with safety and to the advantage of many endangered species.

The availability of large numbers of normal IVP embryos would be a very useful research tool for researchers wishing to study early embryonic development. The morula and blastocyst stages of development are characterized by the first development of cell junctions, the initiation of tissue differentiation with the formation of a functioning transporting epithelium, the trophectoderm, and by the start of true embryonic growth. All of these processes are of fundamental biological interest and, while they can readily be studied in mouse embryos, it is important to have at least one other animal model such as one of the livestock species in which these processes can be studied (Kane 2003).

Different constituents and conditions are essential for the development of the embryos of different species. Kane and Foote (1970) discovered that amino acids were essential for blastocyst formation in the rabbit, as well as for the cleavage (Edwards *et al.* 1970) and development to the blastocyst stage of *in vitro* fertilized human embryos (Steptoe *et al.* 1971). A major breakthrough for farm animal embryo culture was the discovery in First's laboratory in 1983 of the importance of using a temperature equal to the animal's core body temperature (about 39 °C for cattle) for *in vitro* maturation and fertilization of cattle oocytes (Lenz *et al.* 1983). This led to the later discovery that the same temperature should be used for sheep (Gandolfi and Moor 1987; Fukui *et al.* 1988) and cattle embryo culture (Fukui and Ono 1988; Fukuda *et al.* 1990). A further very significant factor in the development of embryo culture in farm animals was the finding that embryos tended to develop better in gas phases with oxygen concentrations as low as 5% (Hejlasz et al 1987), especially when cultured in the absence of somatic cell support (Tervit *et al.* 1972; Fukui *et al.* 1991).

The pre-implantation embryos of cattle, sheep and pigs differ radically from those of the mouse and most other laboratory species with respect to the degree of growth that takes place before implantation, true for both *in vitro* or *in vivo* development. The 1-cell mouse embryo contains about 20 ng of protein and the blastocyst just before implantation also has only about 20 ng (Brinster 1967; Schiffner and Spielmann, 1976). This means that true growth before implantation is minimal or non-existent. In contrast to this the 2-cell cattle embryo has about 132 ng of protein and the day 8 early blastocyst about 185 ng of protein, while the day 16 elongated blastocyst (depending on size) can have a protein content of more than 900 μg (Grealy *et al.* 1996). A similar picture is seen in the pig (Wright *et al.* 1981; Wright *et al.* 1983). This difference in the degree of pre-implantation growth has major consequences for embryo culture. It

could possibly explain why it is possible to culture mouse embryos over a period of about 3–4 days from 1-cell to blastocyst in a simple medium devoid of amino acids, vitamins or trace elements, but cannot be done with livestock embryos, and therefore why it is difficult to obtain good blastocyst development of the embryos of farm animals *in vitro*. An average to good success rates with livestock embryos seem to lie in the range of 30–45% of matured oocytes reaching the blastocyst stage following IVF and embryo culture, with pregnancy rates of 40–60% following embryo transfer (Hasler 1962; Hasler *et al.* 1995). Results depend to some extent on the degree of oocyte selection. Careful selection of oocytes before maturation start will dramatically increase blastocyst rate (Hawk and Wall 1994). In contrast to the situation for embryo cleavage and early blastocyst formation, there appear to be no reports of *in vitro* culture of cattle early blastocysts to elongated blastocysts. However, *in vivo* culture of IVP early cattle blastocysts to expanded and hatching blastocysts in the sheep oviduct followed by culture to elongated blastocysts in the cow uterus, is possible (Galli *et al.* 1997).

Improvements in the *in vitro* culture conditions of livestock embryos could be considered in the following variables:

1. The addition of co-culture cells to the complex culture medium. Various cell types can be used for this purpose as mentioned, even from a non-related species (Fukuda *et al.* 1990, Thibodeaux and Godke 1992, Wiemer *et al.* 1997).
2. If a simple cell-free culture medium such as synthetic oviductal fluid (SOF) is used, the medium has to be supplemented with amino acids, vitamins, serum, and other components essential for embryonic development (Kane and Foote 1997).

3. In the absence of fetal bovine serum (FBS) or bovine serum albumin (BSA), growth factors have to be supplemented (Nasr-Esfahani *et al.* 1992, Shultz and Heiner 1992).

4. Oviductins, a family of glycoproteins synthesized and secreted by oviduct cells, which bind to the zona pellucida of the oocyte after ovulation, might play a major role in embryo protection after fertilization, but may be involved in hatching (Malette and Bleau 1993).

5. Adjusting the operating temperature of the laboratory incubator to actual core temperature of the animal, plays an important role in the success achieved with *in vitro* culture (Lenz *et al*. 1983).

6. The volume of medium in which embryos are cultured is a factor that needs examination. Currently embryos are cultured in relatively large volumes of medium in spite of the fact that a mammalian embryo of just over 150 μm diameter, and has a volume of only 1.5–2.0 nL and in the oviduct it is probably surrounded by mere picoliters of fluid at any one time. The embryo is therefore lying in an ocean of fluid into which it is leaking its own internal nutrients (Kane 2003). It is therefore possible that culture of cleavage stage embryos in volumes of medium 1 μL or less might markedly improve development in culture. The possible problems with this approach is (1) exhaustion of nutrients in the medium due to usage by the embryos, and therefore more frequent medium changes, (2) the effect of surface tension of such a small volume, (3) the physical problem of managing such small culture volumes and (4) the danger that these small culture droplets with a high surface area to volume ratio might facilitate uptake of toxic substances from the environment.

7. Very mild agitation could be used to mimic the movement of the female reproductive tract although there is evidence that in some circumstance at least, agitation is harmful (Hickman *et al.* 2002).

8. Another curious finding was the report by Elliott *et al.* (1974) that increased atmospheric pressure in culture improved rabbit embryo development.

9. To improve blastocyst expansion, co-culture on polarized uterine epithelial monolayers by growing the cells on collagen-impregnated filters with culture medium above and below the filter could be beneficial (Dickens *et al.* 1993). It is possible that such monolayers might constitute an environment analogous to the uterine environment.

10. For embryonic development at a later stage, a perfusion system such as that used by Pincus and Werthessen (1938) on the growth of the rabbit blastocyst, might be useful in promoting growth of cattle and sheep blastocysts.

11. When adding new constituents to a medium, consideration should always be given to possible negative as well as beneficial effects. Bavister (1995) has emphasized the importance of the possible toxic effects of relatively ordinary constituents of complex culture media at the concentrations normally used in these media. A very early indication of this problem was the finding that the nucleic acid precursor, thymidine, at the concentration used in Ham's F10 medium, was inhibitory to rabbit blastocyst formation (Kane and Foote 1971). Thus, toxic contaminants in culture medium ingredients, including the water, are always a major concern. This could possibly be addressed by adding agents such as EDTA to remove toxic contaminants or metabolites is one area of possible improvement. This role is possibly also carried out by albumin (when adding serum or

BSA to the culture medium) and to help prevent free radical production (Nasr-Esfahani *et al.* 1992).

12. Another problem has been the production of toxic metabolites from medium constituents, such as the production of toxic ammonia from amino acids in the medium. An enzyme, glutamate dehydrogenase which removes the ammonia by converting it to glutamic acid, has been added to culture media to deal with this problem (Lane and Gardner 1995).

13. Free oxygen radicals produced in culture media might block embryo development (Legge and Sellens 1991). Various methods of remedying this problem have been used. Reducing the oxygen concentration in the gas phase to about 5% probably helps (Umaoka *et al.* 1992). Other approaches have included the use of antioxidants, e.g. thioredoxin and superoxide dismutase (Nonogaki *et al.* 1991), EDTA (Nasr-Esfahani *et al.* 1992), catalase (Nasr-Esfahani and Johnson 1992) and vitamins C and E (Vermeiden and Bast 1995). Recently, Olson and Seidel (2000) examined the effects of EDTA and vitamins E and C on the culture of IVP cattle embryos; vitamin E markedly improved blastocyst development but EDTA and vitamin C had no obvious beneficial effect.

14. Possible toxic factors other than medium constituents, for example, the number of times the incubator door is opened, and the conditions under which embryos are taken out of the incubator for examination, may be important. Taking embryos out of the incubator exposes them to altered conditions of temperature, light and gas phase CO_2 to which they would not be exposed to *in vivo*. According to Hegele-Hartung *et al.* (1991) and Noda *et al.* (1994) they found evidence that exposure to light and room temperature negatively influences rabbit and human embryo development, and exposure to a CO_2-free gas phase (as is the case outside the incubator) and therefore changes in pH, is rapidly toxic to hamster

embryos. Although short-term cooling of cattle oocytes to 25 °C does not appear to be inhibitory to later embryo viability (Hasler *et al.* 1995), it is probable that the number of removals of cultured embryos from the incubator should be as limited as possible, as short as possible, and the nature of the environment in which they are handled and examined kept as non-toxic as possible.

15. There is evidence that *in vitro* embryo culture and manipulation causes abnormal DNA methylation which in turn causes abnormal gene expression both in pre-implantation and fetal stages resulting in fetal overgrowth and large offspring syndrome (Niemann and Wrenzycki 2000; Young *et al.* 2001). Changing the culture conditions so that normal gene methylation is present in IVP embryos might reduce or abolish the incidence of large offspring syndrome.

Culture systems that would allow the development of > 90% of normal 1-cell cattle, sheep or pig embryos to early blastocysts would have a huge general impact on the development of related areas of reproductive biotechnology (Kane 2003). Such a culture system would be a very powerful research tool. Reports from large commercial groups using ovum pick up and optimal sources of oocytes (pregnant heifers and first-parity cows), unfortunately indicate figures of at best about 60% of cleaved IVP embryos reaching the stage of transferable morulae or blastocysts (Galli *et al.* 2001). The study of oocyte maturation and the development of optimal oocyte maturation systems, the study of fertilization and the development of optimal IVF systems, and the study of cloning and development of optimal cloning systems would benefit enormously from the availability of such culture systems for testing the normality and viability of oocytes or embryos developed from the

system under study (Kane 2003). Any experimental manipulation which affected the oocyte or embryo so as to reduce development to the blastocyst stage, would readily be detected with minimal numbers of oocytes or embryos. Such a culture system, however, would not detect the deleterious effect which affects development after the blastocyst stage was reached.

Perhaps the most important and certainly the most obvious biotechnological implication at this time is that in addition to facilitating the production of better embryo cloning systems, the availability of such an optimal culture system would greatly increase the availability of cloned embryos simply because very few embryos would be lost due to damage by the culture process. With current systems, the embryo undergoes the double insult of the cloning procedure and a less than optimal culture procedure. These two insults are probably not just additive but rather negatively synergistic in their effects. One can expect an embryo which has been injured by one procedure to be much less capable of surviving injury from a second procedure (Kane 2003).

Currently almost all cattle embryo transfer is carried out non-surgically using blastocysts and this necessitates the use of a reliable culture system. In combination with optimal methods of super-ovulation, ovum pick up, *in vitro* maturation (IVM) of oocytes and IVF, optimal methods of embryo culture would facilitate greatly the use of IVP embryos. In dairy cattle this would lead to a better use of the genetic merit of high yielding dairy cows.

As most researchers have foreseen, and many breeders have hoped, *in vivo* and *in vitro* production of livestock embryos and the birth of subsequent offspring never really replaced

artificial insemination during the past 30 years. This was, to a large extent, due to very variable and unreliable numbers of embryos produced using these two methods. The present study was therefore undertaken to investigate certain aspects of assisted reproductive techniques (ART) to try and solve some of these difficulties. Problems addresses were the management of follicular development on the ovary by controlling the dominant follicle, and investigating alternative and more cost-effective culture media and conditions for embryo culture. The reports on the stimulating effect of chick amniotic fluid on the development of mammalian embryos (Blakewood and Godke 1989) lead to this study where conditioned medium from the amniotic cavity of first trimester pregnant cows were used as the culture medium.

REFERENCES

Ali, J., Shabata, M.A.M., and Al-Natsha, S.D. (2000). Formulation of a protein-free medium for human assisted reproduction. *Human Reproduction* **15** (1), 145-156.

Bavister, B.D. (1995). Culture of preimplantation embryos: facts and artifacts. *Hum. Reprod. Update* **1,** 91–148.

Blakewood, E.G., Jaynes, J.M., Johnson, W.A. and Godke, R.A. (1989). Using the amniotic cavity of a developing chick embryo for the *in vivo* culture of mammalian embryos. *Poultry Science* **68**, 1695-1702.

Blakewood, R.G. and Godke, R.A. (1989). A method using the chick embryo amnion for mammalian embryo culture. *Journal of Tissue Culture Methods* **12**, 73-76.

Boland, M.P. (1984). Use of the oviduct as a screening tool for the viability of mammalian eggs. *Theriogenology* **21**, 126-137.

Brachet, A. Development *in vitro* de blastodermes et jeunes embryons de mammiferes. (1912). *CR Acad Sci* **155,** 1191-1193.

Brinster, R.L. (1967). Protein content of the mouse embryo during the first five days of development. *J. Reprod. Fertil.* **13,** 413–420.

Brinster, R.L., Chen, H.Y., Warren, R., Sarthy, A. and Palmiter, R.D. (1982). Regulation of metallothionein–thymidine kinase fusion plasmids injected into mouse eggs. *Nature* **296,** 39–41.

Campbell, K.H.S., McWhir, J., Ritchie W.A. and Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380,** 64–66.

Camous, S., Heyman, Y., Meziou, W. and Menezo, Y. (1984). Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. *Journal of Reproduction and Fertility* **72**, 479-485.

Carnevale, E.M., Ramirez, E.L., Squires, E.L., Alvarenga, M.A., Vanderwall, D.K. and McCue, P.M. (2000). Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. *Theriogenology* **54**, 965-979.

Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de Leon F.A. and Robl, J.M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280,** 1256–1258.

Critser, E.S. and First, N.L. (1986). Use of a fluorescent stain for visualization of nuclear material in living oocytes and early embryos. *Stain Technology* **61**, 1-5.

Dickens, C.J., Southgate J. and Leese, H.J. (1993). Use of primary cultures of rabbit oviduct epithelial cells to study the ionic basis of tubal fluid formation. *J. Reprod. Fertil.* **98,** 603–610.

Ebert, K.M. and Schindler, J.E.S. (1993). Transgenic farm animals: progress report. *Theriogenology* **39,** 121–135.

Edwards, R.G., Steptoe P.C. and Purdy, J.M. (1970). Fertilization and cleavage *in vitro* of pre-ovulatory human oocytes. *Nature* **227,** 1307–1309.

Ellington, J.E., Farrell, P.B., Simkin, M.E., Foote, R.H., Goldman, E.E. and McGrath, A.B. (1990). Development and survival after transfer of cow embryos cultured from 1-2 cells to morulae or blastocysts in rabbit oviducts or in a simple medium with bovine oviduct epithelial cells. *Journal of Reproduction and Fertility* **89**, 293-299.

Elliott, D.S., Maurer R.R. and Staples, R.E. (1974). Development of mammalian embryos in vitro with increased atmospheric pressure. *Biol. Reprod.* **11,** 162–167.

Eppig, J.J. and O'Brien, M.J. (1996). Development *in vitro* of mouse oocytes from primordial follicles. *Biol. Reprod.* **54,** 197–207.

Faundez, R., Spohr, I. and Boryczko, Z. (1988). Effect of follicle cells on maturation and *in vitro* fertilization of cattle oocytes. *Proceedings of the 11th International Congress on Animal reproduction and Artificial Insemination,* Dublin Ireland **3**, 325 (Abstract).

Fukuda, Y., Ichikava, M., Maito, K. and Toyoda, Y. (1990). Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells *in vitro* up to the blastocyst stage. *Biology of Reproduction* **42**, 114-119.

Fukui, Y., Glew, A.M., Gandolfi F. and Moor, R.M. (1988). *In vitro* culture of sheep oocytes matured and fertilized *in vitro*. *Theriogenology* **29,** 883–891.

Fukui, Y. and Ono, H. (1988). *In vitro* development to blastocyst of *in vitro* matured and fertilized bovine oocytes. *Vet. Rec.* **122,** 282.

Fukui, Y., McGowan, L.T., James, R.W., Pugh P.A. and Tervit, H.R. (1991). Factors affecting the *in vitro* development to blastocysts of bovine oocytes matured and fertilized *in vitro*. *J. Reprod. Fertil.* **92,** 125–131.

Galli, C., Duchi, R., Crotti, G. and Lazzari, G. (1997). Elongated bovine blastocysts can be obtained from IVM-IVF embryos cultured *in vivo* from day 2 to day 7 in the sheep oviduct and from day 7 to day 14–16 in the synchronized cow uterus. *J. Reprod. Fertil.* **19**, 11 (Abstract).

Galli, C,. Crotti, G., Notari, C., Turini, P., Duchi R. and Lazzari, G. (2001). Embryo production by ovum pick up from live donors. *Theriogenology* **55,** 1341–1357.

Gandolfi F. and Moor, R.M. (1987). Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. *J. Reprod. Fertil.* **81,** 23–28.

Gordon, I. and Boland, M.P. (1978). Cattle twinning by non-surgical egg transfer. In: Sreenan, J.M. (Ed.), Control of Reproduction in the Cow, vol. 1. Martinus Nijhoff, The Hague, pp. 336–355.

Gordon, I. and Lu, K.H. (1990). Production of embryos *in vitro* and its impact on livestock production. *Theriogenology* **33,** 77–87.

Grealy, M., Diskin, M.G. and Sreenan, J.M. (1996). Protein content of cattle oocytes and embryos from the two-cell to the elongated blastocyst stage at day 16. *J. Reprod. Fertil.* **107,** 229–233.

Hammond, J. (1949). Recovery and culture of tubal mouse ova. *Nature* **163**, 28-29.

Hansel, W. (2003). The potential for improving the growth and development of cultured farm animal oocytes. *Anim Reprod Sci* 79 (3-4), 191-201.

Hasler, J.F. (1962). Commercial applications of *in vitro* fertilization in cattle. *The Compendium* **16,** 1062–1073.

Hasler, J.F., Henderson, W.B., Hurtgen, P.J., Jin, A.D., McCauley, Z.Q., Mower, S.A., Neely, B., Shuey, L.S., Stokes J.E. and Trimmer, S.A. (1995). Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* **43,** 141–152.

Hawk H.W. and Wall, R.J. (1994). Improved yields of bovine blastocysts from *in vitro* produced-oocytes. II. Selection of oocytes and zygotes. *Theriogenology* **41,** 1571–1583.

Hegele-Hartung, C., Schumacher A. and Fischer, B. (1991). Effects of visible light and room temperature on the ultra-structure of pre-implantation rabbit embryos: a time course study. *Anat. Embryol.* **183,** 559–571.

Hejlasz, Z., Nicpon, J., Rauluszkiewicz, S. & Samborski Z. (1987). Buffer systems, electrolytes, pH and erythrocyte indices in the blood of cows and calves in the peri-natal period. *Pol Arch Weter* **25** (2-3), 225-235.

Hickman, D.L., Beebe, D.J., Rodriguez-Zas S.L. and Wheeler, M.B. (2002). Comparison of static and dynamic medium environments for culturing of pre-implantation mouse embryos. *Comp. Med.* **52,** 122–126.

Jocobsen, H., Schmidt, M., Holm, P., Sangild, P.T., Greve, T. and Callesen, H. (2000). Ease of calving, blood chemistry, insulin and bovine growth hormone of newborn calves derived from embryos produced *in vitro* in culture systems with serum and co-culture or with PVA. *Theriogenology* **54**, 147-15.

Kane M.T. and Foote, R.H. (1970). Culture of two-and four-cell rabbit embryos to the expanding blastocyst stage in synthetic media. *Proc. Soc. Exp. Biol. Med.* **133,** 921–925.

Kane M.T. and Foote, R.H. (1971. Factors affecting blastocyst expansion of rabbit zygotes and young embryos in defined media. *Biol. Reprod.* **4,** 41–47.

Kane, M.T. (2003). A review of *in vitro* gamete maturation and embryo culture and potential impact on future animal biotechnology. *Animal Reproduction Science* **79** (3-4), 171-190.

Lane M. and Gardner, D.K. (1995). Removal of embryo-toxic ammonium from the culture medium by in situ enzymatic conversion to glutamate. *J. Exp. Zool.* **271,** 356–363.

Legge M. and Sellens, M.H. (1991). Free radical scavengers ameliorate the 2-cell block in mouse embryo culture. *Hum. Reprod.* **6,** 867–871.

Lenz, R.W., Ball, G.D., Leibfried, M.L., Ax R.L. and First, N.L. (1983). *In vitro* maturation and fertilization of bovine oocytes are temperature-dependent processes. *Biol. Reprod.* **29,**173–179.

Lewis, W.H. and Gregory, P.W. (1929). Cinematographs of living developing rabbit-eggs. *Science* **69**, 226-229.

Malette B. and Bleau, G. (1993). Biochemical characterization of hamster oviductin as a sulphated zona pellucida-binding glycoprotein. *Biochem. J.* **295,** 437–445.

McLaren, A. and Biggers, J.D. (1958). Successful development and birth of mice cultivated *in vitro* as early embryos. *Nature* **182**, 877-878.

Nasr-Esfahani M.H. and Johnson, M.H. (1992). Quantitative analysis of cellular glutathione in early pre-implantation mouse embryos developing in vivo and in vitro. *Hum. Reprod.* **7,** 1281–1290.

Nasr-Esfahani, M.H., Winston N.J. and Johnson, M.H. (1992). Effects of glucose, glutamine, ethylenediaminetetraacetic acid and oxygen tension on the concentration of reactive oxygen species and on development of the mouse pre-implantation embryo in vitro. *J. Reprod. Fertil.* **96,** 219–231.

Nelson, L.D. and nelson, C.F. (2001). Handling of bovine embryos: survey of media used in 26 embryo transfer companies in the USA. *Theriogenology* **56** (9), 1377-1382.

Niemann H. and Wrenzycki, C. (2000). Alterations of expression of developmentally important genes in pre-implantation bovine embryos by in vitro culture conditions: implications for subsequent development. *Theriogenology* **53**, 21–34.

Noda, Y., Shiotani, M., Goto, Y., Nakayama, T., Umaoka Y. and Mori, T. (1994). Culture of human embryos in alpha modification of Eagle's medium under low oxygen tension and low illumination. *Fert. Steril.* **62,** 1022–1027.

Nonogaki, T., Noda, Y., Narimoto, K., Umaoka Y. and Mori, T. (1991). Protection from oxidative stress by thioredoxin and superoxide dismutase of mouse embryos fertilized in vitro. *Hum. Reprod.* **6,** 1305–1310.

Olson S.E. and Seidel, G.E. (2000). Culture of *in vitro*-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients. *Biol. Reprod.* **62,** 248–252.

Park, K.-W., Lai, L.X., Cheong, H.-T., Cabot, R., Sun, Q.-Y., Wu, G.M., Rucker, E.B., Durtschi, D., Bonk, A., Samuel, M., Rieke, A., Day, B.N., Murphy, C.N., Carter D.B. and Prather, R.S. (2002). Mosaic gene expression in nuclear transfer-derived embryos and the production of cloned transgenic pigs from ear-derived fibroblasts. *Biol. Reprod.* **66,** 1001–1005.

Pieterse, M.C., Szenci, O., Willemse, A.H., Bajcsy, C.S.A., Dieleman, S.J. and Taverne, M. (1990). Early pregnancy diagnosis in cattle by means of linear-array real-time ultrasound scanning of the uterus and a qualitative and quantitative milk progesterone test. *Theriogenology* **33** (3), 697-707.

Pincus, G. and Enzmann, E.V. (1935). The comparative behaviour of mammalian eggs *in vivo* and *in vitro*: I. The activation of ovarian eggs. *Journal of Experimental Medicine* **62**, 655-676.

Pincus, G. and Werthessen, N.T. (1938). The comparative behaviour of mammalian eggs *in vivo* and *in vitro*: III. Factors controlling the growth of the rabbit blastocyst. *Journal of Experimental Zoology* **78**, 1-18.

Prichard, J.F., Pool, S.H., Blakewood, E.G. and Godke, R.A. (1990). Culture of early-state caprine embryos using goat oviductal and uterine cell monolayers *Theriogenology* **33** (1)*, 300 (Abstract)

Rexroad, C.E. (1989). Co-culture of domestic animal embryos. *Theriogenology* **31**, 105-114.

Rodriguez, H.F., Luvoni, G.C., Denniston, R.D. and Godke R.A. (1991). The co-culture of equine demi-embryos using bovine granulosa cells. *Assisted Reproductive Technology and Andrology* **2**, 142-144.

Schiffner, J. and Spielmann, H. (1976). Fluorometric assay of the protein content of mouse and rat embryos during pre-implantation development. *J. Reprod. Fertil.* **47**, 145–147.

Shultz, G.A. and Heiner, S. (1992). Gene expression in pre-implantation mammalian embryos. Mutation research/*Reviews in Genetic Toxicology* **296**, 17-31.

Seidel, G.E., Allen, C.H.. Johnson, L.A.. Holland, M.D.. Brink, Z.. Welch, G.R.. Graham J.K. and Cattell, M.B. (1997). Uterine horn insemination of heifers with very low numbers of non-frozen and sexed spermatozoa. *Theriogenology* **48,** 1255–1264.

Seidel, G.E.. Jr., Schenk, J.L.. Herickhoff, L.A../ Doyle, S.P.. Brink, Z.. Green R.D. and Cran, D.G. (1999). Insemination of heifers with sexed sperm. *Theriogenology* **52**, 1407–1420.

Seidel G.E. and Garner, D.L. (2002). Current status of sexing mammalian spermatozoa. *Reproduction* **124,** 733–743.

Steptoe, P.C.. Edwards R.G. and Purdy, J.M. (1971). Human blastocysts grown in culture. *Nature* **229,** 132–133.

Stringfellow, D.A. (1998). Recommendations for the sanitary handling of *in vivo*-derived embryos. *Manual of the International Transfer Society 1998*, 3rd edition.

Tervit, H.R.. Whittingham D.G. and Rowson, L.E.A. (1972). Successful culture *in vitro* of sheep and cattle ova. *J. Reprod. Fertil.* **30,** 493–497.

Thibodeaux, J.K. and Godke, R.A. (1992). *In vitro* enhancement of early-stage embryos with co-culture. *Arch Pathol Lab Med* **116**, 364-372.

Thompson, J.G. and Duganzich, D. (1996). Analysis of culture systems for bovine *in vitro* embryo production reported in abstracts of the proceedings of the International Embryo Transfer Society from 1991 to 1995. *Theriogenology* **45**, 195 (Abstract).

Umaoka, Y., Noda, Y., Narimoto K. and Mori, T. (1992). Effects of oxygen toxicity on early development of mouse embryos. *Mol. Reprod. Dev.* **31,** 28–33.

Vermeiden J.P.W. and Bast, A. (1995). Antioxidants in IVF culture media. *Hum. Reprod.* **10,** 697–698.

Voelkel, S.A., Amborski, G.F., Hill, G.K. and Godke, R.A. (1985). Use of uterine-cell monolayer culture system for micro-manipulated bovine embryos. *Theriogenology* **24**, 271-281.

Wall, R.J., Hawk H.W., and Nel, N. (1992). Making transgenic livestock: genetic engineering on a large scale. *J. Cell. Biochem.* **49,** 113–120

Whitten, M. (1957). Culture of tubal ova. *Nature* **179**, 1081-1082

Wiemer, K.E/, Denniston, R.S., Amborski, G.F., White, K.L. and Godke, R.A. (1987). A fetal fibroblast monolayer system of *in vitro* culture of bovine embryos. *Journal of Animal Science* **65** (supplement 1), 122 (Abstract).

Wilmut, I., Schnieke, A.E., McWhir, J., Kind A.J. and Campbell, K.H.S. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–813.

Wright, R.W., Grammer, J., Bondioli, K., Kuzan F.and Menino, A.J. (1981). Protein content of porcine embryos during the first nine days of development. *Theriogenology* **15,** 235–239.

Wright, R.W., Grammer, J., Bondioli, K., Kuzan F. and Menino, A. (1983). Protein content and volume of early porcine blastocysts. *Anim. Reprod. Sci.* **5,** 207–212.

Young, L.E., Fernandes, K., McEvoy, T.G., Butterwith, S.C., Gutierrez, C.G., Carolan, C., Broadbent, P.J., Robinson, J.J., Wilmut I. and Sinclair, K.D. (2001). Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat. Genet.* **27,** 153–154.

Zhang L., Denniston, R.D. and Godke, R.A. (1991). The effect of insulin on the development of IVF porcine oocytes. *Journal of Animal Science* **69** (supplement 1), 49 (Abstract).

Zhang, L., Barry, D.M., Denniston, R.D., Bunch, T.D. and Godke, R.A. (1992). Successful transfer of frozen-thawed IVF-derived bovine embryos. *Theriogenology* **37** (1), 331 (Abstract).

CHAPTER 2

SYNCHRONIZATION OF FOLLICULAR WAVES
IN SHEEP, GOATS AND CATTLE
FOR OPTIMAL EMBRYO PRODUCTION

2.1 Abstract

A method to control the development of the dominant follicle in the cohort of follicles, as well as the waves of follicular development in the ovaries of sheep, goats and cows with an estrogenic product was investigated. Estradiol cypionate (ECP) was used for this purpose, injected intramuscularly after the insertion of the norgestomet implant. The animals of the different species were randomly divided into two groups each, the ECP-group receiving the estradiol cypionate injection, and the control group receiving a saline injection. Five days after the ECP injection a program of follicular multi-stimulation with FSH hormone was initiated. The different female species were bred by either natural service (goats) or inseminated by laparoscopy (sheep) or trans-cervically (cows) to fertilize the ovulated ova. Embryos and unfertilized ova were collected surgically at the 8 to 16-cell stage three to four days after breeding in the sheep and goats, and trans-cervically in the cows. Significantly more CL formed, and a significantly greater number of ova and embryos, as well as transferable embryos, were collected from the ECP-group of sheep ewes and goat does compared to the control group that received no ECP ($p<0.01$). There was, however, no difference in the average number of unfertilized ova that were collected in the two sheep or goat groups. In the cows the number of CL counted, the total number of embryos and ova and of transferable embryos collected, were significantly greater ($p<0.05$) in the group that were injected with ECP compared to the group that received no ECP. The control group also had a significantly larger number of unfertilized ova than the ECP-group ($p<0.05$).

2.2 Introduction

For experimental procedures and culture studies on animal embryos, large numbers of embryos are required (Coe *et al.* 1987). A heifer calf is born with approximately 150,000 primordial follicles in the cortices of both ovaries. This diminishes rapidly by degeneration during the animal's life until only approximately 3,000 remains at the age of 15 to 20 years (Erickson 1966). According to Driancourt (2000), there are between 100 and 1,000 follicles at the pre-antral stage of development and between 50 and 300 follicles at the antral stage of development regardless of the stage of the estrus cycle in livestock. The antral follicles become mobilized when they measure 0.8 to 2.0 mm and 1.0 to 3.0 mm in diameter in sheep and cows respectively, and gonadotropin dependent when they are 4.0, 2.0, 1.0, and 10 mm in diameter in cows, sheep ewes, sows and mares, respectively. These gonadotropin size follicles will regress when follicle stimulating hormone (FSH) and luteinizing hormone (LH) is withdrawn from the circulation by hypophysectomy, or if an antagonist to their releasing hormone, GnRH, is injected (Driancourt 2000).

Follicular development already starts in the fetus before birth. Recruitment of the dormant primordial follicles at the prophase 1 stage of development is by an unknown factor. The size of the pool of primordial follicles seem to influence the rate at which primordial resting follicles are recruited (Krohn 1967; Karup *et al.* 1969), although there seem to be other factors, including those secreted by the mural granulosa cells into the follicular fluid that will reduce the number of follicles in a cohort. Once a follicle has been recruited, its development is continuous and sequential until it undergoes either ovulation or apoptosis (Peters 1994). If collection of the embryos are done after a natural estrous cycle in farm animals, without multi-stimulation of the cohort of follicles on the two ovaries, a maximum of two to three embryos in goats or one embryo

in cows can be collected every 21 days (Merton *et al.* 2003), while only on to two embryos can be collected in sheep ewes every 17 days (Hafez 1993). According to Webb & Armstrong (1998) ≥ 91% of cows will only ovulate one ovum every estrus cycle. Due to large numbers of ruminant embryos required for experimental purposes, a more efficient but cost effective method had to be found for the production of larger numbers of embryos for this and other research projects.

In ruminants, a relatively large number of follicles develop during each cycle in a follicular wave fashion (Adams *et al.* 1992; Fortune 1994, Ginther *et al.* 1996; Driancourt 2000; Fortune *et al.* 2004). These are the follicles that can be stimulated to develop and ovulate during a follicular multi-stimulation and subsequent ovulation program (super-ovulation). Each wave of growing follicles is characterized by the emergence, from a pool or cohort of growing follicles, of a larger ovulatory dominant follicle. All follicles in a cohort are potentially capable of ovulating, since selective removal of all the follicles in the cohort except one will not postpone ovulation in the sheep (Tsonis *et al.* 1984) or cattle (Gibbons *et al.* 1997). The follicle selected to become dominant appears to be the one that first develops LH receptors on its granulosa cells. Follicles develop LH receptors when they reach 4.0 and 6.0 mm in diameter in sheep and cattle respectively. There seem to be a correlation between the size of the follicles when they start to develop LH receptors, and the size when they are selected for dominance (Driancourt 2000). It should be noted that not all follicles in a cohort are similar in granulosa cell proliferation, and that there is even a variation between follicles of the same size to FSH and LH stimulation (McNatty *et al.* 1990). There are also large differences among female livestock animals in follicle populations and gonadotropin concentrations. This inevitably leads to a large variation in the number of ovulations during a super-ovulation program (Driancourt & Fry 1992).

The follicular waves are preceded by recurrent increases in follicle stimulating hormone (FSH) secretion from the adenohypophysis (Adams *et al.* 1992, Sunderland *et al.* 1994) which provides the initial stimulation for the pool of growing follicles (Turzillo & Fortune 1990). The dominant follicle of the cohort of developing follicles, however, produces the hormones inhibin and estrogen that prevents/inhibits the further development of the other follicles in the wave, by suppressing the release of FSH by the adenohypophysis (Driancourt 2000, Ginther *et al.* 2001). The effect of dominance by the largest follicle on the subordinate follicles on both ovaries can be illustrated by the fact that the next wave of follicular growth cannot be detected until after the start of the regression of the previous dominant follicle. No recruitment of growing follicles takes place during the period of dominance (Driancourt *et al.* 1993). Furthermore, the time of selection of the dominant follicle corresponds with a marked reduction on both the number and growth of the subordinate follicles on both ovaries (Webb & Armstrong 1995). The inhibitory effect of the dominant follicle on the release of FSH can be, to a large extent, be overridden by parenteral administration of FSH by injection. Although the exact mechanism involved in dominant follicle suppression of the subordinate follicles are not fully understood, the effect appears to be systemic, as the dominant follicle on the one ovary will also suppress the subordinate follicles on the other ovary (Webb & Armstrong 1998). The possible negative paracrine effect of the dominant follicle on the development of the other follicles in the same cohort can only be eliminated by permanent removal of the dominant follicle.

