The Possible Ameliorating Effects of Rooibos, Honeybush and Sutherlandia on Diabetes-Induced Reproductive Impairment in Adult Male Wistar Rats

by

Temidayo Siyanbola Omolaoye

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Supervisor: Prof Stefan S du Plessis

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Declaration

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Abstract

The global prevalence of infertility is on the rise, and so is male factor infertility. Out of the approximately 72.4 million infertile couples worldwide, male factor is responsible for 50%. It has been reported that sperm quality is on the decline, even in healthy men. Several aetiologies have been identified, and this includes disease-related male infertility, such as diabetes mellitus (DM). DM is a chronic non-communicable disease, marked by increased hyperglycaemia which occurs as a result of lack in insulin secretion or due to the insensitivity of the target tissue to the metabolic effect of insulin. It has been reported that 422 million people are affected with DM globally, of which ≤10% is type 1 diabetic and ≤90% is type 2. Studies have shown that although most autoimmune diseases usually affect a higher percentage of women, type 1 diabetes is more prevalent in boys and men. The consequential detrimental effects of DM on male fertility have been reported in both experimental animals and diabetic men. This includes, decreased sperm motility, reduced normal sperm morphology, increased fragmented spermatozoa and many more defects. In the course of unravelling the pathways through which DM affect male fertility, studies have elucidated the role of endocrine dysfunction, formation of reactive oxygen species (ROS) and the subsequent development of oxidative stress (OS). However, other possible related pathways such as the involvement of glycated proteins, are yet to be explored. Since there are evidences that DM is detrimental to male fertility, it is important to firstly understand the underlying pathophysiology and then develop and explore strategies to combat this disease and its associated male reproductive complications.

Many natural and artificial agents have been proposed to have antioxidant properties due to their potential in reducing oxidation. Antioxidants work either by scavenging ROS or by

preventing its formation. Rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and sutherlandia (*Lessertia frutescens*) are plants endemic to Southern Africa. Herbal teas are derived from the leaves and stems of these plants. These infusions have been shown to be caffeine-free. Studies have also reported that the infusions from these plants contain bioactive chemical compounds with therapeutic benefits. Additionally, studies have shown their beneficial role in preventing the development of diseases and some have shown their ameliorative effects. However, their role in male reproduction is little understood as this represents the first study to report the effects of honeybush and sutherlandia on male reproductive function. Based on the expressed gaps in knowledge, the study was divided into two main parts, with each having several objectives.

For the first part, thirty adult male rats were randomly divided into three groups of ten, which included a vehicle and two streptozotocin (STZ) groups receiving either 30mg/kg (STZ30) or 60mg/kg (STZ60) intraperitoneally. For the second part, ninety animals were divided into nine groups of ten without bias. The groups included a control (water only), vehicle (STZ control, citrate buffer + water), rooibos (2% fermented rooibos), honeybush (4% fermented honeybush), sutherlandia (0.2% unfermented sutherlandia), diabetic control (STZ45mg/kg + water), diabetic + rooibos (STZ45mg/kg + 2% rooibos), diabetic + honeybush (STZ45mg/kg + 4% honeybush), and diabetic + sutherlandia (STZ45mg/kg + 0.2% sutherlandia) group. Animals in the diabetic infusion treated groups were pre-treated with the respective infusion one week before DM induction. Animals were monitored closely throughout the treatment period and were sacrificed seven weeks after DM induction. Blood, testes and epididymides were collected for further analysis.

The results of the first part of this study showed the negative effects of DM on male reproductive function, as diabetic animals presented with a decrease in spermatozoa with normal morphology and an increased number of spermatozoa with a higher deformity index. Histological and histomorphometrical analysis of the testis showed alteration in the seminiferous tubules' cellular association in diabetic animals. Additionally, the first section further sheds some light on the involvement/expression of advanced glycation end products (AGE), its receptor (RAGE), mitogen-activated protein kinases and the activation of apoptosis in diabetes, which may contribute to the impairment seen in male fertility. Briefly, in DM, there is altered antioxidant enzyme activity (↓Catalase) which may result in OS. The development of OS can on the one hand instigate the breakdown of polyunsaturated fatty acids (PUFA), which can be measured by the levels of malondialdehyde (MDA) and also indirectly increase AGE formation. The peroxidation of PUFAs attracts more ROS formation, which can activate apoptotic induction, resulting in impaired sperm function. AGEs work by binding to their receptor (RAGE). The AGE-RAGE complex initiates the activation of the MAPKs, which may induce apoptosis by increasing the cleavage of PARP, hence resulting in nDNA damage. All these effects collectively result in reduced male fertility.

To properly understand the role of the infusions (rooibos, honeybush and sutherlandia) on male reproduction of both healthy and diabetic animals, the second part of the study was divided into four sub-studies.

Subsection one of the second part of the study investigated whether treatment with rooibos, honeybush and sutherlandia will impact sperm functional parameters positively or otherwise, in healthy rats. Animals treated with the respective infusions presented with a percentage increase in SOD activity but showed reduced sperm motility and decreased normal

morphology. Paradoxically, they presented with increased sperm concentrations. Hence, rooibos, honeybush and sutherlandia may enhance sperm concentration, which represents the sperm quantity. However, they may impair sperm quality (sperm motility and morphology), when consumed by healthy animals.

Subsection two of the second part of the study investigated the testicular oxidative stress and apoptosis status in diabetic rats following treatment with rooibos, honeybush and sutherlandia infusions. This section highlighted the negative impact of DM on sperm functional parameters through increased lipid peroxidation and reduced antioxidant activity. The infusion treatment groups displayed increased antioxidant enzyme activity, which may be partly responsible for the observed improvement in sperm motility and morphology of diabetic animals receiving rooibos (DRF) and diabetic animals receiving sutherlandia (DSL). Additionally, the current study have shown the increased expression of apoptotic biomarkers in the diabetic control (DC) animals, which were not alleviated by the infusions. This suggests that these infusions play a role in alleviating diabetes-induced sperm function impairment through suppression of OS, but their role in apoptosis is still unclear.

Subsection three of the second part of the study investigated the role of rooibos, honeybush and sutherlandia on insulin signalling in the testes of diabetic rats. All diabetic groups presented with a significant increase in blood glucose levels after diabetes induction. However, the diabetic animals treated with the infusions showed only a mild decrease in fasting blood glucose. The diabetic control animals showed a decrease in testis protein expression of IRS-1, PkB/Akt and GLUT4. Diabetic animals treated with rooibos (DRF) and honeybush (DHB) displayed an upregulation in IRS-1, while diabetic animals treated with sutherlandia (DSL) showed improvement in IRS-2. Although, DRF animals presented with a

decrease in PkB/Akt, DHB and DSL animals displayed upregulation compared to control. All diabetic animals showed increased phosphorylated ERK1/2 and reduced total ERK1/2 when compared to control and vehicle. Additionally, diabetic control animals presented with a non-significant decrease in plasma testosterone concentration compared to control, while DRF and DSL showed a significant decrease and DHB showed upregulation. This suggests that (i) the activation of IRS, PkB/Akt, ERK and GLUT translocation is important in testicular insulin signalling (ii) rooibos, honeybush and sutherlandia may play a role in testicular insulin signalling, however, through different pathways (iii) honeybush may mildly enhance testosterone production in diabetes.

Lastly, the fourth subsection of the second part of the study investigated the descriptive histological evaluation of the testis and cauda epididymis after treating with rooibos, honeybush and sutherlandia in both healthy and diabetic rats. The infusion control groups (RF, HB and SL) showed normal seminiferous tubule cellular association, presence of spermatozoa in the epididymal lumen and normal overall architecture. Both the testicular and epididymal morphology were altered in DM, but these disruptions were mildly alleviated by rooibos, honeybush and sutherlandia.

It appears, from appreciating the results of the entire study, that diabetes does have detrimental effects on male reproduction, both at the tissue and cellular levels. The cellular impact may be exerted through protein glycation, development of OS and apoptosis induction. The use of rooibos, honeybush and sutherlandia, when healthy, should be taken moderately and with caution, as too much may be detrimental and may impair sperm functional parameters. However, in disease conditions such as diabetes, these infusions may be beneficial, as:

(i) Rooibos and sutherlandia may mildly improve sperm quality (motility and morphology),

(ii) Rooibos, honeybush and sutherlandia may mildly reduce hyperglycaemia by enhancing insulin signalling,

(iii) Honeybush may enhance testosterone production,

(iv) Rooibos, honeybush and sutherlandia may mildly improve the histomorphological architecture of the seminiferous tubule and cauda epididymal tubule.

Opsomming

Die wêreldwye voorkoms van onvrugbaarheid, en in spesifiek manlike-faktor-onvrugbaarheid, is besig om toe te neem. Die manlike faktor is verantwoordelik vir 50% van alle gevalle van onvrugbaarheid in die ongeveer 72,4 miljoen onvrugbare paartjies wêreldwyd. Gepaardgaande hiermee word daar berig dat spermkwaliteit aan die afneem is, selfs in gesonde mans. Verskeie etiologieë is geïdentifiseer, en dit sluit in siekteverwante manlike onvrugbaarheid soos diabetes mellitus (DM). DM is ’n chroniese nie-oordraagbare siekte, gekenmerk deur hiperglukemie, wat voorkom as gevolg van ’n gebrek aan insulienafskeiding of weens die ongevoeligheid van die teikenweefsel vir die metaboliese effek van insulien. Daar is berig dat 422 miljoen mense wêreldwyd deur DM geaffekteer word, waarvan ≤10% tipe 1-diabete en ≤90% tipe 2-diabete is. Studies het getoon dat hoewel die meeste outo-immuun siektes gewoonlik ’n hoër persentasie vroue aantas, tipe 1-diabetes meer algemeen voorkom in seuns en mans. Die gevolglike nadelige gevolge van DM op manlike vrugbaarheid is in sowel eksperimentele diere as in diabetiese mans aangemeld. Dit sluit in verlaagde spermmotiliteit, verminderde normale spermorfologie, verhoging in spermatosoa met DNS-fragmentasie en nog vele meer defekte. In die proses om die roetes waardeur DM ’n invloed op manlike vrugbaarheid het te ontrafel, het studies die rol van endokriene disfunksie, die vorming van reaktiewe suurstofspesies (RSS) en die daaropvolgende ontwikkeling van oksidatiewe spanning (OS) toegelig. Ander moontlike verwante roetes, soos die betrokkenheid van geglikeerde proteïene, moet egter nog ondersoek word. Aangesien daar bewyse is dat DM nadelig is vir manlike vrugbaarheid, is dit belangrik om eers die onderliggende patofisiologie te verstaan en dan strategieë te ontwikkel

en te ondersoek om hierdie siekte en die gepaardgaande komplikasies vir manlike voortplanting te bestry.

Daar word voorgehou dat baie natuurlike en kunsmatige middels antioksidant-eienskappe het as gevolg van hul potensiaal om oksidasie te verminder. Antioksidante werk óf deur RSS op te vang óf deur die vorming daarvan te voorkom. Rooibos (*Aspalathus linearis*), heuningbos (*Cyclopia intermedia*) en sutherlandia (*Lessertia frutescens*) is plante wat inheems is aan Suider-Afrika. Kruietee word gemaak van die blare en stamme van hierdie plante. Daar is bewys dat hierdie infusies kafeïenvry is. Studies berig ook dat die infusies van hierdie plante bioaktiewe chemiese verbindings met terapeutiese voordele bevat. Daarbenewens het studies getoon dat hulle ’n voordelige rol speel in die voorkoming van die ontwikkeling van siektes, en sommige het verbeterende effekte getoon. Hulle rol in manlike voortplanting word egter nog nie goed verstaan nie, en hierdie is die eerste studie wat die gevolge van heuningbos en sutherlandia op manlike voortplantingsfunksie aantoon. Op grond van die gemelde kennisleemtes is die studie in twee hoofafdelings verdeel, elk met verskeie doelstellings.

Vir die eerste deel is dertig volwasse manlike rotte lukraak in drie groepe van tien elk verdeel, wat ’n draer en twee streptosotosien (STS) groepe insluit wat óf 30 mg/kg (STS30) óf 60 mg/kg (STS60) intraperitoneaal ontvang het. Vir die tweede deel is negentig diere sonder vooroordeel in nege groepe van tien elk verdeel. Die groepe het ’n kontrole- (slegs water), draer- (STS-kontrole, sitraatbuffer + water), rooibos- (2% gefermenteerde rooibos), heuningbos- (4% gefermenteerde heuningbos), sutherlandia- (0,2% ongefermenteerde sutherlandia), diabeteskontrole- (STS45 mg/kg + water), diabetes + rooibos- (STS45 mg/kg + 2% rooibos), diabetes + heuningbos- (STS45 mg/kg + 4% heuningbos), en diabetes +

sutherlandia- (STS45 mg/kg + 0,2% sutherlandia) groep ingesluit. Diere in groepe wat behandel is met ’n diabetiese infusie is ’n week voor die DM-induksie met die onderskeie infusies behandel. Diere is noukeurig gemonitorgedurende die behandelingsperiode en is sewe weke ná DM-induksie geoffer. Bloed, testes en epididimisse is ingewin vir verdere ontleding.

Die uitslae van die eerste deel van hierdie studie het die negatiewe gevolge van DM op manlike voortplantingsfunksie getoon, aangesien daar ’n afname in spermatosoa met normale morfologie en ’n verhoogde aantal spermatosoa met ’n hoër deformiteitsindeks in diere met diabetes voorgekom het. Histologiese en histomorfometriese analise van die testis het veranderinge in die sellulêre assosiasie van die seminale tubules in diabetiese diere getoon. Daarbenewens lig die eerste afdeling die betrokkenheid/uitdrukking van gevorderde glikasie-eindprodukte (AGE), sy reseptor (RAGE), mitogeen-geaktiveerde proteïenkinases (MGPK) en die aktivering van apoptose in diabetes, wat kan bydra tot die afwykings gesien in manlike vrugbaarheid, toe. Kortliks, in DM is daar ’n veranderde antioksidant-ensiemaktiwiteit (↓ Katalase) wat kan lei tot OS. Die ontwikkeling van OS kan enersyds die afbreek van poli-onversadigde vetsure (POVS) aanhelp, wat gemeet kan word aan die vlakke van malondialdehied (MDA), en andersyds ook die vorming van AGE indirek verhoog. Die peroksidasie van POVS lok meer RSS-vorming, wat apoptotiese induksie kan aktiveer, wat lei tot beskadigde spermfunksie. AGE's werk deur te bind aan hul reseptor (RAGE). Die AGE-RAGE-kompleks inisieer die aktivering van die MGPK’s, wat apoptose kan veroorsaak deur die splitsing van PARP te verhoog, wat gevolglik lei tot nDNA-skade. Al hierdie effekte lei gesamentlik tot ’n verlaagde vrugbaarheid in mans.

Om die rol van die infusies (rooibos, heuningbos en sutherlandia) op manlike voortplanting van beide gesonde en diabetiese diere behoorlik te verstaan, is die tweede deel van die studie in vier substudies verdeel.

Onderafdeling een van die tweede deel van die studie het ondersoek of behandeling met rooibos, heuningbos en sutherlandia sperm se funksionele parameters in gesonde rotte positief of andersins sal beïnvloed. Diere wat met die onderskeie infusies behandel is, het met ’n persentasie toename in superoksieddismutase-aktiwiteit gepresenteer, maar het ’n verlaging in spermmotiliteit en normale morfologie getoon. Paradoksaal genoeg het hulle verhoogde spermkonsentrasies gehad. Rooibos, heuningbos en sutherlandia kan dus moontlik spermkonsentrasie, wat die hoeveelheid sperma verteenwoordig, verhoog. Die gebruik hiervan kan egter moontlik die spermkwaliteit (spermmotiliteit en morfologie) van gesonde diere benadeel.

Onderafdeling twee van die tweede deel van die studie het die testikulêre OS en apoptose status in diabetiese rotte ondersoek na behandeling met rooibos, heuningbos en sutherlandia-infusies. Hierdie afdeling het die negatiewe impak van DM, as gevolg van verhoogde lipiedperoksidasie en verlaagde antioksidant aktiwiteit, op die funksionele parameters van sperms beklemtoon. Die infusiebehandelingsgroepe het ’n verhoogde antioksidant-ensiemaktiwiteit getoon, wat moontlik deels verantwoordelik kan wees vir die waargenome verbetering in spermbeweeglikheid en -morfologie van diabetiese diere wat rooibos (DRF) ontvang, en diabetiese diere wat sutherlandia (DSL) ontvang. Daarbenewens het die huidige studie die toenemende uitdrukking van apoptotiese biomerkers in die diabeteskontrole(DK)-diere getoon, wat nie deur die infusies verlig is nie. Dit dui daarop dat

hierdie infusies ’n rol speel in die verligting van die verswakking van diabetes-geïnduseerde spermfunksies deur onderdrukking van OS, maar hulle rol in apoptose is nog onduidelik.

Onderafdeling drie van die tweede deel van die studie het die rol van rooibos, heuningbos en sutherlandia op insulienseingewing in die testes van diabetiese rotte ondersoek. Al die diabetesgroepe het ’n beduidende toename in bloedglukosevlakke gehad ná die induksie van diabetes. Die diabetesdiere wat met die infusies behandel is, het egter slegs ’n effense afname in die vastende bloedglukose getoon. Die DK diere het ’n afname in testisproteïenuitdrukking van IRS-1, PkB/Akt en GLUT4 getoon. Diabetiese diere wat met rooibos (DRF) en heuningbos (DHB) behandel is, het ’n opregulering in IRS-1 getoon, terwyl diabetiese diere wat met sutherlandia (DSL) behandel is, verbetering in IRS-2 getoon het. Alhoewel DRF-diere ’n afname in PkB/Akt getoon het, het DHB- en DSL-diere opregulering getoon in vergelyking met kontrole. Alle diabetiese diere het verhoogde gefosforileerde ERK1/2 en verlaagde totale ERK1/2 getoon in vergelyking met die kontrole- en draerdiere. Daarbenewens het DK-diere ’n nie-noemenswaardige afname in plasma-testosteroonkonsentrasie in vergeleke met kontrole getoon, terwyl DRF en DSL ’n beduidende afname en DHB opregulering getoon het. Dit dui daarop dat (i) die aktivering van IRS, PkB/Akt, ERK en GLUT translokasie belangrik is in testikulêre insulienseingewing (ii) rooibos, heuningbos en sutherlandia moontlik ’n rol kan speel in testikulêre insulienseingewing deur verskillende roetes en (iii) heuningbos moontlik testosteroonproduksie in diabetes effens kan verhoog.

Laastens het die vierde onderafdeling van die tweede deel van die studie die beskrywende histologiese evaluering van die testis en cauda epididimis ondersoek na behandeling met rooibos, heuningbos en sutherlandia in beide gesonde en diabetiese rotte. Die infusie-kontrolegroepe (RF, HB en SL) het normale seminale tubulêre sellulêre assosiasie,

teenwoordigheid van spermatosoa in die epididimale lumen en normale algehele argitektuur getoon. Beide die testikulêre en epididimale morfologie was veranderd in DM, maar hierdie ontwrigtings is effens verlig deur rooibos, heuningbos en sutherlandia.

Met inagname van die resultate van die hele studie blyk dit dat diabetes wel ’n nadelige uitwerking op manlike voortplanting het, beide op die weefsel- en sellulêre vlak. Die sellulêre impak kan uitgeoefen word deur proteïenglikasie, ontwikkeling van OS en induksie van apoptose. Rooibos, heuningbos en sutherlandia moet matig en met omsigtigheid deur gesonde persone geneem word, aangesien te veel daarvan nadelig kan wees en die funksionele parameters van spermatosoa kan benadeel. In siektetoestande soos diabetes kan hierdie infusies egter voordelig wees, aangesien:

(i) Rooibos en sutherlandia die spermkwaliteit (beweeglikheid en morfologie) effens kan verbeter,

(ii) Rooibos, heuningbos en sutherlandia hiperglukemie effens verlaag deur insulien seingewing te verbeter,

(iii) Heuningbos testosteroonproduksie moontlik kan verhoog,

(iv) Rooibos, heuningbos en sutherlandia moontlik die histomorfologiese argitektuur van die seminale buisie en die cauda epididimale buisie effens kan verbeter.

Dedication

This dissertation is dedicated to

Women who have been unjustly treated due to infertility

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Psalm 126:1

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List of abbreviations

%	= Percentage
↑	= Increase
↓	= Decrease
°C	= Degree Celsius
µl	= Microliter
µm	= Micrometer
3βHSD2	= 3β-hydroxysteroid dehydrogenase
6-OHD	= 6-hydroxydopamine
AGE	= Advanced glycation end product
AIDS	= Acquired immunodeficiency syndrome
ALH	= Amplitude lateral head
AMPK	= Adenosine 5´monophosphate-activated protein kinase
ANOVA	= Analysis of variance
ANS	= Autonomic nervous system
AR	= Acrosome reaction
ART	= Assisted reproductive technology
ASK-1	= Apoptosis signal-regulating kinase 1
ATP	= Adenosine triphosphate
Bax	= Bcl-2-associated X protein

BCA	= Bicinchoninicacid
BCF	= Beat/cross frequency
Bcl2	= B-cell lymphoma 2
BHT	= Butylated hydroxytoluene
BIR	= Baculovirus (inhibitor of apoptosisAIP) repeat
CASA	= Computer aided sperm analysis
CASMA	= Computer-aided sperm morphometry analysis
CAT	= Catalase
CD4	= Cluster of differentiation 4
CoQ10	= Coenzyme Q10
cP450	= Cytochrome P450
CYP11B1	= Cytochrome P450 Family 11 Subfamily B Member 1
CYP17A1	= Cytochrome P450 Family 17 Subfamily A Member 1
CYP21A2	= Cytochrome P450 Family 21 Subfamily A Member 2
DC	= Diabetic control
DETAPAC	= Diethylenetriaminepentaacetic acid
DHB	= Streptozotocin (45mg/kg) +Honeybush
DM	= Diabetes mellitus
DMEM	= Dulbecco’s modified eagle’s medium-low glucose
DN	= Diabetic neuropathy

DNA	= Deoxyribonucleic acid
DRF	= Streptozotocin (45mg/kg) + Rooibos
DSL	= Streptozotocin (45mg/kg) + Sutherlandia
ED	= Erectile dysfunction
ELISA	= Enzyme-linked immunosorbent assay
EPCs	= Endothelial progenitor cells
ERK1	= Extracellular regulated kinase 1
ERK2	= Extracellular regulated kinase 2
FAAD	= Fas-associated death domain
FASL	= Fas ligand
FASN	= Fatty acid synthetase
FSH	= Follicle stimulating hormone
g	= Gram
GABA	= y-aminobutyric acid
GLUT2	= Glucose transporter 2
GLUT8	= Glucose transporter 8
GLUTs	= Glucose transporters
GnRH	= Gonadotropin-releasing hormone
GPx	= Glutathione peroxidase
GrB	= Growth factor receptor-bound protein

GSH	= Reduced glutathione
GPx	= Glutathione peroxidase
GSSG	= Oxidized glutathione
H & E	= Haematoxylin and eosin
H_2O	= Water molecule
H_2O_2	= Hydrogen peroxide
HB	= Honeybush
HbA1c	= Haemoglobin A1c
HIV	= Human immunodeficiency virusa
HL-60 cells	= Human promyelocytic leukaemia
HPG	= Hypothalamic-pituitary gonadal axis
IAP	= Inhibitor of apoptosis
ICAM-1	= Intercellular adhesion molecule-1
ICSI	= Intracytoplasmic sperm injection
IDDM	= Insulin-dependent diabetes mellitus
IkB-α	= I Kappa B alpha
IPGTT	= Intra-peritoneal glucose tolerance test
IVF	= In *vitro* fertilization
JNK	= C-Jun N-terminal kinase
K	= Potassium

KCl	= Potassium chloride
kDa	= KiloDalton
Kg	= Kilogram
KPi	= Potassium phospahte
L	= Liter
LH	= Luteinizing hormone
LIN	= Linearity index
mA	= Mili-ampere
MAPK	= Mitogen-activated protein kinase
MAPKK	= Mitogen-activated protein kinase kinase
MAPKKK	= Mitogen-activated protein kinase kinasekinase
MCP-1	= Monocyte chemoattractant protein-1
MDA	= Malondialdehyde
mg	= Milligram
ml	= Milliliter
mM	= Milimolar
mm^2	= Milimeter square
MPTM	= MiliporeImmobilon-P transfer membrane
mRNA	= Messenger ribonucleic acid
mtDNA	= Mitochondria Deoxyribonucleic acid

N	= Number
Na	= Sodium
Na_3PO_4	= Trisodium phosphate/Sodium orthophosphate
NAD	= Nicotinamide adenine dinucleotide
NADH	= Reduced Nicotinamide adenine dinucleotide
NADPH	= Nicotinamide adenine dinucleotide phosphate
nDNA	= Nuclear DNA
NF-κB	= Nuclear factor kappa B
NIDDM	= Non-insulin-dependent diabetes mellitus
nm	= Nanometer
NO	= Nitric oxide
O_2	= Oxygen molecule
OH^-	= Hydroxyl ion
$ONOO^-$	= Peroxynitrite
OS	= Oxidative stress
p38MAPK	= p38 mitogen-activated protein kinase
PARP	= Poly adenosine diphosphate-ribose polymerase
PBS	= Phosphate buffered saline
PKC	= Protein kinase C
pP38	= Phosphorylated p38

PPARγ	= Peroxisome proliferator-activated receptor gamma
PPARα	= Peroxisome proliferator-activated receptor alpha
PUFA	= Polyunsaturated fatty acid
RAGE	= Receptor for advanced glycation end product
RCF	= Relative Centrifugal Force
RF	= Rooibos
RNS	= Reactive nitrogen species
ROS	= Reactive oxygen species
rpm	= Revolution per minute
SCA	= Sperm Class Analyzer
SD	= Standard deviation
SDI	= Sperm deformity index
SDS	= Sodium dodecyl sulfate
SDS-PAGE electrophoresis	= Sodium dodecyl sulfate-Polyacrylamide gel
SEM	= Standard error of mean
SL	= Sutherlandia
SOD	= Superoxide dismutase
SOS	= Son of sevenless
SREBF1	= Sterol regulatory binding factor-1

STR	= Straight line index
STZ	= Streptozotocin
SURRG	= Stellenbosch University Reproductive Research Group
TAC	= Total antioxidant capacity
TBA	= Thiobarbituric acid
TBARS	= Thiobarbituric acid reactive substances
TBS	= Tris-buffered saline
TCA	= Trichloroacetic acid
Ul	= Micrometer
UV	= Ultraviolet
V	= Voltage
VAP	= Average path velocity
VCAM-1	= Vascular cell adhesion molecule-1
VCL	= Curvilinear velocity
Veh	= Vehicle
VSL	= Straight line velocity
WHO	= World Health Organization
WOB	= Oscillation index
XIAP	= X-linked inhibitory apoptotic protein

“There is nothing more difficult to take in hand, more perilious to conduct, or more uncertain in its success, than to take the lead in the introduction of a new order of things”.

-Emile Cady-

Chapter 1

Introduction and problem statement

1.1 Introduction and Rationale

Infertility, which is described as a disease of the reproductive system has become an integral part of global health, as fertility, measured by the number of children per woman declined by ±44% (from 4.5 to 2.5 children per woman) between 1970 and 2015 (United Nations, Department of Economic and Social Affairs, 2017). Jacky et al. estimated that globally, infertility is prevalent among 72.4 million women of infertile couples, serving as a representative of infertile couples (Jacky *et al.*, 2007). Because of the difference in culture and belief systems, studies in the past neglected the role of male factor in infertility. However, contrary to the previous beliefs, that infertility majorly stems from the female partner of infertile couples, it is now evident that infertility is equally attributable to both male and female. Recent studies have reported that male factor is exclusively responsible for 30% of infertility cases, and another 20% is associated to both partners (Agarwal *et al.*, 2015). The prevalence of male infertility is on the rise (Collet *et al.*, 1988; Lunenfeld and Van Steirteghem, 2004; Agarwal *et al.*, 2015; Datta *et al.*, 2016), as it was recently estimated that 1 in every 10 men between ages 16 and 74 years have experienced infertility (Datta *et al.*, 2016). This report suggests that more men are becoming infertile during their reproductive years. Several diseases, including diabetes mellitus (DM), have been implicated in deteriorating male fertility.

DM is a metabolic disorder that affects 422 million people globally as of 2014. It has been ranked as the 7th highest cause of death in humans (World Health Organization, 2014). Hyperglycaemia is a known effect of uncontrolled DM that can consequently lead to damageing of various systems and tissues (Vanstone *et al.*, 2015). Diverse pathogenic processes are included in the development of DM. This ranges from the autoimmune destruction of the pancreatic β-cells with resulting insulin insufficiency, to abnormalities that causes resistance to insulin action (Burén *et al.*, 2002). The fundamental effect of insulin loss or insulin ineffectiveness on glucose homeostasis is the inefficient uptake and usage of glucose by glucose dependent cells, resulting in hyperglycaemia (World Health Organization, 2014). The numerous cases of DM fall into two broad categories, which are classified as either Type I DM or Type II DM. In both types of DM, metabolism of carbohydrates, lipids and protein are affected.

DM may influence male reproductive function at multiple levels. This occurs as a result of its effects on the endocrine control of the hypothalamic-pituitary-gonadal (HPG) axis (Sexton and Jarow, 1997)), or because of the inequality between ROS production and antioxidant defence mechanisms subsequently leading to OS (Du Plessis *et al.*, 2010, 2015). Apoptosis induction (Said *et al.*, 2004; Zhao *et al.*, 2011), and the alteration in the modulation of glucose transporters have also been implicated (Lampiao and du Plessis, 2010). Baccetti et al. showed that the gonadotropins released in response to GnRH were lower in diabetic men. This was accompanied by a decrease in sperm motility and a higher percentage of sperm immaturity (Baccetti *et al.*, 2002). These findings indicate that DM may affect male reproduction by interfering with the hypothalamic-pituitary-testicular axis. DM has been shown to increase testicular oxidative damage, which occurs when reactive oxygen species (ROS) exceeds the seminal antioxidant defence ability (Du Plessis et al., 2015). Thereby resulting in many

cascades of reactions, which subsequently lead to sperm DNA damage, mitochondrial DNA fragmentation, altered sperm parameters and ultimately, male infertility (Mangoli et al., 2013). Kanter et al. showed that after DM induction in rats, there was an increase in testicular malondialdehyde (MDA) levels with reduced antioxidant enzyme activities (glutathione peroxidase (GPx) and superoxide dismutase (SOD). Which subsequently resulted in severe testicular damage as evidenced by disrupted seminiferous tubule structure. Also observed was the increased number of spermatozoa with fragmented DNA following apoptosis activation (Kanter, Aktas and Erboga, 2012).

Additionally, Jiang et al. reported that streptozotocin (STZ)-induced diabetic rats displayed an increase in testicular tunnel positive cells, Bax/Bcl2 ratio and oxidative damage (Jiang *et al.*, 2013). In the same vein, Roessner et al. reported increased cleaved caspase 3 in the ejaculate of diabetic men (Roessner et al., 2012). These findings show that DM may affect male reproduction via apoptotic signalling. Furthermore, increased ROS production has also been implicated in the generation of advanced glycation end products (AGEs). AGEs are products of non-enzymatic reactions between sugars and the amino groups of proteins, lipids and DNA under hyperglycaemic conditions. AGEs can alter the normal functioning of macromolecules by generating ROS independently, or by activating the receptors for advanced glycated end products (RAGE), which leads to a further increase in ROS production by these mutated proteins. RAGE is a multi-ligand member of the immunoglobulin family which interact with specific ligands. Binding of RAGE to its ligands leads specifically to altered gene expression. The role of AGE-RAGE activation in the induction of apoptosis has been widely evaluated. Many of these studies were performed on various cell types such as neuronal and endothelial cells (Chen et al., 2010; Ramasamy et al., 2005), as well as periodontal ligament fibroblasts (Li et al., 2014). Additionally, the binding of AGE-RAGE could trigger mitogen-activated protein

kinase (MAPK) signalling and the nuclear factor kappa B (NF-κB) pathway, thereby leading to elevated production of ROS and upregulation of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and monocyte chemoattractant protein-1 (MCP-1) (Hoefen and Berk, 2002). Interestingly, AGEs have been suggested to play a role in instigating harm and is believed to act as a mediator of damage to the reproductive system of diabetic men (Mallidis et al., 2009).

Seeing that DM may inflict damage on male fertility at various levels, it is pertinent to counter-act this action by exploring every possible strategy to combat DM and its associated male reproductive complications.

Many natural and artificial agents have been proposed to have antioxidant properties due to their ability in inhibiting oxidation. Antioxidants work either by scavenging ROS or by preventing the formation of ROS (Lampiao, 2013).

Rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and sutherlandia (*Lessertia frutescens*) are plants endemic to the Southern Africa (Morton, 1983; McGaw, Steenkamp and Eloff, 2007; Joubert *et al.*, 2008, 2011; van Wyk and Albrecht, 2008; Schloms and Swart, 2014). Herbal teas derived from their leaves and stems have been shown to be caffeine-free. Studies have also shown that the infusions from these plants contain high bioactive chemical compounds with therapeutic benefits.

1.2 Statement of problem

A large amount of data is available on the effects of DM on sperm function. However, the mechanism through which the effects are exerted is less clearly understood. To gain further insight into the pathophysiology of DM-related male reproductive impairment, this study

aimed to shed some light with regards to the pathway by which OS induced AGE formation is correlated with apoptosis and the resultant effect of the association.

Additionally, rooibos, honeybush and sutherlandia have been shown to have antioxidant properties and several studies have shown their beneficial role in diverse disease states. Regarding male reproduction, very few studies have investigated the role of rooibos tea and this represent the first study to determine the effects of honeybush and sutherlandia on male reproductive function. Thus, this study also aimed to investigate the individual effect(s) of these infusions on male reproductive function and to determine their possible ameliorative role in DM-impaired male reproductive function.

1.3 Aims and objectives

As highlighted in the statement of problem, this study was divided into two, having different independent aims.

1.3.1 Aim 1

The first aim was to investigate the mechanism and correlation between apoptotic pathway activation, accumulation of AGEs and the expression of the receptor for AGEs (RAGE) in the streptozotocin (STZ) rat models of pre-diabetes and diabetes, as well as the independent and collective impact on male reproductive function.

Objectives

I. To establish an in vivo rat model of pre-diabetes and diabetes,

II. To investigate the effects of these two models on male reproductive parameters,

III. To investigate the apoptotic pathway activation, accumulation of AGEs and expression of receptor for AGEs in the testis of pre-diabetic and diabetic rats and their respective impact on male reproductive function.

1.3.2 Aim 2

The second aim of this study was to determine the effects of rooibos, honeybush and sutherlandia on diabetes induced male reproductive impairment in Wistar rats.

Objectives

I. To establish an in vivo model of streptozotocin induced diabetes,

II. To determine the effects of rooibos, honeybush and sutherlandia on sperm functional parameters,

III. To determine the effects of rooibos, honeybush and sutherlandia on the antioxidant status of diabetic rats and the plausible ameliorative effects on the impaired male reproductive functions in DM rats.

1.4 Study Layout

To give a background to the study, chapter 1 briefly introduces the study by highlighting the rationale, statement of the problem, aims and objectives and the study layout. Chapter 2 explains the general overview of the literature regarding diabetes mellitus, male infertility, rooibos, honeybush and sutherlandia. Chapter 2 is accompanied by two addenda (2A and 2B), focusing on the mechanisms through which diabetes impact male fertility and the resultant outcomes. This is followed by a comprehensive basic materials and methods section (Chapter 3). The results of the study are reported in different chapters, as published papers or as

manuscripts in preparation (prepared according to journal guidelines). Both Chapters 4 and 5 addresses aim 1, while another three chapters (Chapters 6-8) focused on the role of rooibos, honeybush and sutherlandia on male reproductive function, either independently or their role in diabetes impaired male reproductive function. Chapter 9 describes the qualitative histological evaluation of the testis and cauda epididymis. The final chapter (Chapter 10) include the conclusion and recommendation for future studies.

Addendum 2A and Chapter 4 are presented as published papers. Addenda 2B and Chapters 5 and 7 are shown as submitted for publication, while Chapters 6, 8 and 9 are displayed as manuscripts in preparation. Hence, repetition may occur in the introductory section of each chapter. In addition, applicable references are included in each chapter.

Attached to this dissertation are the original copies of the published articles.

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Chapter 2

Literature review

2.1 Introduction

This chapter entails a broad overview of the literature regarding, diabetes mellitus (DM), male infertility, mechanisms through which DM impact male fertility, rooibos, honeybush, sutherlandia and their potential impact on general health, including male reproductive health. The mechanisms/relationship between DM and male infertility are discussed in addendum 2A as published, while the mechanistic role of AGE/RAGE pathway in the investigation of male infertility is elucidated in addendum 2B as submitted for publication.

2.2 Diabetes mellitus

DM is a chronic disease that result from the inability of the pancreas to produce enough insulin or from the incapability of the body to effectively use the produced insulin. DM has become an important public health problem, because its prevalence is on the rise and has recently been included as one of the four prioritized non-communicable diseases that requires immediate attention (World Health Organization, 2014). It can be said that the global prevalence of DM has escalated since the 1980s, as it was reported to affect 108 million people in 1980, 177 million in 2002, 346 million in 2004 and 422 million in 2014 (World Heallth Organization, 2010; World Health Organization, 2014) (Figure 1, 2). Hence, the global

prevalence relative to age-standard has tripled since 1980. It was reported that DM and DM-related complications caused 3.7 million deaths in 2012, of which 43% are people younger than 70 years (World Health Organization, 2014). This increase in DM prevalence has led to a substantial loss to both health systems and national economies.

Diverse pathogenic processes are involved in DM development. This includes the autoimmune destruction of the pancreatic β-cells with resultant deficient insulin synthesis and secretion and other disorders that lead to insulin resistance. The basis for metabolic abnormalities seen in DM is the deficient action of insulin on target tissues (American Diabetes Association, 1970). Symptoms of DM include, hyperglycaemia, polydipsia, polyuria, polyphagia, reduced body weight, impaired growth, vulnerability to infections, fatigue and blurred vision (American Diabetes Association, 1970; World Health Organization, 2014). DM can be classified into two, namely type 1 (type 1 DM) and type 2 (type 2 DM).

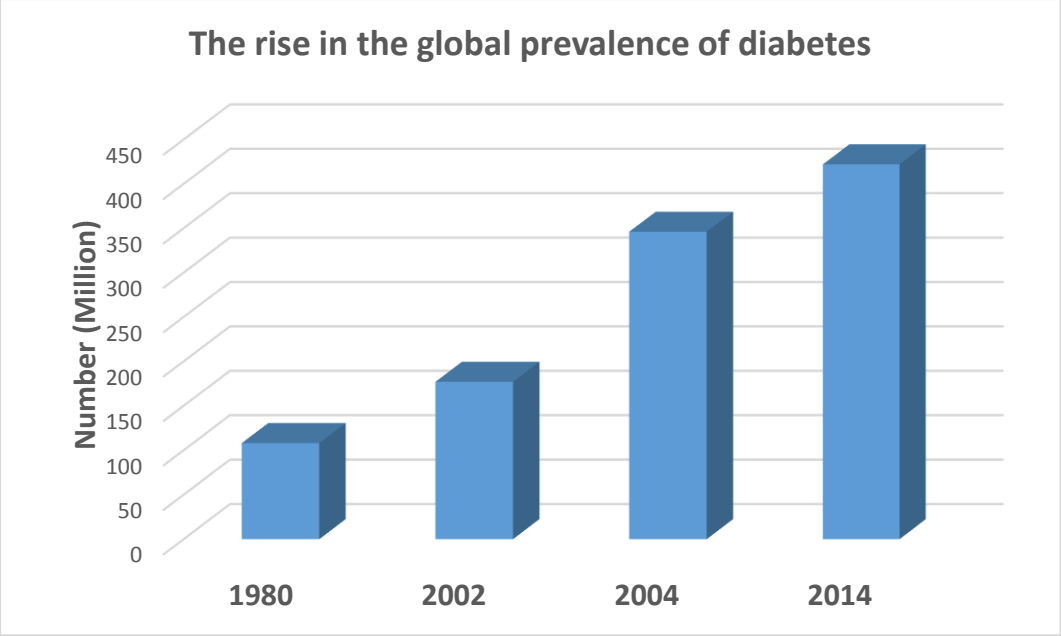

Figure 1: The rise in the global prevalence of diabetes mellitus from 1980-2014. Adapted from (World Health Organization, 2014).

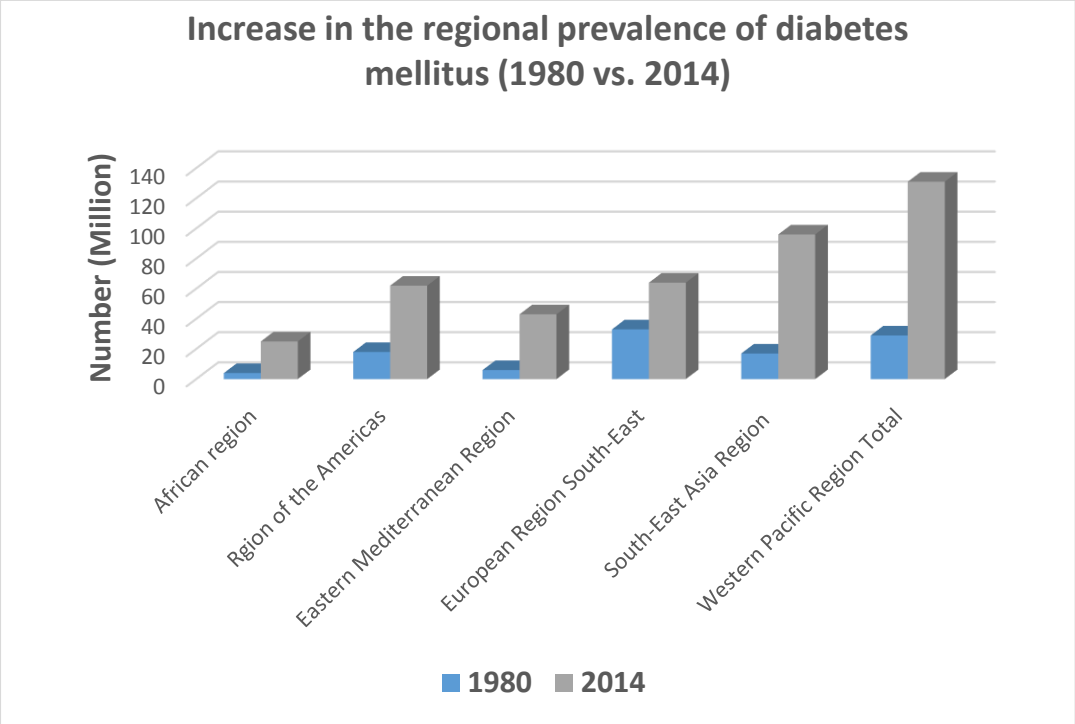

Figure 2: Showing regional distribution of diabetes prevalence (1980 vs. 2014). Adapted from (World Health Organization, 2014).

2.3 Type 1 DM

2.3.1 Prevalence

Out of the 422 million people affected by DM globally in 2014, ≤10% is attributed to type I DM. About 85% of this population are diagnosed before the age of 20 and ≥15% of the cases are ascribed to adults (≥30years). The WHO through the DIAMOND project estimated that 4.5% of the world's population as at 2000 between age ranges 0-14 have type I DM following an estimation from 50 different countries. However, a >350-fold difference was seen in the incidence of type I DM after inclusion of another 50 countries and that it increases with age in most populations with the highest occurrence observed in 10–14 year olds (Karvonen *et al.*, 2000; Maahs, David M; West, Nancy A; Lawrence, Jean M; Mayer-Davis, 2010). Although, most autoimmune diseases usually affect a higher percentage of women, type 1 DM is more prevalent in boys and men (Östman *et al.*, 2008).

2.3.2 Pathogenesis of type 1 DM

Type 1 DM is instigated by injury or cellular-controlled autoimmune destruction of the pancreatic β-cells. Heredity, race or ethnicity, age and gender are some of the associated risk factors that play a role in ascertaining the susceptibility of the insulin producing cells to abrasion. It may develop swiftly over a period of a few days or weeks, following this sequence: (1) decreased insulin, (2) elevated usage of fats for energy, and (3) degradation of the body's proteins and amino acid oxidation due to insulin deprivation (Caso and McNurlan, 2010)

Studies have shown the importance of genetic susceptibility in the development of type 1 DM (Lambert *et al.*, 2004; Pociot *et al.*, 2010; Redondo, Steck and Pugliese, 2018). The genes for type 1 DM are unique in some ways when compared to other autoimmune disease genetic

components. This is because they provide both vulnerability towards and protection against the development of type 1 DM. These genes are located within the major histocompatibility complex (MHC) HLA class II region on chromosome 6p21 and they accounts for 45-50% susceptibility for the disease (Buzzetti, Quattrocchi and Nisticò, 1998). These same genes protect against developing type 1 DM. Therefore, it can be said that type 1 DM is not just a disease instigated by the autoimmune destruction of the pancreatic β-cells, it is a heterogeneous and polygenic disease (Lernmark and Ott, 1998).

2.4 Type 2 DM

2.4.1 Prevalence

The prevalence and incidence of type 2 DM is rapidly increasing throughout the world and it accounts for 90–95% of those with DM. Type 2 DM was known to be an adult or old age disease, but in the last few decades, its prevalence has increased among youth, which predicts for higher estimate of occurrence in the future. Nijpels in 2016 predicted a 71.5% increase in the prevalence of type 2 DM amidst age 20-79 by 2035, including 6% increase in Africa, 7.1% in Europe, 11.3% in Middle East, 12.3% in North America and Caribbean, 8.2% in South and Central America, 9.4% in South East Asia and 8.4% in the Western Pacific (Nijpels, 2016).

2.4.2 Pathogenesis of type 2 DM

It occurs because of decreased perceptivity of target tissues to the metabolic effects of insulin. Type 2 DM is also described as a ‘‘modern day disease’’ because it is caused by lifestyle factors, such as, diet and obesity. Ethnicity, environmental exposure and socio-economic factors are also evident risk factors. In comparison to type 1 DM, it is correlated with elevated plasma insulin concentrations (hyperinsulinemia). This occurs as a compensatory feedback by

the pancreatic β-cells for reduced sensitivity of responsive tissues to the metabolic effects of insulin. The diminished insulin sensitivity debilitates carbohydrate usage and storage, increasing blood glucose and spurring a compensatory increase in insulin secretion.

Since the basis for abnormalities in carbohydrate, fat, and protein metabolism in DM is the insufficient action of insulin on the target tissues, it is important to know what insulin is, what it does and how it works.

2.5 Insulin

Insulin, synthesized and secreted by the pancreatic β-cell, is a fundamental peptide hormone with physiological importance in the regulation of carbohydrates, fats and protein metabolism (D.Melloul, S.Marshak and E.Cerasi, 2002). Insulin is secreted in its inactive form, preproinsulin. The proteolytic cleavage of insulin precursor produces proinsulin and as a compensatory response to an increase in blood glucose or amino acid concentration, proinsulin is secreted and proteolytically converted to active insulin (Rains and Jain, 2011). Active insulin consist of two polypeptide chains connected by disulphide bridges (D.Melloul, S.Marshak and E.Cerasi, 2002). The function of insulin is to regulate glucose, lipid and protein homeostasis. Insulin controls glucose metabolism by stimulating glucose transport into the muscle and adipose cells and concurrently reducing gluconeogenesis and glycogenolysis by the liver. While lipid homeostasis is achieved by increasing lipid synthesis in the liver and adipocytes, and concurrently reducing lipolysis to enhance cell growth.

2.5.1 Insulin signalling in health

Insulin receptors, such as insulin-like growth factor 1-receptor (IGF1-R) and insulin receptor related-receptor (IRR), are tetrameric proteins having four functional subunits (2α and 2β subunits). These receptors can form functional complexes with insulin, such that the inhibitory mutation in one receptor subunit can inhibit the activity of the others (Butler and LeRoith, 2001). When there is an increase in blood glucose, insulin is released and it binds the α-subunit of insulin receptor. This leads to the suppression of β subunit kinase activity and subsequent stimulation of tyrosine auto-phosphorylation of the β subunit (Alan and Kahn, 2001; Bloch-Damti and Bashan, 2005). Hence, the insulin receptor is activated/phosphorylated. The activation of the receptor leads to the phosphorylation of insulin receptor substrates (IRS 1-4), although only IRS 1/2 are important in glucose transport (Kriauciunas, Myers and Kahn, 2000). Activated IRS proteins acts as binding sites for molecules that contains src-homology-2 (SH-2) domains such as phosphoinositide-3-kinase (PI3K), growth factor receptor-bound protein-2 (GRB-2)/ Son-of-sevenless proteins (SOS) and phosphotyrosine phosphatase (SHP2). Binding of these molecules to IRS1/2 form a complex that mediate the down-stream signalling.

It has been shown that PI3K is the major signal mediator of the metabolic (catabolic and anabolic) and mitogenic actions of insulin. PI3K is composed of a regulatory p85 subunit and a p110 catalytic subunit. Binding of the p85 subunit to IRS enhances the catalytic action of p110 which then allows the phosphorylation of its substrate, Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to Phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) (Bloch-Damti and Bashan, 2005). PtdIns(3,4,5)P_3 recruits serine kinases, protein kinase-B (PKB/Akt), protein kinase-C (PKC) and phospho-inositide-dependent kinase -1 (PDK-1) through the PH domain to the plasma membrane. Activation of these kinases leads to: (i) the

translocation of glucose transporter 4 (GLUT 4) from the intracellular vesicles (storage pools) to the plasma membrane, (ii) phosphorylation of glycogen synthase kinase-3 (GSK-3) to synthesize glycogen and (iii) upregulation of fatty acid synthase gene to induce lipogenesis.

Additionally, insulin can activate the mitogen activated protein kinases (MAPK), especially the ERKs through the binding of GRB-2 and SOS to IRS1/2. Activation of ERK induces cell proliferation and differentiation gene expression. GRB-2, SOS and SHP-2 binds IRS1/2 to activate membrane bound Ras. The phosphorylation of Ras stimulates a stepwise activation of kinase signalling of Raf, MEK and ERK. ERK is then translocated into the nucleus where it stimulates the phosphorylation of transcription factors to initiate gene expression needed for cell proliferation and differentiation (Alan and Kahn, 2001)

Another pathway through which insulin enhances glucose uptake is by stimulating the tyrosine phosphorylation of the Cb1 proto-oncogene. Cb1 functions by binding to CAP. CAP is an adaptor protein that contains SH3 domains and Sorbin homology (SoHo) domain. Binding of Cb1 to CAP, forms a complex called Cb1-CAP. The interaction of the SoHo domain of the complex with protein flotillin, stimulates the phosphorylation of Cb1-CAP complex and its translocation to the lipid raft domains on the plasma membrane (Baumann *et al.*, 2000). The presence of the Cb1 complex on the lipid raft results in the recruitment of adapter protein CrkII, which leads to the formation of complex signalling that aid the activation of G protein, TC10. Once TC10 is activated, it acts as a second signal (in addition to the signalling of PI3K) in recruiting GLUT4 from the intracellular pools to the plasma membrane (Baumann *et al.*, 2000).

However, when the blood glucose levels is uncontrolled due to lack in secretion or insensitivity of tissue to insulin, hyperglycaemia ensues, which is a marked characteristic of

DM. In recent years, the views that DM has inconsequential effects on male reproductive functions have been questioned by conclusive data from various studies (Zhao *et al.*, 2011; Roessner *et al.*, 2012)

2.6 Infertility: male infertility

The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the WHO describes infertility as the reproductive system disease marked by the failure to achieve a clinical pregnancy after 1year or more of consistent unprotected sexual intercourse (Zegers-Hochschild *et al.*, 2009). Infertility has become an integral part of reproductive health as it is estimated to affect about 60-80 million couples of reproductive age, of which 56% seeks health care (Jacky *et al.*, 2007). Male infertility on the other hand, describes a male's inability to cause fertilization in a fertile female over a period of 12 months of consistent and unprotected intercourse. It is estimated that about half (50%) of all the infertility cases is attributed to the problem of the male with 20-30% exclusively attributed to the problem of the male and another 20-30% are due to a combination of male and female anomalies (Sharlip *et al.*, 2002). A recent study revealed that 1 in every 10 men between the age of 16 and 74 have experienced infertility (Datta *et al.*, 2016), and that the cause of infertility due to the male factor ranged from 20-70% across different groups. This include 50% in North America (Agarwal *et al.*, 2015), 60-70% in the Middle East (Collet *et al.*, 1988), 20-40% in Sub-Saharan Africa (Lunenfeld and Van Steirteghem, 2004) and 37% in Asia (Elussein *et al.*, 2008).

Several studies have highlighted the negative impact of DM on male fertility. This include endocrine function dysregulation, testicular dysfunction, disruption of spermatogenesis, reduced sperm motility and decreased normal sperm morphology (Amaral *et al.*, 2006; Khaki

et al., 2010; Chen *et al.*, 2016). The adverse effects of DM on male fertility and the mechanism through which the effect are exerted are broadly discussed in addenda 2A and 2B as published and submitted respectively.

Since it has been shown that DM affects 422 million people globally (World Health Organization, 2014), including men in their reproductive age (Nijpels, 2016), it is therefore important to explore every possible strategy to combat DM and its associated male reproductive complications.

Many natural and artificial substances have been proposed to have antioxidant properties due to their ability in scavenging free radicals (ROS) or their ability to prevent the formation of ROS (Lampiao, 2006), in diseases such as DM.

2.7 Rooibos

Rooibos is a low-tannin, caffeine free beverage (Morton, 1983). It was first discovered in the Cederberg Mountains of the Western Cape region of South Africa (Joubert et al., 2008; McGaw et al., 2007). Rooibos is a remarkable polymorphic straight plant with differing ecology, morphology, geographical distribution and chemical contents (Hawkins et al., 2011; Van Wyk & Gorelik, 2017). It has a rigid, densely clustered shoot system with varying aspalathin content (leaves>stems>dried shoots), even, between plants from the same plantation (Joubert and Schulz, 2006) (Figure 3).

There are varieties of wild types of rooibos tea that are all considered part of the *Aspalathus linearis* species complex. These various forms were first made known by the Conservator of Forests (conservator of Forest; 1949). They include (i) Swart type (dark in colour, grows in the

rocky region of Cederberg mountain with less aroma). (ii) Vaal type (it is pale and greyish green, grows in Cederberg and the Olifants River, with undesirable honey aroma). (iii) Rooi-bruin type (reddish-brown in colour, grows on the sand flats in the northern region of Clanwilliam division, it is slightly coarse and has mild flavour). (iv) Cederberg type (grows in the northern part of Cederberg) and lastly, the Rooi type. (v) The Rooi type originated from the wild forms found mainly in the northern part of the Cederberg Mountains. Although all forms of rooibos tea were previously commercially available only Rooi Tea is obtainable today, which is generally known as the rooibos tea (Van Wyk and Gorelik, 2017).

Rooibos is traditionally produced in two ways, which include the fermented and the unfermented products (Figure 4A, B). The fermented tea is achieved by oxidation that result in the distinctive reddish-brown leaf colour with woody-fynbos-floral honey flavour (Koch et al., 2012). The unfermented type is also called the green rooibos. Hot water extracts prepared from both fermented and green rooibos are used as food and/or cosmetic ingredients due to the presence of high aspalathin content, especially in the unfermented. Fermented rooibos aspalathin content is 2% while that of green rooibos is 8% (de Beer et al., 2017; Joubert & de Beer, 2012), this is because the amount of aspalathin decreases during fermentation. However, fermented rooibos is more preferred in food because of the flavour and it is considered more economical as there is greater demand for it.

Figure 3: *Aspalathus linearis* (Rooibos) plant in bloom (Wyk, 2008)

A

B

Figure 4: Different types of processed rooibos (Medical News Today www.medicalnewstoday.com/articles/323637.php)

A=fermented rooibos, **B**= unfermented /green rooibos

2.7.1 Fermentation/degradation process of Rooibos

The red-brown colour formation of rooibos during fermentation has been linked largely to the degradation of aspalathin, a process known to be a non-enzymatic oxidative breakdown. After shredding, bruising and wetting of the plant material in heaps, the fermentation process begins. In brief, aspalathin is oxidized to O-quinone. This O-quinone rearranges to quinine methide, which allows fast conversion of isolated single eriodictyols into a stable equilibrium. Quinine methide undergo further oxidation to give (S)-eriodictyol-6-C-β-D-glucopyranoside (**A**), (S)-eriodictyol-8-C-β-D-glucopyranoside (**B**), (R)-eriodictyol-6-C-β-D-glucopyranoside (**C**) and (R)-eriodictyol-8-C-β-D-glucopyranoside (**D**) (Krafczyk and Glomb, 2008). Further oxidation of **A+C** results in the formation of isoorientin and orientin while oxidative breakdown of **B+D** results in the formation of isoorientin (Figure 5). During this process, the oxidative coupling intermediates produced are known to be colourless dimers (Bramati, Aquilano and Pietta, 2003). However, Heinrich et al., reported the presence of two additional coloured dimers with dibenzofuran skeleton ((S)-and (R)-3-(7,9-dihydroxy-2,3-dioxo-6-β-D-glucopyranosyl- 3,4-dihydrodibenzo[b,d]furan-4a(2H)-yl) propionic acid) during the fermentation process of rooibos (Heinrich, Willenberg and Glomb, 2012).

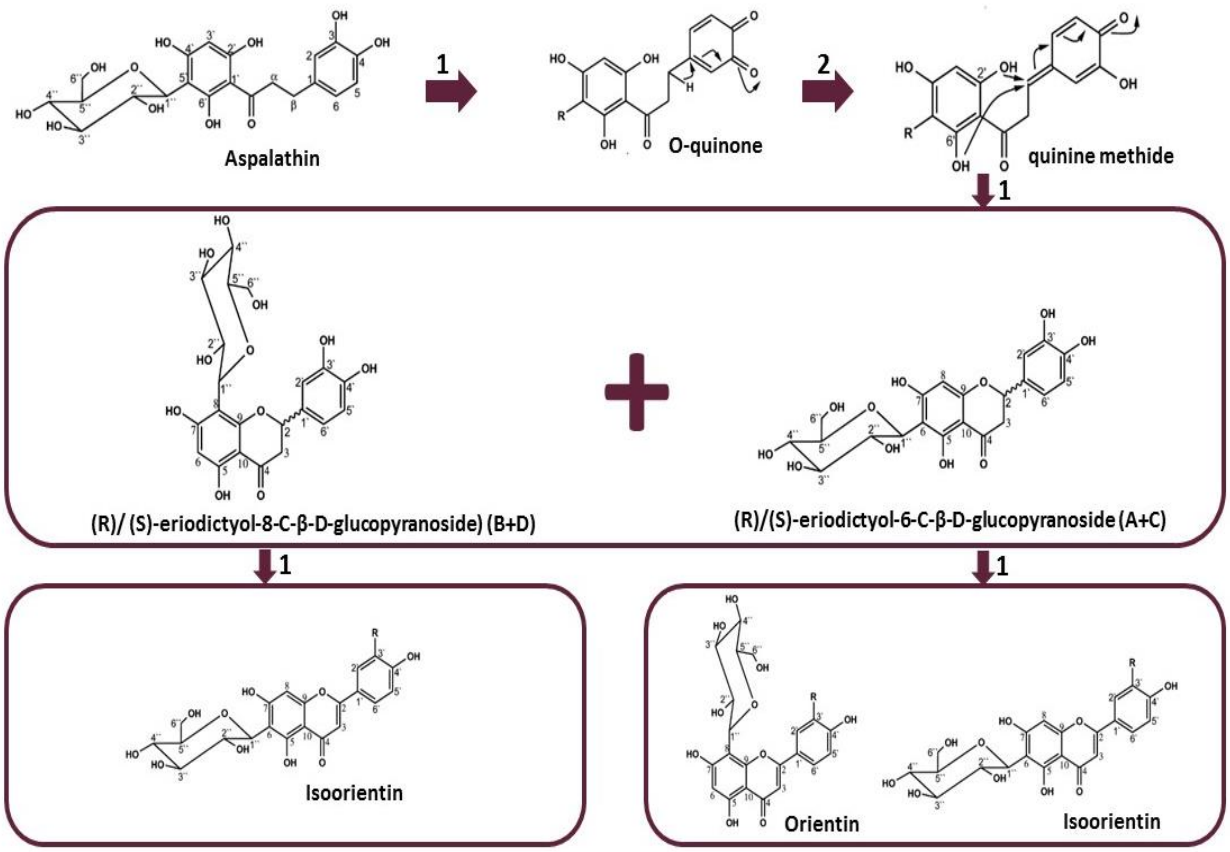

Figure 5: Fermentation process of rooibos.

1=oxidation, **2**=rearrangement, **A**=(S)-eriodictyol-6-C-β-D-glucopyranoside, **B**=(S)-eriodictyol-8-C-β-D-glucopyranoside, **C**=(R)-eriodictyol-6-C-β-D-glucopyranoside, **D**=(R)-eriodictyol-8-C-β-D-glucopyranoside

2.7.2 Chemical composition of Rooibos

The recognized health promoting advantages of rooibos are partly attributed to its phenolic composition. The phenolic constituents of rooibos comprises of the dihydrochalcones (nothofagin, aspalathin, C-5-hexosyl derivative of aspalathin), cyclic dihydrochalcone (aspalalinin), phenylpropenoids (phenypyruvic acid-2-0-glucoside), flavones (orientin, isoorientin, vitexin, isovitexin, luteolin, luteolin-7-0-glucoside, chyrysoeriol) and the flavonols (quercetin, isoquercetin, hyperoside, rutin, quercetin-3-0-ribinobioside), flavanones ((S)-Eriodictyol-6-C-glucosidee), (R)-Eriodictyol-6-C-glucosidee), (S)-Eriodictyol-8-C-glucosidee and (R)-Eriodictyol-8-C-glucosidee) (Figure 6) (Beelders et al., 2012).

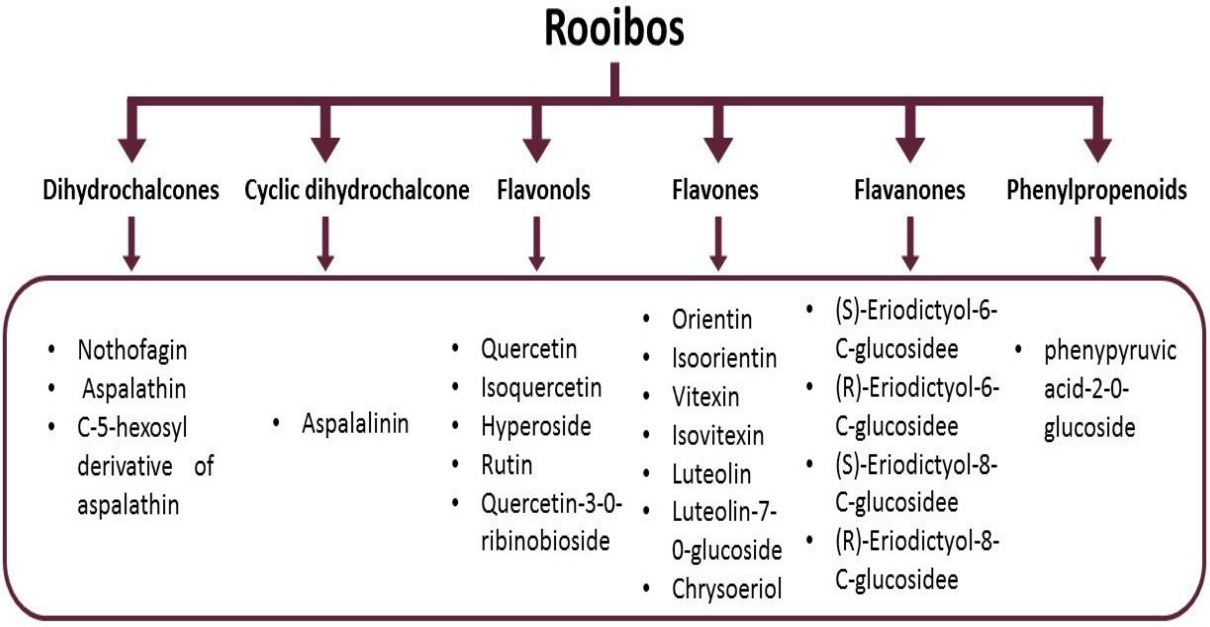

Figure 6: The chemical composition of rooibos tea.

2.7.3 Benefits of Rooibos

Several studies have investigated and elucidated the antioxidant activities of the flavonoid contents of rooibos on different systems (Awoniyi *et al.*, 2012; Pheiffer, de Beer, *et al.*, 2013; Hong, Lee and Kim, 2014) and cells (Schloms and Swart, 2014; Moosa *et al.*, 2018; Shabalala *et al.*, 2019). Pheiffer et al. reported that treatment of 3T3-L1 adipocytes with fermented rooibos inhibited intracellular lipid build-up. It was further stated that the inhibition of adipogenesis was accompanied by reduced mRNA expression of peroxisome proliferator-activated receptor gamma (PPARy), peroxisome proliferator-activated receptor alpha (PPARα), sterol regulatory binding factor (SREBF1) and fatty acid synthetase (FASN). This collectively suggest its likely potency to prevent obesity.