Although it is more than 40 years since the birth of the first calf by embryo transfer, the efficacy of embryo transfer has been limited by the unpredictable and unreliable supply of good quality ova due to the effect of the inhibitory substances produced by the dominant follicle. One of the major problems in a super-ovulation program is the large variation in the number and quality of

embryos produced in such a program (Boland *et al.* 1991; Armstrong *et al.* 1993). This could be due to the fact that, although fertilization of the ovulated ova has taken place, a significant percentage of early-stage embryos are lost due to reduced quality of the oocyte prior to ovulation and fertilization (McEvoy *et al.* 1995**;** 1997, Bishonga *et al.* 1995). To achieve an optimal embryo collection rate for research or transfer purposes, synchronized follicular growth is needed which allows viable oocytes to be fertilized (Webb & Armstrong 1998).

Dominant follicle removal can be achieved by different means (Bo *et al.* 1995; Baracaldo 2000; Bo *et al.* 2003), including by physical means (electrocauterization or ultrasound-guided follicle ablation) or hormonally with different hormones like GnRH, estradiol and progesterone (Bo *et al.* 1994; 1995, Colazo 2003).

The study deals with the control of follicular waves including the dominant follicle with an estrogenic compound to optimize embryo production. Estradiol cypionate (ECP, Upjohn) was used for this purpose.

2.3 Hypothesis

Estradiol cypionate administration will suppress follicular development, including the growth of the dominant follicle by suppressing FSH secretion from the pituitary. Administration of estradiol 17β, like estradiol cypionate, before the multi-ovulation treatment is started, will therefore increase the number and quality of embryos harvested one week after standing estrus and fertilization in ruminants.

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2.4 Material and Methods

2.4.1 Sheep

2.4.1.1 Experimental design

The following program was used to control and stimulate follicular development in the sheep ewes for embryo production.

DAYS

0	4		9 10 11 12	13	14		17	

Fluorogestone sponge in

ECP or saline injection

FSH treatment

Laparoscopic AI

Implant removal
+ Cloprostenol inj.

Surgical embryo collection
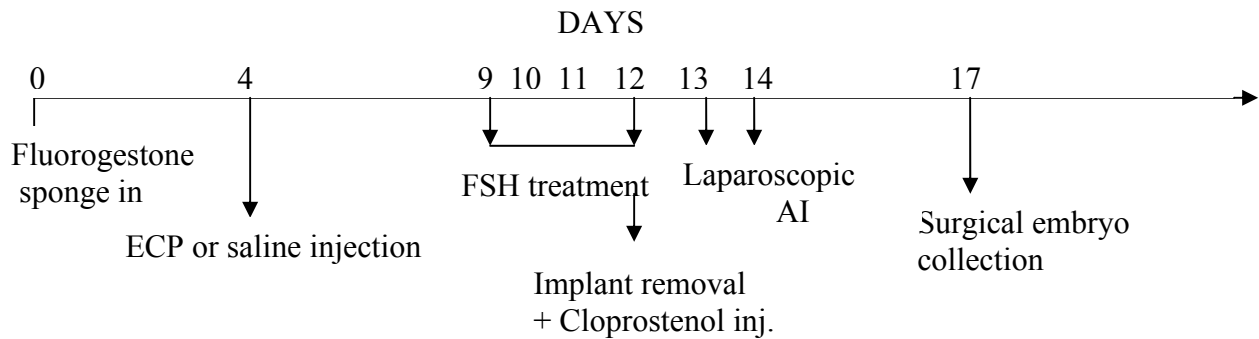
2.4.1.2 Method

A total of 94 Dorper mutton breed sheep at body condition score of between 3/5 to 4/5 were randomly divided into two groups (Treatment A, Group I, n = 54; Treatment B, Group II, n = 40). The sheep were kept on kukuju (*Pennisetum clandestinum*) pastures during the day and additionally fed a ration of 1 kg of a 12% protein pellets at night. Water was available *ad libitum*. The embryo collections were done between March and June in the southern hemisphere at latitude of 33.57 degrees and a longitude of 18.50 degrees. Females in Group I and Group II were multi-ovulated with FSH (Ovagen, ICP, New Zealand). Females in Group I received an ECP injection before the onset of the super-stimulation program, while those in Group II were treated with physiological saline (Sabax, South Africa) using the following program:

Day 0   Insertion of an intra-vaginal fluogestone acetate sponge

Day 4   Estradiol cypionate (Group I) or saline injection (Group II – control)

D 9-12   Multi-ovulation treatment with FSH administered intramuscularly twice daily

Day 12   Sponge removal and 125 µg cloprostenol intramuscular

Day 14   Laparoscopic AI at 36 and 52 h after sponge removal

Day 17   Surgical embryo collection 5 d after sponge removal

2.4.1.3 Progesterone implant

A cylindrical sponge impregnated with 40 mg fluogestone acetate (Chronogest, Intervet The Netherlands) was inserted with a special plastic applicator (Intervet) into the anterior vagina of the donor ewes. The applicator was disinfected between ewes with an antiseptic solution (10% Hibitane, Astra Zeneca, South Africa) to prevent the development of vaginitis during the duration of the sponge inside the ewe.

2.4.1.4 Estradiol cypionate injection

The 54 donor ewes (Group I) were each injected intramuscularly with 1 mg of estradiol cypionate (ECP) into either the splenius, longissimus capitus, semispinalis capitus or obliquus capitus caudalis muscles of the neck to suppress the present wave of follicular development, including the dominant follicle (Bo *et al.* 1994) and allow a new wave to emerge. The 40 control ewes (Group II) were given 1 mL of isotonic saline (Sabax, Johannesburg, South Africa) as a placebo by intramuscular injection into the same area of the neck.

2.4.1.5 Follicular multi-stimulation

The 16.7 mg of FSH was dissolved in 20 mL of isotonic saline. Each donor ewe was then injected 0.0835 mg (1 mL) of FSH intramuscular twice daily for 4 d (total FSH dose = 8 mL). With the last FSH injection, the intra-vaginal sponges were removed and the each ewe injected intramuscular, with 125 µg (0.5 mL) of cloprostenol (Estrumate, Schering-Plough, Isando, South Africa).

2.4.1.6 Fertilization

At 36 and 52 h after withdrawal of the intra-vaginal implants, the donor ewes were laparoscopically inseminated with fresh electro-ejaculated ram semen. The same ram of proven fertility and good semen quality (>2 x 10^9 sperm per mL) was used for all of the laparoscopic inseminations. No semen extender was used. The ewes were placed in dorsal recumbency and tilted 45º cranially during insemination. The ventral abdominal wall was clipped, washed and disinfected. The first cannula and trocar was passed through the abdominal wall 2 cm lateral to the linea alba and ~4 cm below the udder attachment. The trocar was withdrawn and the laparoscope passed through the cannula. If the end of the laparoscope was inside the abdominal cavity between the omentum and the ventral abdominal wall, the abdominal cavity was inflated with air through the cannula. A second trocar and cannula was then passed through the abdominal wall perpendicular to the first cannula insertion but on the contra-lateral side of the midline. By means of a sheath and aspic (IMV, L'Aigle, France), 0.05 mL of undiluted semen (~1 x 10^8 sperm) was injected into the lumen of each uterine horn about 7 cm distal to the uterine horn bifurcation through the second cannula. Sterile procedures were maintained at all times.

2.4.1.7 Embryo collection

The donor ewes were administered intravenous thiopentone sodium (Intraval Sodium, Rhone-Poulenc, Merial, Johannesburg, South Africa) dissolved in sterile water (0.8 to 1 g per ewe). The females were then intubated with an endotracheal tube (Portex, Berch Sur Mer, France) with the aid of a curved stainless steel rod, and the cuff inflated with air. Anesthesia was maintained with 2 to 4% halothane gas (Fluothane Astro Zeneca, Johannesburg, South Africa) and oxygen by connecting the endotracheal tube to the anesthetic apparatus. The animals were put in dorsal recumbency at a 30º incline with the head down in a special cradle and the abdominal area in front of the udder clipped, shaved and disinfected with a mixture of alcohol, water and Hibitane (Astro Zeneca) (7:2:1). The uterus and ovaries were exteriorized through a mid-ventral incision starting 2 cm anterior to the mammary glands and extending cranially ~8 cm. The corpora lutea (CL) were counted and recorded. Dulbecco's phosphate buffered saline (D-PBS) (Sigma, USA, Cat P-3813) with 1% fetal bovine serum (FBS) (Highveld Laboratories, Johannesburg, South Africa) and 1% antibiotic-antimycotic solution (Sigma, USA, Cat A-5995) was injected with a sterile plastic 20 mL syringe (Normo-Ject Zentrisch, Henke Sass Wolf, Germany) with a nylon plunger through a 20-gauge intravenous catheter (Abbocath-T, Venisystems, Sligo, Ireland) inserted at the uterine-tubal junction, and the tip of the uterine horn thoroughly massaged. The D-PBS flushing medium was collected through a curved, tapered glass catheter (outside diameter 7 mm, inside diameter 4 mm) placed through the uterine wall at the uterine horn bifurcation (held in position by a modified bowel clamp), into a sterile 250 mL glass beaker . A total of 60 mL of D-PBS medium was used to flush each uterine horn.

2.4.1.8 Embryo handling

The 120 mL of collected D-PBS medium was concentrated to about 30 mL by pouring the medium through an Em-Con filter (Agtech, Kansas, USA) with membrane pore size of 75 μ. The collected D-PBS medium that flowed through the filter membrane was discarded. The final 30 mL remaining in the Em-Con filter was then decanted into a 100 mm grid-lined petri dish. The membrane was thoroughly rinsed with D-PBS and 1% antibiotic-antimycotic, but without FBS, into the same petri dish. The embryos were identified under a stereo microscope at 15X magnification, and washed by transferring them individually through a 4-well dish (Nunc, Roskilde, Denmark). Each well contained at least 1 mL of D-PBS with 10% of FBS and antibiotic-antimycotic (holding medium). Not more that 10 μl of medium was transferred with each individual embryo to the next well, with a 1 in 100 dilution with every transfer. In this way the holding medium was diluted more than 1 million times after four transfers, increasing the potential of the antibiotic and antimycotic to reduce micro-organisms. The embryos were then transferred to a well containing sterile-filtered holding medium.

2.4.1.9 Embryo grading

Two independent technicians graded the washed embryos under an inverted microscope at between 80X and 200X. The following criteria were used:

Symmetry: the zona pellucida had to be spherical, and not oval or flattened, and be intact without any cracks or rents.

Embryo contents: the embryo inside the zona pellucida must, at the pre-compacted morula stage, not fill the entire space within the zona pellucida.

Blastomere damage: individual blastomeres must be transparent without any sign of vacuolization, granularity or pigmentation.

Extruded cells: this is an indication that some of the blastomeres died during the developmental period. The percentage of extruded cells influenced the grading of the embryo.

Embryo grades:

Grade 1: Less than 5% of the total number of blastomeres were damaged or extruded. The zona pellucida must be perfectly spherical, intact and clean.

Grade 2: Between 5 and 25% of the blastomeres could be damaged or extruded. The zona pellucida was sometimes not perfectly spherical.

Grade 3: More than 25% of the blastomeres of the embryo were damaged or extruded. The zona pellucida was often not spherical.

Grade 4: The embryos were totally degenerated, or were unfertilized ova.

2.4.2 Goats

2.4.2.1 Experimental design

The following program was used to control the previous wave of follicles in the goat does and to stimulate the next wave for embryo production.

DAYS

| 0 | 10 | 15 16 17 18 | 19 20 | 24 |

Progesterone implant

Replace progesterone implant + ECP or saline

FSH treatment

Breed

Progesterone implant removal

Surgical embryo collection
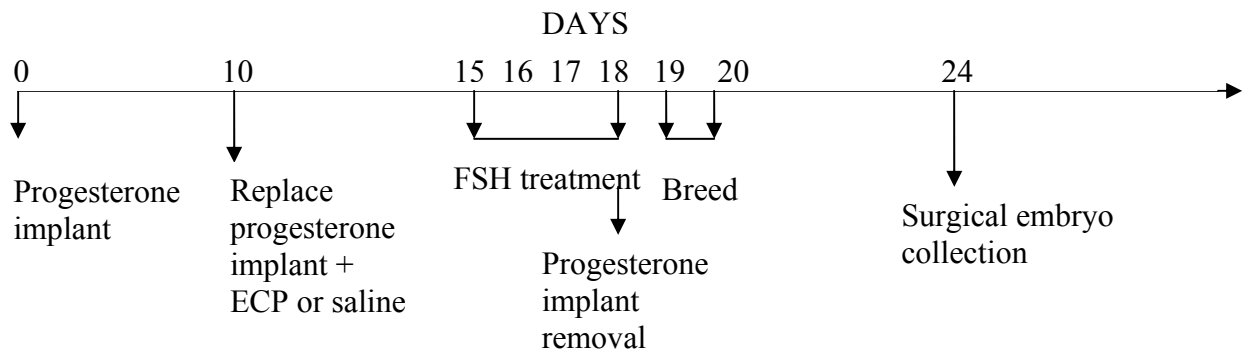
2.4.2.2 Method

A total of 228 Boer goats were randomly divided into two groups   (Treatment A, Group I, n = 114; Treatment B, Group, II = 114). The goats were kept on kukuju pastures during the day, and fed 1.0 kg of a 12% protein pelleted ration and 1 kg of lucern hay each at night. The condition score of the animals was all between 3/5 and 4/5. Water was available *ad libitum*. The Group I ewes were injected intramuscular with ECP 10 d after the insertion of the first CIDR, while the Group II control ewes received an intramuscular injection of an isotonic saline solution. The female animals were super-ovulated with FSH (Ovagen) using the following program between March and June at the same location as the sheep:

Day 0   Insertion of intra-vaginal progesterone implant (CIDR)

Day 10   1 mg ECP intramuscular (Group I) or saline injection (Group II – control)

Day 10   Replace the intra-vaginal progesterone source with a new CIDR

Day 15 – 18   Multi-ovulation treatment with FSH

Day 18   CIDR removal and 125 µg cloprostenol injection

Day 19 – 20   Natural breeding every 12 h, until the ewe no longer accepted the ram

Day 24   Surgical embryo collection was performed 4 d after the onset of standing estrus.

2.4.2.3 Progesterone implant

The Y-shaped progesterone implant (CIDR, InterAg Hamilton, New Zealand) was inserted into the anterior part of the vagina with a special sterile plastic applicator (InterAg). The nylon string attached to the CIDR was cut at the vulva opening, to prevent the donor ewes from pulling at

the implant with their teeth and dislodging it. This implant was removed and replaced with a new CIDR 10 d later, the second CIDR which was removed with the last FSH injection.

2.4.2.4 Estradiol cypionate injection

The 114 donor goat females in Group I were injected with 1 mg (0.5 mL) of estradiol cypionate intramuscularly (ECP) into the muscle at the back of the hind leg (semimembranous and semitendinous muscles) 5 d before the start of the FSH follicular multi-stimulation treatment. The control ewes were injected with 500 μl of sterile saline (Sabax, South Africa).

2.4.2.5 Follicular multi-stimulation

The FSH injections started 5 d after the second progesterone implant was inserted. The 16.7 mg of FSH hormone (Ovagen) was dissolved in 20 mL of sterile saline, and each ewe injected with a total of 10 mL of the solution in the following schedule every 12 h:

Day 15	am	1.67 mg (2 mL) FSH
	pm	1.67 mg FSH
Day 16	am	1.25 mg (1.5 mL) FSH
	pm	1.25 mg FSH
Day 17	am	0.835 mg (1 mL) FSH
	p.m.	0.835 mg FSH
Day 18	am	0.42 mg (0.5 mL) FSH and 125 μg cloprostenol injected i.m.
	pm	0.42 mg (0.5) mL FSH and the implant removed.

2.4.2.6 Fertilization

The ewes were mated to a proven fertile buck from 12 h after the onset of standing estrus and every 12 h after that, until they reached the end of their estrous period and would not allow further mating. Three buck of proven fertility and excellent sperm count ($>2.5 \times 10^9$ sperm per mL) were used to hand-mate all of the ewes during a 2 m period.

2.4.2.7 Embryo collection

The embryos were collected using a similar surgical procedure as described for the sheep donors.

2.4.2.8 Embryo handling and grading

The embryos were handled and graded as described for the sheep donor ewes.

2.4.3 Cows

2.4.3.1 Experimental design

The program below was used to control and stimulate follicular development in the cows for multiple embryo collection.

DAYS

| 0 | 4 | | 9 10 11 12 | 13 | 14 | | 18 |

Norgestomet
ear implant

ECP or
saline

FSH treatment

Cloprostenol
injection
+ implant removal

AI   AI

Trans-cervical
embryo
collection
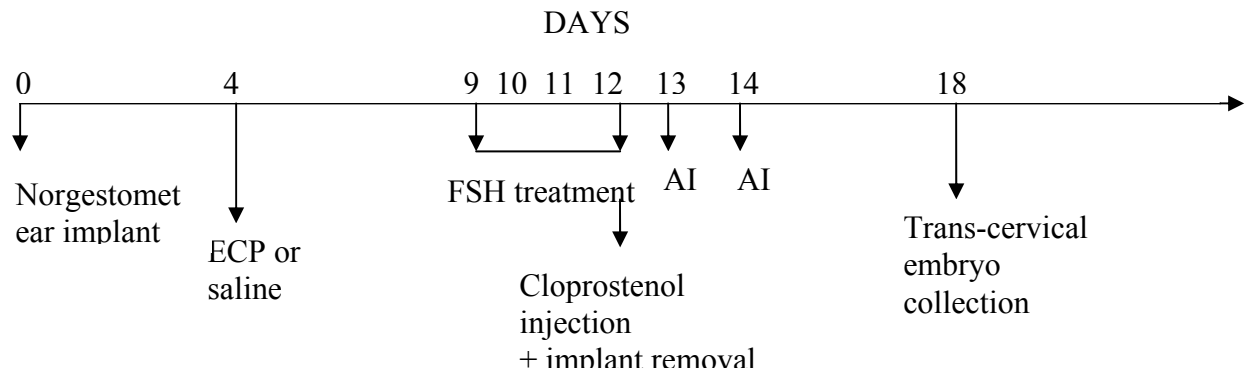
2.4.3.2 Method

A total of 95 mixed breed non-suckling beef cows between three and eight years old were randomly divided into two groups (Treatment A, Group I, n = 43; Treatment B, Group II, n = 52). The cows were kept on free-ranging natural grass pastures and had *ad libitum* access to a salt-protein lick and clean drinking water. The condition score of the cows were between 2.5/5 and 3.5/5. The cows in both groups were multi-stimulated with FSH (Ovagen) using the following program:

Day 0   Insertion of a subcutaneous progesterone implant (Crestar)

Day 4   Estradiol cypionate (ECP) in Group I, saline injection in Group II (control)

Day 9 - 12   Multi-ovulation treatment with FSH

Day 12   Cloprostenol injection (500 µg) with the second to last FSH injection

Day 12   Implant removal with the last FSH injection

Day 14 - 15   Artificial insemination at 12 h and 24 h after the onset of standing estrus

Day 18   Trans-cervical embryo collection 4.5 d after the onset of estrus.

2.4.3.3 Progesterone implant

The donor cow was restrained in a neck clamp and movement of the head restricted by means of a nose holder. The hair on the dorsal surface of the right ear was shaven, and the skin disinfected with a mixture of 10% Hibitane and 90% methanol. The 3 mg norgestomet impregnated silicone cylinder (Crestar, Intervet, The Netherlands) was then placed between the dorsal skin and the cartilage of the ear with a special applicator (Intervet), halfway between the tip and the base of the ear. The accompanying injection of 5 mg estradiol valerate and 3 mg norgestomet was not given.

2.4.3.4 Estradiol cypionate injection

Estradiol cypionate (ECP) was injected intramuscularly (2mg) in the gluteus medius muscle in the case of the Group I donor cows (n = 43) to control the dominant follicle and the cohort of developing follicles. The control cows (Group II, n = 52) received an intramuscular injection of 1 mL of isotonic sterile saline solution.

2.4.3.5 Follicular multi-stimulation

A total of 17.6 ± 2 mg of active follicle stimulating hormone (Ovagen) was dissolved in 20 mL of isotonic saline. The donor cows were then intramuscularly injected into the gluteus medius muscle twice daily using the following program (total FSH dose = 20 mL):

Day 9 am   3.34 mg (4 mL) FSH

Day 9 pm   3.34 mg FSH

Day 10 am   2.5 mg (3 mL) FSH

Day 10 pm   2.5 mg FSH

Day 11 am   1.67 mg (2 mL) FSH

Day 11 pm   1.67 mg FSH

Day 12 am   0.83 mg (1 mL) FSH and 500 μg (2 mL) cloprostenol (Estrumate, Schering-Plough) intramuscularly injected

Day 12 pm   0.83 mg FSH and removal of the progesterone ear implant with a mosquito forceps.

2.4.3.6 Fertilization

After thawing, 50% of the semen of one straw containing a minimum of 15×10^6 live sperm per straw, with a total volume of ~0.25 mL per straw, was deposited into the proximal part of each uterine horn ~4 cm after the bifurcation 12 h after the onset of standing estrus. The caudal part of the cervix and the clitoris was thoroughly massaged on withdrawal of the insemination pistolet. This procedure was repeated after 12 h.

2.4.3.7 Embryo collection

The embryos were collected trans-cervically, as described by Drost *et al.* (1976), under epidural anesthesia. About 500 ml of Dulbecco's phosphate buffered saline (D-PBS) with 1% FBS and 1% antibiotic-antimycotic (as used in the sheep) was instilled into each uterine horn, the horn massaged thoroughly, and the medium with the embryos drained into a 1 L empty, sterile, intravenous infusion glass bottle.

2.4.3.7 Embryo handling and evaluation

The embryos were handled and graded as described for the sheep donor ewes.

2.5 Statistical analysis

Data reported in this manuscript are as mean ± SD. All data were analyzed using the Student's t-test assuming two-sample means with unequal variances (Microsoft Excell).

2.6 Results

2.6.1 Sheep

The results from the ECP-treated and the control donor ewes are presented in Table 2.1. Ewes in the ECP-treated group had 14.3 ± 4.3 CL per female compared with 7.2 ± 3.9 CL per female in the group that did not receive ECP (p<0.01). This significant difference was also demonstrated in the total number of ova/embryos, where the ECP-treated group developed 12.6 ± 4.4 ova/embryos, while 6.0 ± 3.8 ova/embryos were collected in the control group (p<0.01). The number of transferable embryos (Grade 1 and Grade 2 embryos) produced in the ECP-treated group were 10.9 ± 4, compared with 4.4 ± 3.5 transferable embryos in the control group (p<0.01).

In the production of non-fertilized ova there was no significant difference between the two groups of sheep

2.6.2 Goats

The results on the Boer goat donors are represented in Table 2.2. Here the standard deviation was more pronounced. Significantly more CL were formed in the ECP-treated group (p<0.05) than in the control group (11.7 ± 8.5 and 7.5 ± 6.5 for the ECP-treated group and control group, respectively). There were also significantly more ova/embryos produced in the ECP-treated group (13.2 ± 8.8) than in the control group (9.5 ± 7.6) (p<0.01), and significantly more of the embryos were of transferable quality in the ECP-treated group (9.4 ± 9.3) compared to the control group (6.6 ± 6.9) (p<0.05). The number of unfertilized ova did, however, not differ significantly (p=0.45)

2.6.3 Cows

The results on the beef cow donors are represented in Table 2.3. Significantly more CL were formed (p<0.05) in Group II (ECP-treated group) (13.6 ± 3.7) than in the control group (11.9 ± 2.7). In the total number of ova/embryos the ECP-treated group had significantly higher numbers (p<0.05) (11.9 ± 3.5) than the control group (10.2 ± 2.4), and the number of transferable embryos in the ECP-treated group (8.2 ± 2.8) were significantly greater than the controls (4.4 ± 1.5) at a p-value of less than 0.01 The number of unfertilized ova in the cows was, however, significantly greater in the control cows than in the ECP-treated group (3.4 ± 1.9 vs.1.8 ± 1.1, respectively) (p<0.01).

Table 2.1. Average number of CL, ova and embryos, unfertilized ova and transferable embryos for the two groups of sheep treated or not treated with estradiol cypionate

Group	n	Ave.# of CL	Ave. # of ova/embryos	Ave. # of unfertilized ova	Ave. # of transferable embryos
Group I (with ECP)	54	14.3 ± 4.3 [a]	12.6 ± 4.4 [c]	1.3 ± 1.4	10.9 ± 4.0 [e]
Group II (no ECP)	40	7.2 ± 3.9 [b]	6.0 ± 3.8 [d]	1.2 ± 1.2	4.4 ± 3.5 [f]

[ab] $p<0.01$ Values with different superscripts within a column are significantly different
[cd] $p<0.01$
[ef] $p<0.01$.

Table 2.2. Average number of CL, ova and embryos, unfertilized ova and transferable embryos for the two groups of goats treated or not treated with estradiol cypionate

Group	n	Ave.# of CL	Ave. # of ova/embryos	Ave. # of unfertilized ova	Ave. # of transferable embryos
Group 1 (with ECP)	114	11.7 ± 8.5 [a]	13.2 ± 8.8 [c]	3.2 ± 5.9	9.4 ± 9.3 [e]
Group 2 (no ECP)	114	7.5 ± 6.5 [b]	9.5 ± 7.6 [d]	2.6 ± 3.8	6.5 ± 6.9 [f]

[ab] $p<0.05$ Values with different superscripts within a column are significantly different
[cd] $p<0.01$
[ef] $p<0.05$.

Table 2.3. Average number of CL, ova and embryos, unfertilized ova and transferable embryos for the two groups of cows treated or not treated with estradiol cypionate

Group	n	Ave. # of CL	Ave. # of ova/embryos	Ave. # of unfertilized ova	Ave. # of transferable embryos
Group 1 (with ECP)	43	13.6 ± 3.7 [a]	11.9 ± 3.5 [c]	1.8 ± 1.1 [e]	8.2 ± 2.8 [g]
Group 2 (no ECP)	52	11.9 ± 2.7 [b]	10.2 ± 2.4 [d]	3.4 ± 1.9 [f]	4.4 ± 1.5 [h]

[ab] $p=0.05$ Values with different superscripts within a column are significantly different
[cd] $p<0.05$
[ef] $p<0.01$
[gh] $p<0.01$

In Figure 2.1 and 2.2 are bovine pre-compacted morula stage grade 1 embryo collected at 3.0 to 3.5

d post ovulation. Figure 2.3 represent a retarded degenerating bovine embryo.

Figure 2.1 Bovine pre-compacted morula stage grade 1 embryo (16-cell stage).
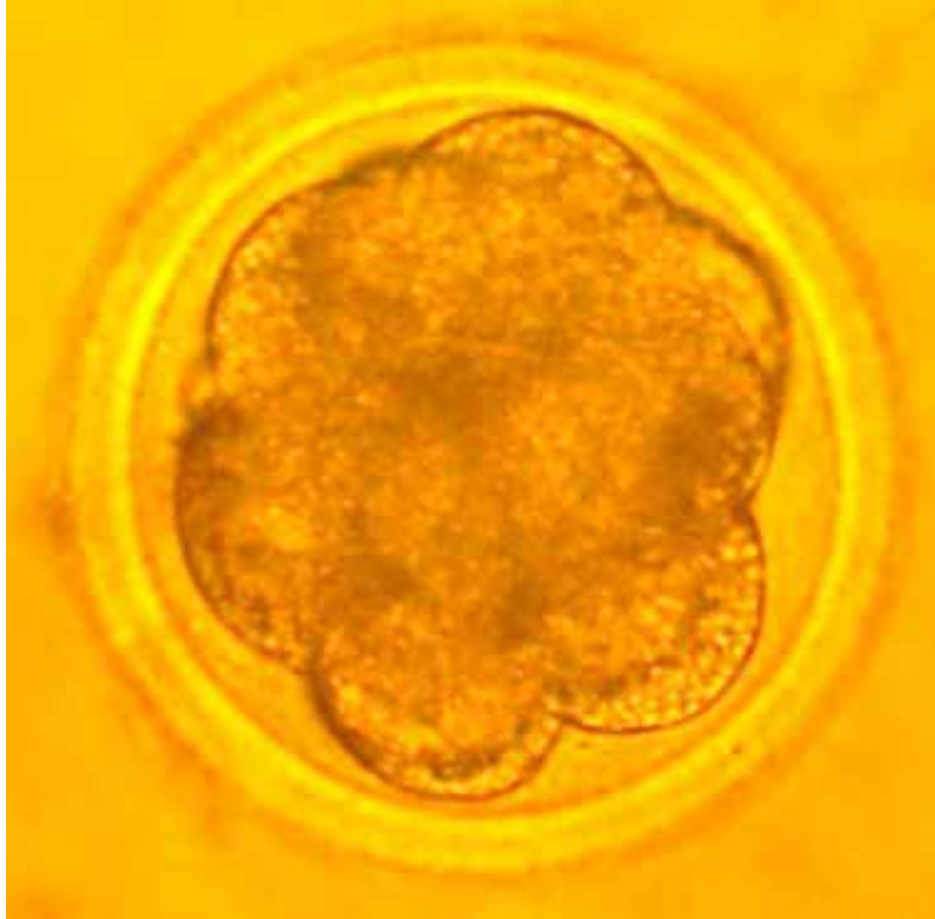
Figure 2.2 Pre-compacted morula stage bovine embryo.

Figure 2.3 Retarded 8-cell stage bovine embryo.

2.7 Discussion and conclusion

The addition of estradiol cypionate to the follicular multi-stimulation program had a beneficial effect on the production of gametes in all three species. The administration of estradiol cypionate seemed to have blocked the present wave of follicular development, including the development of the dominant follicle (Bo *et al.* 1994; Diershke *et al.* 1994). When the FSH treatment was therefore started, there was the emergence of a new wave of follicular development with all the follicles probably at the same size and same stage of development. This prevented the suppressing effect of the dominant follicle on the development of the recessive follicles. When the follicles were therefore stimulated by the administered FSH hormone injected twice daily, they all developed at approximately the same rate, while none of them were at a stage of atresia or undergoing apoptosis. This is especially shown in the number of unfertilized ova in the group of cows not treated with estradiol cypionate (3.2 ± 2) compared to the treatment group (1.8 ± 1.1) (<0.01). More follicles were probably at a stage of atresia when the FSH treatment was started in the control group of cows, and there ova could therefore not be salvaged, as compared to the treatment where the follicles were mostly in a developmental stage and the ova therefore fertilizable. It was further noted that the pre-treatment with ECP gave larger total number of embryos collected, as well as greater numbers of transferable embryos. This was probably due to the emergence of a new wave of follicles that followed on the estradiol cypionate injection, and that was stimulated by the FSH injections. Due to the absence of the effect of the dominant follicle in the ECP-group of animals, a greater number of ovulated ova were fertilized. The greater number of ovulations and subsequent CL is in accordance with findings by Bo *et al.* (1995) who concluded that after estradiol 17β treatment, there was a significant increase in the fertilization rate in the treatment group to that of the control group of cows ($p<0.05$). He also observed that when a

progestagen implant was used without an estradiol 17β injection, the embryos were of a poorer quality. This he attributed to the recessive follicles under the influence of the dominant follicle that were undergoing degeneration at the time of the follicle multi-stimulation treatment.

It can therefore be concluded that an ECP injection during a follicular multi-stimulation program will suppress the present wave of follicular development to such an extent that a new wave will develop that can be simulated with regular FSH injections. The number and quality of embryos produced and collected in a follicular multi-stimulation program can therefore be increased.

2.8 References

Adams, G.P., Matteri, R.L., Kastelic, J.P., Ko, J.C.H. & Ginther, O.J. (1992). Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. *Journal of Reproduction and Fertility* **94**, 177-188.

Armstrong, D.G., Baxter, G., Gutierrez, C.G., Wood, K.J., Sinclair, K.D., Robinson, J.J., McEvoy, T.G. & Webb, R. (1993). Ovarian IGF-II MRNA expression in cattle fed different energy and protein diets. *Journal of Reproduction and Fertility Abstract series* **19**, Abstract 31.

Baracaldo, M.I., Martinez, M., Adams, G.P. & Mapletoft, R.J. (2000). Super-ovulatory response following trans-vaginal follicle ablation in cattle. *Theriogenology* **53**, 1239-1250.

Bishonga, C., Robertson, J.J., McEvoy, T.G., Robinson, J.J., Aitken, R.P., Finlay, P.A. & Robertson, I.S. (1995). The effects of excess rumen degradable protein in ewes on ovulation rate, fertilization and embryo survival *in vivo* and during *in vitro* culture. *Animal Production* **58**, Abstract 81.

Bo, G.A., Adams, G.P., Pierson, R.A., Tribulo, H.E. & Mapletoft, R.J. (1994). Follicular wave dynamics after estradiol-17 β treatment of heifers with or without a progesterone implant. *Theriogenology* **41**, 1555-1569.

Bo, G.A., Adams, G.P., Pierson, R.A. & Mapletoft, R.J. (1995). Exogenous control of follicular wave emergence in cattle. *Theriogenology* **43**(1), 31-40.

Bo, G.A., Baruselli, P.S. & Martinez, M.F. (2003). Pattern and manipulation of follicular development in *Bos indicus* cattle. *Animal Reproduction Science* **78**, 307-326.

Boland, M.P., Goulding, D. & Roche, J.F. (1991). Alternative gonadotropins for super-ovulation in cattle. *Theriogenology* **35**, 5-17.

Coe, P.H., Gibson, C.D., Kaneene, J.B., Morrow, D.A. & Marinez, R.O. (1987) The use of embryo collection techniques in Holstein heifers: A model to study early embryonic death. *Theriogenology* **27** (5), 729-736.

Colazo, M.G., Kastelic, J.P. & Mapletoft, R.J. (2003). Effects of estradiol cypionate (ECP) on ovarian follicular dynamics, synchrony of ovulation, and fertility in CIDR-based, fixed-time AI programs in beef heifers. *Theriogenology* **60** (5), 855-865.

Diershke, D.J., Chaffin, C.L. & Hutz, R.J. (1994) Role and site of estrogen action in follicular atresia. *Trends in Endocriological Metabolism* **5**, 215-219.

Driancourt, M.A. (2000).   Regulation of ovarian follicular dynamics in farm animals : Implications for manipulation of reproduction. *Theriogenology* **55** (6), 1211-1239.

Driancourt, M.A. & Fry, R.C. (1992). Effect of super-ovulation with p-FSH or PMSG on growth and maturation of the ovulatory follicles in sheep. *Animal Reproduction Science* **27**, 279-292.

Driancourt, M.A., Gougeon, A., Royere, D. & Thibault, C. (1993). Ovarian function. In "Reproduction in Mammals and Man". C Thibault, M-C Levasseur & RHF Hunter as Editors, Paris, Ellipses. 281-305.

Drost, M., Brand, A. & Aarts, M.H. (1976). A device for non-surgical recovery of bovine embryos. *Theriogenology* **6**, 503-507.

Erickson, B.H. (1966). Development and the radio-response of the prenatal bovine ovary. *Journal of Reproduction and Fertility* **11**, 97-105.

Fortune, J.E., Rivera, G.M. & Yang, M.Y. (2004). Follicular development: the role of follicular microenvironment in selection of the dominant follicle. *Animal Reproduction Science* **82-83**, 109-126.

Fortune, J.E. (1994). Ovarian follicular growth and development in mammals. *Biology of Reproduction* **50**, 225-232.

Gibbons, J.R., Witbank, M.C. & Ginther, O.J. (1997). Functional relationships between follicles greater than 4 mm and the FSH surge in heifers. *Biology of reproduction* **57**, 1066-1073.

Ginther, O.J., Kot, K., Kulick, L.J., Martin, S. & Witbank, M.C. (1996). Relationship between FSH and ovarian follicular waves during the last six months of pregnancy in cattle. *Journal of Reproduction and Fertility* **108**, 271-279.

Ginther, O.J., Beg, M.A., Bergfelt, D.R., Donadeu, F.X. & Kot, K. (2001). Follicle selection in monovular species. *Biology of Reproduction* **65**, 638-647.

Hafez ESE. (1993) Reproduction in Farm Animals. Lea & Febiger, 6th Edition, pp461-502.

Karup, T., Pederson, T. & Faber, M. (1969). Regulation of oocyte growth in the mouse ovary. *Nature* **224**, 187-188.

Krohn, P.L. (1967). Factors influencing the number of oocytes in the ovary. *Archives of Anatomy and Microscopic Morphological Experiments* **56**, 151-159.

McEvoy, T.G., Robinson, J.J., Aitken, R.P., Finlay, P.A. & Robertson, I.S. (1995). Relationship between pre-ovulatory feed intake, progesterone priming and the subsequent *in vitro* development of ova collected from super-ovulated ewes. *Theriogenology* **43**, 276.

McEvoy, T.G., Robinson, J.J., Aitken, R.P., Finlay, P.A. & Robertson, I.S. (1997). Dietary excess of urea influences the viability and metabolism of pre-implantation sheep embryos and may affect fetal growth among survivors. *Animal Reproduction Science* **47**, 71-90.

McNatty, K.P., Heath, D.A., Hudson, N. & Clarke, I.J. (1990). Effect of long-term hypophysectomy on ovarian follicle population and gonadotropin-induced adenosine cyclic

3,5-monphosphate output by follicles from Booroola ewes with or without the F gene. *Journal of reproduction and Fertility* **90**, 515-522.

Merton, J.S., de Roos, A.P.W., Mullaart, E., de Ruigh, L., Kaal, L., Vos, P.L.A.M. & Dieleman, S.J. (2003). Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology* **59** (2), 651-674.

Sunderland, S.J., Crowe, M.A., Boland, MP., Roche, J.F. & Ireland, J.J. (1994). Selection, dominance and atresia of follicles during the estrous cycle of heifers. *Journal of Reproduction and Fertility* **101**, 547-555.

Tsonis, C.G., Cahill, L.P., Carson, R.S. & Findlay, J.K. (1984). Identification at the onset of luteolysis of follicles capable of ovulation in the ewe. *Journal of Reproduction and Fertility* **70**, 609-614.

Turzillo, A. & Fortune, J. (1990). Suppression of the secondary FSH surge with bovine follicular fluid is associated with delayed ovarian follicular development in heifers. *Journal of reproduction and Fertility* **89**, 643-653.

Webb, R. & Armstrong, D.G. (1998) Control of ovarian function; effect of local interactions and environmental influences on follicular turnover in cattle: a review. *Livestock Production Science* **53**, 95-112.

CHAPTER 3

CULTURING OF BOVINE PRE-COMPACTED MORLAE IN TCM-199 AND BAF IN A STANDARD 5% CO_2 LABORATORY INCUBATOR AND IN THE VAGINA OF A GOAT DOE

3.1 Abstract

Since more than half a century ago, attempts have been made to culture cells and embryos outside the body (*in vitro* or *ex vivo*). This was done with different culture media and in various "incubators". In the present study two different culture media were used: a standard TCM-199 culture medium and first trimester amniotic fluid (BAF) collected sterilely from pregnant cows after slaughter. Two different culture conditions were also investigated, the standard laboratory CO_2 incubator versus culturing bovine embryos in the vagina of a goat doe. Two experiments were done: Firstly the permeability of different receptacles to CO_2 gas was analyzed for possible culture in the vagina. Four-well plates and straws were used to incubate TCM-199 and BAF for a period of 120 h in the presence or absence of 5% CO_2 gas. The pH values were measured and recorded every 24 h. In the second experiment pre-compacted morula stage bovine embryos were cultured in the above culture media in sealed 0.25 mL straws in a standard laboratory incubator and in the vagina of a goat doe. Evaluation was done on (1) stage of development and (2) number of blastomeres after 96 h of culture. In the first experiment it was shown that the CO_2 gas diffused out of the 4-well plate as well as through the wall of the straws in the absence of CO_2 gas, while in the presence of CO_2 the pH of both media stabilized between 7.3 and 7.5. This meant that the semen straws were permeable to CO_2 gas and could therefore be used as receptacles for culturing early stage bovine embryos. In the second experiment no statistical differences ($p > 0.05$) were found in the number of pre-compacted bovine embryos that developed to the blastocyst stage, or the hatched blastocyst stage, neither for the culture medium used, or the method of culturing in the

two incubators. Neither was there any difference ($p>0.05$) in the number of blastomeres that developed at the blastocyst stage between the two types of incubators. The bovine embryos tended to develop more blastomeres when cultured in BAF than when cultured in TCM-199 in both the standard laboratory incubator and when using the vagina of a goat doe as an incubator.

3.2 Introduction

During the process of *in vitro* embryo production and nuclear transfer, the gametes and embryos have to be cultured under optimal conditions to develop to viable blastocysts that can produce live offspring after transfer to a surrogate female animal. Finding optimal media and incubator conditions for cell, ova and embryo culture have been thoroughly investigated during the last five decades. This include reports of the culturing of rabbit ova as far back as 1959 (Chang 1959), and of culturing chicken cells in residues of horse serum and chick-embryo extracts (Sanford *et al.* 1952). Holtzer *et al.* (1960) reported on media and conditions used to culture cartilage cells, while Onuma *et al.* (1968) cultured early cleavage stage rabbit embryos to the blastocyst stage.

Blakewood *et al.* (1989, 1990) cultured 2- to 8-cell goat embryos to the hatching blastocyst stage. During this study an unusual culture medium, chicken amniotic fluid, as well as a remarkable incubator, a developing chicken egg, were used. Further studies using the amniotic sack of a developing chicken egg were done by Ocampo *et al.* (1993, 1994) when porcine oocytes were matured and embryos were cultured. They concluded that the amniotic cavity of a chicken egg could be used to mature porcine oocytes, although the efficacy can be influenced by the developmental stage of the chicken egg. The developing egg amniotic sack could therefore be

valuable for culturing porcine embryos to the blastocyst stage where a CO_2 controlled incubator is not available.

Duodenal villi were cultured by Calvert (1981), and Beaulieu and Calvert (1985) in different media. He found that if a commercial culture medium was used to culture duodenal explants, no villi would develop in a 72 h period. When 25% of mouse amniotic fluid was added to the same control culture medium, short villi would appear after only 12 h of culture, and medium length villi after 48 h. The trophic effect of rabbit amniotic fluid used as a culture medium was also observed when fetal gastric mucosal cells were cultured *in vitro* by Mulvihill *et al.* (1986, 1989).

Amniotic fluid contains a series of growth factors that will stimulate cell growth. Kong *et al.* (1998) reported on the stimulating effect of rabbit amniotic fluid on rabbit fetal gastric epithelial cell growth. Culture medium containing 20% of rabbit amniotic fluid showed significant ($p < 0.05$) increases in cell growth numbers compared to the control. Wang *et al.* (1990) have characterized an insulin-like growth factor (IGF)-binding protein present in ovine amniotic fluid. Ovine amniotic fluid contains an unsaturated, non-glycosylated IGF-binding protein with high affinity for insulin-like growth factor II (IGF-II), the latter being a potent developmental stimulant in the body with 30x the potency of IGF-I. Murine amniotic fluid may also contain multiple growth factors for murine fetal liver cells, one of which may be a unique molecule related to stem cell growth factor (Heidari *et al.* 1996a, 1996b).

Human amniotic fluid was used as a culture medium (Dorfmann *et al.* 1989; Oettlé & Wiswedel 1989) for developing mouse and human embryos, and as a freezing medium with propanediol as cryoprotectant to freeze 2-cell murine embryos (Ng *et al.* 1988). When using human amniotic fluid collected by 16-week routine amniocenteses, they found that significantly ($p<0.05$) more murine embryos developed to the blastocyst stage than in standard Earle's growth

medium. No significant difference in human pre-implantation embryo cleavage was observed when human amniotic fluid and Hams F-10 were used as culture mediums for human embryos. Ng *et al.* (1988), however, found that when he compared the developmental rate of 2-cell frozen-thawed murine embryos to the blastocyst stage, pure human serum had no advantage when the 2-cell murine embryos were frozen in serum compared to embryos that were frozen in human amniotic fluid. Montoro *et al.* (1991) reported very promising results for the use of amniotic fluid as a culture medium when he showed that fresh amniotic fluid stored for up to 30 days, stimulated up to 89% of 2-cell murine embryos to develop to the blastocyst stage even in the absence of CO_2. There was no significant difference in the development of human IVF embryos cultured in amniotic fluid collected from different human patients and Whittingham's T6 plus 10% of fetal calf serum. When cultured to the blastocyst stage in amniotic fluid, 4/9 pregnancies were obtained after transfer of the embryos compared to 2/12 when cultured in T6 medium plus maternal serum (Gianaroli *et al.* 1986).

Hansel (1986) predicted that by the turn of the millennium, an animal breeding system will have been developed in which artificial insemination will be replaced by a system where a transferred embryo will be used to fertilize livestock. These embryos will derive from *in vivo* as well as *in vitro* fertilization. The ovum and sperm donors will be genetically superior animals, and the embryos in most cases will be sexed and frozen-stored until transferred. He also predicted that embryos will be genetically engineered by the year 2000, containing acquired genes that will result in faster growth, leaner carcasses, greater disease resistance, and even better milk production. To optimize the production of these embryos and clones, the best favorable culture media and conditions had to be found (Hansel 1986).

Nuclear transfer of adult somatic cells to produce cloned offspring predicted by Hansel & Godke (1992) to occur soon was reported early in 1997 by Wilmut *et al.* (1997) when the first animal, a sheep lamb called Dolly, cloned from a somatic cell derived from an udder cell of an adult sheep ewe was born. Preceding that there were many reports on embryonic cell nuclear transfer successes (Willadsen 1986; Prather *et al.* 1988; Stice & Robl 1988; Willadsen *et al.* 1991; Yang & Anderson 1992,) producing viable embryos and even live offspring. Today nuclear transfer (cloned) offspring from adult somatic cells has been produced in many species, including sheep, mouse (Wakayama *et al.* 1998), cow (Kato *et al.* 1998; Galli *et al.* 1999), goat (Baguisi *et al.* 1999; Behboodi *et al.* 2001), pig (Polejaeva *et al.* 2000), rabbits (Chesne *et al.* 2002), cat (Shin *et al.* 2002), mule (Woods *et al.* 2003), horse (Galli *et al.* 2003) and the rat (Zhou *et al.* 2003). This was made possible by the knowledge accumulated on the *in vitro* culture requirements of developing animal embryos during the last decade.

Most of the embryos are cultured in open wells (four to 96 wells per plate), and cell cultures in flasks that can be closed with a screw-on lid. If the cells or embryos are cultured in open wells, the surface of the medium is usually overlaid with sterile, washed oil, like paraffin oil (Kane 1987; Dode & Adona 2001). The oil will then prevent evaporation of the medium and therefore osmolarity changes. A lid is put over the open wells to prevent dust and micro-organisms from falling into the medium (Nelson & Nelson 2001), but it does not prevent CO_2 from escaping from the medium. The screw-on lid of the flask will only prevent evaporation of the watery part of the medium, and will therefore prevent osmolarity changes of the medium, but not pH changes. When the flask is put into the incubator for culturing of cell lines, the screw-on lid must not be tightly closed to allow CO_2 to enter the culture medium and stabilize the pH.

The CO_2 of the incubator which dissolves in the medium – normally at a 5% to 7% level - changes the pH of the medium as carbonic acid is formed. The CO_2 can penetrate the oil overlay, as well as diffuse past the lid of the 4-well dish, and also past the screw-on cap of the flask if it is not screwed on tightly.

Often a laboratory incubator is not available, for example where somatic cells, oocytes or embryos are cultured after thawing in non-laboratory conditions to increase the survival of the cells. This takes place when the laboratory is too far removed from the place of transfer under extensive farming or wildlife conditions (Vajta *et al.* 1997, 2004).

Animal and human cells can be cultured and oocytes matured in the vagina of a woman or female animal (Ranoux *et al.* 1988; Hewitt 1991). This can be done in a suitable container like a 0.25 mL or 0.5 mL semen straw or glass ampoule. The CO_2 gas must preferably be able to penetrate the wall of the container to allow the CO_2 to dissolve in the medium surrounding the cells, embryos or oocytes. In the present study, the first experiment was carried out to evaluate the permeability of a 0.25 mL semen straw for CO_2 and water to allow for a stable pH and osmolarity of the medium within the straw. The pH changes in the straw were then compared to the pH changes in the medium cultured under similar conditions in a 4-well plate in a CO_2 incubator.

When embryos are cultured under laboratory conditions, it is mostly done in 5% to 7% CO_2 in air. The air in the incubator is usually atmospheric air consisting of ~20% oxygen, ~79% nitrogen and less than 1% of other gases. The CO_2 level is then increased to 5% by the frequent injection of CO_2, and controlled at this level. *In vivo*, however, especially in the oviduct and uterus, the concentration of oxygen is much less than 20%, and more in the region of 5% (Hejlasz *et al.* 1987)

Alternative techniques were therefore investigated to culture embryos at a lower oxygen tension without having to inject a prefabricated gas mixture into the incubator, or lower the oxygen tension by increasing the nitrogen level of the gas mixture inside the incubator. The vagina of the female animal was chosen for this purpose.

Maturing oocytes and culturing embryos in the vagina of a female animal compared to a standard laboratory incubator has some advantages:

1. If a laboratory female, like a rabbit doe, is used, it is much more "portable" than a full-size laboratory incubator.

2. The O_2 and CO_2 levels in the vagina is much closer to the partial pressures of these gases in the oviduct and uterus, than the 20% O_2 and 5% CO_2 levels in a laboratory incubator.

There are, however, also disadvantages to culturing embryos in the vagina, e.g.

1. The embryos and their development can only be thoroughly evaluated if they are outside the straw, and not regularly as when they are inside a dish and cultured in an incubator. This implies that the straw has to be emptied every time the embryos have to be evaluated, which makes it a laborious procedure if all the developmental stages of the embryo have to be followed. That also implies that the culture medium cannot be changed or replenished during the culturing process, except if the receptacle containing the embryos, is first emptied and the embryos are then reloaded into a new straw;

2. Pathogenic vaginal micro-organisms, like fungi, can contaminate the medium when the embryos are removed from the straw, if the outside of the straw is not thoroughly cleansed with an antiseptic before cutting off the sealed ends. The low pH of the vagina, however, prevents most micro-organisms from multiplying inside in the lumen of the vagina, and therefore controls contamination of the outside of the straw while it is inside the vagina.

3.3 Hypothesis

1. A semen straw can be used to culture early stage bovine embryos in an incubator as well as in the vagina of a female animal, as CO_2 gas can penetrate the wall of the straw allowing for an optimal pH level inside the straw.

2. First trimester bovine amniotic fluid will support early stage bovine embryo development to the blastocyst stage.

3.4 Material and Methods

3.4.1 Experiment 1

3.4.1.1 Experimental design

pH changes of bicarbonate buffered media (TCM-199 and first trimester bovine amniotic fluid - BAF) in different receptacles were evaluated during incubation in the presence and absence of CO_2 gas as indicated below.

Receptacle	Medium incubated	CO 2 gas	Period of incubation	pH measured every
4-Well plate	TCM-199	Present	120 h	24 h
0.25 mL straw	TCM-199	Present	120 h	24 h
0.25 mL straw	BAF	Present	120 h	24 h
0.25 mL straw	TCM-199	Absent	120 h	24 h
0.25 mL straw	BAF	Absent	120 h	24 h

3.4.1.2 Method: Experiment 1

Tissue culture medium-199 (TCM-199, Sigma, USA, Cat. # M7528) containing 10% heat inactivated (56 ºC for 30 minutes) fetal bovine serum and 100 iu Penicillin and 100 µg Streptomycin per mL was sterilely transferred in a laminar flow hood to the following containers:

(a) 4-well plates (Nunc) with 0.5 mL in each well. The surface was not covered with oil.

(b) 0.25 mL semen straws (IMV) (n=20) with ~200 μL of medium in each straw and heat-sealed at both ends.

A pregnant bovine reproductive tract containing a fetus of 16.25 cm head-tail base length (~80 days pregnancy) was collected under sterile and cooled conditions from the local abattoir. The uterine wall was dissected open and the amniotic fluid aspirated with a sterile rubberless 50 mL syringe and an 18 gauge needle. The bovine amniotic fluid (BAF) was then heat inactivated at 56°C for 30 minutes, and stored at 4 °C in screw cap 15 mL plastic vials (Cellstar, Greiner Bio-One) until needed. Twenty semen straws were filled with the same volumes of frozen-thawed BAF containing 10% FBS and antibiotics, as used in the TCM-199 experiment.

The receptacles with either TCM-199 or BAF were then placed in a fully automatic incubator (Forma Scientific, Marietta, USA) with or without 5% CO_2 at 38.5 °C and 100% humidity, and the pH measured every 24 hours with a pH meter (Hanna Instruments, Woonsocket, USA) for a period of 120 hours. The pH was measured within 10 seconds after the container was removed from the incubator to minimize pH changes due to CO_2 diffusion. The experiment was repeated three times. The osmolarity was monitored with an osmometer (Vapro, Ontario, Canada) and recorded. The mean pH values are presented in Table 3.1.

3.4.2 Experiment 2

3.4.2.1 Experimental design

Bovine pre-compacted morula stage embryos were cultured in TCM-199 and BAF in a standard CO_2 incubator and in the vagina of a goat doe for 96 hours as indicated below.

n (Embryos)	Incubator	Culture medium	Culture period	Evaluation
55	5% CO2 lab	TCM-199	96 h	Stage + # blastomeres
60	Vagina - goat	TCM-199	96 h	Stage + # blastomeres
75	5% CO2 lab	Bovine amniotic fluid	96 h	Stage + # blastomeres
75	Vagina - goat	Bovine amniotic fluid	96 h	Stage + # blastomeres

3.4.2.2 Method

Pre-compacted bovine morulae were collected from multi-ovulated cows as described in Chapter 2. After washing the embryos three times in D-PBS, and finally in the appropriate culture medium, the grade 1 embryos were cultured in sterile heat sealed semen straws in two different media and two different "incubators". The embryos were aspirated in groups of not more than five in a 5 cm length of a 0.25 mL sterile semen straw (IMV) by means of a 16G needle attached to the cotton wool plug end of the straw, and a 1 mL syringe by pulling on the plunger of the syringe. The straws were then heat-sealed at both ends with a heated mosquito hemostat. The straws, containing the grade 1 bovine embryos, were then incubated for 96 hours in either (1) a standard 5% CO_2 laboratory incubator at 38.5 ºC and saturated humidity, or (2) inside a cylindrical sponge, and the sponge with its straw, inserted in the anterior part of the vagina of a goat doe in diestrus by means of a sponge applicator. The media in both "incubators" were not changed during the 96 hour culture period.

After the *in vivo* and *in vitro* culture periods had elapsed, the sponges containing the straws were pulled from the vagina, and the straws cleaned on the outside with methanol. Both ends of

the sealed straws cultured in the vagina and incubator were cut and the embryos flushed into a 35 mm petri dish for evaluation of the developmental stage under an inverted microscope. The embryos were stained with DNA-specific fluorochrome stain (Hoechst stain No 33342), and the stained nuclei of the live blastomeres counted on a fluorescent UV light inverted microscope at 400X magnification.

3.4.2.2.1 Staining of the embryos

The embryos, after evaluation of their developmental stage, were placed in a 50 μL drop of culture medium on a glass slide, and covered with a glass cover slip with a drop of a 50:50 heated mixture of Vaseline and candle wax at each corner. The cover slip was then very carefully lowered and pushed down until the cover slip made contact with the embryos. This could be visualized by a halo forming around the embryo. Care was taken not to put to much pressure on the cover slip, because it will cause the embryo to disintegrate and disperse all of its cellular material. The sides of the cover slip was then sealed with a special rubber cement, and only a small "window" left open on both sides for the administration of the fluorochrome stain, bisbenzimide (Hoechst 33324, USA, Cat B-2261) . The stain was added at the one window by means of a micro-pipette, and dispersed underneath the cover slip by absorbing medium at the other window with a paper towel. The two windows were then closed with the rubber cement. Counting of the blastomeres commenced within 48 hours after staining, as a fluorochrome stain tends to loose its staining ability over time.

3.5 Statistical analysis

Data reported in this manuscript are as mean ± SD. All data were analyzed using the Student's t-test assuming two-sample means with unequal variances (Microsoft Excell).

3.6 Results

3.6.1 Experiment 1

The pH values are given in Table 3.1 and Figure 3.1

Table 3.1. pH values of TCM-199 and BAF cultured in straws and 4-well plates with or without CO_2.

Medium	Receptacle	0 hours	24 hours	48 hours	72 hours	96 hours	120 hours
TCM w/ CO_2	Straw	7.44	7.41	7.36	7.30	7.40	7.44
TCM w/ CO2	4-Well	7.44	7.44	7.45	7.42	7.35	7.36
TCM w/out CO_2	Straw	7.44	7.58	7.72	7.80	7.88	8.04
BAF w/ CO_2	Straw	7.46	7.43	7.44	7.40	7.37	7.44
BAF w/out CO_2	Straw	7.46	7.57	7.74	7.76	7.80	7.91

The osmolarity of the two media stayed at a constant value of 285 and 322 for TCM-199 and BAF respectively during the 120-hour period.
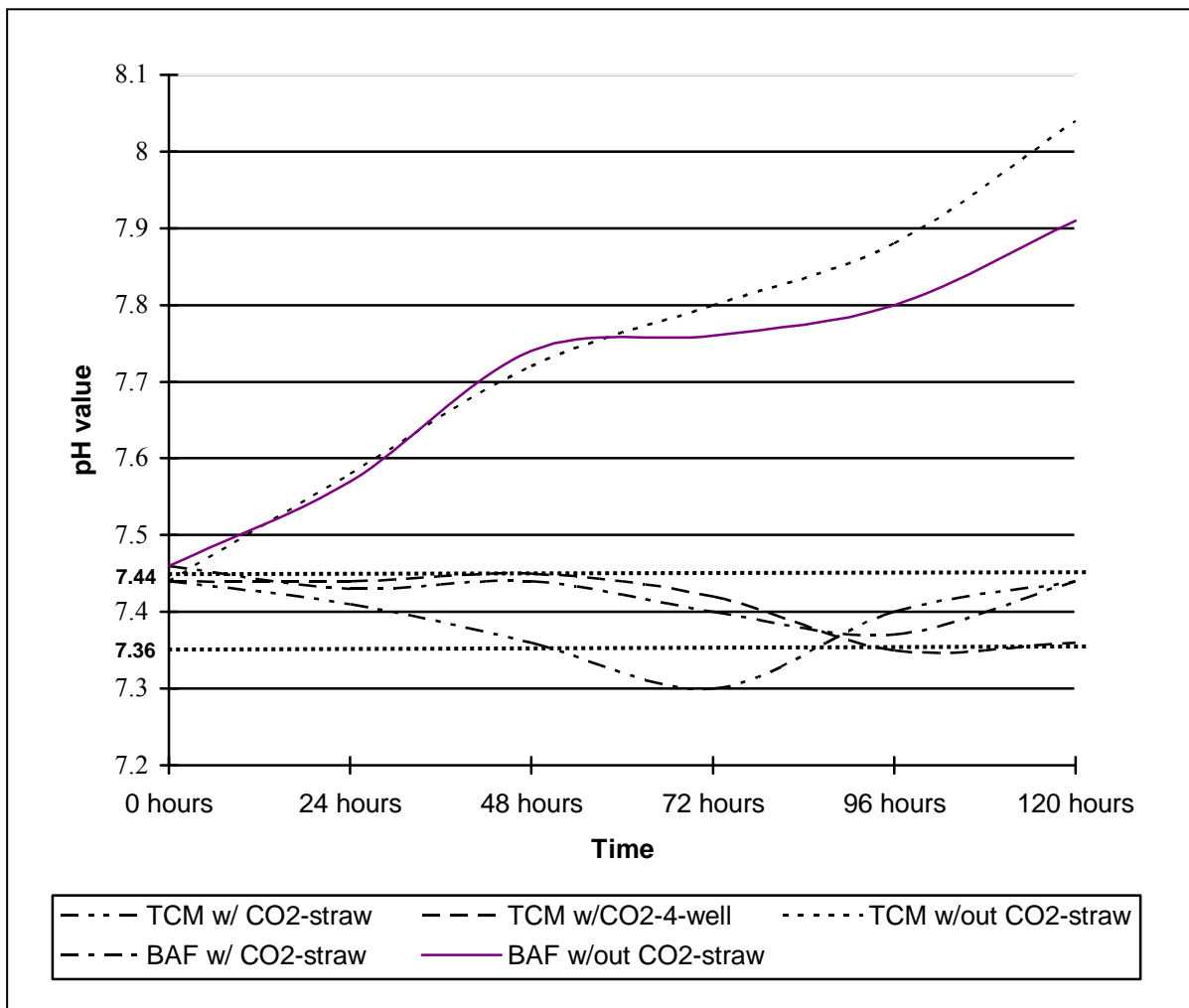
Figure 3.1 pH values of TCM-199 and BAF cultured in straws and 4-well plates with or without CO_2.

3.6.2 Experiment 2

The development of the bovine pre-compacted morula stage embryos in TCM-199 and BAF in the laboratory incubator and the vagina of the goat doe over the 96 hour culture period is given in Table 3.2.

Table 3.2. Embryo development in TCM-199 vs. BAF in an incubator vs. goat vagina

Treatment group	n	Morula (%)	Blast -Expanded Blastocysts (%)	Hatched Blastocysts (%)
Incubator – TCM-199	55	12 (21.8)^{ab}	22 (40.0)^d	21 (38.1)^e
Vagina – TCM-199	60	19 (31.7)^a	16 (26.7)^d	25 (41.7)^e
Incubator – BAF	75	12 (16.0)^b	28 (37.3)^d	35 (46.7)^e
Vagina – BAF	75	26 (34.7)^{ac}	20 (26.7)^d	29 (38.7)^e

^{abcde} Columns with different superscripts differ significantly ($p<0.05$)

The number of blastomeres for each developmental stage of the embryos from compacted morula to hatched blastocyst is listed in Table 3.3

Table 3.3. Number of blastomeres at each developmental stage of the embryos cultured in TCM-199 and BAF in a 5% CO_2 incubator and in the vagina of a goat doe

Treatment groups	Morula stage	Blastocyst stage	Hatched blastocyst
Incubator – TCM-199	40.3 ± 2.4^a	64.8 ± 11.8^c	94.1 ± 7.8^e
Vagina – TCM-199	38.5 ± 2.6^a	63.9 ± 5.5^c	95.9 ± 8.9^{eg}
Incubator – BAF	48.1 ± 4.0^b	72.6 ± 12.7^d	103.2 ± 15.4^f
Vagina - BAF	47.3 ± 4.5^b	68.4 ± 9.5^{cd}	101.7 ± 14.9^{fg}

Columns with different superscripts differ significantly (^{ab} $p<0.01$,, ^{cd} $p<0.05$, ^{efg} $p<0.05$)

Figure 3.1 represents a bovine pre-compacted stage morula before culture. Graphic images of the developed embryos cultured for 96 h in TCM-199 and BAF in both the incubator and the vagina of the goat are illustrated in Fig 3.2 to Fig 3.8 below.
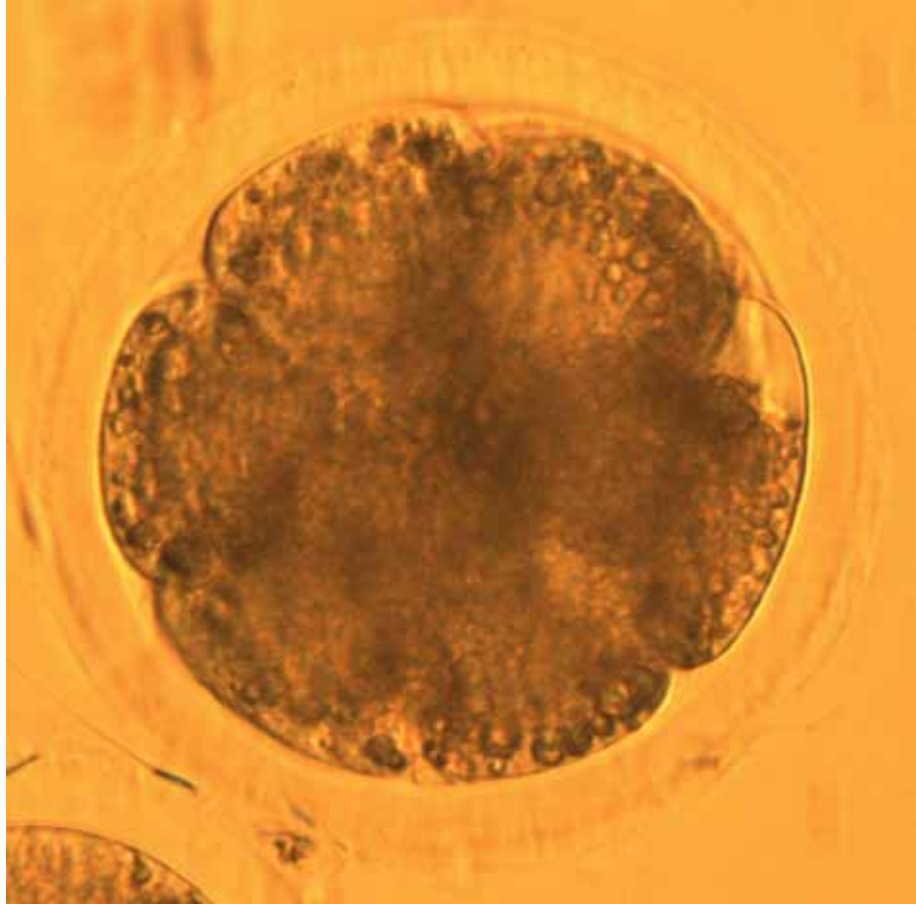
Figure 3.1. Bovine pre-compacted morula stage embryo before culture.
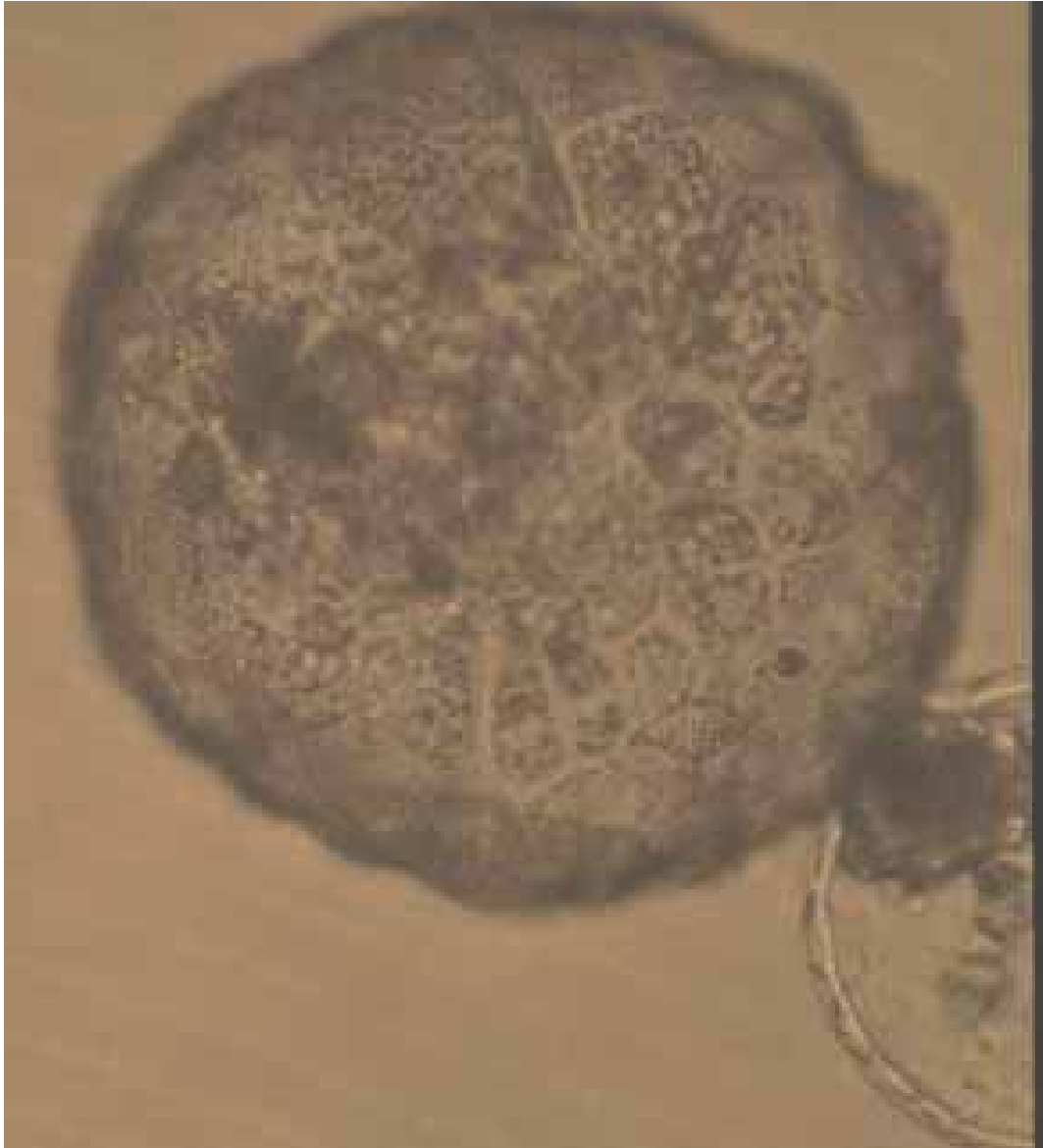
Figure 3.2. Bovine hatched blastocyst stage embryo cultured in TCM-199 in the vagina of a goat doe.
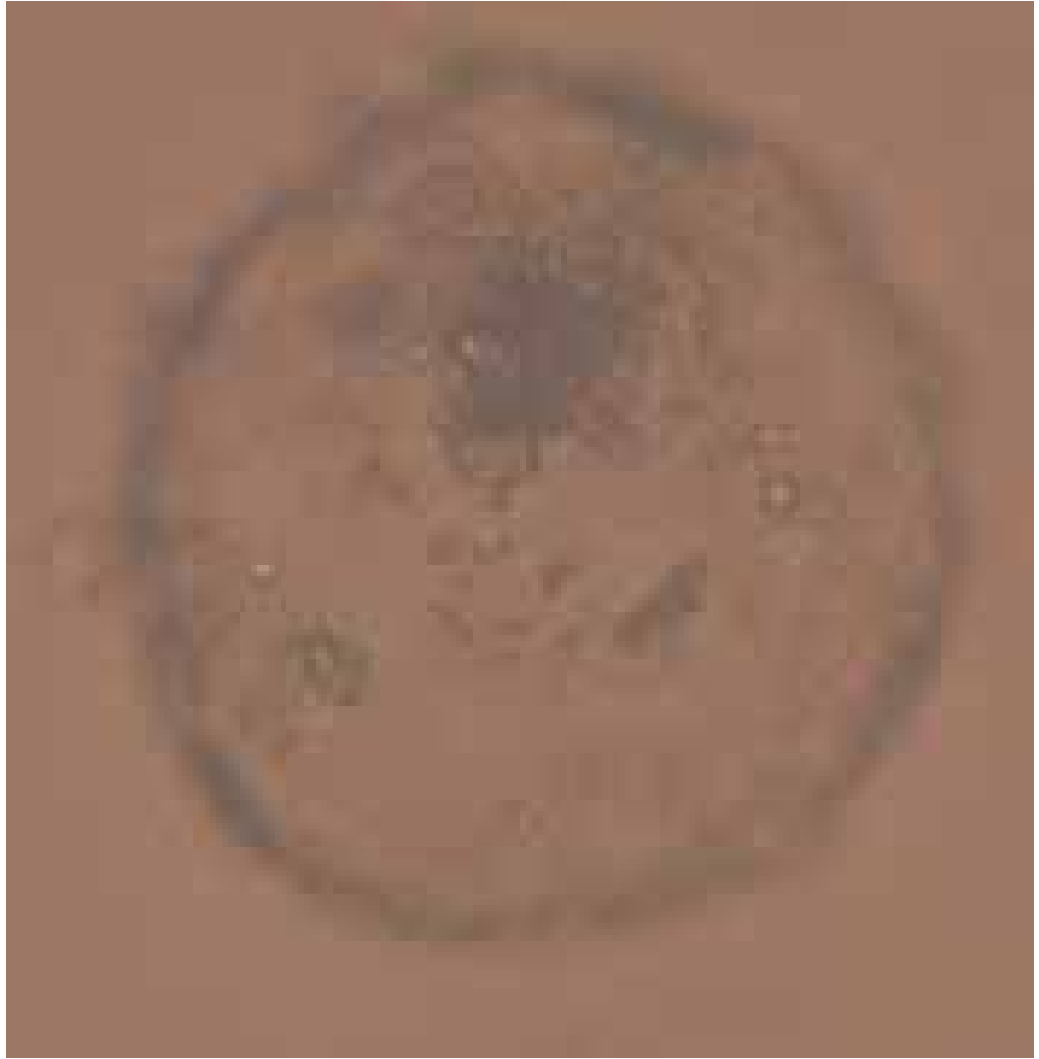
Figure 3.3.   Hatched blastocyst stage bovine embryo cultured in BAF in an incubator.