Additionally, the effects of major rooibos flavonoids such as, dihydrochalcones (aspalathin and nothofagin) and the flavones (orientin and vitexin) were examined on the adrenal steroidogenic enzymes (3β-hydroxysteroid dehydrogenase (3βHSD2), cytochrome P450 (cP450), P450 17α hydroxylase/17,20-lyase (CYP17A1), P450 21-hydroxylase (CYP21A2) and P450 11β-hydroxylase (CYP11B1). It was concluded that flavonoids of interest inhibited 3βHSD2 and CYP11B1 thereby causing structural changes of the enzymes. This suggest that flavonoids influence the activity of adrenal steroidogenic enzymes by causing structural plasticity (Schloms and Swart, 2014). Furthermore, Hong et al. reported the antioxidant effect of rooibos on immobilization induced OS in rat brain (Hong, Lee and Kim, 2014).

Several in vivo studies have reported the anti-diabetic effect of rooibos (Son *et al.*, 2013; Dludla *et al.*, 2018; Layman *et al.*, 2019). Some authors showed that aspalathin treatment in diabetic vervet monkeys improves glucose tolerance and also decreases low-density lipoproteins. It was further reported that after treatment with rooibos, a reduction in the

percentage of oxidized CoQ10 and circulating oxidized low-density lipoproteins were observed. This collectively suggest that rooibos can ameliorate OS and impaired glucose metabolism (Orlando *et al.*, 2019). Additionally, rooibos was able to repair the impaired insulin signalling by activating 5´-adenosine monophosphate-activated protein kinase (AMPK) pathway and also improve lowered mitochondria respiration rate in induced hepatic insulin resistance (Mazibuko-Mbeje *et al.*, 2019). In addition to the antioxidant, anti-diabetic and anti-inflammatory effects of rooibos, studies have further highlighted its long-term beneficial effect on long-term spatial memory in adult male rats (Pyrzanowska *et al.*, 2019).

However, despite the numerous available data on rooibos and its health benefits, few studies have investigated its effect on male reproductive function. This includes a study that reported improvement in sperm motility, sperm concentration and sperm viability after treatment with rooibos (Opuwari and Monsees, 2014). Additionally, Ros-Santaella and Pintus reported that the in vitro treatment of boar semen with rooibos protected the sperm acrosome structure accompanied by improved sperm velocity (Ros-Santaella and Pintus, 2017).

Despite the enormous health benefits of rooibos, studies have highlighted some adverse effects when the consumption is excessive. It was shown that lengthened exposure to rooibos induces acrosome reaction in rats, which can subsequently lead to impaired reproduction (Opuwari and Monsees, 2014).

2.8 Honeybush

Honeybush is native to the Southeast and Southwest coastal areas of South Africa and it forms a part of the fynbos biome with the family name Fabaceae. It has been shown to be used as

a traditional tea since the 19th century (Joubert *et al.*, 2011; Schloms and Swart, 2014). Honeybush is a short woody perennial plant with the life span of 15 years (North *et al.*, 2017). About 23 different species of honeybush have been identified, however, only five species are commercially available. This includes, *Cyclopia intermedia*, *Cyclopia genistoides*, *Cyclopia maculata*, *Cyclopia sessiliflora* and *Cyclopia subternata.* A mature honeybush plant has the flowers, leaves, stem and seeds. The flowers are bright yellow, fluorescent and sweetly scented, having a characteristic sweet honey flavour. The leaves are in trifoliolate with differing shapes and sizes relative to specie variability. In general, the leaves are thin, needle-like and elongated. Honeybush have golden yellow woody stem and grows to about 1-3 m in height (Figure 7). The seeds are formed in small pods that turns brown when ripe. Honeybush tea is made from the flowers, leaves and twigs and it is mostly preferable in the fermented form, however, the unfermented product is also commercially available (Joubert *et al.*, 2011).

2.8.1 Chemical composition of Honeybush

Honeybush tea does not only have a pleasant taste and scent, it also contains phytochemicals especially polyphenols and some volatile organic compounds that are beneficial to health (Joubert *et al.*, 2008). Le Roux et al. found 183 volatile organic compounds in honeybush (C.subternata) which included compounds that are believed to be responsible for the sweet aroma after fermentation. The odour-active compounds found in honeybush are (E)-β-Damascenone, (R/S)-linalool, (E)-β-damascone, geraniol, (E)-β-ionone, (7E)-megastigma-5,7,9-trien-4-one, (6E,8Z)-megastigma-4,6,8-trien-3-one, (6E,8E)-megastigma-4,6,8-trien-3-one, (7E)-megastigma-5,7,9-trien-4-one, 10-epi-γ-eudesmol, epi-α-muurolol, and epi-α-cadinol (Le Roux *et al.*, 2012).

The known health benefits of honeybush are ascribed to its phytochemicals, which includes the flavanones (eriodictyol, hesperetin, isokuranetin, naringenin, chrysoeriol), inositols (pinitol), flavones (luteolin), flavonols (kaempferol), phenolic acid (tyrosol and derivatives), xanthones (isomangiferin, mangiferin), coumestans (flemichapparin, medicagol, sophoracoumestan) and isoflavonols (afrormosin, calycosin, formononetin, fujikinetin, pseudobaptigen, wistin) (Figure 8) {Formatting Citation}.

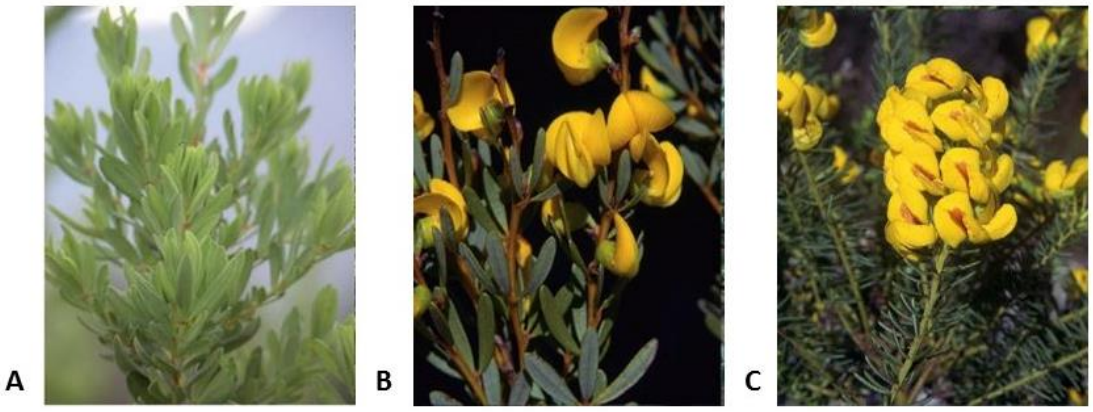

Figure 7: Different species of honeybush.
A. *Cyclopia intermedia*, **B**. *Cyclopia maculate*, **C**. *Cyclopia genoside* (Van Wyk, 2015).

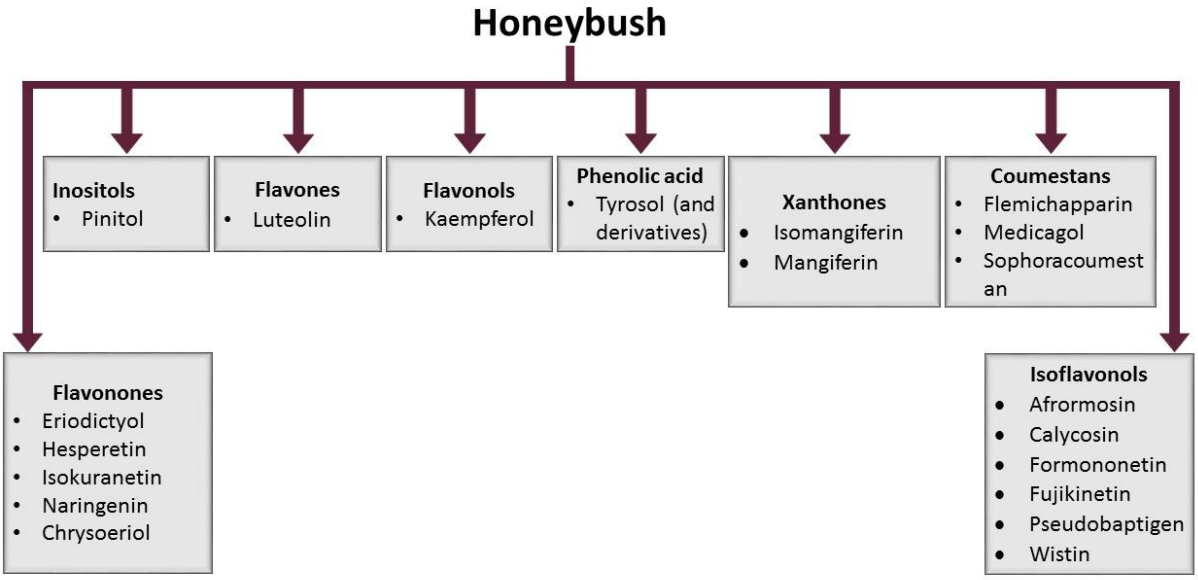

Figure 8: The chemical composition of honeybush tea.

2.8.2 Benefits of Honeybush

The *C. maculata* and *C. subternata* specie of honeybush have been reported to inhibit adipogenesis (Dudhia *et al.*, 2013) and stimulate lipolysis (Pheiffer, Dudhia, *et al.*, 2013). Studies have highlighted the *in vitro* (Moreira, Carlos and Vilegas, 2001; Leiro *et al.*, 2003; Marnewick *et al.*, 2005; Hubbe and Joubert, 2010) and *in vivo* (Sánchez *et al.*, 2000; Marnewick *et al.*, 2003) antioxidant activity of honeybush.

Marnewick et al. reported that honeybush increases the activities of cytosolic glutathione S-transferase alpha and microsomal glucuronosyl transferase with a significant elevated ratio of oxidized glutathione (GSSG) and reduced glutathione (GSH). This suggest that honeybush may modulate metabolizing enzymes and OS (Marnewick *et al.*, 2003). Additionally, honeybush was reported to protect against ultraviolet-B induced damage in HaCaT

keratinocytes by stimulating the activities of antioxidant enzymes, inhibiting phosphorylation of mitogen-activated protein kinase (MAPK) and suppressing the expression of inflammatory cytokines (Im *et al.*, 2016; Magcwebeba *et al.*, 2016). This shows that honeybush exhibit antioxidant and anti-inflammatory properties that can be used as a skin anti-photoageing agent. *Cyclopia intermedia* specie of honeybush was shown to display chemoprotective properties against fumonisin B1 induced cancer in rat liver (Marnewick *et al.*, 2009) and anti-wrinkle effects in hairless mice (Im *et al.*, 2014). Additionally, honeybush has been reported to have antidiabetic effects (Miura *et al.*, 2001; Muruganandan *et al.*, 2002; ICHIKI *et al.*, 2011) and phytoestrogenic activity (Verhoog, Joubert and Louw, 2007; Louw, Joubert and Visser, 2013). Unfortunately, to the best of our knowledge, no studies reported the effect of honeybush on male reproduction. Hence, this is the first study investigating the effect of honeybush on male reproductive function**.**

2.9 Sutherlandia

Sutherlandia is a plant with diverse species that are widely spread across specific geographic regions of Southern Africa. It is indigenous to the northern, eastern and western Cape areas of South Africa, southern areas of Namibia and south eastern regions of Botswana and Lesotho (van Wyk and Albrecht, 2008). *Lessertia frutescens* is an erect short-lived perennial plant with varying heights range of <0.2-2.5m. The stems are smooth having numerous leaves. The leaves are shortly attached to the stalk with ≤8 pairs of opposite leaflets. The leaflets have an oval to oblonglated shape, depending on the specie. The flowers are oblong in shape and are mostly red in colour but some are white.

Six species of sutherlandia were previously identified, namely *Lessertia frutescens, Sutherlandia humilis, Sutherlandia microphylla, Sutherlandia montana, Sutherlandia speciose and Sutherlandia tomentosa.* However, in 1998, a group of authors suggested that the classes of sutheralndia sub species should be reduced to two, as some of the sub species have similar characteristics after analysing the plant with enzyme electrophoresis (Moshe *et al.*, 1998). The suggested two classes are *Sutherlandia tomentosa and Lessertia frutescens,* as all the remaining sub species are now regarded as S. *frutescens*.

Sutherlandia is used traditionally to treat varying ailments (fever, wounds, stress, internal cancer and diabetes) among diverse cultural groups in the regions of southern Africa. In the old days, traditional medicine practitioners prepares the infusion from the leaves, flowers, stems and roots for diverse diseases (Figure 9). That is, the different type of diseases presented are treated with infusions from the separate part of the plant. However, in recent years, sutherlandia is available in various forms, including capsules and tablets, syrups and some cosmetic products (van Wyk and Albrecht, 2008; Aboyade *et al.*, 2013).

Figure 9: Image of *Lessertia frutescens (van Wyk and Albrecht, 2008)*.

2.9.1 Chemical composition of Sutherlandia

Sutherlandia has a variety of phytochemicals (Figure 10), which are known to have health benefits. The chemical composition includes free amino acids (asparagine, proline and arginine) (Moshe *et al.*, 1998; van Wyk and Albrecht, 2008), non-protein amino acids (y-aminobutyric acid (GABA), and L-carvanine) (Ortega, 2003), flavonoids (sutherlandin A, sutherlandin B, sutherlandin C, and sutherlandin D) (Avula *et al.*, 2010), cycloartanol glycosides (sutherlandioside A, sutherlandioside B, sutherlandioside C, sutherlandioside D) (Avula *et al.*, 2010), and triterpenol saponins (cycloartane-type triterpene glycoside) (van Wyk and Albrecht, 2008).

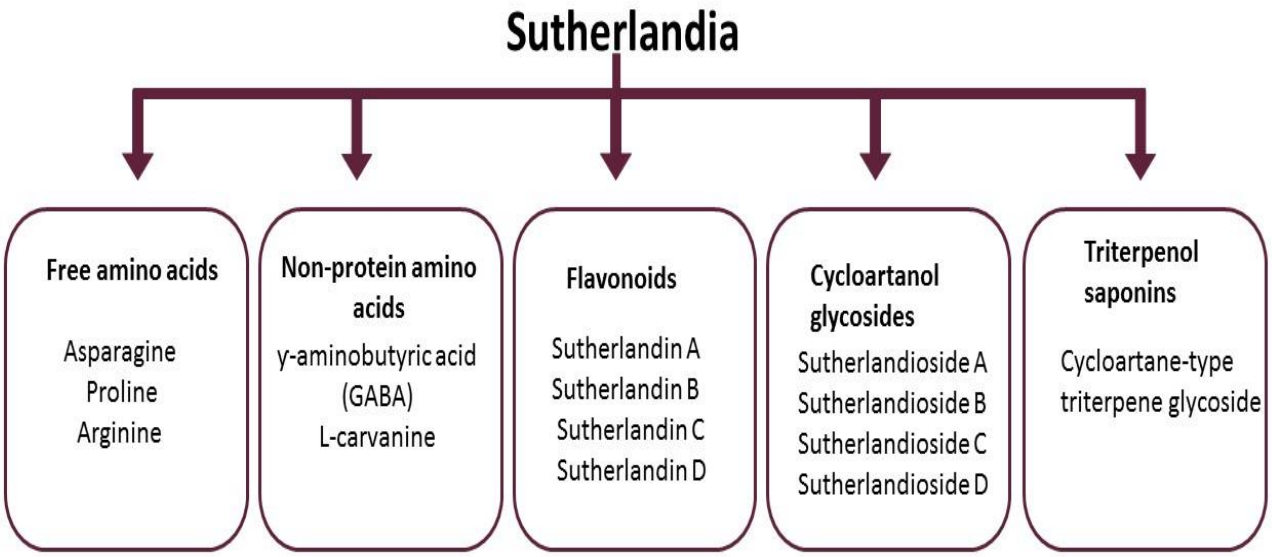

Figure 10: The chemical composition of sutherlandia.

2.9.2 Benefits of Sutherlandia

Based on the array of pharmacological products present in sutherlandia, studies have highlighted its role in cancer (Tai *et al.*, 2004; Grandi, Roselli and Vernay, 2005), Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (Harnett, Oosthuizen and Van De Venter, 2005; Mills *et al.*, 2005; Wilson *et al.*, 2015), diabetes (Sia, 2004; Chadwick *et al.*, 2007), inflammation (Lei *et al.*, 2015; Vasaikar *et al.*, 2018) stress (Chuang *et al.*, 2015; Sergeant *et al.*, 2017) and oxidative stress (Fernandes *et al.*, 2004; Tobwala *et al.*, 2014).

Although the mechanism through which sutherlandia exert it effect is still unclear, a group of authors reported a reduced fatigue and improved life quality in cancer patients after taking

sutherlandia (Grandi, Roselli and Vernay, 2005). Some in vitro studies reported the anti-proliferative and pro-apoptotic effects of sutherlandia on several human tumour cell lines (Tai *et al.*, 2004; Skerman, Joubert and Cronjé, 2011; Van Der Walt, Zakeri and Cronjé, 2016). This suggest that *Lessertia frutescens* extracts may be used in anti-cancer therapy, although further investigation is required to ascertain its effects (Chinkwo, 2005). Additionally, the inhibitory effect of sutherlandia on HIV target enzymes were reported in in vitro studies (Harnett, Oosthuizen and Van De Venter, 2005; Bessong *et al.*, 2006), hence, displaying a prospective pathway to treating HIV. Studies have also shown improved CD4 counts and reduced viral loads in AIDS patients who used sutherlandia tonic (Johnson *et al.*, 2007).

The anti-diabetic effect of sutherlandia have been described in experimental studies. Some in vivo studies showed that pinitol, a component of sutherlandia exerted an insulin-like effect in diabetic mice and thus reduces blood glucose levels (Bates, Jones and Bailey, 2000; Ojewole, 2004). Additionally, Chadwick et al. reported that sutherlandia increases glucose uptake in peripheral tissues and also reduces intestinal glucose uptake in rats fed with diabetogenic diet (Chadwick *et al.*, 2007).

Although studies have shown the relatively safe usage of sutherlandia, the toxicity of canavanine has been implicated in lupus erythematosus syndrome (Capasso et al., 2000). Additionally, the cross-links of proteins after long exposure to sutherlandia was reported and that it can result in autoimmunity (Prete, 1985) and teratogenicity. Ngcobo et al. showed that the in vitro chronic treatment of normal T lymphocyte cells with sutherlandia was toxic (Ngcobo *et al.*, 2012), hence , too much of sutherlandia may be bad. It was also reported by Adrienne et al. that the concurrent use of sutherlandia with an antiretroviral protease inhibitor (atazanavir) reduces the bioavailability of the protease inhibitor, which means that

sutherlandia may reduce the anti-HIV efficacy of atazanavir (Müller, Skinner and Kanfer, 2013). Up-to-date, the effect of sutherlandia has not been reported on male reproductive function in either healthy /diseased humans or animals.

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Addenda to Chapter 2

Addendum 2A

Diabetes Mellitus and Male Infertility

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[1]Omolaoye Temidayo S (BTech Hons.), [*1]Du Plessis Stefan S (PhD)

Affiliation:

[1]Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa

*Corresponding author: SS du Plessis, Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Francie van Zijl Drive, Tygerberg 7505, South Africa.

E-mail: ssdp@sun.ac.za

Key words: Diabetes mellitus, Male infertility, Diabetic neuropathy, Oxidative stress, Reactive oxygen species, Advanced glycated end products

Abstract

Infertility is prevalent in about 10%-25% of couples in their reproductive age, analogous to 60-80 million infertile couples globally. Of these infertility cases, 10%-30% are exclusively attributed to a problem of the male. Several diseases have been implicated as contributors to deteriorating male fertility and diabetes mellitus (DM) is included. DM, a chronic non-communicable disease, is one of the most appreciable health threats, as it affects 9% (422 million) of the world's population as of 2014. It is characterised by hyperglycaemia, which can result from the inability of the pancreatic β-cells to secrete insulin or from the target tissue becoming insensitive to insulin. DM has been reported to influence male reproductive function through diverse pathways and mechanisms. The adverse effects of reactive oxygen species and successive development of oxidative stress that occur due to DM have been investigated and implicated by several studies. The products of non-enzymatic glycosylation are reported to be widely distributed in the reproductive tract of diabetic men. Additionally, DM has been implicated to impair the processes of male sexual acts. Data reported in this review were extracted from PubMed, Google Scholar, Science Direct and Scopus with diabetes and male infertility as the key search words.

In light of the aforementioned, the aim of this review is to provide brief background information on DM as well highlight and explain the likely mechanisms of male fertility which DM impacts.

2A.1 Introduction

Diabetes mellitus (DM) is an embodiment of diverse metabolic disorders marked by chronic hyperglycaemia that can result from lack in insulin synthesis and secretion or reduced sensitivity of tissues to insulin [1]. In present-day societies, DM represents one of the most noticeable health perils and its prevalence is increasing swiftly. In 2014, the World Health Organization (WHO) reported that 422 million people have DM, connoting a 60% global increase relative to 2002 [2]. The WHO previously projected that the number will rise to about 300 million by 2025 [1]. Our calculation, however, showed that the projected value of DM for 2025 has already been surpassed by 28.9% in 2014. In recent years, the views that DM has inconsequential effects on male reproductive function have been questioned by conclusive data from various studies.

Male infertility describes a male's inefficiency to cause fertilization in a fertile female over a period of 12 month of consistent and unprotected intercourse [3]. It is estimated that 56% of infertile couples of childbearing age seek medical help [4]. Amidst these couples, 10%-30% of infertility cases are attributed exclusively to a problem of the male and another 15%-30% of cases showed significant anomalies in both partners [5]. Studies on prevalence of infertility in DM male partners of infertile couples revealed diminished sperm motility and increased abnormal sperm morphology [6-7]. Additionally, the increased production of highly potent free radicals and subsequent production of advanced glycation end products (AGEs) as well as an upsurge in the expression of receptor for AGE (RAGE) have been reported in DM [8,9]. These molecules are likewise implicated amongst other pathology of diabetic neuropathy (DN) [10]. With the data available, there is no uncertainty that DM is responsible for various pathological and biochemical modifications that reduce male fertility [11].

The aim of this review is to provide an abrupt background on DM and introduce its relationship to male infertility. It will as well make specific reference of the possible mechanisms via which DM elicits its impact on the male reproductive system. Data reported in this review were extracted from PubMed, Google Scholar, Science Direct and Scopus with diabetes and male infertility as the key search words.

2A.2 Overview of diabetes mellitus

DM prevalence has increased substantially in the last three decades, and has been ranked to be the 7th cause of death in the human race [2]. Hyperglycaemia is a known effect of uncontrolled DM and consequently can lead to damage to various systems and tissues, especially the nerves and blood vessels [12]. Diverse pathogenic processes are included in the development of DM ranging from pancreatic β-cells autoimmune destruction with insulin insufficiency to abnormalities that causes resistance to insulin action [13]. The fundamental effect of insulin loss or insulin ineffectiveness on glucose homeostasis is the inefficient uptake and usage of glucose by glucose-dependent cells, resulting in hyperglycaemia.

In the healthy state, the presence of insulin causes the stimulation of the glucose transporters (GLUTs), allowing glucose to bind to the extracellular portion of these transporters, which results in the translocation of the protein, thus having rapid glucose diffusion into the cell [14-15]. In DM, due to decreased insulin, there is dysregulation of the processes of glucose metabolism and utilization that concurrently alters the stimulatory effect of insulin on glucose transporter translocation.

The numerous cases of DM fall into two broad categories that are classified as Type Ⅰ and Type Ⅱ. In both types of DM, metabolism of carbohydrates, lipids and protein are impaired.

2A.2.1 Type Ⅰ DM

Out of 422 million people affected by DM globally, ≤10% is attributed to Type Ⅰ DM. About 85% of this population are diagnosed before the age of 20 and ≥15% of the cases are ascribed to adults (≥ 30 years old). It is instigated by injury or cellular-controlled autoimmune destruction of the pancreatic β-cells. Heredity, race or ethnicity, age and gender are some of the associated risk factors that play a role in ascertaining the susceptibility of the insulin producing cells to abrasion. It may develop swiftly over a period of a few days or weeks, following this sequence: (1) decreased insulin; (2) elevated usage of fats for energy and for formation of cholesterol by the liver; (3) reduction of the body's proteins [16].

2A.2.2 Type Ⅱ DM

The prevalence and incidence of Type II DM is rapidly increasing throughout the world and it accounts for 90%-95% of those with DM. Type II DM was known to be an adult or old age disease. But in the last few decades, its prevalence has increased among youth, which predicts for higher estimate of occurrence in the future. It occurs as a result of decreased perceptivity of target tissues to the metabolic effects of insulin. Type Ⅱ DM is also described as a 'contemporary disease' because it is caused by lifestyle factors, such as diet and obesity. Ethnicity, environmental exposure and socio-economic factors are also evident risk factors. In comparison to Type Ⅰ DM, it is correlated with elevated plasma insulin concentrations (hyperinsulinemia). This occurs as compensatory feedback by the pancreatic β-cells for reduced sensitivity of marked tissues to the metabolic effects of insulin. The diminished insulin sensitivity debilitates carbohydrate usage and storage, increasing blood glucose and spurring a compensatory increase in insulin secretion.

It was predicted in 2016 that there would be a 71.5% prevalence increase of Type Ⅱ DM amidst age 20-79 by 2035, including 6.0% in Africa, 7.1% in Europe, 11.3% in Middle East, 12.3% in North America and Caribbean, 8.2% in South and Central America, 9.4% in South East Asia and 8.4% in Western Pacific [17]. Considering the above futuristic prevalence evaluation, DM will affect more men ahead of and amid their reproductive years.

2A.3 Impact of diabetes on male fertility

Diabetes has been substantiated to have adverse effects on both male and female reproductive function [16,18] and its impacts can be seen in increased prevalence of infertility [6,19-21]. About 90% of diabetics experience upheaval in sexual function, including a decrease in libido, impotence and infertility [22]. Furthermore, diabetic men are vulnerable to different sexual problems, though progressive physical disorders and deteriorative psychological response are contributors [23]. Several studies have investigated and reported different pathologies commonly experienced by diabetic men and have also highlighted the subsequent reproductive defects. Some of the extracted findings highlighting the impacts of DM on male reproductive functions in human and animal models were represented in Table 2A.1. Selected mechanisms through which DM impact male reproductive function were summarized in Figure 2A. 1.

Table 2A.1: Findings highlighting the impact of DM on male reproductive functions in human and animal models.

Ref	Author	Year	Numbers	Type	Model	DM	Results
[24]	Mallidis *et al*.	2007	52	*In vivo*	Human	Type Ⅰ/Type Ⅱ	↓Sperm count
[25]	Agbaje *et al*.	2007	56	*In vivo*	Human	Type Ⅰ	↓Semen volume, ↑sperm nDNA fragmentation, ↑deletion of mtDNA
[18]	American Society	2009	12	*In vivo*	Human	Type Ⅰ	↓nDNA damage
[26]	Roessner *et al*.	2012	45	*In vivo*	Human	Type Ⅰ/Type Ⅱ	↑nDNA fragmentation, ↑lipid peroxidation, ↑disrupted mitochondria potential
[27]	Sudhindra *et al*.	2014	103	*In vivo*	Human	Type I	↓motility, ↓semen volume, ↑DNA damage
[28]	Murray *et al*.	1985	91	*In vivo*	Rats	Type Ⅰ	↑Testosterone, ↓LH, Altered Sertoli cells, ↑abnormal morphology
[29]	Maresch *et al*.	2017	26	*In vivo*	Mice	Type I	↓Normal morphology, ↓seminiferous tubule diameter , ↑ spermatogenic disruption
[30]	Ballester *et al*.	2004	44	*In vivo*	Rats	Type Ⅰ, STZ	↓Testosterone, ↓Leydig cells, ↓FSH, ↓LH
[31]	Shrilatha *et al.*	2007	24	*In vivo*	Mice	Type Ⅰ, STZ	↑nDNA damage, ↓sperm count
[32]	Vikram *et al*.	2008	45	*In vivo*	Rats	Type Ⅰ, STZ	↓Testosterone, ↑Lipid peroxidation
[33]	Jelodar *et al.*	2009	16	*In vivo*	Rats	Type I, Alloxan	↓ Leydig cells, ↓Sertoli cells, ↓seminiferous tubule diameter
[34]	Soudamani *et al*.	2009	36	*In vivo*	Rats	Type Ⅰ, STZ	↓Motility
[35]	Navarro-Casado *et al*.	2010	86	*In vivo*	Rats	Type Ⅰ, STZ	↓Testosterone, ↓motility
[36]	Mangoli *et al*.	2013	20	*In vivo*	Mice	Type Ⅰ, STZ	↓Motility, ↓viable cells; ↑abnormal morphology

STZ=streptozotocin.↑= increase, ↓= decrease.

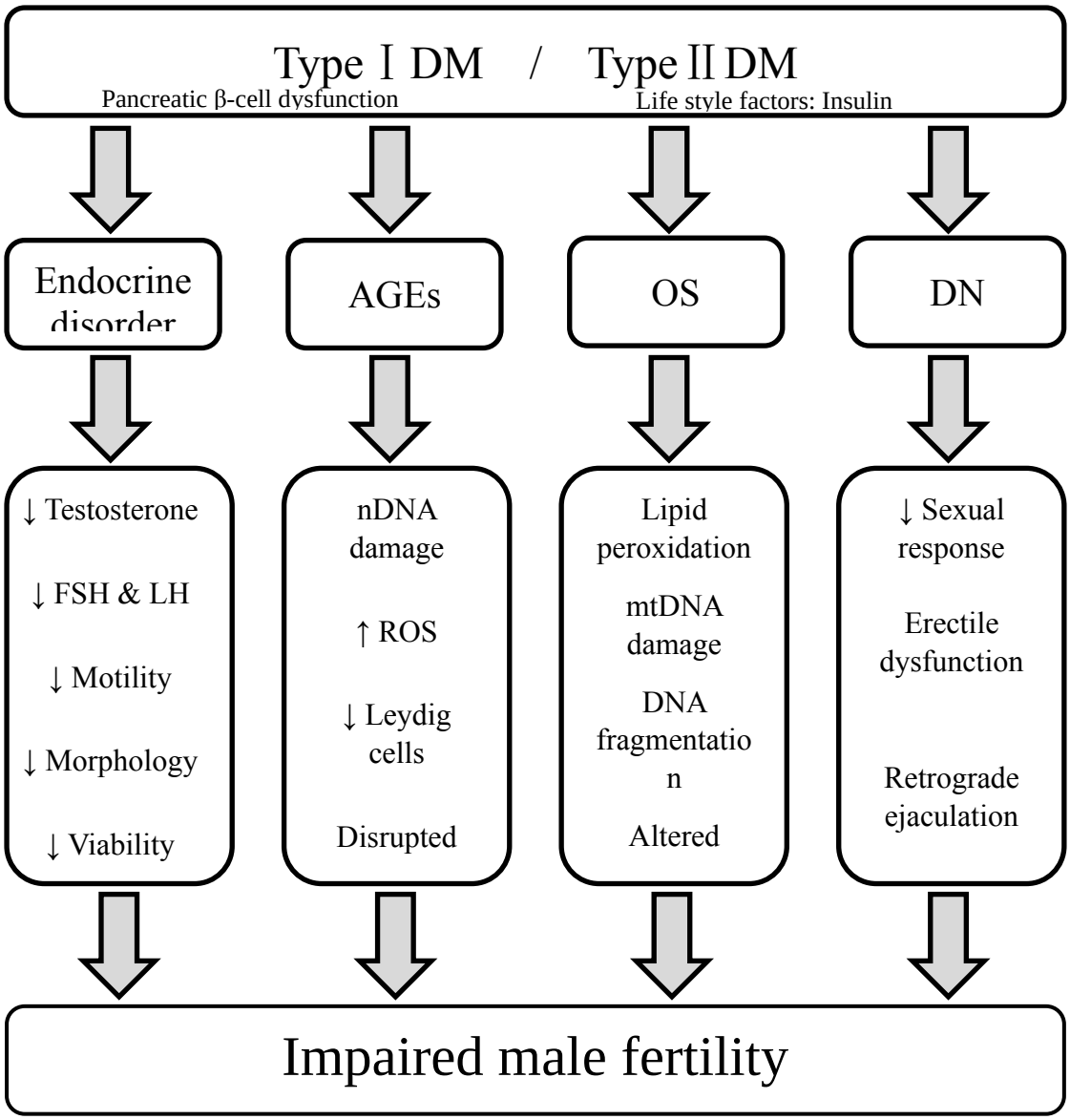

Figure 2A.1 Mechanisms through which DM affects male reproductive functions.
↑= increase, ↓= decrease

2A.3.1 DM effects on spermatogenesis: role of endocrine disorder

Under normal circumstances, the hypothalamus releases gonadotropin-releasing hormone, thereby stimulating the anterior pituitary to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates the Leydig cells to secrete testosterone and

dihydrotestosterone, while FSH stimulates the Sertoli cells of seminiferous tubules to assist the process of spermatogenesis.

Unarguably, spermatozoa are capable of using glycolysis and/or oxidative phosphorylation in generating energy. They are as well structured to be capable of using external hexoses (glucose, fructose and mannose), and smaller substrates (lactate, citrate amino acids and lipids) for energy production [30]. Though spermatozoa are known to secret their insulin, they are however sensitive to hormonal fluctuations [37]. Therefore, deficiency of insulin or insensitivity to insulin in DM alters the endocrine pathway (negative feedback mechanism), resulting in impaired male reproductive function.

Animal studies on induced hyperglycaemia revealed some adverse effects on male reproductive function relative to altered endocrine control (Table 2A. 1). Additionally, decreased Sertoli cell vacuolization [26], decreased sperm production [29,33,38-40], decreased fertility [33,41], alteration of epididymis morphology and density [39], decreased LH, FSH and testosterone serum levels [42,43], decreased number of Leydig and Sertoli cells and decreased number of spermatogonia [33] were observed in induced DM. The effects of DM on spermatogenesis have not only been shown in animal models, but have also been shown in men (Table 2A. 1).

Furthermore, Ballester *et al* [30] reported a reduction in Leydig cell number and impaired cell function in streptozocin (STZ) induced mice model of DM. The decreased Leydig cell number linked to the decrease in serum LH, which in part explained the stimulatory effects of LH on Leydig cells. This also indicated that the Leydig cells production involving insulin and insulin-like growth factor 1 signal mechanisms is mediated by LH [44,45]. While the impaired cell function was measured by the loss of tyrosine phosphorylation, as well as decreased

expression of GLUT-3 receptors, androgen receptors and insulin-like growth factor 1 receptors [36]. These findings are supported by several other animal studies that investigated the effect of DM on male fertility [35,39,46,47]. In addition, DM alters spermatogenesis through an FSH-related mechanism. Insulin deficiency present in Type Ⅰ DM does not appear to affect spermatogenesis through a direct effect on the epithelium of seminiferous tubules, but instead by an alteration in serum FSH levels [30,48]. Decreased FSH is followed by a reduction in tubular FSH receptors in STZ induced Type Ⅰ DM, thus causing a diminished response of the epithelium of the seminiferous tubules to FSH stimulation. Therefore, DM alters spermatogenesis by disrupting the modulating effect of insulin on the regulation of serum FSH levels [30,48].

Likewise, glucose has been shown to be important for spermatogenesis and the acrosome reaction (AR). This was evidenced when a medium deprived of glucose inhibited the spontaneous AR, which was swiftly restored after the subsequent addition of glucose [49]. These substrates are conveyed into the cell by GLUTs [50].

GLUTs are specific transporters that catalyse the passive diffusion of glucose into the mammalian cells along a concentration gradient. The GLUT family consists of 14 members and can be divided into three groups based on their sequence similarities [51].

GLUT8 belongs to the class 3 transporters and is expressed predominantly in the testis [52,53]. Research on GLUT8 expression in human spermatozoa revealed its presence in the acrosome and mid-piece region of mature spermatozoa [54]. It was also found in the acrosome and mid-piece region of mouse mature spermatozoa [55]. While some researches detected GLUT8 in differentiating spermatocytes of the stage 1 type, but not in mature spermatozoa [52]. Glucose transported into the cell is converted to energy, which is needed

for spermatogenesis and motility. The disruption of GLUT8 activity caused by decreased insulin resulted in reduced sperm motility and impaired fertilization [56]. This can as well be a result of lower gonadotropin response to gonadotropin releasing hormone in diabetics [44].

2A.3.2 DM effect on sperm parameters: role of oxidative stress (OS) and AGEs

Studies have shown that DM induces subtle molecular changes that are essential for sperm quality and function. In a study carried out on 52 diabetic men, semen analysis revealed a significant decrease in sperm motility, including the number of rapid progressive cells [57]. Furthermore, in a comparative study on sperm cryopreservation, semen samples collected from diabetic men showed a significant decrease in sperm parameters when compared to groups of men with autoimmune disorders, kidney diseases, ulcerative colitis and heart diseases [58]. Another study on prevalence of infertility carried out by Delfino *et al.* [7], revealed a significant alteration in sperm kinetic properties and sperm morphology of male diabetic partners. A few more studies also revealed a significant decrease in semen volume, sperm motility and morphology in the semen of diabetic men [25,59]. All these outcomes are associated to the development of OS.

Effect of DM on male reproductive function can also be explained through the impact of OS, caused by the inequality between reactive oxygen species (ROS) production and antioxidant defence mechanisms [60]. The main origins of ROS in the male reproductive system are known to be the immature spermatozoa and leukocytes [60,61]. Additionally, mechanisms that involve repeated mild changes in cellular metabolism may result in tissue damage within a brief occurrence of hyperglycaemia. An enormous bulk of data give priority to certain metabolic pathways as being dominant contributors to hyperglycaemic induced cell damage,

e.g. elevated glycolysis, glucose autoxidation, increased polyol pathway flux, increased AGE formation, activation of protein kinase C isoforms and increased hexosamine pathway flux [62,63]. It has been shown that excessive production of O_2^- by mitochondria in hyperglycaemia is the trigger that propels these pathways. Excessive production of O_2^- momentously inhibits glyceraldehyde-3-phosphate dehydrogenase activity, which in turn activates all the pathways of hyperglycaemic damage by diverting upstream glycolytic metabolites to these pathways [62]. Furthermore, when the highly potent ROS exceeds the seminal antioxidant defence ability, many cascades of reactions will occur, which can lead to sperm DNA damage and mitochondrial DNA fragmentation, then altered sperm parameters and subsequently male infertility.

In OS, there is excessive production of NO^-, which is detrimental to sperm motility. NO^- may react with O_2^- or H_2O_2 to form $ONOO^-$ or OH^-, which will cause oxidation of sperm membrane lipids and thiol proteins [45]. It can also cause a decreased ATP levels, thereby affecting the kinematics of spermatozoa.

The high polyunsaturated fatty acids contents in the sperm plasma membrane are susceptible to ROS, its invasion thereof, leads to lipid peroxidation [64]. Lipid peroxidation occurs in three stages including, initiation, propagation, and termination. During initiation, free radicals react with fatty acid chains to form the lipid peroxyl radical. Peroxyl radicals in turn react with fatty acids to produce free radicals and the reaction is thus propagated. In termination, the two radicals react with each other which lead to lipid break down [64]. Furthermore, oxidation of sugar by OH^- has been shown to be the main cause of DNA strand breaks. Oxidative damage can further cause base degradation, DNA fragmentation, and cross-linking of proteins. The proportion of DNA strand break is increased in the sperm of infertile diabetic men [9].

Apoptosis, also regarded as programmed cell death, can be instigated by ROS-induced oxidative damage. High levels of ROS alter integrity of mitochondrial membrane [65-68], resulting in mitochondria DNA (mtDNA) damage and subsequently affects sperm functions negatively.

Elevated ROS production has also been implicated in the generation of AGEs. AGEs are products of non-enzymatic reaction between sugar and the amino groups of proteins, lipids and DNA under hyperglycaemic conditions [69,70]. AGEs can alter the normal functioning of macromolecules directly, by generating ROS independently, or indirectly, by activating the receptors for advanced glycated end products (RAGE) [71]. AGEs may play a key role in instigating harm and further act as mediator of damage to reproductive system of diabetic men [72].

RAGE is a ligand binding receptor that increases cellular dysfunction in inflammatory disorders such as DM. RAGE is expressed at low levels in normal tissues. However, in diseased conditions such as DM, its increased expression leads to tissue damage [73,74]. Immunohistochemistry on the testes, epididymis and spermatozoa of 21 diabetic men revealed the wide distribution of RAGE in their reproductive tracts as compared to non-diabetics [24]. An increased prevalence of immunoreactive cells was revealed in the seminiferous epithelium in the testes of diabetic men and sections of the epididymis displayed various degrees of RAGE immunoreactivity. Increased RAGE expression was also found in the spermatozoa acrosomal cap of these men, as determination of the specific location of RAGE was examined during different stages of the AR [72]. It therefore suggests a major role for glycation processes in sperm nDNA damage and cellular damage [24,72].

Furthermore, it has been shown that seminal plasma has important antioxidant systems that can supply the spermatozoa with a defensive environment against OS [45]. However, it was demonstrated that diabetic men have significantly lower seminal total antioxidant capacity (TAC) levels compared to their non-diabetic counterparts. This was supported by another study that showed that seminal TAC has an effect on male fertility and that increased ROS levels leads to low TAC levels [75]. The reduced TAC level in DM is consistent with higher malonaldehyde levels, which suggests a possible role for AGEs in instigating lipid peroxidation levels.

2A.3.3 Diabetic neuropathy

DN is one of the most prevalent complications of DM. It has been reported to affect about 50% of patients with Type Ⅰ and Type Ⅱ DM. It can be categorized into autonomic neuropathy and peripheral neuropathy because of its effect on either autonomic or peripheral nervous systems. Both neuropathies results from microvascular dysfunction which can affect the autonomic nervous system (ANS), leading to autonomic neuropathy and can as well impair peripheral nerves causing peripheral neuropathy. Various pathogenic pathways involved in the development of DN and subsequent damage of the male reproductive function were summarized in Figure 2A.2. Since the ANS is involved in the regulation of sexual response cycle, impairment thereof by DN can result in reduced sexual response, erectile dysfunction (ED) and retrograde ejaculation [76].

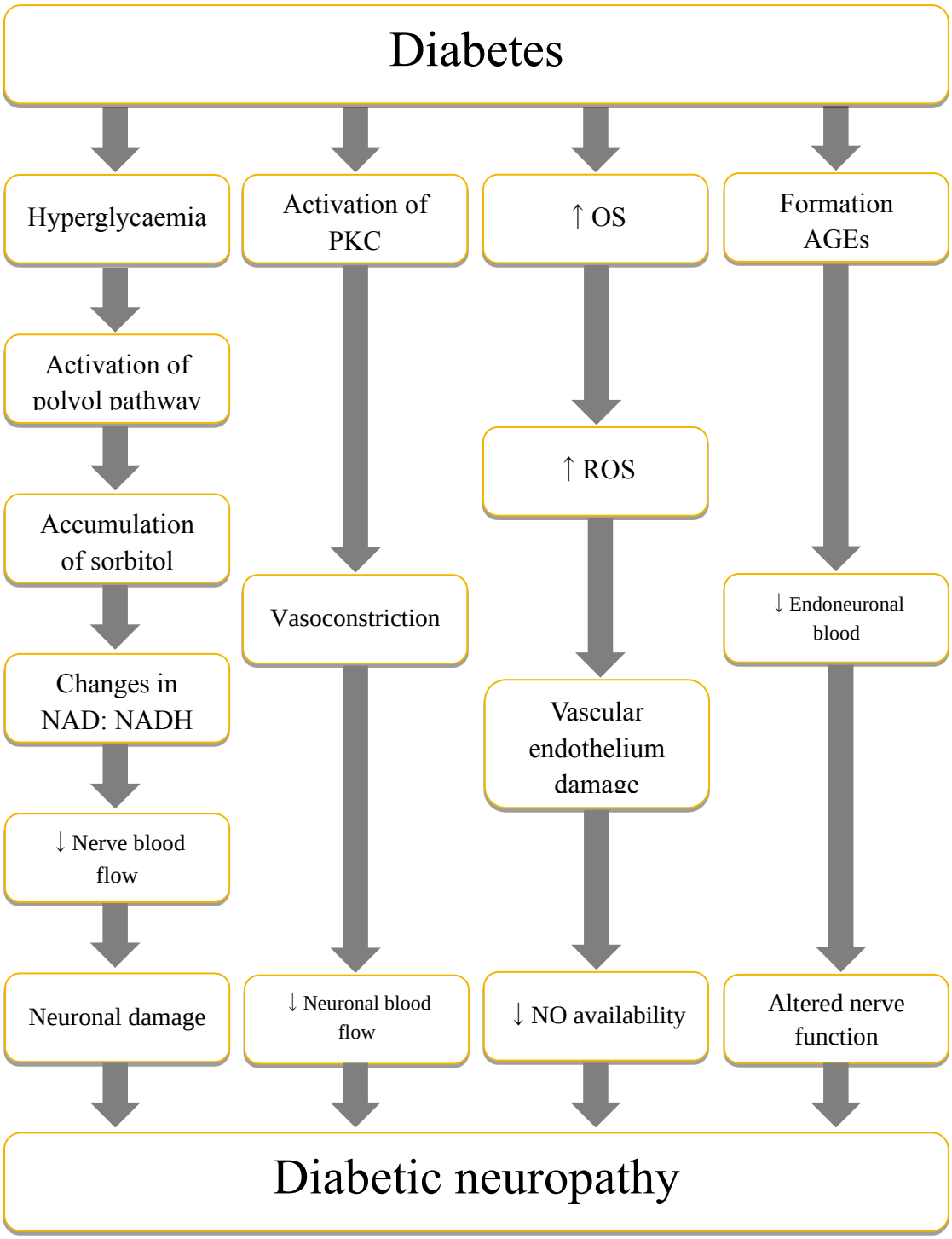

Figure 2A.2: Pathogenic pathways of DN leading to male reproductive function impairment. NAD=nicotinamide adenine dinucleotide; NADH= reduced nicotinamide adenine dinucleotide; PKC= Protein kinase C.↑= increase, ↓= decrease.

2A.3.3.1 Reduced sexual response

Male sexual response is a result of physical or psychological stimulation which leads to vasodilation and subsequent increased blood flow into the penis. Low libido has been reported to be associated with DM and that it worsens with progressive diabetic state [77]. The reduced sexual response/libido can be related to the physical and psychological susceptibility to deterioration in DM. For instance, Fairburn *et al.* [77] reported an absence of pumping sensation that usually follow ejaculation in over a third of diabetic subjects in their study, with these patients describing semen flowing from their erect or drooping penis' either at or prior to orgasm.

2A.3.3.2 ED

ED is rampant in millions of men, and has been reported to be more prevalent amongst diabetic men. However, ED's prevalence depends on variant factors, such as the population reviewed, as well as the definition and methods used [78]. For instance, it was reported that 41.3% of adult men (above 18 years old) in France have ED, 33.2% in Brazil and 41.8% in China[79]; 18.0%-48.0% among middle-aged men in Germany [80] and about 50.0% of ageing diabetic men (aged 56-85) have ED in the United States [81].

Usually, neurotransmitters, especially NO are released either from the penile nerve endings or from endothelium during normal sexual activity, which triggers the relaxation of the cavernosa arteries and the surrounding smooth muscles. This in turn, promotes an increase in penile arterial blood flow, thereby causing an erection. However, diabetic men have impairment in both endothelium dependent smooth muscles and autonomic mechanism that mediate the relaxation of corpora cavernosa [37]. In addition, endothelial dysfunction reflects the loss of NO activity and biosynthesis at the endothelial level, thus leading to ED [82,83].

2A.3.3.3 Retrograde ejaculation

The emptying of semen in the prostatic urethra results in various reflex actions controlled by sensory nerves from the prostatic urethra. This excites centres in the sacral and lumbar regions of the spinal cord, which then transmits impulses to autonomic and somatic pathways, thereby causing ejaculation. However, in DM, retrograde ejaculation occurs due to ANS impairment and subsequent loss of constriction by the external urethral sphincter and loss of other reflex actions involved in ejaculation. Retrograde ejaculation can be defined as the retro-influx of semen into the bladder rather than emptying into the anterior urethra [84]. This can result in the subject experiencing the pumping sensation associated with ejaculation without semen emerging from the penis. Urine collected immediately after ejaculation in these men appears cloudy and the diagnosis of retrograde ejaculation can be confirmed by the numerous spermatozoa in a post-orgasmic urine sample.

2A.4 Treatment of male infertility caused by DM

Once an individual has been identified as having fertility related issues due to diabetic complications, treatment should be aimed at treating the disease through amelioration of the underlying cause and subsequently treating the consequences.

2A.4.1 Treatment of DM

Treatment of DM focuses on controlling the blood glucose levels without causing hypoglycaemia.

2A.4.1.1 Treating Type Ⅰ DM

Effective treatment of Type Ⅰ DM requires administration of sufficient exogenous insulin to maintain glucose metabolisms and to prevent hyperglycaemia. Insulin present in several forms, such as short acting insulin and protein derivative precipitated insulin. The half-life of short acting insulin is 3-8hr, while that of the protein derivatives is 10-48hr [16]. However, treatment should be given with an individualized pattern.

2A.4.1.2 Treating Type Ⅱ DM

Management of Type Ⅱ DM can be accomplished through a strict adherence to healthy lifestyle, diet control, exercise, weight loss and the use of suitable medication in an attempt to reverse insulin resistance. Examples of prescribed drugs for Type Ⅱ DM are metformin and thiazolidinediones. Metformin works by improving the sensitivity of the body tissues to insulin. Thiazolidinediones make the body's tissues more sensitive to insulin, while sulfonylureas and meglitinides stimulate the pancreas to secret more insulin.

2A.4.2 Treating the consequences

2A.4.2.1 Antioxidant therapy

Antioxidants operate by arresting the oxidative chain reaction, removing, or reducing the formation of ROS [85]. Hughes *et al.* [86] reported a significant protection of a media containing ascorbic acid (600 µmol/L), alpha-tocopherol (30 and 60 µmol/L), and urate (400 µmol/L) from sperm DNA damage by the non-enzymatic antioxidants during an *in vitro* fertilization (IVF) procedure. Studies on antioxidant treatment of OS related male infertility reported an improvement of the sperm quality and greater assisted reproductive technology

(ART) procedures success rate [87-90], but antioxidant therapy remains highly debated and controversial.

2A.4.2.2 ART

Infertility can be reduced in diabetic men with ED or retrograde ejaculation through intra cytoplasmic sperm injection (ICSI) or IVF.

Spermatozoa of diabetic men with ED can be obtained via testicular biopsy. Since ICSI-IVF requires at least one sperm, the sperm retrieved can be injected into the female gamete for fertilization. The fertilized embryo can then be transferred inside the uterus.

In addition, ICSI-IVF can as well be applied to treatment of diabetic men with retrograde ejaculation. This can be achieved by recovering the spermatozoa from the post ejaculatory urine of these men. Nakolettos *et al.* [54] advised that retrograde ejaculation resistant to long term medication can be managed using the ART, following an outcome of 51.2% fertilization rate amongst their study subjects.

2A.4.2.3 Treating diabetic neuropathy

The aim of manageing reduced sexual response, ED and retrograde ejaculation is to help reduce infertility and also aid the affected enjoy their sexual activities irrespective of the limits set by the diseased factors. The treatment can be focused on physical, psychological and surgical treatment as well as medication. Physical treatment improves the subject's general state of health by changing any reversible physical activity contributing to the sexual problem. Psychological treatment is that primary and secondary psychological reactions contributing to the problem should be tackled. Surgical treatment means surgical management of ED includes implantation of penile prostheses and penile vascular regeneration. Surgical

procedure to correct retrograde ejaculation can be done by reconstructing the bladder vesical sphincter [91]. Medication refers to drugs commonly used to manage ED that are Avanafil (Stendra), Sildenafil (Viagra), Tadalafil (Cialis) and Vardenafil (Levitra, Staxyn). All these work by relaxing the smooth muscles and boosting vasodilation, thereby making it easier to achieve and maintain erection.

2A.4.3 Conclusion

DM has been implicated in the impairment of male fertility. Studies have revealed the different mechanisms involved in this pathology. The mechanisms include endocrine disorder, alteration in GLUT8 activity, OS development, AGEs formation and occurrence of DN. It is important that endocrinologists and physicians educate their patients on the possible impact of DM on male fertility, while reproductive endocrinologists should carefully consider the impact of DM as part of their strategies for fertility treatment.

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Addendum 2B

Male Infertility: A Proximate Look at the Advanced Glycation End Products

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Temidayo S Omolaoye[1], Stefan S du Plessis[1], [2*]

1. Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa
2. Department of Basic Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine of Health Sciences, Dubai, UAE

* Corresponding author: Prof SS du Plessis, Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University. Francie van Zijl Drive, Tygerberg, 7505. South Africa.

OR

Department of Basic Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine of Health Sciences, Dubai, UAE.

E-mail: ssdp@sun.ac.za / stefan.duplessis@mbru.ac.ae

Abstract

Advanced glycation end products (AGEs) are products of cascades of non-enzymatic glycosylation. They are formed over a period of hours to days, depending on the protein lifetime. AGEs acts by independently producing reactive oxygen species (ROS) or by binding to their receptors. Binding of AGE to the receptor for advanced glycation end products (RAGE) has been shown to play a role in physiological processes, including lung homeostasis, bone metabolism, neuronal systems and the immune system. When in excess, they take part in the pathogenesis of diseases such as diabetes mellitus, cardiovascular diseases, neurodegenerative diseases, and etcetera.

The cause of male infertility is considered unexplained in many cases, suggesting that there are gaps in the mechanistic knowledge of sperm production and function, especially, pathways involved in the physiochemical protein regulation of spermatogenesis. It is therefore important to consider areas of research highlighting protein modification and identification and their implication for male fertility.

Keywords: Advanced glycation end products, Receptor for advanced glycation end products, Hyperglycaemia, Male infertility, Infertility, Assisted reproductive technology

2B.1 Introduction

Male infertility describes a male's inability to impregnate a fertile female over a period of 12 months of consistent and unprotected intercourse [1]. It has been estimated that 20-30% of infertility cases is solely attributed to male factor and another 20% associated with problems from both partners [2], cumulatively, it accounts for 50% of the total cases [3,4].

Various studies have attempted to elucidate the impact of molecular alterations, in terms of genetic and epigenetic modifications, on male fertility [5,6]. It has been shown that genetic modification, including chromosomal abnormalities and single-gene mutation, explains for about 10-15% of infertility cases [7]. Chromosomal abnormalities comprise of both the numerical and structural variations of chromosomes. The numerical chromosomal variation such as Klinefelter syndrome has been reported to be the most persistent karyotype abnormality in infertile men [8]. On the other hand, the structural modification of the Y chromosome, including translocation between the Y chromosome and autosomal chromosome, deletions, and inversion of the Y chromosome is prevalent in oligozoospermic patients [9]. Furthermore, the changes caused by molecular modifications of DNA or histones rather than changes in the DNA sequence resulting in the phenotype alteration have been reported to impact male fertility [10–13]. These findings collectively serve as a gateway to further investigations on the implication(s) of expressed modified proteins either during spermatogenesis or fertilization.

2B.1.1 Brief Evidence of Protein Build-up in the Male Reproductive System

Proteins are large biomolecules that are involved in several physiological processes. Michael and Michael (1983) reported that the Sertoli cells synthesize and secrete a protein referred to as the testicular ceruloplasmin. This protein has a similar immunological function as the

serum ceruloplasmin [14]. Ceruloplasmin is a protease, oxidase sensitive serum protein, which is also essential for copper transport protein. It has further been suggested to be a protein required for germ cell viability [14].

Additionally, it has been shown that the Sertoli cells secrete a protein called testicular transferrin, which allows for iron transport and helps in the development of germ cells. It was reported to be regulated by follicle-stimulating hormone (FSH), insulin, testosterone and Vitamin A [15]. Furthermore, the importance of aquaporin (s) in the transport of water and solutes across cell membranes has been elucidated. Aquaporin proteins (AQPs) are integral transmembrane proteins, functioning as selective water, glycerol, urea and non-electrolytes channels [16–24]. Thirteen subunits of AQPs have been identified in mammalian cells and are categorized into three groups based on sequence similarity and substrate selectivity. The first group (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8) are water-selective channels having high permeability to water. They are also known as orthodox AQPs. Aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) are the second subunit of AQPs, having high permeability not only to water but also to glycerol, urea and other small non-electrolytes. Superaquaporins (AQP11 and AQP12) are also water-selective channels but differ from both orthodox and aquaglyceroporins in localization. Superaquaporins are found in the membrane of intracellular organelles and are therefore involved in regulating volume, vesicle homeostasis and water transport intracellularly [25]. However, the male reproductive tract including the testes, epididymides and efferent ducts contains various AQPs except for AQP6 and AQP12. The most abundant AQPs in the epididymides are AQP1 and in the efferent ducts, AQP9. They have been shown to play a crucial role in the dynamics of luminal fluid secretion and reabsorption during sperm transport and maturation. Additionally, AQP3, AQP7, AQP8 and AQP11 have been shown to be present in sperm and are essential for the differentiation of

spermatids to spermatozoa during spermiogenesis. They also help in regulating osmolality during sperm transit [26].

Furthermore, the AQPs present in the male reproductive tract (epididymides and efferent ducts) have been shown to be regulated by estrogen. That estrogen controls water reabsorption in the epididymides and efferent ducts via AQP1 and AQP9 [27]. While Bernardino et al. showed that estrogen modulates glycerol permeability in the Sertoli cells through the regulation of AQP9 [28]. This suggests that AQPs are essential for normal male reproductive function and alteration in their expression or function may result in subfertility or infertility [26].

Thus, this is indicative that proteins are involved in the physiological processes of spermatogenesis and fertilization. Seeing that the organs and cellular components involved in male reproduction are loaded with proteins, it is pertinent to further investigate the implication of protein modification due to physiological or pathophysiological stress in male fertility.

2B.1.2 Why Advanced Glycation End Products?

Under both normal and abnormal conditions, proteins undergo enzymatic and non-enzymatic glycosylation. Glycosylation is a form of post-translational and co-translational modification, which are mostly involved in the production of amino acids, RNA and DNA [29]. During enzymatic glycosylation, the carbonyl group of reducing sugars covalently attach to proteins and lipids, aiding folding and physiochemical stability of glycoproteins. However, when proteins and lipids attach to reducing sugars covalently, but non-enzymatically, glycation occurs. In 1985, Ahmed et al. reported the presence of a trace product during a glycation reaction between polylysine (N^{α}-formal-N^{ε}-fructoslysine) and amino acids. The trace

compound observed was said to be N^{ε}-carboxymethylysine (CML) representing the first advanced glycation end product (AGEs) discovered [30]. After a 15-day period, 40% CML formation was observed following incubation at physiological pH and temperature. The rate of formation, however, was intensified with increased phosphate buffer concentration in the incubation mixture. This suggests that AGE formation surges with increased exposure to sugar. The role of AGEs have been widely investigated in several pathologies, including neurodegenerative diseases, chronic inflammatory diseases, diabetes mellitus (DM) and DM related disorders. However, its role in infertility, specifically, male infertility, is less understood. It is worth noting that most studies that reported the impact of AGEs on male fertility investigated DM (type 1 and type 2 DM) related infertility. This is because AGEs are shown to increase under hyperglycaemic conditions and reducing sugars are important key players in their formation. Mallidis et al. reported a significantly higher percentage of spermatozoa with nuclear DNA fragmentation and elevated levels of AGEs in the testis, epididymis and sperm of diabetic men [31], while Agbaje et al. showed increased nuclear and mitochondrial DNA damage in the spermatozoa of diabetic men [32]. DM and/ or disease related male infertility is not the only factor involved in sperm function decline. In their very detailed systematic review, Levine et al. [33], reported a significant (50-60%) decrease in sperm count across different populations including North America, South America, Europe, Australasia, Asia and Africa over a period of 4.6 decades [33].

Unfortunately, in many cases, the cause of infertility is not identified and hence considered unexplained [34]. This suggests that there are gaps in the mechanistic knowledge of sperm production and function. Especially, pathways involved in the physiochemical/protein regulation of spermatogenesis. It is therefore appropriate to consider areas of research highlighting, protein modification and identification.

Hence, this review aimed to evaluate the existing literature on the impacts of protein modification, especially the AGEs on male reproduction and to briefly highlight their role in certain pathologies.

2B.2 Overview/ formation of Advanced Glycation End Products

AGEs are products of non-enzymatic glycosylation of endogenous and exogenous proteins. Various routes of formation have been identified (Figure 2B.1). Firstly, AGEs can be formed during a conventional Millard reaction through glycoxidation. Briefly, the electrophilic carbonyl group of reducing sugars react with the free amino acids [35] to form unstable Schiff bases. The Schiff bases undergo a reversible rearrangement, thereby producing to a greater extent stable Amadori products. Amadori products are glycated proteins. Hence, when glycated proteins undergo oxidative cleavage (glycoxidation), they give rise to glycated protein products. While the Schiff bases are undergoing rearrangement, some highly reactive molecules called the α-dicarbonyl compounds (α-oxoaldehyde), including, glyoxal (GO), methylglyoxal (MGO) and 3-deoxyglucosone (3-DG) are formed [36,37]. These compounds are regarded as AGE precursors as they can further undergo auto-oxidative, oxidative or non-oxidative degradation to form AGEs. Hence, α-dicarbonyls are important in the formation of AGEs. Briefly, GO is formed from the oxidative cleavage of glucose [38]. Or, from the breakdown of glycated proteins, monosaccharide autoxidation [39], and lipid peroxidation [40]. While MGO is formed by both enzymatic and non-enzymatic degradation of triose phosphates, the breakdown of acetone, and the catabolism of threonine [41]. 3-DG, on the other hand, is formed by the degradation of glycated proteins [42] and from the fragmentation of fructose 3-phosphate [43].

Secondly, AGEs can be indirectly formed from the autoxidation of monosaccharides. Glucose can be oxidized under physiological conditions in the presence of transition metals, which reduces molecular oxygen to form α-dicarbonyls (GO specifically), hydrogen peroxide (H_2O_2) and free radical intermediates. The α-dicarbonyls formed binds to the lysine groups of amino acids to form AGEs [38]. Thirdly, AGEs can also be formed from lipid peroxidation. The cell plasma membrane contains polyunsaturated fatty acids (PUFA), which are vulnerable to free radicals. Attack of PUFA by ROS results in the formation of lipid and peroxyl radicals. Due to the highly reactive nature of the peroxyl molecules, lipids further undergo oxidation, thereby forming lipid hydroperoxides (4-hydroxy-trans-2-nonenal, acrolein), α-oxoaldehydes, such as GO, MGO, and 3-deoxyglucusone and H_2O_2 [44,45]. The α-oxoaldehydes formed can further undergo auto-oxidative, oxidative or non-oxidative degradation to form AGEs [46]. The role of α-dicarbonyls in the direct and indirect formation of AGEs is thus important.

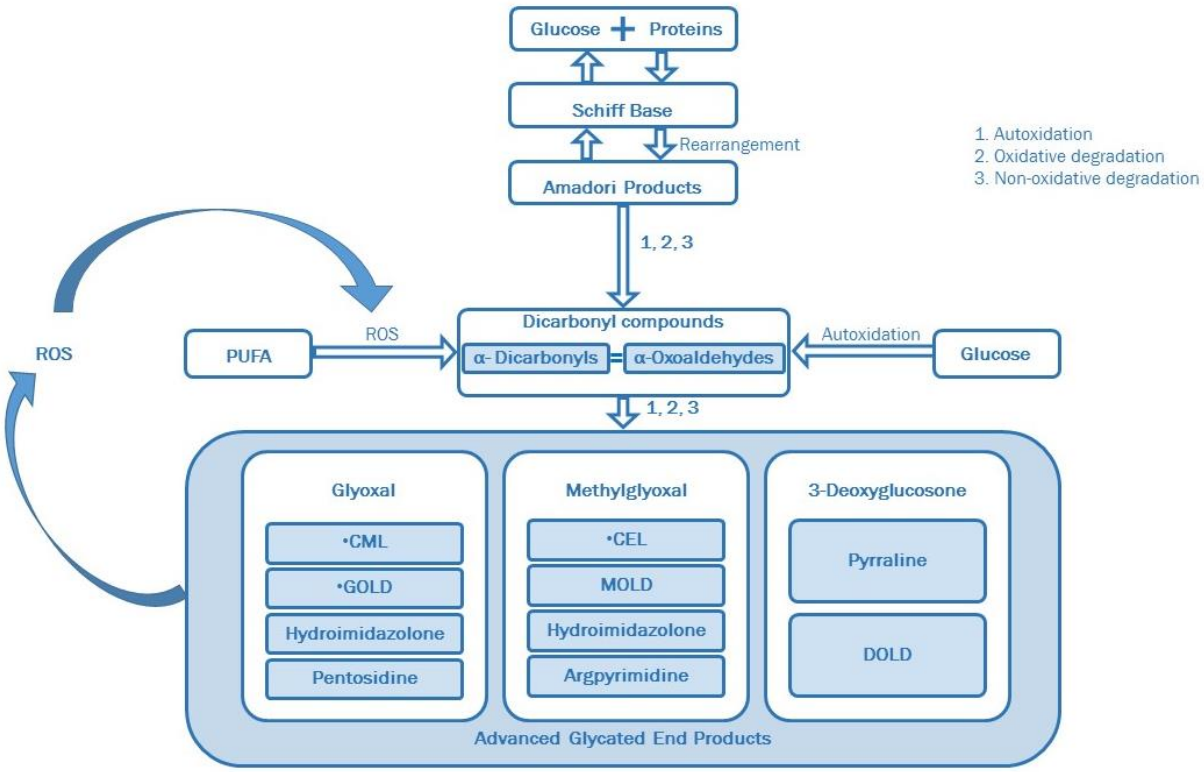

Figure 1: Identified route of AGEs formation.

CML=N-carboxymethyllysine, CEL=N-carboxyethyllysine, GOLD=glyoxal lysine dimer, MOLD=methylglyoxal lysine dimer, DOLD=deoxyglucosone-lysine dimer, ROS=reactive oxygen species. **1**= Autoxidation, **2**= Oxidative degradation, **3**= Non-oxidative degradation.

2B.2.1 Classification of Advanced Glycation End Products

The categorization of AGEs is partly based on examining the level of protein modification, aggregation state of glycated proteins and the fructosamine content. These characteristics are measured by fluorescence and colorimetric. For instance, modifications on arginine and lysine side chains result in protein cross-link [47]. Cross-linkers are molecules with two or more reactive ends, capable of chemically attaching to specific functional groups (amines, sulfhydryls) on proteins or other particles. Therefore, depending on the presenting features of AGEs, they are classified into three groups: (i) Non cross-linking AGEs (CML, N-carboxyethyllysine (CEL) and pyrraline), (ii) Non-fluorescent cross-linking AGEs such as imidazolium derivatives, (glyoxal lysine dimer (GOLD), methylglyoxal lysine dimer (MOLD), alkyl-formyl-glycosyl pyrrole and argininelysine imidazole), and (iii) Fluorescent cross-linking AGEs (crossline and pentosidine) [35].

CML is a heterogeneous non-fluorescent protein compound formed by the oxidative glycation of its precursor GO. Since its discovery, it has been the most studied biomarker of AGEs, including food sources. Various studies have elucidated the accumulation of CML in different systems, both in health and disease [48–55]. GO, and free CML have been reported to be involved in serotonin release. Serotonin is known for its role in wellbeing and happiness. However, it also regulates feeding and satiety when present in small quantities [56]. Imidazolium derivative cross-links (MOLD and GLOD) are formed by reactions between MGO and GO derivatives and lysine residues. Hiroko et al. reported an increase in the levels of MOLD and GOLD in the sera of non-diabetic uremic patients. This suggests that, even in the absence of hyperglycaemia, an increase in the production of dicarbonyls caused by OS can in-part result in tissue damage seen in uremia [57]. Crossline is one of the primary AGEs

produced from the reaction between ϵ-one in lysine and D-glucose in vitro. It has been identified to be the first protein cross-link, having fluorescent features similar to the in vivo products. Crossline levels have further been reported to increase with age in the lenses of diabetic rats [58].

2B.2.2 Sources of Advanced Glycation End Products

AGEs have both endogenous and exogenous sources; endogenous as in biological systems (skin collagen, neonate urine, human lens, etc.) and exogenous as in food. AGEs are present in food such as meat, fish, cereal-derived products, coffee, soups, sauces, dairy products, cooked fruit and vegetables [59–61]. An increase in the production of AGEs has been documented in thermally treated products, especially after cooking at high temperatures (frying, roasting, and toasting). Hull et al. reported a higher concentration of CML in heat-cooked foods compared to non-to low thermally treated foods [62]. Uribari et al. reported the moderate formation of AGEs after cooking at low temperatures (boiling, steaming and stewing) [63]. This increase in AGE formation during food processing may lead to a decrease in nutritional value and can further generate toxic compounds [63].

2B.2.3 Elimination/ Excretion of Advanced Glycation End Products

AGEs are part of human life from birth to death. Hence, it is essential that there is a coping strategy by which biological systems regulate their formation and accumulation. AGEs can be removed from tissues through extracellular proteolysis, degradative glyoxalase enzymes and tissue macrophages (lysosomes and proteasomes). Lysosomal degradation is a process whereby macromolecules are internalized from the extracellular space through endocytosis. The endosomes then fuse with phagosomes, which cumulatively result in complete

degradation of the macromolecules. Proteasomes, on the other hand, are important in regulating the degradation of proteins [44]. In brief, AGEs are engulfed by the cell-surface receptor, mediated by endocytosis. They are then broken down intracellularly to release second-generation AGEs [49]. These second generation AGEs are subsequently filtered and excreted by the kidney.

However, an imbalance between the rate of formation and excretion of AGEs may lead to accumulation. These raised concentrations are implicated in the pathology of several diseases as multiple pathways have been identified through which AGEs can affect biological systems.

2B.2.4 Mechanism of Action of AGEs

AGEs are often the root cause of various pathologies and can affect tissues and organs through several mechanistic pathways. Firstly, as they change protein structures, it leads to a functional modification. Secondly, because of the molecular protein cross-linking that occurs during AGE formation, it affects the tissues. Thirdly, it can result in increased free radical formation due to the accumulation of damaged proteins and the subsequent imbalance in molecular homeostasis. Fourthly, AGE accumulation can acutely activate signalling pathways due to heightened binding to their receptors, that is, the receptor for advanced glycated end products (RAGE), or even other cell membrane receptors. Several other AGE receptors have also been identified, but with RAGE being the most well-known and widely studied. These other receptors include AGE-R1/oligosaccharyl transferase-48 (OST-48), AGE-R2/80K-H, AGE-R3/galectin-3 and macrophage scavenger receptors (SR-A, SR-B: CD36, SR-BI, SR-E: LOX-1, FEEL-1; FEEL-2) [64–68].

RAGE is a multi-ligand receptor that is composed of three extracellular domains, one V and two C-type areas. The V-type has ligand binding properties, and the two C-types are immunoglobulin domains [69]. In addition to the activation of RAGE by AGE, RAGE can also be stimulated by the expression of other ligands including amphoterins, S100-calgranulins, amyloid fibrils, β-amyloid, β-integrin MAC-1 and HMGB-1 [70]. Furthermore, the binding of AGE to RAGE has been suggested to trigger the activation of mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (ERK 1/2), phosphatidyl-inositol 3 kinase (PI3K), p21Ras, stress-activated protein kinase/c-Jun-N-terminal kinase and nuclear factor kappa B (NF-κB) pathways. Activation of these pathways ultimately lead to elevated ROS production, and the upregulation of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and monocyte chemoattractant protein-1 (MCP-1)[71]. Increased expression of RAGE has been correlated to elevated accumulation of AGEs, and subsequent enhancement of tissue damage as well as the development and progression of diseases. The spectrum of diseases include DM and DM related pathologies (nephropathy, retinopathy, and neuropathy), neurodegenerative diseases (Parkinson's disease, Alzheimer's disease), arthritis, periodontitis, ovarian ageing and male infertility. Interestingly, AGE-RAGE activation is not only involved in the aetiology of diseases, but it also participates in some physiological processes including embryogenesis, ageing, lung homeostasis, bone metabolism, neuronal systems and the immune system.

2B.3 Physiological role of AGE-RAGE

Christiane et al. reported the expression of RAGE-mRNA on day 4 in the early blastocyst in the rabbit embryo [72]. Although its role is still largely unknown, RAGE was found in the

embryoblast during blastocyst formation. Another group of authors discovered RAGE-mRNA in embryonic cortical neurons in rats, and it was shown to be associated with neurite outgrowth [73]. RAGE expression during embryogenesis has been largely linked with fetal lung development in rats, as it helps in the proliferation of type 1 alveolar epithelial cells. Reynolds et al. reported a systematic increase in the expression of RAGE from embryonic day 13.5 to birth (18.5) in type 1 alveolar epithelial cells in mouse lungs [74]. Interestingly, RAGE expression was down regulated in the malignant tumour of the lungs, which further validate its physiological role in the development of fetal lungs.

In bovine tissues, RAGE was expressed in the pulmonary endothelium, alveolar macrophages, leiomyocytes, visceral pleural surface, bronchial and vascular smooth muscle [75]. In RAGE knockout mice, Al-Robaiy et al. reported upregulation of dynamic lung compliance and a reduction in maximum expiratory air flow, which subsequently affected cell coupling [76]. Additionally, the role of RAGE in the enhancement of cell to matrix adhesion has been shown, as inhibition of RAGE decreases alveolar epithelial cells adhesion to the basement membrane [77].

In RAGE knockout mice, bone biomechanical strength and mineral compactness were increased, which led to a reduction in the number of osteoclasts formed. It was suggested that the lack of RAGE deteriorate osteoclast function as a gene involved in osteoblast differentiation was downregulated [78–80].

RAGE expression has been identified on various immune cells, including neutrophils, monocytes/macrophages, lymphocytes and dendritic cells [75]. Kislinger et al. reported a reduced inflammatory response subsequent to RAGE blockade [81]. Brisslert et al. showed elevated leucocyte counts in the peritoneal cavity of naïve mice treated with soluble RAGE

(sRAGE). Also observed was the increase in the number of $CD19^+$ B cells in the spleen and a reduced rate of $CD19^+$ B cells in the bone marrow. Splenocytes showed higher IgG production with elevated levels of interleukin-6, interleukin-10, and interferon-γ in response to lipopolysaccharide stimulation. It was concluded that since the administration of sRAGE in healthy mice led to rearrangements in cellular composition in the bone marrow and spleen, this indicates that RAGE may play a role in adaptive immunity [82].

2B.4. Pathophysiological role of AGE-RAGE

Increased expression of RAGE has been correlated to elevated accumulation of AGEs, and subsequent enhancement of tissue damage as well as the development and progression of diseases. The spectrum of diseases Include DM and DM related pathologies (nephropathy, retinopathy, and neuropathy), neurodegenerative diseases (Parkinson's disease, Alzheimer's disease), arthritis, periodontitis, ovarian ageing and male infertility.

2B.4.1 AGEs and Diabetes Mellitus

Studies have shown the importance of AGEs in the pathogenesis of diabetic complications [83]. In DM, AGEs are formed more rapidly, which can be up to a 14-fold increase [84].

Intracellular reducing sugars and their derivatives have been shown to participate in glycation at a much faster rate than glucose [84]. The detrimental effect of AGEs arises from the modification of protein structures and function and the cross-linking of modified proteins.

In DM, increased concentrations of reducing sugars lead to elevation of downstream intracellular accumulation of GO, MGO, 3-GD [85,86] and subsequently, AGEs formation.

Increase in AGEs leads to elevated RAGE with subsequent ROS production. In brief, AGE-RAGE interaction stimulates NADPH-oxidase activity, leading to activation of Nf-κB and ultimately result in increased expression of iNOS and $ONOO^-$ [87]. AGEs have been implicated in several DM-related complications, including diabetic retinopathy, diabetic atherosclerosis, diabetic cataract, diabetic nephropathy, diabetic embryopathy, and diabetic neuropathy. Please refer to the manuscripts of Ahmed [35] and Yamagishi [49] for a comprehensive review on these complications.

2B.4.2 AGEs and Male Infertility

The hypothesis that AGEs is one of the underlying causative factors of infertility has provided a new facet to researching this pathology on different levels, including its role in disease induced male reproductive function impairment. As previously explained, the formation of AGEs depends on the quantity of proteins available for glycoxidation, the level of oxidants, and primarily, the presence of reducing sugars which is increase in hyperglycaemia. Hence the rate of AGE production increases with long term hyperglycaemia. Based on this rationale, the majority of studies that investigated AGEs, explored whether its formation is increased in male infertility and if it is, where is it located. Very few studies have highlighted its mechanism *per se*, hence, the pathway by which AGEs impact male fertility is still less understood.

However, to achieve the key aim of this review, a comprehensive literature search was performed on the two foremost research databases, that is, Scopus and PubMed. The main keywords “advanced glycation end products” was used in combination with “sperm”, “semen”, “fertility” and “male infertility” and the initial search produced 18, 12, 9, and 13 studies respectively for a total of 52 manuscripts. However, after excluding duplicate articles, review

articles and those not related to the topic, while further limiting the studies to the English language and date of publication (1 January 1986 and 31 May 2019), a total of only 13 studies remained and thus form the basis of the remainder of this review (Table 1).

Table 1: Studies illustrating the effects of AGEs on male fertility

Ref no	Authors	Year	Model	Main findings
94	Bagheri et al.	2016	Infertile men with Varicocele	↑S100A12 (RAGE ligand) in semen of infertile men
				↓sperm motility
87	Chen et al.	2016	Leydig cells treated with AGEs (in vitro)	AGEs induce ROS production, apoptosis and activates HIF-1α and HO-1
99	Chen et al.	2016	Diabetic rats	Impairment of male fertility through activation AGE-RAGE/p38MAPK pathway
89	Chen et al.	2019	Rodents (Mice and Rat Models)	AGEs diet induced histopathological damage of the testes and epididymides
				↓normal sperm morphology
				↓epididymal reserve
				↑RAGE
				↑MDA levels
93	Charalampidou et al.	2017	Normo-oligo-zoospermic men	↓soluble RAGE in seminal plasma
49	Karimi et al.	2010	Diabetic men	↑AGEs in seminal plasma
				↑lipid peroxidation
86	Karimi et al.	2012	Diabetic men	↑spermatoza RAGE expression

				↑sperm nDNA fragmentation
29	Mallidis et al.	2007	Diabetic men	↑in spermatozoa displaying RAGE ↑ RAGE in seminal plasma
50	Mallidis et al.	2010	Diabetic men	AGE accumulates in the reproductive tract of diabetic men
100	Neil et al.	2010	Diabetic mouse	↑formation of CML ↑sperm DNA fragmentation
46	Nevin et al.	2018	Spermatozoa + GO Sperm + MGO (in vitro)	↑DNA damage
98	Oborna et al.	2011	Normozoospermic male partners from Infertile relationship	AGE-RAGE negatively impact male fertility through oxidative stress (OS)
90	Tóthová et al.	2013	OS- induced Rats	↑AGEs ↑MDA levels

Table 1: Studies illustrating the impacting effects of AGEs on male fertility. AGEs=advanced glycation end products, MDA=malondialdehyde, CML=N=carboxylmethyllysine MGO=methylgloxal, GO=glyoxal, RAGE=receptor for advanced glycation end products, nDNA=nuclear deoxyribonucleic acid, MAPK=mitogen activated protein kinase, HIF-1α= hypoxia-inducible factor -1α, HO-1= Heme oxygenase-1, ROS=reactive oxygen species.

Mallidis et al. reported the presence of CML in the epithelium of seminiferous tubule, nuclei of spermatogonia and spermatocytes. CML was also found in the caput epididymis, cytoplasmic droplets, sperm acrosomal cap, mid-piece and tail. The quantity of this AGE was significantly increased in the spermatozoa of diabetic men [52]. Bearing in mind the identified damaging effects of AGEs in other systems, the presence, localization, and quantity of CML suggest that they may play a role in male infertility, especially, DM related male infertility. This group of authors also reported in a different manuscript, elevated seminal plasma concentrations of RAGE and an increase in the number of spermatozoa displaying RAGE in diabetic men. RAGE was also expressed throughout the testis and caput epididymis of both diabetic and non-diabetic men but was higher in diabetic men [31]. Karimi et al. likewise reported an increase in the expression of RAGE in the spermatozoa of diabetic men and went on to show a positive correlation between RAGE levels and nDNA fragmentation [88].In a different manuscript, Karimi et al. also reported elevated total AGEs in the seminal plasma of diabetic men with spermatozoa showing increased lipid peroxidation [51]. It is known that men with DM display increased OS parameters accompanied by elevated sperm nDNA fragmentation [32]. Considering the interplay between AGE formation, RAGE expression and ROS production it is plausible that an increase in the accumulation of AGEs and RAGE can cause an upsurge in ROS which can lead to lipid peroxidation and eventually sperm damage.

Additionally, after treating murine Leydig TM3 cells with AGEs for 48 hours, Ying et al. reported increased ROS production, apoptosis induction and activation of HIF-1α and HO-1 [89]. HIF-1α is a protein required for apoptosis regulation in physiological systems. The testis is a relatively hypoxic organ [90], hence, HIF-1α is required for the control of primary transcriptional responses to hypoxic stress, plays an adaptive role and also regulate apoptosis. Its alteration, however, may in part lead to male infertility. Therefore, the authors concluded

that weakened expression of HIF-1α exacerbated OS injury by AGEs [89]. This suggests that HIF-1α protein regulate the apoptotic effect of AGEs. However, when proteins are altered, testicular damage and ultimately male infertility ensue.

In a recent study conducted by Min et al. (2019), histopathological damage was observed in the testes and epididymides of mice fed with an AGE rich diet. This was accompanied by a decrease in normal sperm morphology and epididymal sperm reserve [91]. There was a concomitant increase in the level of RAGE, accompanied by elevated MDA levels. Conclusively, dietary AGE may promote testicular dysfunction through the OS pathway.

Furthermore, Clare et al. reported that GO (CML precursor), induced a significant increase in CML levels in spermatozoa with a resultant increase in DNA damage. It was further reported that AGEs were located in all regions of the sperm, but motility and hyaluronidase activity were not negatively affected [48]. Considering the reported damaging effects of elevated levels of AGE on DNA integrity, without an effect on motility and hyaluronidase activity, suggests that sperm may preserve some fertilizing capacity under adverse conditions or that the in vitro assay did not mimic in vivo processes.

Tothova et al. reported a significant increase in the formation of AGEs after administration of exogenous testosterone to rat testes [92]. In this model, elevated AGE levels were accompanied by increased MDA levels. It was suggested that since AGEs, a biomarker of carbonyl stress, were increased, the signalling of testicular oxidative damage was also increased. This may lead to a decrease in total antioxidant capacity and further impairment of sperm function [93,94].

A report from one of the few studies that investigated the role of soluble RAGE (sRAGE, a RAGE subunit) in non-diabetes related male infertility showed rather contrasting findings in

comparison to what is known in DM. Charalampidou et al. reported increased serum sRAGE in fertile men compared to infertile men [95]. However, it was interesting to see that there was a significant decrease in sRAGE levels in the seminal plasma of both fertile and infertile groups compared to the levels in sera. It was therefore suggested that these reduced levels of sRAGE in seminal plasma may be due to the increased clearance of AGE-sRAGE complexes [95]. However, they suggested that further research is required to enumerate the role and mechanism of sRAGE in male infertility and why it was increased in the sera of fertile men.

Furthermore, Vahid et al. [96] investigated the link between S100A12 (RAGE ligand) and infertility and also examined the amount of S100A12 in the semen of infertile men who suffered from varicocele [96]. S100A12 (EN-RAGE) is a member of the S100 multigene family of calcium-binding proteins involved in Ca^{2+}-dependent synchronization of a variety of intracellular activities, including protection against oxidative cellular damage, protein breakdown and inflammation. They are primarily secreted by activated neutrophils in humans [97]. Increase in the number of WBC in semen have been associated with male reproductive tract infections and/or inflammation. During inflammation, the WBC are activated with subsequent release of an array of products including ROS, inflammatory molecules such as S100 proteins and cytokines [98].

The authors, therefore, argue that the significant increase in S100A12 in the semen of infertile men may be as a result of neutrophil accumulation in the lower part of the genital tract, which may further be correlated with the reduction in sperm motility observed [96].

Additionally, Hakimi et al. [99] reported the increased levels of other pro-inflammatory molecules, including CXCL1 and CXCL9 during upper genital tract infections. This indicates that

increased expression of RAGE in any part of the male reproductive tract can be bad as several ligands such as AGEs and S100A12, can bind to inflict damage.

A randomized controlled trial that investigated the effect of lycopene (an antioxidant) on sRAGE levels in the blood and seminal plasma of normozoospermic male partners of infertile couples showed that lycopene reduced sRAGE in seminal plasma. The decrease in the seminal sRAGE levels may be a result of lycopene suppression of oxidative stressors [100]. The improvement in fertility reported by this group of authors following antioxidant administration can serve as further evidence that AGE-RAGE negatively impacts male fertility through OS.

Additionally, Yuping et al. reported the suppression of the AGE-RAGE /p38 MAPK pathway in streptozotocin (STZ) induced DM after iridoid glycoside from *C. officinalis* (IGCO) treatment, a traditional Chinese medicine. It was shown that the IGCO reduced ROS and MDA levels, restored SOD and CAT activities, and decreased spermatogenic cell apoptosis [101], which cumulatively reduced the formation of AGEs, RAGE and phosphorylated p38 MAPK levels. This suggests that AGE-RAGE can impair male fertility through the p38 MAPK pathway.

Neil et al. [102], in their quest to build on human studies that explored the role of AGEs in male infertility, and to also establish a model of DM that is similar to human findings, investigated the formation and accompanying adverse effects of CML in two different DM animal models (heterozygous $Ins2^{Akita}$ and the STZ induced DM mice). It was shown that both the control and $Ins2^{Akita}$ mice presented with identical immunohistochemical localization of CML in the testis. Though, the degree of the staining was noticeably higher in the $Ins2^{Akita}$ mice. Additionally, a speckled-like structure appearance was observed throughout the cytoplasm of all cells in the seminiferous epithelium as well as in the constituent of the

interstitium. Furthermore, the nuclei of spermatogonia and differentiating spermatogenic cells exhibited little immunoreactivity for CML with the most prominent stain found in the lumen border of the tubules, especially the phase where elongating and mature spermatids were present [102].

The formation of CML in the STZ treated mice was significantly increased when compared to the control and $Ins2^{Akita}$ groups. Additionally, the round spermatids (especially the area related to the developing acrosome) exhibited immunoreactivity for CML. In relation to what was observed in the testis, the efferent ducts and the caput (head) epididymis were immunoreactive for CML. Furthermore, the sperm head of both diabetic groups had prominent immunofluorescent bands for CML and RAGE. This was accompanied by a significant increase in the percentage of sperm with single DNA strand breaks with a more elevated alteration in the $Ins2^{Akita}$ mice [102]. Cumulatively, these results mirror what is observed in human studies, which further support the detrimental effects of AGEs in DM related male infertility.

2B.4.3 AGEs and other Fertility Related Effects

Seeing that about 20% of infertility cases are attributed to both male and female, it is important to make brief mention of the involvement of AGEs and RAGE in female infertility. Studies have shown that from this 20%, ovarian ageing is the most frequent cause for female partners.

During fetal life, the ovaries have approximately 6 million oocytes surrounded by somatic granulosa cells. The primordial follicles are slowly depleted by atresia, such that about 1 million follicles remain at birth. The follicles further decrease to 300,000 at menarche, and

about 1000 follicles are lost each month during reproductive life [103–106]. The decrease in the ovarian follicle pool due to senescence is proportional to the reduction of ovarian function and a decline in the ability to fall pregnant [105].

Mette et al. reported the presence of AGE and RAGE in granulosa-lutein cells as well as ovarian monocytes in cells isolated from aspirates of ovarian follicles from women who underwent assisted reproduction treatment (ART) [107]. It was suggested that the reduction in ovarian function that comes with ageing may be caused by the structural long-lived extracellular matrix AGEs [107].

Additionally, AGEs has been reported to be present in the granulosa, theca and luteinized cells of healthy women. However, in women with polycystic ovarian syndrome (PCOS), AGEs are expressed in the granulosa, theca interna and the ovarian endothelial cells with increased accumulation compared to healthy counterparts [108]. Interestingly, there was a mild RAGE expression in the ovaries of healthy women and this activated the NF-Kβ (p50/65) in the cytoplasm of the theca interna and granulosa cells [108]. However, increased RAGE expression was observed in the ovaries of women with PCOS. This appears to further stimulate the activation of NF-Kβ (p65) in the cytoplasm of the theca interna and granulosa cells. Women with PCOS have been shown to have endothelial dysfunction which have been previously related to insulin resistance [109], androgen levels and inflammation [110,111], but the presence of increased AGEs in the endothelial cells of PCOS ovaries may be a plausible mechanism through which this dysfunction occurs.

Embryogenesis, which refers to the formation and development of human embryo, involves both the male and female gamete. Therefore, it is appropriate to consider the involvement of AGEs in the process of artificial fertilization.

AGE accumulation has been reported to affect embryonal development adversely and to as well endanger the development and achievement of pregnancy by ART.

Jinno et al. [112] investigated the role of AGEs during in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) and their consequence in achieving pregnancy. Toxic AGE (TAGE), pentosidine and CML were measured in both follicular fluid (FF) and serum of ART patients. It was reported that increase in TAGE, pentosidine and CML in FF and TAGE in serum significantly decreases the level of estradiol as well as the number of follicles larger than 12mm in diameter, retrieved oocytes, fertilized oocytes and embryos. Furthermore, it was interesting to note that the correlation between the degree of TAGE, pentosidine, CML accumulation and the resultant negative effect on achieving pregnancy was an independent determinant despite normal fertility parameters (reproductive age and normal day-3 FSH). It was thus concluded that an increase in serum TAGE is an indicator of ovarian dysfunction [112].

Additionally, a study investigated the influence of reproductive age on the accumulation of AGE, sRAGE and VEGF in the plasma and FF of women undergoing ART. The increase was subsequently correlated to pregnancy outcomes. A negative correlation was found between the age of the participant and the number of oocytes retrieved [113]. This suggests that the sRAGE secreted in older women (>35 years) is more involved in binding to AGEs which are increased with age. Studies have reported that the increase in FF concentration of VEGF may decrease the pregnancy rate in ART [114,115].

Furthermore, Lin et al. [116] reported the negative effect of AGEs on embryonic development from the pronuclear to the blastocyst stage. Embryos from flushed fallopian tubes of rats after superovulation and mating were treated with GO (AGEs precursor) and BSA-AGE at different

concentrations over time. It was shown that GO, in a concentration-dependent manner, repressed cleavage of embryos at every stage (2n-cell, 4n-cell, morula and blastocyst). The blastocyst yield was significantly reduced and morphological changes, such as fragmentation, were observed after treatment with GO at higher concentrations. Additionally, BSA-AGEs also suppressed the cleavage of embryos at every stage in a concentration-dependent manner. It was Interesting to note that, N-acetyl-L-cysteine (L-NAC) inhibited both GO and BSA-AGE induced embryonic toxicity. Hence, AGE accumulation may influence the pregnancy rate after IVF and embryo transfer, which ultimately increase infertility [116].

2B.5 Conclusion and Recommendation

In small quantity, AGEs play a physiological role in embryogenesis, ageing, lung homeostasis, bone metabolism, neuronal systems and the immune system. However, when the formation rate is higher than the elimination rate, pathophysiological activities ensue. As illustrated in this manuscript, studies have reported the adverse impacts of these biomolecules on several systems and cells, at least in part. The importance of ROS production, activation of cellular signalling and subsequent gene or protein modification in response to increased AGEs have been highlighted. In the study of male infertility however, the conventional seminal analysis in categorizing fertility is becoming inadequate. It is evident that AGE impact male reproductive function and subsequently, male fertility, through ROS and/or activation of other mechanistic pathways, which at this time is largely unknown.

Seeing that normal male reproductive function depends on: (I) the balanced physiochemical dynamics of proteins involved in the process of spermatogenesis and (ii) the controlled expression and interaction of proteins which are involved in the production of amino acids,

RNA and DNA. It is thus essential to investigate the basic molecular protein interaction, which may provide more information about infertility as a whole.

Understanding the role of proteins involved in fertility will provide clinicians with the fundamental information to translate research findings from the bench to the bedside.

Therefore, we recommend that future studies investigating idiopathic infertility and disease-induced infertility should further elucidate the impacts of protein modification and glycation. Additionally, studies investigating its mechanistic role in infertility are required as this will aid the therapeutic approach.

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Chapter 3

Materials and Methods

3.0 Introduction

Findings from this study are discussed in separate chapters. Included in each chapter is the materials and methods used, as they are formatted according to the requirement of different journals. However, an in-depth overview of the materials and methods employed in the entire study are outlined below.

3.1 Preparation of streptozotocin and the infusions

3.1.1 Streptozotocin (STZ) preparation

STZ (S0130-IG) procured from Sigma was prepared by dissolving it in freshly prepared sodium citrate buffer at a pH 4.5 to make a 30mg/ml stock of STZ. The volume of STZ injected was relative to the bodyweight.

3.1.2 Infusion preparation

Rooibos (fermented, 2%), honeybush (fermented, 4%) and sutherlandia (unfermented 4%) were prepared according to standard protocols. These concentrations are commonly used for tea making purposes. Preparation methods conformed to the experimental established protocols for rooibos (Marnewick *et al.*, 2011), honeybush (Toit and Joubert, 1999; Marnewick *et al.*, 2003), and sutherlandia (Tobwala *et al.*, 2014).

The plants used in preparing the infusions were processed during the same season. The same batch of the individual plant material was used throughout the study. That is, the plant batch that was prepared at the start of experiment was used throughout the treatment period. All infusions (rooibos, honeybush and sutherlandia) were prepared every other day (48 hours). The infusions served as the only drinking fluid for the animal groups receiving the respective infusions.

3.1.2.1 Rooibos

Rooibos infusion was prepared by adding 20g of dried fermented stems and leaves of rooibos (Carmien SA PTY LTD, South Africa) in 1litre of tap boiling water to make a 2% concentration and a dose of 20mg/ml. The mixture was allowed to steep/rest for 30 minutes and then filtered three times using a cheesecloth, number 4 Whatman filter paper and Whatman number 1 filter paper (WhatmanTM, Buckinghamshire, UK) respectively. Filtered infusions were transferred to a dark bottle plastic container and stored at 4°C.

3.1.2.2 Honeybush

Honeybush infusion was prepared by adding 40g of fermented dried stem and leaves of honeybush (Afrinaturals, South Africa) in 1litre of boiled tap water to make a concentration of 4% at dose 40mg/ml. The mixture steeped for 30 minutes and thereafter filtered with cheese cloth and Whatman filter paper (number 4 and 1). The infusion was then transferred to a dark bottle plastic container and stored at 4°C.

3.1.2.3 Sutherlandia

Sutherlandia was prepared by adding 40g of unfermented dried stem and leaves of sutherlandia (Afrinaturals, South Africa) in 1litre of boiled tap water to make an initial

concentration of 4% at dose 40mg/ml. The mixture was also allowed to steep for 30 minutes and then filtered with cheese cloth and Whatman filter paper. The infusion was transferred to a dark bottle plastic container and stored at 4°C. The 4% sutherlandia infusion concentration was further diluted to 0.2% by adding 2.5ml of 4% in 50ml of tap water, making a final dose of 2mg/ml. This is because the initial 4% infusion was bitter and animals refused to drink it.

3.2 Experimental procedures

3.2.1 Ethics and animal care

Ethics approval for this project was obtained from the Stellenbosch University Animal Ethics Committee (Project numbers: SU-ACUD16-00101 and SU-ACUD17-00016). Animals were treated according to the recommendations of the Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council, 2010).

Healthy adult male Wistar rats weighing 240-300g at start of experiment were housed individually in standard ventilated cages and were exposed to a 12 hour light: 12 hour dark cycle at 23°C±2°C in the Animal Housing Unit of the Faculty of Medicine and Health Sciences, Stellenbosch University. Animals had free access to food and water throughout the duration of the study. During the first week of acclimatization, animals were fed with pellets (standard Epol™ rat chow, Table 1) and water only for the control groups. While, the diabetic + infusion groups were fed with pellets and given the individual infusion as per the group. After one week of acclimatization, rats were fed with standard rat chow, water and the different

infusions as appropriate. The only source of fluid intake for the infusion control groups and the diabetic + infusion groups are the infusions (that is, the infusions serve as the drinking water).

Table 1: The nutritional values of the standard rat chow pellets

Contents	AS(g/kg)	-Dm-(g/kg
Crude protein (min)	200	240
Moisture (max)	10	
Crudeoils and fats (min)	50	53
Linoleic acid (min)	12	14
Crude fiber (max)	40	45
Crude ash (max)	70	75
Ca:P ratio	1.1-2.1	1.1-2.1
Calcium (min)	12	14
Phosphorus (min)	7.5	8
Vitamin A (min)	16000 (IU/kg)	16000(IU/kg)
Vitamin D (min)	2000 (IU/kg)	2000 (IU/kg)
Vitamin E (min)	100 (mg/kg)	100 (mg/kg)

3.2.2 Diabetes induction

From the STZ solution prepared as described in section 3.1.1, animals were immediately injected intra-peritoneally and administered doses at (30mg/kg, 45mg/kg and 60mg/kg body weights). Animals were feed fasted overnight before injecting with STZ. The successful induction of diabetes was confirmed after one week, with animals showing a justifying blood glucose level of ≥14mmol/L, using a Glucoplus™ glucometer (Ayeleso, Brooks and Oguntibeju, 2014; Dludla *et al.*, 2014).

3.2.3 Treatments

Animals were treated with tap water, rooibos, honeybush or sutherlandia as appropriate (Table 2). They had free access to feed and fluid. However, before performing test such as glucose tolerance, animals were feed fasted overnight (16 hours). The body weights, food and fluid intake of the animals were measured three times weekly. The diabetic state was confirmed throughout the 8 weeks treatment period as glucose levels were measured once a week. Intra-peritoneal glucose tolerance test was performed between 48-72 hours before sacrifice.

3.2.4 Study design

The study was designed relative to the aims, hence, study design is in two parts (Figure 1). First part, thirty animals were randomly divided into three groups of vehicle and two STZ groups (STZ30 and STZ60). For the second part, ninety animals were divided into nine groups without bias. The groups include control, vehicle, rooibos, honeybush, sutherlandia, diabetic control (STZ45mg/kg), diabetic + rooibos, diabetic + honeybush, and diabetic +sutherlandia (Table 1). Since one of the objectives for aim 2 (second part) includes determining the effect of the infusions on male reproductive function, hence the control (tap water only) was included. To investigate these infusions in both health and diabetic states, the second part was structured as summarized in figure 2.

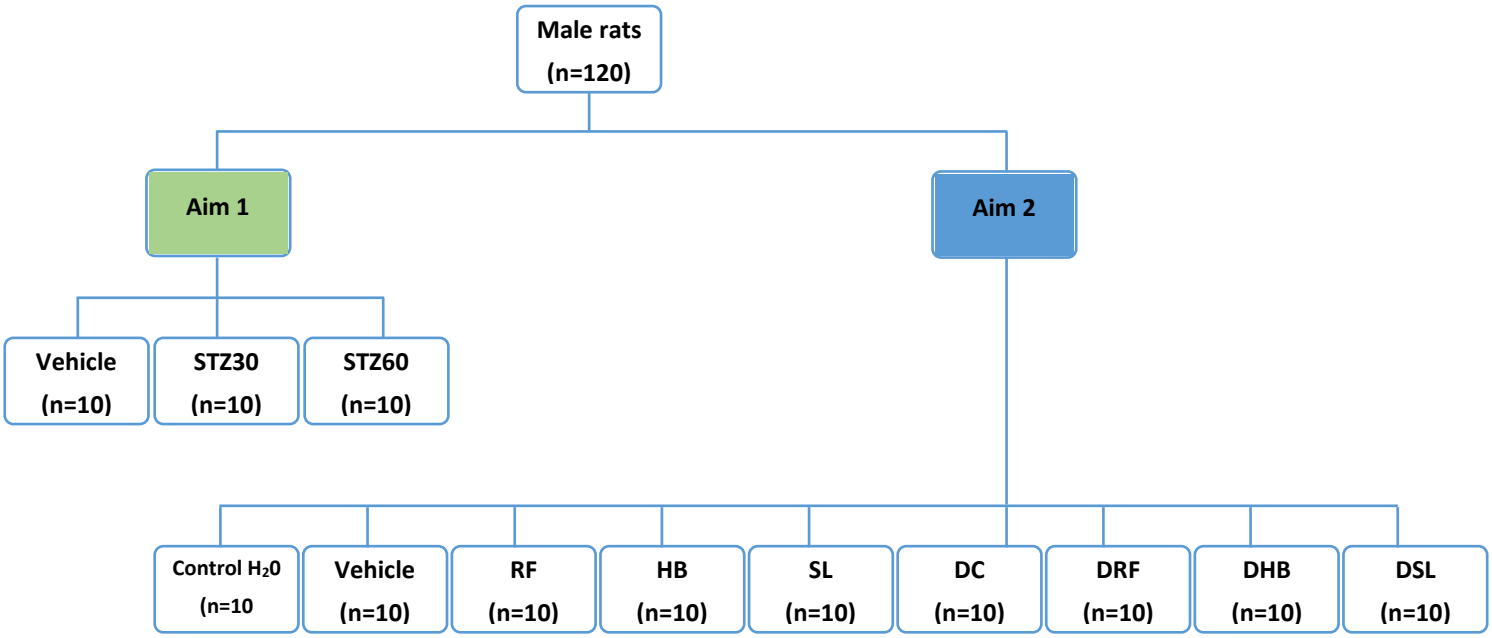

Figure 1: Schematic diagram of the overall study design. Green box=Aim 1 or Part 1 of the study while the Blue box=Aim 2 or Part 2 of the study.

Table 2: Overall abbreviations and treatment groups.

Groups abbreviation	Group Full Names	Intervention	Treatment
Veh	Vehicle	Citrate buffer	Tap water
STZ30	Streptozotocin 30mg/kg	STZ 30mg/kg	Tap water
STZ60	Streptozotocin 60mg/kg	STZ 60mg/kg	Tap water
Control H_20	Control	-	Tap water
Veh	Vehicle	Citrate buffer	Tap water
RF	Rooibos	-	2% Rooibos
HB	Honeybush	-	4% Honeybush
SL	Sutherlandia	-	0.2% Sutherlandia
DC	Diabetic control	STZ 45mg/kg	Tap water
DRF	Diabetic+rooibos	STZ 45mg/kg	Rooibos
DHB	Diabetic+honeybush	STZ 45mg/kg	Honeybush
DSL	Diabetic+sutherlandia	STZ 45mg/kg	Sutherlandia

Green box=Aim 1 or Part 1 of the study while the Blue box=Aim 2 or Part 2 of the study.

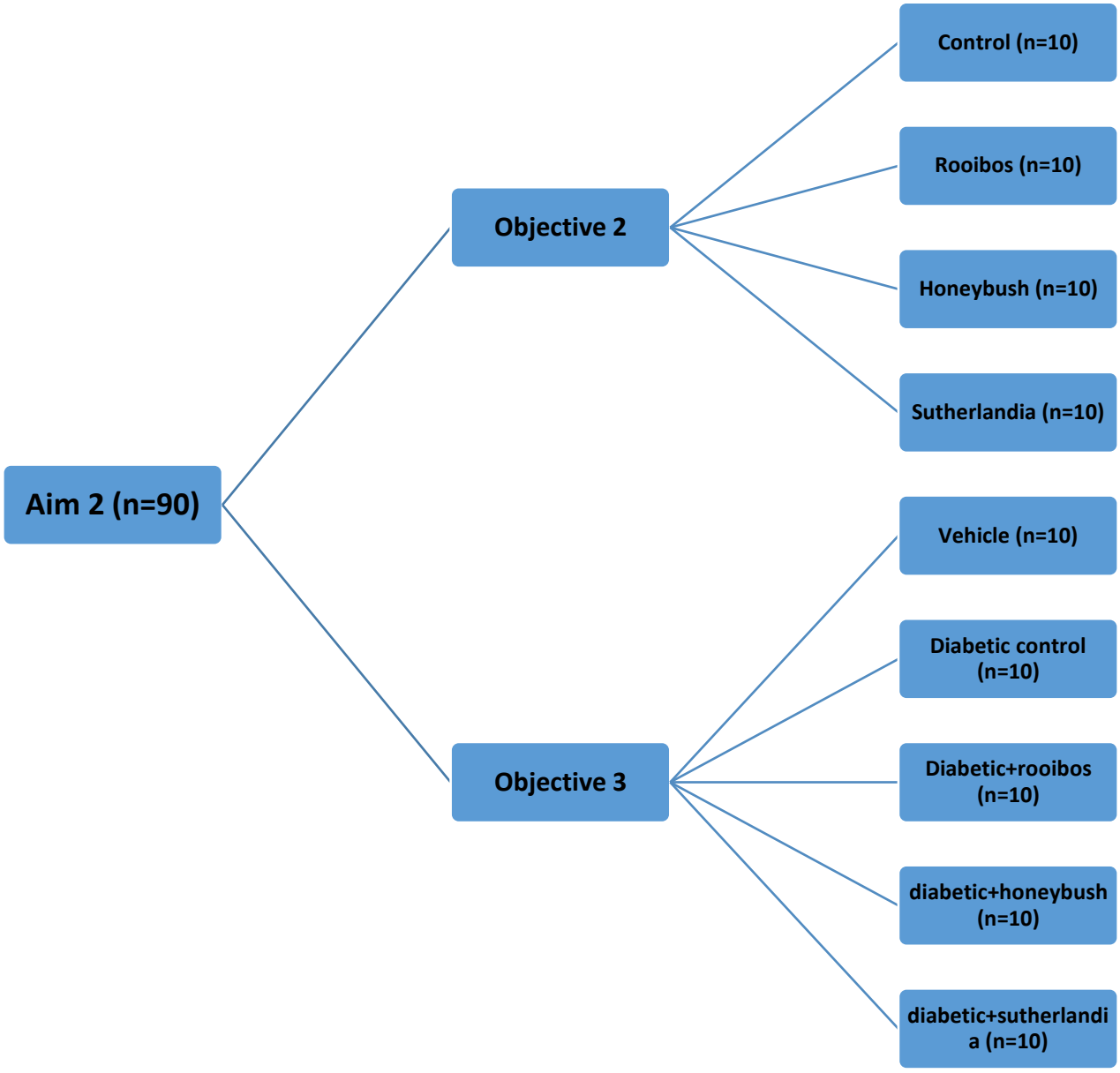

Figure 2: Summarizing the study design for aim 2

3.2.5 Intra-Peritoneal Glucose Tolerance Test (IPGTT)

Animals were feed fasted overnight, a 20% glucose solution was prepared, and a dose of 2g/kg body weight (10µl/g) was administered intra-peritoneally. Glucose levels were measured at 12 different time points (0, 3, 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 minutes) after administering glucose solution.

3.2.6 Sacrifice and sample collection

After 8 weeks of treatment, animals were sacrificed by administering an overdose of sodium pentobarbital (160mg/kg body weight). Blood was collected and allowed to clot on ice for 15-30 minutes, after which it will be centrifuged at 3000rpm for 30 minutes at 4°C and serum was collected. Serum aliquots were stored at -80°C for further analyses. The testes, epididymides and visceral fats were harvested, rinsed and weighed.

3.3 Experimental analysis

3.3.1 Sperm parameters

3.3.1.1 Sperm retrieval

The harvested left epididymis was defatted and placed in a Petri dish that contains a 2ml solution of Hams F-12 nutrient media (Sigma Chemicals, St Louis, MO, USA), at 37°C. After rinsing, the cauda area of the epididymis was dissected into a separate dish containing 2ml Hams and spermatozoa were allowed to swim out. After 30 seconds of sperm retrieval, sperm motility was analysed. Sperm solution for concentration, morphology and viability analysis were obtained by dissecting the caudal area into smaller pieces after motility was analysed and left for further 5 minutes allowing a maximum number of spermatozoa swim out.

3.3.1.2 Sperm motility

After 30 seconds of retrieval, 2µl of the sperm solution from the edge of sperm cloud was infused into a 20µl depth chamber slide (Leja, Netherlands) and placed on a Nikon Eclipse E200 microscope with an in-built heating stage (37°C). Sperm motility was measured via computer-aided sperm analysis (CASA) using the Sperm Class Analyser (SCA 6.3, Microptic, Barcelona, Spain). The sperm kinematic parameters (Figure 3) (curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straight-line index (STR), linearity index (LIN), sperm oscillation index (WOB), amplitude lateral head (ALH) and beat frequency (BCF), total motility and progressive motility were analysed. Motility was analysed at 4x (p^-phBM) phase contrast 1, no filter and CASA settings, 50 frames/sec, minimum area (μm^2) =300, maximum area (μm^2) =1600, drifting (µm/s) =20, static (µm/s) =<50, slow-medium (µm/s) =80, rapid (µm/s) =>120, progressive STR=>70, VAP points (pixels) =9, grid distance (µm/s) =100, connectivity (pixels) =80, VCL/VAP =VCL.

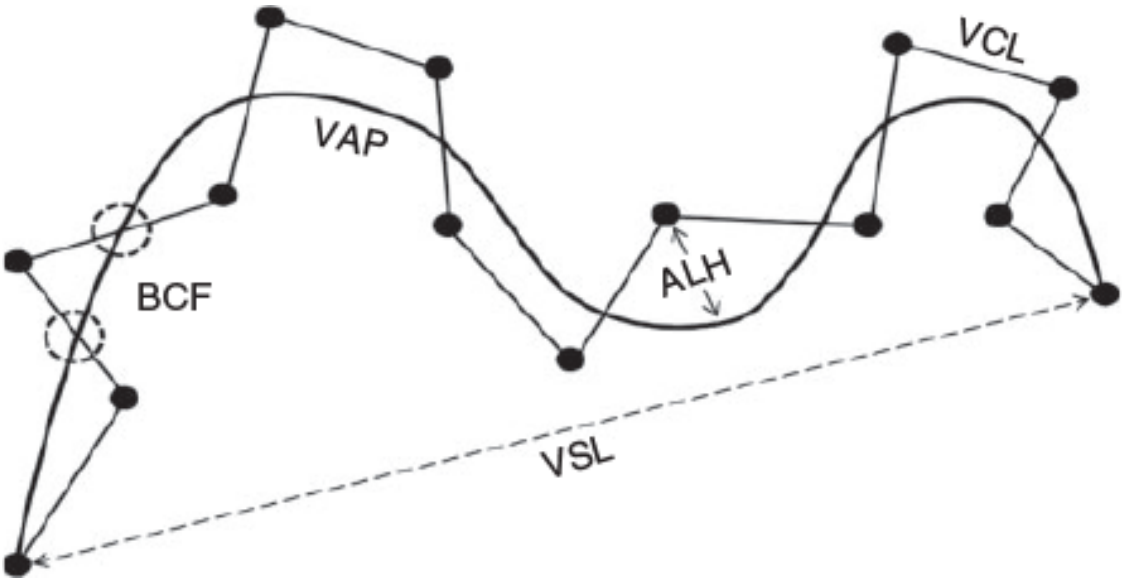

Figure 3: Sperm kinematic parameters (spermcell.net/2018/01/19/casa-terminology).

3.3.1.3 Sperm concentration

After analysing sperm motility at 30 sec, sperm concentration was measured by dissecting the caudal epididymis into smaller pieces and left for further 5 minutes for maximum spermatozoa swim out. The pieces were removed after 5 minutes and the sperm solution was mixed homogenously. Of the 2ml solution, 10µl was diluted in 50µl Hams. From this solution, 2µl was infused into a chamber slide and analysed via CASA.

3.3.1.4 Morphology

Morphology was carried out in accordance to the protocol of the SURRG laboratory. Two staining protocols were employed as the manufacturer upgraded the stains to be time effective. From the sperm solution retrieved after 5 minutes, 10µl was smeared on a glass slide and left to dry at room temperature. First protocol, air-dried slides were fixed for 20 minutes in a staining tray containing Sperm Blue® fixative (Microptic SL, Barcelona, Spain). Fixed slides were then stained for 15-17 minutes with Sperm Blue Stain. Second protocol, the new Sperm Blue is made up of both the fixative and stain. Hence, air-dried slides were fixed and stained in Sperm Blue for 3 minutes. Stained slides were gently submerged in water for 3 seconds to eliminate excessive stains and then allowed to air-dry. Dried slides were mounted using a mounting medium (DPX) and cover slip. Sperm morphometry was analysed through computer-aided sperm morphometry analysis (CASMA) via Sperm Class Analyser (SCA®; Microptic, Barcelona, Spain) and a Nikon E-200 microscope with bright field optics at x60 magnification under a blue filter. The SCA software automatically analyse sperm morphometric parameters including, head-arc, width, chord, ecetera. These parameters are measured to determine whether sperm are morphologically normal or abnormal (Figure 4).

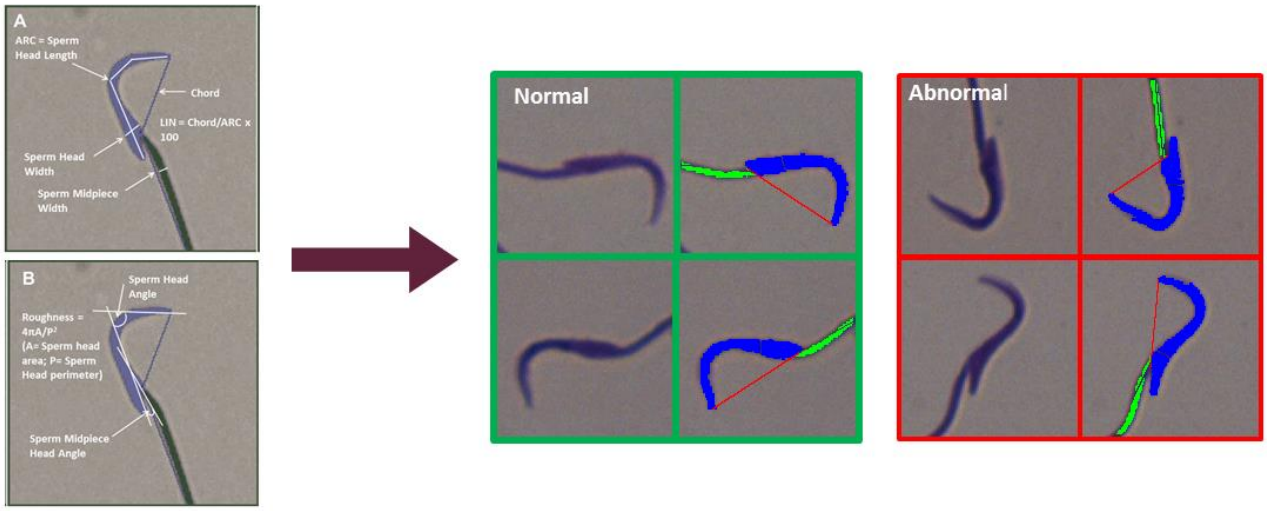

Figure 4: Sperm morphology as measured by CASA

3.3.2 Histology

The testis and the epididymis were fixed in 10% formalin solution. The tissues were kept in the solution for a minimum of 48hours to allow for complete fixation. The fixated tissues were dehydrated with a series of alcohols, cleared with xylene and then infiltrated with paraffin wax. Tissues were processed using automated processor (Duplex Processor, Shandon Elliot).

Processed tissue pieces were embedded in paraffin wax at 60°C using the Leica EG1160 embedder. Tissue blocks were kept at 20-25°c until sectioning takes place. Tissue blocks were trimmed and sectioned using a Leica RM 2125 microtome, to obtain a uniform 5µm sections. Sections were placed in a water bath (40-55°C), allowing the tissue to stretch. Tissues were then stained with haematoxylin and eosin (H&E) using an autostainer (Leica Auto stainer XL). Stained tissue slides were mounted in DPX mounting medium and left to dry for 48 hours to allow for proper visual quality while viewing under microscope.

The Zenlite histology analyser software was used to measure the seminiferous tubule diameter, luminal diameter, epithelial height and luminal area (Figure 5).

Additionally, for proper visualization of the tissues towards qualitative analysis, testicular tissues were stained with alcian blue-periodic acid schiff (AB/PAS). In brief, sectioned tissues were deparaffinised and hydrated in distilled water. Sections were stained with alcian blue for 15 minutes, rinsed thoroughly in tap water for 2 minutes and further rinsed in distilled water for another 2 minutes. Sections were oxidized in 1% periodic acid solution for 10 minutes and then rinsed in distilled water. Rinsed sections were placed in Schiff's reagent for 15 minutes and then wash in lukewarm tap water for 7 minutes 30 second. At this point, sections turned dark pink. Sections were counter stained with haematoxylin solution for 30 seconds, wash in tap water for 3 minutes and then dehydrated and cleared with xylene. Sections were mounted with DPX mounting medium and allowed to air dry.

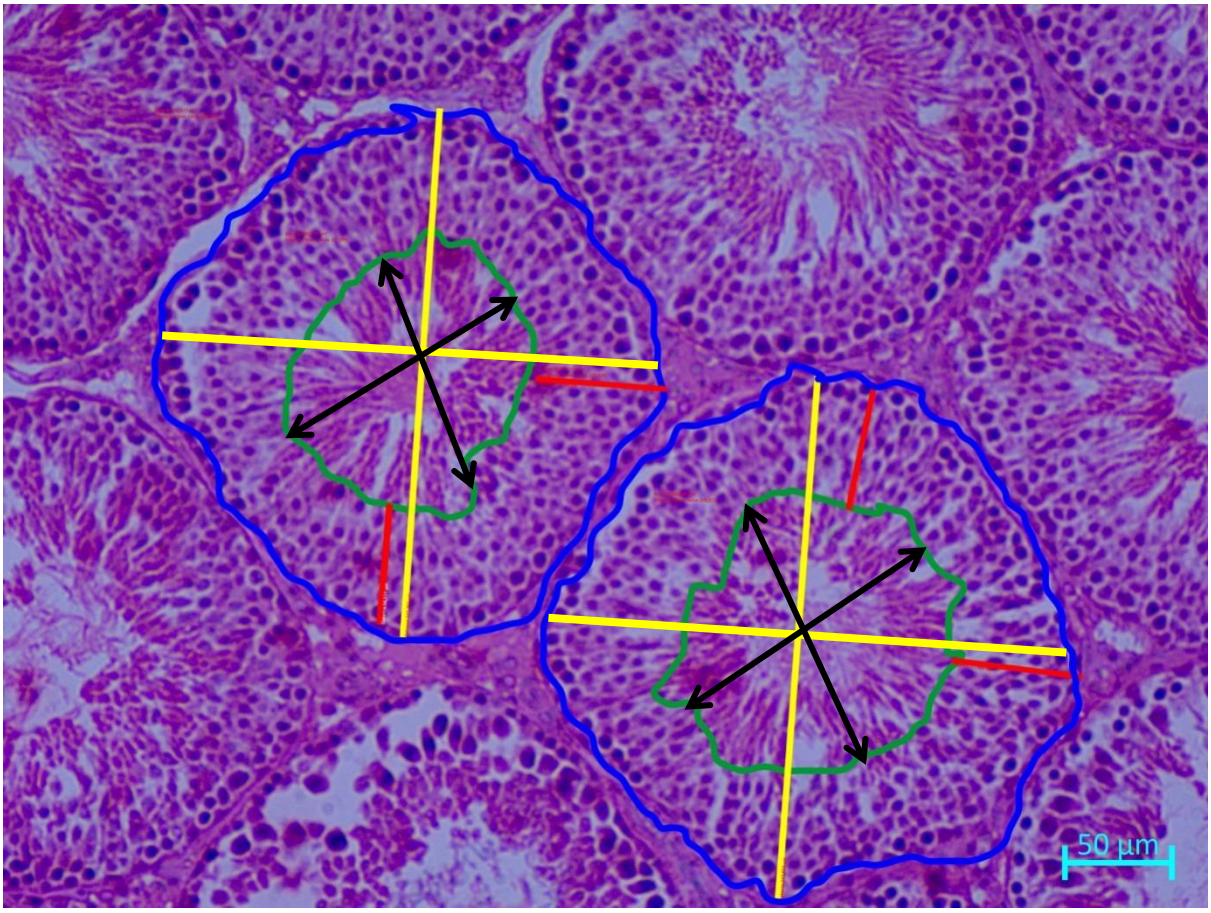

Figure 5: Micrograph showing measurement of seminiferous tubule parameters. Seminiferous tubule diameter (yellow line); tubular area (blue spine contour), epithelial height (red line), luminal diameter (black line) and luminal area (green spine contour).

3.3.3 Protein determination

3.3.3.1 Bicinchoninic Acid (BCA)

BCA assay was used for protein determination for oxidative stress analysis. In brief, Frozen testicular tissue were pulverized and thereafter homogenized in cold lysis buffer (Na3PO4, 0.5% Triton X-100) by bullet blending at speed 9 for 3 minutes with a 1-minute interval in-between. The solution was centrifuged at 15000rpm (25200rcf/xg) for 20 minutes at 4°C. Supernatant were fractioned. Bovine serum Albumin fraction V (BSA) was prepared and was used as standards (µl) (0, 20, 40, 60 and 80). BCA Reagent A and B were prepared at ratio 50:1. Samples were diluted in ionized water (x10). After preparing and vortexing standards and samples, 25µl of each solution was loaded appropriately into microplate vials, 200µl of Reagent AB was added and the microplate was vortexed for 5 seconds. Thereafter, samples were incubated at 37°C for 30 minutes and absorbance was read at 450nm on a plate reader.

3.3.3.2 Bradford protein determination

To determine the quantity of proteins in a sample through the Bradford method 0.05-0.07g of tissue were pulverized and placed into 700µl lysis buffer. Seven stainless homogenization beads (1.6mm, Next Advance Inc., USA) were added into each tube to allow for complete homogenization (Bradford, 1976; Marais *et al.*, 2001). The pulverized tissue was bullet blended at speed 8 for 3 minutes at 5 minutes interval in-between, at 4°C. The homogenates were centrifuged for 20 minutes (15000rpm (25200rcf/xg), 4°C) and the supernatants were removed for further analysis.

3.3.4 Oxidative stress parameters

3.3.4.1 Catalase

Tissue homogenates were obtained as described for BCA. For the assay, tissue homogenates were diluted to 10x in deionized water. From the diluted samples and standards, 5µl were loaded in triplicate into UV microplate wells. Catalase assay buffer (170µl) were added into each well and lastly, 50µL of H_2O_2 was added into the wells and analysis was performed immediately on a plate reader (Multiskan spectrum) at 240nm every 60 seconds over a 5 minute period using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software.

3.3.4.2 Superoxide dismutase (SOD)

Tissue homogenates were obtained as described for BCA. From the diluted standards and samples, 10µl were dispensed into the microplate wells in triplicate, followed by adding 170µl of diethylenetriaminepentaacetic acid (DETAPAC) and 5µl of SOD assay buffer (50mM Na/K Phosphate buffer at PH 7.4). Immediately before reading, SOD activity was activated by adding 15µl of freshly prepared 6-hydroxydopamine (6-OHD) into the wells and instantly analysed on a plate reader (Multiskan spectrum) at 490nm, 25°C for 5minutes at 1 minute interval using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software.

3.3.4.3 TBARS assay

Frozen testicular tissue samples were homogenized in lysis buffer (0.1M KPi, 1.15% KCl) by bullet blending at speed 9 for 3 minutes with a 1-minute interval in-between. To measure the levels of malondialdehyde (MDA) in testicular tissue, 100µl of standards and samples were

pipetted into corresponding 10ml glass tubes, followed by adding 1ml of SDS and 2ml of 10%TCA-BHT buffer solution. Samples were vortexed, and after resting for 10 minutes, 2ml of TBA was added and vortexed again. The standards and samples were covered with marbles and incubated in a water bath (1 hour at 100^{o}C), where after it was cooled on ice for 15 minutes. The standards and samples were centrifuged (3000rpm, 15 minutes 4°C) and the supernatants retrieved. From the supernatants, 250µl of each standard and sample were loaded in triplicate into microplate wells and analysed on a plate reader (Multiskan spectrum) at a 532nm wavelength within 30 minutes after centrifugation.

3.3.5 Western blot

Sample protein concentration was determined using Bradford method as described in section 3.3.3.2. Tissue lysates were prepared by diluting the sample in Laemmli sample buffer and lysis buffer, boiled for 5 minutes and 50ug protein/µl was separated by electrophoresis on a 12% SDS-PAGE mini-proteon gel or on a 4-20% stain-free precast gel (Criterion TGX, Biorad, USA). The running protocol consisted of an initial 10 minutes electrophoresis at 100V and 200mA followed by 40minutes at 200V and 200mA. The running protocol for the precast gel consist of an initial 10 minutes electrophoresis at 100V and 200mA followed by 25-30 minutes at 140V and 140mA. Gels were activated using ChemiDoc (BioRad). Thereafter, the proteins were transferred onto a milipore Immobilon-P transfer membrane (0.45µm) (Immobilon®-P, Merck Millipore Ltd, Germany). Non-specific sites were blocked with 5% fat-free milk in TBS-tween.

The following proteins were probed for, AGE (ab23722; Abcam, SA), RAGE (ab361; Abcam, SA), Caspase 3 (Sigma-Aldrich), Caspase 7 (Abcam, SA), PARP (Cell Signalling Technology),

p38MAPK (Cell Signalling Technology), C-Jun-N-terminal kinase (JNK) (Cell Signalling Technology) and IkBα (Cell Signalling Technology), GLUT4 (Cell Signalling Technology), PKB/Akt (Cell Signalling Technology), XIAP (Abcam, SA), ERK1/2 (Cell Signalling Technology), IRS1/2 (Cell Signalling Technology), p53 (Cell Signalling Technology), pTEN (Cell Signalling Technology), Bad (Cell Signalling Technology). A goat anti- rabbit-horseradish peroxidase-conjugated antibody (Sigma-Aldrich) or goat anti-mouse-horseradish peroxidase-conjugated antibody were used as the secondary antibody appropriately. Proteins were detected and visualized using a ChemiDoc (BioRad, USA) and were further normalized (Stain-free blots as housekeeping proteins) and analysed with ImageLab 5.0 (ImageLab software, USA). All primary antibodies were diluted in TBS-Tween in a 1:1000 ratio while the secondary antibody was diluted in TBS-Tween in a 1:4000 ratio. All data points are from independent biological repeats (n=4-5).

3.3.6 Hormone analysis

3.3.6.1 Testosterone

Testosterone in the plasma was measured using a commercially available ELISA kit (Elabscience Biotechnology, Hubei). All reagents were brought to room temperature (18-25°C) before use. Fifty microliters of each standard and sample were loaded into their corresponding wells, and then another 100µl of HRP-labelled testosterone was added to each sample well. From Detection A and Detection B, 50µl was added and the solution was thoroughly mixed and cover with the plate sealer. The mixture was incubated for 1 hour at 37°C. The solution from each well was decanted and thoroughly washed with a 350µl of washing buffer and left for 1-2 minutes. After 2 minutes, the solution was decanted and pat

dry. This was be repeated x3. After the washing process, 100µl of Substrate A and Substrate B was added to each well and mix thoroughly. This was then incubated for 15 minutes at 37°C. After a colour change, 50µl of STOP solution was added to each well and the absorbance was be measured at 450nm using the pre heated microplate reader.

3.3.6.2 Estradiol

Estradiol in the plasma was measured using a commercially available ELISA kit (Elabscience Biotechnology, Hubei). All steps were as described for testosterone.

3.3.6.3 Insulin

Serum insulin was measured using a commercially available ELISA kit (Elabscience Biotechnology, Hubei). All reagents were brought to room temperature (18-25°C) before use. One hundred microliters of standard and samples were added to each well and incubated for 90 minutes at 37°C. The liquid was removed without washing and 100µl of biotinylated detection antibody was added to each well and allow to incubate for another 60 minutes at 37°C. After incubation, the solution was aspirated and washed 3 times for 5 minutes. After washing, 100µl of HRP conjugate was added and left to incubate for 30 minutes before washing for 5 minutes. Ninety microliters of substrate reagent was added and left to incubate for 15 minutes, after which 50µl of stop solution was added. The absorbance was determined immediately at 450nm on a microplate reader.

3.4 Statistical analysis

GraphPad Prism™ software (GraphPad™ Software, Version 5 and 8.2, CA, USA) was used. Normal data distribution was measured using the Anderson-Darling and Kolmogorov-Smirnov normality tests. When data passed all normality tests, a one-way ANOVA of variance with a Tukey's or Bonferroni's Post-hoc Tests were performed. Where data were not evenly distributed, a Kruskal-Wallis test and a Dunns Post-hoc Test were carried out. A probability level of $p < 0.05$ was considered statistically significant and results are expressed as mean ± SD

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Chapter 4

Diabetes Mellitus- Induction: Effect of different Streptozotocin doses on Male Reproductive Parameters

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Temidayo S Omolaoye, Bongekile T Skosana Stefan S du Plessis*

Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa

Corresponding author:

Prof SS du Plessis

Division of Medical Physiology

Faculty of Medicine and Health Sciences

Stellenbosch University

Francie van Zijl Drive

Tygerberg

7505

South Africa

E-mail: ssdp@sun.ac.za

Abstract

Diabetes mellitus (DM) is reported to be involved in male reproductive impairment, and its impact is evident in the increased prevalence of infertility. Various studies have reported that a single parenteral injection of <40mg/kg Streptozotocin (STZ) is ineffective in ablating pancreatic β-cells and creating a rat model to investigate the effect of DM on the male reproductive system. This study therefore aims to validate these claims.