Figure 3.4. Hatching stage bovine embryo cultured in TCM-199 in the
vagina of a goat doe.
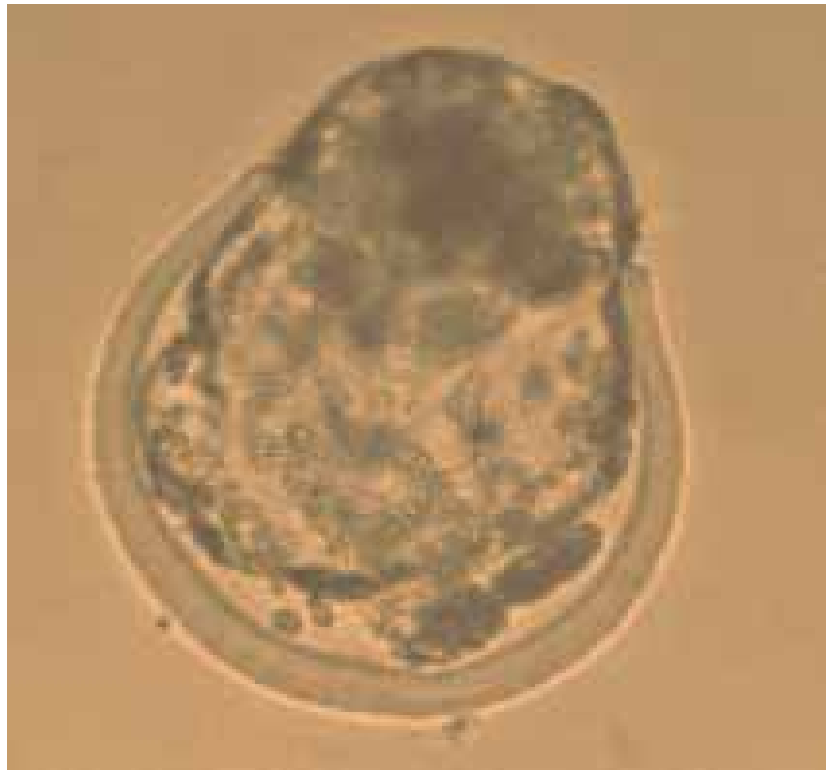
Figure 3.5. Hatching blastocyst stage bovine embryo cultured in
BAF in the vagina of a goat doe.

Figure 3.6. Hatched blastocyst stage bovine embryo culture in
TCM-199 in the vagina of a goat doe.

Figure 3.7. Two hatched blastocyst stage bovine embryos cultured
in BAF in the vagina of a goat doe.

Figure 3.8. Expanded blastocyst stage embryo cultured in TCM-199
in the vagina of a goat doe.

3.7 Discussion and Conclusion

3.7.1 Experiment 1

The present results show that CO_2 can penetrate the plastic wall of a 0.25 mL semen straw. In the first experiment the pH of the TCM-199 dropped from an initial 7.44 to 7.30 over a 72 hour period. This is due to the CO_2 penetrating the wall of the straw, binding with the water in the straw, and forming carbonic acid. The carbonic acid then dissociates into H^+ and HCO_3^-. The pH of the BAF in the straw dropped from an initial 7.46 to 7.40 over the same time period. The pH changes in the straw were not significantly different from that observed in the 4-well plates. The BAF kept a more constant pH value than TCM-199 in the environment inside the straw where no water evaporation was possible. The optimal pH for culturing embryos is between 7.36 and 7.44 (Gianaroli 1986).

In the absence of CO_2 in the incubator, the pH of both the TCM-199 and BAF rose to above physiological normal limits. The pH of the TCM-199 rose from an initial 7.44 to 8.04 over 5 days, showing that the CO_2 diffused through the wall of the sealed semen straw from the inside towards the outside (with the concentration gradient) decreasing the concentration of carbonic acid inside the straw. The pH of the BAF in the straw in the incubator without CO_2 rose from an initial 7.46 to its highest level of 7.91 over the 5 day culture period.

The stable osmolarity of the two media inside the semen straws showed that the straws were not permeable to the water molecules of the respective media.

This experiment clearly indicated that sterile semen straws could be used as a receptacle for culturing ova or embryos, as the wall of the straw would allow CO_2 gas, but not water molecules, to penetrate.

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3.7.2 Experiment 2

The results showed that early stage (pre-compacted) bovine embryos will develop to the hatched blastocyst stage in both TCM-199 and in first trimester bovine amniotic fluid. It was shown that the same embryos developed to the hatched blastocyst stage in both a standard laboratory incubator and in the vagina of a goat doe. There were no statistical differences found in the number of pre-compacted bovine embryos that developed to the blastocyst stage, or the hatched blastocyst stage, neither for the culture medium used, or the method of culturing in the two incubators. These results compare favorably with Blakewood *et al.* (1989), who found that significantly more bovine embryos developed in the chick embryo amniotic cavity than in the control medium. He concluded that the amnion of a developing chick embryo can support murine, caprine and bovine early-stage embryos through culture to the blastocyst stage. In the present experiment it was also true for the conditioned medium (amniotic fluid) collected during the first trimester of pregnancy in cows. Growth factors and favorable conditions present in the amniotic cavity of the chick embryo, were also present in the bovine amniotic fluid.

As for the blastomeres numbers in the different developmental stages of the embryos in the two media, it was shown that the embryo will develop more cells when it is cultured in BAF then when cultured in TCM-199. This is true when both the blastocyst stage (64.4 vs 70.5, respectively) and the hatched blastocyst stage (95.0 vs 102.5 respectively) of the embryos are compared ($p<0.05$) when cultured in both the incubator and the vagina of the goat doe. Vlad *et al.* (1996) have found that human embryos at the 2-cell stage that are transferred to co-culture containing human oviductal (ampullary) epithelial cells, developed to the blastocyst stage with an average of 120.7 blastomeres per embryo after 3.5 d of co-culture in a standard laboratory incubator. If the transfer to co-culture took place at the 4-cell stage, the blastocysts developed 62.9 blastomeres per embryo

(p=0.023) during the same length of incubation, while the embryos cultured in standard culture medium without co-culture only developed 42.1 blastomeres at the blastocyst stage (p<0.05) under similar conditions.   In another study by the same authors the percentage of hatching blastocysts was significantly higher (P<0.05) for human zygotes co-cultured with human oviductal epithelial cells from day 1 post-insemination (38%) than for zygotes which had not been co-cultured (7%). The blastocyst hatching rate for embryos co-cultured from day 2 post-insemination was 15%. It can therefore be concluded that (1) co-culture of human embryos with oviductal cells could improve the development of the embryos *in vitro* (2) the degree of improvement was more pronounced when the co-culture started at an earlier stage (Yeung *et al.* 1992), and (3) bovine amniotic fluid from early-stage pregnancies probably contains some or all of the stimulants and growth factors secreted by the amniotic sac of a developing chick embryo, and also secreted by the cells during *in vitro* co-culture.

When the two incubators are compared, it was shown that there was no statistical difference between the numbers of blastomeres at the blastocyst stage when the embryos were cultured in the incubator compared to the vagina, or when cultured in BAF in the vagina. There was, however, a significant difference (p<0.05) when the number of blastomeres at the blastocyst stage of the embryos cultured in TCM-199 in the incubator is compared to the number of blastomeres of embryos at the blastocyst stage when cultured in BAF in the same incubator. This significance was not present between the blastomere numbers of embryos at the blastocyst stage cultured in BAF in the incubator vs cultured in the vagina of the goat doe.

If the number of blastomeres in the hatched blastocysts after 96 hours of culture are compared, the situation is slightly different. Again there was no significant difference between the numbers of blastomeres of the embryos cultured in TCM-199 in the incubator vs the vagina of a

goat doe. There was also no significant difference between the two culture media when both groups were cultured in the vagina of a goat doe. There was, however, a significantly lower number of blastomeres at the hatched blastocyst stage ($p < 0.05$) when the embryos were cultured in TCM-199 compared with BAF as a culture medium in the incubator. There were also significantly more blastomeres at the hatched blastocyst stage when the embryos were cultured in BAF in the incubator than when cultured in TCM-199 in the vagina or in the incubator ($p < 0.05$).

It can therefore be concluded that pre-compacted stage bovine embryos can be cultured and will develop to the hatched blastocyst stage when both TCM-199 and BAF are used as culture media. It can also be concluded that pre-compacted stage bovine embryos develop just as well in a sealed straw in the vagina of a goat doe as in a standard laboratory incubator.

3.8 References

Baguisi, A., Behboodi, E., Melican, D.T., Pollock J., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Perter, C.A. & Midura, P. (1999). Production of goats by somatic cell nuclear transfer. *Natural Biotechnology* **17**, 456-461.

Beaulieu, J-F. and Calvert, R. (1985). Influences of dexamethasone on the maturation of fetal mouse intestinal mucosa in organ cultures. *Comparitive Biochemistry and Physiology, Part A: Physiology* **82** (1), 91-95.

Behboodi, E., Melican, D.T., Liem, H. & Chen, L.H. (2001). Production of transgenic goats by adult somatic cell nuclear transfer. *Theriogenology* **55**, 254.

Blakewood, E.G., Jayenes, J.M., Johnson, J.M. & Godke, R.A. (1989). Using the amniotic cavity of the developing chick embryo for *in vivo* culture of early-stage mammalian embryos. *Poultry Science* **68** (12), 1695-1702.

Blakewood, E.G., Pol, S.H., Prichard, J.F. & Godke, R.A. (1990). Culturing two- to eight-cell caprine embryos using domestic chicken eggs. *Mol Reprod Dev* **27** (4), 288-294.

Calvert, R. (1981). Effect of amniotic fluid and fetal bovine serum on the morphogenesis of mouse duodenal villi in organ culture. *Experientia* **37** (4), 417-418

Chang, M.C. (1959). Fertilization of rabbit ova *in vitro*. *Nature* **8**, 466-467

Chesne, P., Adenot, P.G., Viglietta, C., Barette, M., Boulanger, L. & Renard, J.P. (2002). Cloned rabbits produced by nuclear transfer from adult somatic cells. *Natural Biotechnology* **20**, 366-369.

Dode, M.A.N. & Adona, P.R. (2001). Development capacity of *Bos indicus* oocytes after inhibition of meiotic resumption by 6-dimethylaminopurine. *Animal Reproduction Science* **65**, 171-180.

Dorfmann, A.D., Bender, S.D., Robinson, P., Fugger, E.F., Bustillo, M., Reed, G & Schulman, J.D. (1989). Cell-free human amniotic fluid as culture medium for mouse and human embryos. *Fertility and Sterility* **51** (4), 517.

Gianaroli, L., Seracchioli, R., Ferraretti, A.P., Trounson, A., Flamigni, C. & Bovicelli, L. (1986). The successful use of human amniotic fluid for mouse embryo culture and human *in vitro* fertilization, embryo culture, and transfer. *Fertility and Sterility* **46** (5), 907-913.

Galli, C., Duchi, R., Moor, R.M. & Lazzari, G. (1999). Mammalian leucocytes contain all the genetic information necessary for the development of a new individual. *Cloning* **1**, 161-170.

Galli, C., Lagutina, G., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R. & Lazzari, G. (2003). A cloned horse born to its dam. *Nature* **424**, 635

Hansel, W. (1986). Animal agriculture for the year 2000 and beyond. William Henry Hatch memorial Lecture, National Association for State University, Land Grant Colleges, USDA, CSRS.

Hansel, W & Godke, R.A. (1992). Future perspectives on animal biotechnology. *Animal Biotechnology* **3**, 111-137.

Heidari, Z., Isobo, K., Kiuchi, K., Nakashima, I. & Tomoda, Y. (1996a). Promotion of proliferation of murine haematopoietic stem cell growth factors in murine amniotic fluid. *Journal of Reproduction Iimmunology* **31** (1-2), 51-64.

Heidari, Z., Isobo, K., Goto, S., Nakashima, I., Kiuchi, K. & Tomoda, Y. (1996b). Characterization of growth factor activity of amniotic fluid cells from haematopoietic and lymphoid organs of different life stages. *Microbiol Immunology* **40** (8), 583-589.

Hejlasz, Z., Nicpon, J., Rauluszkiewicz, S. & Samborski Z. (1987). Buffer systems, electrolytes, pH and erythrocyte indices in the blood of cows and calves in the peri-natal period. *Pol Arch Weter* **25** (2-3), 225-235.

Hewitt J, 1991. The intra-vaginal culture technique for supernumerary oocytes from gamete intra-Fallopian transfer. *Human reproduction* **16** (1): 76-78.

Holtzer, H., Abbot, J., Lash, J. & Holtzer, S. The loss of phenotypic traits by differentiated cells *in vitro*. I. Dedifferentiation of cartilage cells. *Proceedings of the Academy of Natural Sciences* **46**, 1533-1542.

Kane, M.T. (1987). Culture media and culture of early embryos. *Theriogenology* **27**, 49-57.

Kato, Y., Tani, T., Sotomaruy., Kurokawa, K. (1998). Eight calves cloned from a somatic cell from a single adult. *Science* **282**, 2095-2098.

Kong, W., Yee, L.F. & Mulvihill, S.J. (1998). Hepatocyte growth factor stimulates fetal gastric epithelial cell growth *in vitro*. *J Surg Res* **78** (2), 161-168.

Montoro. L., De la Fuente, J., Baeza, F. & Cocero, M.J. (1991). Mouse embryos cultured in bovine amniotic fluid. *Theriogenology* **35**, (1): 246.

Mulvihill, S.J., Stone, M.M., Fonkalsrud, E.W. & Debas H.T. (1986). Tropic effect of amniotic fluid on fetal gastrointestinal development. *J Surg Res* **40** (4), 291-296.

Mulvihill, S.J., Hallden, G. & Debas, H.T. (1989). Tropic effect of amniotic fluid on cultured fetal gastric mucosal cells. *J Surg Res* **46** (4), 327-329.

Nelson, L.D. & Nelson, C.F. (2001). Handling and culture of bovine embryos: Survey of media used by 26 embryo transfer companies in the USA. *Theriogenology* **56**, 1377-1382.

Ng, S.C., Sathananthan, H., Bongso, A., Lee, M.N., Mok, H., Wong, P.C. & Ratnan, S.S. (1988). The use of amniotic fluid and serum with propanediol in freezing murine 2-cell embryos. *Fertility and Sterility* **50** (3), 510-513.

Ocampo, M.B., Ocampo, L.C., Suzuki, K., Mori, T., Ueda, J., Shimizu, H. & Kanagawa, H. (1993). Development to the blastocyst stage of pig embryos cultured in the amniotic fluid of developing chick embryos. *Journal of Veterinary Medical Science* **55** (5), 889-891.

Ocampo, M.B., Ocampo L.C., Suzuki, K., Mori, T., Ueda, J. & Kanagawa, H. (1994). Nuclear and cytoplasmic maturation of pig oocytes cultured in the amniotic fluid of developing chick embryos. *Journal of Veterinary Medical Science* **56** (1), 173-176.

Oettle, E.E. and Wiswedel, K. (1989). Mouse embryos cultured in amniotic fluid. *South African Medical Journal* **61** (1), 63-64.

Onuma, H., Maurer, R.R. & Foote, R.H. (1968). *In vitro* culture of rabbit ova from early cleavage stages to the blastocyst stage. *Journal of Reproduction and Fertility* **16**, 491-493.

Polejaeva, I.A., Chen, S., Vaught, T.D., Page, R.L., Mullins, J, Ball, S., Dai, Y., Boone, J., Walker, S. & Ayares, D.L. (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* **407**, 505-508.

Prather, R.S., Sims, M.L. & First, N.L. (1988). Nuclear transplantation in the early porcine embryo**.** *Theriogenology* **29***,* Abstract, 290.

Ranoux C, Aubriot FX, Dubuisson JB and Cardone V, 1988. A new *in vitro* fertilization technique: intra-vaginal culture. *Fertility and Sterility* **49** (4): 654-657.

Robl, J.M. & Stice, S.L. (1989). Prospects for the commercial cloning of animals by nuclear transplantation *Theriogenology* **31***,* 75-84.

Sanford, K.K., Waltz, H,K., Shannon, J.E., Earle, W.R. & Evans, V.J. (1952). The effects of ultra-filtrates and residues of horse serum and chick-embryo extract on proliferation of cells *in vitro*. *Journal of the National Cancer Institute* **13** (1), 121-137.

Shin, T., Kraemer, D., Pryor, J., Liu, L., Rugila, J., Howe, L., Buck, S., Murphy, K., Lyons, L. & Westhusin, M. (20020). A cat cloned by nuclear transplantation. *Nature* **415**, 859.

Stice, S.L. and Robl, J.M. (1988). Nuclear reprogramming of nuclear transplant rabbit embryos. *Biology of Reproduction* **39** (3), 257-264.

Vajta, G., Holm, P., Breve, T. & Callesen, H. (1997). The submarine incubation system, a new tool for *in vitro* embryo culture: A technique report. *Theriogenology* **48**, 1379-1385.

Vajta, G., Bartels, P., Joubert, J., de la Rey, M., Treadwell, R & Callesen, H. (2004). Production of a healthy calf by somatic cell nuclear transfer without micro-manipulators and carbon dioxide incubators using the handmade cloning (HMC) and the submarine incubation system (SIS). *Theriogenology* **62**, 1465-1472.

Vlad, M., Walker, D. and Kennedy, R.C. (1996). Nuclei number in human embryos co-cultured with human ampullary cells. *Human Reproduction* **11**, 1678-1686.

Wakayama, T., Perry, A.C., Zuccotti, M., Johnson, K.R. & Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**, 369-374.

Wang, J.F., Fraher, L.J. & Hill, D.J. (1990). Characterization of insulin-like growth factor-binding protein in ovine amniotic fluid. *Journal of Endocrinology* **127** (2), 325-333.

Willadsen, S.M. (1986). Nuclear transplantation in sheep embryos. *Nature* **320**, 63-65.

Willadsen, S.M., Jansen, R.E., McAlister, R.J., Shea, B.F., Hamilton, G. & McDermand, D. (1991). The viability of late morula and blastocysts produced by nuclear transplantation in cattle. *Theriogenology* **35** (1), 161-170.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H.S. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810-813

Woods, G.L., White, K.L., Vanderwall, D.K., Li, K.I., Bunch, T.D., Meerdo, L.N. & Pate, B.J. (2003). A mule cloned form fetal cells by nuclear transfer. *Science* (in print).

Yang, X. & Anderson G.B. (1992). Micromanipulation of mammalian embryos: Principles, progress and future possibilities. *Theriogenology* **38** (2), 315-335.

Yeung, W.S., Ho, P.C., Lau, E.Y. and Chan, S.T. (1992). Improved development of human embryos *in vitro* by a human oviductal cell co-culture system. *Human Reproduction* **7** (8), 1144-1149.

Zhou, Q., Renard, J.P., Le Friec, G., Brochard, V., Beaujean, N., Cherifi, Y., Fraichard, A. & Cozzi, J. (2003). Generation of fertile cloned rats by regulating oocyte activation. *Science* (in press).

CHAPTER 4

CULTURING OF OVINE, CAPRINE AND BOVINE EMBRYOS
OF DIFFERENT QUALITY GRADES
IN TCM-199 AND BAF IN A 5% CO_2 INCUBATOR

4.1 Abstract

After the collection of *in vivo* produced livestock embryos, they are normally evaluated under high magnification (minimum of 80X) with the aid of an inverted or stereo microscope. The Grade 1 embryos will give the best conception results when transferred to synchronize recipient female animals, while the Grade 3 embryos will give the worst results. The aim of the present study was to culture all three quality grades of *in vivo* produced pre-compacted morula-stage embryos of sheep, goats and cows in two different culture media and then compare the development of the embryos by evaluating the number of embryos reaching the hatched blastocyst stage. The results have shown that there were no significant differences between the development of the Grade 1 and the Grade 2 embryos from any of the three species when either cultured in TCM-199 or heat inactivated early pregnancy-stage (<60 d) bovine amniotic fluid (BAF) as culture media. Significantly more *in vivo* produced Grade 3 pre-compacted morula-stage sheep, goat and cow embryos, however, developed to the hatched blastocyst stage when cultured in BAF with 10% FBS and antibiotics, compared to culture in TCM-199 with 10% FBS and antibiotics ($p < 0.05$).

4.2 Introduction

Embryos from different species of animals and man have been cultured in different media for a variety of reasons. Some of the media that has been used for culturing mammalian embryos are:

- Amniotic fluid (Gianaroli 1986; Ng *et al.* 1988; Oettle & Wiswedel 1989; Coetzee *et al.* 1989; Dorfmann *et al.* 1989);

- Combination of DMEM and F12 (Lai *et al.* 1992; Zeng and Zeng 1998; Ornoy *et al.* 2003);

- Complex Serum-free Medium (CSM) (Sakkas and Trounson 1991);

- Connaught Medical Research Laboratories medium (Zheng *et al.* 2001);

- Culture medium plus human follicular fluid (Richards *et al.* 1990) ;

- CZB (Carney *et al.* 1990; Chatot *et al.* 1990a; 1990b, Zhu *et al.* 2004);

- Dulbecco's minimum essential medium (DMEM) (Kuzan & Wright 1982; Petters & Wells 1993; Roudebush *et al.* 1994);

- Eagle's basal medium (EBM) (Spindle 1980);

- Earle's Balanced Salt Solution (Dandekar and Glass 1987);

- Gardner's G1 and G2 (Krisher *et al.* 1999);

- Ham's F-10 (Seidel *et al.* 1976; Kuzan & Wright 1982; Ackerman *et al.* 1984; Dandekar and Glass 1987; Roudebush *et al.* 1994; Jin *et al.* 2000);

- Ham's F12 (Ball *et al.* 1991; Conaghan *et al.*1998);

- Hamster embryo culture medium (HECM) (Krisher *et al.* 1999; Zheng *et al.* 2001; Lee *et al.* 2003; Wirtu *et al.* 2004);

- Hoppe and Pitt's Medium (Dandekar and Glass 1987);

- Human tubal fluid (hTF) (Olar & Potts 1993; Erbach *et al.* 1993; Conaghan *et al.*1998; Ornoy *et al.* 2003);

- KSOM (potassium simplex optimization medium) (Liu *et al.* 1994, Erbach *et al.* 1994; Summers *et al.* 1995, 2000; Bhuiyan *et al.* 2004);

- Medium 199 (Nakao and Nakatsuji 1990; Carney & Foote 1991; Sparks *et al.* 1992);

- Medium RD (mixture of RPMI and D-MEM, defined, serum-free) (Carney & Foote 1991; Li *et al.* 1996; Jin *et al.* (2000,);

- Modified Earle's balanced salts solution (EBSS and MEBSS) (Scott *et al.* 1997; Conaghan *et al.* 1998; Han & Kiessling 1988);

- Modified T6 medium (mT6) (Spindle 1990);

- RPMI (Cavanagh 1984, Carney and Foote 1991; Rondeau *et al.* 1996);

- Spindle's Medium (Dandekar and Glass 1987);

- Synthetic oviduct fluid (SOF) (Krisher *et al.* 1999; Lee *et al.* 2003);

- Whittingham's M16 Medium (Lawitts and Biggers 1991);

- Whittingham's T6 Medium (Kruger and Stander 1985; Gianaroli *et al.* 1986; Dandekar and Glass 1987).

Some of the advantages to co-culture early stage livestock embryos are:

- Increase transplant pregnancy rates of *in vivo*-derived and IVF-derived embryos;

- To select out embryos with early-stage defects;

- To allow for embryo sexing and biopsy procedures prior to transfer (Handyside *et al.* 1989 1990; Tominaga 2,004);

- To get the embryos through the *in vitro* block stage, which in the case of cattle, sheep and goats is at the 8-cell to 16-cell stage;

- To rescue stressed embryos, especially after collection of the partially retarded and damaged embryos from the uterus (Putney *et al.* 1989);

- To increase the survival rate of demi-embryos after splitting (Heyman & Renard, 1996);

- To increase the post-thaw survival rate of frozen embryos (Vajta *et al.* 1999);

- To allow genetic screening for quantitative trait loci (QTLs) of the early stage embryo before transfer (Hardy and Handyside 1992; Delhanty 1994);

- To increase the implantation rate of human embryos transferred to the uterus of the donor (Veeck *et al.* 2004).

The quality of an *in vivo* produced embryo plays a major role in its ability to become a pregnancy. Schneider *et al.* (1980), Tervit *et al.* (1980) and Lindner and Wright (1983) have all shown that good quality embryos have a greater chance of conception and development to term when transferred than lesser quality grade embryos collected at different stages of development. Revival of *in vivo* produced damaged embryos (Grades 2 and 3) after collection, but before transfer to a synchronized recipient female animal, is therefore of great economic importance in a commercial setup of embryo collection and transfer (Elsden *et al.*, 1978).

Culturing early stage embryos will allow the *in vitro* produced embryo to develop to a stage where not only implantation rates are increased, but the blastomeres of the embryo are of a smaller volume which increases the chance of the embryo to survive cryo-preservation (Cohen *et al.* 1985; Cuello *et al.* 2004).

Biopsy of mammalian embryos causes damage to the embryo, as some of the blastomeres are removed from the early stage embryo during biopsy to allow for sexing, genetic screening of defects, genetic manipulation, cloning, etc. Co-culturing the embryo after the biopsy procedure allows the embryo to overcome the stressful procedure by supplying the developing embryo with most of the nutrients, as well as supporting cells secreting the necessary growth factors and stimulants for normal development.

Mammalian embryos change from the maternal genome to the embryonic genome at different levels of development (Schultz and Heyner 1992). The mammalian pre-implantation

embryo is under the control of maternal informational macromolecules that are accumulated during oogenesis. At a certain stage (the 8-cell to 16-cell stage in livestock species), the further development of the embryo becomes dependent upon new transcription derived from the embryonic genome. This change in transcription leads to a block in *in vitro* development, if certain essential growth factors and stimulants are not present in the culture medium. If culture takes place on a co-culture layer of somatic cells (mostly of reproductive organ origin), then these cells will supply the developing embryo of the crucial factors and stimulants to develop past the block stage. In the absence of co-culture, the essential growth factors and stimulants must be added to the *in vitro* culture medium in a sequential manner.

Blakewood *et al.* (1989) cultured early-stage mammalian embryos in the amniotic cavity of developing chicken eggs to the blastocyst stage. The wall of the amniotic sac served as a co-culture layer secreting the necessary growth stimulants into the amniotic fluid that stimulated the mammalian embryos to develop. The objective of the present experiment was therefore firstly to evaluate the potential of collected first trimester amniotic fluid to sustain mammalian embryo development to the blastocyst stage, that is: would there be sufficient growth factors in the amniotic fluid to supply in the needs of a developing livestock embryo?

When *in vivo* produced animal embryos are collected from the uterus, some of the embryos might be developmentally damaged and retarded. The bovine embryo is sensitive to maternal heat stress during the first 7 d after estrus. Embryo cultured past the 7-day period and then transferred may bypass this period of embryonic sensitivity and provide an alternative to AI to partially circumvent heat stress-induced infertility in cattle (Putney *et al.* 1989).

When frozen embryos are thawed, their blastomeres are similarly stressed due to freeze-damage to the structures of the cells. This damage can be repaired to a large extent if the embryos are cultured on an appropriate co-culture or in a medium with essential growth factors.

Genetic defects occur in less than 10% of livestock species. In humans, however, it can be as high as 35%. To identify a genetic defect, a biopsy of the embryo is done by removing a number of blastomeres from the embryo before transfer to the uterus, and identifying the abnormal gene by FISH (fluorescent in situ hybridization). While the screening process is in progress, the embryo is cultured to maintain viability. The biopsy process, however, causes cellular damage and stress to the live embryo. To overcome the cellular damage, the embryo can be cultured for a length of time before being transferred to a recipient surrogate.

In the past human embryos were transferred after the second or third cell cycle, therefore transferring a 4-cell to 8-cell embryo back into the donors uterus, while the embryo should at that stage still be in the oviduct. The uterus of a donor is not an appropriate environment for an embryo at this early stage, because it was under the influence of very high estrogen levels just two to four days prior when the ova were collected after a hyper-stimulation cycle. If the embryo could be cultured *in vitro* for another couple of days to reach the blastocysts stage, it would increase its chances of implantation to a large extent. Due to the smaller size of the blastomeres of the blastocyst stage of the embryo, compared to the 8-cell stage of the embryo, the embryos will also survive the freezing process better. This *in vitro* culture period has to take place on co-culture (a monolayer of somatic cells), or when in the absence of co-culture, in the presence of essential growth factors and stimulants.

4.3 Hypothesis

First trimester amniotic fluid can support development of different quality grade ruminant embryos from the pre-compacted morula stage to the hatched blastocyst stage.

4.4 Material and methods

4.4.1 Experimental design

Pre-compacted morula stage embryos of sheep, goats and cows were cultured in two different culture media in a standard laboratory incubator for in 4-well plates for 120 h as follows:

Embryo Grade	Culture medium	Culture period
1	TCM-199	120 h
2	TCM-199	120 h
3	TCM-199	120 h
1	BAF	120 h
2	BAF	120 h
3	BAF	120 h

4.4.2 Method

Pre-compacted morulae (16-cell stage) were collected from sheep, goats and cattle as described in Chapter 2. In summary: The estrous cycles of the female animals were synchronized with fluorogestone acetate intra-vaginal sponges (Chronogest, Intervet, The Netherlands – sheep), or progesterone intra-vaginal Y-pieces (Eazi-Breed CIDR, InterAg, New Zealand – goats) or norgestomet ear implants (Crestar, Intervet, The Netherlands - cows). The development of the present wave of follicles, with or without its dominant follicle, was then suppressed with an intramuscular injection of estradiol cypionate (ECP. ICI, Johannesburg, South Africa). Five days

after the ECP injection at the emergence of the next wave of follicular development, the follicles were stimulated to develop to the ovulatory stage with a series of FSH (Ovagen, ICP, New Zealand) injections. The embryos were collected surgically under general anaesthesia (sheep and goats), or trans-cervically (cows) 3 d to 4 d after ovulation.   The collected medium was concentrated through an EmCon filter (Agtech, USA) and the pre-compacted stage embryos and unfertilized ova identified and isolated, and then transferred to fresh filter-sterilized holding medium (D-PBS with 10% of fetal bovine serum and 1% anti-biotic-antimycotic).

After washing the pre-compacted embryos three times in holding medium, they were carefully evaluated by two independent technicians at 80X or more with an inverted microscope. The following criteria were used when the embryos were evaluated for different quality grades:

Symmetry: the zona pellucida had to be spherical, and not oval or flattened, and be intact without any cracks or rents.

Embryo contents: the embryo inside the zona pellucida must, at the pre-compacted morula stage, not fill the entire space within the zona pellucida.

Blastomere damage: individual blastomeres must be transparent without any sign of vacuolization, granularity or pigmentation.

Extruded cells: this is an indication that some of the blastomeres died during the developmental period. The percentage of extruded cells influenced the grading of the embryo.

Embryo grades:

Grade 1: Less than 5% of the total number of blastomeres were damaged or extruded. The zona pellucida must be perfectly spherical, intact and clean.

Grade 2: Between 5 and 25% of the blastomeres could be damaged or extruded. The zona pellucida was sometimes not perfectly spherical.

Grade 3: More than 25% of the blastomeres of the embryo were damaged or extruded. The zona pellucida was often not spherical.

Grade 4: The embryos were totally degenerated, or were unfertilized ova.

The different quality grades of embryos were cultured in 4-well plates (Nunc, UK) in groups of not more than five embryos. The wells contained either 1 mL of pre-incubated TCM-199 with heat inactivated 10% fetal bovine serum (FBS, Highveld Biologicals, Johannesburg, South Africa) and 1% antibiotic-antimycotic (Sigma, USA, Cat A-5995), or pre-incubated heat inactivated first trimester bovine amniotic fluid (BAF) with the same additives as in TCM-199. The first trimester bovine amniotic fluid was collected as described in Chapter 2. In summary: Several pregnant bovine reproductive tracts containing a fetus of ≤ 10 cm head-tail base length (~60 days pregnancy) was collected under sterile and cooled conditions from the local abattoir. The uterine wall was dissected open and the amniotic fluid aspirated with a sterile rubberless 50 mL syringe (Normo-Ject Zentrisch, Henke Sass Wolf, Germany) and an 18 gauge needle. The individual bovine amniotic fluid (BAF) collections were then pooled, heat inactivated at 56 ºC for 30 minutes, and stored at 4 ºC in screw cap 15 mL plastic vials (Cellstar, Greiner Bio-One) until needed.