Adult male Wistar rats received either a single intraperitoneal injection of STZ (30mg/kg or 60mg/kg) or saline (0.9%, Control). Diabetes was confirmed after 72hours if plasma glucose levels were ≥14mmol/L. Body weight, glucose level, fluid and food intake were measured weekly. Animals were sacrificed after 8 weeks of treatment by an overdose of sodium pentobarbital (160mg/kg body weight). The testis and epididymis were harvested and weighed prior to preparation for histological evaluation. Epididymal sperm morphology was analysed using computer aided sperm analysis (CASA). STZ60 animals presented with significantly lower body weights compared to both control and STZ30 groups. Animals in both STZ30 and STZ60 groups showed decreased normal sperm morphology compared to control. Histological evaluation of the testes showed a decrease in the number of spermatozoa in the seminiferous tubules of animals in the STZ30 and STZ60 groups compared to control. A complete absence of spermiogenesis was observed in the seminiferous tubules of STZ60 animals. These findings prove that an STZ concentration of 30mg/kg, which is much lower than the reported 40mg/kg, has adverse effects on the male reproductive system via its diabetogenic effect and can therefore be used to study the impact of DM on male fertility.

Keywords: Streptozotocin (STZ); Diabetes Mellitus (DM); Testicular histology; Spermatozoa; Morphology; Motility

Introduction

Streptozotocin (STZ) is a chemical compound exhibiting antibiotic and antineoplastic properties, and it is produced by the bacterium Streptomyces achromogens (Vavra et al., 1959). Since the diabetogenic introductory report on STZ in 1963 (Rakieten et al., 1963), several work has been done to showcase its mechanism of action and also why it damages the pancreatic β-cells. STZ has a dynamic chemical structure that contains deoxy- glucose molecules which allows it to identify the GLUT2 receptors that is ample on the plasma membrane of pancreatic β- cells (Lenzen, 2008). These molecules are also associated to a highly reactive methylnitrosourea group, which is responsible for the cytotoxic effect of STZ.

Due to STZs high affinity for binding to the glucose receptors present on the pancreatic β-cells, its ingestion relish the cytotoxic effect upon these cells and lead to dysfunction or cell death. This subsequently leads to alteration of insulin levels and blood glucose concentrations (Wei *et al.* 2003), thereby indicating that STZ may be a suitable diabetes inducing agent. To further validate its potency, Eleazu et al. (2013) reported that there is a rapid elimination of its components by the liver and that any functional impairment observed may be ascribed to the hyperglycaemic effect (Eleazu et al. 2013). In lieu of this, STZ can therefore be used to induce experimental Type I or Type II diabetes mellitus, either independently, or in combination with high fat diet and/or in combo with nicotinamide administration (Skovso, 2014; Szkudelski, 2012).

Diabetes mellitus (DM) is a chronic non-communicable disease that is characterized by hyperglycaemia, which can result from a lack of insulin secretion by the pancreatic β-cells or from target tissues becoming insensitive to insulin. It can be classified into two broad categories: (i) Type I DM, also called insulin-dependent diabetes mellitus (IDDM), is caused by

lack of insulin secretion; (ii) Type II DM, also called non-insulin-dependent diabetes mellitus (NIDDM), is caused by the decrease in sensitivity of the target tissues to the metabolic effects of insulin. DM have been shown to affect male reproductive function at multiple levels as a result of its effects on the endocrine control of the hypothalamic-pituitary gonadal (HPG) axis, spermatogenesis itself or by impairing penile erection and ejaculation (Sexton and Jarow, 1997). In experimental animals, STZ induced diabetes have been reported to cause decreased serum testosterone, decreased serum FSH and decreased serum LH levels as well as a decrease in the total number of Leydig cells (Ballester et al., 2004; Murray et al., 1985). Another study done on the inhibition of glucose-stimulated insulin secretion in rats' islets of Langerhans by STZ revealed that the main reason for the STZ-induced β-cell death is the alkylation of DNA (Szkudelski, 2001). Shrilatha (2007) further reported increased sperm DNA damage and decrease in sperm count of STZ induced diabetic animals (Shrilatha, 2007). Additionally, Ganda et al., (1976) reported that the use of a single intravenous or intraperitoneal dose in adult rats to induce Type I DM is between 40 and 60mg/kg body weight (Ganda et al., 1976). Katsumata and Katsumata (1992) supported the above findings and further added that a single dose less than 40 mg/kg body weight may be ineffective. Furthermore, Vikram et al. (2008) reported a biochemical alteration in rat accessory sex organs after administration of STZ at 45mg/kg body weight Also, Navarro-Casado et al. (2010) reported a decrease in sperm motility, testicular and epididymal weight, following administration of STZ at both 45mg/kg and 60mg/kg to investigate its effects on male reproductive function of rats. However, we could not find any study that investigated the effects of a lower dose of STZ on male reproductive function in experimental rats. Therefore, this study was designed to investigate the effects/impact of a lower dose of STZ on male

reproductive function and to compare these outcomes with the effects of a known efficacious dose in Wistar rats.

Materials and Methods

Animals

Adult male Wistar rats (240-290g) were housed in standard ventilated cages, and were exposed to a 12hour light: 12hour dark cycle at 23°C±2°C. Animals had free access to food (standard Epol™ rat chow) and water. Ethics approval for this research was obtained from the Stellenbosch University Animal Ethics Committee.

Diabetes Induction

STZ (S0130-IG) was obtained from Sigma and prepared by dissolving it in freshly prepared sodium citrate buffer at a pH 4.5 to make a 30mg/ml stock of STZ. The STZ solution was immediately injected intraperitoneally and administered at doses of 30mg /kg body weight or 60mg/kg body weight. The injection volume was calculated according to the dose required and the weight of the animal.

Experimental Design

Animals were randomly assigned to treatment groups to avoid bias and were acclimatized for two weeks. Fifteen rats were divided into three (3) groups (n=5), namely, a control group and

two treatment groups receiving STZ at either 30mg/kg body weight (STZ30) or 60mg/kg body weight (STZ60). Animals in the control group were treated with vehicle (0.9% saline). The successful induction of diabetes was confirmed after 72 hours, with animals showing a justifying blood glucose level of >14mmol/L, using a Glucoplus™ glucometer (Ayeleso et al., 2014; Dludla et al., 2014). The diabetic state was confirmed throughout the 8 weeks treatment period and glucose levels were measured weekly in all groups. The fluid and food intake of the animals were also measured weekly, while the body weights were measured at start and end of the experiment. The relative testicular weight is the sum of both testes divided by the final body weight of the specific animal multiplied by 100. Values are expressed as percentage of body weight.

Experimental Procedure

Rats were humanely treated at all times and sacrificed after 8 weeks of treatment, by an overdose of sodium pentobarbital (160mg/kg body weight). The testis and epididymis were harvested, rinsed and weighed. The testis was fixed in 10% formalin solution for histological evaluation. The Cauda area of the epididymis was isolated and spermatozoa retrieved, which was subsequently used for motility and morphological analysis.

Histology

The testicular tissues were fixed in a 10% formalin solution. The tissues were kept in the solution for 48hours minimum to allow for complete fixation. The fixated tissues were

dehydrated with series of alcohols, cleared with xylene and infiltrated with paraffin wax. Tissues were processed using automated processor (Duplex Processor, Shandon Elliot).

Processed tissue pieces were embedded in paraffin wax at 60˚C using the Leica EG1160 embedder. Processed tissue blocks were kept at 20-25˚C until sectioning. Sections were placed in a water bath (approximately 40˚C), allowing the tissue to stretch. Tissues were stained with Haematoxylin and eosin (H & E) using an Auto stainer (Leica Auto stainer XL). Stained tissue slides were mounted with DPX mounting medium and left to dry for 48hours; allowing for proper visualization.

Histological Evaluations

Descriptive and quantitative histological evaluations of the testes were carried out. The quantitative aspect involves the histomorphometric analysis of the seminiferous tubules using the ZENlite histological analyser. This method involves measuring all seminiferous tubules seen in a specific field at a given time point. Seminiferous tubules were measured randomly at x10 magnification and field area 234.3713mm^2, knowing that all seminiferous tubules cannot be in the same stage of spermatogenesis at the point of analysis and that seminiferous tubules have different sizes at different stages. The following parameters were measured; seminiferous tubule (Tubular) diameter (Fig. 1 yellow line); tubular area (Fig.1 blue spine contour), epithelial height (Fig.1 red line) and luminal diameter and area (Fig.1 black line and green spine contour respectively).

The descriptive analysis was performed by randomly counting 100 seminiferous tubules across different fields in each rat with x20 and x40 magnification. Seminiferous tubules were

analysed at field area 58.649mm^2 evaluating maximum of 2 tubules per field to avoid bias and inaccurate analysis. In each animal the tubules were examined and categorized as normal, atrophic, sloughing or undergoing cellular degeneration. Tubules classified as normal are characterized by presenting with the complete spermatogenic phases, normal cellular organization, typical cellular association and regular interstitial spaces. Atrophy represents tubules with very few or absence of germ cells and cellular disorganization, while sloughing is characterized by the immature accumulation of cells in the lumen. Cellular degeneration is characterized by absence of some or all of the spermatogenic phases as well as degeneration of cells (presence of vacuole).

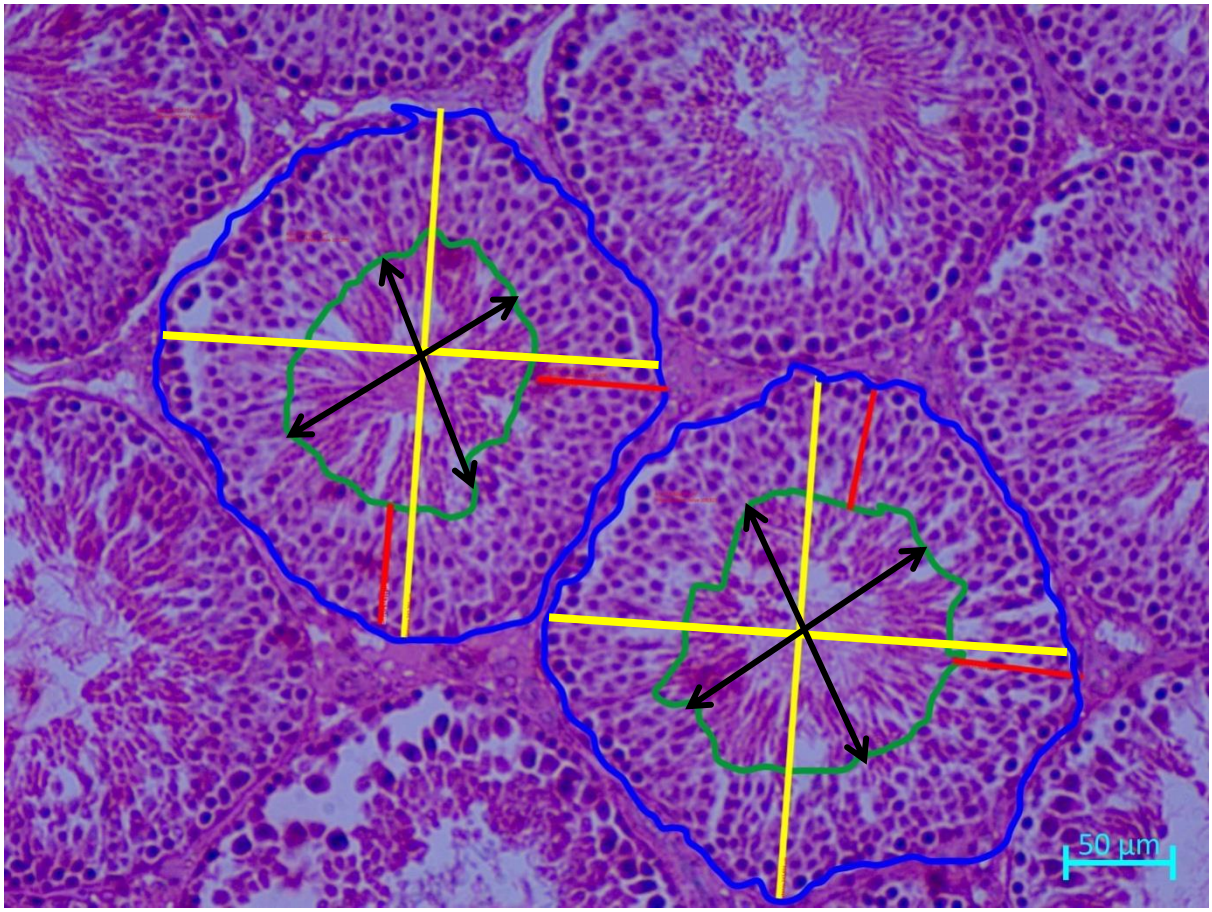

Fig. 1. Micrograph showing measurement of seminiferous tubule parameters. Seminiferous tubule diameter (yellow line); tubular area (blue spine contour), epithelial height (red line), luminal diameter (black line) and luminal area (green spine contour).

Evaluation of Sperm Parameters

Sperm Retrieval

The Cauda area of the epididymis was extracted inside a separate HAMs solution (2ml) forming a sperm solution.

Motility

Sperm motility was analysed through computer aided sperm analysis (CASA) using the Sperm Class Analyzer (SCA, Microptics, Barcelona, Spain) and Nikon Eclipse E200 microscope. Between 500-600 spermatozoa were analysed per animal across five different fields. Total motility, progressive, non-progressive and immotile spermatozoa were expressed as a percentage. Sperm velocities and kinematic parameters were also analysed and recorded.

Morphology

The various sperm mixtures were used to make smears, fixed and then stained with Sperm Blue® (Microptic SL, Barcelona, Spain) according to the manufacturer's guidelines. Sperm morphology was analysed using the morphology module of the SCA®; (Microptic, Barcelona, Spain). Acrosome, head, mid-piece and tail defects were accurately measured, whilst other morphometric variables (head Arc, width, area, linearity, angle, roughness and chord) were also analysed in order to determine whether sperm are morphologically normal or abnormal. Additionally, the sperm deformity index was evaluated by summing the total number of defects divided by the total number of spermatozoa analysed. This index provides a further measure of sperm quality in terms of morphological appearance.

Statistical Analysis

Results are expressed as mean ± SEM. Data were analysed using Shapiro-Wilk test to check for normality and even data distribution. Where data showed normal distribution, a oneway ANOVA of variance was performed and where global p is significant, a Bonferroni's post-hoc comparison test between the groups was performed. Kruskal-Wallis test was employed when data failed normality and Dunn's post-hoc comparison test was carried out between groups if the global p is significant (Graph Pad Prism 5 (GraphPad Software, CA, USA)). A probability level of $p< 0.05$ is considered statistically significant.

Results

Anthropometry Parameters

After 8 weeks of treatment, STZ60 animals lost a significant amount of body weight compared to control ($p<0.001$) and STZ30 ($p<0.001$) as well as their own initial weights ($p<0.01$), while STZ30 animals presented with increased body weight [Table 1]. There was a significant increase in the fluid and food intake of STZ60 compared to control ($p<0.001$) and STZ30 ($p<0.001$). STZ30 animals presented with increased fluid and food intake compared to control ($p=0.09$). Additionally, STZ60 presented with a significant increase in glucose levels compared to control ($p<0.001$) and STZ30 ($p<0.001$) [Table 1], while STZ30 presented with a subtle increase in glucose levels compared to control. Furthermore, STZ60 showed a decrease in testicular weight compared to control and STZ30 [Table 1].

Table 1: Comparison of anthropometry Parameters

Groups	Start body weight (g)	End body weight (g)	Food Intake (g)	Fluid Intake (ml)	Glucose Level (mmol/L)	Testicular weight (g)	Relative testicular weight (%)
Control	264.70±12.55	338.30±19.76	21.46±0.86	28.79±1.34	6.53±0.07	1.40±0.09	0.43±0.01
STZ30	271.74±7.46	391.26±13.60	24.23±1.32	33.12±1.44	6.95±0.14	1.48±0.08	0.37±0.02
STZ60	283.70±3.94	234.36±14.92$^{@\&}$	36.40±1.15$^{@\&}$	183.00±9.17$^{@\&}$	23.73±0.65$^{@\&}$	1.36±0.10	0.57±0.02$^{@\&}$

$^{@}$ $p<0.001$ vs. control; $^{\&}$ $p<0.001$ vs. STZ30

Quantitative histological evaluation

Following histomorphometric analysis of the seminiferous tubules, there was a significant increase of seminiferous tubule diameter of STZ60 compared to control ($p<0.05$) representing 121% of control and 117% of STZ30 [Table 2].

Table 2: Quantitative histological parameters of the testes

Groups	**Tubular diameter (µm)**	**Tubular area ($µm^2$)**	**Epithelial height (µm)**	**Luminal diameter (µm)**	**Luminal area ($µm^2$)**
Control	224.8±8.28	39987.0±2857.00	70.0±11.98	120.1±11.23	11763.0±2132.00
STZ30	263.1±16.13	55480.0±6605.00	69.3±4.99	138.4±7.02	15613.0±1514.00
STZ60	272.1±5.33*	58603.0±2311.00*	69.4±1.44	143.8±6.81	16734.0±1551.00

*$p<0.05$ vs. control

Descriptive histological evaluation

STZ60 presented with a significant decrease in the number of normal seminiferous tubules compared to control ($p<0.001$) representing 41% of control and STZ30 ($p<0.05$). STZ30 as well presented with a significant decrease in the number of normal seminiferous tubules compared to control ($p<0.05$) representing 21% of control. STZ60 presented with an increased number of atrophic tubules compared to control ($p<0.001$) and STZ30 ($p<0.05$) [Table 3].

Table 3: Descriptive histological parameters of the seminiferous tubules. Values are expressed in percentage

Groups	Normal (%)	Atrophy (%)	Sloughing (%)	Cell degeneration (%)
Control	94.6±0.92	3.0±1.483	1.8±1.11	0.6±0.60
STZ30	75.2±3.49*	10.0±5.57	8.8±3.99	6.0±1.30
STZ60	55.8±6.27$^{@\#}$	24.0±1.70$^{@\#}$	9.0±2.84	11.0±4.56

*p<0.05 vs. control; @ p<0.001 vs. control; # p<0.05 vs. STZ30

Sperm motility

Regarding the effect of STZ on male reproductive parameters, STZ60 showed a significant decrease in the number of motile spermatozoa compared to control ($p<0.05$) and also displayed a decrease in progressive motility compared to control [Table 4]. Additionally, STZ30 presented with a decrease in the number of motile spermatozoa and progressive motility compared to control [Table 4]. Thirty seconds post retrieval from the cauda epididymis, STZ60 spermatozoa presented with a decrease in the curvilinear velocity (VCL)

and average path velocity (VAP) compared to control and STZ30 [Fig.2]. Additionally, STZ60 displayed a decrease in oscillation index (WOB) compared to control [Fig. 3].

Table 4 Sperm motility as measured 30 seconds post retrieval from the cauda epididymis

Groups	Motile (%)	Progressive Motility (%)
Control	85.17±7.40	47.79±9.56
STZ30	78.88±3.62	35.79±10.84
STZ60	67.40±8.44	41.92±3.96

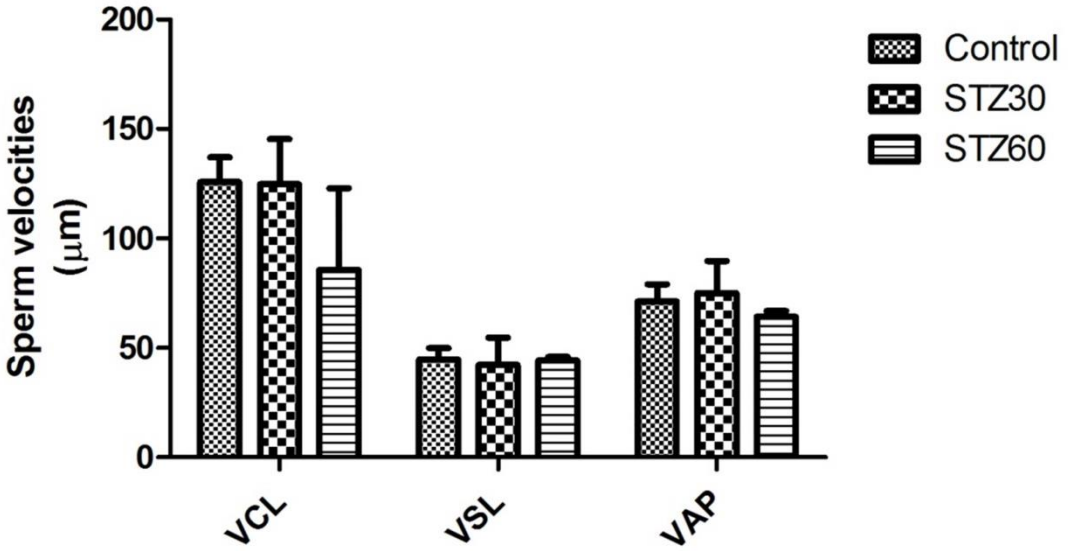

Figure 2 Sperm velocity parameters for the three groups. VCL= Curvilinear velocity; VSL= Straight-line velocity; VAP=Average path velocity. VCL ANOVA p=0.4; VSL ANOVA p=0.9; VAP ANOVA p=0.7

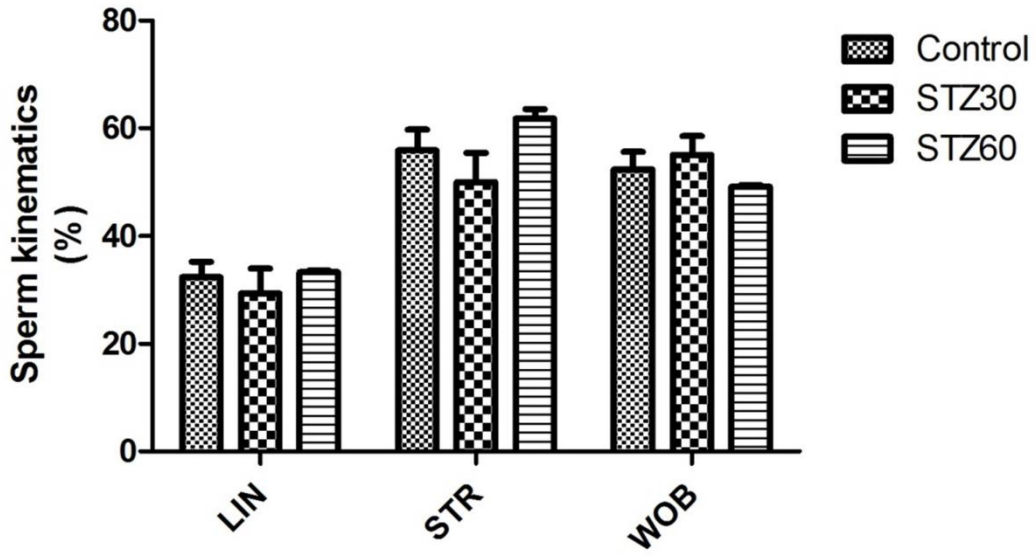

Figure 3: Sperm kinematic parameters for the three groups. LIN= Linearity index; STR= Straightness index; WOB= Oscillation index. LIN ANOVA p=0.6; STR ANOVA p=0.1; WOB ANOVA p=0.3

Sperm morphology

STZ30 and STZ60 exhibited a significant decrease in the number of morphologically normal spermatozoa compared to control ($p<0.05$ and $p<0.001$ respectively) [Fig. 4 & Fig. 5], while the sperm deformity index [Fig. 6] of STZ30 and STZ60 were increased compared to control.

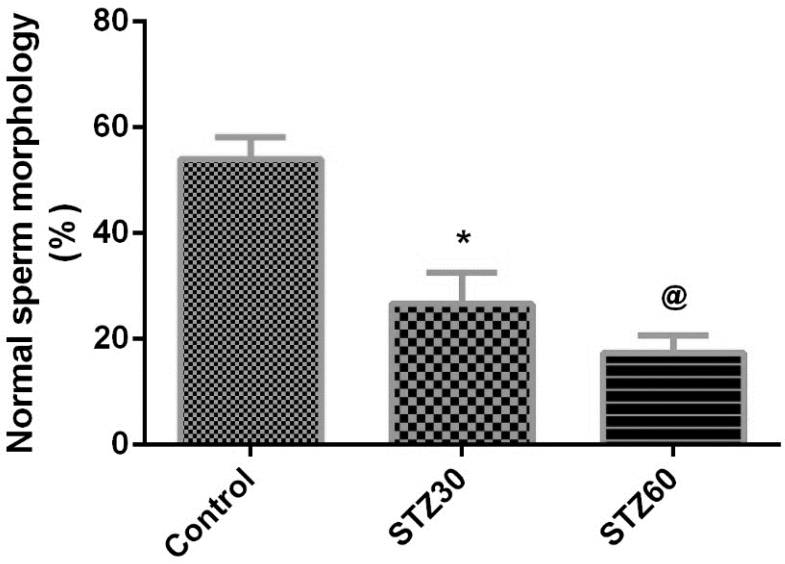

Figure 4: The percentage of spermatozoa displaying normal morphology. *$p<0.05$ vs. control; @$p<0.001$ vs. control, Normal sperm morphology ANOVA $p=0.003$ ($n=3$). Sperm is considered abnormal if one or more listed characteristics are altered.

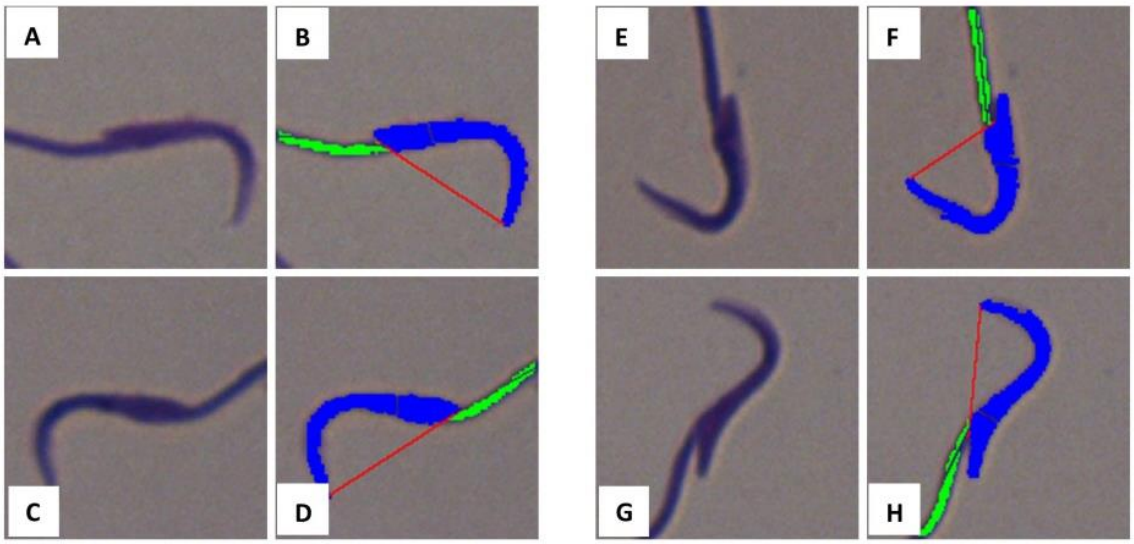

Figure 5: Stained rat sperm, automatically analysed for morphology. Each image on the left is of an actual stained spermatozoon and directly to the right is the subsequent SCA analysis with blue overlay showing the head and green overlay the midpiece. Figures a-d are of morphologically normal spermatozoa and Figure e-h are of morphologically abnormal spermatozoa. Morphology assessment is based on thresholding for head size, head shape (arc, width, area, perimeter, linearity, angle, roughness and chord), acrosome size, midpiece (size, angle and insertion) and tail (length and regularity).

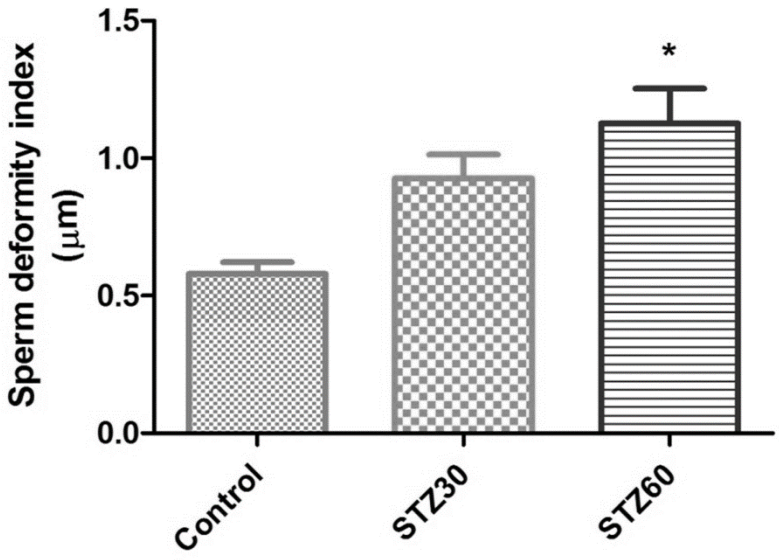

Figure 6 Sperm deformity index (SDI) for control, STZ30 and STZ60. Sperm deformity index measures sperm quality in terms of morphological appearance. *p<0.05 vs. control. Sperm deformity index ANOVA p=0.015 (n=3)

Discussion

STZ has been a known diabetes inducing agent since its discovery in 1963 (Rakieten et al., 1963) and have been reported to be effective only when administered at concentrations of ≥40mg/kg (Ganda et al., 1976; Katsumata and Katsumata, 1992). To further validate its experimental use, several authors have worked to annul the possible meddling effects of its components on male reproductive function (Navarro-Casado et al., 2010). In this study we investigated the effects of a lower STZ dose (30mg/kg) on male reproductive function and also compare these outcomes with the effects of a known effective STZ dose (60mg/kg).

Observed was an increase in the intake of fluid and food, as well as the blood glucose levels of STZ30, though the elevated blood glucose remained within the normal physiological range (as opposed to STZ60 which reached pathophysiological levels) (Oliveira et al.,2015). However, there was an increase in body weight of these animals. The elucidated parameters are typical characteristics of pre-diabetes (Soewondo and Pramono, 2011), which concur with findings, from Rato et al. (2013) and Oliveira et al. (2015). In comparison, STZ60 displayed heightened fluid and food intake as well as elevated blood glucose levels. However, these animals presented with a decrease in body weight; all of which are pathophysiological characteristics of diabetes (Vikram et al., 2008; Navarro-Casado et al., 2010). The decreased body weight might be due to the inability to utilize the glucose derived from feeds as a result of the ablated pancreatic β-cells and subsequent lack of insulin signalling.

The testes of STZ30 showed mild abnormal morphology of the seminiferous tubules characterized by the presence of immature and irregular accumulation of cells in the lumen. Furthermore, an increase in vascular structures was also observed. Likewise this was observed in STZ60. Additionally, STZ30 tubules have diverse rate of spermatogenic phase arrest while

cell degeneration was also seldom observed. STZ60 however, presented with severe absence of spermatogenic phases as well as persistent cellular degeneration. Furthermore, STZ60 tubules were greatly disorganized and germ cells were severely dissociated [Fig.7]. Absence of spermatogenic phases was also reported by Ricci et al. (2009) following STZ diabetes induction, who further reported that the abnormal localization or absence of Occludin in the tight junction of Sertoli-Sertoli cells observed in diabetes prevents the formation of the blood-testis barrier, which is necessary for sustaining spermatogenesis (Lui et al., 2003; Ricci et al., 2009; Wong et al., 2005). In the same vein, Hess and Nakai (2000) reported that the presence of seminiferous tubules with persistent atrophy could indicate infertility. Observed in STZ30 is the reduced relative testicular weight and increased seminiferous tubules diameter which was consistent with increased epithelium width , small lumen, scattered or compressed germ cells, presence of vacuoles in the basal region of the tubule and absence of phases II &III of spermatogenesis. STZ60 however, exhibited a decrease in testicular weight which is in agreement with Ferah (2007) and several other authors that also reported reduction in testicular weights of diabetic rats treated with high doses of STZ (Anderson and Thliveris, 1986, 1987). In addition, we observed the enlargement and increased number of STZ30 and STZ60 testicular blood vessels. This is consistent with the findings of Anderson and Thliveris (1986) and Amaral et al., (2006) who reported that diabetes is implicated with the increased number of testicular blood vessels.

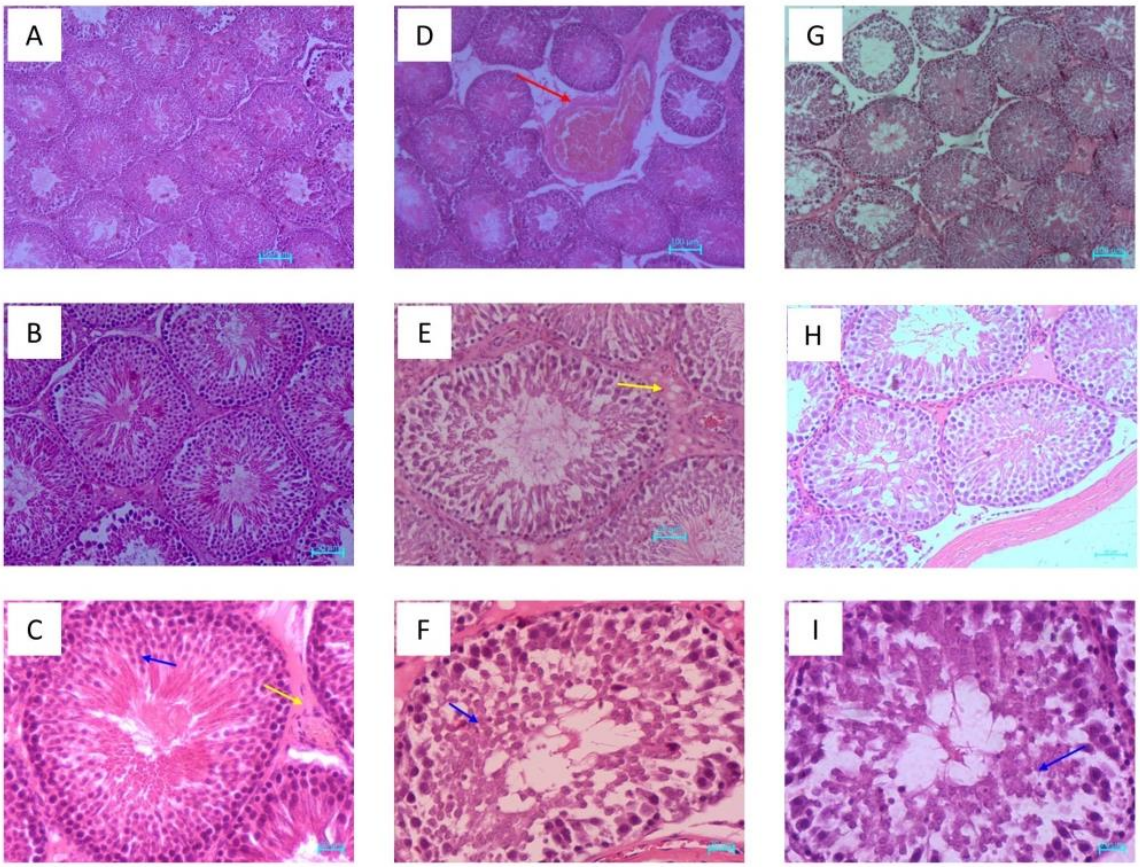

Figure 7 Testicular Sections of Control, STZ30 and STZ60 Rats. Testicular sections with different magnification of control rats (a-c) shows normal spermatogenesis and normal cell arrangement, STZ30 (d-f), shows enlarged interstitial space (arrow e), increased number of blood vessels (d) and tubular disorganization (e, f) while testicular sections of animals treated with STZ (60mg/kg body weight) (g -i), presented with further seminiferous tubule disorganization (i) and incomplete spermatogenic phases (h, i).

Regarding the effect of a lower STZ dose on sperm parameters, our results showed a decrease in total sperm motility and reduced progressive motility. In comparison to a known effective dose, total sperm motility and progressive motility were more negatively affected. A known potent STZ dose (≥40mg/kg) has been suggested to adversely affect sperm motility (Mangoli et al., 2013; Vignera et al., 2012) due to various physiological alterations such as hormonal changes, oxidative stress enhancement and as well as neuropathy development, all caused by hyperglycaemia. Additionally, there was a decrease in sperm velocity and kinematic parameters of diabetic and prediabetic animals. Interestingly, in a recent study conducted by Ayad et al. (2017), sperm kinematic parameters were also reduced after a long ejaculatory abstinence period and that short ejaculatory abstinence period of 4hours was associated with a substantial increase in human sperm motility and velocity parameters.

Also observed is the increase in abnormal sperm morphology of STZ30 spermatozoa and a more elevated abnormality of STZ60 sperm morphology. This result concurs with the findings reported by Murray et al. (1985) and Mangoli et al. (2013). Spermatozoa are known to undergo different phases of spermiogenesis (Golgi phase, Cap phase, Acrosome phase, and Maturation phase) for normal morphological structure development. However, we observed the absence of phases II and III of spermatogenesis in both STZ30 and STZ60, which indicate that some tubules did not even undergo spermiogenesis. This result suggests that the absence of these phases position the testicular morphological structures formation for abnormalities.

Moreover, to our utmost knowledge this is the first study reporting the adverse effect of a relatively lower STZ dose (30mg/kg) on male reproductive parameters and that the observed effects are moderate compared to a higher STZ dose (60mg/kg). The moderate adverse effect

seen may be associated to the incomplete ablation of the pancreatic β-cells, which might allow for partial functioning and secretion of insulin by these cells.

Conclusion

Based on this present study, we conclude that STZ administration of a lower dose (30mg/kg) adversely affects male reproductive parameters via its diabetogenic effect and can therefore be used to study the impact of pre-diabetes on male fertility and that damage is more prominent in a higher dose. We also recommend that future studies should focus on the reason for an increase in testicular blood vessels in diabetes and prediabetes.

Conflict of Interest

The authors have no conflict of interest to declare.

Contributors

TSO help design the study, collected and analysed the data, drafted the manuscript; BTS assisted with data collection and analysis; SSDP help design the study and assisted in drafting the manuscript; all authors reviewed and approved the final manuscript.

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Chapter 5

The effect of streptozotocin induced diabetes on sperm function: A closer look at AGEs, RAGEs, MAPKs and activation of the apoptotic pathway

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Omolaoye Temidayo S [1], du Plessis Stefan S [1, 2]*

1. Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa
2. Department of Basic Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates

* Corresponding author:

Prof SS du Plessis

Division of Medical Physiology

Faculty of Medicine and Health Sciences

Stellenbosch University

Francie van Zijl Drive

Tygerberg

7505

South Africa

E-mail: ssdp@sun.ac.za

List of abbreviations

AGE	advanced glycation end product
BCA	bicinchoninic acid
CASA	computer aided sperm analysis
DM	diabetes mellitus
DNA	deoxyribonucleic acid
GnRH	gonadotropin-releasing hormone
ICAM-1	intercellular adhesion molecule-1
JNK	C-Jun N-terminal kinase
MAPKs	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MDA	malondialdehyde
Nf-kB	nuclear factor kappa B
OS	oxidative stress
PARP	poly adenosine diphosphate-ribose polymerase
PUFA	polyunsaturated fatty acid
RAGE	receptor for advanced glycation end product
ROS	reactive oxygen species
SF	supplementary figure
STZ	streptozotocin

TBA	thiobarbituric acid
VCAM-1	vascular cell adhesion molecule-1

Abstract

This study was designed to investigate the possible mechanisms through which diabetes-induced advanced glycation end products (AGEs) and receptor for AGEs (RAGE) activation can affect male reproductive function and to corroborate the interaction of previously established independent pathways.

Male albino Wistar rats (14weeks old) weighing 250-300g received either a single intraperitoneal injection of streptozotocin (30mg/kg or 60mg/kg), regarded as STZ30 or STZ60 respectively or citrate buffer (Control). DM was confirmed if plasma glucose levels were ≥14mmol/L after one week. Animals were sacrificed after 8 weeks of treatment by an overdose of sodium pentobarbital (160mg/kg body weight). The testes and epididymides were harvested. The testis was used for biochemical and Western blot analysis, while sperm was retrieved from the epididymis and analysed with computer-aided sperm analysis (CASA).

The blood glucose levels of STZ60 animals were above the cut-off point and hence will be regarded as diabetic animals. Diabetic animals presented with a non-significant increase in AGE and RAGE expression. Diabetic animals showed a significant increase in the expression of cleaved caspase 3 compared to control ($p<0.001$), which was accompanied by an increase in the expression of JNK ($p<0.05$), PARP ($p=0.059$) and p38MAPK ($p=0.1$). Diabetic animals also displayed decreased catalase activity accompanied by a non-significant increase in malondialdehyde (MDA) levels. Additionally, there was a significant decrease in the percentage of progressively motile spermatozoa ($p<0.05$) in diabetic animals.

This study has shed some light on the interplay between DM, AGE, RAGE and mitogen-activated protein kinase signalling in the testis of diabetic rats, which can result in altered

sperm function and may further contribute to male infertility. However, more studies are needed to better understand this complicated process.

Keywords: Advanced glycation end products, Apoptosis, Diabetes-Mellitus, Mitogen-activated protein kinases, Receptor for advanced glycation end products and Sperm

Introduction

Diabetes mellitus (DM) is an embodiment of various metabolic pathologies. It represents one of the most appreciable health hazards as its prevalence has increased from 177 million to 422 million in only 12 years (2002-2014). This further connotes a 60% increase (1,2). Infertility, a disease of the reproductive system has become an integral part of global health as it is estimated to affect 60-80 million couples of which 50% is directly attributed to a male factor (3,4). Studies have shown that DM affects male reproductive function adversely. Some of the reported effects include a decrease in sperm motility (5,6), alteration in sperm kinematic properties, increased abnormal sperm morphology (7,8), decreased seminiferous tubule diameter, increased spermatogenic disruption (9–11) and decreased number of Leydig and Sertoli cells (9,12).

Baccetti et al. reported that the gonadotropins released in response to GnRH were lower in diabetic men. This was accompanied by a decrease in sperm motility and a higher percentage of sperm immaturity (13), which indicates that DM may affect male reproduction by interfering with the hypothalamic-pituitary-testicular axis. Additionally, Jiang et al. reported that streptozotocin (STZ)-induced diabetic rats displayed an increase in testicular tunnel positive cells, Bax/Bcl2 ratio and oxidative damage (14). In the same vein, Roessner et al. reported increased cleaved caspase 3 in the ejaculate of diabetic men (15). These findings show that DM may affect male reproduction via apoptotic signalling. Furthermore, DM has been shown to increase testicular oxidative damage, which occurs when reactive oxygen species (ROS) exceeds the seminal antioxidant defence ability (16). Thereby resulting in many cascades of reactions, which subsequently lead to sperm DNA damage, mitochondrial DNA fragmentation, altered sperm parameters and ultimately male infertility (17). Increased ROS

production has also been implicated in the generation of advanced glycation end products (AGEs). AGEs are products of non-enzymatic reactions between sugars and the amino groups of proteins, lipids and DNA under hyperglycaemic conditions. AGEs can alter the normal functioning of macromolecules by generating ROS independently, or by activating the receptors for advanced glycated end products (RAGE), which leads to a further increase in ROS production by these mutated proteins. RAGE is a multi-ligand member of the immunoglobulin family, which interact with specific ligands. Binding of RAGE to its ligands leads specifically to altered gene expression.

The role of AGE-RAGE activation in the induction of apoptosis has been widely evaluated. Many of these studies were performed on various cell types such as neuronal and endothelial cells (18,19), as well as periodontal ligament fibroblasts(20). Additionally, the binding of AGE-RAGE could trigger mitogen-activated protein kinase (MAPK) signalling and the nuclear factor kappa B (NF-κB) pathway, thereby leading to elevated production of ROS and upregulation of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and monocyte chemoattractant protein-1 (MCP-1) (21). Interestingly, AGEs have been suggested to play a role in instigating harm as they are known to produce ROS and subsequently cause OS, of which sperm has high susceptibility to the excessive production of ROS. It is believed that AGEs may act as a mediator of damage to the reproductive system of diabetic men (22).

Although there are substantial data available on the effects of DM on sperm function, the mechanism through which the effects are exerted is less clearly understood. The present study, therefore, aimed to investigate the possibility of AGE-RAGE pathway activation in DM related sperm function impairment and to furthermore corroborate the interaction of previously established independent pathways.

Materials and Methods

Animals

Male albino Wistar rats (14weeks old) weighing 250-300g were used in this study. Animals were individually housed in standard cages in the Stellenbosch University Animal Unit. Both room temperature (23°C±2°C) and light (12 hours light/12 hours dark) were controlled. Ethical approval was obtained from the Stellenbosch University Animal Ethics Committee (SU-ACUD16-00101). Rats were treated according to the recommendations of the Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (23). Animals were acclimatized for one week before treatment commenced and had free access to food (standard Epol™ rat chow, Nutritionhub (PTY) LTD, South Africa) and water.

Study Design

Thirty rats were randomly divided into three groups (n=10), namely, a control group and two treatment groups (receiving STZ). STZ (S0130-IG) was obtained from Sigma (South Africa), and a stock solution (30mg/ml) was prepared by dissolving it in freshly prepared sodium citrate buffer (pH 4.5). Animals were treated with a single intraperitoneal injection of STZ, either 30mg/kg body weight (STZ30) or 60mg/kg body weight (STZ60), while the control group were treated with an equivalent volume (0.5ml) of citrate buffer. The successful induction of diabetes was confirmed after one week in animals presenting with a non-fasting blood glucose level of ≥14mmol/L, using a Glucoplus™ glucometer. Glucose levels were monitored throughout the 8 weeks treatment period by measuring (non-fasting) once a week. Blood

used for the weekly glucose level measurement was collected by pricking the tail vein of the restrained animal. Blood glucose levels are the mean of consecutive measurements.

Experimental Procedures

After 8 weeks of treatment, animals were sacrificed by administering an overdose of sodium pentobarbital (160mg/kg body weight). The testes and epididymides were harvested, weighed and rinsed. The left testis was frozen at -80°C until further analysis.

Sperm Motility

The harvested left epididymis was placed in a Petri dish containing a 2ml solution of Hams F-12 nutrient media (Sigma Chemicals, St Louis, MO, USA), at 37°C. The mature spermatozoa were retrieved into a separate dish containing 2ml Hams by dissecting the caudal area of the epididymis and allowing sperm to swim out for 30 seconds. The retrieved spermatozoa (2µl) was infused into a chamber slide (20µm, Leja, Netherlands) and placed on a microscope (Nikon Eclipse E200) with an in-built heating stage (37°C). Sperm motility was measured via computer-aided sperm analysis (CASA) using the Sperm Class Analyser (SCA, Microptic, Barcelona, Spain). Various motility and kinematic parameters were analysed (24).

Biochemical Analysis

Oxidative Stress Parameters

Catalase

Frozen testicular tissue samples were pulverized in liquid nitrogen to prevent thawing and prevent enzymatic reactions from taking place. An equal amount (0.05g) of each sample was placed in an Eppendorf tube containing 50mg of homogenization beads and 100µl of cold lysis buffer (Na_3PO_4, 0.5% Triton X-100). Samples were homogenized by bullet blending at speed 9 for 3 minutes with a 1-minute interval in-between. After bullet blending, an additional 400µl of lysis buffer was added to allow for total tissue breakdown. Homogenates were centrifuged at 15000rpm for 20 minutes at 4°C. The supernatants were transferred to different Eppendorf tubes and kept on ice. Tissue homogenates were diluted to 10x in deionized water and Bicinchoninic Acid (BCA) assay was used to determine the protein concentration. From the diluted samples and standards, 5µl were loaded in triplicate into UV microplate wells. Catalase assay buffer (170µl) were added into each well using a multichannel pipette. Finally, 50µL of H_2O_2 was added into the wells and analysis was performed immediately on a plate reader (Multiskan spectrum) at 240nm every 60 seconds over a 5-minute period using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software.

Lipid Peroxidation (Thiobarbituric acid reactive substances (TBARS/MDA)

Frozen testicular tissue samples were pulverized in liquid nitrogen and 0.05g of each sample were placed in an Eppendorf tube containing 0.05g of homogenization beads and 0.5ml of cold lysis buffer (0.1M KPi, 1.15% KCl). Samples were homogenized by bullet blending at speed 9 for 3 minutes with a 1-minute interval in-between. Supernatants were retrieved for further

analysis (BCA and TBARS assays). In order to measure the MDA levels, 100µl of standards and samples were pipetted into corresponding 10ml glass tubes. Subsequently, 1ml of SDS and 2ml of 10%TCA-BHT buffer solution was added. Samples were vortexed, and after resting for 10 minutes, 2ml of thiobarbituric acid (TBA) was added and vortexed again. The standards and samples were covered with marbles and incubated in a water bath (1 hour at 100^{o}C), where after it was cooled on ice for 15 minutes. The standards and samples were centrifuged (3000rpm, 15 minutes 4°C) and the supernatants retrieved. From the supernatants, 250µl of each standard and sample were loaded in triplicate into microplate wells and analysed on a plate reader (Multiskan spectrum) within 30 minutes after centrifugation at a wavelength of 532nm.

Western Blot Analysis

To determine the quantity of proteins in a sample through the Bradford method 0.05-0.07g of tissue were pulverized and placed into 700µl lysis buffer. Seven stainless homogenization beads (1.6mm, Next Advance Inc., USA) were added into each tube to allow for complete homogenization (25,26). The pulverized tissue was bullet blended at speed 8 for 3 minutes at 5 minutes interval in-between. The homogenates were centrifuged for 20 minutes (15000rpm, 4°C) and the supernatants were removed for further analysis. Tissue lysates were prepared by diluting the sample in Laemmli sample buffer and lysis buffer, boiled for 5 minutes and 50ug protein/µl was separated by electrophoresis on a 12% SDS-PAGE mini-proteon gel. The running protocol consisted of an initial 10 minutes electrophoresis at 100V and 200mA followed by 40 minutes at 200V and 200mA. Gels were activated using ChemiDoc (BioRad). Thereafter, the proteins were transferred onto a milipore Immobilon-P transfer

membrane (0.45µm) (Immobilon®-P, Merck Millipore Ltd, Germany). Non-specific sites were blocked with 5% fat-free milk in TBS-tween.

AGE was measured using Anti-AGE antibody (ab23722; Abcam, SA) and the expression of RAGE was quantified using anti-RAGE antibody (ab361; Abcam, SA). The apoptotic markers measured are Caspase 3 (Sigma-Aldrich), Caspase 7 (Abcam, SA), PARP (Cell Signalling Technology), p38MAPK (Cell Signalling Technology), C-Jun-N-terminal kinase (JNK) (Cell Signalling Technology) and IkBα (Cell Signalling Technology). A goat anti-mouse/rabbit-horseradish peroxidase-conjugated antibody (Sigma-Aldrich) was used as the secondary antibody. Proteins were detected and visualized using a ChemiDoc (BioRad, USA) and were further normalized (Stain-free blots as housekeeping proteins) and analysed with ImageLab 5.0 (ImageLab software, USA). All primary antibodies were diluted in TBS-Tween in a 1:1000 ratio while the secondary antibody was diluted in TBS-Tween in a 1:4000 ratio. All data points are from independent biological repeats (n=4-5).

Statistical Analysis

GraphPad Prism™ software (GraphPad™ Software, Version 8.1.2(332), San Diego, CA, USA) was used for statistical analysis. Normal data distribution was measured using the Shapiro-Wilk, Anderson-Darling, Kolmogorov-Smirnov and D'Agostino & Pearson, normality tests. When data passed all normality tests, a one-way ANOVA of variance with a Tukey's Post-hoc Test were performed. Where data was not evenly distributed, a Kruskal-Wallis test and a Dunns Post-hoc Test were carried out. A probability level of $p < 0.05$ was considered statistically significant and results are expressed as mean ± SEM.

Results

Basic Biological Parameters

After 8 weeks of treatment, STZ60 animals lost a significant amount of body weight compared to both control and STZ30 ($p<0.0001$) [Table 1]. Additionally, STZ60 animals presented with a significant increase in blood glucose levels compared to both control and STZ30 ($p<0.0001$) (n=8) [Table 1 and Figure 1].

Table 1: Basic biological parameters

Parameters	Control	STZ30	STZ60	Global p-value
Start body weight (g)	272.1±7.076	277.2±4.599	283.0±3.692	0.3
End body weight (g)	340.7±10.09	377.9±8.777*	252.3±11.80$^{\$\&}$	<0.0001
Difference in body weight (g)	86.4±19.32	100.8±7.95	-27.6±11.39$^{*\&}$	<0.0001
Glucose level (mml/L)	6.299 ±0.169	6.532±0.162	22.60±1.009$^{\$\&}$	<0.0001

$^{*}p<0.05$ vs. control; $^{\$}$ $p<0.0001$ vs. control; $^{\&}$ $p<0.0001$ vs. STZ30. Blood glucose levels is the mean of consecutive measurements from induction to termination.

Figure 1: Non-fasting blood glucose levels of experimental animals as monitored during the 8 week treatment period

Sperm Motility

STZ60 animals displayed a significant decrease in the percentages of motile and progressively motile spermatozoa compared to the control group ($p<0.05$) ($n=8$) [Figure 2 A-B; supplementary figure (SF) 1]. Although there was a decrease in the progressive motility as well as total motility of STZ30 animals compared to control, the reduction was not statistically significant. Additionally, there was a substantial, but non-significant decrease in the sperm kinematic parameters [Table 2] of STZ60 animals compared to both the control and STZ30 groups respectively, (Curvilinear velocity (VCL): -30%, -30%; Straight-line velocity (VSL): -21%, -17%; Average path velocity (VAP): -18%, -9%). The kinematic parameters of STZ30 animals, despite all six being lower, remained very similar to that of control animals [Table 2].

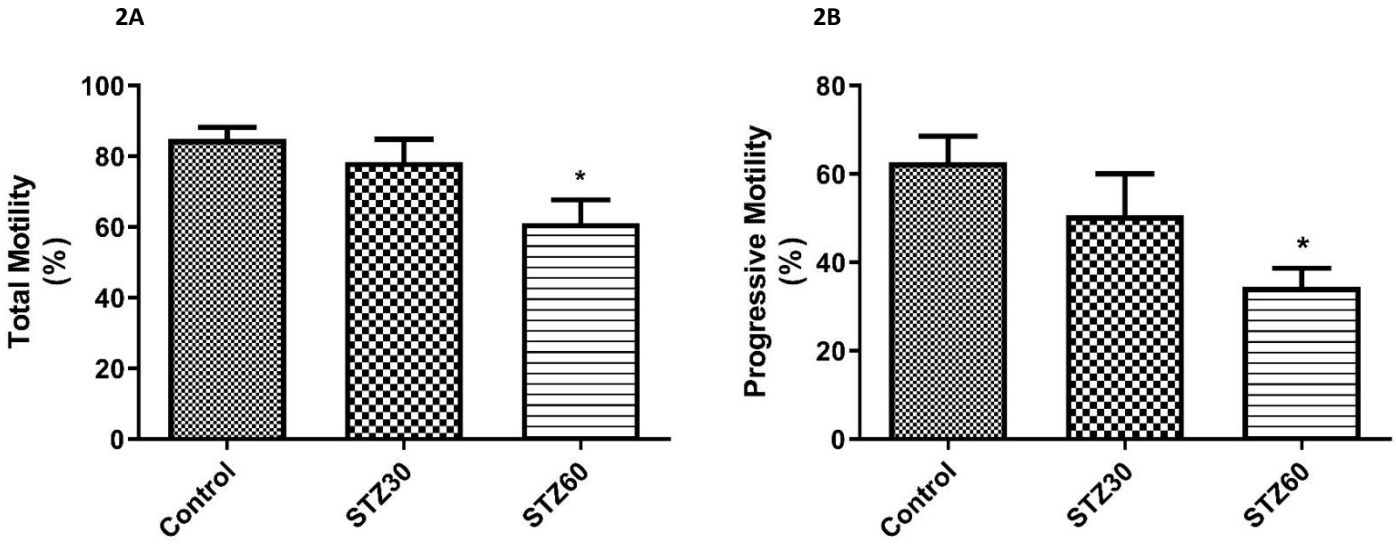


Figure 2: The effect of STZ induction on sperm motility. **A**. percentage of total motility; **B**. percentage of progressive motility. *$p<0.05$ vs control. Global p for progressive motility is $p=0.0285$; global p for total motility $p=0.0132$.

Table 2: Sperm kinematic parameters

Group	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)	STR (%)	LIN (%)	WOB (%)
Control	179.0±54.19	83.29±26.02	50.69±18.29	57.37±5.192	28.65±5.113	46.62±6.127
STZ30	178.2±86.85	79.73±31.36	45.56±18.83	52.46±10.23	25.09±7.736	44.58±10.15
STZ60	124.6±49.91	65.81±12.74	41.53±9.435	56.30±7.103	28.24±5.816	45.59±5.362

Curvilinear speed (VCL), Average path velocity (VAP), Straight Line velocity (VSL), Straight line index (STR), Linearity index (LIN), and Oscillation index (WOB).

Oxidative Stress Parameters

STZ60 animals presented with a non-significant decrease (-65%) of catalase activity compared to the control group, while STZ30 animals displayed a 57.7% reduction of catalase activity compared to control (p=0.14) (n=8). This was accompanied by a 96.6% increase in MDA levels in the STZ60 animals and 10% increase in the STZ30 group compared to controls (p=0.18) [Figure 3 A-B; SF2].

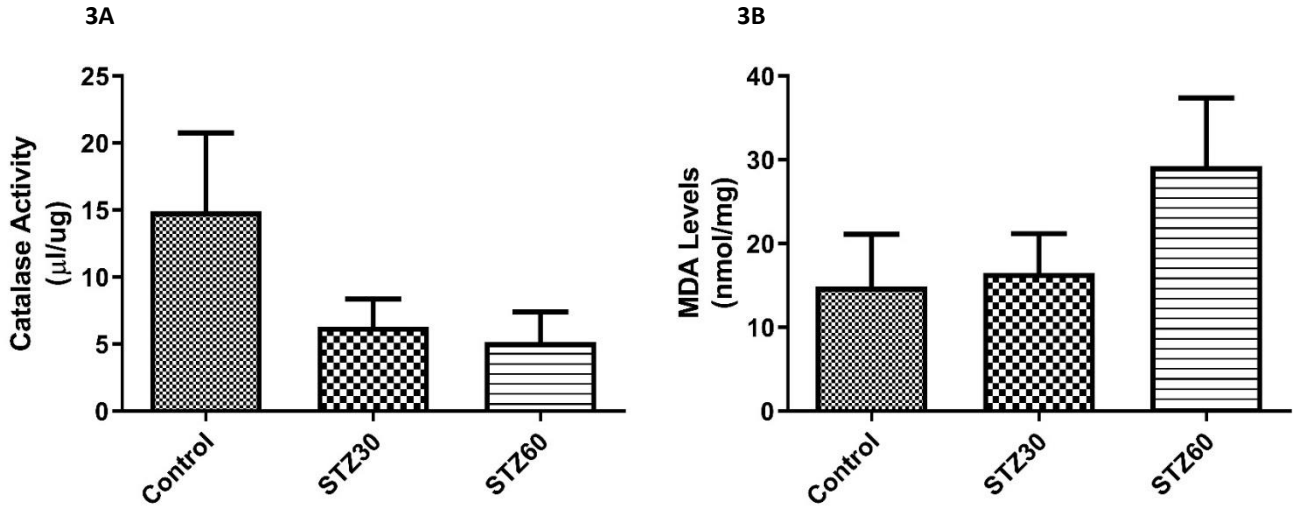
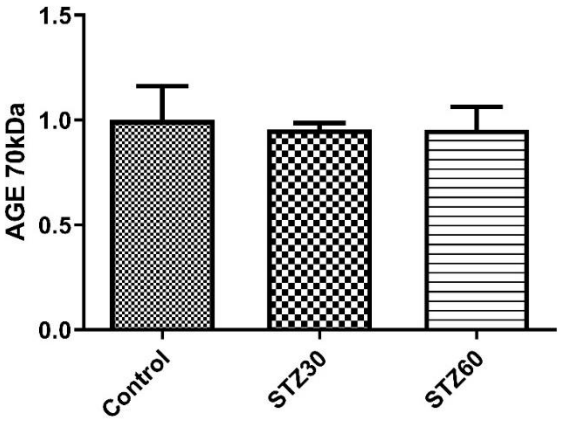


Figure 3: The effect of STZ induction on oxidative stress parameters. **A**. the effect of STZ induction on catalase activity; **B**. the effect of STZ induction on MDA levels. The global p for catalase activity p=0.13, global p for MDA p=0.18.

AGE Accumulation and RAGE Expression

Four different isoforms of AGE proteins were observed, with STZ60 animals showing a non-significant increase in accumulation of each AGE isoform except for the 70kDa protein compared to control [Figure 4 A-F; SF3]. Additionally, STZ60 animals displayed a non-significant increase in the total accumulation of AGE isoforms (6.4%) compared to control and STZ30 presented a 2.2% increase compared to the control group (p=0.6).

4A



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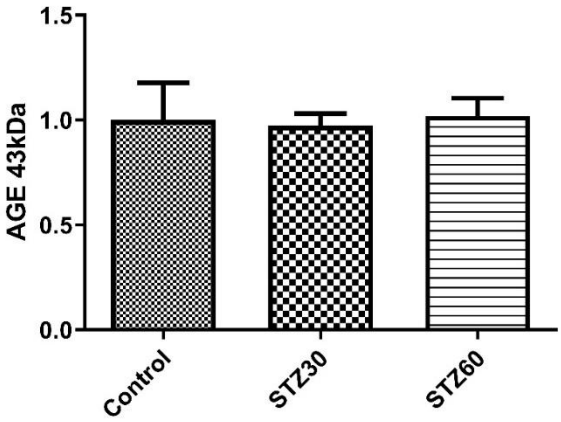

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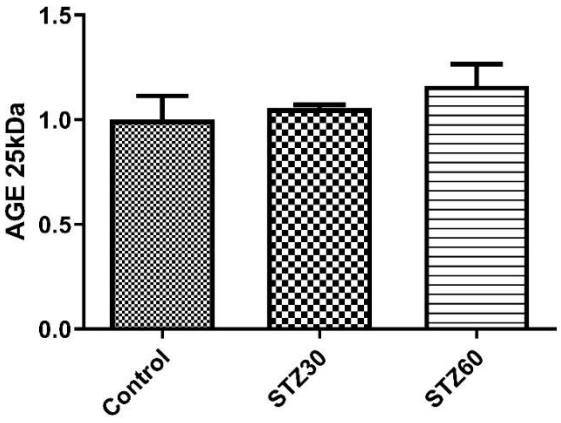

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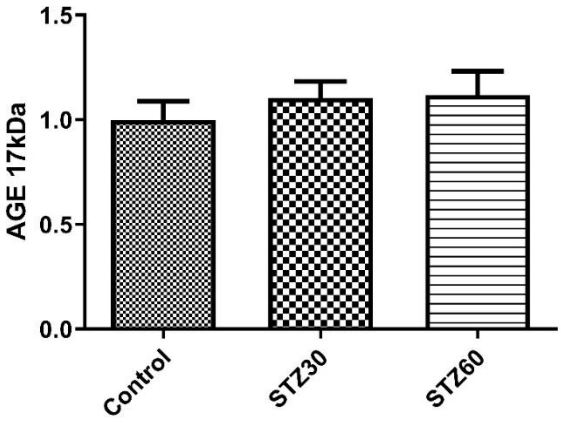

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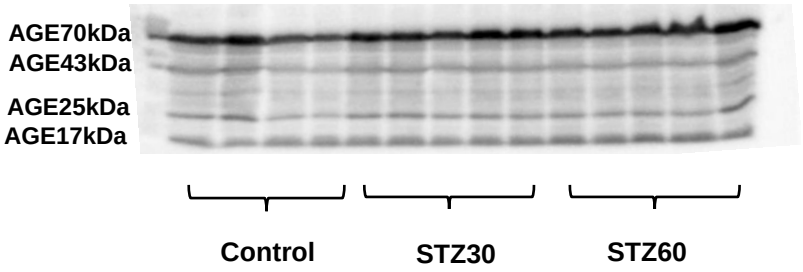
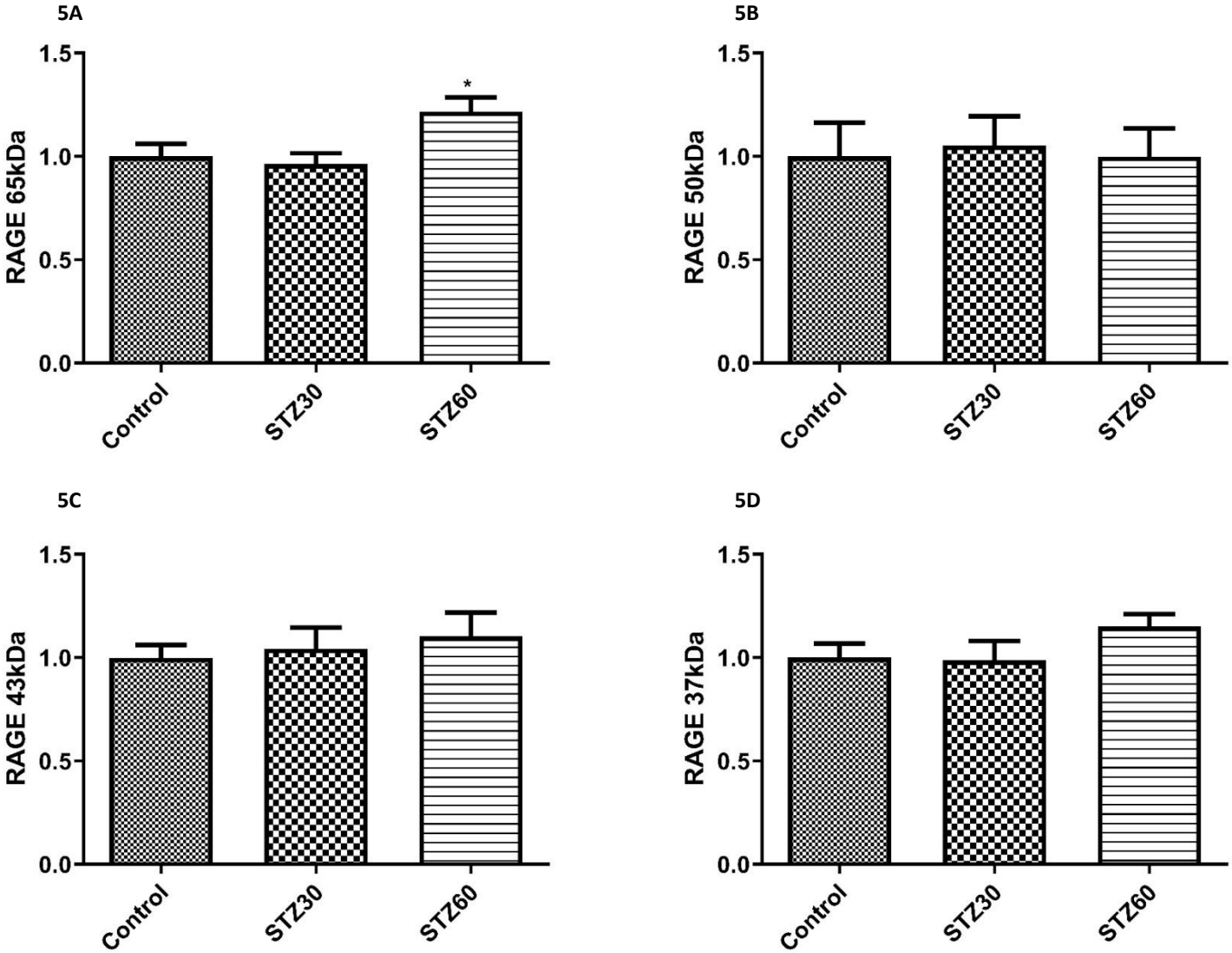


Figure 4: The effect of STZ induction on advanced glycation end products accumulation.

Four different isoforms of AGE proteins were observed A=70kDa, B=43kDa, C=25kDa, D=17kDa, E=Radar plot showing relative distribution of total and individual AGE isomers as a ratio of their respective controls; F=Blot (probed). The global p for A p=0.9; B p=0.9; C p=0.4; D p=0.6.

In the same vein, four different isoforms of RAGE proteins were observed. The testicular tissue of STZ60 animals showed a 21.7% (p=0.054) increase in the expression of RAGE (65kDa) compared to control and a significant increase compared to STZ30 ($p<0.05$). The total combined expression of the four RAGE isoforms was 11.7% higher than that of the control group, while STZ30 animals presented with 1.2% non-significant increase compared to the control group [Figure 5 A-F; SF4].



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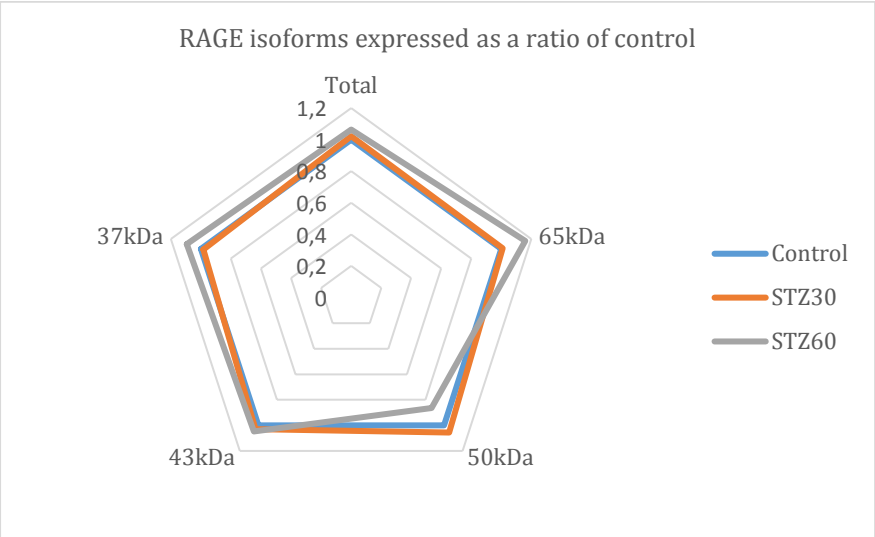

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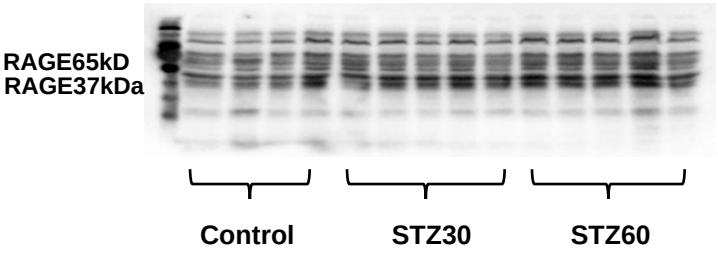


Figure 5: The effect of STZ induction on receptor for advanced glycation end products accumulation. Four different isoforms of RAGE proteins were observed A=65kDa, B=50kDa, C=43kDa, D=37kDa, E= Radar plot showing relative distribution of total and individual RAGE isomers as a ratio of their respective controls; F=Blot (probed). *p<0.05 vs. STZ30. The global p for A p=0.02; B p=0.9; C p=0.7; D p=0.2.

Apoptotic Markers

STZ60 animals exhibited a highly significant increase in the expression of cleaved caspase 3 compared to both control ($p<0.001$) and STZ30 ($p<0.001$). Additionally, there was a 30% increase in the total expression of caspase 7 of STZ60 animals compared to control and a 20% increase in STZ30 group compared to the control group ($p=0.056$). Although not significant, both of the STZ groups showed increased PARP cleavage compared to the control ($p=0.059$) [Figure 6 A-F; SF5].

Also observed was a non-significant gradual increase in p38MAPK expression relative to the increase in STZ concentration administered ($p=0.2$). STZ60 animals further presented with a significant increase in the expression of JNK 46kDa ($p<0.05$) compared to the control group. A non-significant increase in JNK55kDa was also observed compared to control (25.6%) and STZ30 (36.3%) respectively ($p=0.1$) [Figure 7 A-F; SF6]

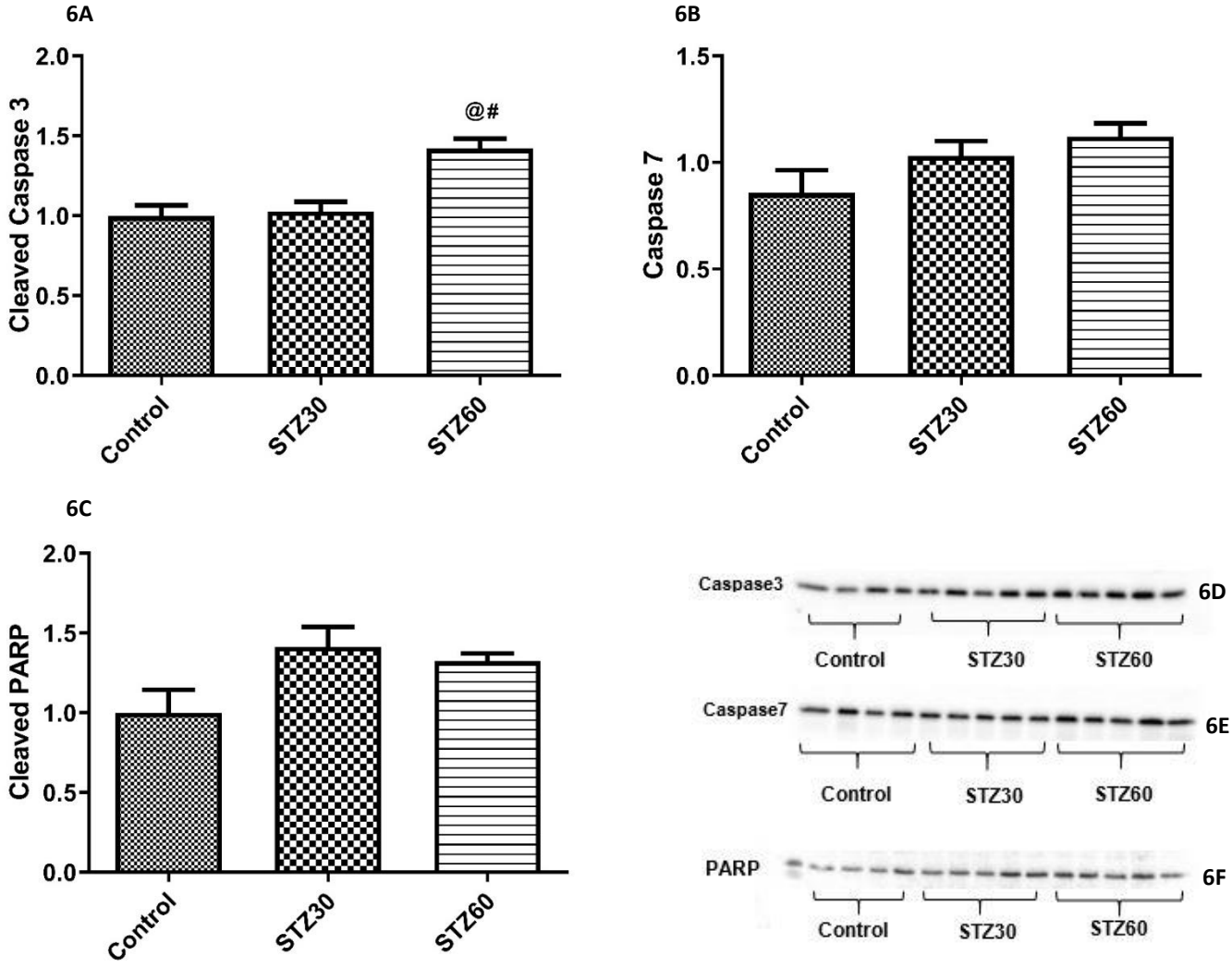
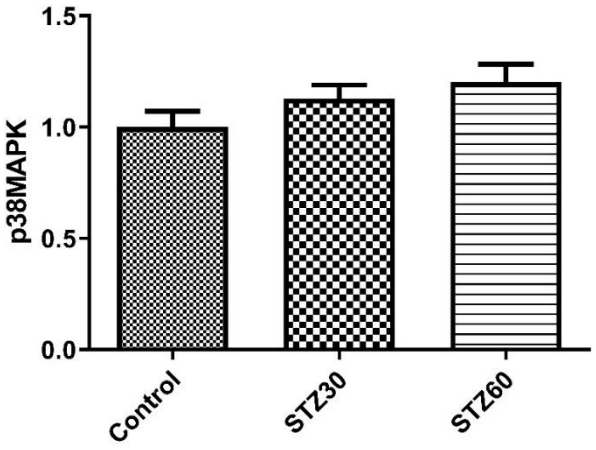


Figure 6: The effect of STZ induction on apoptosis activation. A=Cleaved Caspase 3; B=Caspase 7; C= PARP D=Blot for caspase 3 (probed); E= Blot for caspase 7 (probed) F=Blot for PARP (probed). $^{@}p<0.001$ vs. control; $^{\#}p<0.001$ vs. STZ30. The global p for cleaved caspase 3 p=0.0008, caspase 7 p=0.1, PARP p=0.059.

7A



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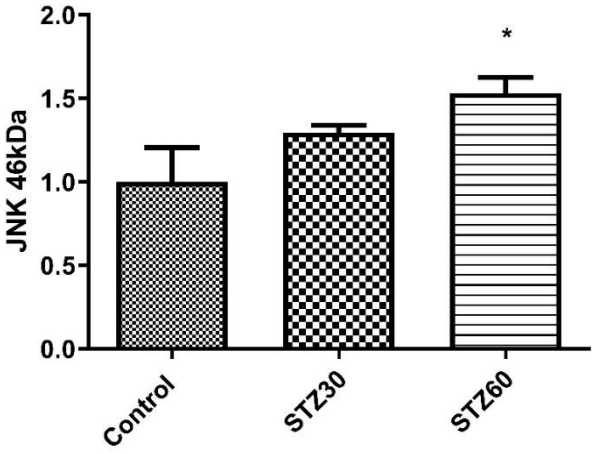

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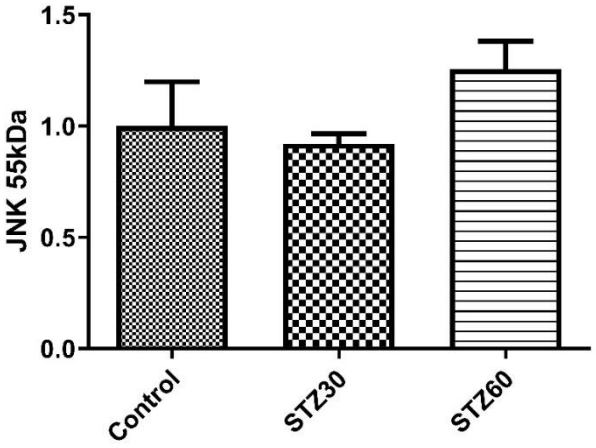

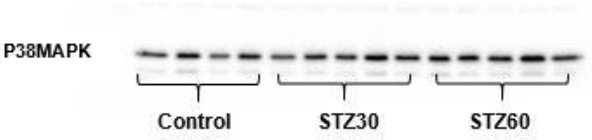

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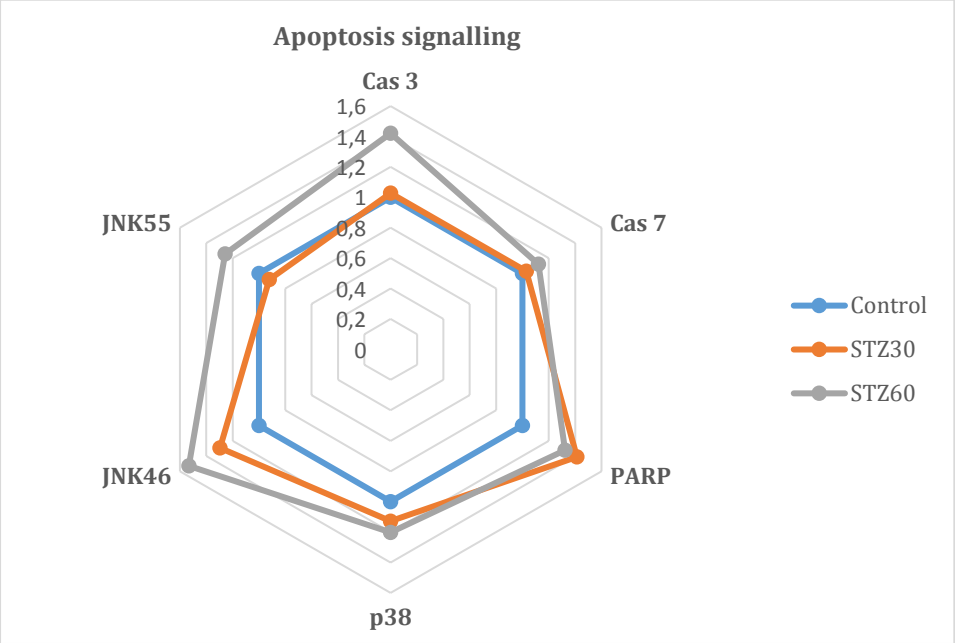

Figure 7: The effect of STZ induction on and the MAPKs signalling. A=p38MAPK; B=JNK46kDa; C=JNK55kDa; D=Blot for p38MAPK; E=Blot for JNK, F=Radar plot for all measured apoptotic and MAPK biomarkers. *p<0.05 vs. control; global p for p38 p=0.1; JNK 55kDa p=0.1; JNK 46kDa p=0.03.

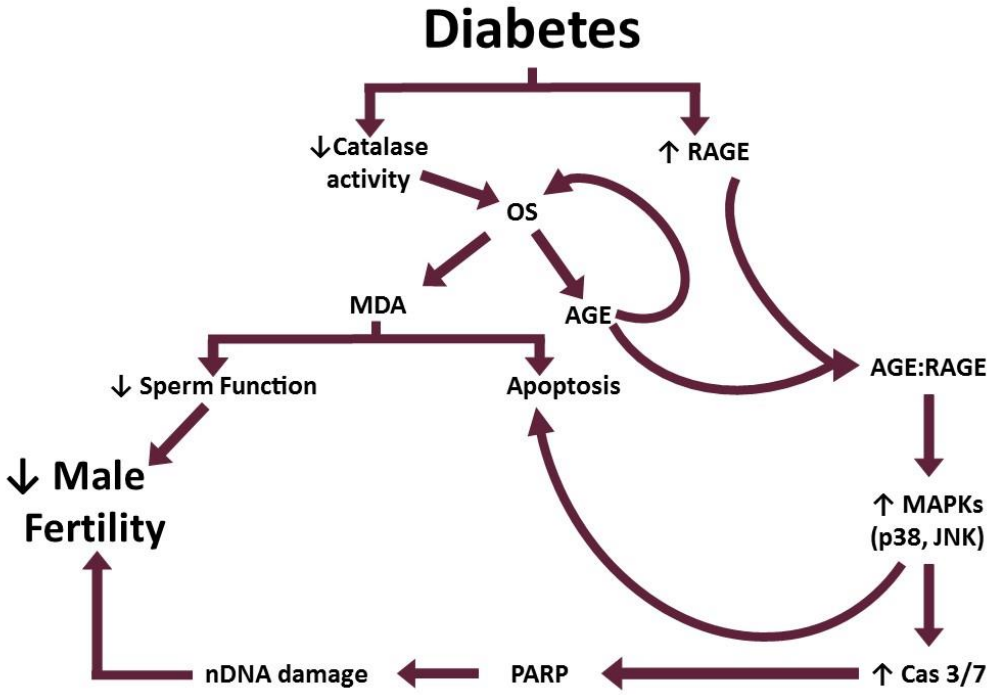

Figure 8. The proposed mechanisms through which diabetes may affect male reproductive function.

↓=decrease, ↑=increase. In DM, there is altered antioxidant enzyme activity (↓Catalase) which may result in OS. The development of OS can on one arm instigate the breakdown of poly unsaturated fatty acids (MDA) and also indirectly increase AGE formation. The peroxidation of PUFA attracts more ROS formation which can activate apoptotic induction, resulting in impaired sperm function. Due to the presence of RAGE ligand, AGE, binding occurs. The AGE-RAGE complex initiates the activation of the MAPKs which may induce apoptosis by increasing the cleavage of PARP, hence resulting in nDNA damage. All these effects collectively result in reduced male fertility.

Discussion

Diabetes has been shown to affect male reproductive function by altering hormone regulation and increasing OS, thereby causing testicular dysfunction and altering spermatogenesis (10,27,28). These effects subsequently result in decreased total and progressively motile spermatozoa and reduced normal sperm morphology (11,17,29). However, the pathway(s) through which the effects are exerted is little understood. Hence, the current study was performed, in order to investigate the possibility of alternative pathways through which DM could affect sperm function.

In the current study, STZ60 animals presented with significantly elevated blood glucose levels to far above the normal reference range, accompanied by a substantial loss in body weight. These are typical features of type 1 DM. STZ30 animals, on the other hand, presented with only a 6.4% non-significant increase in blood glucose, which remained within the normal physiological range. Interestingly, these animals furthermore displayed a significant increase in body weight. The increase in glucose levels and decreased body weight observed in STZ60 animals might be explained by the inability to utilize the glucose derived from the food as a consequence of the ablated pancreatic β-cells and subsequent lack of insulin signalling. Hence, the STZ60 group could be regarded as a typical type 1 DM model (Singh et al., 2009; Vikram et al., 2008).

Diabetic animals from the current study presented with a decrease in the percentage of total and progressively motile spermatozoa. This concurs with Bhattacharya et al. who reported decreased sperm motility parameters in diabetic men (Bhattacharya et al., 2014) and as well supported by Singh et al. who showed impaired sperm motility in STZ induced diabetic rats (30). DM animals of the present study displayed a decrease in catalase activity and increased

MDA levels. This is in agreement with the findings of Adaramoye and Lawal, who similarly reported increased lipid peroxidation and decrease in the activities of catalase, SOD and GPx in diabetic rats (33). Hyperglycaemia has been reported to cause increased OS in various tissues (34), including testicular tissue (35) and spermatozoa (36). This is evidenced by another study that reported elevated MDA levels, lipid hydroperoxides and lipid peroxides in the plasma of type 1 diabetic patients (37). In the diabetic state, lipid peroxidation can be induced by protein glycation and glucose auto-oxidation that can further lead to the formation of free radicals (38). The damage that these free radicals impose on cells may be quantitatively determined by measuring MDA levels which is a product of lipid peroxidation (39). The increase in lipid peroxidation may be as a result of the imbalance between the oxidant and antioxidant activities. This is supported by Buttke and Sandstrom who reported that under an elevated OS status, ROS cause cellular injury via several mechanisms including lipid peroxidation and oxidative damage of proteins and DNA (40). These findings are backed up by other studies which also reported a relationship between ROS and DNA damage (41,42)

Furthermore, an increase in the production of ROS has been shown to cause the non-enzymatic and progressive glycation of proteins with the consequently increased formation of glucose-derived AGEs. The modified proteins further cause an increase in ROS formation and aggravation of OS (43,44) after binding to the ligand-receptor, RAGE.

AGE-RAGE activation has been implicated in the development and progression of several diseases, both metabolic and non-metabolic. This is evidenced by Chen et al. who showed that the excessive production of oxidants in the endothelial progenitor cells (EPCs) resulted in RAGE-mediated AGE accumulation with resultant inhibition of cell proliferation, migration and adhesion which were all attenuated after RAGE blockage (18). In the same vein, inhibition

of RAGE aided the protective effect of glucagon-like peptide-1 on AGE-induced apoptotic neuronal cells (45). Observed in the current study is an increase in the expression of RAGE in the testis of diabetic animals. This result concurs with Mallidis et al. who reported increased RAGE, not only in the testis but also in the epididymis, sperm and seminal plasma of diabetic men. This study further reported that the increased RAGE expression contributed to the elevated levels of damaged nDNA observed in the spermatozoa (46).

An association has been reported between AGE-RAGE activation and MAPKs signalling (47–49). MAPKs are activated in response to external stimuli. Upon external stimulation, mitogen-activated protein kinase kinase kinase (MAPKKK) is phosphorylated thereby activating two different subgroups of mitogen-activated protein kinase kinase (MAPKK) (MKK3/MKK6 and MKK4). These dual kinases phosphorylate and activate p38MAPK and JNK (50). P38MAPK and JNK have been implicated in the development and progression of human diseases. Adhikary et al. described the accumulation of p38MAPK in the interstitial macrophages and myofibroblasts in the kidneys of both diabetic male mice and human. It is associated with increased HbA1c levels, albuminuria and ultimately lead to interstitial fibrosis (51). Additionally, p38MAPK and JNK accumulation have been associated with apoptosis (52,53). Another study reported the activation of p38MAPK followed by stimulation of cytochrome C release, Caspase 8 and 3 activation, PARP cleavage and DNA fragmentation in induced apoptosis of human promyelocytic leukaemia HL-60 cells. The apoptotic events were repressed when p38 was blocked, which suggest that p38 phosphorylation occurred prior to caspase activation (54). It was also showed that p38MAPk is an instigator of Bcl2-x (Bax) activation and mitochondrial apoptosis in primary keratinocytes (55). In the current study, we investigated if diabetes can affect male reproductive function through the p38MAPK-apoptotic pathway. Similar to the previous studies, there was an increase in the level of JNK

and Caspase 3 and an increasing trend in p38MAPK, Caspase 7 and PARP cleavage in the testis of diabetic rats. The activation of p38 has been described to phosphorylate, activate and translocate Bcl2, Bax and Bcl-xl. The translocation causes a change in the mitochondrial membrane potential and thus lead to cytochrome C release which further activates the cleavage of pro-apoptotic caspases. Additionally, JNK has been shown to induce apoptosis through FASL signalling. Increase in JNK expression stimulates FAS/FASL and subsequent phosphorylation of Bid (a Bcl-2 family) and the release of cytochrome C or it recruits caspase 8 through adaptor protein FAAD and thus cleaves caspase 3. Jiang et al. reported activation of the apoptotic pathway, as evidenced by increased expression of cleaved caspase 3 and caspase 8 and subsequently altered spermatogenesis (14). Additionally, Metzler et al reported that spermatocyte loss in AZFc-deleted patients occurs through apoptotic pathway activation (56). Furthermore, a significant association was found between caspase 3 activation and increased sperm DNA fragmentation (57). In corroboration with previous studies, the current study suggests a possible pathway and association between DM and male infertility, as explained in Figure 8.

The current study highlighted the possible inter-connected mechanism through which diabetes may confer its impact on the male reproductive function, which may further contribute to male infertility (Figure 8). We, therefore, conclude that there is an association between AGE-RAGE activation and MAPKs signalling. The increase in the expression of JNK and Caspase 3 and the non-significant increase in the expression of p38MAPK, Caspase 7 and PARP cleavage in the testis of diabetic rats reveals a deadly liaison, which can result in altered sperm function and may further contribute to male infertility.

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Conflict of Interest

The authors have no conflict of interest to declare

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Chapter 6

The effect of rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and sutherlandia (*Lessertia frutescens*) on sperm functional parameters: good, bad or ugly

Will be submitted to Animal Reproduction Science

Temidayo S Omolaoye[1], Stefan S du Plessis[1, 2]*

1. Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa

2. Department of Basic Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates

* Corresponding author:

Prof SS du Plessis

Division of Medical Physiology

Faculty of Medicine and Health Sciences

Stellenbosch University

Francie van Zijl Drive

Tygerberg

7505

South Africa

E-mail: ssdp@sun.ac.za

Abstract

The current study was designed to investigate whether treatment with rooibos, honeybush and sutherlandia will impact sperm functional parameters positively or otherwise, in healthy rats.

Fourteen week old pathogen-free adult male Wistar rats (25-300g) were randomly divided into four groups of ten, including a control group (receiving tap water), rooibos group (RF, receiving 2% fermented rooibos), honeybush (HB, receiving 4% fermented honeybush) and a sutherlandia group (SL, receiving 0.2% unfemented sutherlandia). Animals were sacrificed, the testes and the epididymides were harvested and weighed. Spermatozoa were retrieved from the cauda epididymis for motility, morphology and concentration analysis and the testis was used for all biochemical assays.

The infusion treated animals (RF, HB, and SL) presented with a non-significant decrease of (-14.3%, -18.2%, -17.2%) (-24.8%, -20.7%, -27.3%) in total motility and progressive motility when compared to the control group respectively. There was a significant increase in the number of spermatozoa with slow speed (P=0.03), especially SL treated group compared to the control (P=0.03). There was a percentage reduction in the sperm kinematic parameters of the infusion treated animals. However, they (RF, HB, SL) showed an increase of 2.3%, 14.3%, 19.7% in sperm concentration compared to the control. Additionally, there was an increase of 28.8%, 31.7%, 23% in SOD activity of RF, HB and SL compared to control. This was accompanied with a percentage decrease of -21.1%, -23.7%, 45.9% in MDA levels compared to the control group.

Animals treated with the respective infusions presented with a percentage increase in SOD activity but have reduced sperm motility and decreased normal morphology. Paradoxically,

they presented with increased sperm concentration. Hence, it is concluded that rooibos, honeybush and sutherlandia may enhance sperm concentration which represent the sperm quantity, but, they may impair sperm motility and morphology (sperm quality) when consumed by healthy animals.

Keywords: Sperm; Motility; sperm concentration; Rooibos; Honeybush; Sutherlandia

Introduction

The use of medicinal plants in treating diseases have been used over many decades in traditional medicine. It is interesting to know that the world is becoming more intrigued in exploring the benefits of phytochemicals and to also investigate their importance in health.

Rooibos, honeybush and sutherlandia are indigenous Southern Africa plants, and they are caffeine-free beverages. Rooibos originate from the Cederberg Mountains of the Western Cape region of South Africa (Joubert et al., 2008; McGaw et al., 2007). The leaves and stems are used for the commercially available rooibos tea. Rooibos is generally available either as the unfermented (green rooibos) or the fermented (reddish-brown rooibos) form. The fermented tea is achieved by oxidation that result in the unique reddish-brown leaf colour with woody-fynbos-floral honey flavour (Koch et al., 2012). Whether unfermented or fermented rooibos, studies have shown the presence of numerous bioactive chemical compounds, which include nothofagin, aspalathin, C-5-hexosyl derivative of aspalathin, aspalalinin, phenypyruvic acid-2-0-glucoside, orientin, isoorientin, vitexin, isovitexin, luteolin, luteolin-7-0-glucoside, chyrysoerio, and many more (Beelders et al., 2012). Due to the presence of aspalathin and other phytochemical contents, hot water extracts prepared from green and fermented rooibos are used as food and/or cosmetic ingredients. Although the aspalathin content of unfermented rooibos is greater (8%) than the fermented (2%), the latter is still more preferable in food because of the flavour, and it is considered more economical as there is greater demand for it (de Beer et al., 2017; Joubert and de Beer, 2012). Studies have elucidated the role of rooibos in protecting against disease development (Lawal et al., 2019) and also the ameliorative effects on diverse disorders (Ajuwon et al., 2018; Marnewick et al., 2005). Akinrinmade et

al. suggested that the long-term continuous consumption of fermented rooibos tea confer a mild neuroprotection against ischaemic brain injury in rats. This was evidenced by the decrease in brain oedema, neuronal apoptosis, decreased lipid peroxidation and increase total antioxidant capacity, which cumulatively improved neuro-behavioural outcomes in rats (Akinrinmade et al., 2017). Nash et al. reported that osteoblast (Saos2 cells) treated with luteolin and orientin (from rooibos) displayed elevated alkaline phosphatase and mitochondrial activities. This was associated with reduced toxicity and decreased expression of anti-inflammatory cytokines, which collectively suggest that these rooibos metabolites improved osteoblast mineral content (Nash et al., 2015). Other studies have reported its antioxidant (Hong et al., 2014), anti-diabetic (Dludla et al., 2018; Layman et al., 2019; Orlando et al., 2019; Son et al., 2013) and anti-inflammatory (Pyrzanowska et al., 2019) properties.

Honeybush is native to the Southeast and Southwest coastal areas of South Africa and it forms a part of the fynbos biome with the family name Fabaceae. It has been shown to be used as a traditional tea since the 19th century (Joubert et al., 2011; Schloms and Swart, 2014). Honeybush tea does not only have a pleasant taste and scent, it also contains phytochemicals and volatile organic compounds that are beneficial to health (Joubert et al., 2008). The known health benefits of honeybush are ascribed to its rich bioactive compounds such as Eriodictyol, Hesperetin, Isokuranetin, Naringenin, Chrysoeriol, Luteolin, Kaempferol, Afrormosin, Calycosin, Formononetin, Fujikinetin, Pseudobaptigen, Wistin, Flemichapparin, Medicagol, Sophoracoumestan, Pinitol, Isomangiferin and Mangiferin (Diane L. McKay and Jeffrey B. Blumberg, 2007; Kamara et al., 2007).

Choi et al. reported that honeybush improved skin wrinkles, elasticity and hydration

in patients with crow's feet wrinkles after daily supplementation during their randomized double-blinded controlled trial (Choi et al., 2018). The anti-wrinkle effect of honeybush has been reported in animals (Im et al., 2014) and in vitro studies (Magcwebeba et al., 2016). Furthermore, studies have also shown its antioxidant (Marnewick et al., 2003; Sánchez et al., 2000), anti-mutagenic (Kokotkiewicz and Luczkiewicz, 2009) and mucosa immune therapy (Murakami et al., 2018) effects in both in vivo and in vitro studies.

Sutherlandia has been used in traditional medicine since the 1800s. Sutherlandia has a variety of health benefits which are associated to its bioactive phytochemicals. The chemical compounds include, asparagine, proline and arginine (Moshe et al., 1998; van Wyk and Albrecht, 2008), γ-aminobutyric acid (GABA), L-carvanine, (Ortega, 2003), sutherlandin A, sutherlandin B, sutherlandin C, and sutherlandin D) (Avula et al., 2010), sutherlandioside A, sutherlandioside B, sutherlandioside C, sutherlandioside D (Avula et al., 2010) and cycloartane-type triterpene glycoside (van Wyk and Albrecht, 2008). Based on the array of pharmacological products present in sutherlandia, studies have highlighted its role in cancer (Grandi et al., 2005; Tai et al., 2004), Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (Harnett et al., 2005; Mills et al., 2005; Wilson et al., 2015), diabetes (Chadwick et al., 2007; J. et al., 2012; Sia, 2004), inflammation (Lei et al., 2015; Vasaikar et al., 2018) stress (Chuang et al., 2015; Sergeant et al., 2017) and oxidative stress (Fernandes et al., 2004; Tobwala et al., 2014).

With regards to the effects of these infusions on male reproduction, very few studies are available. This include a study that reported improvement in sperm motility, sperm concentration and sperm viability after treatment with rooibos in rats (Opuwari and Monsees, 2014). Ros-Santaella and Pintus showed that the in vitro treatment of boar

semen with rooibos protected the sperm acrosome structure, which was accompanied by improved sperm velocity (Ros-Santaella and Pintus, 2017). Additionally, Awoniyi et al. suggested that rooibos may provide a measure of protection against induced oxidative damage by elevating antioxidant activities and thus improve sperm function (Awoniyi et al., 2012).

However, to the best of our knowledge, there are no studies reporting the effect of honeybush or sutherlandia on male reproduction. Despite the numerous health benefits of these infusions, there are concerns as to their safety in long-term use (Fantoukh et al., 2019). Opuwari and Monsees reported that lengthened exposure to rooibos induced acrosome reaction in rats, which can subsequently lead to impaired reproduction (Opuwari and Monsees, 2014). Additionally, the toxicity of canavanine (from sutherlandia) has been implicated in lupus erythematosus syndrome. Long-term exposure to sutherlandia have likewise been reported to cause protein cross-links and it can as well result in autoimmunity and teratogenicity. Ngcobo et al. showed that the in vitro chronic treatment of normal T-lymphocyte cells with sutherlandia was toxic (Ngcobo et al., 2012), which means too much of sutherlandia may be bad. Therefore, the current study was designed to investigate whether treatment with rooibos, honeybush and sutherlandia will impact sperm functional parameters positively or otherwise, in healthy rats.

Materials and methods

Animal care

Fourteen week old healthy pathogen-free adult male Wistar rats (250-300g) were housed at the Stellenbosch University's Faculty of Medicine and Health Sciences' Animal Unit at room temperature (18-23°C), under a normal 12:12 light/dark cycle. Animals were caged individually, had free access to food (standard raw chow) and water/infusions and were treated according to the recommendations of the Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council, 2010). Ethics approval was obtained from the Stellenbosch University Animal Ethics Committee (SU-ACUD17-00016).

Infusion preparation

Fermented rooibos (2%, Carmien SA PTY LTD, South Africa), fermented honeybush (4%, Afrinaturals, South Africa) and unfermented sutherlandia (0.2%, Afrinaturals, South Africa) were prepared according to standard protocols. Preparation procedure conformed to the experimental established protocols for rooibos (Marnewick et al., 2011), honeybush (Toit And Joubert, 1999) and sutherlandia (Tobwala et al., 2014). In brief, 2% rooibos was prepared by adding 20g of dried fermented rooibos in 1litre of boiling water and allowed to steep/rest for 30-45 minutes. The mixture was filtered three times using a cheesecloth, number 4 filter paper and number 1 filter paper (Whatman™, Buckinghamshire, UK) respectively. Filtered infusions were transferred to a dark bottle plastic container and stored at 4°C. Honeybush

(4%; 40g in 1L) was prepared following the similar steps as used for making rooibos tea. However, due to the bitter taste of sutherlandia, the concentration was reduced to 0.2%. Briefly, 40g of unfermented sutherlandia was prepared as described for rooibos, from the initial 4% solution, 2.5ml was diluted in 50ml of water to give the working concentration of 0.2%.

All infusions (rooibos, honeybush and sutherlandia) were prepared every other day (48 hours). The herbal teas serve as the only drinking fluid for these infusion groups. The fluid intake of the animals was measured three times a week.

Experimental design

Forty animals were randomly divided into four groups of ten. The groups include a control group, which received tap water, rooibos group (RF) (2% fermented), honeybush (HB) (4% fermented) and a sutherlandia group (SL) (0.2% unfermented). Animals were sacrificed after 7 weeks of treatment. Blood, testes and epididymides were harvested. Blood was collected into a heparized tube, placed on ice for 30 minutes and afterwards centrifuged (3000rpm at 4°C for 30 minutes). Plasma was stored at -80°C until further analysis. The testis was used for all biochemical analysis, while the caudal area of the epididymis was dissected for sperm retrieval. Relative testicular/epididymal weight were calculated by expressing the tissue weight as a percentage of body weight.

Sperm functional parameters

Motility

Sperm retrieval and motility analysis were carried out as described by (Omolaoye et al., 2018). Briefly, the left epididymis was rinsed in a petri dish containing 2ml Hams F-12 nutrient media (Sigma Chemicals, St Louis, MO, USA) at 37°C. The caudal area of the rinsed epididymis was dissected and spermatozoa were retrieved into a separate 2ml Hams, at 37°C. After 30 seconds of retrieval, 2µl of the sperm solution was infused into a 20µl depth chamber slide (Leja, Netherlands) and placed on a Nikon Eclipse E200 microscope with an in-built heating stage (37°C). Sperm motility was measured via computer-aided sperm analysis (CASA) using the Sperm Class Analyser (SCA 6.3, Microptic, Barcelona, Spain). Total motility, progressive motility and the sperm kinematic parameters (curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straight-line index (STR), linearity index (LIN), sperm oscillation index (WOB), amplitude lateral head (ALH) and beat frequency (BCF) were analysed. After analysing sperm motility at 30 sec, sperm concentration was measured by dissecting the caudal epididymis into smaller pieces and was left for a further 5 minutes allowing a maximum number of spermatozoa to swim out. The pieces were removed after 5 minutes and the sperm solution was mixed homogenously. Of the 2ml solution, 10µl was diluted in 50µl of Hams and sperm concentration was analysed through CASA.

Morphology

From the sperm solution used for sperm concentration analysis, 10µl was smeared on a glass slide and allowed to air dry. The dried slides were stained with Sperm Blue® fixative

and stain (Microptic SL, Barcelona, Spain) for 3 minutes, rinsed for 3 seconds to remove excessive stain, air dried and then mounted (DPX, HiMedia Laboratories Pvt.Ltd., Mumbai, India). Sperm morphology was analysed through computer-aided sperm morphometry analysis (CASMA) using the SCA® (Maree et al., 2010)

Testosterone and Estradiol

The plasma concentration of testosterone (E-EL-0072) and estradiol (E-EL-0065) were measured using a commercially available ELISA kit (Elabscience Biotechnology, Hubei) as per manufacturer's instructions.

Biochemical analysis

Superoxide dismutase activity

Testicular tissue samples were homogenized in lysis buffer (Na3PO4, 0.5% Triton X-100) and centrifuged at 15000rpm for 20 minutes at 4°C. Tissue homogenates were diluted to 10x in deionized water. From the diluted standards and samples, 10µl were dispensed into the microplate wells in triplicate, followed by adding 170µl of diethylenetriaminepentaacetic acid (DETAPAC) and 5µl of SOD assay buffer (50mM Na/K Phosphate buffer at PH 7.4). SOD activity was measured on a plate reader (Multiskan spectrum) at 490nm, 25°C for 5minutes at 1 minute interval using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.immediately after adding 15µl of freshly prepared 6-hydroxydopamine (6-OHD) into the well.

Catalase

Tissue homogenates were obtained as described for SOD. From the diluted samples and standards, 5µl were loaded in triplicate into UV microplate wells. Catalase assay buffer (170µl) were added into each well and lastly, 50µL of H_2O_2 was added into the wells and analysis was performed immediately on a plate reader (Multiskan spectrum) at 240nm every 60 seconds over a 5 minute period using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software.

MDA levels

Testicular tissue samples were homogenized in lysis buffer (0.1M KPi, 1.15% KCl) by bullet blending at speed 9 for 3 minutes with a 1-minute interval in-between. MDA levels in the testis was determined by pipetting 100µl of standards and samples into corresponding 10ml glass tubes, followed by adding 1ml of SDS and 2ml of 10%TCA-BHT buffer solution. Samples were vortexed, and after resting for 10 minutes, 2ml of TBA was added and vortexed again. The standards and samples were covered with marbles and incubated in a water bath (1 hour at 100^{o}C), where after it was cooled on ice for 15 minutes. The standards and samples were centrifuged (3000rpm, 15 minutes 4°C) and the supernatants retrieved. From the supernatants, 250µl of each standard and sample were loaded in triplicate into microplate wells and analysed on a plate reader (Multiskan spectrum) at a 532nm wavelength within 30 minutes after centrifugation.

Statistics

GraphPad Prism™ software (GraphPad™ Software, Version 8.2, CA, USA) was used. Normal data distribution was measured using the Anderson-Darling and Kolmogorov-Smirnov normality tests. When data passed all normality tests, a one-way ANOVA of variance with a Tukey's Post-hoc Test were performed. Where data were not evenly distributed, a Kruskal-Wallis test and a Dunns Post-hoc Test were carried out. A probability level of $P< 0.05$ was considered statistically significant and results are expressed as mean ± SD.

Results

Anthropometric data

After 7 weeks of treatment, no significant differences were observed in the body, testicular and epididymal weights between any of the groups [Table 1]. However, the SL group presented with a higher (7%) percentage epididymal weight mean compared to the RF group (*P*=0.07). The RF treated animals displayed a significantly lower (-7.5%) relative testicular weight compared to the HB group (*P*=0.04), while there was no significant difference in relative epididymal weight between the groups (*P*=0.3) (Table 1).

Table 1: Body and tissue weights

Parameters	Control	Rooibos (RF)	Honeybush (HB)	Sutherlandia (SL)	Global *P*
Bodyweight (g)	344,6±19,04	349,4±21,41 (1.4%)	345,3±25,55 (0.2%)	352,6±19,37 (2.3%)	0,8224
Testicular weight (g)	1,414±0,12	1,346±0,08 (-4.8%)	1,438±0,12 (1.7%)	1,423±0,11 (0.6%)	0,1492
Epididymal weight (g)	0,4702±0,03	0,4560±0,025 (-3.0%)	0,4753±0,046 (1.1%)	0,4892±0,022 (4.0%)	0,0618
Relative testicular weight (%)	0,4107±0,03	0,3858±0,025[a] (-6.1%)	0,4174±0,034 (1.6%)	0,4034±0,018 (-1.8%)	0,0588
Relative epididymal weight (%)	0,1367±0,01	0,1308±0,008 (-4.3%)	0,1381±0,015 (1.0%)	0,1389±0,004 (0.6%)	0,3406

[a]*P*<0.05 vs. Honeybush, values in brackets () denotes the percentage change from control, n=10

Sperm functional parameters

The infusion treated animals (RF, HB, and SL) presented with a non-significant decrease (-14.3%, -18.2%, -17.2%) (-24.8%, -20.7%, -27.3%) in total motility and progressive motility when compared to the control group respectively [Figure 1A-B]. Although not significant, the rapid progressive motility of RF, HB and SL animals reduced by 23%, 28% and 37% respectively (Table 2), and there was a significant increase in the number of spermatozoa with slow speed (P=0.03), especially the SL treated group compared to the control (P=0.03) (Table 2). All sperm kinematic parameters of the infusion treated animals were non-significantly reduced compared to the control (Table 3). RF, HB and SL treated animals displayed a decrease in VCL (-14.3%, -18.8%, -23.7%); VAP (-16.5%, -22%, -27.6%); VSL (-20%, -25.5%, -33.2%); STR (-11.1%, -8.5%, -14.9%); LIN (-15.8%, -14.6%, -22.6%) WOB (-8%, -9%, -10%); ALH (-14.8%, -15.9%, -20.5%) and BCF (-16.3%, -18.7%, -26.3%), compared to the control group respectively (Table 3). Interestingly, infusion treated groups displayed a non-significant increase in sperm concentration compared to the control group [Figure 2]. However, there was no significant differences in the percentage of morphologically normal spermatozoa between the groups, but RF and SL are on the decrease compared to the control (Table 4).

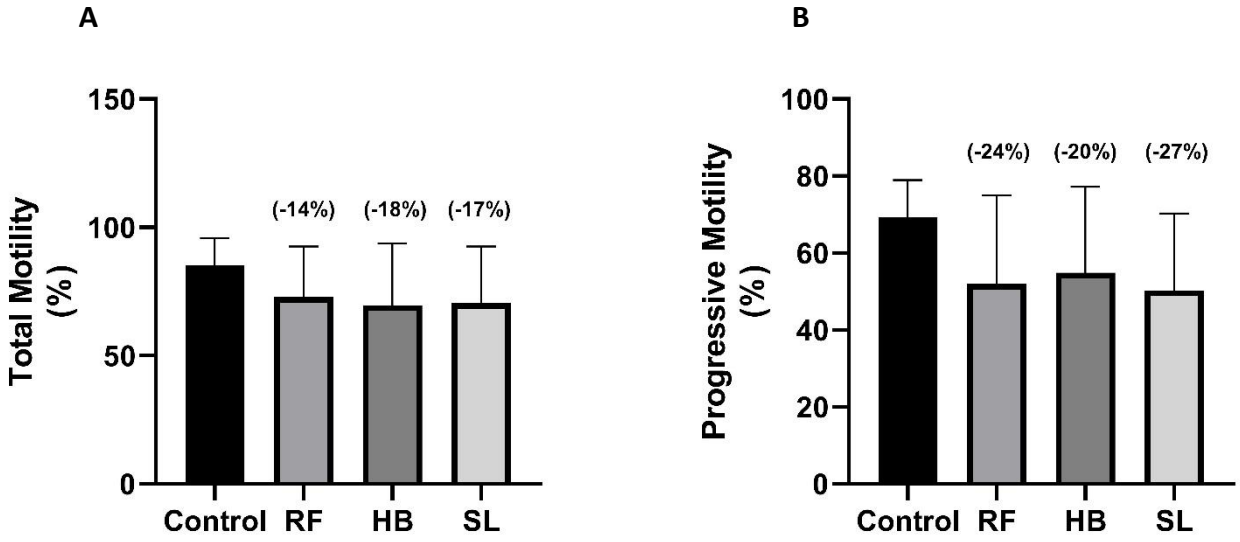

Figure 1: Sperm total and progressive motilities at 30 seconds. **A.** total motility, **B**. progressive motility, RF=rooibos, HB=honeybush, SL=sutherlandia, values in brackets () denotes the percentage change from control, n=10

Table 2: Sperm speed and progressive motilities as measured at 30 seconds

Parameters	Control	Rooibos (RF)	Honeybush (HB)	Sutherlandia (SL)	Global *P*
Rapid speed (%)	53,71±18,53	42,88±24,49 (-20.2%)	38,68±24,10 (-31.7%)	36,94±22,33 (-31.2%)	0,3534
Medium speed (%)	17,64±9,681	9,319±6,486 (-47.2%)	12,43±6,924 (-29.5%)	10,24±6,747 (-41.9%)	0,0820
Slow speed (%)	13,79±5,225	20,68±8,814 (49.9%)	18,52±5,651 (34.3%)	23,27±8,272[a] (68.7%)	0,0369
Rapid progressive motility (%)	24,19±11,35	18,61±12,91 (23%)	17,38±12,00 (28%)	15,08±9,40 (37%)	0,2919

Medium progressive motility (%)	41,59±10,73	29,83±22,41 (-28.2%)	29,77±17,24 (-28.4%)	27,15±16,92 (-34.7%)	0,2619
Non-progressive motility (%)	19,36±6,893	24,45±8,454 (26.2%)	22,48±6,192 (16.1%)	28,23±7,948 (45.8%)	0,0746

[a]*P*<0.05 vs. control, values in brackets () denotes the percentage change from control, n=10

Table 3: Sperm kinematic parameters as measured at 30 seconds of retrieval

Parameters	Control	Rooibos (RF)	Honeybush (HB)	Sutherlandia (SL)	Global *P*
VCL (µm/s)	191,1±48,70	163,7±40,97	155,1±35,23	145,8±30,83	0,0799
VAP (µm/s)	87,85±25,67	73,29±28,39	68,47±20,24	63,52±21,67	0,2031
VSL (µm/s)	53,55±18,42	42,75±15,01	39,87±12,19	35,77±13,33	0,1217
STR (%)	58,55±5,960	52,03±6,885	53,56±7,608	49,80±7,788	0,0577
LIN (%)	28,53±3,798	24,00±7,050	24,36±6,019	22,08±6,994	0,1348
WOB (%)	45,99±3,302	42,29±9,588	41,84±6,011	41,25±8,967	0,4825
ALH (µm)	7,360±1,363	6,269±1,688	6,186±1,451	5,846±1,063	0,1088
BCF (Hz)	16,65±3,116	13,92±3,311	13,53±2,933	12,26±4,292[a]	0,0504

[a]*P*<0.05 vs. control, n=10

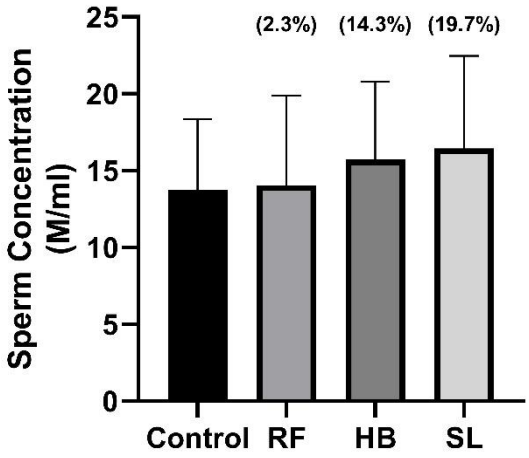

Figure 2: Sperm concentration measured after 5 minutes of retrieval. RF=rooibos, HB=honeybush, SL=sutherlandia, values in brackets () denotes the percentage change from control, n=10

Table 4: Sperm normal morphology and morphometric parameters

Parameters	Control	Rooibos (RF)	Honeybush (HB)	Sutherlandia (SL)	Global *P*
Head arc	21,70±0,491	21,47±0,770 (-1.0%)	21,59±0,440 (-0.5%)	21,96±0,319 (1.2%)	0,2159
Linearity	53,10±2,126	52,43±2,857 (1.3%)	52,35±1,550 (-1.4%)	52,50±1,984 (-1.1%)	0,8636
Width	1,436±0,103	1,467±0,083 (2.15%)	1,451±0,117 (1.0%)	1,409±0,148 (-1.88%)	0,5116
Normal morphology (%)	59,60±11,350	58,70±9,262 (-1.5%)	59,80±16,210 (0.3%)	54,20±17,780 (-8.3%)	0,7907

Values in brackets () denotes the percentage change from control, n=10

Hormones and biochemical assays

There were no statistical difference in the concentration of testosterone and estradiol between the groups [Figure 3A-B]. However, there was an increase of 28.8%, 31.7%, and 23% in SOD activity, decrease of -11.3%, -16.6%, 23.7% in catalase activity and a decrease of -21.1%, -23.7%, 45.9% in MDA levels of RF, HB and SL groups when compared to the control group respectively [Figure 4A-C].

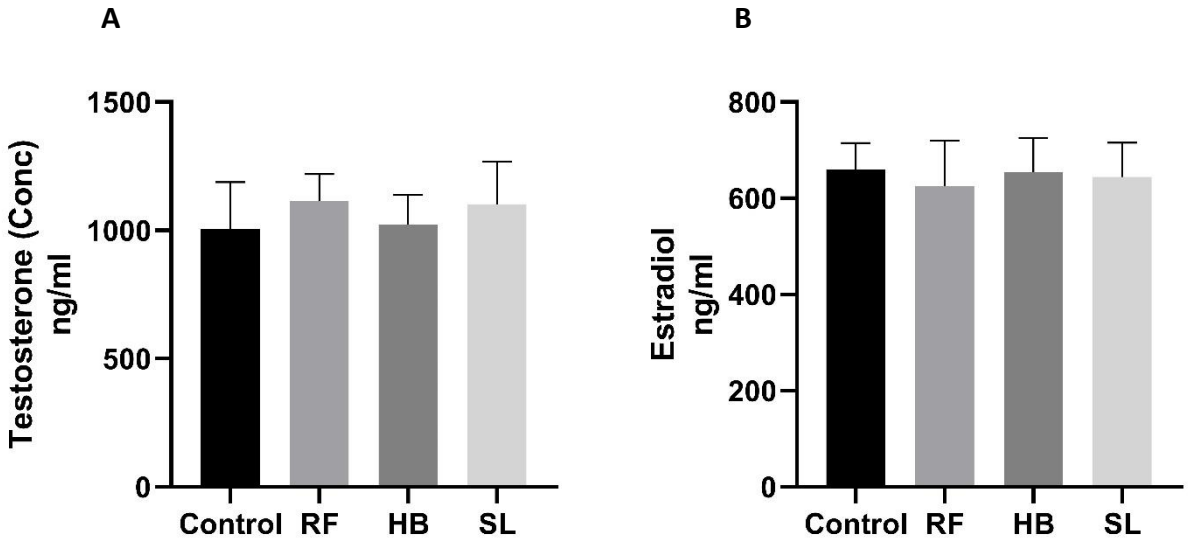

Figure 3: Testosterone and estradiol concentration. **A**. testosterone, **B**. estradiol, RF=rooibos, HB=honeybush, SL=sutherlandia, n=9

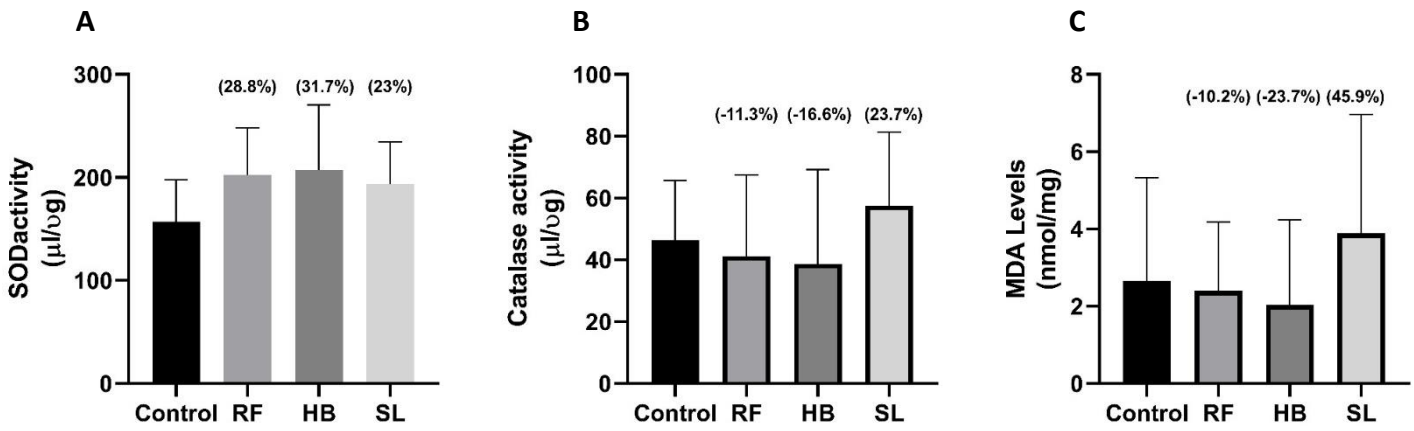

Figure 4: Oxidative stress parameters. **A**. SOD activity, **B**. catalase activity, **C**. MDA levels, RF=rooibos, HB=honeybush, SL=sutherlandia, values in brackets () denotes the percentage change from control, n=10

Discussion

The use of natural products in the prevention and treatment of diverse diseases is increasing. This is due to the availability of phytochemical bioactive compounds in these plants, which are able to mimic the pharmacokinetics of synthetic drugs with fewer resultant adverse effects (Diane L. McKay and Jeffrey B. Blumberg, 2007; Mills et al., 2005; van Wyk and Albrecht, 2008). Rooibos, honeybush and sutherladia are plants with bioactive compounds and they have been shown to have numerous health benefits (van Wyk and Albrecht, 2008). However, only a few studies have been performed regarding their effects on male reproductive function. From these few studies, concerns regarding long term use have already been highlighted. Opuwari and Monsees reported that long-term consumption of rooibos resulted in spontaneous acrosome reaction, which may impair male reproduction (Opuwari and Monsees, 2014). The current study investigated the effects of rooibos, honeybush and sutherlandia on male reproductive functional parameters in rats.

After 7 weeks of treatment, there was no significant difference in body and organ (testes and epididymides) weights. This is similar to the report of Opuwari and Monsees, who also showed no significant difference in the body and tissue weight gain after 52 days of rooibos consumption in rat (Opuwari and Monsees, 2014). Many natural products of plant origin contains flavonoids, and flavonoids are considered endocrine disruptors (Patisaul and Jefferson, 2010). Chandra et al. reported that green tea administered in relatively high dose inhibited the activities of testicular 3β and 17β-hydoxy-steriod dehydrogenase, decreased serum testosterone and reduced testicular weight, which cumulatively resulted in inhibition of spermatogenesis. This suggest that high dose of this

tea may impair the morphological and normal function of the testis (Das and Karmakar, 2015). Regarding rooibos, an in vitro study reported decrease in the production of testosterone by the Leydig cells after treatment with rooibos (Opuwari and Monsees, 2015). However, In the current, although RF animals presented with a decrease in relative testicular weight, there was no significant difference in the serum concentration of testosterone, but the estradiol levels decreased by 5%. The observed result of testosterone in the current study is supported by a study that reported no significant difference in serum testosterone after administration of both fermented and unfermented forms of rooibos in rats (Opuwari and Monsees, 2014). The role of rooibos as endocrine disruptor still remains inconclusive, because, recently, Noh reported that MR-10, a novel complex of dandelion and rooibos increases testosterone levels and also improved sperm production in older (>45) men (Noh, 2018). Hence, more studies are required to ascertain the role of rooibos on the endocrine regulation of male reproduction. Honeybush has been shown to have mild oestrogenic activity as it was reported to induce proliferation of oestrogen-insentive MDA-MD-231 cells (Verhoog et al., 2007). As previously stated, studies are lacking on its role in male reproduction, thus more studies are required to investigate the role on male reproduction.

In the current study, the effects of rooibos, honeybush and sutherlandia on the sperm motility, concentration and morphology represent an ilicit/confusing situation. Animals treated with the respective infusion (RF, HB, SL) presented with a decrease in all sperm kinematic parameters. This is in contrast to the report of Ros-Santaella and Pintus. They showed that after treating boar semen with rooibos, there was an increase in sperm kinematic parameters (Ros-Santaella and Pintus, 2017). The contrasting result may be due to the difference in models. Additionally, there was a decrease in the total motility (-14%,

-18%, -17%), progressive motility (-24%, -20%, -27%) and normal sperm morphology (-1.5%, 0.3% and -9%) of animals treated with RF, HB and SL, with SL animals showing a significant increase in the percentage of spermatozoa with slow speed. The results of the total and progressive motilities of the current study are in contrast with the reports of Awoniyi et al. and Ayeleso et al.(Awoniyi et al., 2012; A. O. Ayeleso et al., 2014). The difference may be due to the health status of animals analysed. Awoniyi et al. reported an increase in the total and progressive motilities of diabetic animals treated with rooibos and Ayelso et al. showed an improvement in the total and progressive motilities of animals that were induced with OS. Additionally, from data obtained in our laboratory (SURRG Stellenbosch University Reproductive Research Group), we observed that diabetic animals treated with fermented rooibos displayed an increase that was up to 5% in total and progressive motilities when compared to non-diabetic animals (un-published work). This suggest that rooibos consumption may be beneficial in a diseased state, especially, DM-related male reproductive function impairment in rats. However, when consumed by healthy rats, it may reduce sperm motility. Since studies on honeybush and sutherlandia are lacking, little can be said about them. We can speculate that when administered to healthy animals, there is a possibility of sperm function impairment since they follow the same trend as rooibos.

Interestingly, animals treated with these infusions displayed a non-significant increase in sperm concentration. This is partly supported by Opuwari and Monsees. They reported that unfermented rooibos supplementation significantly enhanced sperm concentration in rats (Opuwari and Monsees, 2014). Several studies have shown the antioxidant potential of rooibos, honeybush and sutherlandia on diverse diseases (A. Ayeleso et al., 2014; Fernandes et al., 2004). In like manner, the RF, HB and SL animals of the current study

presented with an increase in SOD activity accompanied with reduced MDA levels in RF and HB groups, with SL group showing higher MDA levels. The former is supported by several studies that have shown increased testicular antioxidant enzyme activity after treatment with rooibos either in disease or health (Awoniyi et al., 2012; A. O. Ayeleso et al., 2014; Opuwari and Monsees, 2014). Although, SOD activity was increased in the infusion treated groups of the current study, the sperm motility and morphology were adversely affected. The mechanisms behind these outcomes were not investigated, but it can be speculated that, the increased SOD and reduced catalase activities observed are compensatory responses. As SL that displayed higher SOD and catalase activities, resultantly showed a non-significant 45% increase in MDA levels.

Conclusion

The present study have evaluated the role of rooibos, honeybush and sutherlandia on sperm functional parameters in healthy rats. Animals treated with the respective infusions presented with percentage increase in antioxidant enzyme activity but have reduced sperm motility and decreased normal morphology. Paradoxically, they presented with increased sperm concentration. Hence, it is concluded that rooibos, honeybush and sutherlandia may enhance sperm concentration, which represent sperm quantity, but they may impair sperm motility and morphology (sperm quality) when consumed by healthy animals.

Thus, caution should be taken regarding the amount of teas' consumed when healthy, as it can affect the antioxidant shift (antioxidant paradox). That is, too much of antioxidants can push into the opposite state of oxidative stress and rather lead to reductive stress.

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Conflict of interest

The authors have no conflict of interest

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Chapter 7

Testicular Oxidative Stress and Apoptosis Status in Streptozotocin-induced Diabetic Rats after Treatment with Rooibos, Honeybush and Sutherlandia Infusions

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Temidayo S Omolaoye[1], Stefan S du Plessis[1, 2]*

1. Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa

2. Department of Basic Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates

* Corresponding author:

Prof SS du Plessis

Division of Medical Physiology

Faculty of Medicine and Health Sciences

Stellenbosch University

Francie van Zijl Drive

Tygerberg

7505

South Africa

E-mail: ssdp@sun.ac.za

Abstract

This study aimed to investigate the testicular oxidative stress and apoptosis status in streptozotocin (STZ) induced diabetic rats following treatment with rooibos, honeybush and sutherlandia infusions. Diabetes was induced by injecting fourteen week old adult male Wistar rats (250-300g) with a single intraperitoneal injection of STZ (45mg/kg). Fifty rats were randomly divided into five groups. The groups included a vehicle (Veh) (citrate buffer), a diabetic control (DC) (STZ45mg/kg), a diabetic + rooibos (DRF) (STZ45mg/kg + 2% rooibos), a diabetic + honeybush (DHB) (STZ45mg/kg + 4% honeybush) and a diabetic + sutherlandia (DSL) (STZ45mg/kg + 0.2% sutherlandia) group. Animals in the infusion treatment groups started receiving the respective infusion one week before diabetes induction for acclimatization. Animals were sacrificed after 7 weeks of DM induction. The testes and the epididymides were harvested and weighed. Spermatozoa was retrieved from the cauda epididymis for motility, morphology and concentration analysis and the testis was used for all biochemical assays. After diabetes induction, DC, DRF, DHB and DSL animals presented with a significantly elevated blood glucose levels compared to the Vehicle group ($p<0.0001$). DC animals presented with a reduction in sperm progressive motility (-15.17%), while DRF and DSL animals displayed a respective increase (26.2%, 15%) in progressive motility compared to DC. DHB, on the other hand, showed a decrease (-17%) in progressive motility. Additionally, the percentage of morphologically normal spermatozoa was increased by 13%, 16%, and 15% after treatment with rooibos, honeybush and sutherlandia respectively. DRF, DHB and DSL animals displayed an increase (37.4%, 53.7%, and 48%) in SOD activity compared to DC respectively, which was accompanied by a reduction in lipid peroxidation, as measured by malondialdehyde (MDA) levels. However, they (DRF, DHB and DSL) showed an increase in the expression of Caspase 7, PARP and p38MAPK and a decrease in the X-linked inhibitor of

apoptosis protein (XIAP). The results of the current study suggests that rooibos, honeybush and sutherlandia infusions may partly play a role in alleviating DM-induced sperm function impairment through the suppression of OS, but their role in apoptosis is still unclear, hence further investigations are required.