The 1 mL of medium in each well was covered with washed, sterile mineral oil, and then cultured in an automatic 5% CO_2 incubator with 100% humidified air for 120 hours at 38.5 ºC. The embryos were evaluated every 24 hours under an inverted microscope at 80X to 200X for further development. Half of the medium was changed every 24 hours. No co-culture was used at any stage.

4.5 Statistical analysis

Data reported in this manuscript are as mean ± SD. All data were analyzed using the Student's t-test assuming two-sample means with unequal variances (Microsoft Excell).

4.6 Results

The results of the development of the three different grades of pre-compacted morulae of the different species (sheep, goats and cows) and the numbers of embryos that reached the blastocyst and hatched blastocyst stages after 5 days of culturing in the two culture media are represented in Table 4.1 (sheep), Table 4.2 (goats) and Table 4.3 (cows).

Table 4.1. Development of ovine embryos of three different quality grades cultured in TCM-199 and BAF

TCM-199					Bovine Amniotic Fluid				
Embryo Quality	n	Mor (%)	Blast (%)	HB (%)	Embryo Quality	n	Mor (%)	Blast (%)	HB (%)
Grade 1	45	3 (6.6)	4 (8.9)	38 (84.4)^a	Grade 1	46	7 (15.2)	6 (13.0)	33 (71.4)^a
Grade 2	58	8 (13.7)	24 (41.4)	26 (44.8)^a	Grade 2	60	10 (1.6)	21 (35.0)	29 (48.2)^a
Grade 3	61	18 (29.5)	32 (52.5)	11 (18.0)^a	Grade 3	65	11 (16.9)	26 (40.0)	28 (43.1)^b
Total	164	29 (17.7)	60 (36.6)	75 (53.6)^a	Total	171	28 (16.3%)	53 (31.0)	90 (56.1)^a

[ab] Different superscripts in the same row are significant (p<0.01)
Mor = Morula stage; Blast = blastocyst stage; HB = Hatched blastocyst stage of the embryo

Table 4.2. Development of caprine embryos of three different quality grades cultured in TCM-199 and BAF

	TCM-199				Bovine Amniotic Fluid				
Embryo Quality	n	Mor (%)	Blast (%)	HB (%)	Grade	n	Mor (%)	Blast (%)	HB (%)
Grade 1	121	5	22 (18.2)	94 (77.7)[a]	1	124	4	19 (15.3)	101 (81.5)[a]
Grade 2	87	17	33 (37.9)	37 (42.5)[a]	2	85	16	31 (36.5)	38 (44.7)[a]
Grade 3	51	15	24 (47.1)	12 (23.5)[a]	3	55	9	19 (34.5)	27 (49.1)[b]
Total	259	37	79 (30.5)	143 (55.2)[a]	Total	264	29	69 (26.1)	166 (62.9)[a]

[ab] Different superscripts in the same row are significant ($p < 0.05$)
Mor = Morula stage; Blast = blastocyst stage; HB = Hatched blastocyst stage of the embryo

Table 4.3. Development of bovine embryos of three different quality grades cultured in TCM-199 and BAF in a standard laboratory incubator

	TCM-199				Bovine Amniotic Fluid				
Embryo quality	n	Mor (%)	Blast (%)	HB (%)	Grade	n	Mor (%)	Blast (%)	HB (%)
Grade 1	98	10	22 (22.4)	66 (67.3)[a]	1	97	11	19 (19.6)	67 (69.1)[a]
Grade 2	98	3	32 (32.7)	63 (64.3)[a]	2	98	14	24 (24.5)	60 (61.2)[a]
Grade 3	72	25	35 (48.6)	12 (16.7)[a]	3	75	12	23 (30.7)	40 (53.3)[b]
Total	258	38	89 (33.2)	141 (52.6)[a]	Total	270	37	66 (24.4)	194 (71.6)[b]

[ab] Different superscripts in the same row for HB are significant ($p < 0.01$)
Mor = Morula stage; Blast = blastocyst stage; HB = Hatched blastocyst stage of the embryo

Figures 4.1 to 4.3 represent different quality grades of bovine embryos before culture.

Figure 4.4 represents a hatched blastocyst stage embryo cultured in TCM-199.

Figure 4.5 shows a hatched blastocyst stage bovine embryo cultured in BAF.

Figure 4.6 represents an ovine hatched blastocyst stage embryo that developed in BAF from the pre-compacted morula stage.

Figures 4.7 and 4.8 represent two caprine embryos reaching the hatching blastocyst stage in TCM-199 and BAF, respectively.
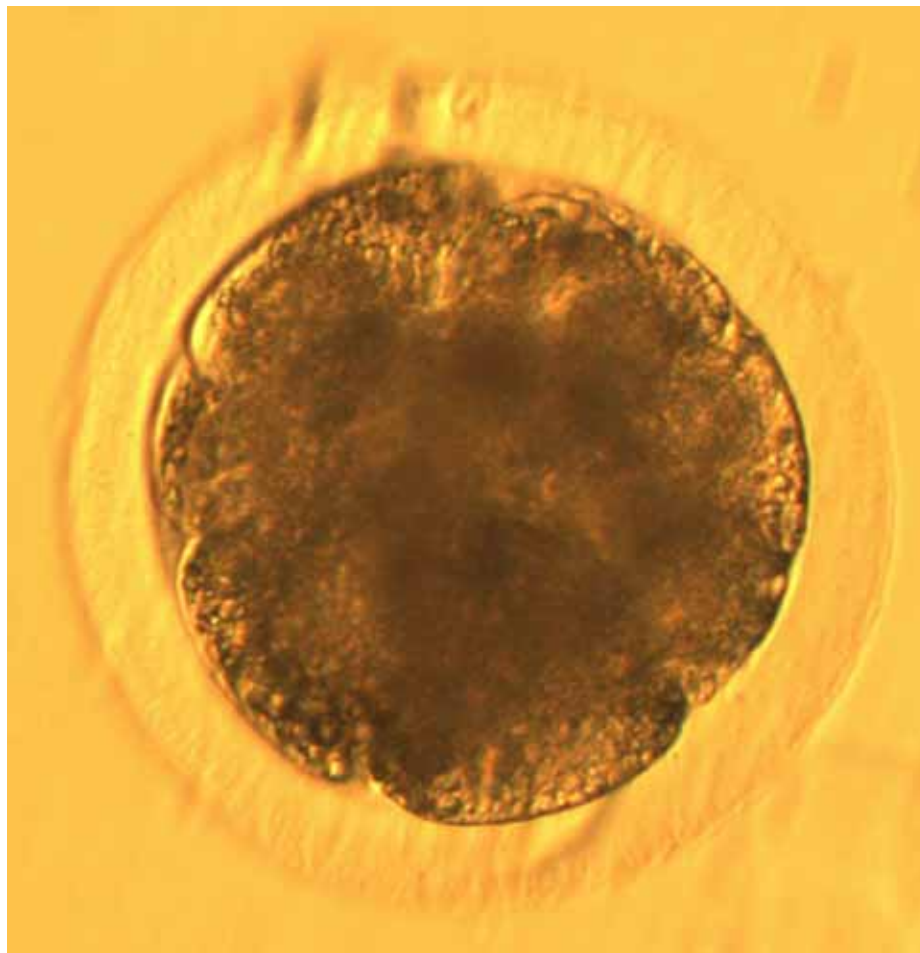
Figure 4.1. Grade 1 bovine pre-compacted morula stage embryo.

Figure 4.2. Grade 2 bovine pre-compacted morula stage embryo.

Figure 4.3. Grade 3 bovine pre-compacted morula stage embryo.
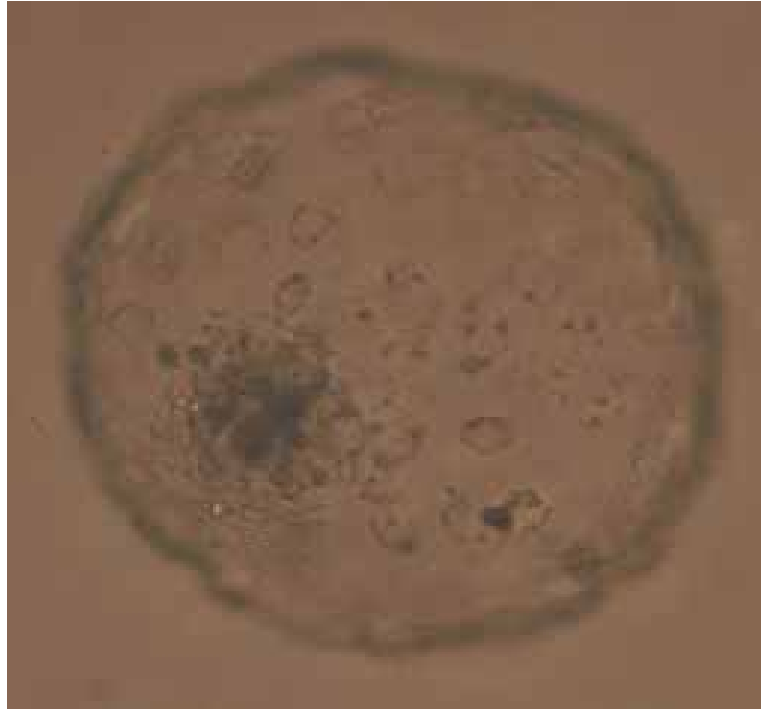
Figure 4.4 Bovine hatched blastocyst stage embryo cultured in TCM-199.

Figure 4.5. Bovine hatched blastocyst stage embryo cultured in BAF.
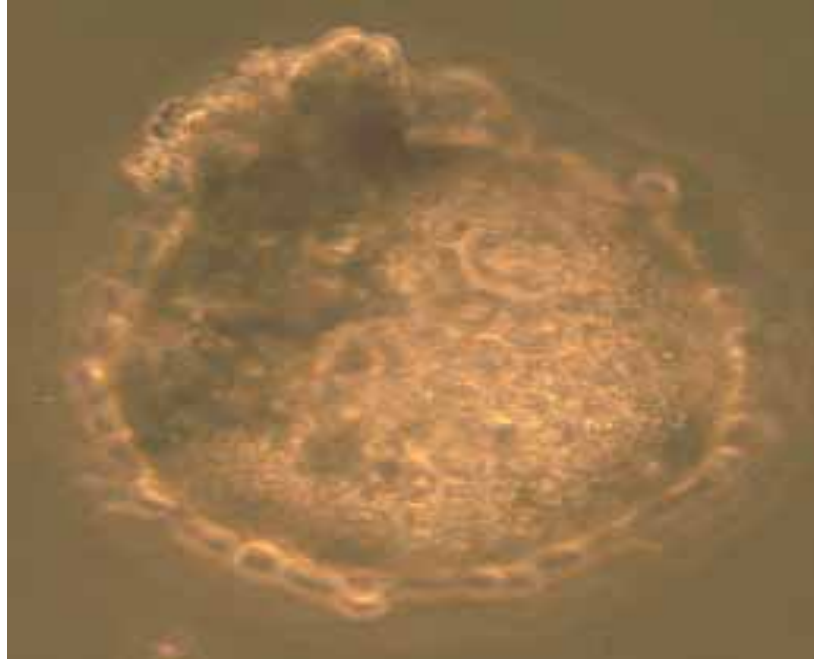
Figure 4.6. Ovine hatched blastocyst stage embryo developed from a grade 3 pre-compacted morula stage embryo in BAF.
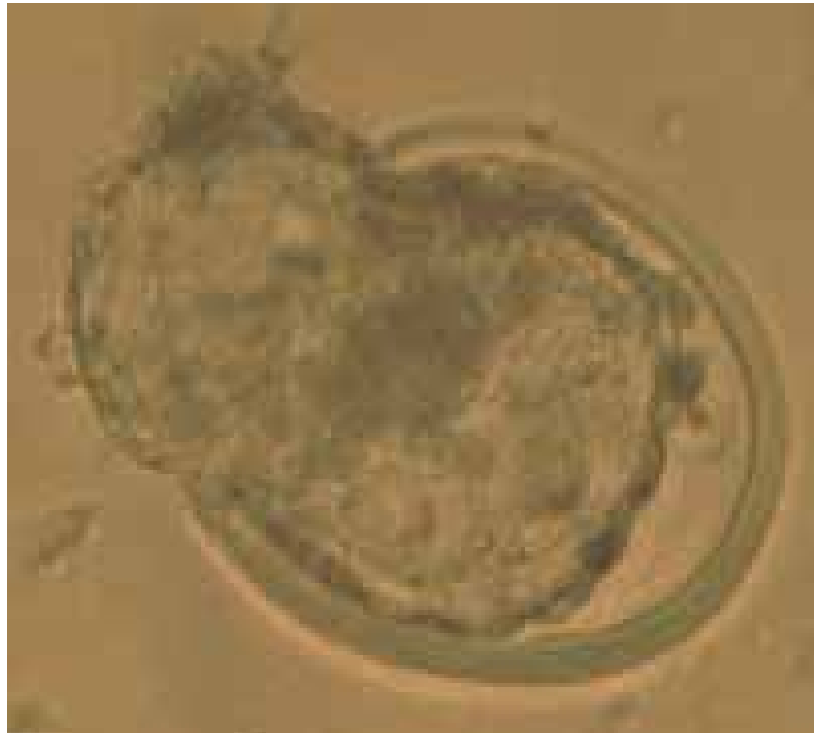
Figure 4.7. Hatching blastocyst stage caprine embryos developed from a grade 3 pre-compacted morula stage embryo in TCM-199 after 72 h of culturing.

Figure 4.8. Hatching blastocyst stage caprine embryo cultured in
BAF for 72 h.

4.7 Discussion

4.7.1 Sheep

A high number of the Grade 1 ovine embryos cultured in both the TCM-199 and the first trimester bovine amniotic fluid (BAF) reached the hatched blastocyst stage (84.4% and 71.4% respectively). There was, however, no significant difference between the number of these embryos that developed to the blastocyst or hatched blastocyst stage in the TCM-199 and the BAF groups (p=0.14). In the Grade 2 ovine embryos cultured in TCM-199, 41.4% reached the blastocyst stage and 44.8% reached the hatched blastocyst stage. This was, however, not different (p=0.70) from the number of embryos cultured in BAF that reached the blastocyst (35.0%) or hatched blastocyst stage (48.2%). When the Grade 3 group of embryos that were cultured in TCM-199 is compared to the Grade 3 embryos that were cultured in first trimester amniotic fluid, there was a significant difference (p<0.01) between the number of embryos that developed to the hatched blastocyst stage (18.0% vs 43.1%, respectively). If the three grades of embryos are added together, there is no significant difference in the total number of ovine embryos that hatched between the two culture mediums.

4.7.2 Goats

In the three quality grades of caprine embryos, the same tendency as in sheep was noticed. Less Grade 1 caprine embryos that were cultured in TCM-199 (77.7%) developed to the hatched blastocyst stage than embryos that were cultured in bovine first trimester amniotic fluid (81.5%), but this difference was not significant (p=0.46) A lower number of Grade 2 caprine embryos that were cultured in TCM-199 (42.5%) developed to the hatched blastocyst stage, than the Grade 3 embryos that were cultured in BAF (44.7%), but again this was not significant (p=0.44). Significantly more Grade 3 pre-compacted morula-stage caprine embryos that were cultured in

bovine first trimester amniotic fluid developed to the hatched blastocyst stage (49.1%) than similar Grade 3 caprine embryos that were cultured in a standard animal embryo culture medium, TCM-199 (23.5%) (p<0.05). When the three embryos grades were added together, there was no significant difference in the total number of caprine embryos that developed to the hatched blastocyst stage between the two culture mediums.

4.7.3 Cows

The same trend as was seen in the sheep and goat embryo development in the TCM-199 and the BAF, was also found when the development of the pre-compacted morula stage bovine embryos in the two culture media were compared. Although slightly more Grade I bovine pre-compacted-stage morula embryos developed to the hatched blastocyst stage (69.1%) in first trimester amniotic fluid, than similar embryos cultured in TCM-199 (67.3%), this difference was not significant (p=0.79). When the Grade 2 bovine pre-compacted-stage embryos were cultured in TCM-199, more embryos developed to the hatched blastocyst stage (64.3%) than when similar embryos were cultured in fresh first trimester bovine amniotic fluid. Significantly more Grade 3 bovine pre-compacted morula-stage embryos reached the hatched blastocyst stage when cultured in first trimester bovine amniotic fluid (53.3%) than those cultured in TCM-199 (16.7%) (p<0.01). When the three different quality-grades of embryos are added together, and then compared, the number of bovine pre-compacted morula-stage embryos that developed to the hatched blastocyst stage in early pregnancy-stage bovine amniotic fluid (71.6%), were significantly greater than those cultured in TCM-199 (52.6%) (p<0.01).

4.8 Conclusion

It was shown that both TCM-199, a standard embryo culture medium, as well as bovine amniotic fluid collected from early pregnancies (less than 60 d), will support embryo development of different quality grades of embryos of sheep, goats and cows from the pre-compacted morula stage to the hatched blastocyst stage. When the two culture mediums are compared in the development of all the quality grades of pre-compacted morula-stage embryos, only the Grade 3 (most damaged) embryos in sheep, goats and cows will developed significantly better to the hatched blastocyst stage when cultured in BAF than in TCM-199. When the three quality grades of embryos in each species were pooled, it was only in the bovine species that significantly more pre-compacted morula-stage embryos developed to the hatched blastocyst stage when cultured in BAF than in TCM-199 ($p<0.01$).

The results indicate the probability of a possible "rescue" factor present in amniotic fluid secreted by the amniotic wall, or by cells (stem cells?) present in the amniotic fluid. Two important molecules were identified (Fraidenraich *et al.* 2004) implicated in signaling from embryonic stem cells to damaged or partly damaged cells. These molecules are insulin-like growth factor 1 (IGF-1) and WNT5a protein. The latter is an oncogene protein that belongs to the family of presumptive signaling molecules that have been implicated not only in the regulation of normal pattern formation during embryogenesis and differentiation of cell lineages, but also in oncogenic events (Clark *et al.* 1993). IGF-1, on the other hand, plays an active role in embryonic development by having both a paracrine effect on neighboring cell development and autocrine effect on itself (Evain-Brion 1993). Wang *et al.* (1990) identified an insulin-like growth factor in ovine amniotic fluid that plays a significant role in embryo development. It is postulated that these and other proteins present in first trimester bovine amniotic fluid played an active role in the

revival of especially the Grade 3 pre-compacted morula-stage embryos of the sheep, goat and cow when cultured in the bovine amniotic fluid from early pregnancies. In these <60 d pregnancies (crown-rump length < 10 cm), the amniotic fluid is small in volume and therefore have high concentrations of the growth factors that play a role in the development of the fetus.

4.9 References

Ackerman, S.B., Taylor, S.P., Swanson, R.J. & Laurell, L.H. (1984). Mouse embryo culture for screening in human IVF. *Arch Androl* **12** Suppl. 129-136.

Ball, B.A., Altschul, M. and Ellington, J.E. (1991). *In vitro* development of Day-2 equine embryos co-cultured with oviductal explants or trophoblastic vesicles. *Theriogenology* **35** (3), 669-682.

Bhuiyan, M.M.U., Jong-ki, C., Jang, G., Park, E-s., Kang, S-k., Lee, B-c. and Hwang W-s. (2004). Effect of protein supplementation in potassium simplex optimization medium on pre-implantation development of bovine non-transgenic and transgenic embryos. *Theriogenology* **62**, 1403-1416.

Blakewood, E.G., Jayenes, J.M., Johnson, J.M. & Godke, R.A. (1989). Using the amniotic cavity of the developing chick embryo for *in vivo* culture of early-stage mammalian embryos. *Poultry Science* **68** (12), 1695-1702.

Carney, E.W., Tobback, C. & Foote, R.H. (1990). Co-culture of rabbit one-cell embryos with rabbit oviduct epithelial cells. *In vitro Cell Dev Biol* **26** (6), 629-635.

Carney, E.W. and Foote, R.H. (1991). Improved development of rabbit one-cell embryos to the hatching blastocyst stage by culture in a defined, protein-free culture medium. *Journal of Reproduction Fertility* **91** (1), 113-23.

Caro, C.M., Trounson, A. and Kirby, C. (1987). Effect of growth factors in culture medium on the rate of mouse embryo development and viability *in vitro*. *Journal of In vitro Fertilization and Embryo Transfer* **4** (5), 265-268.

Cavanagh, A.C. (1984). Production *in vitro* of mouse early pregnancy factor and purification to homogeneity. *Journal of Reproduction and Fertility* **71** (2), 581-592.

Chatot, C.L., Lewis, J.L., Torres, I. and Ziomek, C.A. (1990a). Development of 1-cell embryos from different strains of mice in CZB medium. *Biology of Reproduction* **42** (3), 432-440.

Chatot, C.L., Tasca, R.J. and Ziomek, C.A. (1990b). Glutamine uptake and utilization by pre-implantation mouse embryos in CZB medium. *Journal of Reproduction and Fertility* **89** (1), 335-346.

Clark, C.C., Cohen, U., Eichstetter, I., Cannizzaro, L.A., McPherson, J.D., Wasmuth, J.J. and Iozzo, R.V. (2003). Molecular cloning of the human proto-oncogene Wnt-5A and mapping of the gene (WNT5A) to chromosome 3p14-p21. *Genomics* **18** (2), 249-260.

Coetzee, J.C., Sevenster, C.B., Fourie, F.L., van der Merwe, J.V. & Helberg, L.A. (1989). *In vitro* culture of mouse embryos in human amniotic fluid. *South African Medical Journal* **76** (2), 62-63.

Cohen, J., Simons, R.F., Edwards, R.F., Fehilly, C.B. & Fishel, S.B (1985). Pregnancies following the frozen storage of expanding human blastocysts. *Journal of In vitro fertilization and Embryo Transfer* **2** (2), 59-64.

Conaghan, J., Hardy, K., Leese, H.J., Winston, R.M. & Handyside AH. (1998). Culture of human pre-implantation embryos to the blastocyst stage: a comparison of three media. *Int J Dev Biol* **42** (7 Spec No), 885-893.

Cuello, C., Antonia Gil, M., Parilla, I., Tornel J., Vazquez, M., Roca, J., Berthelot, F.M-B. & Martinez, A. (2004). Vitrification of porcine embryos at various developmental stages using different ultra-rapid cooling procedures. *Theriogenology* **62**, 353-361.

Dandekar, P.V. and Glass, R.H. (1987). Development of mouse embryos *in vitro* is affected by strain and culture medium. *Gamete Research* **17** (4), 279-285.

Delhanty, J.D. (1994) Pre-implantation diagnosis. *Prenat Diagn* **14** (13), 1217-1227

De Vos, A. and Steirteghem, A. (2001). Aspects of biopsy prior to pre-implantation genetic diagnosis. *Prenat Diagn* **21** (9), 767-780.

Dorfmann, A.D., Bender, S.D., Robinson, P., Fugger, E.F., Bustillo, M., Reed, G. & Schulman, J.D. (1989). Cell-free human amniotic fluid as culture medium for mouse and human embryos. *Fertility and Sterility* **51** (4), 671-674.

Elsden, R.P.,. Nelson, L.D. and Seidel, G.E. (1978). Super-ovulating cows with follicle stimulating hormone and pregnant mare's serum gonadotropin. *Theriogenology* **9** (1), 17-26.

Erbach, G.T., Lawitts, J.A., Papaioannou, V.E. & Biggers, J.D. (1994). Differential growth of the mouse pre-implantation embryo in chemically defined media. *Biology of Reproduction* **51** (2), 345.

Evain-Brion, D. (1993). Reproduction in Mammals and Man, Editors C Thibault, M-C Levasseur and RHF Hunter, Ellipses, Chapter 9, P167-177.

Fraidenraich D., Stelwell, E., Romero, E., Wilkes, D., Manova, K., Basson, C. and Benezra, R. (2004). Rescue of cardiac defects in *Id* knockout embryos by injection of embryonic stem cells. *Science* **306** (5694), 247-252.

Gardner, D.K., Lane M. (1996). Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters. *Human Reproduction* **11** (12), 2703-2712.

Gianaroli, L., Seracchioli, R., Ferraretti, A.P., Trounson, A., Flamigni, C. & Bovicelli, L. (1986). The successful use of human amniotic fluid for mouse embryo culture and human *in vitro* fertilization, embryo culture, and transfer. *Fertility and Sterility* **46** (5), 907-913.

Han, H.D. and Kiessling, A.A. (1988). *In vivo* development of transferred mouse embryos conceived *in vitro* in simple and complex media. *Fertility and Sterility* **50** (1), 159-163

Handyside, A.H., Pattison, J.K., Penketh, R.J., Delhanty, J.D., Winston, R.M. and Tudenham, E.G. (1989). Biopsy of pre-implantation embryos and sexing by DNA amplification. *Lancet* **18** (1), 347-349.

Handyside, A.H., Kontigianni, E.H., Hardy, K and Winston, R.M. (1990). Pregnancies from biopsied pre-implantation human embryos sexed by Y-specific DNA amplification. *Nature* **344**, 768-770.

Hardy, K. and Handyside, A.H. (1992). Biopsy of cleavage stage human embryos and diagnosis of single gene defects by DNA amplification. *Arch Pathol Lab Med* **116** (4), 388-392.

Heyman, Y. & Renard, J.P. (1996). Cloning of domestic species. *Animal reproduction Science* **42**, 427-436.

Huisman, G.J., Fauser, B.C.J.M., Eijkemans, M.J.C., and Pieters, H.E.C. (2000). Implantation rates after *in vitro* fertilization and transfer of a maximum of two embryos that have undergone three to five days of culture. *Fertility and Sterility* **73** (1), 117-122.

Jin, D.I., Kim, D.K., Im, K.S. and Choi, W.S. (2000). Successful pregnancy after transfer of rabbit blastocysts grown *in vitro* from single-cell zygotes. *Theriogenology* **54**, 1109-1116.

Krisher, R.L., Lane, M. & Bavister, B.D. (1999). Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. *Biology of Reproduction* **60** (6), 1345-1352.

Kruger, T.F. and Stander, F.S. (1985). The effect of cleavage of two-cell mouse embryos after a delay in embryo retrieval in a human *in vitro* fertilization program. *South African Medical Journal* **68** (10), 743-744.

Kuzan, F.B. & Wright, R.W. (1982). Observations on the development of bovine morulae on various cellular and non-cellular substrata. *Journal of Animal Science* **54** (4), 811-816.

Lai, Y.M., Stein, D.E., Soong, Y.K., Tang, Y.X., Grifo, J., Malter, H.E., Talansky, B.E., Cohen, J., Liu, H.C. & Rosenwaks, Z. (1992). Evaluation of Vero cell co-culture system for mouse embryos in various media. *Human Reproduction* **54** (4), 811-816.

Lawitts, J.A. and Biggers, J.D. (1991). Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium. *Biology of reproduction* **45** (2), 245-251.

Lee, B., Wirtu, G.G., Damiani, P., Dresser, B.L., Hwang, W. and Bavister, B.D. (2003). Blastocyst development after intergeneric nuclear transfer of mountain bogo antelope somatic cells into bovine oocytes. *Cloning and Stem Cells* **5** (1), 25-33.

Li, J., Foote, R.H., Liu, Z. and Giles, J.R. (1996). Development of rabbit zygotes into blastocysts in defined protein-free medium and offspring born following culture and embryo transfer. *Theriogenology* **47**, 1103-1113.

Lindner, G.M. and Wright, R.W. (1983). Bovine embryo morphology and evaluation. *Theriogenology* **20** (4), 407-416.

Liu, Z., Foote, R.H. and Yang, X. (1994). Development of early bovine embryos in co-culture with KSOM and taurine, superoxide dismutase or insulin. *Theriogenology* **44**, 741-750.

Nakao, H. and Nakatsuji, N. (1990). Effects of co-culture, medium components and gas phase on *in vitro* culture of *in vitro* matured and *in vitro* fertilized bovine embryos. *Theriogenology* **33** (3), 591-600.

Ng, S.C., Sathananthan, H., Bongso, A., Lee, M.N., Mok, H., Wong, P.C. & Ratnam, S.S. (1988). The use of amniotic fluid and serum with propanediol in freezing of murine 2-cell embryos. *Fertility and Sterility* **50** (3), 510-513.

Oettle, E.E. & Wiswedel, K. (1989). Mouse embryos cultured in amniotic fluid. *South African Medical Journal* **76** (2), 63-64.

Ogawa, S. & Tomoda, S. (1976). Survival of 16-cell and morula stage rabbit embryos frozen to -196 degrees C. *Jikken Dobutso* **25** (4), 273-282.

Olar, T.T. & Potts, A.S. (1993). Effects of medium composition on murine and human blastocyst formation and hatching rate. *Journal of Assisted Reproductive Genetics* **10** (3),192-196.

Ornoy, A., Yacobi, S. & Yaffee P. (2003). A simple method of culture of 11.5-day-old rat embryos in DMEM/F12 and 20% fetal bovine serum. *Journal of Anatomy* **203** (4), 419-423.

Petters, R.M. & Wells, K.D. (1993). Culture of pig embryos. *Journal of Reproduction and Fertility* **48**, 61-73.

Putney, D.J., Drost, M. and Thatcher, W.W. (1989). Influence of summer heat stress on pregnancy rates of lactating dairy cattle following embryo transfer or artificial insemination. *Theriogenology* **31** (4), 765-778.

Richards, D.W., Quinn, P., Stone, B.A. and Marrs, R.P. (1990). Effect of human follicular fluids from pregnant and non-pregnant patients on the development of mouse zygotes *in vitro*. *J In vitro Fert Embryo Transf* **7** (1), 22-27.

Rondeau, M., Guay, P., Goff, A.K. & Cooke, G.M. (1996). Growth and metabolism of murine and bovine embryos in bovine uterine flushing-supplemented culture media. *Can J Vet Res* **60** (1),14-20.

Roudebush, W.E., Often, N.L. & Butler, W.J. (1994). Alpha-minimum essential medium (MEM) enhances *in vitro* hatched blastocysts development and cell number per embryo over Ham's F-10. *Journal of Assisted Reproductive Genetics* **11** (4), 203-207.

Sakkas, D. and Trounson, A.O. (1991). Formulation of a complex serum-free medium (CSM) for use in the co-culture of mouse embryos with cells of the female reproductive tract. *Reproduction and Fertility* **3** (1), 99-108.

Schneider, H.J., Castleberry, R.S. and Griffin, J.L. (1980). Commercial aspects of bovine embryo transfer. *Theriogenology* **13** (1), 73-85.

Scott, L., Mackow, K., Hosid, S., Dekkelbaum, R. and Smith, S. (1997). The association between the glucose-inorganic phosphate mediated and the hypoxanthine-mediated 2-cell block in mouse pre-implantation embryos *in vitro*. *Fertility and Sterility* **68** (supplement 1), S54 (Abstract).

Shultz, G.A. and Heiner, S. (1992). Gene expression in pre-implantation mammalian embryos. Mutation research/*Reviews in Genetic Toxicology* **296**, 17-31.

Seidel, G.E., Bowen, R.A. & Kane, M.T. (1976). *In vitro* fertilization, culture, and transfer of rabbit ova. *Fertility and Sterility* **27** (7), 861-870.

Sparks, A.E.T., Gwazdauskas, F.C. and McGilliard, M.L. (1992). Culture of one-cell bovine embryos in explanted mouse oviducts and bovine epithelial cells. *Theriogenology* **37** (3), 587-594.

Spindle A. (1980). An improved culture medium for mouse blastocysts. *In vitro Cellular Developmental Biology* **16** (8), 669-674.

Spindle A. (1990). *In vitro* development of one-cell embryos from outbred mice: influence of culture medium composition. *In vitro Cellular Developmental Biology* **26** (2), 151-156.

Summers, M.C., Bhatnagar, P.R., Lawitts, J.A. and Biggers, J.D. (1995). Fertilization *in vitro* of mouse ova from inbred and outbred strains: complete pre-implantation embryo development in glucose-supplemented KSOM. *Biology of Reproduction* **53** (2), 431-437.

Summers, M.C., McGinnis, L.K., Lawitts, J.A., Raffin, M. and Biggers, J.D. (2000). IVF of mouse ova in a simplex optimized medium supplemented with amino acids. *Human Reproduction* **15** (8), 1791-1801.

Tervit, H.R., Cooper, M.W., Goold, P.G. and Haszard, G.M. (1980). Non-surgical embryo transfer in cattle. *Theriogenology* **13** (1), 63-71.

Tervit, H.R. and Elsden, R.P. (1981). Development and viability of frozen-thawed cattle embryos. *Theriogenology* **15** (4), 395-403.

Tominaga, K. (2004). Cryopreservation and sexing of *in vivo* and *in vitro*-produced bovine embryos for their practical use. *Reprod Dev* **50** (1), 29-38.

Vajta, G., Rindom, N., Peura, T.T., Holm, P., Greve, T. & Callesen, H. (1999). The effect of media, serum and temperature on the *in vitro* survival of bovine blastocysts after open pulled straw (OPS) vitrification. *Theriogenology* **52**, 939-948.

Veeck, L.L., Bodine, R., Clarke, R.N., Berrios, R., Libraro, J., Moschini, R.M., Zaninovic, N. and Rosenwaks, Z. (2004). High pregnancy rates can be achieved after freezing and thawing human blastocysts. *Fertility and Sterility* **82** (5), 1418-1427.

Wang, J.F., Fraher, L.J. & Hill, D.J. (1990). Characterization of insulin-like growth factor-binding protein in ovine amniotic fluid. *Journal of Endocrinology* **127** (2), 325-333.

Wirtu, G., Pope, C.E., Damiani, P., Miller, F., Dresser, B.L., Short, C.R., Godke, R.A and Bavister, B.D. (2004). Development of *in vitro* derived bovine embryos in protein-free media: effects of amino acids, glucose, pyruvate, lactate, phosphate and osmotic pressure. *Reproduction and Fertility Development* **15** (8), 439-449.

Zeng,W. and Zeng, Z. (1998). Improvement of mouse early embryo development *in vitro* by co-culture with human oviductal epithelial cells in serum-free medium. *Zhonghua Fu Chan Ke Za Zhi* **33** (10), 604-606.

Zheng, P., Wang, H., Bavister, B.D. and Ji, W. (2001). Maturation of rhesus monkey oocytes in chemically defined culture media and their functional assessment by IVF and embryo development. *Human Reproduction* **16** (2), 300-305.

Zhu, B.K., Walker, S.K. and Maddocks, S. (2004). Optimization of *in vitro* culture conditions in B6CBF1 mouse embryos. *Reprod Nutr Dev* **44** (3), 219-231.

CHAPTER 5

THE EFFECT OF CULTURING FROZEN-THAWED CAPRINE EMBRYOS
IN THE VAGINA OF A GOAT DOE
ON THEIR SURVIVAL POST TRANSFER

5.1 Abstract

The effect of co-culture on the survival of caprine embryos post transfer to a synchronized recipient female goat was assessed. A total of 120 Kashmir embryos at the blastocyst stage were divided into three groups after thawing and reconstitution in four steps in glycerol and sucrose medium. The first group of embryos (G1, n=40) were singly transferred semi-laparoscopically in D-PBS with 10% FBS and antibiotics to the ipsi-lateral horn, as the CL over a period of three days. The second group of caprine blastocysts (G2, n=40) was similarly transferred in TCM-199 with FBS and antibiotics. The third group of frozen-thawed caprine blastocyst-stage embryos (G3, n=40) were first co-cultured for ~24 h in TCM-199 with serum and antibiotics in groups of up to five embryos inside a ~50 mm length of a semen straw in a cylindrical sponge in the anterior part of the vagina of a goat doe in her luteal phase. After the culture period, these embryos were transferred in a similar way in TCM-199 without the co-culture as in G1 and G2. Ultrasound scanning showed that significantly more of the blastocyst embryos that were co-cultured in the vagina (G3) before transfer, developed to a pregnancy compared with the embryos transferred in D-PBS (G1). The co-culture Group 3 blastocyst-stage caprine embryos produced significantly more offspring than the non-cultured embryos transferred in both D-PBS (G1) and TCM-199 (G2).