Keywords: Diabetes, Sperm, oxidative stress, apoptosis, Rooibos, Honeybush, Sutherlandia

1.0 Introduction

Diabetes mellitus (DM) is a metabolic disorder which results either from a lack of insulin secretion or the insensitivity of the target tissue to the effect of insulin (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). DM is one of the non-communicable diseases that poses threat to the general health, including male reproductive health (Agbaje et al., 2007; Bhattacharya et al., 2014; Delfino et al., 2007; Giacco and Brownlee, 2010; Li et al., 2004; Mallidis et al., 2009; Roessner et al., 2012). Studies have highlighted some of the negative impacts of DM on male fertility, this include endocrine function deregulation, testicular dysfunction, spermatogenesis disruption, reduced sperm motility and decreased normal sperm morphology (Amaral et al., 2006; Chen et al., 2016; Khaki et al., 2010). One of the pathways through which DM exert its effect on male fertility is through the development of oxidative stress (OS) and the subsequent induction of apoptosis (Allan et al., 1992; Reiter et al., 2000). Kanter et al. showed that after DM induction in rats, there was an increase in testicular malondialdehyde (MDA) levels with reduced antioxidant enzyme activities in glutathione peroxidase (GPx) and superoxide dismutase (SOD). This subsequently resulted in severe testicular damage as evidenced by disrupted seminiferous tubule structure. Also observed was the increased number of spermatozoa with fragmented DNA following apoptosis activation (Kanter et al., 2012). Additionally, Chen et al. reported a significant decrease in the testicular antioxidant activities (SOD, catalase (CAT)) and increase in MDA and ROS levels in DM rats. This was followed by an elevated Bax/Bcl-2 ratio, which is indicative of apoptosis (Chen et al., 2016). This phenomenon has been described by several other authors (Guneli et al., 2008; Jiang et al., 2013; Roessner et al., 2012). Hence, it is evident that DM negatively impact male fertility. Since it has been reported that DM affects 422 million people globally (World Health Organization, 2014), including men of reproductive age

(Nijpels, 2016), it is therefore essential to explore every possible strategy to combat DM and its associated male reproductive complications.

Rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and sutherlandia (*Lessertia frutescens*) are plants endemic to Southern Africa (Joubert et al., 2011, 2008; McGaw et al., 2007; Morton, 1983; Schloms and Swart, 2014; van Wyk and Albrecht, 2008). They are caffeine-free beverages derived from the leaves and stems of their respective plants.

The infusion derived from rooibos contain diverse bioactive compounds, including dihydrochalcones, cyclic dihydrochalcone, phenylpropenoids, flavones, flavonols, and the flavanones (Beelders et al., 2012). Honeybush contains compounds such as flavanones, flavones, isoflavonols, phenolic acid, Inositols and Xanthones (Diane L. McKay and Jeffrey B. Blumberg, 2007; Kamara et al., 2007), while, sutherlandia has phytochemicals such as flavonoids, non-protein amino acids, free amino acids and cycloartanol glycosides (Avula et al., 2010; Ortega, 2003).

Due to the presence of rich bioactive compounds in these teas, studies have explored their role in different systems and cells as antioxidants (Awoniyi et al., 2012; Hong et al., 2014; Marnewick et al., 2003; Pheiffer et al., 2013), anti-inflammatory agents (Marnewick et al., 2009), anti-carcinogens (Skerman et al., 2011; Tai et al., 2004; Van Der Walt et al., 2016), and anti-diabetic agents (Bates et al., 2000; Ojewole, 2004). Although there are several known health benefits of these infusions, only a few studies have investigated the role of rooibos on DM-impaired male reproductive function. To the best of our knowledge, no studies have reported the effect of honeybush and sutherlandia on male fertility or DM related male reproductive complications. Awoniyi et al. reported that rooibos increases SOD and glutathione activities in rat sperm and subsequently improved sperm function in OS–induced rats (Awoniyi et al., 2012). Additionally, sperm motility, sperm viability and sperm

concentration were reportedly improved in diabetic rats treated with rooibos. However, long-term treatment and excessive consumption induced the acrosome reaction, which subsequently may lead to impaired reproduction (Opuwari and Monsees, 2014). Seeing that the reports on the effect of these infusions are still elusive, this study aimed to determine both the testicular oxidative stress status and apoptosis status in diabetic rats following treatment with rooibos, honeybush and sutherlandia.

2.0 Materials and Methods

2.1 Animal care

Healthy adult male Wistar rats (fourteen weeks old) weighing 250-300g were housed at the Animal Unit of the Faculty of Medicine and Health Sciences, Stellenbosch University (18-23°C, 12:12 light/dark cycle). Animals were individually caged, had free access to food and water/infusions and were treated according to the recommendations of the Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council, 2010). Ethics approval was obtained from the Stellenbosch University Animal Ethics Committee (SU-ACUD17-00016).

2.2 Infusion preparation

Fermented rooibos (2%; Carmien SA PTY LTD, South Africa), fermented honeybush (4 %; Afrinaturals, South Africa) and unfermented sutherlandia (0.2%; Afrinaturals, South Africa) were prepared according to standard protocols. Preparation methods conformed to the experimental established protocols for rooibos (Marnewick et al., 2011), honeybush (Du Toit and Joubert, 1999) and sutherlandia (Tobwala et al., 2014). Briefly, 2% rooibos was prepared by adding 20g of dried rooibos in 1litre of boiling water and allowed to rest for 30 minutes. The mixture was filtered three times using a cheesecloth initially, whereafter it was respectively filtered through a number 4 and number 1 Whatman filter paper (Whatman™, Buckinghamshire, UK). Filtered infusions were transferred to a dark plastic containers and stored at 4°C. Honeybush (4%; 40g in 1L) and sutherlandia (0.2%) were prepared following

the same protocol. All infusions (rooibos, honeybush and sutherlandia) were prepared every other day (48 hours). The herbal teas serve as the only drinking fluid for these infusion groups. The fluid intake of the animals was measured three times a week.

2.3 Experimental design

Diabetes was induced with streptozotocin (STZ, S0130-IG, Sigma, South Africa) and a stock solution (30mg/ml) was prepared by dissolving it in freshly prepared sodium citrate buffer (pH 4.5). From the stock solution, rats were administered single intraperitoneal injection of STZ (45mg/kg body weight). Fifty rats were randomly divided into five groups (n=10). The groups included a vehicle group (Veh) (received citrate buffer), a diabetic control group (DC) (received STZ45mg/kg), a diabetic + rooibos (DRF) (received STZ45mg/kg +2 % rooibos), a diabetic + honeybush (DHB) (received STZ45mg/kg + 4% honeybush) and a diabetic + sutherlandia (DSL) (received STZ45mg/kg +4 % sutherlandia) (Table 1). Animals in the infusion treatment groups started receiving the respective infusions one week before diabetes induction occurred for accclimatization. Body weights were measured three times a week and blood glucose was measured weekly (for diabetic status confirmation). Animals were sacrificed 7 weeks after DM induction. The testes and the epididymides were harvested and weighed. The testis was used for all biochemical assays and spermatozoa was retrieved from the epididymis for motility, morphology and concentration analysis. The relative testicular weight was measured by dividing the testicular weight by end bodyweight and multiplied by 100. The reported fasting blood glucose levels were recorded before sacrifice.

Table 1: The experimental design.

Abbreviation	Intervention	Treatment
Veh	Citrate buffer vehicle	Tap water
DC	45mg/kg STZ	Tap water
DRF	45mg/kg STZ	2% fermented rooibos
DHB	45mg/kg STZ	4% fermented honeybush
DSL	45mg/kg STZ	0.2% unfermented sutherlandia

Veh=vehicle, DC=diabetes control, DRF=diabetes+rooibos, DHB=diabetes+honeybush, DSL=diabetes+sutherlandia, STZ=streptozotocin.

2.4 Experimental procedures

2.4.1 Sperm functional parameters

At 30 seconds, sperm motility was analysed using computer-aided sperm analysis (CASA) (SCA version 6.3, Microptic, Barcelona, Spain) in accordance with the protocol previously described by Omolaoye et al. (Omolaoye et al., 2018). Sperm morphology was analysed using computer-aided sperm morphology analysis (CASMA) (SCA 5.4) and it conformed with the formerly established methods by van der Horst et al. (van der Horst et al., 2018). Sperm concentration was also measured using CASA. In brief, spermatozoa were retrieved from the epididymis by dissecting the caudal part into small pieces in a 2ml Dulbecco's modified eagle's medium-low glucose (DMEM) and allowing sperm to swim out during a 5 minute period. The tissue pieces were removed after 5 minutes and the sperm solution was mixed homogenously. Of the 2ml solution, 10µl was removed and diluted in 50µl of DMEM, where after the sperm concentration was measured via CASA.

2.4.2 Oxidative stress parameters

2.4.2.1 Catalase

Frozen testicular tissue samples were homogenized in cold lysis buffer (Na_3PO_4, 0.5% Triton X-100) and centrifuged at 15000rpm for 20 minutes at 4°C. Tissue homogenates were diluted 10x in deionized water. From the diluted samples and standards, 5µl were loaded in triplicate into UV microplate wells. Catalase assay buffer (170µl) were added into each well and lastly, 50µL of H_2O_2 was added into the wells and analysis was performed immediately on a plate reader (Multiskan spectrum) at 240nm every 60 seconds over a 5 minute period using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software.

2.4.2.2 Superoxide dismutase (SOD)

Tissue homogenates were prepared as described for catalase. From the diluted standards and samples, 10µl were dispensed into the microplate wells in triplicate, followed by adding 170µl of diethylenetriaminepentaacetic acid (DETAPAC) and 5µl of SOD assay buffer (50mM Na/K Phosphate buffer at PH 7.4). Immediately before reading, SOD activity was activated by adding 15µl of freshly prepared 6-hydroxydopamine (6-OHD) into the wells and instantly analysed on a plate reader (Multiskan spectrum) at 490nm, 25°C for 5minutes at 1 minute interval using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software.

2.4.2.3 TBARS assay

Frozen testicular tissue samples were homogenized in lysis buffer (0.1M KPi, 1.15% KCl) by bullet blending at speed 9 for 3 minutes with a 1-minute interval in-between. To measure the levels of MDA in testicular tissue, 100µl of standards and samples were pipetted into corresponding 10ml glass tubes, followed by adding 1ml of SDS and 2ml of 10%TCA-BHT buffer solution. Samples were vortexed, and after resting for 10 minutes, 2ml of TBA was

added and vortexed again. The standards and samples were covered with marbles (to prevent contamination from the boiling medium) and incubated in a water bath (1 hour at 100°C), where after it was cooled on ice for 15 minutes. The standards and samples were centrifuged (3000rpm, 15 minutes 4°C) and the supernatants retrieved. From the supernatants, 250µl of each standard and sample were loaded in triplicate into microplate wells and analysed on a plate reader (Multiskan spectrum) at a 532nm wavelength within 30 minutes after centrifugation.

2.4.3 Apoptotic markers

Apoptotic markers were measured using Western blot procedures. Tissue homogenates and protein determination were obtained as described by (Bradford, 1976; Marais et al., 2001). Tissue lysates were prepared by diluting sample in Laemmli sample buffer and lysis buffer, boiled for 5 minutes and 50ug protein/µl was separated by electrophoresis on a 12% SDS-PAGE mini-proteon gel. The running protocol consisted of an initial 10 minutes electrophoresis at 100V and 200mA followed by 30-40 minutes at 200V and 200mA. Gels were activated using ChemiDoc (BioRad). Thereafter, the proteins were transferred onto a milipore Immobilon-P transfer membrane (0.45µm) (Immobilon®-P, Merck Millipore Ltd, Germany). Non-specific sites were blocked with 5% fat free milk in TBS-tween. Primary antibodies were diluted in TBS-Tween in a 1:1000 ratio while the secondary antibody was diluted in TBS-Tween in a 1:4000 ratio. All data points are from independent biological repeats (n=4-5). Measured apoptotic biomarkers are Caspase 3 (Sigma-Aldrich), Caspase 7 (Abcam, SA), PARP (Cell Signalling Technology), p38MAPK (Cell Signalling Technology), C-Jun-N-terminal kinase (JNK) (Cell Signalling Technology) and X-linked inhibitory apoptotic protein (XIAP). A goat anti-mouse/rabbit-horseradish peroxidase-conjugated antibody (Sigma-Aldrich) was used as the secondary antibody.

2.5 Statistics

GraphPad Prism™ software (GraphPad™ Software, Version 8.2, San Diego, CA, USA) was used for the statistical analysis. Normal data distribution was measured using the Shapiro-Wilk, Anderson-Darling, Kolmogorov-Smirnov and D'Agostino & Pearson, normality tests. When data passed all normality tests, a one-way ANOVA of variance with a Tukey's Post-hoc Test was performed. Where data were not evenly distributed, a Kruskal-Wallis test and a Dunns Post-hoc Test were carried out. Additionally, a Pearson's two-tailed correlation matrix was performed. A probability level of $p < 0.05$ was considered statistically significant and results are expressed as mean ± SD.

3.0 Results

3.1 Anthropometric data

Although, DC and DHB animals gained less body weight, while DRF and DSL animals loss a significant amount of body weight compared to Vehicle ($p<0.0001$; $p<0.0001$; $p<0.05$; $p<0.0001$) respectively. DHB animals showed an increase in the change in body weight compared to DC (23,65±62,52 vs. 2,510±21,58). DRF and DSL animals showed a significant increase in relative testicular weight compared to the Vehicle group ($p<0.05$). After one week of diabetes induction, DC, DRF, DHB and DSL animals presented with significantly elevated blood glucose levels compared to Vehicle ($p<0.0001$) (Table 2).

Table 2: Basic anthropometric data

Parameters	Vehicle	DC	DRF	DHB	DSL	Global p Value
Start body weight (g)	273,5±18,23	278,3±13,11	282,9±15,80	264,7±80,32	274,2±14,20	0.9516
End body weight (g)	332,4±30,19	280,8±20,89$^{@}$	266,7±32,95$^{@}$	288,3±37,86*	247,4±30,60$^{@\&}$	<0.0001
Change in bodyweight (g)	58,94±20,78	2,510±21,58	-16,23±29,07$^{@}$	23,65±62,52	-26,80±25,63$^{@}$	<0.0001
Testicular weight (g)	1,351±0,09	1,380±0,11	1,357±0,14	1,342±0,15	1,213±0,22	0.3
Relative testicular weight (%)	0,4090±0,04	0,4937±0,05	0,5165±0,08*	0,4679±0,03	0,4921±0,08*	0.0023

Blood glucose levels (average of 7 weeks) (mmol/L)	6,308±0,36	25,76±2,59[@]	24,73±5,01[@]	21,93±5,77[@]	24,71±3,07[@]	<0.0001
Fasting blood glucose at sacrifice (mmol/L)	5,660±1,02	19,62±7,49[@]	18,91±9,14*	14,98±9,43*	16,60±8,92*	<0.0001

*p<0.05 vs. vehicle; [@]p<0.001 vs. vehicle; [&]p<0.05 vs. DHB

DC=diabetes control, DRF=diabetes+rooibos, DHB=diabetes+honeybush, DSL=diabetes+sutherlandia

3.2 Sperm functional parameters

Although not significant, DC animals had a nearly 7% decrease in total motility compared to the Vehicle. DRF animals displayed a 4.3% increase in total motility compared to the Vehicle group and a 12% increase compared the DC group (Figure 1A). Additionally, there was a 15.17% decrease in the percentage of progressively motile spermatozoa of DC animals compared to the Vehicle group, while DRF displayed a 7% increase compared to the Vehicle and 26.2% compared to the DC group. DHB animals displayed the largest decrease (-17%) in the progressive motility compared to Vehicle and a mild reduction compared to the DC group. DSL animals on the other hand, presented with a 15% increase in progressive motility compared to DC (Figure 1B), and a significant decrease in sperm concentration compared to the Vehicle ($p<0.05$) (Figure 1C). There were no significant differences in sperm kinematic and speed parameters between the groups (Table 3, 4). The DRF group showed a 25% increase in sperm concentration compared to DC, while DHB animals presented with a 30% increase in sperm concentration compared to the DC animals (Figure 1C). Although not significant, DC

animals showed a decrease (-26%) in the percentage of normal sperm morphology compared to Vehicle. DRF, DHB and DSL animals also presented with a decrease in normal sperm morphology compared to the Vehicle group (-8%, -14%, -13%) respectively. However, when compared to the DC animals they showed a mild improvement in the percentage of morphologically normal spermatozoa (13%, 16%, and 15%) respectively (Figure 1D).

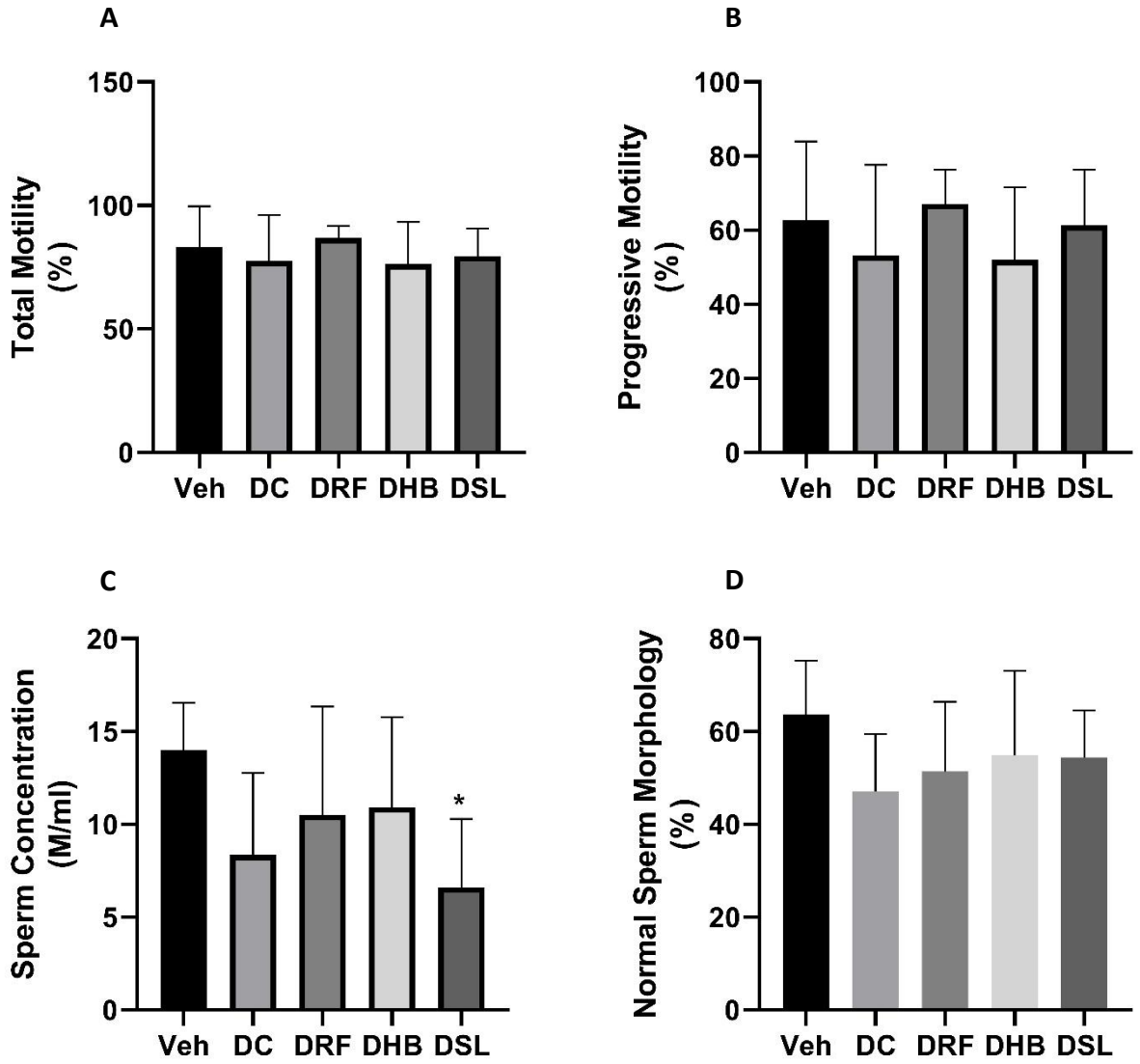

Figure 1: The effects of DM and infusions on sperm functional parameters. A. Total sperm motility (%), **B**. Progressive motility (%), **C**. Sperm concentration (M/ml), **D**. Normal sperm morphology (%). *p<0.05 vs. veh; Gp value for total motility=0.5, progressive motility=0.3, sperm concentration=0.018, sperm morphology=0.2. Veh=vehicle, DC=diabetes control, DRF=diabetes+rooibos, DHB=diabetes+honeybush, DSL=diabetes+sutherlandia

Table 3: Sperm kinematic parameters at 30 seconds.

Parameters	Vehicle	DC	DRF	DHB	DSL	Global p Value
VCL (µm/s)	161,6±66,59	163,3±42,49	170,0±27,87	155,7±35,00	152,2±50,79	0.9247
VAP (µm/s)	70,48±34,04	71,00±20,83	78,12±14,57	70,25±15,30	67,90±26,78	0.6862
VSL (µm/s)	41,89±22,31	42,54±15,69	48,13±10,46	42,79±9,328	44,26±19,43	0.7741
STR (%)	54,65±7,99	54,30±9,63	58,49±6,48	56,64±3,40	60,32±9,33	0.3033
LIN (%)	23,81±8,83	24,75±7,48	28,11±5,52	26,91±3,52	27,71±7,90	0.3113
WOB (%)	40,81±10,88	42,31±5,13	45,19±7,48	44,36±5,08	42,91±7,55	0.7609
ALH (µm)	6,430±2,21	6,471±1,41	6,539±0,96	5,898±1,16	5,713±1,53	0.4925
BCF (Hz)	13,58±5,53	14,29±3,42	16,35±3,29	16,35±2,39	16,06±6,60	0.3577

Curvilinear speed (VCL), Average path velocity (VAP), Straight Line velocity (VSL), Straight line index (STR), Linearity index (LIN), and Oscillation index (WOB), Amplitude Lateral Head (ALH), Sperm Beat Frequency (BCF).

DC=diabetes control, DRF=diabetes+rooibos, DHB=diabetes+honeybush, DSL=diabetes+sutherlandia.

Table 4: Sperm speed and progressive motilities.

Parameters	Vehicle	DC	DRF	DHB	DSL	Global p Value
Rapid speed (%)	42,87±30,40	45,78±22,76	51,75±18,66	41,93±20,40	42,56±24,97	0.8110
Medium speed (%)	12,74±8,17	11,71±5,15	14,80±10,75	16,06±6,09	11,16±9,56	0.6361
Slow speed (%)	17,15±8,03	20,02±7,52	13,32±5,14	18,33±5,28	15,86±6,58	0.2683
Rapid progressive motility (%)	19,61±16,10	22,82±13,95	26,16±14,56	20,01±12,59	25,13±16,20	0.8088
Medium progressive motility (%)	31,26±18,07	30,35±12,66	34,91±14,95	32,00±10,84	24,90±14,79	0.6372

Non progressive motility (%)	21,89±9,16	24,34±7,40	18,80±7,82	24,24±7,83	19,54±7,38	0.3931

DC=diabetes control, DRF=diabetes+rooibos, DHB=diabetes+honeybush, DSL=diabetes+sutherlandia

3.3 Oxidative stress parameters

There was a significant difference in SOD activity as measured in the testicular tissue between the groups ($p<0.05$). The DC group presented with a decrease of 13.5% in SOD activity compared to the Vehicle group. While DRF animals displayed an increase (18.8%) in SOD activity compared to Vehicle and 37.4% compared to the DC group. DHB animals displayed an increase of 32.9%, 53.7% in SOD activity compared to Vehicle and DC respectively ($p=0.06$). DSL animals also presented with 28.3%, 48% increase in SOD activity compared to the Vehicle and DC groups respectively (Figure 2A). Additionally, DHB animals presented with a significant increase in catalase activity compared Vehicle (133.4±42.81µl/µg vs. 20.64±16.38µl/µg, $p=0.005$) and 170% increase compared to the DC group (133.4±42.81µl/µg vs. 49.24±21.59µl/µg, $p=0.3$) (Figure 2B). Although not significant, DC group displayed more than a doubling increase (119%) in the MDA levels compared to Vehicle ($p=0.2$), while it was reduced by 41%, 60%, and 27% respectively in the DRF, DHB and DSL groups compared to the DC group (Figure 2C).

3.4 Apoptotic markers

The expression of caspase 7 in the testicular tissues of DC animals increased by 75.7% compared to vehicle ($p=0.2$), while DRF, DHB and DSL showed 5%, 11%, -17% increase in

caspase 7 compared to DC (Figure 3A). DC animals displayed an increase (28%, 34%, 29%, 27.5%) in the expression of cleaved PARP, pP38, JNK55kDa and JNK44kDa respectively (Figure 3B-F). The testicular tissues of DRF, DHB and DSL animals showed an increase in cleaved PARP (19%, 41%, and 15%) compared to DC. DHB animals displayed a significant increase in the phosphorylation of p38 compared to Vehicle (3.630±2.576 vs. 0.459±0.3749, $p<0.05$). In addition, DRF, DHB, DSL animals showed a -25%, -53.5%, -41.5% decrease in the expression of JNK55kDa compared to the DC group, with DHB showing significance ($p<0.05$). To further evaluate apoptotic activity, the total expression of XIAP was measured. DC animals presented with nearly 20% decrease in the total expression of XIAP compared to Vehicle, while DRF showed a significant decrease compared to Vehicle (0.5789±0.427, 0.2939±0.2266 vs. 0.6903±0.2108, $p<0.05$) respectively. DRF, DHB and DSL animals displayed a further decrease in the expression of total XIAP (-52.6%, -33.9%, -50.6%) compared to the DC group (Figure 3G).

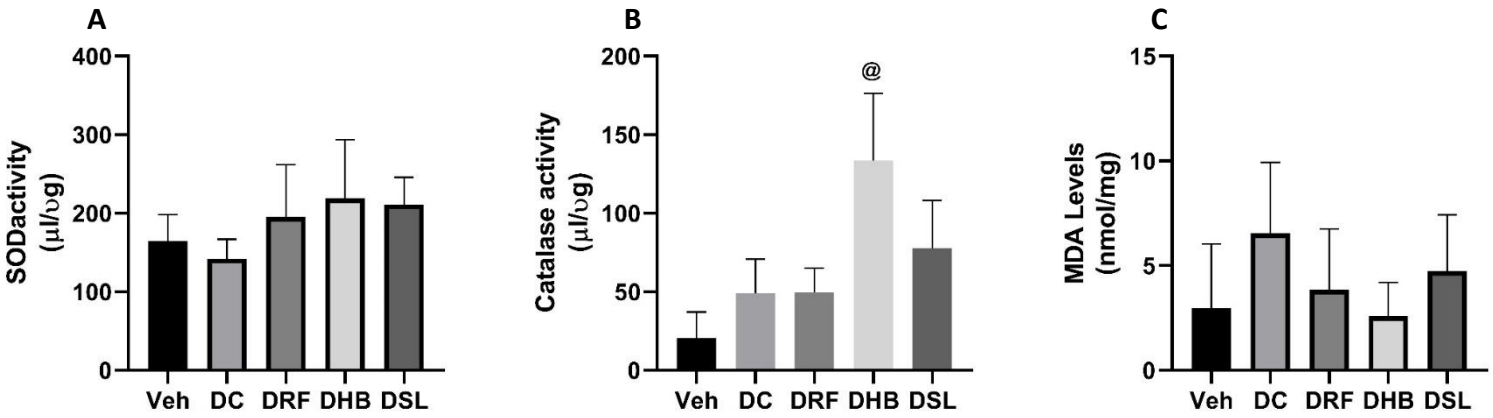

Figure 2: The effect of DM and infusions on testicular oxidative stress. A. SOD activity, **B**. Catalase activity, **C**. MDA levels. $^{@}p<0.001$ vs. veh. Global p value for SOD=0.04, catalase=0.008, MDA levels=0.07. Veh=vehicle, DC=diabetes control, DRF=diabetes+rooibos, DHB=diabetes+honeybush, DSL=diabetes+sutherlandia

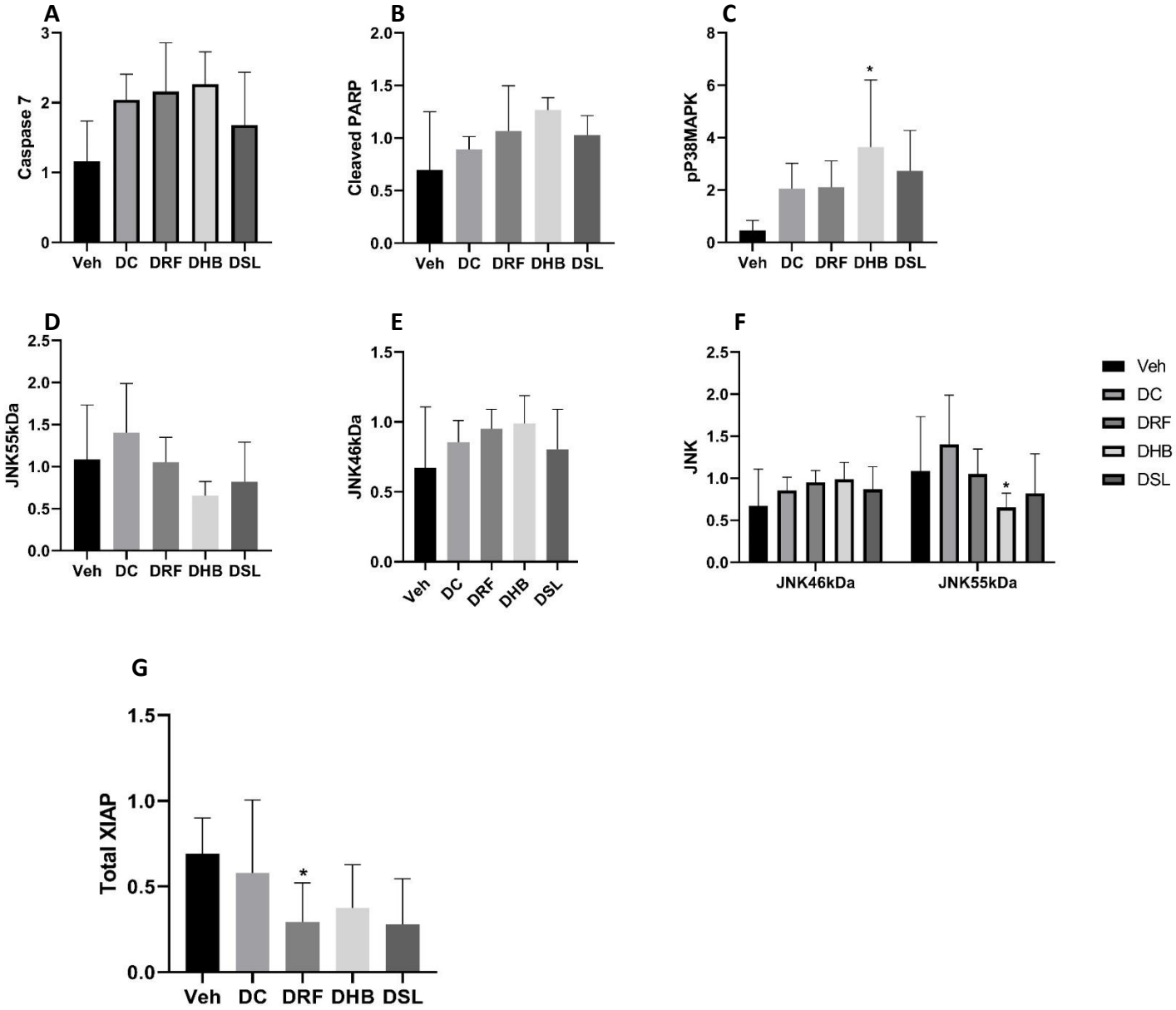

Figure 3: The effects of DM and infusions on testicular apoptotic biomarkers. A. caspase 7, B. cleaved PARP, C. pP38MAPK, D. JNK55kDa, E. JNK46kDa, F. JNK (grouped data), G. total XIAP (XIAP1+XIAP2), and H. Blots (probed). *p<0.05 vs. veh, $^{@}$p<0.001 vs. veh. Global p value for caspase 7=0.054, cleaved PARP=0.08, pP38MAPK=0.02, JNK55kDa=0.1, JNK46kDa=0.4, XIAP=0.02. Veh=vehicle, DC=diabetes control, DRF=diabetes+rooibos, DHB=diabetes+honeybush, DSL=diabetes+sutherlandia

4.0 Discussion

The use of phytochemicals containing natural products has been a crucial part of traditional medicine. The presence of the diverse bioactive compounds in these products have initiated new insights on how they can be beneficial to humanity, either by extracting the bioactive compounds (Dludla et al., 2018; Kokotkiewicz et al., 2013; Son et al., 2013) or by using the whole plant (Hong et al., 2014; Marnewick et al., 2011; Schloms and Swart, 2014). Rooibos, honeybush and sutherlandia are plants containing diverse bioactive compounds, with associated health benefits (Awoniyi et al., 2012; Harnett et al., 2005; Hong et al., 2014; Marnewick et al., 2003; Mills et al., 2005; Pheiffer et al., 2013). Several studies have highlighted their role in preventing the development of cell toxicity (Tandeka U. Magcwebeba et al., 2016; Tandeka Unathi Magcwebeba et al., 2016), alleviating OS in different organ systems including the brain (Hong et al., 2014), liver (Layman et al., 2019), skin (Tiedtke et al., 2002), and many more. However, little is known about the effects of these infusions in DM-induced male reproductive function impairment. Hence, the current study investigated the plausible ameliorative role of rooibos, honeybush and sutherlandia on DM-induced sperm function impairment and also set out to determine testicular OS and apoptosis status after treating diabetic rats with these infusions.

As expected, following DM induction, animals in the DC, DRF, DHB and DSL groups presented with elevated blood glucose levels and they gained less body weight/loss weight over the treatment period. These are known characteristic of type 1 DM as shown by several studies (Singh et al., 2009; Vikram et al., 2008). Hence, animals became diabetic. The DC animals of the current study presented with a non-significant decrease in the percentage of total and progressive motilities. This is in agreement with several studies that showed reduced sperm motility in both diabetic men (Bhattacharya et al., 2014) and diabetic animals (Singh et al.,

2009). Additionally, DC animals presented with a decrease in the percentage of sperm with normal morphology. This concur with other studies who reported reduction in the number of spermatozoa with normal morphology after inducing diabetes in rodents (Mangoli et al., 2013; Maresch et al., 2017). Interestingly, in the current study, DRF animals presented with an increase in progressive motility when compared to DC. However, what is more interesting is that DRF animals had a mild increase in progressive motility than the non-diabetic group. The former result is supported by findings from Awoniyi et al. who reported a significant increase in sperm motility after treating OS-induced rats with both fermented and green rooibos (Awoniyi et al., 2012). Regarding the effect of honeybush and sutherlandia on sperm functional parameters of diabetic rats, the result of the current study showed that DHB animals presented with a decrease in sperm motility. However, there was a mild increase in sperm motility of DSL animals and this represent the first study reporting the effect of honeybush and sutherlandia on sperm motility. Although not significant, and not as normal as the vehicle rats, DRF, DHB and DSL animals had increased number of spermatozoa with normal sperm morphology.

Studies have shown that DM induce subtle molecular changes that are essential for sperm quality and function through the development of OS, caused by the imbalance in the ratio of ROS production and the antioxidant activities (Agarwal et al., 2014; Bhattacharya et al., 2014). Observed in the DC animals of the current study, is a decrease in the activity of SOD, accompanied by an increased level of MDA, which were negatively correlated (Supplementary figure 1). This is supported by studies that reported a significant increase in the plasma level of TBARS in diabetic rats (Ayeleso et al., 2014). This also concur with the findings of Uličná et al. who showed increased levels of MDA in the plasma, liver, kidney and lens of diabetic rats (Uličná et al., 2006). However, DRF, DHB and DSL animals presented with increased SOD

activity. Interestingly, not only did their SOD activity surpasses the DC group, there was an increase in SOD activity compared to the healthy group. Additionally, DHB group presented with increased catalase activity. The increase in SOD and catalase activities were accompanied by a reduction in the MDA levels. Studies have reported the ameliorative effects of these infusions and how they can act as antioxidants in diverse pathologies (Bramati et al., 2003; Tobwala et al., 2014) . In OS, sperm motility is affected because of the increased ROS production (Lanzafame et al., 2009). The sperm plasma membrane has a very high percentage of polyunsaturated fatty acids (PUFA) (Lenzi, 2000; Lenzi et al., 1996) which are essential for sperm motility. These PUFAs are vulnerable to ROS, hence, the invasion of the sperm plasma membrane leads to lipid peroxidation (Liu et al., 2015). Lipid peroxidation occurs when ROS react with fatty acid chains to form the lipid peroxyl radical. Peroxyl radicals in turn react with fatty acids to produce more ROS. The reaction between the free radicals produced results in lipid breakdown. However, studies have shown that to protect against OS-induced damage, it is necessary to treat the underlying cause (Agarwal et al., 2014) and then supress pro-oxidants by antioxidants (Tremellen, 2008).

Hence, based on the results of the current study, it is suggested that the improved sperm motility observed in diabetic animals treated with rooibos and sutherlandia may in part be due to the boost in antioxidant enzyme activity of these animals which subsequently protect against/alleviate the stress imposed by DM on sperm motility. Although DHB animals presented with improved antioxidant enzyme activities, the sperm motilities were not improved. This shows the involvement of other plausible factors in the process of impaired sperm function observed in DM.

The induction of apoptosis as a result of OS has been shown in several pathologies, including DM (Mahfouz et al., 2010). High levels of ROS alter the integrity of mitochondrial membrane

(Simon et al., 2000) which result in mitochondria DNA (mtDNA) damage and subsequently affects sperm functions negatively. In the current study, DC animals displayed increased caspase 7, cleaved PARP, JNK46kDa and p38MAPK, which are all biomarkers of apoptosis. This is partly in agreement with Roessner et al. who reported increase in the cleavage of caspase 3, elevated ROS production and disrupted mitochondrial potential in diabetic men (Roessner et al., 2012). They further showed that these parameters were negatively correlated with the sperm fertilizing capacity in diabetic men. From the current study, after treating diabetic animals with rooibos, honeybush or sutherlandia, there were no improvement observed. To better understand the presented apoptotic status, XIAP was measured. For the control of apoptosis, mammals developed some regulatory proteins which are classified as members of the inhibitor of apoptosis (IAP) family. The pro-type member of the family is XIAP. XIAP has three baculovirus IAP repeat (BIR) domains and a C-terminal RING finger. It is known to inhibit caspases at the initiation (caspase-9) and execution phases (caspase 3 and 7) of apoptosis (Scott et al., 2005). In lieu of this, XIAP was quantified. The expression of XIAP in the testicular tissue of DC animals was decreased, while diabetic animals treated with rooibos, honeybush or sutherlandia showed further decrease in the expression of this protein. From the result of the current study, the reduction of XIAP in DC animals may indicate that caspases were not inhibited and thus apoptosis ensue. However, since the same trend was observed in the infusion treated groups, it can be said that rooibos, honeybush and sutherlandia did not alleviate testicular apoptosis seen in diabetic rats.

5.0 Conclusion

The current study have highlighted the negative impact of DM on sperm functional parameters through increased lipid peroxidation and reduced antioxidant activity. Observed

in the infusion treatment groups was increased antioxidant enzyme activity, which may be partly responsible for the observed improvement in sperm motility and morphology in the DRF and DSL groups. Additionally, the current study have showed the increased expression of apoptotic biomarkers in DM, which were not alleviated by the infusions. This suggest that these infusions play a role in alleviating DM-induced sperm function impairment through suppression of OS, but their role in apoptosis is still unclear. Therefore, based on the results of the current study, it can be speculated that in disease related male reproductive impairment such as DM, rooibos and sutherlandia may be beneficial in ameliorating male fertility complications.

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Conflict of Interest

The authors have no conflict of interest to declare

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Chapter 8

The effect of rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and sutherlandia (*Lessertia frutescens*) on testicular insulin signalling in streptozotocin induced diabetes in Wistar rats

Will be submitted to Phytotherapy Research

Temidayo S Omolaoye[1], Stefan S du Plessis[1, 2]*

1. Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa

2. Department of Basic Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates

* Corresponding author:

Prof SS du Plessis

Division of Medical Physiology

Faculty of Medicine and Health Sciences

Stellenbosch University

Francie van Zijl Drive

Tygerberg

7505

South Africa

E-mail: ssdp@sun.ac.za

Abstract

The current study was designed to investigate the role of rooibos, honeybush and sutherlandia on insulin signalling in the testicular tissue of diabetic rats.

Animals (n=60) were randomly divided into six groups. The groups include a control group (no treatment + water) and vehicle group (STZ control, injected intraperitoneally with citrate buffer and treated with water). Diabetes was induced in the remainder of animals via a single intraperitoneal injection of STZ at 45mg/kg. The remaining four groups are, a diabetic control group (DC, received water), a diabetic + rooibos (DRF, received 2% fermented rooibos), a diabetic + honeybush (DHB, received 4% fermented honeybush) and a diabetic + sutherlandia group (DSL, received 0.2% unfermented sutherlandia). Animals in the infusion treatment groups were pre-treated for one week before diabetes induction and all animals were sacrificed after seven weeks of DM induction. Blood and testes were collected.

All diabetic groups (DC, DRF, DHB, DSL) presented with a significant increase in blood glucose levels after diabetes induction compared to the control and vehicle ($p<0.001$). However, the infusion treated animals displayed a mild decrease in fasting blood glucose compared to the DC group at sacrifice. The DC animals showed a decrease in testis protein expression of IRS-1, PkB/Akt and GLUT4 compared to control. DRF and DHB animals displayed an acute upregulation in IRS-1, while DSL group showed improvement in IRS-2 compared to DC. Although, DRF animals presented with a decrease in PkB/Akt, DHB and DSL animals displayed upregulation (22.3%, 48%) compared to control respectively. All diabetic animals showed significantly increased phosphorylated ERK1/2 and reduced total ERK1/2 when compared to control ($p<0.001$) and vehicle ($p<0.001$). Additionally, DC animals presented with a non-significant decrease in plasma testosterone concentration compared to control, while DRF

and DSL showed significant decrease (p=0.01) and DHB showed mild upregulation compared to DC. In conclusion, it can be suggested that these infusions may enhance insulin signalling through diverse pathways, but further investigations are required to elucidate the specific upstream and downstream proteins that are plausibly activated during this process.

Keywords: Testis, Rooibos, Honeybush, Sutherlandia, Diabetes, insulin signalling

Introduction

Diabetes mellitus (DM) is a non-communicable disease that brings about complications in various systems including the male reproductive system. Both experimental and human studies have highlighted diverse adverse effects of DM on male fertility (Hassan *et al.*, 1993; Sanguinetti *et al.*, 1995; López-Alvarenga *et al.*, 2002; Dhindsa *et al.*, 2004; Maneesh *et al.*, 2006; Bondarenko *et al.*, 2011). In experimental diabetic animals, studies have reported altered spermatogenesis, diminished epididymal sperm reserve (Amaral *et al.*, 2009), abnormal Sertoli-Sertoli cells junction complexes, reduction in the testicular weight, sperm concentration, testicular morphology index and decreased fertility potential (Cameron *et al.*, 1990). Lopez-Alvarenga et al. reported the suppression of both endogenous pulsatile and exogenous gonadotropin releasing hormone (GnRH) stimulated the secretion of luteinizing hormone (LH) in men with type I DM, which resulted in hypogonadotropism (López-Alvarenga *et al.*, 2002). Studies have also shown that there is reduced levels of testosterone, LH and follicle stimulating hormone (FSH) in the sera of diabetic men (Maneesh *et al.*, 2006; Hussein and Jawad Al-Qaisi, 2012; Rezvani *et al.*, 2012). Also reported is an increase in spermatozoa with nuclear and mitochondrial DNA fragmentation (Agbaje *et al.*, 2007, 2008), reduced sperm motility (La Vignera *et al.*, 2009) and decreased sperm with normal morphology (Delfino *et al.*, 2007). In the course of unravelling the mechanism(s) through which DM exert these detrimental effects, studies have shown the importance of insulin regulation in glucose homeostasis, insulin signalling and how lack in insulin secretion indirectly led to reduced male fertility (Ballester *et al.*, 2004; Gómez *et al.*, 2009; Schoeller *et al.*, 2012). Gomez et al. reported a decrease in both systemic and testicular insulin concentration in diabetic rats with resultant disrupted spermatogenesis. It was suggested that, although, the testis produces insulin, systemic hypoinsulinemia due to type I DM adversely affect male fertility (Gómez *et*

al., 2009). Since findings have shown that, although the Sertoli cells secretes its own insulin, lack in pancreatic β-cell insulin production still affected male reproduction adversely. Schoeller et al., investigated the role of both systemic and testicular insulin signalling in male fertility using the Akita mouse diabetic model. They validated the presence of insulin in the testes and further reported that insulin affects male fertility through its indirect regulatory effect on the hypothalamic-pituitary-gonadal axis (HPGA). It was hypothesized that in DM, due to hypoinsulinemia/hyperglycaemia, insulin signalling is altered and the function of HPGA is disrupted, which cumulatively resulted in altered spermatogenesis and reduced sperm function. This was evidenced by the decrease in the levels of serum GnRH, LH, FSH and testosterone as well as testicular LH, FSH and testosterone in the diabetic animals. However, when insulin was administered the levels of these hormones were increased and normal spermatogenesis was restored. They concluded that insulin rescue fertility by restoring function to the HPGA, thus normalizing the hormone levels of LH and testosterone (Schoeller *et al.*, 2012). However, other studies have further shown the importance of normal insulin signalling in normal male reproduction (Yagci and Zik, 2006; Griffeth, Carretero and Burks, 2013).

Since the importance of maintaining normal insulin signalling have been highlighted, it is essential to explore natural and artificial agents that has health benefits, and that can possibly enhance this process. Rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and sutherlandia (*Lessertia frutescens*) are teas native to Southern Africa (Morton, 1983; van Wyk and Albrecht, 2008). Studies have highlighted some of their health benefits (Marnewick *et al.*, 2003, 2009; Awoniyi *et al.*, 2012; Pheiffer *et al.*, 2013; Hong, Lee and Kim, 2014), including antidiabetic properties (Bates, Jones and Bailey, 2000; Ojewole, 2004). Kawano et al. reported that aspalathin, a component of rooibos tea, improved impaired glucose tolerance of db/db

mice at 30, 60, 90 and 120 minutes (Kawano *et al.*, 2009). They further added that rooibos, in a dose-dependent manner, enhanced glucose uptake in cultured L6 myotubes and also increased the secretion of insulin by RIN-5F cells. The results of Kawano et al. is supported by several other authors (Muller *et al.*, 2012; Mazibuko *et al.*, 2013, 2015; Son *et al.*, 2013; Kamakura *et al.*, 2015; Mikami *et al.*, 2015; Dludla *et al.*, 2018), with the inclusion that aspalathin promoted activated protein kinase (AMPK) phosphorylation, enhanced GLUT4 translocation and also improved protein kinase B activation. This suggest that rooibos may improve glucose metabolism by enhancing glucose uptake through restoring normal insulin signalling. Studies have also highlighted the anti-diabetic potential of honeybush (Jung *et al.*, 2004; Akiyama *et al.*, 2010; Muller *et al.*, 2011; Saleh *et al.*, 2014; Wang *et al.*, 2014) and sutherlandia (Chadwick *et al.*, 2007; J. *et al.*, 2012; Williams *et al.*, 2013) in both in vitro and in vivo experiments. All these studies showcasing the hypoglycaemic effects of rooibos, honeybush and sutherlandia were carried out in cardiovascular and other systemic diseases. Only few studies have investigated the role of these teas/infusions (rooibos only) on DM-related male reproductive disorder. Hence, this study was designed to investigate the role of rooibos, honeybush and sutherlandia on testicular insulin signalling in diabetic rats.

Materials and methods

Infusion preparation

Rooibos (*Aspalathus linearis,* 2% fermented), was obtained from Carmien SA PTY LTD, South Africa, while honeybush (*Cyclopia intermedia,* 4% fermented) and sutherlandia (*Lessertia frutescens,* 0.2% unfermented) was obtained from Afrinaturals, South Africa. They were prepared according to previously established protocols. Rooibos was prepared

according to the method described by Marnewick et al. (Marnewick *et al.*, 2011), honeybush by Du Toit and Joubert (Du Toit and Joubert, 1999) and sutherlandia by Tobwala et al. (Tobwala *et al.*, 2014). In summary, 2% fermented rooibos was prepared by adding 20g of dried rooibos in 1litre of boiling water and allowed to draw for 30 minutes. The mixture was filtered using a cheesecloth, a number 4 and finally a number 1 Whatman filter paper (Whatman™, Buckinghamshire, UK). Filtered teas/infusions were transferred to a dark plastic containers and stored at 4°C. Infusions were stored in dark plastics and as well prepared freshly every other day, to prevent the degrading of the polyphenols that have short half-life and are light sensitive. Fermented honeybush (4%; 40g in 1L) and unfermented Sutherlandia (0.2%) were prepared following the same procedures. All infusions (rooibos, honeybush and sutherlandia) were prepared every other day (48 hours). The herbal teas serve as the only drinking fluid for these infusion groups. The fluid intake of the animals was measured three times a week.

Animal care and study design

Fourteen-week-old adult male Wistar rats weighing 250-300g were housed in the Animal Unit of the Faculty of Medicine and Health Sciences, Stellenbosch University (18-23°C, 12:12 light/dark cycle). Animals were caged individually, had free access to food and water/infusions and were treated according to the recommendations of the Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council, 2010). Ethics approval

was obtained from the Stellenbosch University Animal Ethics Committee (SU-ACUD17-00016).

Diabetes was induced with streptozotocin (STZ, S0130-IG, Sigma, South Africa) and a stock solution (30mg/ml) was prepared by dissolving it in freshly prepared sodium citrate buffer (pH 4.5). From the stock solution, rats were administered single intraperitoneal injection of STZ (45mg/kg body weight). Animals in the vehicle group were injected with citrate buffer.

Sixty rats were randomly divided into six groups (n=10). The groups include a control group (water), vehicle group (citrate buffer), a diabetic control group (DC, STZ45mg/kg + water), a diabetic + rooibos (DRF, STZ45mg/kg + 2% fermented rooibos), a diabetic + honeybush (DHB, STZ45mg/kg + 4% fermented honeybush) and a diabetic + sutherlandia group (DSL, STZ45mg/kg + 0.2% unfermented sutherlandia). Animals in the infusion treatment groups were pre-treated with the tea for one week before diabetes induction. Food intake, fluid intake and body weights were measured three times a week and blood glucose levels were measured once weekly. Animals were sacrificed after 7 weeks of DM induction. Blood samples were collected immediately after sacrifice, and the testes was harvested and weighed. Plasma was used for hormonal assays, while the testis was used for Western blotting. The relative testicular weight was expressed as a percentage of body weight. The reported average blood glucose levels were the mean of blood glucose measured over seven weeks and the fasting blood glucose levels were recorded immediately before sacrifice.

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test (IPGTT) was performed 2-3 days before sacrifice. Animals were fasted overnight but allowed free access to water/infusion as appropriate. After 14-16 hours of fasting, animals were injected Intraperitoneally with a 20% glucose solution at

a dose of 2g/kg body weight (10µl/g). Blood glucose levels were then measured at 12 different time points (0, 3, 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 minutes).

Western Blot analysis

Tissue homogenates and protein determination were obtained as described by (Bradford, 1976; Marais *et al.*, 2001). Tissue lysates were prepared by diluting sample in Laemmli sample buffer and lysis buffer, boiled for 5 minutes and 50ug protein/µl was separated by electrophoresis on either a 12% SDS-PAGE mini-proteon gel or a 4-20% stain-free precast gel (Criterion TGX, Biorad, USA). The running protocol consisted of an initial 10 minutes electrophoresis at 100V and 200mA followed by 30-40 minutes at 200V and 200mA for the mini-proteon gels, while the running protocol for the precast gel consist of an initial 10 minutes electrophoresis at 100V and 200mA followed by 25-30 minutes at 140V and 140mA. Gels were activated using ChemiDoc (BioRad) and thereafter, proteins were transferred onto a milipore Immobilon-P transfer membrane (0.45µm) (Immobilon®-P, Merck Millipore Ltd, Germany). Non-specific sites were blocked with 5% fat free milk in TBS-tween. Primary antibodies were diluted in TBS-Tween in a 1:1000 ratio while the secondary antibody was diluted in TBS-Tween in a 1:4000 ratio. All data points are from independent biological repeats (n=4-5). Measured biomarkers are GLUT 4, tPkB/Akt, p/tERK1/2 and p/tIRS 1/2. All primary antibodies were obtained from Cell Signalling Technology. A goat anti-mouse/rabbit-horseradish peroxidase-conjugated antibody (Sigma-Aldrich) was used as the secondary antibody.

Hormonal assays

The plasma concentration of testosterone (E-EL-0072), estradiol (E-EL-0065) and insulin (E-EL-R2466) were measured using a commercially available ELISA kit (Elabscience Biotechnology, Hubei) as per manufacturer's instructions.

Statistical analysis

GraphPad Prism™ software (GraphPad™ Software, Version 8.2, San Diego, CA, USA) was used for the statistical analysis. Normal data distribution was measured using the Shapiro-Wilk, Anderson-Darling, Kolmogorov-Smirnov and D'Agostino & Pearson, normality tests. When data passed all normality tests, a one-way ANOVA of variance with a Tukey's Post-hoc Test was performed. Where data were not evenly distributed, a Kruskal-Wallis test and a Dunns Post-hoc Test were carried out. To investigate the plausible mixed effect of the phospho to total ratios, a mixed effect or ordinary two-tailed, two-way ANOVA was performed. Where appropriate, a Pearson's correlation matrix was carried out. Significance was accepted at $p< 0.05$. Results are expressed as mean ± SD.

Results

Diabetic control (DC) animals consumed more food and drank more water compared to the control ($p<0.001$) and vehicle ($p<0.001$) groups. Diabetic animals receiving rooibos (DRF), honeybush (DHB) and sutherlandia (DSL) followed the same trend as DC when compared to the control ($p<0.001$) and vehicle ($p<0.001$). However, the DM animals treated with the infusions (DRF, DHB, and DSL) displayed a significant decrease in food intake when compared to the DC animals (35.38±4.936g, 32.22±5.440g, 34.92±4.144g vs. 40.43±2.872g, $p<0.001$). DC

animals presented with elevated fasting blood glucose levels when compared to the control (19.62±7.49mmol/L vs. 6.150±0.7246mmol/L, $p<0.001$) and vehicle (19.62±7.49 mmol/L vs. 5.660±1.02mmol/L, $p<0.001$) groups. Although the diabetic infusion treatment groups (DRF, DHB and DSL) presented with a significant increase in fasting blood glucose levels when compared to the control and vehicle groups ($p<0.001$), their (DRF, DHB and DSL) respective fasting blood glucose levels mildly decreased when compared to the DC group (Table 1). DC animals showed a significant reduction in body weight when compared to control ($p<0.05$). The respective infusion treatment groups (DRF, DHB and DSL) also presented with a decrease in body weight when compared to the control ($p<0.001$) and vehicle ($p<0.001$) (Table 2).

Regarding glucose tolerance, there was a significant difference in the measured glucose levels between 0 and 120 minutes, in the DC, DRF, DHB and DSL groups ($p=0.001$). Although there was a non-significant improvement in the glucose tolerance of DHB animals (Figure 1A). Additionally, there was a significant positive correlation ($r=0.7631$) between fasting blood glucose levels and the fluid intake per day ($p<0.0001$) (Figure 1B). All diabetic animals (DC, DRF, DHB and DSL) presented with increased phosphorylated ERK1/2 (pERK1/2) when compared to the vehicle (0.988±0.252, 1.226±0.368, 1.028±0.369, 1.408±0.682 vs. 0.766±0.138, $p<0.05$). They (DC, DRF, DHB and DSL) also displayed a reduction in the total ERK1/2 (tERK1/2) when compared to the control and vehicle (0.6438±0.5, 0.51±0.398, 0.316±0.323, 0.56±0.537 vs. 0.847±0.281 and 0.7407±0.094, $p<0.001$) (Figure 2A-C). Furthermore, DHB animals presented with a significant difference in the ratio of phospho to total ERK1/2 compared to control ($p=0.02$). DSL on the other hand showed an increase in the ratio when compared to vehicle ($p=0.02$).

Diabetic control animals presented with a decrease (-48%, -32%) in tIRS-1 when compared to the control and vehicle groups respectively. The diabetic infusion treatment groups (DRF,

DHB, DSL) displayed a non-significant decrease in trend in tIRS-1 when compared to the control and vehicle groups, but DRF and DHB were acutely upregulated when compared to DC (0.515±0.56, 0.5±0.453 vs. 0.48±0.246) (Figure 3). Despite the reduced tIRS-1 displayed by DSL animals when compared to the DC, they showed a nearly 50% upregulation in tIRS-2 when compared to the DC (Figure 3A-B).

Additionally, DC animals presented with a decrease (-41.7%, -34%) in the expression of PkB/Akt when compared to the control and vehicle groups respectively. The diabetic infusion treatment groups (DRF, DHB, DSL) also displayed a percentage decrease in PkB/Akt when compared to the control and vehicle groups, but DRF animals presented with a further decrease in the total PkB/Akt compared to DC group. While, the DHB and DSL animals displayed an upregulation in total PkB/Akt (22.3%, 48%) when compared to DC respectively (Figure 4A-B).

DC animals showed a reduction in tGLUT4 (-30%, -4%) when compared to the control and vehicle respectively. DRF and DHB groups also displayed a decrease in GLUT4 expression when compared to the DC and control groups. However, DSL animals presented with an increase in GLUT4 (70.9%) when compared to the DC group (p=0.08) (Figure 5A-B).

Although, not significant, DC animals presented with a mild decrease in the level of plasma testosterone concentration (-16.5%) when compared to the vehicle, while DRF and DSL groups displayed a significant decrease compared to vehicle (811.2±126.5nmol/L vs. 1085±95.66nmol/L, p=0.01) (793.1±192.9nmol/L vs. 1085±95.66nmol/L, p=0.02) respectively. However, DHB animals showed a mild increase in testosterone concentration compared to DC (Figure 6A). There were no significant differences in the level of plasma 17β-estradiol concentrations between the groups (Figure 6B).

Discussion

The potential hypoglycaemic effects of rooibos, honeybush and sutherlandia extracts have been widely investigated in both *in vivo* (Chadwick *et al.*, 2007; Kawano *et al.*, 2009; Muller *et al.*, 2011) and *in vitro* studies (Williams *et al.*, 2013; Chellan *et al.*, 2014; Mazibuko *et al.*, 2015; Mazibuko-Mbeje, Dludla, Roux, *et al.*, 2019). The mechanisms through which this occur have been mostly determined in *in vitro* studies using cells lines, such as adipocytes, cardiomyocytes, fibroblast, myotubes/myoblast, etcetera. However, extremely few to no studies have reported the effects of rooibos, honeybush and sutherlandia on testicular insulin signalling, hence, the current study was designed.

As expected, the diabetic control animals of the current study displayed polyphagia, polydipsia, excessive weight loss, sustained increased blood glucose and impaired glucose tolerance. These are features typical of type I DM. This is in agreement with several studies reporting the symptomatic characteristics of DM (Szkudelski, 2001). However, the diabetic animals treated with rooibos (DRF), honeybush (DHB) and sutherlandia (DSL) showed a significant decrease in food intake, and polydipsia was suppressed, as evidenced by the percentage reduction in fluid intake and by the significant positive correlation seen between fluid intake and the fasting blood glucose (FBG) ($r=0.7631$, $p<0.0001$). This was accompanied with a reduction of 23.6% and 15% in the FBG levels of DHB and DSL animals respectively. This means that increased FBG can lead to osmotic diuresis thereby stimulating osmoreceptors to increase fluid intake. Although, the glucose tolerance of DRF and DHB animals improved, it did not reach significance and the glucose tolerance of DSL animals remained unchanged. The slight/mild decrease in FBG levels observed in the DRF animals of the current study, is party supported by a study that showed a significant reduction in the FBG levels of obese diabetic mice after treatment with aspalathin (a component of rooibos) (Son *et al.*, 2013) and was

accompanied with improved glucose intolerance. This is also in agreement with the findings of Muller et al., who showed that rooibos offer a glucose lowering effect on diabetic rats by improving the glucose tolerance (Muller *et al.*, 2012). The decrease in the FBG of up 23.6% observed in the DHB animals of the current study concur with Muller et al., who reported reduced FBG levels in obese insulin resistant rats after administration of honeybush extract (Muller *et al.*, 2011). Additionally, the decrease in FBG levels of DSL animals in the current study is supported by Wayne et al. who showed diabetic rats receiving sutherlandia displayed lower blood glucose as evidenced by normoinsulinaemic levels and increased glucose uptake (Chadwick *et al.*, 2007).

In normal insulin signalling, IRS 1/2 are the most important insulin receptor substrate for glucose homeostasis (Withers *et al.*, 1999), as they act as binding sites for Src-homology-2 domain containing molecules such as PI3K. Binding of these molecules to IRS1/2 activates series of cascade signalling which eventually activates PkB. Activation of PkB/Akt in turn stimulate the translocation of GLUT4 from the intracellular pools to the plasma membrane.

However, the absence of GLUT4 in the testis has been reported by several authors (Burant and Davidson, 1994; Schürmann *et al.*, 2002; Kokk *et al.*, 2004). Gomez et al. showed that GLUT8 is the dominant glucose transporter in the testis and that both insulin depletion and hyperglycaemia do not regulate its expression (Gómez *et al.*, 2009). A year after this report, Lampioa and du Plessis reported that insulin stimulates GLUT8 expression in human spermatozoa and they further speculated that GLUT8 translocation is aided through the PkB/Akt pathway (Lampiao and du Plessis, 2010). In the current study, GLUT4 was measured in the testis and all groups showed GLUT4 expression. Briefly, both the control and vehicle groups of this study displayed testicular protein expression of IRS1/2, PkB/Akt, ERK1/2 and

GLUT4. In addition to the stimulation of GLUT8 during testicular glucose metabolism, these results suggest the involvement of GLUT4 in testicular insulin signalling.

Additionally, the testicular tissue of diabetic control animals of the current study showed a decrease in IRS1/2, PkB/Akt and GLUT4. This is in agreement with the group of authors that reported impaired insulin signalling in C3A liver cells induced with insulin resistance, as these cells displayed decreased PtdIns (3, 4, 5)P_2 and subsequent reduction in PkB (Mazibuko-Mbeje, Dludla, Johnson, *et al.*, 2019). In addition to the impaired insulin synthesis and secretion observed in DM, studies have shown that this disorder in part develop because of disruption in IRS 1/2 (Araki *et al.*, 1994; Withers *et al.*, 1998). Araki et al. reported impaired glucose tolerance, decreased insulin/insulin-like growth factor-1 (IGF-1) stimulated glucose uptake, and reduced intrauterine growth, in targeted gene mutated IRS-1 deficient mice (Araki *et al.*, 1994). Withers et al. additionally showed that disruption of IRS-2 impaired both pancreatic β cell function and peripheral insulin signalling in IRS-2 deficient mice. They further reported that these mice displayed dysfunctional glucose homeostasis, and hence concluded that disruption of IRS-2 may contribute to the pathophysiology of human type II DM (Withers *et al.*, 1998). Pertainng to male reproduction, Griffeth et al. reported that deletion of IRS-2 in mice resulted in a 45% reduction in testicular weight with consequent decreases in Sertoli cells, spermatogonia, spermatocytes, spermatids and the epididymal sperm reserve. However, there was normal cellular association (Griffeth, Carretero and Burks, 2013). Yagci and Zik also reported that IGF-1 play a significant role in testicular function and germ cell development. As the receptor for IGF-1 was distributed in the pachytene primary spermatocyte and Leydig cells of aged rats (Yagci and Zik, 2006). This suggest the importance of maintaining normal testicular insulin signalling, as disruption in signalling molecules, especially, IRS 1/2, PkB/Akt and GLUT may impair testicular function. Additionally, the

diabetic control animals of this study had lower plasma testosterone levels. This is in agreement with several studies that reported reduced serum testosterone following diabetes induction (Ballester *et al.*, 2004; Vikram *et al.*, 2008).

Regarding the effects of rooibos on testicular insulin signalling, the diabetic animals receiving rooibos (DRF) presented with an increase in testicular protein expression of IRS ½. Interestingly, there was also a reduction in the expression of PkB/Akt and GLUT4. The decrease in PkB/Akt and GLUT4 observed in the testicular tissue of DRF rats of the current study is in contrast with the findings of Mazibuko et al., which showed the reversal of insulin resistance in 3T3-L1 adipocytes treated with rooibos through the suppression of phosphorylated IRS-1, nuclear factor kappa beta (Nf-kB), and AMPK and increased GLUT4 expression (Mazibuko *et al.*, 2015). In another manuscript, the same group of authors reported the ameliorative effects of rooibos on insulin resistant C2C12 muscle cells. This was evidenced by the enhanced glucose uptake, mitochondrial activity and ATP production after treating the muscle cells with rooibos. Mechanistically, it was shown that rooibos increased the activation of Akt, AMPK and GLUT4, suggesting that rooibos can exert its ameliorative effects through the PkB/Akt/GLUT4 pathway in these cells (Mazibuko *et al.*, 2013). The difference between the results of the current study and the reports of Mazibuko et al., may be due to (i) different models of investigation (*in vivo/in vitro*), (ii) different tissues (testis/adipocytes and muscle cells) and (iii) the plausibility that rooibos does not enhance testicular insulin signalling through the PkB/Akt/GLUT4 pathway.

Studies have shown that insulin can also activate the mitogen activated protein kinases (MAPK), especially the ERKs through the binding of growth factor receptor-bound protein-2 (GRB-2) and Son-of-sevenless proteins (SOS) to IRS1/2. GRB-2 and SOS can bind IRS1/2 because they contain the Src-homology-2 domain. Binding of these proteins to IRS1/2

activates membrane bound Ras. The phosphorylation of Ras stimulates a stepwise activation of kinase signalling of Raf, MEK and ERK. ERK is then translocated into the nucleus where its stimulate the phosphorylation of transcription factors to initiate gene expression needed for cell proliferation and differentiation (Alan and Kahn, 2001). Interestingly, the DRF animals of the current study presented with an upregulation (13.2%) in phosphorylated ERK 1/2. Although, the upstream signalling molecules (GRB-2 and SOS) were not measured, it may be suggested that rooibos may rather exert its effect on diabetic testicular insulin signalling through the IRS/ERK pathway. Regarding testosterone levels, diabetic animals receiving rooibos displayed a non-significant decrease in plasma testosterone levels. This partly concur with Opuwari and Monsees who reported that both fermented and unfermented rooibos reduces testosterone production in TM3 Leydig cells (Opuwari and Monsees, 2015). This suggest that although rooibos may play a role in insulin signalling, it does not have an effect on testosterone levels.

While studies have shown the hypoglycaemic effects of honeybush (ICHIKI *et al.*, 1998; Muruganandan *et al.*, 2002), little to nothing is known about the mechanism/pathway at which these effects are exerted. In the current study, diabetic animals receiving honeybush (DHB) displayed a mild increase in testis protein expression of IRS 1/2 and PkB/Akt while GLUT4 remained unchanged. Since GLUT8 was not measured in the current study, little can be said about it. However, extrapolating from previous reports, it can be speculated that honeybush may enhance insulin signalling through the stimulation of IRS1/2, PkB/GLUT8 pathway. Additionally, the DHB animals presented with a mild increase (7%) in the level of plasma testosterone. Since, this is the first study demonstrating this property, it can be suggested that honeybush may have a positive effect on testosterone production, although the mechanism through this occur still needs further investigation.

Diabetic animals receiving sutherlandia presented with increased tIRS-2, tPkB and tGLUT4. The increase in GLUT4 observed in the current study concur with Dang et al. who reported that pinitol (a sutherlandia component) enhanced GLUT4 translocation from the cytosol (intracellular vesicles) to the plasma membrane in skeletal muscle of C57BL/6 mice (Dang *et al.*, 2010). Bates et al. also showed that pinitol exert a mild but sustained hypoglycaemic insulin-like effect in the murine model of type 1 DM (Bates, Jones and Bailey, 2000). Additionally, Williams et al. reported that sutherlandia prevents changes in DM related gene expression in insulin resistant cell model and that it upregulates GLUT4 translocation to the cell surface (Williams *et al.*, 2013). Hence, it can be suggested that the decrease in the blood glucose levels of diabetic animals receiving sutherlandia observed in the current study may be through the stimulation of the IRS1/2, PkB/Akt, GLUT4 pathway.

In conclusion, the result of the present study have shown the presence of GLUT4 in the testis and its plausible role in insulin signalling as seen in a typical cell. We have also demonstrated the role of rooibos, honeybush and sutherlandia in insulin signalling. Therefore, it can be suggested that these infusions may enhance insulin signalling through diverse pathways, but further investigations are required to elucidate the specific upstream and downstream proteins that are plausibly activated during this process.

Conflict of interest

Authors have no conflict of interests

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List of tables and figures

Table 1: Biometric data

Parameters	Control	Vehicle	DC	DRF	DHB	DSL	Global p Value
Food intake per day (g)	22,40±2,48	21,48±1,61	40,43±2,87$^{\&@}$	35,38±4,94$^{\&@\#}$	32,22±5,44$^{\&@\#}$	34,92±4,14$^{\&@\#}$	<0,0001
Fluid intake per day (ml)	39,86±8,57	45,00±9,33	190,8±30,60$^{\&@}$	156,0±44,17$^{\&@}$	124,4±45,76*	152,6±25,44$^{\&@}$	<0,0001
Average blood glucose levels (mmol/L)	6,379±0,25	6,308±0,36	25,76±2,59$^{\&@}$	24,73±5,01$^{\&@}$	21,93±5,77$^{\&@}$	24,71±3,07$^{\&@}$	<0,0001
Fasting blood glucose at sacrifice (mmol/L)	6,150±0,73	5,660±1,02	19,62±7,49$^{\&@}$	18,91±9,14$^{\&@}$	14,98±9,43$^{\$}$	16,60±8,92*$^{@}$	<0,0001

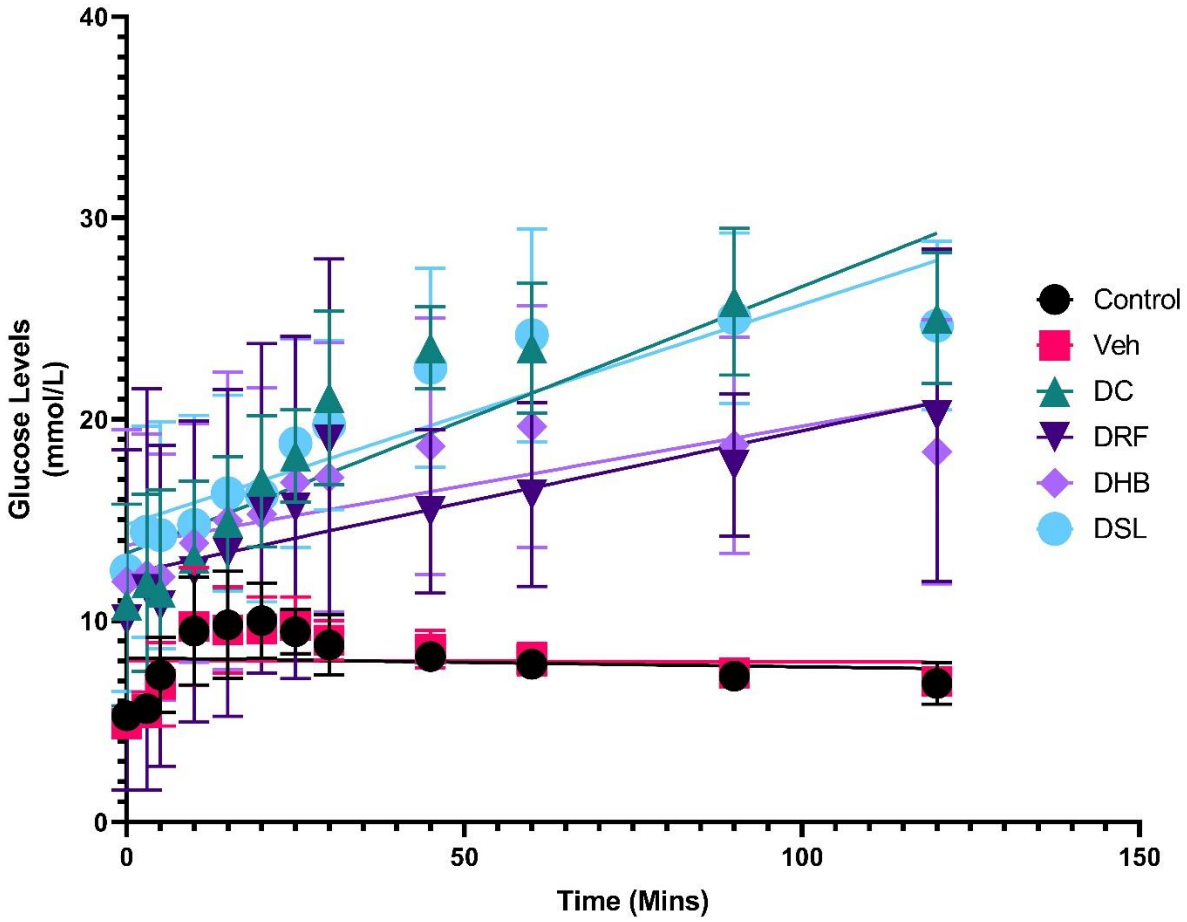
*p<0.05 vs. control; $^{\&}$p<0.001 vs. control; $^{\$}$p<0.05 vs. vehicle; $^{@}$p<0.001 vs. vehicle; $^{\#}$p<0.001 vs. DC. DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic +suthelandia

Table 2: Body, organ and tissue weights

Parameters	Control	Vehicle	DC	DRF	DHB	DSL	Global p Value
End body weight (g)	344,6±19,04	332,4±30,19	280,8±20,89*	266,7±32,95$^{\&@}$	288,3±37,86*	247,4±30,60$^{\&@}$	<0,0001
Testicular weight (g)	1,414±0,12	1,351±0,09	1,380±0,11	1,357±0,14$^{\&}$	1,342±0,15	1,213±0,22	0,2510
Visceral fat weight (g)	13,22±2,94	12,40±3,02	3,073±1,36$^{\&@}$	2,476±1,54$^{\&@}$	4,208±2,51*$^{\$}$	2,378±1,20$^{\&@}$	<0,0001
Relative testicular weight (%)	0,4107±0,03	0,4090±0,04	0,4937±0,05	0,5165±0,08*$^{@}$	0,4679±0,03	0,4921±0,08*	0,0002

*p<0.05 vs. control; $^{\&}$p<0.001 vs. control; $^{\$}$p<0.05 vs. vehicle; $^{@}$p<0.001 vs. vehicle; $^{\#}$p<0.001 vs. DC. DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic +suthelandia

A



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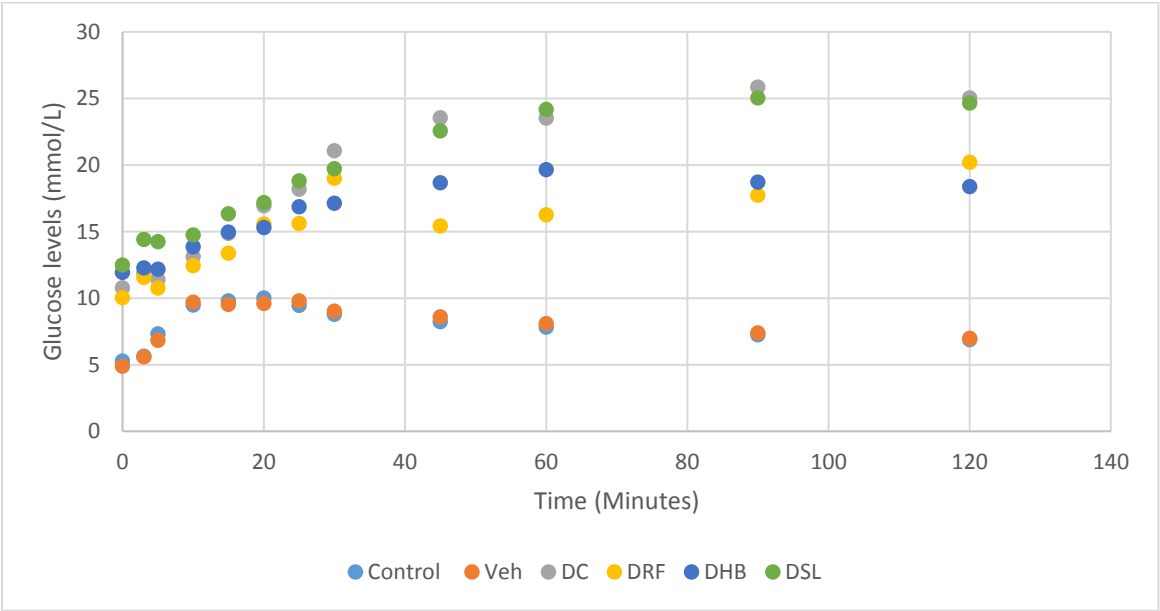

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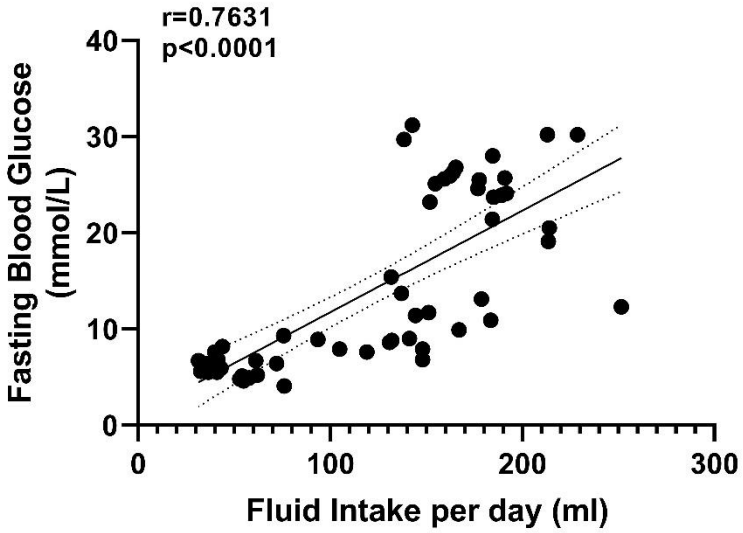

Figure 1: Glucose blood test. **A**. Glucose tolerance test (graph showing mean±SD) **B**. Glucose tolerance test (graph showing mean only) **C**. Correlation between fasting blood glucose levels and fluid intake per day. DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic +suthelandia

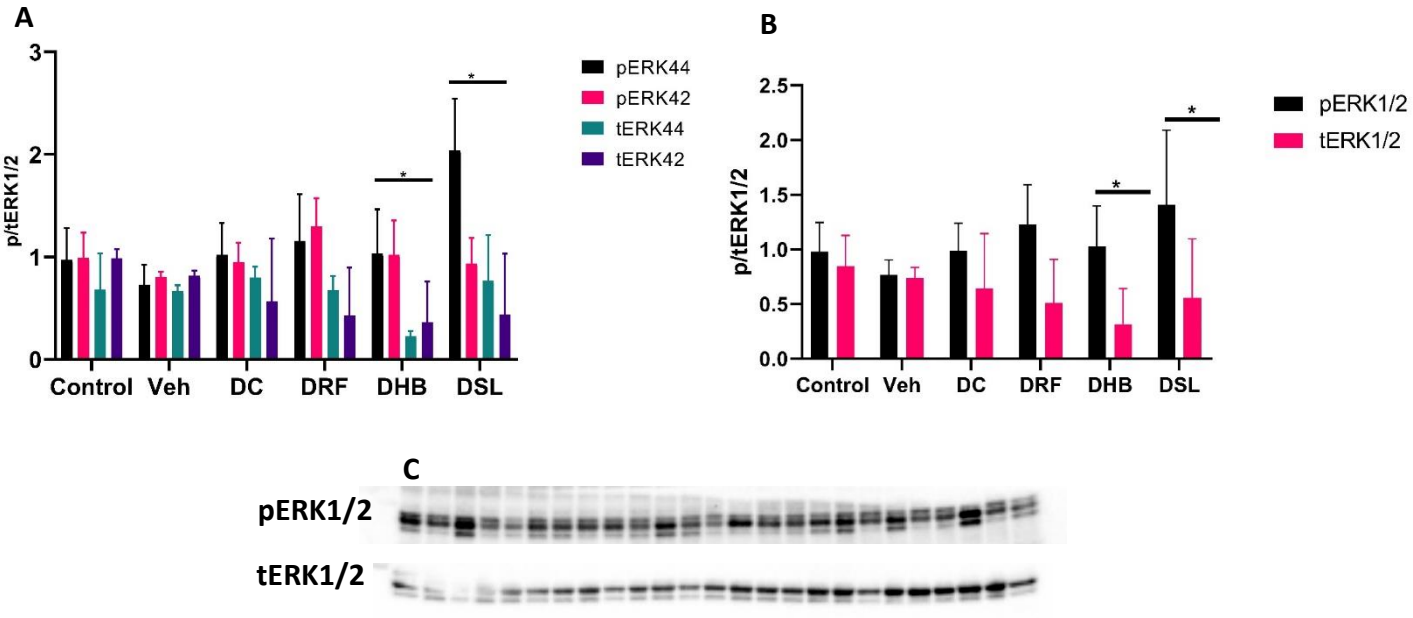

Figure 2: Protein expression of phospho and total ERK1/2. **A**. individual phospho:total ERK1/2, **B**. p/tERK1/2 at a glance, **C**. Probed blot. *p<0.05 vs. control. Veh=vehicle, DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic +sutherlandia, n=4-5.

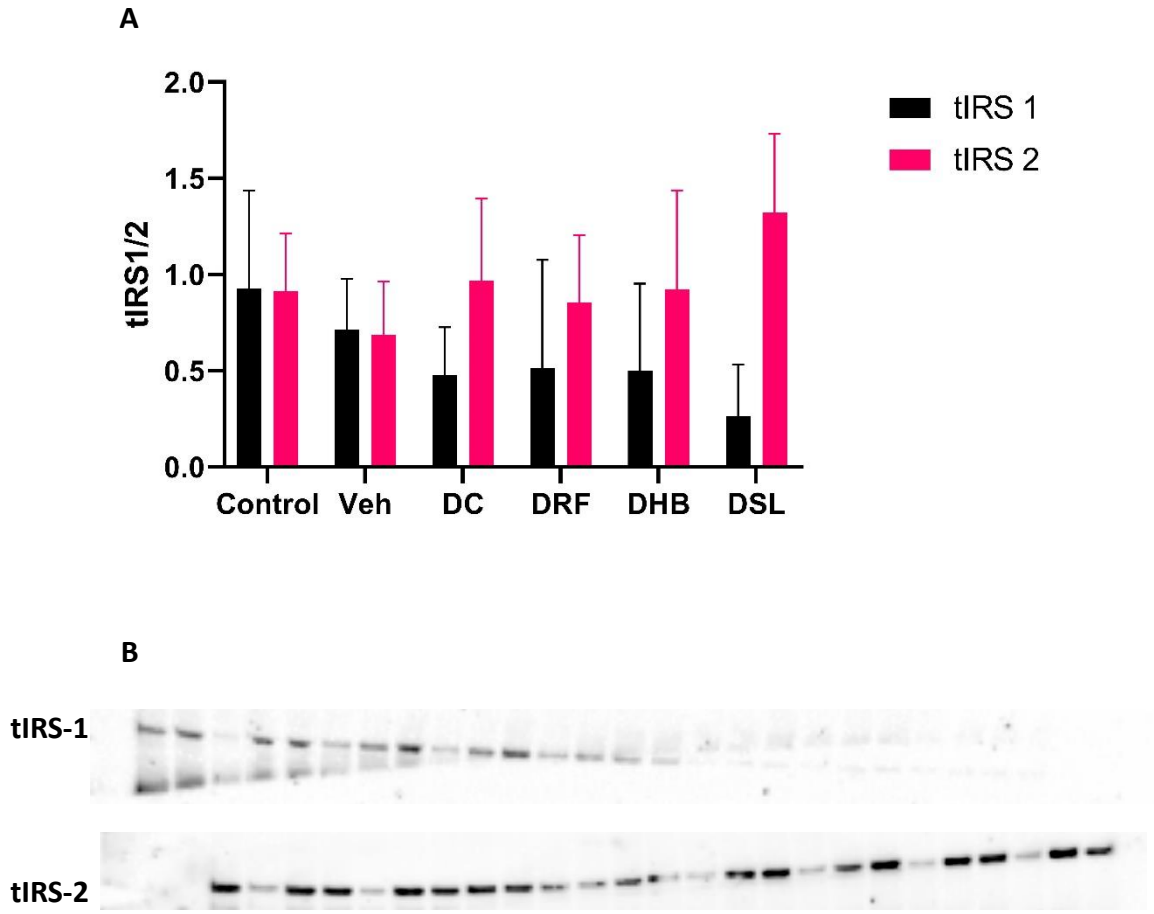

Figure 3: Protein expression of total IRS-1 and IRS-2. **A**. Ratio of IRS1 to IRS 2, **B**. Probed blot. Veh=vehicle, DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic +sutherlandia, n=4-5.

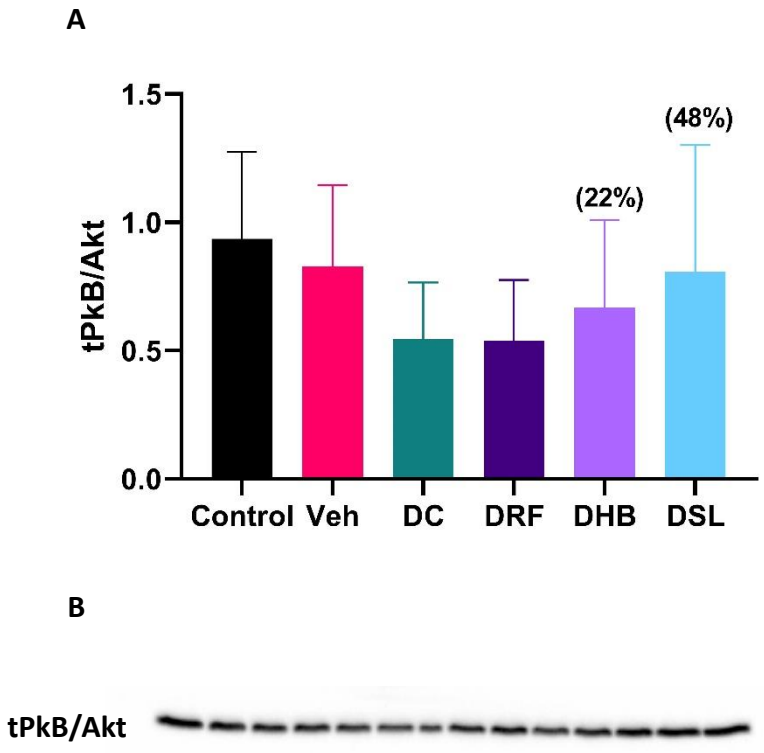

Figure 4: Total protein expression of PkB/Akt. **A.** Graph of PkB/Akt, **B**. Probed blot of PkB/Akt. Values in bracket () denotes percentage change versus DC. Veh=vehicle, DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic +sutherlandia, n=4-5.

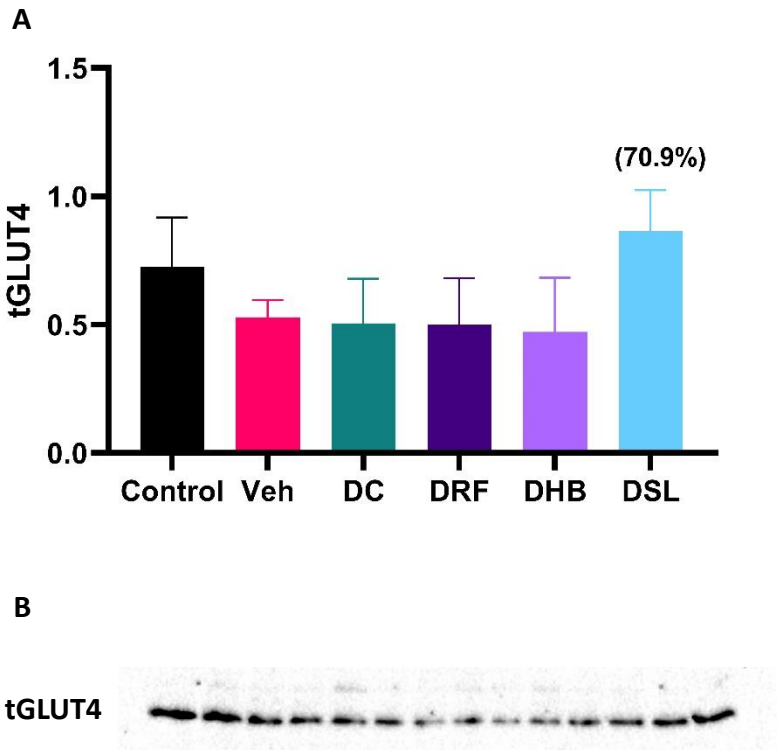

Figure 5: Total protein expression of GLUT4. **A**. Graph showing tGLUT4, **B**. Probed blot. Values in bracket () denotes percentage change versus DC. Veh=vehicle, DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic +sutherlandia, n=4-5.

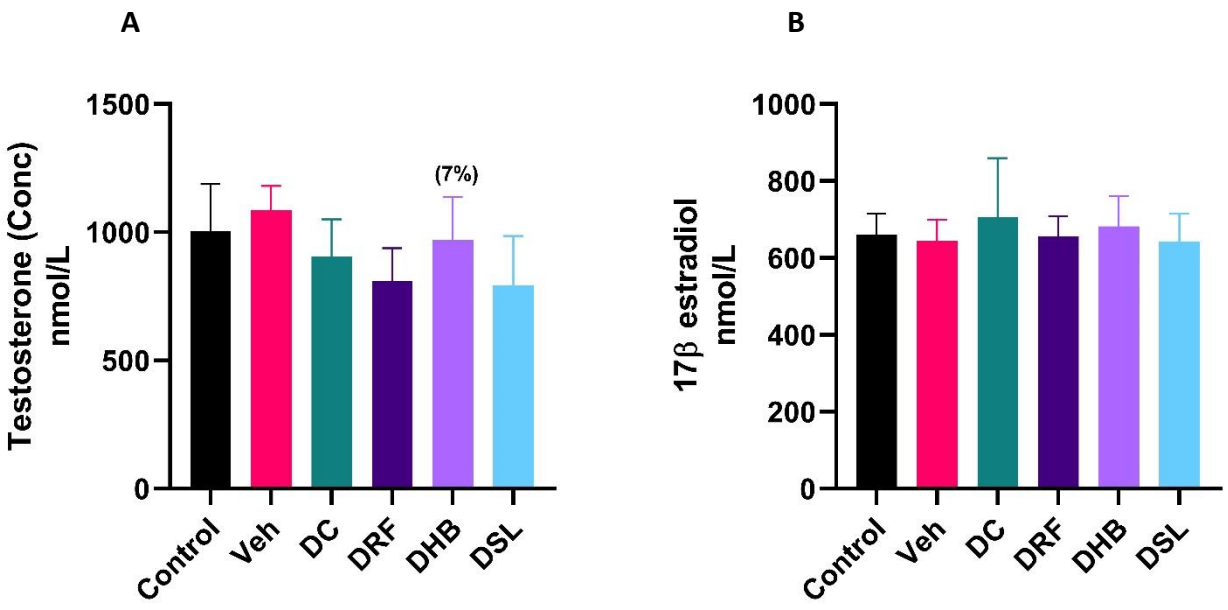

Figure 6: Plasma levels of testosterone and estradiol. **A**. Tetosterone, **B**. Estradiol. Values in bracket () denotes percentage change versus DC. Veh= vehicle, DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic +sutherlandia, n=4-5.

Chapter 9

The descriptive histomorphological evaluation of the testis and caudal epididymis after treating with rooibos, honeybush and sutherlandia in both healthy and streptozotocin-induced diabetic rats.

Will be submitted in Histopathology

Temidayo S Omolaoye[1], Stefan S du Plessis[1, 2]*

1. Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa

2. Department of Basic Sciences, College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates

* Corresponding author:

Prof SS du Plessis

Division of Medical Physiology

Faculty of Medicine and Health Sciences

Stellenbosch University

Francie van Zijl Drive

Tygerberg

7505

South Africa

E-mail: ssdp@sun.ac.za

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Word count: 3948

Abstract

Objective

The aim of the current study was to evaluate the testis and the cauda epididymis after treating both healthy and diabetic rats with rooibos, honeybush and sutherlandia.

Materials and methods

Ninety animals were divided into nine groups without bias (n=10 per group). The groups included five control groups, (control, vehicle, rooibos (RF, 2% fermented rooibos), honeybush (HB, 4% fermented honeybush) and sutherlandia (SL, 0.2% unfermented sutherlandia)) and four diabetic groups. Diabetes was induced via a single intraperitoneal injection of STZ at 45mg/kg. The groups are a diabetic control group (DC, received water), a diabetic + rooibos (DRF, received 2% fermented rooibos), a diabetic + honeybush (DHB, received 4% fermented honeybush) and a diabetic + sutherlandia group (DSL, received 0.2% unfermented sutherlandia). Animals were sacrificed after seven weeks of DM induction. Blood and testes were collected.

Results

Animals in the DC group presented with a significant increase in fasting blood glucose (FBG) levels compared to the control (p=0.02) and vehicle (p=0.0006) groups. DRF and DSL animals presented with a non-significant increase in FBG compared to the control (p=0.07, p=0.1) but was significantly increased compared to the vehicle (p=0.002, p=0.004) groups respectively. However, there was no statistically significant difference between the FBG of DHB compared to the control (p=0.8) and vehicle (p=0.06) groups.

DC animals presented with a significant decrease in the percentage of histologically normal seminiferous tubules compared to the control (49±13.85% vs. 68±7.54%, p=0.049). While DRF, DHB and DSL animals displayed an upregulation (21%, 14%, 5.4%) in the percentage of normal seminiferous tubules compared to the DC group.

Conclusion

The infusion control groups (RF, HB and SL) showed normal seminiferous tubule cellular association, presence of an epididymal lumen filled with spermatozoa and have normal overall architecture. Both the testicular and epididymal morphology were altered in DM, but these disruptions were mildly ameliorated by rooibos, honeybush and sutherlandia

Keywords: Testis, epididymis, rooibos, honeybush, sutherlandia, diabetes, histology

Introduction

The testis is the male reproductive gonad and its function is to produce spermatozoa through a process called spermatogenesis. The produced spermatozoa are transited into the epididymis where maturation occurs. Spermatogenesis is a complex biological process that involves proliferation and differentiation of spermatogonia into spermatids within the seminiferous tubules of the testis. Studies have shown the importance of controlled glucose metabolism and other intermediate metabolites in spermatogenesis [1]. It is known that spermatogenesis takes approximately 74 days in humans [2–4], while it takes 35-52 days in rodents [5]. However, a recent study conducted in a group of normal men concluded that the total time to produce sperm may vary between 42-76 days [6]. The seminiferous tubules are the functional units of the testis. They consist of a basal membrane (the tubular epithelium), Sertoli cells (which are surrounded by the spermatogenic cells on the inside of the epithelium and stem cells exteriorly) , blood-testis barrier (which regulates the passage of substances between rete testis fluid and the lymph or plasma), germ cells at different developmental stages and the lumen that housed early and late spermatids [7]. After spermatozoa production in the seminiferous tubules of the testis, they are transferred into epididymis and move through the caput, and corpus part and are finally stored in the caudal epididymis until ejaculation. The individual sections of the epididymis contributes specifically to the luminal microenvironment stability, which is important for sperm maturation by the time they reach the caudal segment. Due to the array of cellular structures present in the testis and epididymis, diverse biochemical and physiological processes occur. Hence, these organs are easily influenced either positively or otherwise by several factors. Studies have shown the association between an altered cellular organization in the seminiferous tubule, the loss of spermatozoa in the lumen of the epididymis and the subsequently impaired sperm quality in

diverse pathologies including diabetes mellitus (DM). DM is a chronic disease marked by hyperglycaemia. It occurs because of lack in insulin synthesis and secretion and/or due to the insensitivity of the specific tissue to the effect of insulin. DM has been shown to inflict damage on several organs, including the testis [8]. It is estimated that between 35-51% of diabetic men has testicular and or erectile dysfunction [9]. Keyhanmanesh et al. reported a significant decrease in the seminiferous tubule diameter, epithelium height, reduction in the number of Sertoli and Leydig cells, spermatogonia and spermatocytes in diabetic rats [10]. Soudamani et al. on the other hand reported reduction in the size of all the epididymis segments (caput, corpus and cauda) and an increase in the interstitial stroma of diabetic rats. It was further stated that due to the reduction in the epididymal segments, the tubules were shrinked, the epididymal principal cells were tightly packed having clumped nuclei and that the lumen was devoid of spermatozoa [11]. Several other authors have also reported abnormalities in the testis and epididymis of diabetic rats [12,13]. Interestingly, Ayuob et al. reported that after treating diabetic rats with antidiabetic drugs (metformin, pioglitazone and sitagliptin), although the symptoms of DM were improved, these drugs induced histopathological changes on the testicular structures (testis, epididymis, and seminal vesicle) [14]. It was stated that the testis of diabetic rats treated with metformin showed deformed primary spermatocytes, has few spermatozoa in the lumen and also showed cellular vacuolation, while the diabetic animals treated with pioglitazone showed atrophy. The testis of sitagliptin treated rats displayed a reduced mean cross sectional area of the epithelium and the tubular capillaries were congested.

Since the Western/synthetic antidiabetic drugs seem to pose a great danger to the process of sperm production, sperm quality and quantity, it is important to develop different therapeutic strategies in treating and/or manageing DM. In lieu of this, various studies have been

undertaken to explore the phytotherapeutic effects of rooibos, honeybush and sutherlandia in diverse pathologies, including DM.

Rooibos, honeybush and sutherlandia are indigenous Southern African plants. Rooibos originates from the Cederberg Mountains of the Western Cape region of South Africa [15,16]. It is generally available in either the unfermented (green rooibos) or the fermented (reddish-brown rooibos) form. The fermented tea is obtained by oxidation and results in the unique reddish-brown leaf colour and woody-fynbos-floral honey flavour [17]. Honeybush is native to the Southeast and Southwest coastal areas of South Africa. It forms a part of the fynbos biome with the family name Fabaceae. It is used as a traditional tea since the 19th century [18,19]. Sutherlandia is a plant with diverse species that are widely spread across specific geographic regions of Southern Africa. It is indigenous to the Northern, Eastern and Western Cape areas of South Africa, southern areas of Namibia and south eastern regions of Botswana and Lesotho [20]. The infusions from these plants contains assorted phytochemicals that are beneficial to health.

Several authors have reported the suppression of steatosis, liver cirrhosis, reduced inflammation and hepatocellular injury, inhibition of triacylglycerol and reduced plasma levels of aminotransferases in rats treated with either fermented or unfermented rooibos [21,22]. Honeybush has been shown to offer photo protection against UVB-induced skin damage in SKH-1 mice. This was evidenced by the reduction in erythema, peeling and hardening of the skin. It was suggested that honeybush enhanced cell proliferation, hence the observation [23]. Sutherlandia have also been reported to have a neuroprotective effect in C57BL/6J mice. As these mice showed a significant decrease in neuronal cell death with increased activation of microglia in the hippocampus and striatum in the ischemic brains [24].

Regarding testicular tissues, Opuwari and Monsees reported that there were no crucial changes in the histomorphology of the testis and epididymis of healthy rats treated with rooibos. As testis showed complete spermatogenic phases with abundant spermatozoa in the lumen [25]. These authors in another manuscript also reported decreased testosterone production by Leydig cells treated with rooibos in vitro. This suggest that rooibos may have anti-androgenic activities [26]. Hence, the role of rooibos on male reproductive histomorphology remains controversial. Additionally, to the best of our knowledge, till date there are no studies reporting the effects of honeybush and sutherlandia on the histomorphology of the testis and epididymis in experimental animals, in both healthy and diseased states.

Hence, the aim of the current study was to evaluate the testis and the cauda epididymis after treating both healthy and diabetic rats with rooibos, honeybush and sutherlandia.

Materials and methods

Infusion preparation

Fermented rooibos (*Aspalathus linearis*) was procured from Carmien SA PTY LTD, South Africa. Fermented honeybush (*Cyclopia intermedia*) and unfermented sutherlandia (*Lessertia frutescens*) were obtained from Afrinaturals, South Africa. Infusions were prepared according to previously established methods [27]. Briefly, 2% fermented rooibos was prepared by adding 20g of dried rooibos in 1litre of boiled water and allowed to soak for 30 minutes. After 30 minutes, the mixture was initially filtered using a cheesecloth and then filtered with a number 4 and a number 1 Whatman filter paper respectively (WhatmanTM, Buckinghamshire, UK). Filtered teas/infusions were transferred to a dark plastic container to

prevent the degradation of the light sensitive polyphenols and were stored at 4°C. Infusions were prepared freshly every 48 hours. Fermented honeybush (4%) and unfermented sutherlandia (0.2%) were prepared following the same method as described for rooibos. All infusions (rooibos, honeybush and sutherlandia) were prepared every other day (48 hours). The herbal teas serve as the only drinking fluid for these infusion groups. The fluid intake of the animals was measured three times a week.

Experimental design

Ninety adult male Wistar rats of weight range 250-300g were housed at the Stellenbosch University's Faculty of Medicine and Health Sciences Animal Unit (18-23°C, 12:12 light/dark cycle). Animals were randomly divided into nine groups of ten. They were treated according to the recommendations of the Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals [28]. Rats were individually caged and had free access to both food and water/infusions. The Stellenbosch University Animal Ethics Committee approved the study under (SU-ACUD17-00016).

Diabetes was induced by administering a single intraperitoneal injection of streptozotocin (STZ) at 45mg/kg body weight from a stock solution of 30mg/ml (dissolved in sodium citrate buffer pH 4.5) (STZ S0130-IG, Sigma, South Africa). DM was confirmed after one week if the blood glucose level was ≥14mmol/L.

The groups examined in this study include a control group, vehicle group (Veh), rooibos control (RF), honeybush control (HB), sutherlandia control (SL), a diabetic control group (DC), a diabetic + rooibos (DRF), a diabetic + honeybush (DHB) and a diabetic + sutherlandia group (DSL). Animals in the infusion treatment groups were pre-treated with the tea for one week

before diabetes induction. Food intake, fluid intake and body weights were measured thrice a week and blood glucose levels were measured once weekly. After 7 weeks of DM induction, animals were sacrificed and blood samples immediately collected. The testes and epididymis were also harvested and weighed. The relative testicular and relative epididymal weights were expressed as a percentage of body weight. The reported fasting blood glucose levels were recorded immediately before sacrifice and the average blood glucose measured over the treatment period was also reported

Experimental procedures

Testosterone

The plasma concentration of testosterone (E-EL-0072) was measured using a commercially available ELISA kit (Elabscience Biotechnology, Hubei), and analysis was performed as per manufacturer's instructions. Briefly, 100µl of standards and samples were added to each appropriate wells and were incubated for 90 minutes at 37°C. The liquid were removed after incubation and 100µl of Biotinylated detection Ab/Ag was added and incubated for another 60 minutes at 37°C. After incubation, the liquid were aspirated and washed 3 times at 5 minutes interval. Thereafter, 100µl of HRP testosterone conjugate was added, incubated for 30 minutes at 37°C. The liquid were aspirated and the washing steps were repeated 5 times. Hereafter, 90µl of substrate reagent was added and incubated for 15 minutes at 37°C and 50µl of stop solution was finally added. The absorbance was measured at 450nm using a plate reader.

Histology

The right testis and the epididymis were fixed in a 10% formalin solution. The tissues were kept in the solution for a minimum of 48 hours to allow for complete fixation. The fixed tissues were processed according to previously reported protocol [29]. Tissues were stained with haematoxylin and eosin (AUCL/E, Kimix Chemical and Lab. Supplies, South Africa) using an autostainer (Leica Auto stainer XL). Additionally, for proper visualization of the tissues towards qualitative analysis, testicular tissues (testis and epididymis) were stained with alcian blue-periodic acid schiff (AB/PAS). In brief, sectioned tissues were deparaffinised and hydrated in distilled water. Sections were stained with Alcian Blue (AB/PAS powder, Sigma South Africa) for 15 minutes, rinsed thoroughly in tap water for 2 minutes and further rinsed in distilled water for another 2 minutes. Sections were oxidized in 1% periodic acid (Sigma, South Africa) solution for 10 minutes and then rinsed in distilled water. Rinsed sections were placed in Schiff's reagent (CLECA/E, Kimix Chemical and Lab. Supplies, South Africa) for 15 minutes and then wash in lukewarm tap water for 7 minutes 30 second. At this point, sections turned dark pink. Sections were counter stained with haematoxylin solution for 30 seconds, washed in tap water for 3 minutes, then dehydrated, and cleared with xylene. Sections were mounted and allowed to air dry.

Descriptive histomorphology

To properly analyse the morphology of the testis and cauda epididymis, an initial blinded overview of the sections stained with H&E was performed (Figure 1). Thereafter, using AB/PAS stained tissues, tubules were further analysed blindly and categorized as previously described [29]. Briefly, one hundred seminiferous tubules per animal were randomly counted across different fields, with field area ranging between 234.16mm^2-348.97mm^2. Seminiferous

tubules were classified as normal when it showed complete spermatogenic phases, regular cellular organization, normal cellular association and regular interstitial spaces. Tubules were classified as undergoing atrophy when it showed epithelium shrinkage, few or absence of germ cells and cellular disorganization. Tubules undergoing sloughing are characterized by having excessive numbers of immature cells in their lumen and there are absence of some or all of the spermatogenic phases (Figure 2).

The cauda epididymis was considered normal/intact if the following are absent (Figure 3):

(i) Cribriform changes (this represent a hyperplastic modification of the epithelium, e.g infolding of the epithelium)

(ii) Inflammatory infiltrate (excessive accumulation of cells in the interstitium, appearing as a vesicle filled with cells or scattered)

(iii) Debris in the lumen (presence of cytoplasmic shedding in the lumen instead of spermatozoa)

(iv) Clear cells / tubules (clear cells occurs as a consequence of debris in the lumen)

(v) Sperm compaction (this occurs as a result of excessive fluid resorption due to lack in sperm production)

(vi) Epithelial vacuolation (it represents the presence of small and/or large intra and inter cytoplasmic vacuoles, which leads to the alteration of the epithelial organization).

After the blinded overview phase of the cauda epididymis, forty tubules were analysed per animal. Analysis occurred randomly across the whole section at field area ranging between 234.16mm^2-348.97mm^2. The epididymal tubules were then categorized as normal or abnormal (if one of the listed pathologies is present). The percentage of normal epididymides

was obtained by dividing the exact number of normal/intact tubules by the total tubules counted multiplied by 100.

Statistical analysis

GraphPad Prism™ software (GraphPad™ Software, Version 8.2, San Diego, CA, USA) was used for the statistics. Normal data distribution was measured using the Anderson-Darling, Kolmogorov-Smirnov, Shapiro-Wilk and D'Agostino & Pearson, normality tests. When data passed all normality tests, a one-way ANOVA of variance with a Tukey's Post-hoc Test was performed. Where data were not evenly distributed, a Kruskal-Wallis test and a Dunns Post-hoc Test were carried out. Significance was set at $p< 0.05$ and results are expressed as mean ± SD.

Results

Anthropometric parameters

Animals in the DC group presented with a significant increase in fasting blood glucose (FBG) levels compared to the control ($p=0.02$) and vehicle ($p=0.0006$), RF ($p=0.0015$), HB ($p=0.005$) and SL ($p=0.0004$) groups. After seven weeks of infusion treatment, diabetic animals receiving rooibos (DRF) presented with a non-significant increase in FBG compared to the control ($p=0.07$) but was significantly increased compared to the vehicle ($p=0.002$). However, there was no statistically significant difference between the FBG of diabetic animals receiving honeybush (DHB) compared to the control ($p=0.8$) and vehicle ($p=0.06$) groups. The diabetic animals receiving sutherlandia (DSL) followed the same trend as DRF as there was no significant increase in the FBG compared to the control ($p=0.1$) group, while it was significantly higher compared to the vehicle ($p=0.004$) (Table 1)

The DC and DHB animals gained less body weight, while animals in the DRF and DSL groups were significantly lighter compared to the control ($p<0.0001$), vehicle ($p<0.001$), RF ($p<0.001$), HB ($p<0.0001$) and SL ($p<0.0001$) groups. The bodyweight of DHB groups significantly increased compared to DSL ($p=0.02$).

There was no statistically significant difference in testicular weights between the groups. However, the epididymal weight of the DC animals was 10% less compared to the control animals. DRF animals presented with a significant reduction in epididymal weight compared to the control ($p=0.03$). DHB epididymal weight was more than 10% less than the control, while DSL displayed a significant decrease compared to the control ($p=0.001$) group. All diabetic groups presented with a significant reduction in visceral fat weight compared to the control groups ($p<0.0001$).

Although not significant, DC animals presented with an increase of just more than 20% in the relative testicular weight (RTW) compared to control and vehicle respectively, but, there was a significant increase compared to RF (p=0.014) and SL (p=0.045). DRF animals displayed a significant increase in RTW compared to control (p=0.02), vehicle (p=0.014), RF (p=0.0002) and SL (p=0.09). There was no statistical significant difference in relative epididymal weight (REW) between the groups except between the RF and DRF groups (p=0.006) (Table 1).

Testosterone

Although not significant, the DC animals presented with a percentage decrease in plasma testosterone concentration (-9.78%, -16.5%) when compared to the control and vehicle groups respectively. While the DRF animals displayed a significant decrease in plasma testosterone concentration compared to the vehicle group (811.2±126.5nmol/L vs. 1085±95.66nmol/L, p=0.04). The DHB group presented with a non-significant difference in testosterone compared to the DC group. The DSL animals showed a significant reduction in testosterone concentration compared to the RF (793.1±192.9nmol/L vs. 1114±104.5nmol/L, p=0.01) and SL (793.1±192.9nmol/L vs. 1102±165.6nmol/L, p=0.03) (Figure 4).

Histology

DC animals presented with a significant decrease in the percentage of histologically normal seminiferous tubules compared to the control (49±13.85% vs. 68±7.54%, p=0.049). While DRF, DHB and DSL animals displayed an upregulation (21%, 14%, 5.4%) in the percentage of normal seminiferous tubule compared to the DC group (Figure 5). Furthermore, there was a significant difference in the percentage of histomorphologically normal epididymis between groups (p=0.01). There was a significant decrease in the percentage of normal epididymal tubules of the DC group when compared to the SL group (68±12.17% vs. 89.5±10.95%, p=0.04), while they showed a reduction of -23% and -20% when compared to the control and

vehicle respectively. Although not significant, the percentage of normal epididymal tubules was higher in the DRF, DHB and DSL groups when compared to the DC group (23%, 20%, and 10%) respectively (Figure 6).

Discussion

Studies have reported alteration in the cellular arrangement and ultimately disruption of the seminiferous tubule in DM [11]. The absence of spermatozoa in the luminal epididymis has likewise been shown after DM induction or when toxins were administered to rodents [30]. Regarding the effects of drugs used in the treatment of DM, a set of authors investigated the role of some antidiabetic drugs (metformin, pioglitazone and sitagliptin) on the histological organization of the testis and epididymis. Interestingly, they concluded that these drugs confer histopathological changes in the testis and epididymis of these rats [14].

Seeing that synthetic drugs negatively affects male reproductive organs, it is pertinent to develop natural strategies in treating DM and its male reproductive complications. Hence, the current study evaluated the testis and cauda epididymis in both healthy and DM-induced rats after treating with rooibos, honeybush and sutherlandia.

The infusion control groups (RF, HB and SL) of the current study presented with normal FBG relative to the control group. The DC animals showed elevated FBG, reduced visceral fat and body weight. Although there was no statistically significant difference in the testicular weight of the DC animals, they presented with a decrease in epididymal weight. This is in agreement with Soudamani et al. and several other authors who reported the association between hyperglycaemia and reduction in testicular and epididymal weight [10,11,31]. After treating diabetic animals with the respective infusions for seven weeks, DRF animals presented with a non-significant difference in FBG compared to the control but had significance compared to

the vehicle. However, they showed a very mild decrease in FBG compared to DC. The extremely mild decrease in the FBG of the DRF animals of the current study is partly supported by studies that reported the hypoglycaemic effect of rooibos, both in in vivo and in vitro experiments [32,33]. Opuwari and Monsees reported that rooibos had no significant effect on the testicular and epididymal weight of control animals treated for fifty-two days [25]. Another study indicated that after treating rats with rooibos for ten weeks with oxidative stress induction during the last two weeks, there were no changes in the testicular and epididymal weights of these animals [34]. The DRF animals of the current study showed reduction in the testicular, epididymal and visceral fat weights. The DHB animals on the other hand showed no significant difference in the FBG levels when compared to both control and vehicle. When compared to the DC group, they showed a decrease of -23.6%. This concurs with previous studies that have reported the hypoglycaemic effect of honeybush, both in in vivo and in vitro studies [35,36]. Despite the moderate improvement seen in FBG, they still presented with a decrease in body and epididymal weights. Regarding sutherlandia, DSL animals showed improved FBG, but their tissue weight remained on the lower side. Several studies have highlighted the hypoglycaemic potential of sutherlandia [37,38], as Chadwick et al. reported that diabetic rats treated with sutherlandia showed normal serum insulin levels and that it enhanced glucose uptake into the muscle and adipose tissues [38]. Cumulatively, these infusions displayed a very mild hypoglycaemic potential but the animals (DRF, DHB and DSL) showed reduction in the testicular, epididymal and visceral fat weights. It can be speculated that (i) there is increased usage of fat for energy and for the formation of cholesterol (ii) there is a breakdown of the body's protein and amino acid oxidation. Since alteration in the testicular and epididymal morphology observed in DM have been associated with reduction in hormone levels [39–41], we correlated the plasma testosterone

concentration to testicular, epididymal and visceral fat weights. A significant positive correlation was seen between the plasma testosterone concentration and testicular weight (r=0.2482, p=0.02), epididymal weight (r=0.4188, p=0.0001) and visceral fat weight (r=0.4719, p=<0.0001) (Supplementary figure 1). These findings further shows that these animals had less fat and plausibly had less cholesterol and thus leading to altered steroidogenesis.

The DC animals of the current study presented with a decrease in plasma testosterone levels and had altered histomorphology of the testis and epididymis. They showed an increase in the number of disrupted seminiferous tubules, elevated atrophic and sloughy tubules. The epididymis of the DC animals showed an increase in the number of lumen with no spermatozoa, presented with debris in the lumen, vacuoles in the epithelium and moderate inflammatory infiltrates. This cumulatively means that the DC animals showed a reduced percentage of normal/intact epididymides (Figure 6). The histopathological changes observed in both the testis and epididymis of the DC animals of the current study is in agreement with Arikawe et al. who reported altered seminiferous tubule morphology in diabetic rats [42]. Additionally, Soudamani et al. showed the reduction in tubular size (tubular diameter, volume and surface density) of all epididymal segments (caput, corpus and cauda) in diabetic rats. They further reported that due to the shrinkage of tubules, principal cells were packed tightly with clumping of nuclei [11].

Coming to the infusion treated groups, the DRF and DSL animals of the current study presented with a decrease in plasma testosterone concentration, but they showed improvement in the histo-architecture of the seminiferous tubules and epididymis. This is evidenced by the reduced number of seminiferous tubules with accumulation of immature cells in the lumen and they showed normal cellular association and/or organization. The

epididymal epithelium showed lesser vacuolization, and debris in the lumen was reduced. Interestingly, the DHB animals presented with a mild increase (6%) in plasma testosterone concentration with improved histo-morphology of the seminiferous tubules and epididymis. It is worth noting that this is the first study reporting the role of rooibos, honeybush and sutherlandia on the histo-architecture of the testis and epididymis. Although, mechanisms through which these mild changes are exerted is unknown, it can be speculated that honeybush probably enhanced the function of the hypothalamic-pituitary gonadal axis as testosterone was increased.

Conclusion

The current study was designed to evaluate the histomorphology of the testis and epididymis after treating with rooibos, honeybush and sutherlandia, in both healthy and diabetic rats. The infusion control groups (RF, HB and SL) showed normal seminiferous tubule cellular association, presence of an epididymal lumen filled with spermatozoa and have normal overall architecture. Both the testistular and epididymal morphology were altered in DM, but these disruptions were mildly ameliorated by rooibos, honeybush and sutherlandia. Hence, it can be concluded that (i) DM does have detrimental effects on the histomorphological architecture of the testis and epididymis (ii) the individual infusions have no obvious adverse effect on the architecture of the testis and epididymis and (iii) the infusions mildly improve the histomorphology of the testis and epididymis in diabetes. Since, this represent the first study reporting these effects, further investigations are required regarding the mechanisms involved in this process.

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List of table and figures

Table and figure legends

Table 1: Anthropometric data. [a]p<0.0001 vs. control, [b]p<0.0001 vs. vehicle, [c]p<0.0001 vs. rooibos, [d]p<0.0001 vs. honeybush, [e]p<0.001 vs. sutherlandia, [f]p<0.05 vs. DHB, [g]p<0.05 vs. control, [h]p<0.05 vs. vehicle, [i]p<0.05 vs. rooibos, [j]p<0.05 vs. honeybush, [k]p<0.05 vs. sutherlandia. RF=rooibos, HB=honeybush, SL=sutherlandia, DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic+sutherlandia. Average blood glucose = mean of blood glucose measured over 7 weeks.

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Figure 3: Illustration of the cauda epididymal abnormalities (AB/PAS). **A**. Cribiform changes (hyperplastic modification of the epithelium, e.g infolding of the epithelium)**, B and C**.

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Figure 6: Histomorphological overview of cauda epididymis (AB/PAS). **A**. Micrographs scale bar=200µm (the epididymal tubule of the DC animals was frequently void of sperm or have few spermatozoa. Although, the tubule of DSL have spermazoa, there was an in folding of the epithelium)**, B**. Micrographs scale bar=100µm, **C**. Showing the percentage of normal/intact epididymis. [a]p<0.05 vs. SL. Veh=vehicle, RF=rooibos, HB=honeybush, SL=sutherlandia,

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Table 1: **Anthropometric data**

Parameters	Control	Vehicle	RF	HB	SL	DC	DRF	DHB	DSL	Global p
Body weight (g)	344,6±19,04	332,4±30,19	349,4±21,41	345,3±25,55	352,6±19,37	275,1±11,38[a,b,c,d,e]	266,7±32,95[a,b,c,d,e]	288,3±37,86[a,b,c,d,e]	247,4±30,60[a,b,c,d,e,f]	<0,0001
Visceral fat (g)	13,22±2,942	12,40±3,022	12,25±2,867	12,78±3,784	12,58±3,338	3,073±1,363[a,b,e,l,j]	2,476±1,539[a,b,c,d,e]	4,208±2,514[g,h,j,k]	2,378±1,200[a,b,c,d,k]	<0,0001
Testicular weight (g)	1,414±0,123	1,351±0,099	1,346±0,0834	1,438±0,120	1,423±0,111	1,380±0,113	1,357±0,148	1,342±0,154	1,213±0,225	0,0844
Relative testicular weight (%)	0,4107±0,032	0,4090±0,043	0,3858±0,025	0,4174±0,034	0,4034±0,018	0,4937±0,052[c,k]	0,5165±0,081[g,h,l,k]	0,4679±0,036[i]	0,4921±0,080[c]	<0,0001
Epididymal weight (g)	0,4702±0,035	0,4429±0,030	0,4560±0,025	0,4753±0,046	0,4892±0,023	0,4192±0,038[k]	0,3913±0,044[g,j,k]	0,4176±0,049[j,k]	0,3544±0,055[g,l,k]	<0,0001
Relative epididymal weight (%)	0,1367±0,012	0,1344±0,016	0,1308±0,009	0,1381±0,015	0,1389±0,005	0,1500±0,017	0,1684±0,063[c]	0,1453±0,008	0,1438±0,018	0,0047
Fasting blood glucose (mmol/L)	6,150±0,725	5,660±1,024	5,690±0,885	6,120±1,696	5,490±0,682	19,62±7,492[g,b,c,d,e]	18,91±9,148[h,l,j,e]	14,98±9,436[k]	16,60±8,925[b,l,j,e]	<0,0001
Average blood glucose (mmol/L	6,379±0,247	6,308±0,365	6,464±0,352	6,367±0,332	6,410±0,268	25,76±2,592[a,b,c,d,e]	24,73±5,010[a,b,c,d,e]	21,93±5,775[a,b,c,d,e]	24,71±3,074[a,b,c,d,e]	<0,0001

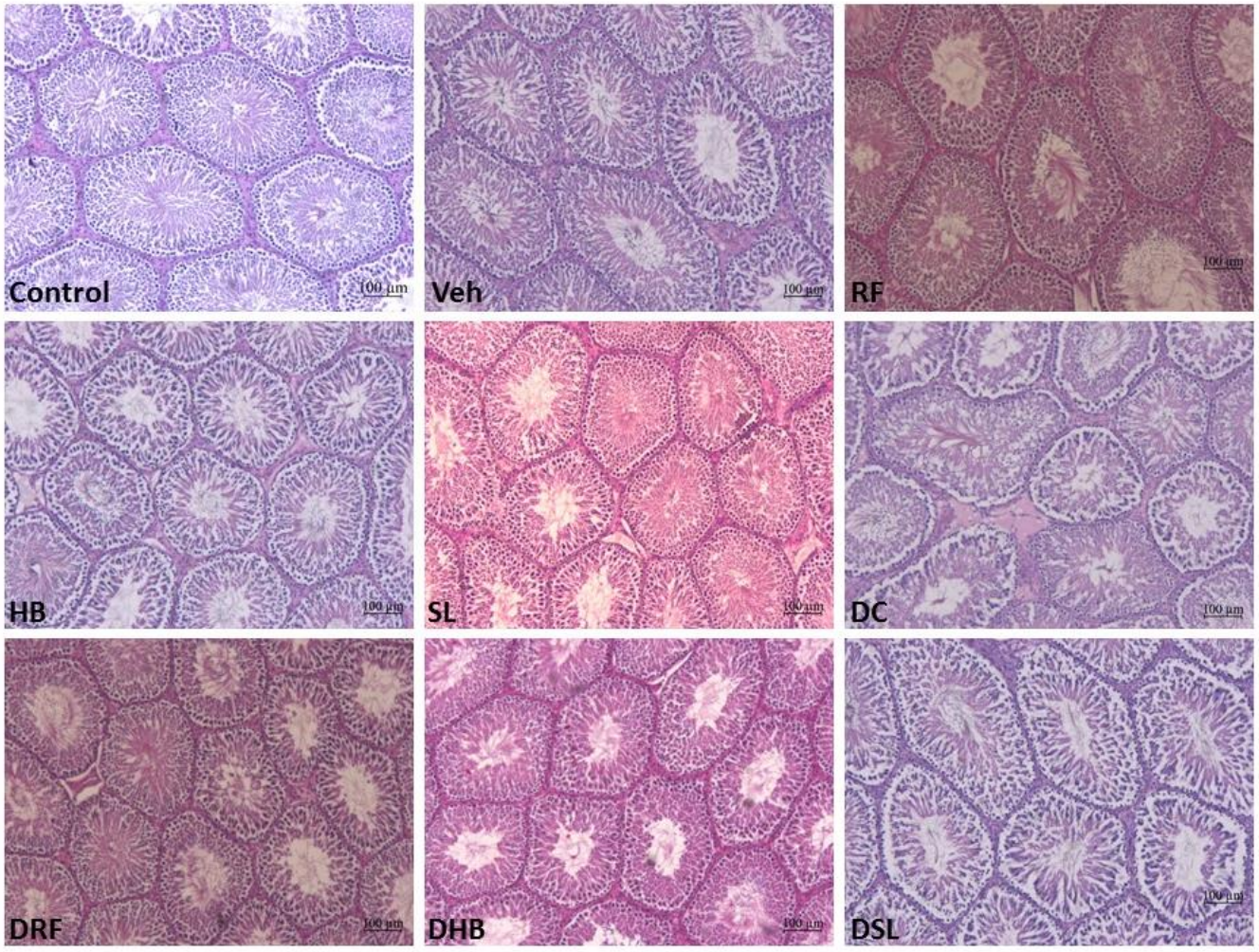
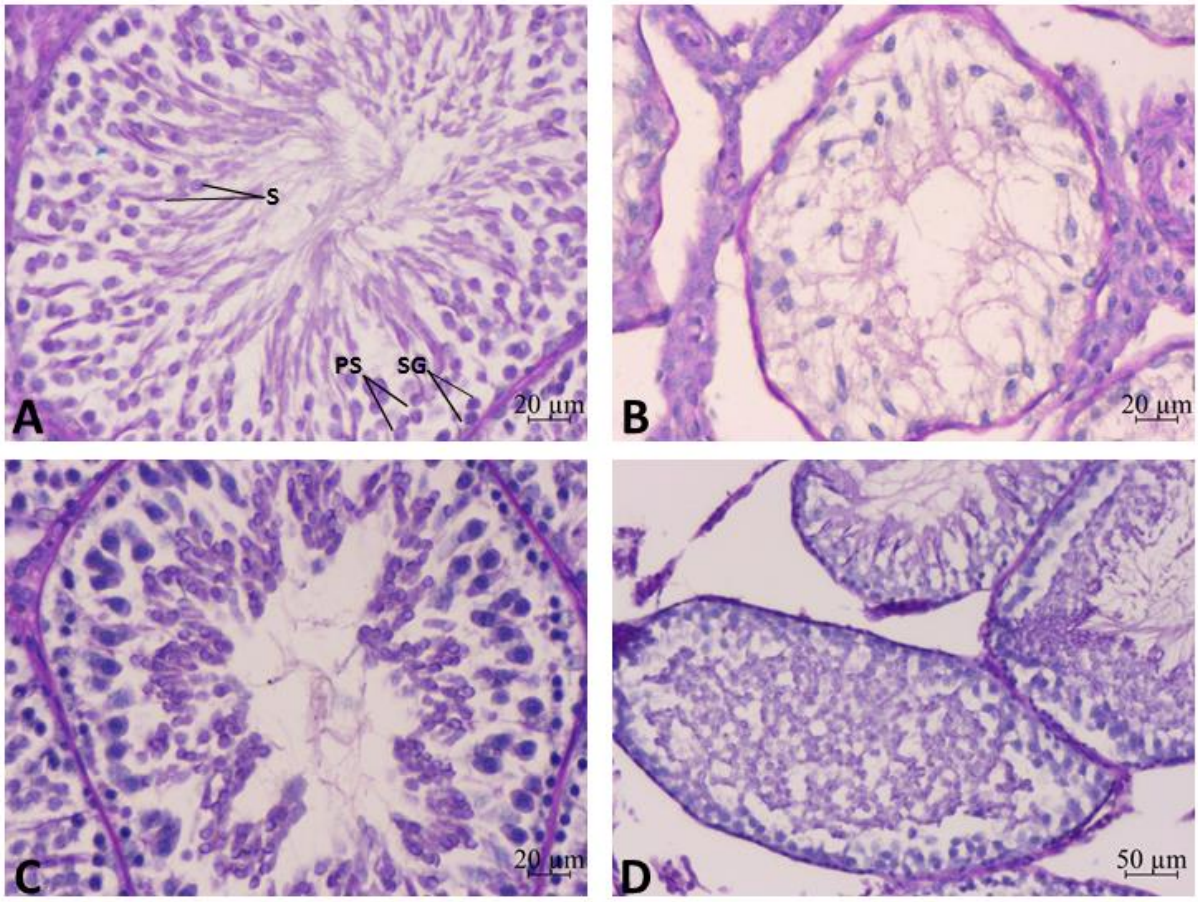


Figure 1**: Histological overview of the seminiferous tubules using H&E.** Veh=vehicle, RF=rooibos, HB=honeybush, SL=sutherlandia, DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic+sutherlandia, Scale bar=100µm.