5.2 Introduction

One of the incentives for culturing mammalian embryos is to increase the chances of a frozen-thawed embryo to develop into a pregnancy and live offspring. After thawing, the thawed embryo is often co-cultured for a period of 6 to 24 hours in a standard laboratory 5% CO_2 incubator, thus overcoming the stress and blastomere damage caused by the freezing process. Research has shown that by co-culturing frozen-thawed mammalian embryos for a period of time (Pugh *et al.* 1998, 2000), the implantation rate of the transferred embryos can be increased. Post-thaw expansion and further development of frozen-thawed embryos during co-culture are also used as indicators of embryo survival rate (Dresser *et al.* 1988; Ziebe *et al.* 1998; Mauri *et al.* 1999; Keskintepe and Brackett 2000).

As early as 1994 Keskintepe *et al.* (1994) reported on the *in vitro* collection, maturation, fertilization and co-culture of caprine embryos, with live offspring born when the embryos were transferred. The highest proportions of cleavage (23/27, 85.2%) and morula-stage embryos (14/27, 51.9%) were obtained by co-culture on caprine cumulus cells. Park *et al.* (2000) found a beneficial effect on the development of co-cultured DNA-injected bovine embryos when he reported that *in vitro* embryo development to the blastocyst stage and subsequent *in vivo* development to term of DNA-injected bovine embryos was improved when the last 5 d of *in vitro* culture were in a murine embryonic fibroblast cell co-culture system in comparison to culture in CR1aa alone.

Desai *et al.* (2000) studied the effect of different growth factors (LIF, IL6, TGF, EGF, IGF-I, IGF-II and TGF ß) and co-culture on the development *in vitro* of frozen-thawed murine embryos. Frozen-thawed murine morula-stage embryos progressed much more rapidly to fully expanded blastocysts either in the presence of these growth factors or in the co-culture environment. Even at

30 h post-thaw, control morulae were lagging behind the co-culture and growth factor-treated embryos. Only 73% of thawed morulae in the control group were able to complete development to the fully expanded blastocyst stage. In contrast, 99% of co-cultured morulae and 90–96% of morulae from growth factor-treated groups were fully differentiated after 30 h of culture. At the 30 h observation point, they also noted a significant difference in embryonic hatching amongst treatment groups. With the exception of PDGF, all growth factors significantly enhanced embryonic hatching. The hatching rate was 61% for control embryos as compared to 82–89% upon supplementation of post-thaw culture medium with individual growth factors (LIF 84%; IL6 85%; TGFα 84%; EGF 87%; IGF-I 82%; IGF-II 89% and TGF ß 83%; $P < 0.005$). Co-culture yielded 96% of hatching, the highest percentage of all the factors. Thawed control embryos cultured for 48 h in medium alone had a mean cell count of 131 ± 36. With the exception of LIF and TGFα, all of the growth factor additives significantly increased total blastomere number in developing embryos when contrasted to the control ($P < 0.005$). Co-culture once again yielded the best results with a mean cell count of 217 ± 76 cells per blastocyst. These results indicate that the post-thaw development of embryos can be modulated by addition of growth factors. It also shows that culture factors can accelerate the progression of thawed morulae to the hatched blastocyst stage, increase total cell number per embryo and affect zona characteristics. It offers further evidence supporting the embryotrophic effect of co-culture. While growth factor additives were clearly beneficial to thawed embryos, no single growth factor was able completely to mimic the results attained with co-culture. It is presumed that the co-culture produces either more growth factors than those added singly in this experiment, or in a more balanced ratio as required by developing murine embryos after freezing.

An enhancement in post-thaw development with co-culture has been observed with both human zygotes (Wiker *et al.*, 1992) and cleavage stage embryos (Tucker *et al.*, 1995). Tucker found that co-culture of human embryos prior to cryopreservation does not appear to improve cryosurvival after thawing, however, it does improve the implantation rate post-thaw compared with embryos cultured with a standard protocol (without co-culture) prior to cryopreservation. If *in vitro* produced vitrified donor embryos at the morula-stage of development are allowed to recover from the damage caused by the vitrification process by co-culturing the embryos for 24 h after thawing, their blastomeres - which are going to be used for nuclear transfer for the production of cloned embryos – will show similar efficiency to develop to cloned blastocysts as with blastomeres from fresh *in vitro* produced embryos (Peura *et al.* 1999).

The purpose of the present experiment was to find an alternative and effective culturing technique for conditions removed from a well-equipped laboratory, where the frozen embryos could be cultured after thawing to increase the chance of each embryo to implant and become a pregnancy. The vagina of a recipient goat doe was used for this purpose. The effect of co-culture of the frozen-thawed caprine embryos on their ability to produce offspring, was also studied.

5.3 Hypothesis

Culturing of frozen-thawed caprine embryos in a suitable co-culture medium in the vagina of a goat doe will increase the viability of the embryos after transfer when compared to non-cultured embryos.

5.4 Material and Methods

5.4.1 Experimental design

A total of 120 frozen-thawed caprine embryos were transferred to synchronized recipient female goats in the following media and conditions:

n	Transfer medium	Cultured before transfer
40	PBS	No
40	TCM-199	No
40	TCM-199	Yes

5.4.2 Method

A total of 120 good quality Kashmir goat blastocysts were frozen with the two-step 10% glycerol method. The collection and freezing process was done in New Zealand and the 120 embryos then exported to Oklahoma, USA. The frozen straws containing up to five embryos each at the blastocyst stage were removed from the liquid nitrogen container, and put horizontally on an empty 100 mm petri dish with the middle column containing the embryos in the middle of the dish, suspended in air, without any object touching it. After air thawing for 2 min, the embryos were reconstituted by serial passage through four mediums with diminishing concentrations of glycerol in a 4-well dish (Nunc, Roskilde, Denmark). The composition of each medium in the four dishes was as follow (all mediums were obtained from Sigma, USA):

1.   The first dish contained D-PBS (Cat # P-3813) with 10% of fetal bovine serum (FBS) (Cat # F-4135), 100 IU of penicillin and 100 μg of streptomycin (1% antibiotics) (Cat # P-0781), 6% of glycerol (Cat # G-5516) and 1 molar of sucrose (Cat # S-1888);

2.   The second dish had the same constituents, but only 3% of glycerol and still 1 molar of sucrose;

3.   The third dish contained D-PBS with the additives, but with no glycerol, and still 1 molar of sucrose;

4. The last dish contained holding medium (but no glycerol or sucrose) consisting of D-PBS with 10% fetal bovine serum and antibiotics as in the first dish.

The sealed end of the frozen-thawed straw was cut with sterile scissors, and the embryos flushed from the straw by inserting a 1 mm diameter metal rod into the plug end of the straw and pushing the plug towards the cut end of the straw. The content of the straw was collected as a 200 µL micro-drop of medium containing the frozen-thawed embryos, in the lid of the 100 mm petri dish, and the embryos identified under a stereo microscope at 80X magnification. The embryos were then transferred to the first dish, and left there for reconstitution for 5 min to 10 min. The embryos were transferred from the first dish in groups of up to five embryos to the second dish, left there for 5 min to 10 min and then transferred to the third dish. After another 5 min to 10 min, the embryos were placed in the holding medium of the fourth dish containing the holding medium.

The 120 caprine embryos were transferred over a three day period at a rate of 40 embryos per day. The embryos were randomly divided into three equal groups of 40 embryos each. A number of each group of embryos was transferred every day.

1. A total of forty embryos were washed three times in Dulbecco's phosphate buffered saline (D-PBS) with 10% fetal bovine serum (FBS) and 1% antibiotic (Group 1). The four wells of Nunc plate was each filled with 1 mL of the D-PBS medium as mentioned. Not more that 10 µl of medium was transferred with each individual embryo to the next well, with a 1 in 100 dilution with every transfer. In this way the holding medium was diluted about 1 million times after three transfers, increasing the potential of the antibiotics to reduce micro-organisms. The embryos were finally transferred to a well containing filter-sterilized D-PBS holding medium.

2. Forty embryos were also washed three times in pre-incubated Tissue Culture Medium-199 (TCM-199) with 10% FBS and 1% antibiotics (Group 2). The four wells of Nunc plate was each filled with 1 mL of the above TCM-199 medium. Not more that 10 µl of medium was transferred with each individual embryo to the next well, with a 1 in 100 dilution with every transfer. The 40 caprine embryos were then transferred to the fourth well containing filter-sterilized TCM-1999 with FBS and antibiotics (holding well). To prevent pH changes of the pre-incubated TCM-199, the embryos were washed and transferred between dishes in groups of not more than three at a time, and transferred to recipient does immediately after moving them to the holding well.

3. Forty embryos (Group 3) were washed as in Group 2. Bovine oviductal epithelial cells, (BOEC) frozen in 0.25 mL straws in a mixture of ethylene glycol and TCM-199, were air-thawed and released from the straw by cutting both ends of the straw and emptying the contents of the straw into a second dish containing pre-incubated TCM-199 and 10% of FBS and 1% of antibiotics. The embryos were then aspirated from the holding well into a ~5 cm length of a 0.25 ml semen straw (IMV, France) in groups of up to five embryos each, as well as some of the BOEC in the second dish. Both ends of the straw were heat-sealed with a heated mosquito forceps, and the straw placed into a cylindrical sponge containing no progesterone. A nylon string was attached to the one end the sponge. The sponges with their straws containing the embryos were then inserted with a plastic sponge applicator into the anterior part of the vagina of a recipient goat doe in the luteal phase of her estrous cycle for ~24 h. The sponge was inserted such that the nylon string protruded through the vulva lips. The applicator was disinfected with alcohol (96% methanol) between sponge insertions to prevent vaginal cross-contamination. After the 24 h culture period, the sponges were

removed from the vaginas by pulling on the nylon string, the straws sterilized on the outside with alcohol, and both ends cut. The forty caprine embryos were flushed into fresh pre-incubated TCM-199 with FBS and antibiotics, and washed twice in this medium to dispose of the BOEC.

The embryos were transferred in their respective mediums semi-laparoscopically to synchronized mixed breed goat does at the stage of their estrous cycle corresponding with the developmental stage of the embryo at transfer. The recipient animals were all in a moderate to good body condition. The does were synchronized with a norgestomet cylindrical ear implant (Synchromate-B, Intervet, The Netherlands), left under the skin of the ear for 8 d. After withdrawal of the norgestomet ear implant, the females were observed for signs of estrus with the aid of a vasectomized buck. The time and date of the onset of estrus were recorded.

The recipient does were used 7 d after the onset of their standing estrus. They were starved for 24 h before the transfer of the embryos. Each doe received an injection of 1 mL of xylazine (Rompun, Bayer, USA) intravenously as sedation and analgesic. The animals were put in dorsal recumbency at a 30º incline with the head down in a special cradle, and the abdominal area in front of the udder clipped, shaved and disinfected with alcohol. The first cannula and trocar was passed through the abdominal wall 2 cm lateral to the linea alba and ~4 cm below the udder attachment. The trocar was withdrawn and the laparoscope passed through the cannula. If the end of the laparoscope was inside the abdominal cavity between the omentum and the ventral abdominal wall, the laparoscope was withdrawn and the abdominal cavity inflated with air through the cannula. A second trocar and cannula was then passed through the abdominal wall perpendicular to the first cannula insertion, but on the contra-lateral side of the midline. A laparoscope connected to a light source was now inserted through the first cannula and the uterine horns with their oviducts and

ovaries visualized. A laparoscopic, spring-loaded forceps was passed through the second cannula. The CL of ovulation on either the right or left ovary was identified with the laparoscope by holding onto the ovarian or intercornual ligament with the aid of the laparoscopic forceps, and the side recorded. The cannula with the holding forceps was withdrawn, and an Allis tissue forceps inserted through the same hole in the body wall made by the trocar. The hole was slightly enlarged with a #10 scalpel blade to allow the 30 cm long Allis tissue forceps to enter the abdominal cavity. While being visualized through the laparoscope, the distal part of the uterine horn ipse-lateral to the CL was grabbed by pushing the grasping ends of the forceps over the uterine horn and holding onto the broad ligament without damaging the tissues of the uterine horn. The distal part of the uterine horn was pulled through the hole in the abdominal cavity and exteriorized. A ~1 mm diameter, sterile, round-ended stainless steel pin was carefully pierced through the uterine wall about 3 cm from and towards the tip of the horn, until it reached the lumen. Care was taken to cause as little damage or bleeding to the endometrium as possible. The washed embryos were individually aspirated under the stereo microscope into the tip of a size 3FG tomcat catheter (Arnolds Veterinary Products, Reading, UK) by means of a 1 mL syringe connected to the other end. The tip of the catheter containing the single embryo was guided through the hole made by the stainless steel rod, and then pushed down the lumen of the uterine horn in the direction of the tip of the horn. The embryo was deposited into the lumen at the tip of the uterine horn by depressing the plunger of the 1 mL syringe still connected to the tomcat catheter.

Pregnancy was determined by trans-abdominal ultrasound (Aloca 500 with a 3.5 MHz probe) from two months after transfer of the embryos, and the kidding rate determined after the kids were born five months after transfer.

5.5 Statistical analysis

All data were analyzed using the Student's t-test assuming two-sample means with equal variances (Microsoft Excell).

5.6 Results

The results of the experiment are represented in Table 5.1. The caprine embryos transferred in PBS resulted in significantly less pregnancies than the embryos that were first co-cultured in TCM-199 (47.5% and 70.0% respectively, $p<0.05$). The pregnancy rate of the co-cultured embryos did, however, not differ significantly from the pregnancy rate of the embryos not cultured but transferred in TCM-199. If the kidding rates are compared, the co-cultured embryos gave significantly ($p<0.05$) more live kids born (65.0%) than the embryos not cultured and transferred in either PBS or TCM-199 (both 42.5%). The reduction in pregnancy rates between the time of scanning and birth of the kids were not significant between the three treatment groups.

Table 5.1. Pregnancy rates and kidding rates after transfer of caprine embryos with three different methods

Group	n	Pregnancy rate (%)	Kidding rate (%)
1 (PBS)	40	19 (47.5)[a]	17 (42.5)[a]
2 (TCM-199)	40	20 (50.0)[ab]	17 (42.5)[a]
3 (TCM-199 + vaginal co-culture)	40	28 (70.0)[b]	26 (65.0)[b]

[ab] Superscripts in the same column are significantly different ($p<0.05$)

5.7 Discussion and Conclusion

It was shown that co-culturing caprine embryos after thawing in a suitable culture medium for as short as 24 hours can be beneficial for the survival of the embryos after transfer to synchronized recipients. This experiment proofed that significantly more frozen-thawed caprine embryos developed in the uterus to pregnancies and to term ($p<0.05$) when first co-cultured with bovine oviductal epithelial cells in a semen straw in the vagina of a goat doe, compared to direct

transfer without culture after thawing. Tucker *et al.* (1995) also found that the co-culture of human embryos has improved the implantation rate post-thaw, when the co-culture was done before freezing. Only 6.9% of pregnancies were encountered when human frozen-thawed embryos, that were not co-cultured before freezing, were transferred, against 13.6% of co-cultured embryos implanted under similar conditions (p<0.05).

When fertilized human zygotes were co-cultured, 68% of them reached the blastocyst stage. Pregnancy rate was 50% per transfer in patients with several previous failures of implantation. A significant increase in clinical pregnancy rate was also demonstrated when zygotes were maintained on Vero cell monolayer for only 24 h. The beneficial effect of the feeder layer may be through the release of embryotrophic factors and the detoxification of the culture medium by the cells (Schillaci *et al.* 1994).

The effect of co-culture on human embryos destined to be zona-drilled to assist hatching was studied by Tucker *et al.* (1994). Female patients of ≥ 38 years and couples who had previously failed to implant embryos three times or more were prospectively and randomly assigned to either an experimental or a control group. In the experimental group all embryos were co-cultured on partial monolayers of bovine oviductal epithelial cells for 2 d followed by assisted hatching by zona drilling (AH+CC). All control embryos were cultured by standard procedures until day 3 when they also underwent zona drilling prior to uterine transfer (AH-alone). There was a marginally lower percentage of fragmentation and a significantly higher degree of zona thickness variability in the AH+CC embryo group. Embryonic implantation was significantly increased (P < 0.05) in the AH+CC group (18%) when compared to the AH-alone group (10%). This difference was reflected in a significantly higher (P < 0.05) initial pregnancy rate (52% versus 32%) in the AH+CC group, and a higher (not significant) viable pregnancy rate (38% versus 22%). This study

proofed that co-culture cells can even be used across species (bovine oviductal cells for human embryos) to stimulate embryonic growth and development.

An interesting study by Freeman *et al.* (1995) showed that even autologous granulosa-lutein cell co-culture for 48 h prior to human embryo transfer improved human embryo development of pronuclear and two-cell zygotes. The live birth rate per embryo transfer was 43.4% with an implantation rate per embryo of 17.6%. Of the non-transferred embryos, 68% developed to the blastocyst stage. In this study, sibling two-pronuclear zygotes were randomly allocated to culture with (co-culture) or without (control) autologous granulosa-lutein cells. After 24 h, embryos were examined for blastomere number and degree of fragmentation. Co-culture had no effect on the average number of blastomeres per embryo at 24 h, however, fragmentation was significantly decreased in co-cultured embryos (0.7 +/- 0.1) compared with controls (1.3 +/- 0.2; $P < 0.05$). The co-culture of the caprine embryos in the present study after thawing and before transfer, could therefore have decreased the fragmentation rate of the frozen-thawed blastomeres and optimized the implantation rate. Naitana *et al.* (1995) describes how vitrified-thawed ovine blastocysts re-formed their blastocoelic cavity after 24 h of co-culture, and gave rise to lambing rates of up to 80% when transferred to synchronized recipient ewes.

Isachenko *et al.* (1994) found a very positive effect of co-culture on the implantation rate of bovine embryos when frozen-thawed bovine embryos were transferred after thawing with or without trophoblastic fragments (TF) from 16-17 d old bovine fetuses. When the thawed embryos were transferred without TF, a 39% pregnancy rate was achieved, while a 56% pregnancy rate was achieved when TF was transferred with the embryos into recipient cows. If the embryos were frozen by vitrification and then transferred after thawing, the pregnancy rates were 33% and 55%,

respectively. The trophoblastic cells either stimulated the development of the embryos *in vivo*, and/or secreted the bTP-1 needed for maternal recognition of pregnancy.

The vagina of a goat doe can be used as an alternative culture system for the development of frozen-thawed caprine embryos. Even fertilization can occur intra-vaginally under favorable conditions (Ranoux *et al.* 1987, 1988). A series of 100 oocyte aspirations in humans gave 22 pregnancies when the oocytes were fertilized and cultured for up to 50 h in the vagina of the recipient before transfer. In a German study 5/22 (22.7%) pregnancies were achieved when human oocytes were fertilized and cultured for 50 h post insemination intra-vaginally, compared to 4/23 (17.4%) when the oocytes were fertilized and cultured using traditional culture in an incubator (Sterzik *et al.* 1989). Sterzik *et al.* (1993) also used the vagina as an incubator for transporting oocytes and sperm over long distances with great efficiency. They found that extracorporal factors such as light and low temperatures could be minimized by intra-vaginal culture. Freude *et al.* (1990) obtained fertilization in 56% of 15 cases of intra-vaginal fertilization. Three intact pregnancies were recorded after embryo transfer. Of these, two occurred in the group of patients with tubal infertility and one in the group with tubal infertility and an additional andrologic abnormality. In another study, fertilization was achieved in 84% of oocytes fertilized intra-vaginally. The cost of intra-vaginal fertilization and culture is approximately one-third that of standard IVF. The advantages of this method are the elimination of the use of gonadotropins, the simplicity of monitoring and oocyte retrieval, and the lack of need for expensive laboratory equipment. Natural oocyte retrieval with intra-vaginal fertilization may prove appropriate for those women requiring IVF who fear multiple pregnancies, have side effects from controlled ovarian hyper-stimulation, or cannot afford standard IVF (Taymor *et al.* 1992).

The technique of intra-vaginal fertilization and culture was also tested in the bovine species (Moyo and Dobson 1995). They used oocytes collected by trans-vaginal, ultrasound-guided oocyte retrieval after multi-stimulation with ovine FSH, and co-cultured the zygotes in capsules in the vagina in oviductal cell co-culture for 48 h. There was no significant difference between the fertilization and cleavage rates of oocytes cultured in the vagina and those cultured in a conventional CO_2 incubator.

The effect of two concentrations of oxygen on the development of bovine embryos was compared using two separate co-culture systems (Voelkel and Hu 1992). Bovine oocytes were matured and fertilized *in vitro* and were then co-cultured for 7 days in 20 µL drops of TCM-199 with 10% fetal calf serum containing oviduct cells. When cultures were performed in an atmosphere of 5% CO_2 in air (20% O_2) or in a mixture of 5% CO_2, 5% O_2 and 90% N_2 (5% O_2), 12% and 31% zygotes developed to or beyond the late morula stage ($P<0.0001$), respectively. After freezing, thawing and 48 hours of additional culture, 10% and 34% embryos were judged viable ($P<0.001$) within the respective treatment groups. The oxygen concentration seems therefore to play an important role in the development of embryos before and after freezing. The oxygen tension in the vagina is similar to that of the rest of the reproductive tract, and intra-vaginal culture therefore favors the development of embryos compared to *in vitro* culture in a standard laboratory incubator.

It can be concluded that co-culturing frozen-thawed caprine embryos before transfer will allow the damage caused by the freezing process to be at least partially repaired. A co-culture period of as short as 24 h after thawing and before transfer seems to be sufficient time for the caprine embryo to recuperate and therefore increase the pregnancy rate after transfer. The co-culture cells seem to produce the necessary growth factors and recuperating proteins for the

embryo to repair its damage blastomeres, to allow implantation and a resultant fetus and live kid at birth.

5.8 References

Desai, N., Lawson, J. and Goldfarb, J. (2000). Assessment of growth factor affects on post-thaw development of cryopreserved mouse morulae to the blastocyst stage. *Human Reproduction* **15** (2), 410-418

Dresser, B.L., Gelwicks, E.J., Wachs, K.B., Keller, G.L. and First, N. (1988). Successful transfer of cryopreserved feline (Felis catus) embryos resulting in live offspring. *Journal of Experimental Zoology* **246** (2), 180-186.

Freeman, M.R., Whitworth, C.M. and Hill, G.A. (1995). Granulosa cell co-culture enhances human embryo development and pregnancy rate following in-vitro fertilization. *Human Reproduction* **10** (2), 408-414.

Freude, G., Artner, B. and Leodolter, S. (1990). Intra-vaginal culture – simplification of IVF. *Wien Med Wochenschr* **140** (20), 498-501.

Isachenko, V.V., Soler, C., Ostashko, F.I. and Isachenko. E.F. (1994). Improvement of attachment rate of bovine embryos after transfer with trophoblastic fragments. *Theriogenology* **42** (2), 339-344.

Keskintepe, L., Darwish, G.M., Kenimer, A.T. and Brackett**,** B. G. (1994). Term development of caprine embryos derived from immature oocytes *in vitro*. *Theriogenology* **42** (3), 527-535.

Keskintepe, L. and Brackett, B.G. (2000). Cryopreservation of bovine blastocysts obtained by intra-cytoplasmic sperm injection. *Theriogenology* **53** (5), 1041-1052.

Mauri, A.L., Petersen, C.G., Baruffi, R.L., Ferreira, R.C. and Franco, J.G. (1999). Clinical experience with ultrarapid cryopreservation of human embryos resulting from intra-cytoplasmic sperm injection: brief communication. *Human Reproduction* **14** (9), 2362-2366.

Moyo, P. and Dobson, H. (1995). Collection, *in vitro* fertilization and culture of bovine oocytes intra-vaginally or in a conventional incubator. *Veterinary Record* **136** (5), 115-118.

Naitana, S., Dattena, M., Gallus, M., Loi, P., Branca, A., Ledda, S. and Cappai. P. (1995). Recipient synchronization affects viability of vitrified ovine blastocysts. *Theriogenology* **43** (8), 1371-1378.

Park, J.S., Han,. Y.M., Lee, C. S., Kim, S.J., Kim, Y.H., Lee, K.J., Lee, K.S. and Lee, K.K. (2000). Improved development of DNA-injected bovine embryos co-cultured with mouse embryonic fibroblast cells. *Animal Reproduction Science* **59** (1-2), 13-22.

Peura, T.T., Lane, M.W., Vajta, G. and Trounson, A.O. (1999). Cloning of bovine embryos from vitrified donor blastomeres. *Journal of Reproduction and Fertility* **116** (1), 95-101.

Pugh, P.A., Ankersmit, A.E., McGowan, L.T. and Tervit, H.R. (1998). Cryopreservation of *in vitro*-produced bovine embryos: effects of protein type and concentration during freezing or of liposomes during culture on post-thaw survival. *Theriogenology* **50** (3), 495-506.

Pugh, P.A., Tervit, H.R. and Niemann, H. (2000). Effects of vitrification medium composition on the survival of bovine *in vitro* produced embryos, following in straw-dilution, *in vitro* and *in vivo* following transfer. *Animal Reproduction Science* **58** (1-2), 9-22.

Ranoux, C., Duboisson, J.B., Foulet, H. and Aubriot, F.X. (1987). Intra-vaginal culture and embryo transfer. A new method for the fertilization of human oocytes. *Rev Fr Gynecol Obstet* **82** (12), 741-744.

Ranoux, C., Aubriot, F.X., Dubuisson, J.B., Cardone, V., Foulot, H., Poirot, C. and Chevallier, O. (1988). A new *in vitro* fertilization technique: intra-vaginal culture. *Fertility and Sterility* **49** (4), 654-657.

Schillaci, R., Ciriminna, R. and Cefalu, E. (1994). Vero cell effect on in-vitro human blastocyst development: preliminary results. *Human Reproduction* **9** (6), 1131-1135.

Sterzik, K., Rosenbusch, B., Sasse, V., Wolf, A., Beier, H.M. and Lauritzen, C. (1989). A new variation of *in vitro* fertilization: intra-vaginal culture of human oocytes and cleavage stages. *Human Reproduction* **4**, 83-86.

Sterzik, K., Rosenbusch, B. and Noss, U. (1993) First pregnancies after intra-vaginal transport and partial zona dissection of human oocytes. *Fertility and Sterility* **60** (3), 582-584.

Taymor, M.L., Ranoux, C.J. and Gross, G.L. (1992). Natural oocyte retrieval with intra-vaginal fertilization: a simplified approach to *in vitro* fertilization. *Obstet Gynecol* **80** (5), 888-891.

Tucker, M.J., Ingargiola, P.E., Massey, J.B., Morton, P.C., Wiemer, K.E., Wiker, S,R. and Wright, G. (1994). Assisted hatching with or without bovine oviductal epithelial cell co-culture for poor prognosis in-vitro fertilization patients. *Human Reproduction* **9** (8), 1528-1531.

Tucker, M.J., Kort, H.I., Toledo, A.A., Morton, P.C., Wright, G., Ingargiola, P.E. and Sweitzer, C.L. (1995). Effect of co-culture on subsequent survival and implantation of cryopreserved human embryos. *J Assist Reprod Genet* **12** (10), 689-692.

Voelkel, S.A. and Hu, Y.X. (1992). Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. *Theriogenology* **37** (5), 1117-1131.

Wiker, S., Tucker, M., Wiemer, K. and Wright, G. (1992) Co-culture of thawed human embryos with bovine oviductal epithelial cells. Editors: Rodriguez-Armas, O., Baumgartner, W. and Burgos-Briceno, L. *XIV World Conference on Fertility and Sterility*. Parthenon Press, Caracas, Venezuela, pp. 407–414.

Ziebe, S., Bech, B., Petersen, K., Mikkelsen, A.L., Gabrielsen, A. and Andersen, A.N. (1992). Resumption of mitosis during post-thaw culture: a key parameter in selecting the right embryos for transfer. *Human Reproduction* **13** (1), 178-181.

CHAPTER 6

MATURATION OF BOVINE OOCYTES IN TCM-199 AND FIRST TRIMESTER AMNIOTIC FLUID IN A LABORATORY INCUBATOR AND IN THE VAGINA OF A GOAT DOE.

6.1 Abstract

The maturation of bovine oocytes to allow the oocyte to resume meiosis, is the first step in *in vitro* fertilization to produce IVMFC embryos. The composition of the maturation medium used, plays an important role in the success achieved with maturation. An investigation was therefore launched to evaluate the maturation ability of first trimester bovine amniotic fluid (BAF) to mature prophase I oocytes collected from abattoir ovaries, to metaphase II oocytes, compared to a standard maturation medium such as TCM-199. In the first experiment three groups of ~100 oocytes each were matured in TCM-199 with estrus cow serum (ECS). The first group of oocytes was matured in a 50 µL drop in an incubator, while the other two groups were matured in semen straws, one group in an incubator and the other group in the vagina of a goat doe in diestrus. Six further groups of ~100 oocytes each, with BAF as maturation medium, three groups with ECS and three without ECS, were matured in the same receptacles and under the same conditions as with the TCM-199. No significant differences in number of oocytes reaching the metaphase II stage could be found for any of the nine treatment groups. In the second experiment, fresh and frozen-thawed BAF with or without ECS, was tested as a maturation medium while the oocytes were matured in micro-drops inside a laboratory incubator. Although the fresh BAF with added 10% ECS was inclined to give better results as a maturation medium for bovine oocytes when compared to fresh BAF without serum, or frozen-thawed BAF with and without added serum, the differences between the four groups were not significant.

6.2 Introduction

In human, livestock and wildlife species assisted reproductive technologies (ART), such as *in vitro* fertilization, embryo transfer, the production of transgenic animals and cloning, have a limited impact because of a lack of superior quality oocytes that can be fertilized. Stimulation of an individual with FSH and LH can increase the number of ovulated oocytes (follicular multi-stimulation and super-ovulation), but the response is variable, large numbers are not generally obtained, and the treatment may have many unwished side-effects. The *in vitro* maturation of immature oocytes from antral follicles is an alternative method to increase the number of fertilizable oocytes. The maturation rate of oocytes from later-stage follicles (>6 mm in diameter) is more rapid than those from earlier-stages ($\leq$ 6 mm), but they are less abundant in mammalian ovaries. The ability to initiate growth in culture of the most abundant follicular stage in the ovary, the primordial follicle, and to maintain this growth to a stage when the oocyte can be matured and fertilized, would dramatically increase the reproductive potential of mammalian species (Van den Hurk and Zhao 2004). Although the generation of fertilizable oocytes from primordial follicles is one of the most challenging goals in reproductive biology and medicine, to date no embryos of domestic species and primates have been produced starting with primordial follicles as oocyte donors. This is due to the lack of knowledge of the factors required for activation of these dormant follicles and further growth and differentiation of the oocytes in these species. Signaling factors exchanged between the oocyte and its surrounding granulosa cells are thought to be essential for inducing and regulating the differentiation of follicular compartments from a specific developmental stage to the next one and thus for the development of an oocyte that will be competent to undergo fertilization and subsequent embryogenesis (Van den Hurk *et al.* 2000; Eppig 2001; Matzuk *et al.* 2002). The oocyte controls follicular development, because from the

time of follicular organization and continuing throughout ovulation, it orchestrates the development of a follicle by controlling granulosa cell proliferation and differentiation into cells producing adequate compounds like proteins and steroids. This leads to increased responsiveness to gonadotropins, theca cell differentiation, cumulus expansion and in the end rupture of the follicular wall. The granulosa cells, in turn, are indispensable for oocyte growth, differentiation, nuclear meiotic state, cytoplasmic maturation and genomic transcriptional activity. This knowledge, together with the finding that, upon reaching a threshold-size, oocytes suppress the ability of granulosa cells to promote its further growth, strongly indicate that the oocyte determines not only follicle growth, but also indirectly its own growth (Matzuk *et al.* 2002).

About sixty years ago our knowledge of oocyte maturation, fertilization and embryo development was based on the study of sea urchin eggs, although Pincus and Enzman (1935), and Pincus and Saunders (1939) reported as early as 1935 on the comparative behavior of mammalian ova cultured *in vivo* as well as *in vitro*. During the 1950's there were several reports by Chang (1951, 1952, 1955) on bovine and rabbit oocyte and sperm changes during the processes around fertilization. It was, however, only when the process of capacitation of mammalian sperm was recognized as a precursor of fertilization (Austin 1951), that real progress was made towards *in vitro* oocyte maturation, fertilization and the birth of the first IVF mammalian offspring (Chang 1968).

Oocyte maturation is a very complex process, involving nuclear as well as cytoplasmic maturation (Yokoo and Sato 2004). From the resting primordial follicles to the growing and dominant follicles, the oocyte remains arrested at the diplotene stage of the first meiotic prophase division, which is comparable with the G2 phase equivalent of mitotic cells. *In vivo*, resumption of meiosis from prophase 1 is initiated by the pre-ovulatory LH surge and only occurs in fully grown,

meiotically competent oocytes from dominant follicles. Before and at the time of the LH surge, the oocyte is surrounded by a compact cumulus investment. From the innermost cumulus cells (corona radiate), numerous projections penetrate the zona pellucida that end on the oocyte cell membrane (oolemma) with gap junctions. Shortly after the LH surge, disruption of these junctions takes place. During the period between the LH surge and ovulation, the oocyte undergoes a series of marked changes, not only in its nucleus, but also in its cytoplasm, a process known as oocyte maturation. Nuclear maturation, lasting about 24 h in cow and sheep, about 44 h in pig and about 36 h in horse, comprises several steps (Van den Hurk *et al.* 1999), that include two consecutive meiotic divisions (M-phases) in the absence of DNA replication (S phase). Oocytes then become arrested at MII until fertilization, when an activation stimulus provided by sperm penetration and binding to the oolemma triggers the completion of the meiotic cycle and initiates embryonic development. Ooplasmic maturation is required to (1) acquire the conditions to block polyspermy in case of fertilization, (2) to decondense penetrated spermatozoa and (3) to form pronuclei after fertilization, (4) for redistribution of cell organelles, (5) for the migration of mitochondria to a peri-nuclear position and (6) the accumulation of granules along the oolemma. Inhibition of LH secretion or inactivation of LH receptors, results in prevention of oocyte maturation and failure in oocyte ovulation. Since no LH receptors are detected in or on the oocyte (Peng *et al.* 1991), the signals that trigger oocyte maturation thus likely originate from their surrounding follicle cells. It is well established that follicles respond to the LH surge with a shift in steroid production by granulosa cells from predominantly estrogen to a progesterone environment. With the production of hyaluronidase enzyme by the cumulus cells, this enzyme leads to the mucification and expansion of the cumulus cells and the accompanying termination of oocyte–cumulus junctional contacts (Picton *et al.* 1998). Disruption of these gap junctions means disappearance of the highly

coordinated bi-directional communication between the oocyte and its surrounding somatic cells. Despite species differences, the basic molecular machinery governing the process of oocyte maturation in response to the LH surge shares several regulatory pathways such as the alteration of protein phosphorylation, cyclic adenosine monophosphate (cAMP) and calcium levels (Maller and Krebs 1977, 1980; Bornslaeger *et al.* 1986; Homa 1995, Conti *et al.* 1998; Wiersma 1998; Carabatsos *et al.* 2000; Gordo *et al.* 2001; Trounson *et al.* 2001; Lincoln *et al.* 2002; Van den Hurk and Zhao 2004).

Apart from oocyte maturation, ovulation *in vivo* requires a precise control of extracellular matrix modification. One of the LH-induced processes culminating in ovulation is cumulus expansion, whereby cumulus cells secrete hyaluronan to form a muco-elastic extracellular matrix with proteins derived from the serum and the follicle. This matrix structure is of importance for oocyte extrusion from the follicle and for pick-up by the fimbria (Talbot *et al.* 2003). In addition, a function of selective barrier for sperm has also been reported for this matrix structure. Apart from LH, oocyte-derived GDF-9 plays an important role in the induction of cumulus expansion (Elvin *et al.* 1999). This action of GDF-9 is remarkable, since it is expressed in oocytes throughout follicular development. In cumulus cells, LH may activate GDF-9 by induction of proteases that loosen it from its inactivating proteinglycan or by stimulating the formation of a specific receptor for GDF-9 (Richards *et al.* 2002). Activated GDF-9 may induce the secretion of hyaluronan by the cumulus cells that, together with proteins derived from the serum and follicle, form the muco-elastic matrix characteristic for cumulus expansion. GDF-9 also induces cyclooxigenase 2 (COX2) and suppresses urokinase-type plasminogen activator (uPA) production in cultured granulosa cells (Elvin *et al.* 1999). COX2 is required for prostaglandin E2 synthesis that binds the EP2 receptor. Knockout experiments showed that these latter compounds are essential for cumulus expansion and

ovulation (Richards *et al.* 2002). Plasminogen activators are serine proteases that cleave plasminogen to form the active protease plasmin (Dow *et al.* 2002). These enzymes are involved in matrix remodeling in several tissues, including the ovary. uPA changes the somatic tissue structure of a pre-ovulatory follicle to evoke ovulation. Apart from GDF-9, the oocyte modulates the response of cumulus cells to an ovulatory dose of LH by producing tissue PA (tPA), which prevents the degradation of cumulus matrix (Salustri 2000). Oocytes accumulate tPA mRNA during their growth phase and translation of this mRNA is triggered upon resumption of meiotic maturation. It therefore seems as if there is a sophisticated interplay between oocyte and cumulus cells in control of the integrity of cumulus–oocyte complex (COC).

Most of the changes that take place during oocyte cytoplasmic and nuclear maturation *in vitro* can be observed under high magnification using a stereo or inverted microscope. Cumulus expansion, especially, can clearly be visualized under a microscope without staining the oocyte and therefore possibly harming it. Good expansion is therefore an indication of possible oocyte maturation, as the oocyte controls its cumulus expansion.

Several attempts have been made to culture bovine and other species primordial and pre-antral follicles to the ovulatory oocyte stage (Hulshof *et al.* 1995; Telfer 1996, 1998, Van den Hurk *et al.* 1997; Gutierrez *et al.* 2000; McCafferey *et al.* 2000). The culture of early-stage bovine follicles has been be maintained for up to 28 days, but no indication is present of oocyte quality during these long-term cultures. So far, no *in vitro* grown bovine oocytes have successfully undergone meiotic maturation to the metaphase II stage during long-term follicle culture. Bovine pre-antral follicles (166 ± 2.15 μm), surrounded by theca cells, have been cultured under conditions known to maintain granulosa cell viability *in vitro*. The effects of EGF, IGF-I, FSH and co-culture

with bovine granulosa cells on pre-antral follicle growth were analyzed and showed a significant increase in follicle and oocyte diameter ($P<0.05$) with time in culture. Antrum formation (confirmed by confocal microscopy) occurred between days 10–28 of bovine follicle culture. These results are promising and demonstrate bovine oocyte growth and granulosa cell differentiation over an extended culture period *in vitro* (Telfer *et al.* 2000).

6.3 Hypothesis

1. Freshly-collected bovine amniotic fluid can support maturation of bovine oocytes up to the metaphase II stage.

2. Semen straws can be used as a receptacle for bovine oocyte maturation.

3. The vagina of a goat doe can support bovine oocyte maturation.

4. Frozen-thawed bovine amniotic fluid can support bovine oocyte maturation.

6.4 Material and Methods

6.4.1 Experimental design

6.4.1.1 Experiment 1

Nine groups of ~100 oocytes each were matured in the different culture media and "incubators" as indicated below:

Group	1	2	3	4	5	6	7	8	9
Culture method	Incu-bator	Incu-bator	Incu-bator	Incu-bator	Incu-bator	Incu-bator	Vagina	Vagina	Vagina
Medium	TCM+ Serum	BAF	BAF+ Serum	TCM+ Serum	BAF	BAF+ Serum	TCM+ Serum	BAF	BAF+ Serum
Culture environment	Micro drop	Micro drop	Micro Drop	Straw	Straw	Straw	Straw	Straw	Straw
Number	~100	~100	~100	~100	~100	~100	~100	~100	~100

6.4.1.2 Experiment 2

Four groups of ~100 oocytes each were matured in fresh and frozen-thawed BAF with or without

ECS as culture media as indicated below:

Medium	BAF	BAF + serum	Frozen BAF	Frozen BAF + Serum
Number	~100	~100	~100	~100
Culture method	Incubator	Incubator	Incubator	Incubator

6.4.2 Method

6.4.2.1 Experiment 1

6.4.2.1.1 Oocyte collection

Bovine ovaries were collected within 30 minutes after culling of the cows of dairy and beef

breeds at the local abattoir and placed in phosphate buffered saline (Sigma, USA, Cat P-3813) at

~30 ºC with 1% antibiotics (PenStrep, Highveld Biologicals, Johannesburg, South Africa) (PBS-

Ab). The ovaries were then transported for about 45 km to the laboratory and the ovaries washed

twice in sterile PBS-Ab. The temperature in the laboratory was ≥ 20 ºC. With a sterile 18 gauge

needle (Promex, NPC, Bergvlei, South Africa) the antral follicles between 2 mm and 10 mm with

their follicular fluid were aspirated from the floor of the follicles by piercing ovarian tissue before

entering the follicles with the bevel of the 18 gauge needle facing towards the antrum of the

follicle. The needle was attached by sterile tubing to a sterile 15 mL conical tube (Cellstar, Griner

Bio-One, Frickenhausen, Germany) that was connected to a 1 L glass bottle vacuum reservoir

which in turn was attached to a water faucet suction system. When each tube was filled with

oocytes and follicular fluid, the oocytes were allowed to sediment for 5 min, then the supernatant

was aspirated and discarded, and the 15 mL conical tube refilled with sterile PBS-Ab and inverted

to suspend the oocytes inside the medium. After another 5 min of sedimentation, this washing

procedure was repeated a second time, the supernatant aspirated and the tube filled with the PBS-Ab a third time. The tube was inverted a number of times, and then emptied into a 100 mm grid-lined petri dish. The inside of the tube was thoroughly rinsed with PBS-Ab and emptied into the same petri dish.

Oocytes with a clear, homogenous cytoplasm with at least three layers of cumulus granulosa cells, were identified under a stereo microscope at ~60X magnification. They were aspirated and then transferred into sterile medium, i.e. PBS with 1% fetal bovine serum (Highveld Laboratories, Johannesburg, South Africa) and 1% antibiotics, with a gel loading pipette tip (Greiner Bio-One, Frickenhausen, Germany, Cat 770290) connected to a 1 mL syringe (Normo-Ject Zentrisch, Henke Sass Wolf, Germany) by means of a rubber connector made from a ~30 mm piece of a catheter (Foley, 20 gauge, Latex Products, Johannesburg, South Africa).

From the PBS medium, the oocytes with surrounding cumulus granulosa cells (cumulus-oocytes complex, COC) were washed by transferring them in groups through a 4-well dish (Nunc, Roskilde, Denmark). Each well contained at least 1 mL of either tissue culture medium 199 (TCM-199) or first trimester bovine amniotic fluid (BAF) with 10% heat-inactivated estrus cow serum (ECS) and 1% antibiotics. Not more that 10 μl of medium was transferred with each group of COC to the next well, with a 1 in 100 dilution with every transfer. In this way the holding medium was diluted more than 1 million times after four transfers, increasing the potential of the antibiotic to reduce micro-organisms. The COC were then finally transferred to a well with holding medium containing either sterile-filtered TCM-199 or BAF with 10% ECS and 1% antibiotics.

6.4.2.1.2 Medium preparation

(i) First-trimester amniotic fluid was sterilely collected from pregnant tracts of cows less than 90 d pregnant after slaughter at the local abattoir. The pregnant tracts were transported to the laboratory on ice, and the uterine wall carefully dissected as not to damage the underlying amniochorion. The amniotic fluid was aspirated sterilely with a 50 mL nylon syringe without rubber plunger (Normo-Ject Zentrisch, Henke Sass Wolf, Germany) and an 18 gauge needle (Promex, NPC, Bergvlei, South Africa) from the amniotic sac by inserting the needle into the amniotic sac through the amniochorion. The amniotic fluid was heat inactivated at 56 ºC for 30 min in 15 mL conical tubes (Greiner Bio-One, Frickenhausen, Germany) in a waterbath.   The amniotic fluid of individual tracts was pooled, and half the volume of the pooled amniotic fluid was frozen for Experiment 2.

(ii) Tissue culture medium 199 (TCM-199, Sigma, USA Cat M-7528) to which was added 0.1 gm L-glutamine per liter of TCM-199.

(iii) Blood was collected from cows early in standing estrus in clot-activator 10 mL glass tubes (Vacutainer SST, Johannesburg, South Africa) and left on the bench top for clotting and serum separation. The tubes with clotted blood were then centrifuged at ~2000 revolutions for ~20 min. The estrus cow serum (ECS) was aspirated under a laminar flow hood, and heat inactivated in 15 mL conical tubes at 56 ºC for 30 min in a waterbath.

6.4.2.1.3 Oocyte maturation

From the two holding mediums the oocytes were divided into nine groups of a minimum of 100 oocytes each. Three groups of oocytes (Group 1 to 3) were matured for 24 h in 50 µL micro-drops covered with oil (Sigma, USA, Cat M-8410) in a 35 mm petri dish (Cellstar, Griner Bio-One,

Frickenhausen, Germany) in a standard laboratory incubator (Binder, Tuttlingen, Germany) at 5% CO_2, 100% humidified air and at 38.5 ℃. No more than 10 oocytes were included in each micro-drop. Six groups (Groups 4 to 9) were cultured in a sterile semen straw, with not more than 10 COC per 50 mm length of straw. The oocytes were aspirated into the straw by means of a 16 gauge needle (Promex, NPC, Bergvlei, South Africa) attached to a 1 mL syringe (Normo-Ject Zentrisch, Henke Sass Wolf, Germany). The needle was fitted into the plug end of the sterile straw, and the oocytes aspirated into the straw by pulling on the plunger of the syringe. Both ends of the straw were sealed with a heated hemostat. The straws with their COC of Groups 4 to 6 were cultured for 24 h in the same laboratory incubator as in Groups 1 to 3, and Groups 7 to 9 were cultured for 24 h in the vagina of a goat doe. The short length of straw with its COC was inserted into a cylindrical sponge without progesterone, and the sponge with its straw inserted into the anterior part of the vagina of a goat female in diestrus with a special plastic sponge applicator (Intervet, The Netherlands). In Groups 1, 4, and 7 TCM-199 with 10% of ECS and 1% antibiotics were used as culture medium. In Groups 2, 5 and 8 the culture medium was bovine amniotic fluid with 1% antibiotics, and in Groups 3, 6 and 9, bovine amniotic fluid with 10% ECS and 1% antibiotics were used as culture medium. The COC maturation in both the incubator and intra-vaginally took 24 to 26 h.

6.4.2.2 Experiment 2

Oocyte collection was similar as in Experiment 1. The collected and washed COC (n=550) were divided into four different groups of not less than 100 COC per group. In two of the groups the COC were matured in fresh BAF, and in two groups the COC maturation took place in frozen-thawed BAF (frozen for a minimum of 30 d). Estrus cow serum (10%) was added to the one

group of fresh BAF and to the one group of frozen BAF. The COC were matured in 50 μL micro-drops with oil overlay, as in Experiment 1, in a 35 mm petri dish in a standard laboratory incubator at 5% CO_2 and saturated air at 38.5 ºC. The COC were matured for 24 h – 26 h.

6.4.3 Oocyte evaluation

After a period of 24 h to 26 h of maturation, the COC were harvested from the micro-drops as well as from the straws inside the vagina. The COC were then vortexed for ~5 min in a 15 mL conical tube, and the contents of the tube emptied into a 60 mm petri dish. If there were still cumulus granulosa cells sticking to the zona pellucida, the oocytes were vortexed a second time. The nude oocytes were then removed and transferred to a 24-well plate containing a 3:1 mixture of acetic acid and methanol. Fixation in this medium took 24 h. A 1% Orcein stain (Merck, Darmstad, Germany) dissolved in the above fixative was then added in a 20 μL drop fashion until the DNA of the metaphase II plate and the first polar body could be visualized.. Evaluation for oocyte maturation was done on an inverted microscope (Olympus 1X70 with Hoffman optics) at 200X to 400X. The presence of both a metaphase plate and first polar body were the indications of successful maturation.

6.5 Statistical analysis

All data were analyzed using the Student's t-test assuming two-sample means with unequal variances (Microsoft Excell).

6.6 Results

6.6.1 Experiment 1

The results of the number of bovine oocytes (%) that matured during the culture period of 24 h in the standard culture medium (TCM-199) and in bovine first trimester amniotic fluid with or without 10% ECS in both the standard laboratory incubator, as well as the oocytes that were cultured in the vagina of a goat doe in her diestrus stage of her estrous cycle in both straws and micro-drops, are presented in Table 6.1.

Table 6.1. Bovine oocytes (%) that matured during a 24 h culture period in TCM-199 and BAF in micro-drops and straws inside an incubator and in the vagina of a goat doe

Medium	TCM+	BAF	BAF+	TCM+	BAF	BAF+	TCM+	BAF	BAF+
Holder	Micro	Micro	Micro	Straw	Straw	Straw	Straw	Straw	Straw
Incubation	Inc	Inc	Inc	Inc	Inc	Inc	Vagina	Vagina	Vagina
Matured Oocytes (%)	69/107 (64%)	63/102 (62%)	75/104 (72%)	66/105 (63%)	70/100 (70%)	75/108 (69%)	66/110 (60%)	58/98 (59%)	78/112 (70%)

TCM+, tissue culture medium with 10% ECS; BAF+, bovine amniotic fluid with 10% ECS; BAF, bovine amniotic fluid without ECS; micro, 50 μL drops.

No significant differences could be found between any of the groups.

6.6.2 Experiment 2

In Table 6.2 the results of the number (%) of bovine oocytes that matured during the 24 h culture period in bovine first trimester amniotic fluid either fresh or frozen, and with or without 10% ECS in standard laboratory incubator, are represented.

Table 6.2 Bovine oocytes (%) that matured during a 24 h culture period in fresh and frozen BAF with or without FBS in a standard incubator

Culture medium	BAF	BAF + EC serum	FBAF	FBAF + EC serum
Matured oocytes	63/102 (62%)	119/165 (72%)	79/125 (63%)	103/158 (65%)

BAF, bovine amniotic fluid; FBAF, frozen bovine amniotic fluid

No significant differences could be found between any of the groups. The addition of estrus cow serum to both the fresh and frozen-thawed amniotic fluid seemed to have increased the number of oocytes that matured, but this was not significant.

6.7 Discussion and Conclusion

In most mammalian species, meiotic competence, as defined by the ability to resume and complete meiosis, is dependent on and increases with follicle size and is not strictly correlated with oocyte diameter (Trounson *et al.* 2001). In all non-rodent species studied, the ability of oocytes to resume meiosis is acquired when oocyte growth is completed, i.e. when the follicle diameter is about 9–13% of the ovulatory diameter (Gilchrist *et al.* 1995). According to Wynn (1998) the minimum follicle diameter from which oocytes mature *in vitro* in the human is 5 mm. In cows, follicles >6 mm have about twice the developmental potential of oocytes from smaller antral follicles, and oocytes acquire full meiotic competence and developmental competence at a diameter of 115 and 120 um, respectively (Otoi 1997). In species ovulating one oocyte at the beginning of the estrous cycle, only one or two follicles enlarge and become dominant during their selection in the final growth phase before ovulation. This leads to a marked increase in the ratio of estrogen to androgen of the dominant follicle(s). However, the size of the leading follicle does not affect the cohort oocytes with regard to their fertilization rate and further embryo development (Wittmaack *et al.* 1994; Trounson *et al.* 2001). This suggests that the developmental competence of the *in vitro* matured oocyte is compromised by some inherent but yet unidentified defect in the maturation process rather than being directly related to reduced follicle size. During the present study bovine follicles between the diameters of 2 mm and 10 mm were aspirated. Most of the follicles were less than 6 mm in diameter. This might explain the relatively low maturation rates of the COC. No

supplementary hormones like FSH, LH and estrogen were added to the maturation media as is often done during IVMFC. The estrous cow serum (ECS) was the only additive to some of the media used for maturation.

The use of a sterile semen straw for the maturation of bovine oocytes has some advantages over the maturation of oocytes in micro-drops. Due to the impermeability of the wall of the semen straw for water molecules, the osmolarity of the medium used for the maturation inside the sealed straw can not change. As the plastic wall of the semen straw will, however, allow CO_2 gas to penetrate, the HCO_3^- based maturation medium will stay at a fairly constant pH level during the 24 h of maturation, as described in Chapter 3. Secondly, the sealed medium inside the semen straw can not be contaminated with micro-organisms from within the incubator. There were no significant differences between the number of oocytes that matured in micro-drops compared to those that matured inside the semen straws, either in the incubator or in the vagina of the goat doe.

The freeze-storage of the amniotic fluid at -20 ºC did not seem to have influenced the maturation capability of the amniotic fluid, as there were no significant differences between the numbers of oocytes reaching maturation in the fresh BAF compared to that of frozen-thawed BAF.

It can therefore be concluded that a semen straw can be used for the maturation of bovine oocytes. The vagina of a goat doe will serve as an incubator for the maturation process, and either TCM-199 with estrus cow serum, fresh bovine amniotic fluid of frozen-thawed bovine amniotic fluid, with or without the addition of 10% estrus cow serum, can be used as maturation media.

6.8 References

Austin, C.R. (1951). Observations on the penetration of sperm into mammalian eggs. *Australian Journal of Scientific Research* **4**, 581-596.

Bornslaeger, E.A., Mattei, P.M. and Schultz R.M. (1986). Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. *Dev Biol* **114,** 453–462.

Carabatsos, M.J., Sellitto, C., Goodenough, D.A. and Albertini, D.F. (2000). Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev Biol* **226**, 167–179.

Chang, M.C. (1951). Fertilizing capacity of spermatozoa deposited into the fallopian tube. *Nature* **168**, 697-698.

Chang, M.C. (1952). Development of bovine blastocyst with a note on implantation. *Anatomical Record* **113**, 143-161.

Chang, M.C. (1955). The maturation of rabbit oocytes in culture and their maturation, activation, fertilization and subsequent development in the fallopian tubes. *Journal of Experimental Zoology* **123**, 379-386.

Chang, M.C. (1968). *In vitro* fertilization of mammalian eggs. *Journal of Animal Science* **27**, 15-22.

Conti, M., Andersen, C.B., Richard, F.J., Shitsukawa, K and Tsafriri, A. (1998). Role of cyclic nucleotide phosphodiesterases in resumption of meiosis. *Mol Cell Endocrinol* **145,** 9–14.

Dow, M.P., Bakke, L.J., Cassar, C.A., Peters, M.W., Pursley J.R. and Smith, G.W. (2002). Gonadotrophin surge-induced up-regulation of the plasminogen activators (tissue plasminogen activator and urokinase plasminogen activator) and the urokinase plasminogen

activator receptor within bovine periovulatory follicular and luteal tissue, *Biol Reprod* **66,** 1413–1421.

Elvin, J.A., Clark, A.T., Wang, P., Wolfman M.N. and Matzuk, M.M. (1999). Paracrine actions of growth differentiation factor-9 in the mammalian ovary, *Mol Endocrinol* **13,** 1035–1048.

Eppig, J.J. (2001). Oocyte control of ovarian follicular development and function in mammals. *Reproduction* **122,** 829–838.

Gilchrist, R.B., Nayudu, P.L., Nowshari, M.A and Hodges, J.K.. (1995). Meiotic competence of marmoset monkey oocytes is related to follicle size and oocyte–somatic cell associations. *Biology of Reproduction* **52,** 1234–1243.

Gordo, A.C., He, C.L., Smith, S. and Fissore R.A. (2001). Mitogen activated protein kinase plays a significant role in metaphase II arrest, spindle morphology, and maintenance of maturation promoting factor activity in bovine oocytes, *Mol Reprod Dev* **59**, 106–114.

Gutierrez, C.G., Ralph, J.R., Telfer, E.E., Wilmut, I. and Webb, R. (2000). Growth and antrum formation of bovine pre-antral follicles in long-term culture *in vitro*. *Biology of Reproduction* **62**, 1322–1328.

Homa S.T. (1995). Calcium and meiotic maturation of the mammalian oocyte. *Mol Reprod Dev* **40**, 122–134.

Hulshof, S.C.J., Figueiredo, J.R., Beckers, J.F., Bevers, M.M., van der Donk, J.A. and van den Hurk, R. (1995). Effects of fetal bovine serum, FSH and 17β estradiol on the culture of bovine pre-antral follicles. *Theriogenology* **44**, 217–226.

Lincoln, A.J., Wickrramasinghe, D., Stein, P., Schultz, R.M., Palko, M.E., De Miguel, M.P., Tessarollo, L. and Donovan, P.J. (2002). Cdc25b phosphatase is required for resumption of meiosis during oocyte maturation. *Nat Genet* **30,** 446–449.

Maller, J.L. and Krebs E.G. (1977). Progesterone-stimulated meiotic cell division in Xenopus oocytes. Induction by regulatory subunit and inhibition by catalytic subunit of adenosine 3':5'-monophosphate-dependent protein kinase, *J Biol Chem* **252,** 1712–1718.

Maller, J.L. and Krebs, E.G. (1980). Regulation of oocyte maturation, *Curr Top Cell Regul* **16**, 271–311.

Matzuk, M.M., Burns, K.H., Viveiros, M.M. and Eppig, J.J. (2002). Intercellular communication in the mammalian ovary: oocytes carry the conversation, *Science* **296,** 2178–2190.

McCaffery, F.H., Leask, R., Riley, S.C., Telfer, E.E. (2000). Culture of bovine pre-antal follicles in a serum-free system: markers for assessment of growth and development. *Biology of Reproduction* **63**, 267–273.

Otoi, T. Yamamoto, K. Koyama, N. Tachikawa S. and Suzuki, T. (1997). Bovine oocyte diameter in relation to developmental competence, *Theriogenology* **48**, 769–774.

Peng, X.R., Hsueh, A.J., LaPolt, P.S., Bjersing, L. and Ny, T. (1991). Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. *Endocrinology* **129**, 3200–3207.

Picton, H., Briggs, D. and Gosden, R. (1998). The molecular basis of oocyte growth and development, *Mol Cell Endocrinol* **145,** 27–37.

Pincus, G. and Enzman, E.V. (1935). The comparative behavior of mammalian eggs *in vivo* and *in vitro*. *Journal of Experimental Medicine* **62**, 665-675.

Pincus, G. and Saunders, B. (1939). The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. VI. The maturation of human ovarian ova. *Anatomical Record* **75**, 737-741.

Richards, J.S., Russell, D.L., Ochsner, S., Hsieh, M., Doyle K.H. and Falender A.E. (2002). Novel signalling pathways that control ovarian follicular development, ovulation, and luteinization, *Recent Prog Horm Res* **57,** 195–220.

Salustri, A. (2000). Paracrine actions of oocytes in the mouse pre-ovulatory follicle, *Int J Dev Biol* **44,**.591–597.

Talbot, P., Shur, B.D. and Myles, D.G. (2003). Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interaction, and sperm-egg fusion. *Biology of Reproduction* **68**, 1-9.

Telfer, E.E. (1996). The development of methods for isolation and culture of pre-antral follicles from bovine and porcine ovaries. *Theriogenology* **45**, 101–110.

Telfer, E.E. (1998). *In vitro* models for oocyte development. *Theriogenology* **49**, 451–460.

Telfer, E,E., Binniem, J.P., McCaffery, F.H. and Campbell, B.K. (2000). *In vitro* development of oocytes from porcine and bovine primary follicles. *Mol Cell Endocrinol* 163, 117-123.

Trounson, A., Anderiesz, C. and Jones, G. (2001). Maturation of human oocytes *in vitro* and their developmental competence. *Reproduction* **121**, 51–75.

Van den Hurk, R., Bevers, M.M. and Beckers, J.F. (1997). *In vivo* and *in vitro* development of pre-antral follicles. *Theriogenology* **47**, 73–82.

Van den Hurk, R., Bevers, M.M. and Dieleman, S.J. (1999). In: *Comparative endocrinology and reproduction*, Editors: K.P. Joy, A. Krishna and C. Haldar, Narosa Publishing House, New Delhi, pp. 296–312.

Van den Hurk, R., Abir, R., Telfer, E.E. and Bevers, M.M. (2000). Primate and bovine immature oocytes and follicles as sources of fertilizable oocytes. *Hum Reprod Update* **6,** 457–474.

Van den Hurk, R. and Zhao, J. (2004). Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. Theriogenology (in press).

Wiersma A., Hirsch B., Tsafriri A., Hanssen R.G., Van de Kant M. and Kloosterboer H.J., Conti, M. and Hsueh, A.J. (1998). Phosphodiesterase 3 inhibitors suppress oocyte maturation and consequent pregnancy without affecting ovulation and cyclicity in rodents. *J Clin Invest* **102**, 532–537.

Wittmaack, F.M. Kreger, D.O. Blasco, L. Tureck, R.W. Mastroianni L. and Lessey, B.A. (1994). Effect of follicular size on oocyte retrieval, fertilization, cleavage and embryo quality in in vitro fertilization cycles: a 6 year data collection, *Fertility and Sterility* **62**,1205–1210.

Wynn, P., Picton, H.M., Krapez, J.A., Rutherford, A.J., Balen A.H. and Gosden, R.G. (1998). Pretreatment with follicle stimulating hormone promotes the numbers of human oocytes reaching metaphase II by in vitro maturation, *Hum Reprod* **13,** 3132–3138.

Yokoo, M. and Sato, E. (2004). Cumulus-oocyte interactions during oocyte maturation. *Int Rev Cytol* **235**, 251-291.

DEVELOPMENT OF OVINE EMBRYOS UNDER MICRO-GRAVITY CONDITIONS AT THE INTERNATIONAL SPACE STATION

7.1 Abstract

During the 33rd launch of the Soyuz capsule and Proton rocket by the Russian Rocket and Space Corporation (Energia) from the Baykonur launch pad in Kazachstan in April 2002, ovine 1-cell embryos were sent in a portable incubator to the International Space Station (ISS) for a culture period of 10 d to investigate embryonic development under micro-gravity conditions. The early-stage embryos were collected after follicular multi-stimulation from indigenous Tegeres breed ewes and artificial insemination with electro-ejaculated undiluted semen. Eight embryos were placed in two special, closed-system, plastic culture test tubes in a portable incubator, and eight embryos were cultured under similar conditions on earth. Pre-conditioned culture medium (TCM-199 with 10% fetal bovine serum, 1% antibiotics and 0.5% essential and non-essential amino acids) was injected into the special culture tubes to change the culture medium every second to third day by the cosmonaut, Mark Shuttleworth, the first African in space. A special 2 µ pore size filter inside the test tube prevented the embryos from being flushed from the tube during the medium changes. After the return of the samples from the ISS, the space and control embryos were frozen-stored in liquid nitrogen and transported to our laboratory in Stellenbosch, South Africa, where further evaluations and cell counts were done. There were no significant difference in developmental rate between the space and ground control samples in 1-cell embryos reaching the blastocyst or hatched blastocyst stage, but significantly more inner cell mass cells developed in the hatched blastocyst stage embryos cultured in space than in the ground control embryos. Due to

government animal health control regulations we were not allowed to transfer any of the embryos to recipient ewes.

7.2 Introduction

On 12 April 1961 Yuri Gagarin, a Russian cosmonaut, where launched into space to be the first human being to experience micro-gravity. Since that time, scientists have been attempting to research the effects of space travel and micro-gravity on the development of living organisms, especially mammalian gametes. Mammalian reproductive physiology, including fertilization and early development, has not yet been studied in depth in a micro-gravity environment. Long space flights and eventual colonization of our solar system will require control of reproductive function and understanding of factors unique to space flight. Will early-stage mammalian embryos survive increased gravity during launching of a space rocket, and will they develop under micro-gravity conditions? A study and an understanding of crucial factors and conditions in outer space will enable researchers to realize the complete life cycle in outer space, from fertilization to delivery and sexual maturation.

Two of the most profound changes that become apparent on leaving the earth is firstly that of macro-gravity during the launching period, and then secondly of micro-gravity when the space vehicle is put in orbit in outer space.. The adaptation to long-term micro-gravity might pose important problems with respect to reproduction and embryonic development in mammals (Kojima *et al.* 2000)

Reproductive experiments have been performed on insects, fish, amphibians, and birds in actual and simulated weightless conditions (Miquel and Philpott 1978; Smith and Neff 1986; Boda

et al. 1991; Ubbels *et al.* 1992; Serova 1993; Snetkova *et al.* 1995; Souza *et al.* 1995). Unfortunately, the effects of micro-gravity on mammalian reproductive function, especially fertilization and early embryonic development, are conflicting and the data is very sparse. One of the first attempts to assess the fertilization capacity of rats *in vivo* in micro-gravity was made during the Cosmos-1129 flight (Serova and Denisova 1982). During the 18.5-day flight, two of the five females became pregnant, but the embryos were absorbed because of the effect of impact acceleration subsequent to the weightlessness-induced decrease of total resistance. This experiment demonstrated only that fertilization occurred in a space environment, but it was unclear whether fertilization occurred normally under those conditions.

Because of technical limitations, cost and availability, space-borne experiments have been limited. Earth-bound simulations have therefore been used to explore the effects of altered gravity. Such a simulation is the clinostat, and rotating culture flask keeping developing material and cells in suspension. The clinostat has been used to produce a vector-averaged gravitational environment. It was first used by plant physiologists more than 100 years ago to study gravitational biology (Larsen 1962). Recently, it has been used to study mammalian cell growth and differentiation and morphogenesis in response to alterations in gravitational conditions (Cogoli *et al.* 1984; Gruener 1985; Kunisada *et al.* 1997; Rulong *et al.* 1998). Moore and Cogoli (1996) recommended that a biologic object proposed for a space-flight experiment (including fertilization and development) should first be investigated in clinostat experiments before it is selected.

De Mazière *et al.* (1996) investigated the effect of micro-gravity on the early embryonic development of *Xenopus laevis* (South African clawed toad) during the launch of the Maser-6 sounding rocket launched from Kiruna in Sweden in 1993. Gravity plays a role in the early

development of the clawed toad shortly after fertilization, when the oocyte becomes loose from in its vitelline membrane and jelly coat. It then rotates until the animal-vegetal axis changes to vertical, because the vegetal hemisphere which is loaded with coarse yolk granules is heavier than the animal hemisphere. Experiments done during the Texus-17 (Ubbels *et al.* 1989) and Maser-3 sounding rocket missions (Ubbels *et al.* 1992), as well as Shuttle missions IML-1 (Ubbels *et al.* 1994) and SL-J (Black & Souza 1994) of the North American Space Agency (NASA) revealed that *Xenopus laevis* oocytes can be successfully fertilized, initiate embryonic development, are symmetrical in shape and will form seemingly normal gastrulae and tadpoles.

In a pilot experiment during the Maser-3 sounding rocket mission, *Xenopus laevis* oocytes were fertilized in micro-gravity (Ubbels *et al.* 1992). After return from space these embryos were further cultured under normal gravity conditions. Most of the embryos from two small samples died during gastrulation. From the one sample, however, a few larvae developed, but with an abnormally developing posterior region. A further experiment was therefore designed during the Maser-6 flight to assess whether the abnormalities seen during Maser-3 were reproducible effects. The cause of the abnormalities was investigated to establish if it was due to (1) the micro-gravity environment where the fertilization took place, due to (2) flight perturbations, or (3) due to inherent properties of the oocytes used. In order to distinguish the effect of flight perturbations on the development of the tadpoles, the fertilization of the oocytes inside the rocket took place in a 1-g centrifuge on board (De Mazière *et al.* 1996). The results of this experiment concluded that seemingly normal *Xenopus* larvae can originate from oocytes fertilized and developed in micro-gravity conditions during the first 5 to 6 min after fertilization. These initially abnormal developing blastulae recover and develop normally from the gastrula stage onward when cultured on earth. This was also true for *Xenopus* embryos continuously cultured in the clinostat (Neff *et al.*

1993). The *Xenopus* embryos developed normally into tadpoles under these constant artificial micro-gravity conditions. The results of the Maser-6 mission were, however, in contradiction with the pilot study during the Maser-3 mission, because none of the abnormalities of the posterior region was seen during the later Maser-6 mission. These differences could be attributed to more stable yolk area of the oocytes used. The intrinsic properties of the eggs are due to the female producing the oocytes, and while different *Xenopus* females were used for the production of the oocytes for the two space missions, the difference in larvae development can be attributed to that (De Mazière *et al.* 1996). The fact that the Maser-6 flight was less vigorous than the Maser-3 flight, because the launcher and rocket were different, could also have contributed to the difference in development of the posterior region of the *Xenopus* larvae under micro-gravity conditions.

Kojima *et al.* (2000) studied mammalian fertilization and further embryonic development making use of a horizontal clinostat devise as described earlier. The clinostat rotates so that the floor of the culture chamber turns continuously around an axis that is perpendicular to the axis of gravity (vertical).

When ovine embryos are cultured under normal gravity conditions on earth, they hatch on day seven to eight after fertilization. The hatched embryo will then, due to gravity, attach to the floor of the receptacle, which can be a well of a petri-dish or the floor of a tissue-culture quality test tube. A single layer of trophoblast cells will such be formed, and the embryo will loose its appearance and ability to give rise to a pregnancy and offspring.

During 2002 the opportunity arose to send early-stage ovine embryos with the South African cosmonaut, Mark Shuttleworth, to the international space station. A specially adapted portable

incubator was designed for this purpose. Specialized equipment had to be developed within a time span of 3 m before the launch of the Soyuz capsule # 33 in April 2002, taking the effects of micro-gravity on liquids into account.

7.3 Hypothesis

Micro-gravity conditions will support ovine embryonic development in a test tube up to the blastocyst stage.

7.4 Material and Methods

Twenty Tegeres breed sheep ewes (hybrid between Edelweiss and Suffolk sheep breeds), indigenous to Kazachstan, were multi-ovulated with a FSH-program as described in Chapter 1. Due to a very severe winter season, the ewes were in a <2/5 body condition score at the onset of the follicular multi-stimulation program. The animals were fed a mixture of lucerne hay and natural grass, supplemented with maize bran. The ewes were cervically inseminated with undiluted semen collected by electro-ejaculation from two Tegeres rams, and then left with the rams for the next 24 h. Insemination took place ~24 h before the animals were slaughtered. About 14 h before the launch of the Russian Proton space rocket and Soyuz capsule from Baykonur in Kazakhstan, the ewes were slaughtered and the oviducts surgically removed. The oviducts with their 1-cell stage embryos were transported in phosphate buffered saline (PBS, Sigma Cat # P-3813) at 37 ºC to a sterile laboratory near the launching site, where the early-stage embryos were flushed from the oviducts with the aid of a 20 gauge intravenous catheter (Abbocath-T, Venisystems, Sligo, Ireland), a 10 mL rubberless syringe and D-PBS medium. The uterine-tubal junction (UTJ) of each tract was cut, and the intravenous catheter inserted into the uterine side of the oviduct. The embryos were retrograde flushed through the infundibulum part of the oviduct and the flushing

medium collected into a 200 mL sterile glass beaker. A total of 16 1-cell ovine embryos were collected from the 20 reproductive tracts.

The embryos were then washed four times by transferring them with less than 1% of tissue culture medium (TCM-199, Sigma, USA, Cat. # M-7528) through a series of five wells. Half of the collected ovine 1-cell stage embryos (n=8) were then transferred in groups of four to two separate, adapted, tissue culture quality 5 mL test tubes (Cellstar, Greiner Bio-One, Friekenhausen, Germany) which were placed inside the portable incubator (Figure 6.1, space embryos). Eight more embryos were placed in similar test tubes into a second similar, portable, battery operated incubator (control embryos). The culture medium used in both the culture and reservoir tubes was TCM-199 with 10% of fetal bovine serum (FBS, Highveld Biologicals, Johannesburg, South Africa), 1% antibiotics (Sigma, USA, Cat A-5995) and 0.5% of both essential and non-essential amino acids (Sigma Cat # M-5550 and M-7145, respectively). The one incubator was sent to the international space station for a period of ten days, and the second incubator were kept on earth for the ten day period as a control.

Certain conditions at the International Space Station had to be taken into consideration when the culture experiment was planned:

1. No sharp objects, like metal needles, were allowed to be handled by the cosmonauts, as they might prick their fingers, causing bleeding. If the floating blood entered the life support systems, it might create a suitable culture medium for micro-organisms in the ISS. We therefore had to use plastic needles (Blunt Plastic Cannula, Becton Dickenson, Franklin Lakes, USA) for all of the culture medium changes.

2. Due to the absence of gravity at the ISS, it is not possible to aspirate a liquid, like culture medium, from a solid tube, such as a test tube. We therefore had to store the culture

medium inside the 14 mL reservoir tubes (Cellstar) in sterile plastic bags that would collapse when culture medium was aspirated from the reservoir tube by means of the 10 mL rubberless syringe and the plastic needle.

3. The injection site of the culture tube had to be such that it would not leak even if it was punctured 10 times or more. Any leakage meant that culture medium might float in the atmosphere of the ISS, ending up in the life support systems with danger to the inhabiting cosmonauts. Medium was changed five times during the duration of the space flight, and every time it was injected through the intravenous fluid-like injection site that would not leak even if it was punctured many times. The injection sites (Injection Site, Becton Dickenson) were machined to perfectly fit into the open end of the 5 mL culture tubes and 14 mL reservoir test tubes. The connection was further sealed with parafilm ("M", American National Can, Greenwich, USA). The empty 5 mL culture tubes and 14 mL reservoir tubes were then gamma ray sterilized.

4. Three barriers to prevent liquid leakage from floating in the atmosphere of the ISS had to be in place: (1) the culture medium was inside a sold walled, plastic culture tube closed of by an injection site that would not leak even if punctured many times; (2) the lid of the incubator (Biotherm, Cryologic, Australia) containing the culture and reservoir tubes were replaced after every medium change (see Figure 6.2); (3) the portable incubator was placed inside a Perspex glove box, and all of the medium changes had to take place with gloved hands inside the glove box (Figure 6.3).

In both incubators, about half of the culture medium in the culture tubes was replaced every two to three days with fresh medium from the reservoir tubes. The pre-conditioned reservoir culture medium as kept inside sterile plastic bags in 14 mL test tubes, was aspirated with a 10 mL

rubberless syringe and a plastic needle pierced through the injection site of the reservoir tube. The stopper at the end of the needle point of the exit line of the culture test tube was removed, and the tube attached to the waste bag (Flexboy, Stedim, USA) connected to the needle point of the culture tube (Fig 6.1). The plastic needle of the rubberless syringe with the reservoir medium was now inserted through the injection site of the culture tube. About 2 mL of the preconditioned culture medium was then injected into each of the culture test tubes. While the fresh culture medium was injected into the culture tube, half of the used culture medium was flowing through the membrane filter at the end of the exit tube, through the straw exit line inside the tube and out through the waste tubing into the waste bag. The 2 μ sterile filter at the tip of the exit tube prevented the embryos from being flushed out of the culture tube when fresh medium was injected. The exit line was now removed, and the stopper replaced to the needle end of the culture test tube.

At the end of the space journey, the culture test tubes were removed from the portable incubator in the ISS and returned to earth in an insulated container (Biokont-T Passive Thermostat, Germany) maintaining the temperature of the samples above 30 ℃.

When the culture test tubes were received at a make-shift laboratory in Star City Moscow, they were emptied into a 35 mm Petri dish and the embryos identified. The embryos were washed three times in conditioned TCM-199 with 10% FBS and 1% antibiotics. Both the micro-gravity cultured embryos as well as the ground control embryos were then frozen in a freezing medium containing 1.5 molar ethylene glycol in straws in a portable liquid nitrogen freezer (CL863 FreezeControl Programmable Freezer, Cryologic, Australia) . The embryos were stored in liquid nitrogen, and transported to our laboratory in Stellenbosch, South Africa.

In the laboratory at the University of Stellenbosch, the frozen embryos cultured at the space station and on earth were taken from the liquid nitrogen, thawed and transferred to dishes with

holding medium for further analysis. The embryos were stained with DNA-specific fluorochrome stain, and the trophoblast and inner cell mass cells counted and recorded. The embryos were placed in a 50 µL drop of culture medium on a glass slide, and covered with a glass cover slip with a drop of a 50:50 heated mixture of Vaseline and candle wax at each corner. The cover slip was then very carefully lowered and pushed down until the cover slip made contact with the embryos. This could be visualized by a halo forming around the embryo. Care was taken not to put to much pressure on the cover slip, because this will cause the embryo to disintegrate and disperse all of its cellular material. The sides of the cover slip was then sealed with a special rubber cement, and only a small "window" left open on both sides for the administration of the fluorochrome stain, bisbenzimide (Hoechst 33324, USA, Cat B-2261) . The stain was added at the one window by means of a micro-pipette, and dispersed underneath the cover slip by aspirating medium at the other window with a paper towel. The two windows were then closed with the rubber cement. Counting of the blastomeres commenced within 48 hours after staining, because a fluorochrome stain tends to loose its fluorescent ability over time.
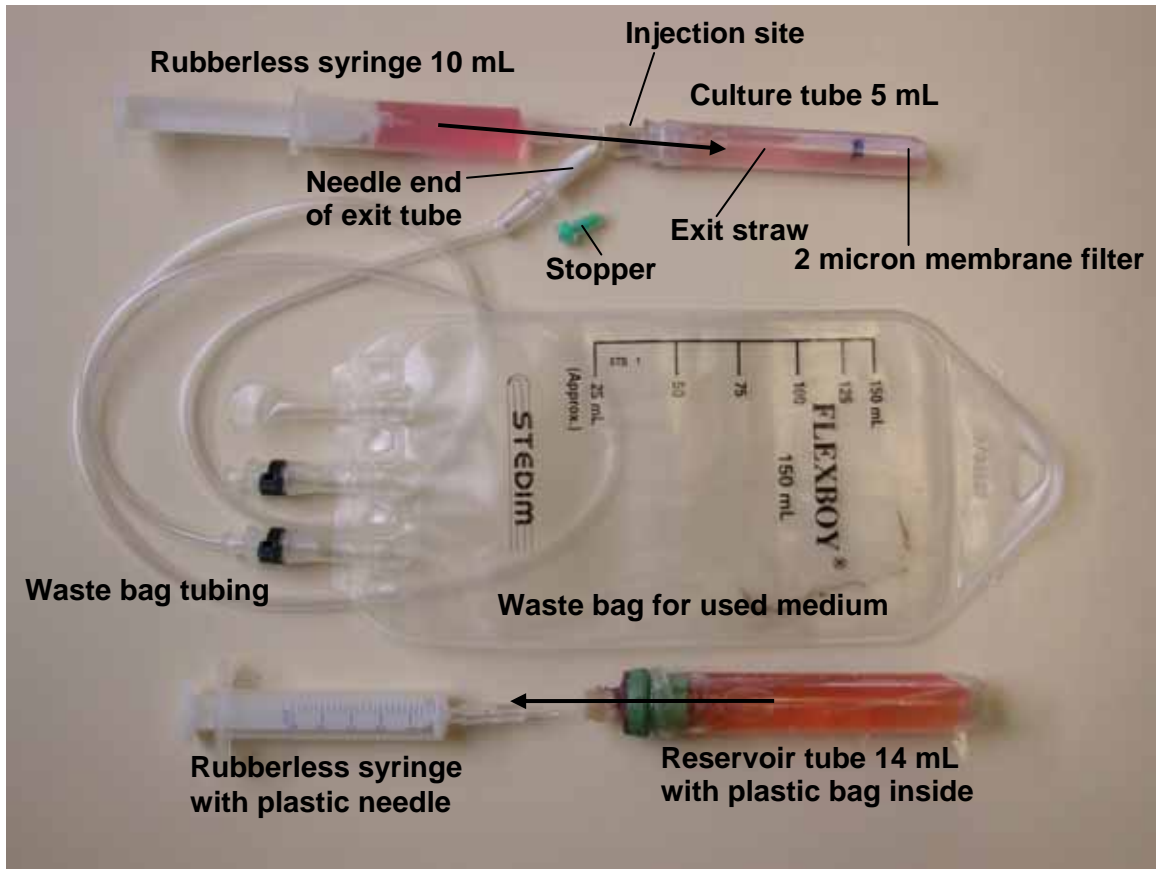
Fig 6.1. Adapted test tubes, tubing and waste bag for culturing 1-cell stage ovine embryos at the International Space Station.

Figure 6.2. Side (A) and top view (B) of the portable incubators with lid used to culture the ovine embryos in space and on earth.
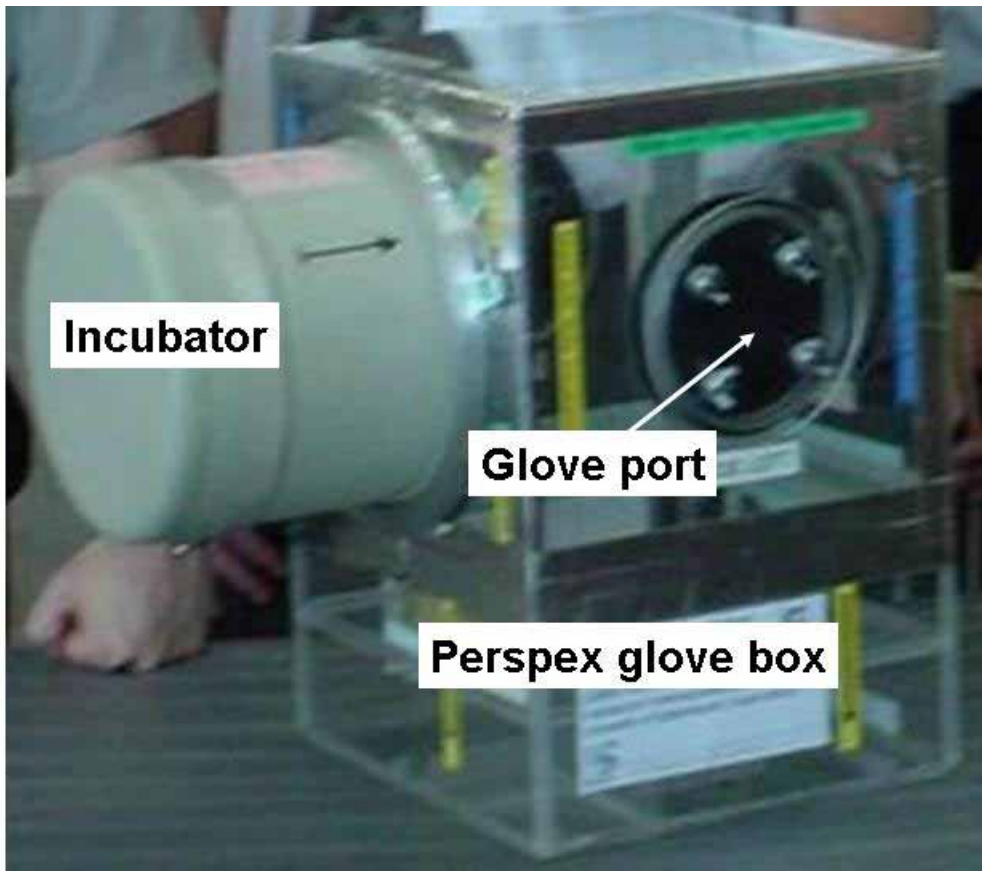
Figure 6.3. Glove box with attached incubator on the left.

7.5 Results

Figure 6.4 was taken through the eyepiece of an inverted microscope of the 1-cell ovine embryos collected and sent to the ISS for the 10 d culture period.
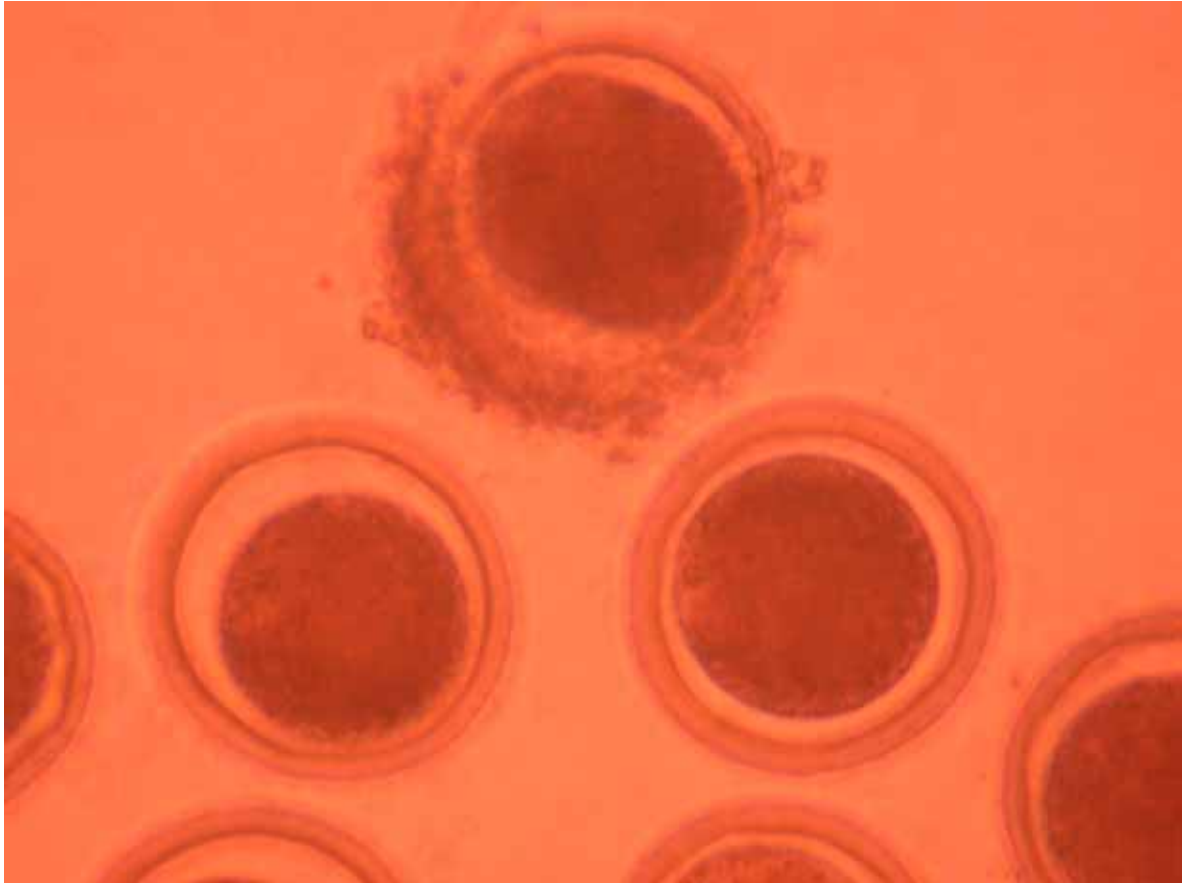
Figure 6.4. 1-Cell ovine embryos at the beginning of the culture period in space and as controls on earth.

The results of the ovine embryos received after 10 d of culture at the ISS, as well as of the control embryos, are presented in Table 6.1. Table 6.2 represents the mean number of blastomeres ± SD for the blastocyst stages of the space and control embryos. The number of cells of the inner cell mass (ICM) for the hatched blastocyst stages are also given.

Table 6.1. Number of ovine embryos in each developmental stage cultured at the international space station and as ground controls

	n	No cleavage (%)	2-Cell to morula (%)	Blastocyst stage (%)	Hatched blastocyst stage (%)
Ground controls	8	2	3 (37.5%)	1 (12.5%)	2 (25%)
Space embryos	8	2	2 (25%)	1 (12.5%)	3 (37.5%)

No significant differences

Table 6.2. Mean number of blastomeres ± SD at the different stages of embryo development

	2-Cell to morula	Blastocyst	Hatched blastocyst	HB: Inner cell mass cells
Ground controls	10±9	66	105±9[a]	19.5±4[c]
Space embryos	10±3	58	148.7±17[b]	49.7±7[d]

[ab,cd] Different superscripts in the same column differ significantly ($p<0.05$)

Six of the eight ovine embryos cultured in space and the earth control embryos cleaved at least once. Three of the six ground control cleaved embryos reached the blastocyst stage of development, while four of the six cleaved embryos cultured in space reached the same stage. Of the three control blastocyst stage embryos, two hatched on earth, while three of the four blastocysts cultured in space hatched from the zona pellucida. Due to low embryo numbers, this was not significant.

In the three ground control embryos, and the two embryos cultured in space that did not reach the blastocyst stage of development, the blastomere numbers were low (10 blastomeres each). The ground control blastocyst stage embryos consisted of 66 blastomeres, and the space blastocyst of 58 cells. In the hatched blastocyst stage embryos there were significant differences between the embryos in both blastomere numbers and ICM numbers. On average, the control

hatched blastocyst stage embryos consisted of 105 blastomeres, while the mean number of blastomeres for the space embryos were 148 cells ($p<0.05$). The control embryos had, on average, 19 cells in the inner cell mass, while the space hatched blastocyst stage embryos had almost 40 cells. This can be seen in Figure 6.5, that shows that the inner cell mass cells of the space embryos kept on multiplying after hatching, increasing the number to more than twofold that of the control embryos after hatching. Figure 6.6 shows a hatched blastocyst stage ovine embryo that was cultured at earth's gravity conditions, without the outgrowth of the inner cell mass cells.
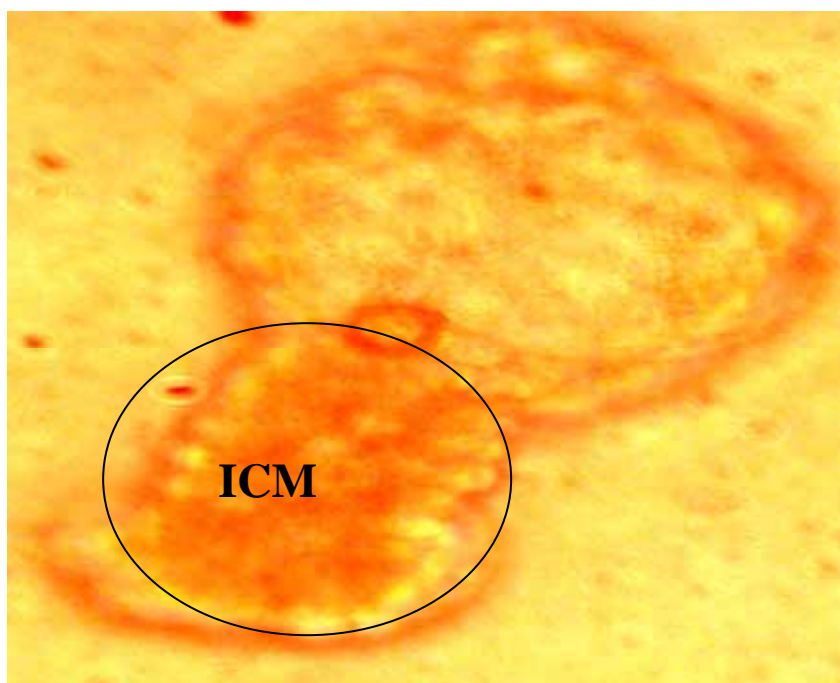

Fig 6.5. Outgrowth of the inner cell mass (ICM) in a space hatched
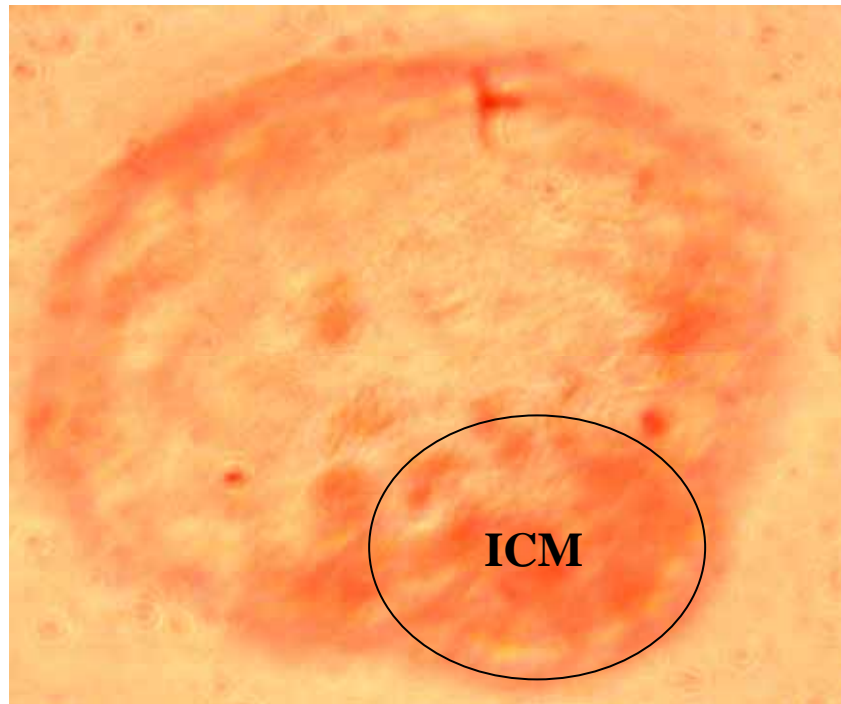blastocyst stage ovine embryo.

Fig 6.6. Ground control hatched blastocyst stage ovine embryo.

7.6 Discussion and conclusion

Kojima *et al.* (2000) used a clinostat to simulate micro-gravity conditions for the fertilization and culture of murine embryos under normal gravity conditions on earth. In their first experiment there were no statistically significant differences in the efficiency of achieving normal fertilization *in vitro* among the clinostat and control conditions. There were also no significant differences in the percentage of post-implantation development of ova fertilized in micro-gravity or as controls. In their second experiment, they found a statistically significant decrease observed in the number of embryos reaching the morula and blastocyst stages after 96 hours of culture under the condition of clinostat rotation, compared to the controls (18% and 25% versus 24% and 38%, respectively, $p < 0.05$). They therefore concluded that the process of fertilization *in vitro* is not sensitive to the

gravitational vector. However, the possibility exists that the frequency of early embryonic lethality of murine embryos is increased by micro-gravity.

In the present study the fertilization of ovine oocytes under micro-gravity conditions were not investigated, because the ovine embryos were already at the 1-cell stage when they were transferred to the portable incubator that was sent to the ISS. The micro-gravity conditions at the international space station did, however, not seem to have played a significant role in the rate of embryonic development of the ovine embryos up to the hatched blastocyst stage. The effect of micro-gravity as expressed by the death of a larger number of murine embryos cultured in the simulated micro-gravity conditions in the clinostat compared to the controls was not experienced in the ovine embryos. On the contrary, a greater number of blastocyst and hatched blastocyst embryos were returned from the micro-gravity conditions than were cultured under control normal gravity conditions. This was, however, due to small embryo numbers, not significant.

When the ground control blastocyst stage embryos hatched about 7 d after fertilization, the inner cell mass numbers remained stationary. In the case of the space embryos, the inner cell mass cells kept on multiplying for the next three days until the samples were returned to earth from the space station, more than doubling their numbers. This is also contrary to the findings of Kojima *et al.* (2000), where no differences in appearance were observed between the clinostat culture murine embryos and the stationary controls.

7.7 References

Black, S.D. and Souza, K.A. (1994). Regulative development of Xenopus laevis at micro-gravity. *ASGSB Bulletin* **8** (1), 6 (Abstract).

Boda, K., Melesko, G.I., Sabo, V., Sepelev, E.J., Gurjeva, T.S. and M. Jurani. (1991). Embryonic development of Japanese quail under micro-gravity conditions. *Physiologist* **34**, 59–61.

Cogoli, A., Tschopp, A. and Fuchs-Bislin, P. (1984). Cell sensitivity to gravity. *Science* **225,** 228–230 .

De Mazière, A., Gonzalez-Jurado, J., Reinen, M., Narraway, J. and Ubbels, G.A. (1996). Transient effects of micro-gravity on early embryos of *Xenopus laevis*. *Adv Space Res* **17** (6/7), 219-223.

Gruener, R. (1985). Stimulated hypo-gravity and synaptogenesis in culture. *Physiologist* **28,** 79–80.

Kojima, Y., Sasaki, S., Kubota, Y., Ikeuchi, T., Hayashi, Y. and Kohri, K. (2000). Effects of simulated micro-gravity on mammalian fertilization and pre-implantation embryonic development *in vitro*. *Fertility and Sterility* **74** (6), 1142-1147.

Kunisada, T., Kawai, A., Inoue, H. and Namba, M. (1997). Effects of simulated micro-gravity on human osteoblast-like cells in culture. *Acta Med Okayama* **51**, 135–140.

Larsen P. (1962). Geotropism: an introduction. In: W. Ruhland, ed. Encyclopedia of plant physiology. Berlin: Springer Verlag, 34–73.

Miquel, J. and Philpott, D.E. (1978). Effects of weightlessness on the genetic and aging process of Drosophila melanogaster. In: Final report: *US experiments flown on the Sowiet satellite Cosmas* **936**, 32.

Moore, D. and Cogoli, A. (1996). Gravitational and space biology at the cellular level. In: D. Moore, P. Bie and H. Oser, Editors, *Biological and medical research in space* (4th ed.),, Springer-Verlag, Berlin, 1–106.

Neff, A.W., Yokota, H., Chung, H.M., Wakara, M. and Malacinski, G. (1993). Early amphibian morphogenesis is sensitive to novel gravitational fields. *Dev Biol* **155**, 270-274.

Nieuwkoop, P.D. and Faber, J. (1975). Normal table of *Xenopus laevis* (Daudin), 2nd Edition, North Holland Publishing Company, Amsterdam.

Rulong, S., Takagishi, Y., Koga, K., Resau, J.H., Inouye, M., Jeffers M. (1998). Clinostat rotation promotes HGF/SF-induced morphogenesis of rat pancreas oval cells. *J Aerospace Env Med* **35,** 55–61.

Serova, L.V. (1993). Changing gravity level and the development of animals. *Physiologist* **36**, 31-33.

Serova L.V. and Denisova, L.A. (1982). The effect of weightlessness on the reproductive function of mammals. *Physiologist* **6**, 9–12.

Smith, R.C. and Neff, A.W. (1986). Organization of Xenopus egg cytoplasm: response to simulated micro-gravity. *J Exp Zool* **239**, 365-378.

Snetkova, E., Chelnaya, N., Serova, J., Saveliev, S., Cherdanzova E. and Pronych, S. (1995). Effects of space flight on Xenopus laevis larval development. *J Exp Zool* **273**, 21-32.

Souza, K.A., Black, S.D. and Wassersug, R.J. (1995). Amphibian development in the virtual absence of gravity. *Proc Natl Acad Sci USA* **92**, 1975–1978.

Ubbels, G.A., Hara, K., Koster, C.H. and Kirschner, M.W. (1983). Evidence for a functional role of the cytoskeleton in determination of the dorsoventral axis in Xenopus laevis eggs. *J Embryol Exp Morph* **77**, 15-37.

Ubbels, G.A., Berendsen, W. and Narraway, J. (1989). Fertilization of frog eggs on a sounding rocket in space. *Adv Space Res* **9** (11), 187-197.

Ubbels, G.A., Berendsen, W., Kerkvliet, S. and Narraway, J. (1992). Fertilization and development of eggs of the South African clawed toad, *Xenopus laevis,* on sounding rockets in space. *Adv Space Res* **12** (1), 181-194.

Ubbels, G.A., Reijnen, M., Meijerink, J. and Narraway, J. (1994). *Xenopus laevis* embryos can establish their spatial bilateral symmetrical body pattern without gravity. *Adv Space Res* **14** (8), 257-269.

CHAPTER 8

CONCLUSION AND RECOMMENDATIONS

To produce *in vivo* embryos with fairly consistent results, one has to control and synchronize the follicular waves that develop on the two ovaries of a female animal. Unlike humans that have only one wave of follicular development and only during the follicular phase, livestock animals have from two to four waves of follicular development during both the follicular phase and the luteal phase of the estrous cycle. The present study has shown that by using estradiol cypionate (ECP) injection to control follicular development, twice as many transferable embryos were produced in sheep ewes (10.9 ± 4.0 vs 4.4 ± 3.5 for ECP vs control group, respectively), 50% more transferable embryos were produced in goat ewes (9.4 ± 9.3 vs 6.5 ± 6.9 for ECP and control groups, respectively), and almost twice as many transferable embryos in cows (8.2 ± 2.8 vs 4.4 ± 1.5 for ECP and control groups, respectively). It is therefore recommended that, if the status of the present wave of follicular development and the absence or presence of a dominant follicle is unknown, that estradiol cypionate be injected 4 d to 5 d before FSH follicular multi-stimulation is started, to suppress the previous wave of follicular development where the presence of a dominant follicle could suppress the development of the other follicles.

The different research studies into the use of heat-inactivated first trimester bovine amniotic fluid (BAF) as a culture medium, has allowed the following conclusions to be made:

5. BAF with 10% fetal bovine serum added will support the development of pre-compacted morula stage sheep, goat and cow embryos to the hatched blastocyst stage as well as or better than a standard embryo culture medium such as TCM-199 with added serum. This is probably

due to the fact that BAF contains the necessary nutrients and growth factors needed for early embryonic development of these three species. Due to the inexpensive collection methods and possible supply of BAF, it is recommended that BAF can replace compound culture media in commercial and experimental setups where early- and later-stage embryos of the three species are cultured for various reasons. The transmission of possible micro-organisms such as viruses and pyrons should, however, be taken into account as a possible risk factor. This could possibly be prevented by filtering the BAF before use, or sterilizing the medium by gamma-irradiation before use.

6. The earlier the stage of pregnancy, the more concentrated seems to be the constituents (growth factors and cytokines) that is beneficial for embryonic development *ex vivo*. This can be seen in the developmental rate of bovine embryos to the hatched blastocyst stage in first trimester bovine amniotic fluid from different origins. In Chapter 3 BAF from a fetus of ~80 d of pregnancy supported 46% (35/75) of grade 1 pre-compacted morula stage bovine embryos to develop to the hatched blastocyst stage. This figure increased to 69% (67/97) when BAF from a ~60 d pregnancy was used as culture medium in Chapter 4 under the same conditions inside an incubator. It is therefore recommended that the amniotic fluid to be used as a culture medium for embryos should be collected from the earliest possible stage of pregnancy. However, at <40 d of pregnancy the volume of amniotic fluid present is very small and not feasible to collect.

7. The concentration of growth or recuperating factors is higher in early-stage BAF than in serum. When TCM-199 with added 10% fetal bovine serum (FBS) was used as culture medium for grade 3 embryos in sheep, goat and cow embryos, 18%, 23% and 16% developed to hatched blastocyst, respectively. However, when BAF from a ~60 d pregnancy with 10% fetal bovine

serum added was used as culture medium for similar grade 3 embryos, 43%, 49% and 53% of the pre-compacted stage embryos developed to the hatched blastocyst stage for sheep, goat and cows embryos, respectively. Because 10% fetal bovine serum was added to both culture media, it can be deducted that the recuperating proteins must have been only in the BAF, or in higher concentrations in the BAF than in the FBS. Further investigations into the effect of BAF from different stages of bovine and other livestock fetal development on the development of embryos should be investigated, in order to find the optimal time for amniotic fluid collection.

8. First trimester bovine amniotic fluid (BAF) will support bovine oocyte maturation. It was proven that pooled BAF collected from pregnancies of < 90 d, stimulated bovine prophase I oocytes in a 26 h culture period to develop to the metaphase II stage as well as TCM-199 with serum but without added hormones. No significant differences could be found between any of the groups even when other receptacles (wells with micro-drops vs straws) were used, or other incubators (laboratory incubator vs the vagina of a female goat) was used. Furthermore, freezing of the BAF after collection but before use did not seem to have been detrimental to the maturation capacity of BAF for bovine oocytes. When frozen-thawed BAF was compared to fresh BAF as culture media for the development of 2-cell murine embryos, there was a marked decrease in the number of murine embryos reaching the blastocyst and hatched blastocyst stage when cultured in frozen-thawed BAF when compared to fresh BAF (unpublished data).

Interesting results were obtained when 1-cell ovine embryos were sent to the International Space Station (ISS) in a portable incubator for further development. It was found that early-stage ovine embryos can develop to the hatched blastocyst stage when firstly subjected to increased gravity for about 30 min, and then to micro-gravity conditions for the next ten days. The

interesting phenomenon of the outgrowth of the embryos subjected to the micro-gravity conditions could not be explained. What is, however, of significance, is that the inner cell mass numbers more than doubled during the 10 day micro-gravity culture period. If taken into consideration that embryonic stem cells, with their toti-potency potential, are derived from the inner cell mass of an embryo, this becomes a very important finding. The fact that stem cells and somatic cells sent with the embryos to the ISS (unpublished data) developed into a three-dimensional fashion, the culture of the inner cell mass, stem cells and somatic cells under micro-gravity conditions could be the first step towards the production of body tissue and organs.