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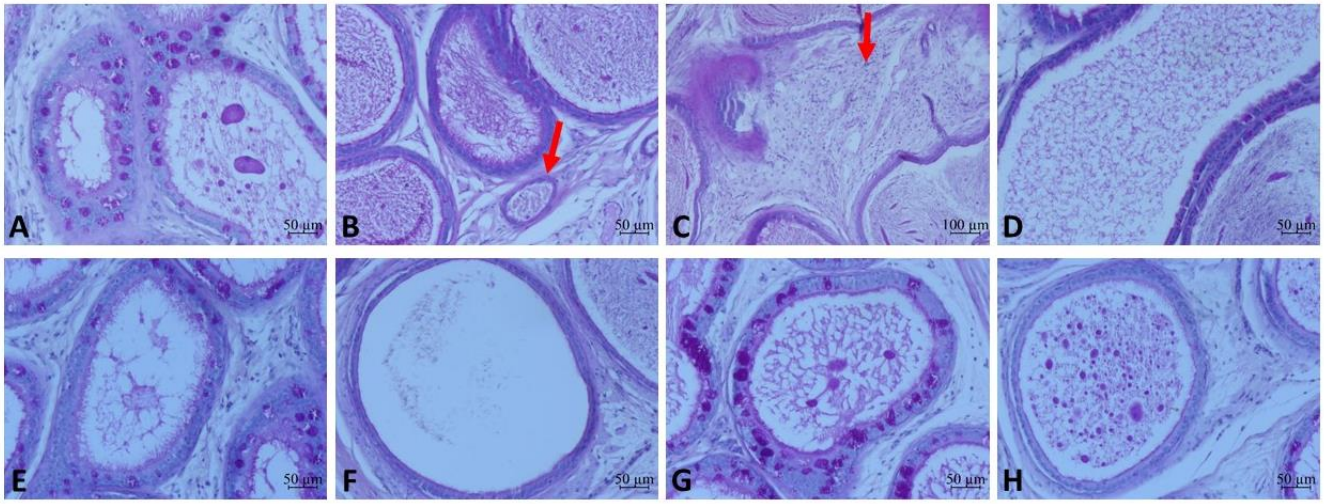

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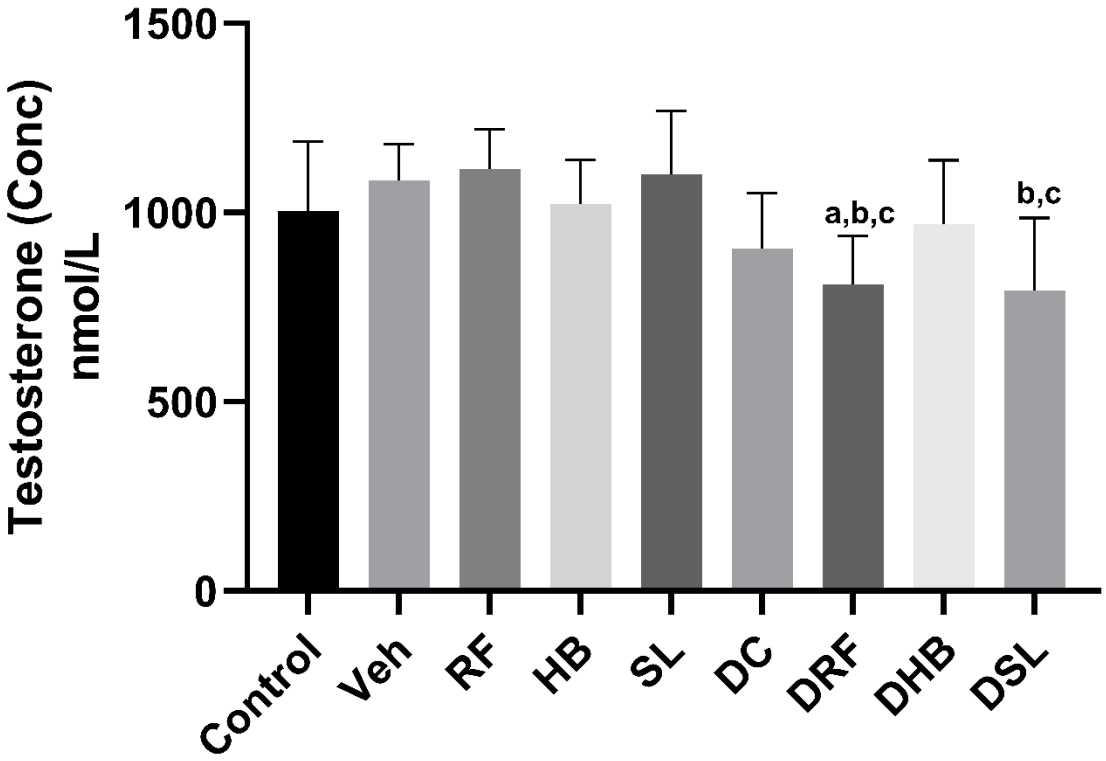

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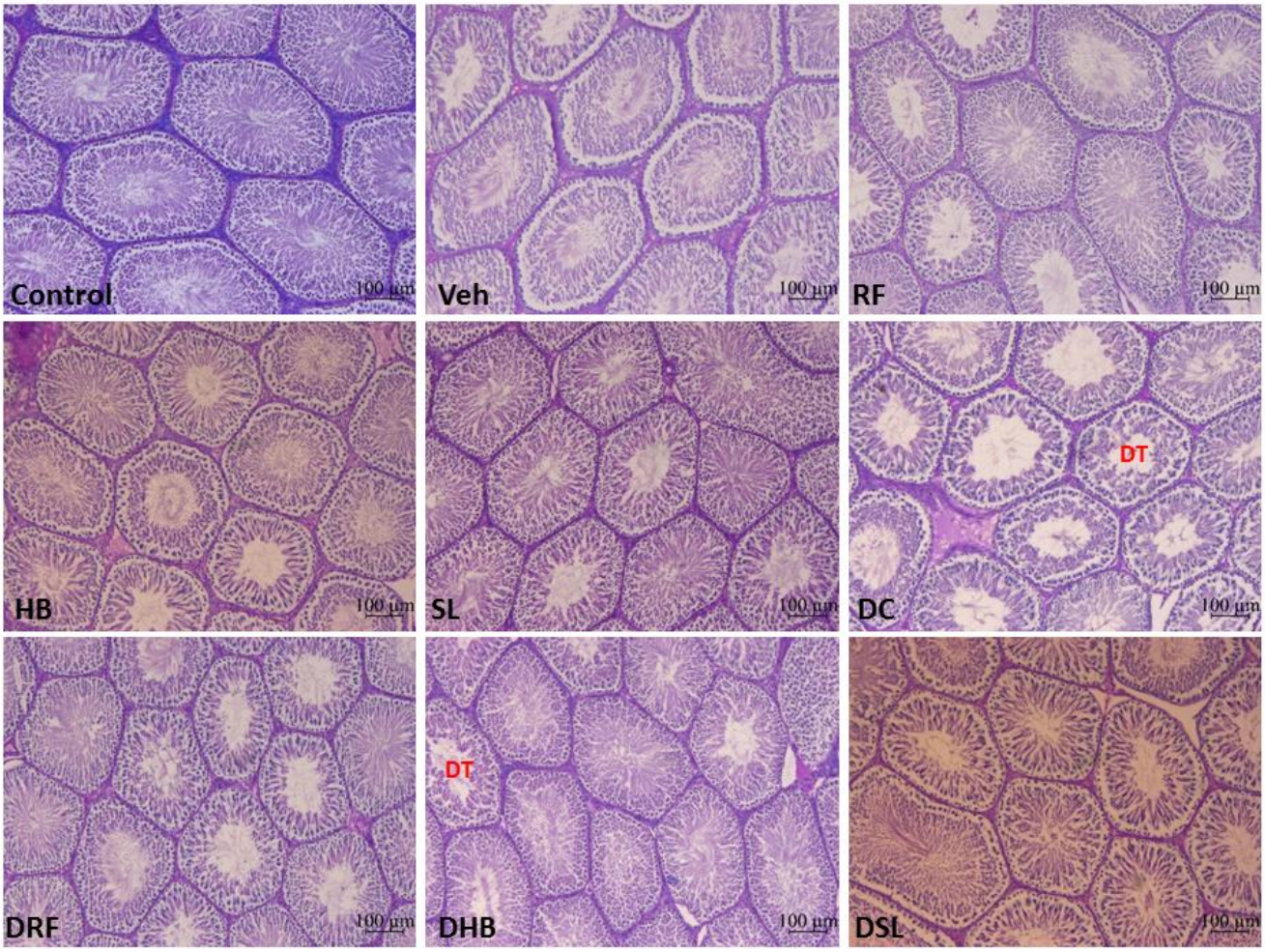
5A



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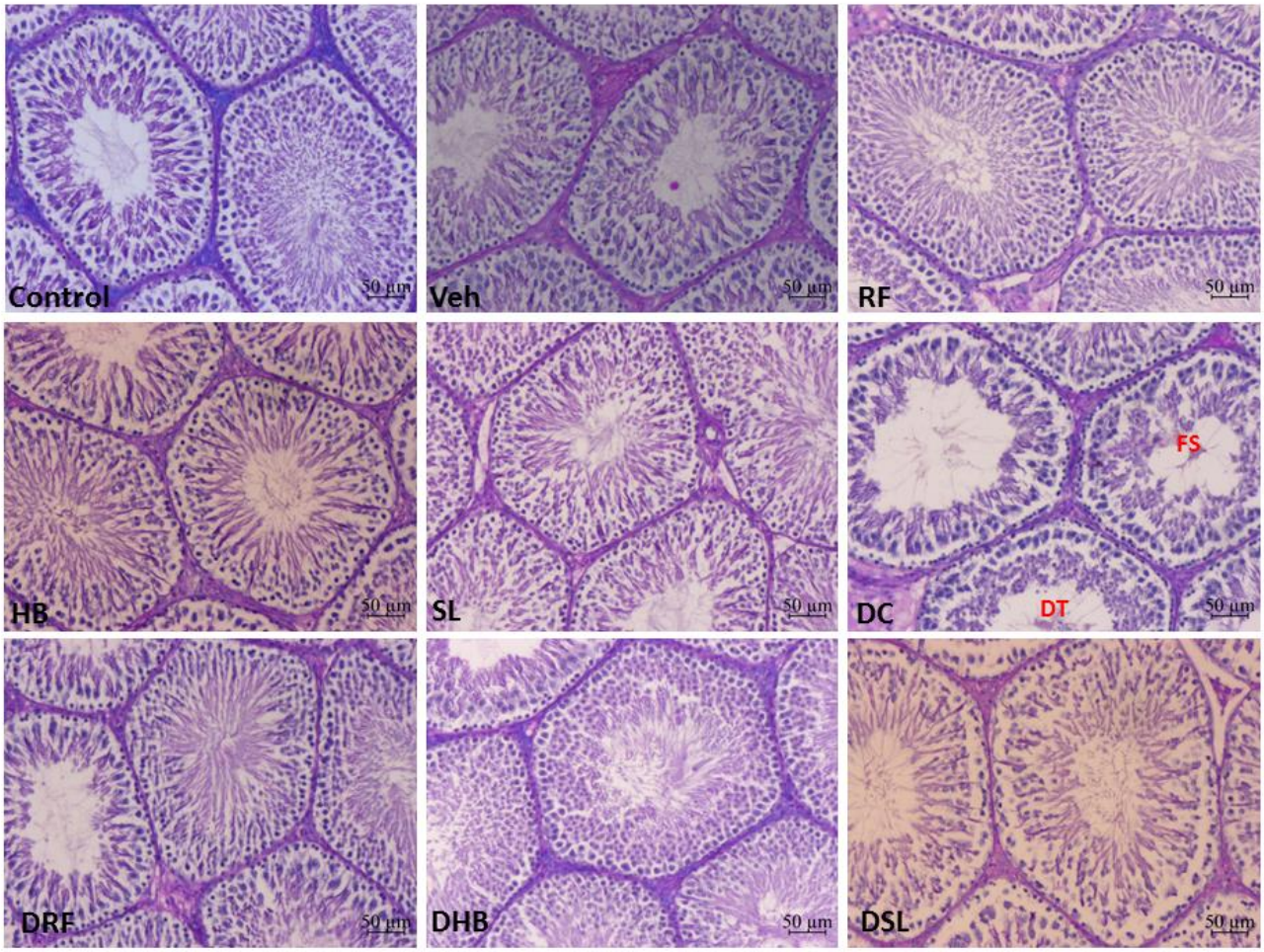

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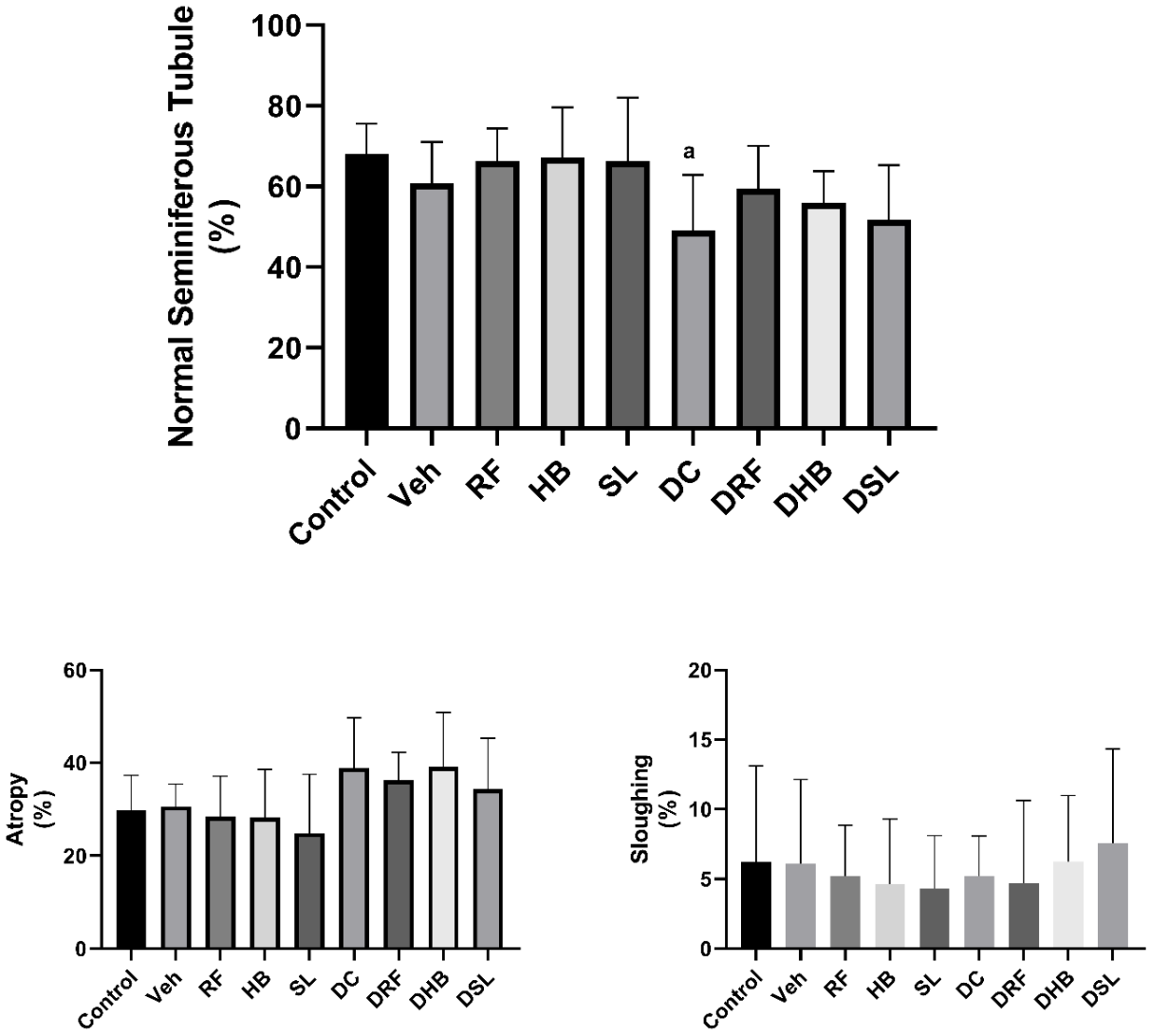

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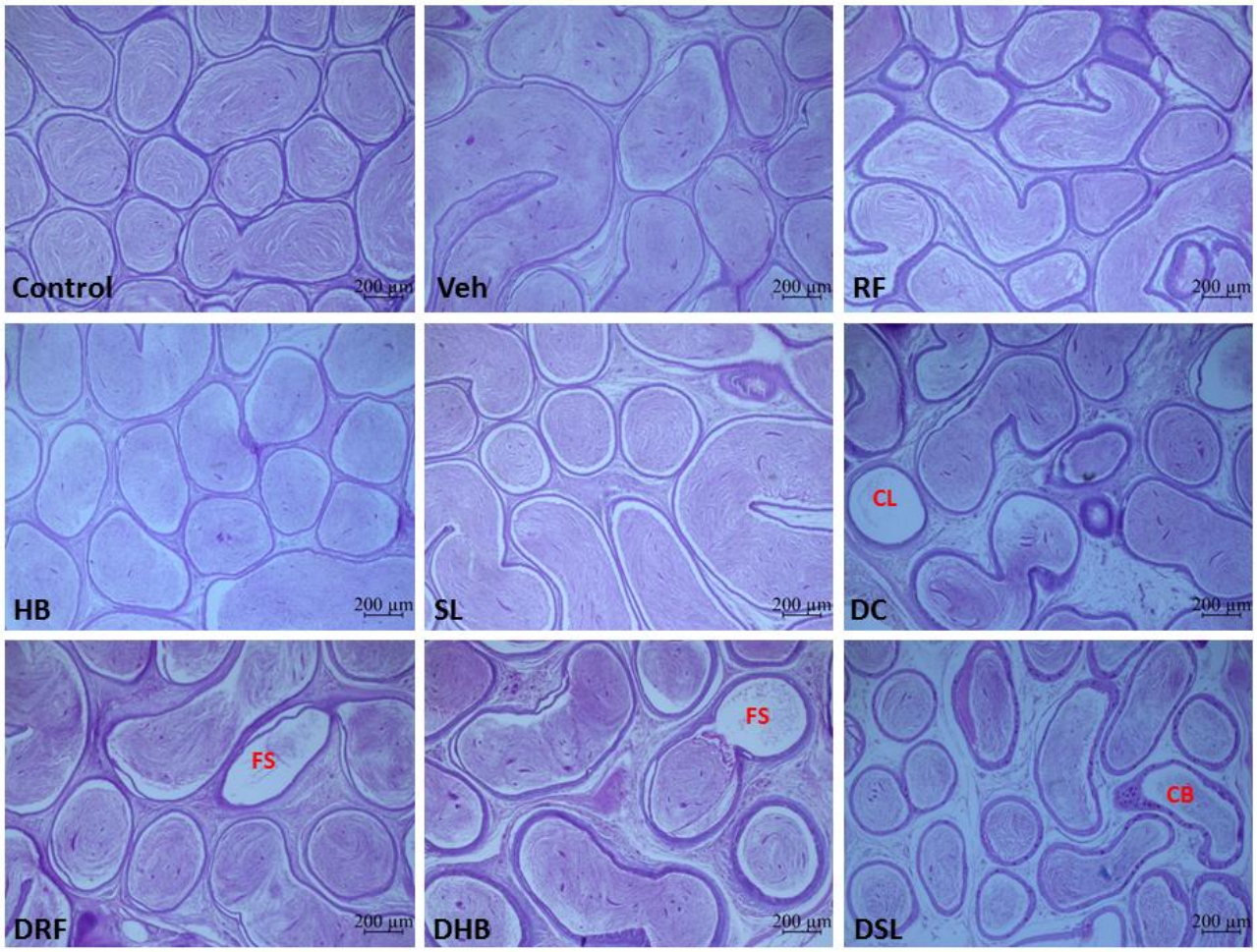
6A



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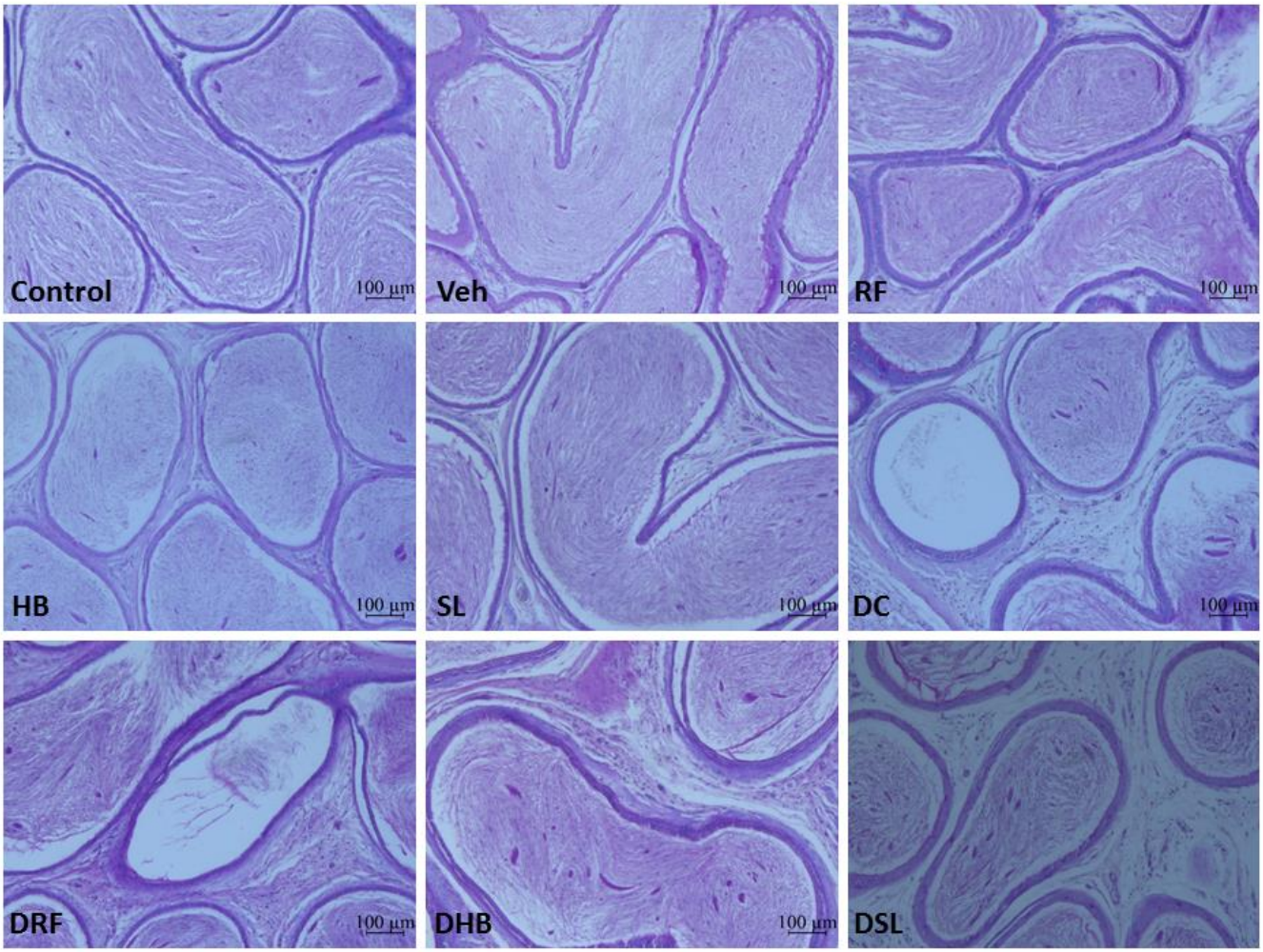

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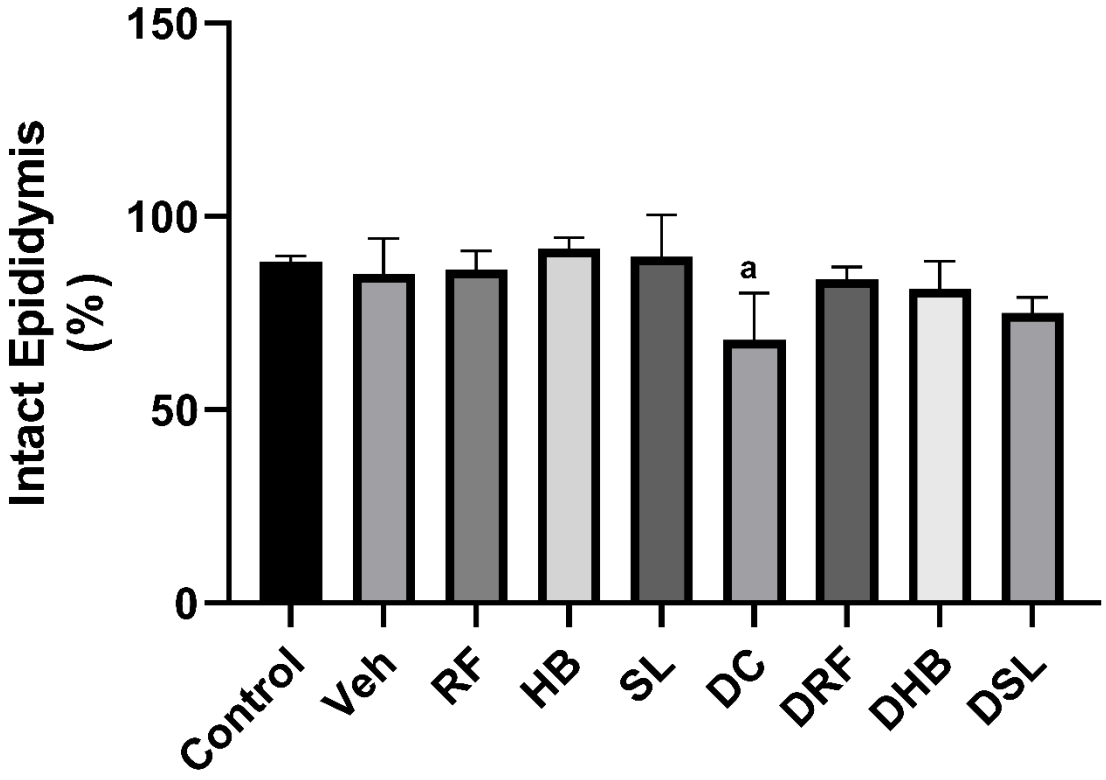

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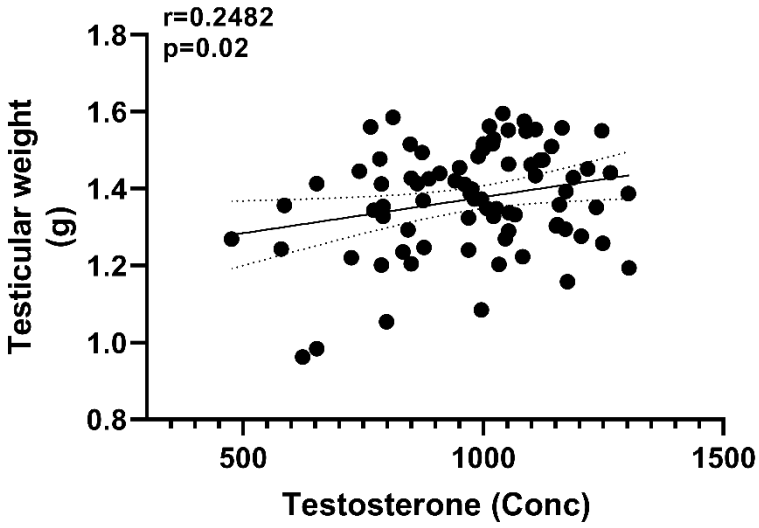
Supplementary figure 1

A



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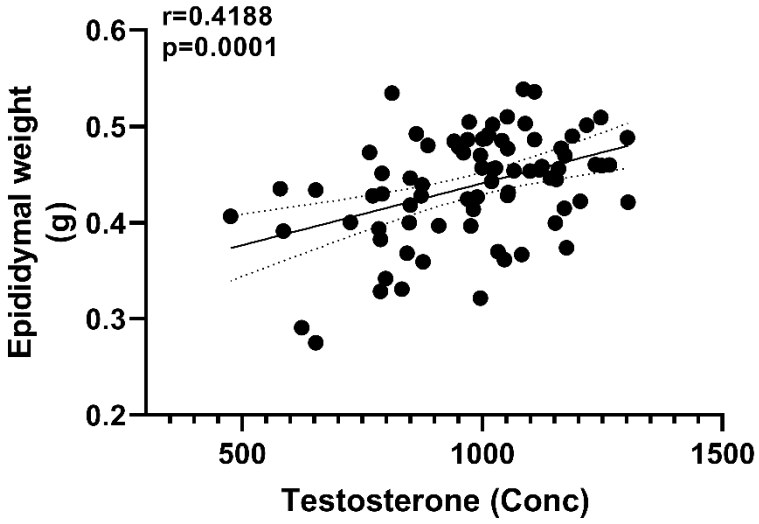

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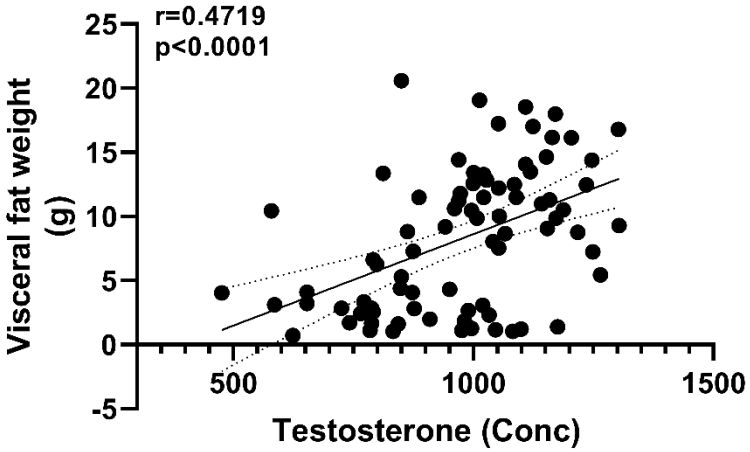

SF1: Correlations between plasma testosterone concentration and (A) testicular weight (B) epididymal weight (C) visceral fat weight.

“Too much of anything is bad, but too much good whiskey is barely enough”

-Mark Twain-

……..“Too much of everything is bad, but too much of rooibos, honeybush and sutherlandia is barely enough in diabetes”……

Chapter 10

Conclusion and Recommendation

Conclusion

Diabetes, characterized by hyperglycaemia does not only affect cardiovascular health, it affects and complicate general health, including male reproductive health. Systematic studies conducted amongst normozospermic men revealed that male fertility is on the decline as sperm quality reduced by 50-60% between 1973 and 2011 (Levine *et al.*, 2017). Given that, DM affects approximately 422 million people globally as of 2014, and it was reported that there is a rise in the number of childhood, adolescent and adulthood diabetic males. This means that as the prevalence of DM increases, there is a plausibility that male subfertility/infertility will increase, because men are mostly affected during their reproductive years (Silink, 2002; Agbaje *et al.*, 2007). Studies have shown that in both diabetic men and animals, there is reduced sperm motility (Singh *et al.*, 2009; Bhattacharya, Ghosh and Nandi, 2014), decreased normal sperm morphology (Maresch *et al.*, 2017) and increased sperm with fragmented nuclear and mitochondria DNA (Agbaje *et al.*, 2007; Shrilatha and Muralidhara, 2007). Although there are significant data available on the effects of DM on sperm function, the mechanism through which the effects are exerted is less clearly understood. Hence, the first part of this study briefly investigated, in corroboration with previously established independent pathways, the possibility of activation and involvement of the AGE-RAGE pathway in DM related sperm function impairment.

The use of medicinal plants in treating diseases have been used for many centuries in traditional medicine. It is intriguing to know that the world is becoming more interested in

exploring the benefits of phytochemicals and to as well investigate their importance in health, as studies have shown that they have fewer negative side effects. Rooibos, honeybush and sutherlandia are plants containing various bioactive chemicals with supposed health benefits. The effects of the infusions/teas from these plants have been widely investigated in diverse disease states, however, very few studies have investigated the role of rooibos in male reproduction and no studies are available regarding honeybush and sutherlandia. Hence, this study investigated the role of rooibos, honeybush and sutherlandia in healthy and diabetes-induced rats, and on the consequent male reproductive function impairment.

Aim 1: Section 1.3.1

Diabetes induction and male reproductive parameters

From the results of this part of the study, it was evident that diabetic animals showed a reduction in sperm motility and normal morphology while the sperm deformity index was increased. Additionally, diabetic animals presented with increased seminiferous tubular diameter, decrease in the number of normal seminiferous tubules and increased number of atrophic tubules. They also presented with severe absence of spermatogenic phases as well as persistent cellular degeneration. The tubules were disorganized and germ cells were severely dissociated.

Ricci et al., who showed the abnormal localization or absence of Occludin in the tight junction between Sertoli-Sertoli cells, which prevents the formation of the blood-testis barrier in DM, also reported the absence of some spermatogenic phases (Ricci *et al.*, 2009) in DM. Hess and Nakai also concluded that the presence of seminiferous tubules with persistent atrophy is correlated with infertility (Hess and Nakai, 2000).

Considering this, the observations from this part of the study suggest that the absence of phases II and III of spermatogenesis and the presence of atrophic and sloughing tubules in diabetic animals may position testicular morphological structures to abnormalities, which ultimately may result in reduced fertility.

Diabetes on sperm function: A closer look at AGEs, RAGEs, MAPKs and activation of the apoptotic pathway

Hyperglycaemia has been reported to cause increased OS in various tissues (Giacco and Brownlee, 2010), including testicular tissue (Ricci *et al.*, 2009) and spermatozoa (Khaki *et al.*, 2010). Another study reported elevated MDA levels, lipid hydroperoxides and lipid peroxides in the plasma of type 1 diabetic patients (Martín-Gallán *et al.*, 2003). In the diabetic state, lipid peroxidation can be induced by protein glycation and glucose auto-oxidation that can further lead to the formation of ROS (Mullarkey, Edelstein and Brownlee, 1990).

Furthermore, an increase in the production of ROS has been shown to cause the non-enzymatic and progressive glycation of proteins with the consequently increased formation of glucose-derived AGEs. The modified proteins further cause an increase in ROS formation and aggravation of OS (Nicholl and Bucala, 1998; Valencia *et al.*, 2004) after binding to the ligand-receptor, RAGE.

AGE-RAGE activation has been implicated in the development and progression of several diseases, both metabolic and non-metabolic. Chen et al. reported that the excessive production of oxidants in the endothelial progenitor cells resulted in RAGE-mediated AGE accumulation with resultant inhibition of cell proliferation, migration and adhesion which were all attenuated after RAGE blockage (Chen *et al.*, 2010). In the same vein, inhibition of RAGE aided the protective effect of glucagon-like peptide-1 on AGE-induced apoptotic neuronal cells (Chen *et al.*, 2016).

From the results of this study, diabetic animals presented with an increase in testicular MDA levels, reduction in catalase enzymatic activities, increase in the expression RAGE, p38MAPK, JNK, caspase 3/7 and PARP. This suggest that the association between AGE-RAGE activation and MAPKs signalling may contribute to the reproductive function impairment seen in DM.

Aim 2: Section 1.3.2

The effect of rooibos, honeybush and sutherlandia on sperm functional parameters

Rooibos, honeybush and sutherlandia are plants with numerous health benefits. However, studies have reported that lengthened exposure to rooibos induced acrosome reaction in rats, which may subsequently lead to impaired reproduction (Opuwari and Monsees, 2014). Additionally, long-term exposure to sutherlandia was reported to cause protein cross-links and that it can as well result in autoimmunity and teratogenicity. Ngcobo et al. showed that the in vitro chronic treatment of normal T-lymphocyte cells with sutherlandia was toxic (Ngcobo *et al.*, 2012). Studying the available evidence regarding the excessive consumption and the long-term intake of these infusions, it is pertinent to further investigate their role in health status. Hence, sperm functional parameters of healthy animals were examined after administration of the respective infusions.

From the results obtained from this study, it was evident that rooibos, honeybush and sutherlandia enhanced testicular antioxidant enzyme activities and reduced MDA levels. However, their role on sperm parameters was confusing. As there was a decrease in sperm kinematic parameters, sperm motility and sperm with normal morphology, however, sperm concentration was increased. This is a novel finding and further investigations are encouraged. We conclude that care should be taken when consuming these teas as a healthy individual, as too much of antioxidants may result in reductive stress.

Testicular oxidative stress and apoptosis status after treating diabetic rats with rooibos, honeybush and sutherlandia

Several studies have highlighted the antioxidant ameliorative potential of these infusions in both human and animal studies (Marnewick *et al.*, 2003; Hong, Lee and Kim, 2014).

Diabetic animals treated with rooibos (DRF), honeybush (DHB) and sutherlandia (DSL) showed an elevation in the antioxidant enzyme activities. It was interesting to see that DRF animals showed increased sperm motility even when compared to the vehicle. DHB animals however displayed a decrease in sperm motility and DSL animals showed an increase in sperm motility compared to the diabetic control. Additionally, these animals (DRF, DHB and DSL) showed an improvement in the number of spermatozoa with normal morphology. It is suggested that the improved sperm motility observed in the DRF and DSL animals may in part be due to the boost in antioxidant enzyme activity, which subsequently protected against/alleviate the stress imposed by DM on sperm motility. Although DHB animals presented with improved antioxidant enzyme activities, the sperm motilities were not improved.

The effects of rooibos, honeybush and sutherlandia on testicular insulin signalling of diabetic rats

The antidiabetic potential of rooibos, honeybush and sutherlandia has been reported. Studies have shown that these infusions may exert their hypoglycaemic effects by improving glucose tolerance and enhancing glucose uptake (Kawano *et al.*, 2009). Some studies showed that rooibos improved glucose metabolism by promoting the activation of PkB and GLUT4 (Mikami *et al.*, 2015; Muller *et al.*, 2018), which suggest its role in insulin signalling. Additionally, the importance of controlled insulin signalling for normal male reproduction have been highlighted (Yagci and Zik, 2006; Griffeth, Bianda and Nef, 2014). Since insulin signalling is impaired in DM, we investigated the role of these infusions on testicular insulin signalling.

The diabetic control group of this study showed impaired testicular insulin signalling. This was evidenced by the decrease in IRS1/2, PkB/Akt and GLUT4 protein expression. Following infusion treatment, DRF animals presented with improved IRS1/2, but PkB/Akt and GLUT4 remained unchanged. DHB animals displayed upregulation in IRS1/2 and PkB/Akt protein expression, but GLUT4 remained unchanged. DSL group showed an increased IRS-2, PkB/Akt and GLUT4. Since there was a very mild decrease in the fasting blood glucose of the DRF, DHB and DSL groups, the following were suggested:

(i) Rooibos may have exerted this mild hypoglycaemic effect through a pathway involving IRS1/2 activation

(ii) Since GLUT8 is believed to be a dominant glucose transporter in the testis, it was suggested that honeybush may have exerted its effect through the IRS1/2,PkB/Akt, GLUT8 pathway

(iii) DSL may improve testicular insulin signalling through the IRS1/2,PkB/Akt, GLUT4 pathway.

These are novel findings, which requires further investigations, to ascertain the specific activated upstream and downstream signalling proteins.

Descriptive histomorphology evaluation of the testis and epididymis following administration of rooibos, honeybush and sutherlandia

The control animals receiving rooibos, honeybush and sutherlandia displayed normal seminiferous tubule cellular association, cauda epididymal lumen were filled with matured spermatozoa and there was normal overall seminiferous tubule and epididymal tubule architecture. Both the testistular and epididymal morphology were altered in DM, but these disruptions were mildly ameliorated following treatment with rooibos, honeybush and

sutherlandia. Since, this represent the first study reporting these effects, further investigations are required regarding the mechanisms.

In conclusion, the results of this study have showed that DM impairs male reproductive parameters and the histo-architecture of the testis and cauda epididymis. The impairment may be exerted through the ROS/OS/AGE-RAGE/MAPKs and apoptosis activation.

Additionally, rooibos, honeybush and sutherlandia may suppress testicular OS in DM by promoting antioxidant enzyme activities but their role in apoptosis needs further investigation. These infusions may also exert mild hypoglycaemic effects by improving insulin signalling and they may enhance the histomorphology of the testis and cauda epididymis. However, when healthy, consumption of these infusions should be proceeded with caution as too much may reduce sperm quality.

In closing, extrapolating from these results, it can be suggested that rooibos, honeybush and sutherlandia are potentially beneficial to general health. Moreover, healthy young men should be careful of how frequent these teas are consumed. In diabetes, rooibos, honeybush and sutherlandia displayed mild hypoglycaemic effect, hence these teas can be used in the management of diabetes. Regarding male reproduction, rooibos seem to have the most beneficial effect on sperm motility, followed by sutherlandia with honeybush showing no positive effect. Hence, rooibos and sutherlandia are recommended for young diabetic men who are planning to have children.

Study limitations

(i) Although a recent study has indicated that the period of spermatogenesis can vary between 42 and 76 days, and the duration of treatment for this study fell within the range, it would have been nice to investigate what happens in a much longer study.

(ii) Infusions were prepared every 48 hours. While some studies have shown that aspalathin, the main component of rooibos have a half-life of 8 hours, others have described the beneficial effects of several bioactive components of rooibos, honeybush and sutherlandia, which have extended half-lives. It is therefore suggested that future studies should prepare the infusions every day. Additionally, since there are wide variations in the phenolic contents of the infusions (rooibos, honeybush and sutherlandia), the determination of the various phytochemical componenets of the herbal teas should be carried out in future studies.

(iii) Since people tend to consume diverse teas simultaneously, a group mimicking this phenomenon maybe included in future studies

Recommendations

(i) In order to properly understand the insulin-signalling pathway in the testis and spermatozoa, future studies can investigate the importance of the activation of the CbI-Proto-oncogene pathway in spermatogenesis.

(ii) In the study of male infertility, the conventional seminal analysis in categorizing fertility is becoming inadequate. It is evident that AGE negatively affect male reproductive function and subsequently male fertility through ROS and/or activation of other mechanistic pathways, which at this time is largely unknown. Therefore, it is essential to investigate the fundamental/basic molecular protein interaction, which may provide more information about infertility as a whole. We recommend that future studies investigating idiopathic infertility and disease induced infertility should further elucidate the impact of protein modification and glycation. Additionally, studies investigating its mechanistic role in infertility are required as this will aid the therapeutic approach

(iii) Since this is the first study reporting the role of rooibos, honeybush and sutherlandia on male reproduction, we were unable to delve deep into the mechanisms through which the effects were exerted. Hence, future studies can investigate the role of these infusions on the upstream and downstream insulin signalling activation.

(iv) Further histological, and immunohistological analysis should be performed, in order to identify the specific stage(s) of spermatogenesis that were improved by the infusions.

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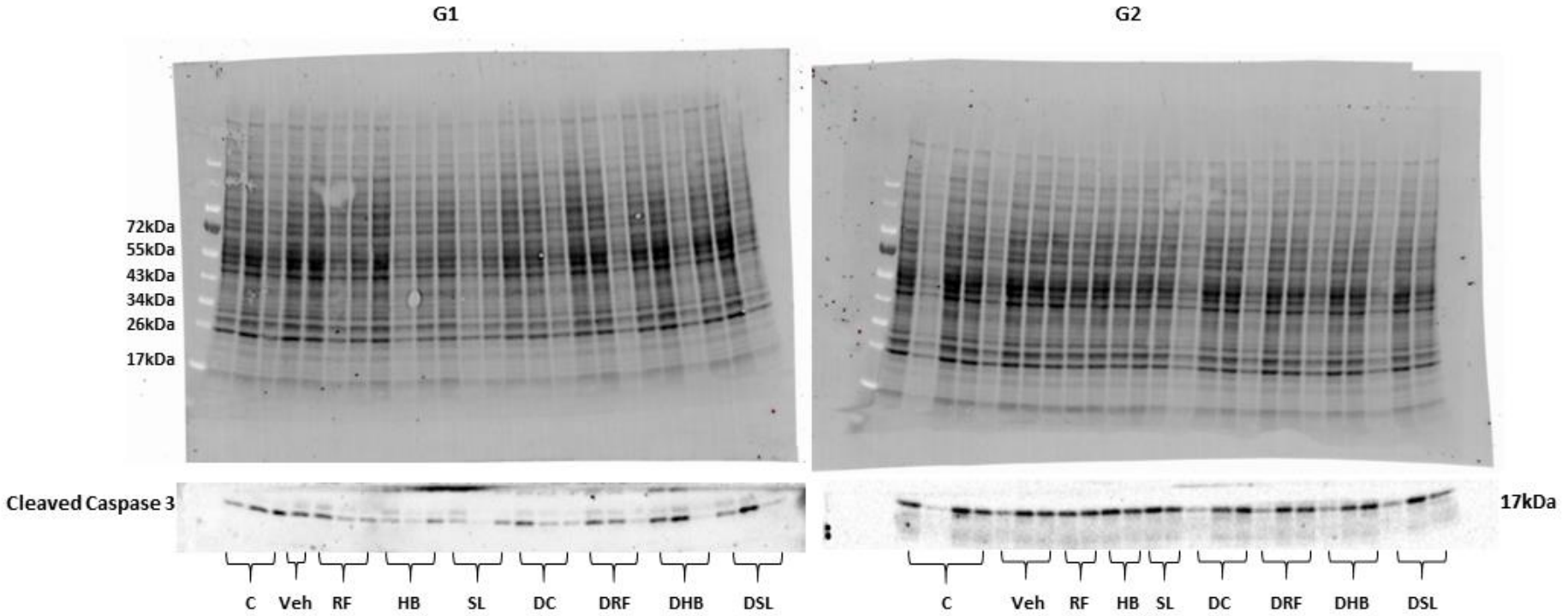
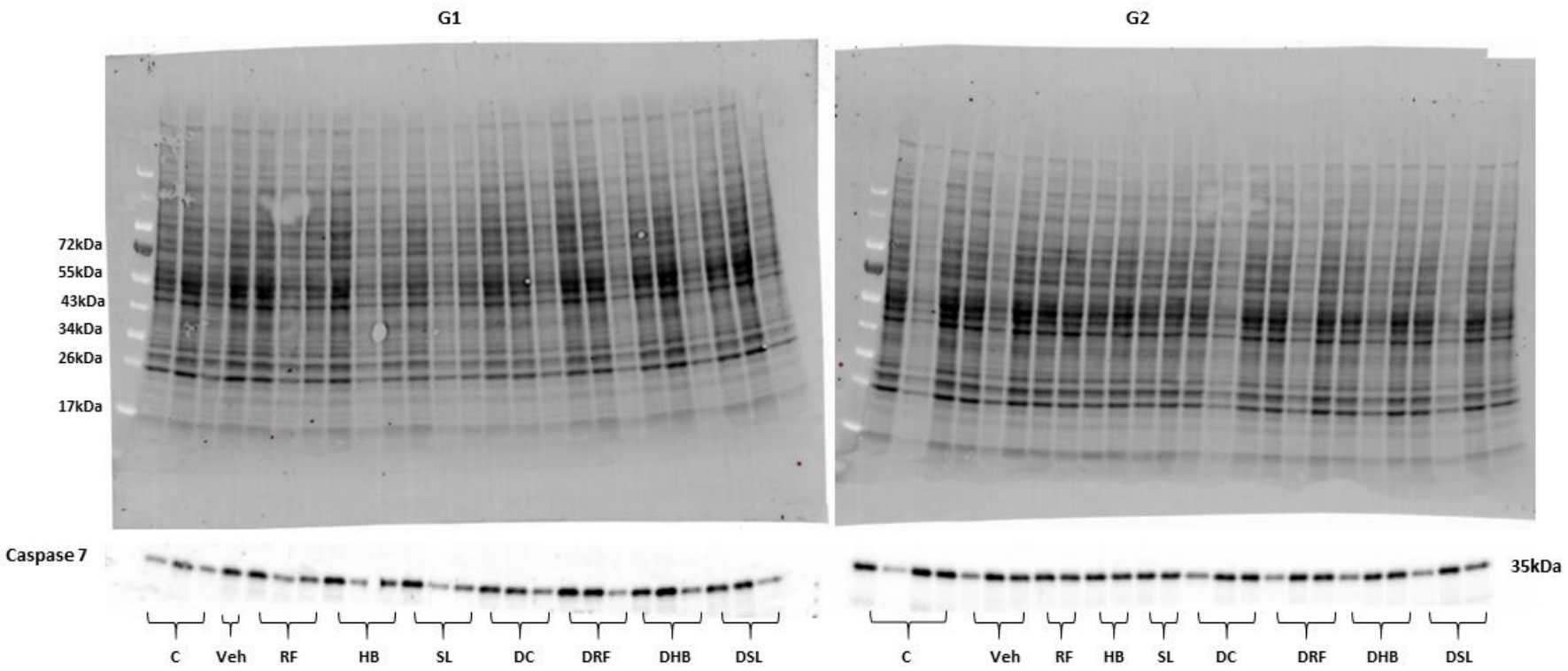
Appendix A

Probed Blots for CHAPTER 7: Testicular Oxidative Stress and Apoptosis Status in Streptozotocin-induced Diabetic Rats after Treatment with Rooibos, Honeybush and Sutherlandia Infusions

A



B



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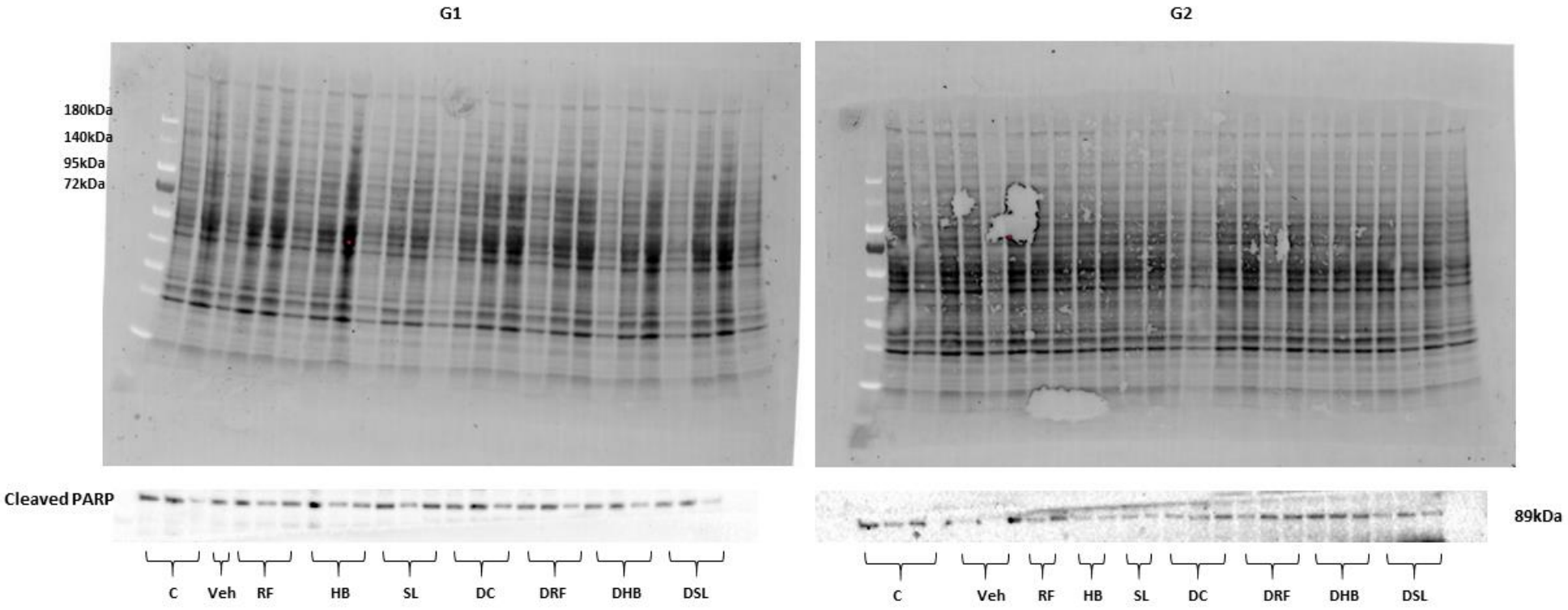

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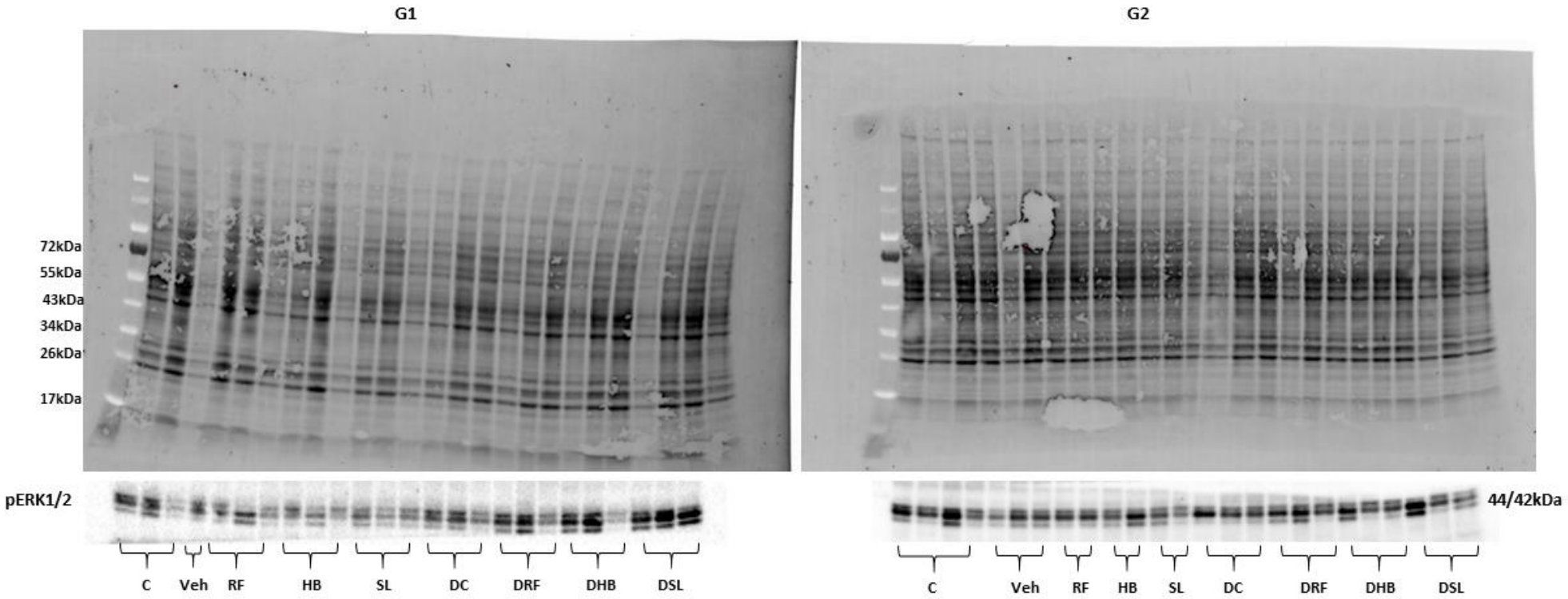

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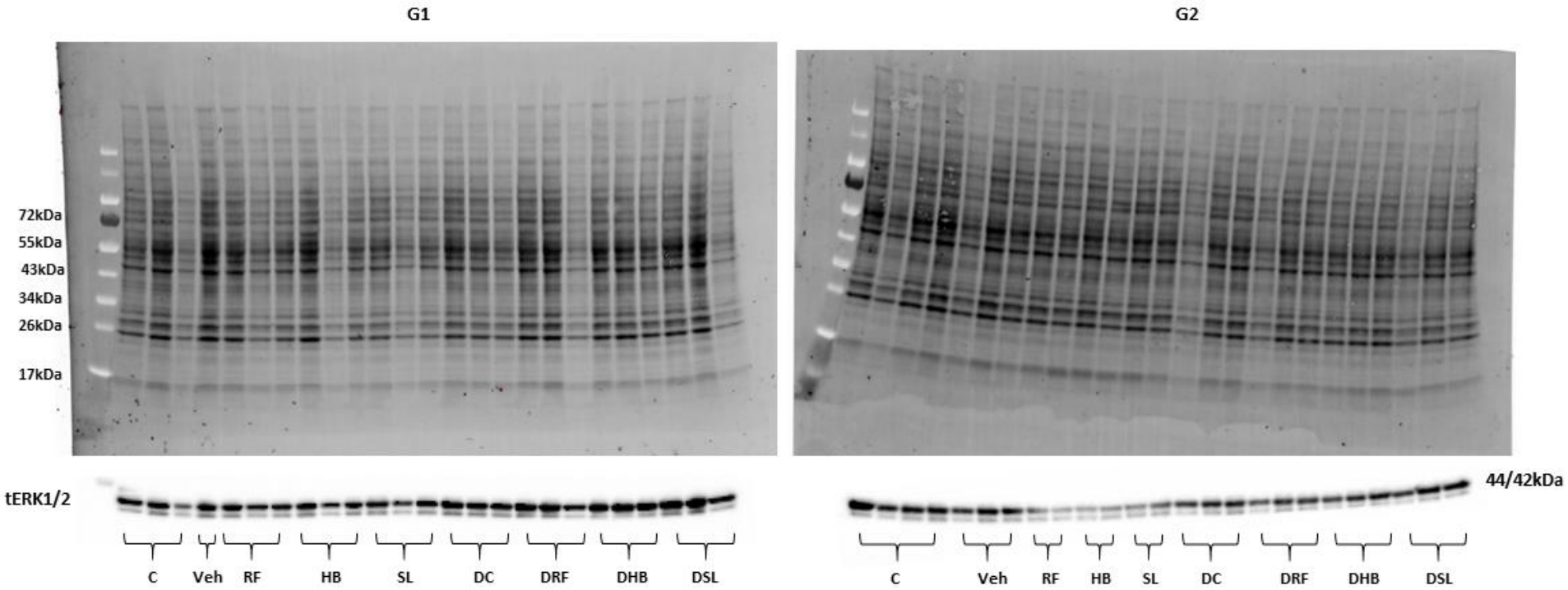

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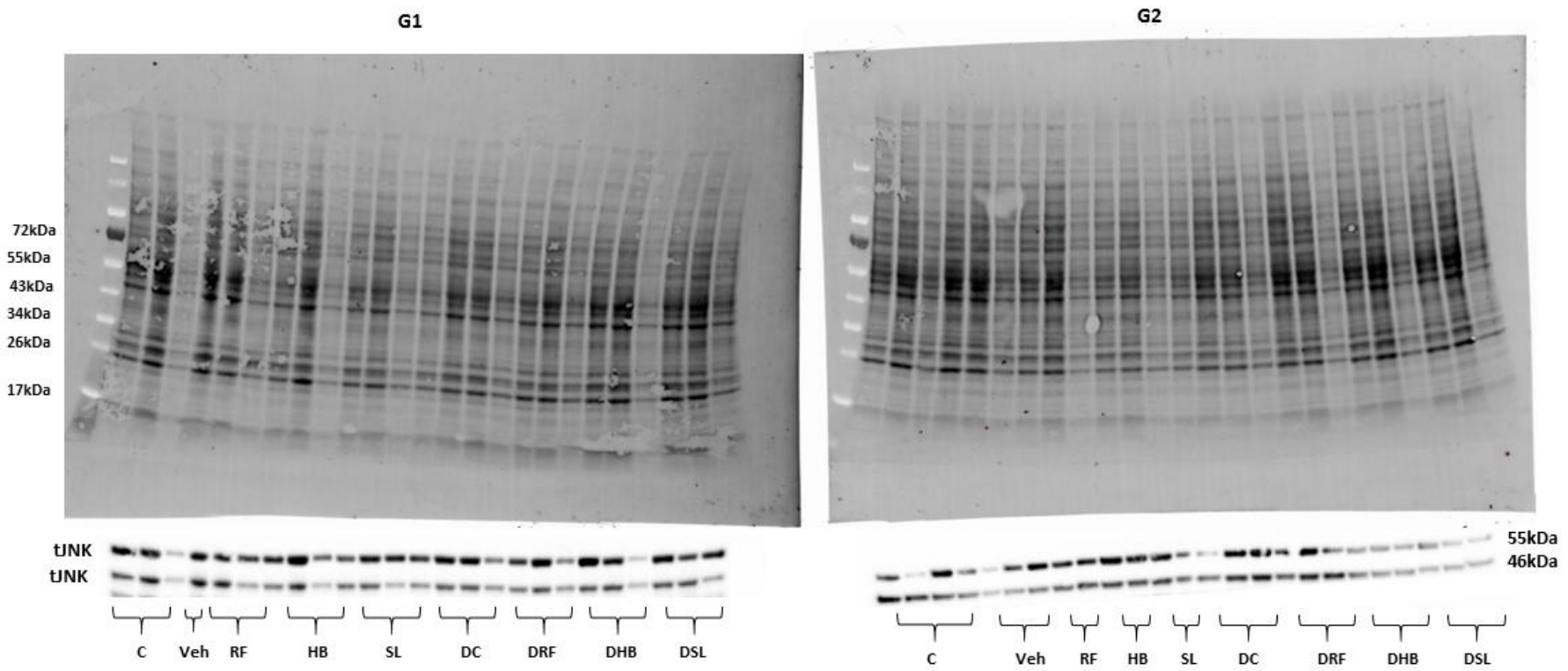

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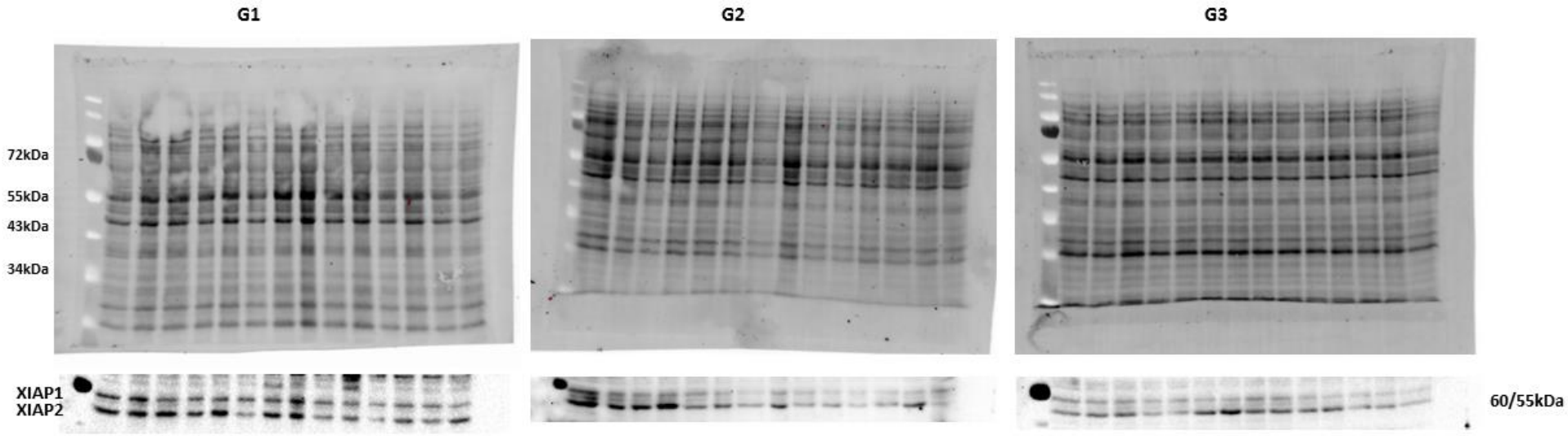
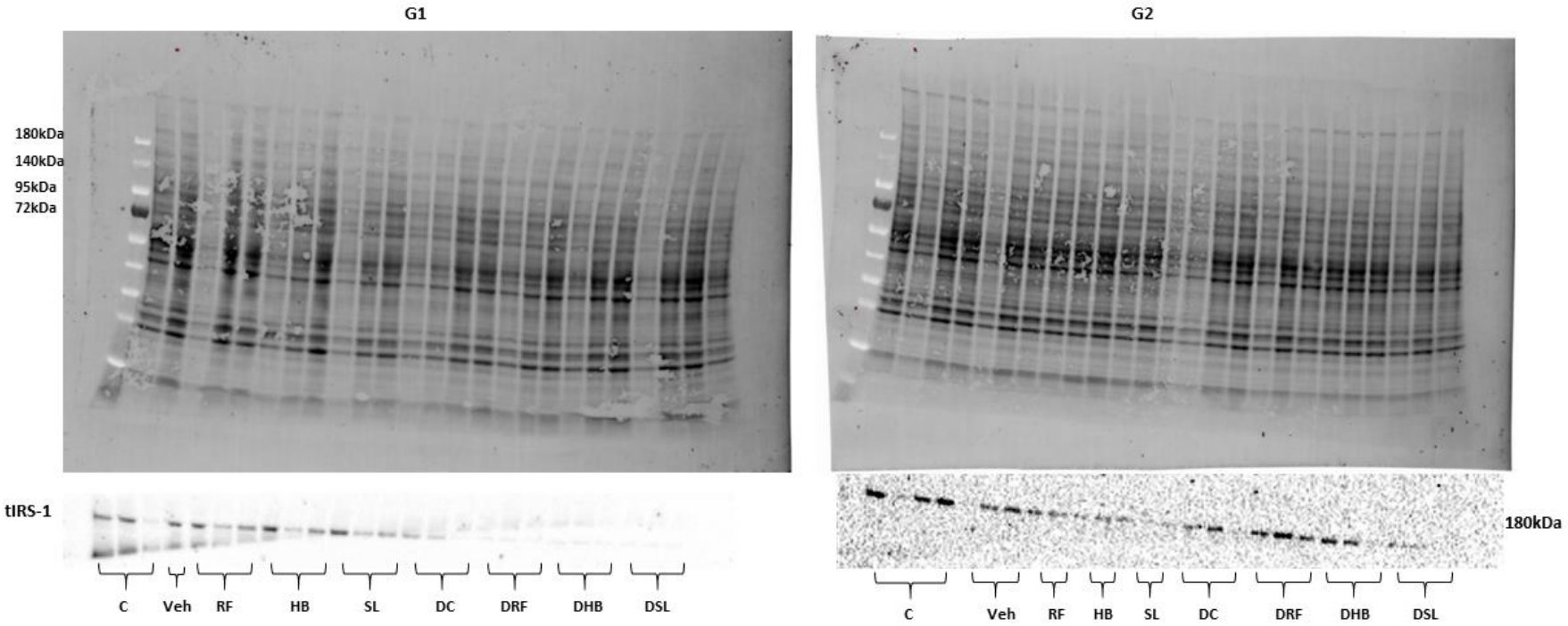


Appendix A: G1=gel 1, G2=gel 2. (G1 and G2 of A-F are 4-20% stain free precast gels (26 lanes)), while G1, G2 and G3 of G are 12% stain free gel (15 lanes). Cumulatively, n=4-6, as each lane represent an animal.

Appendix B

Probed Blots for CHAPTER 8: The effect of rooibos (Aspalathus linearis), honeybush (Cyclopia intermedia) and sutherlandia (Sutherlandia frutescene) on streptozotocin induced diabetes and on testicular insulin signalling in Wistar rats.

A



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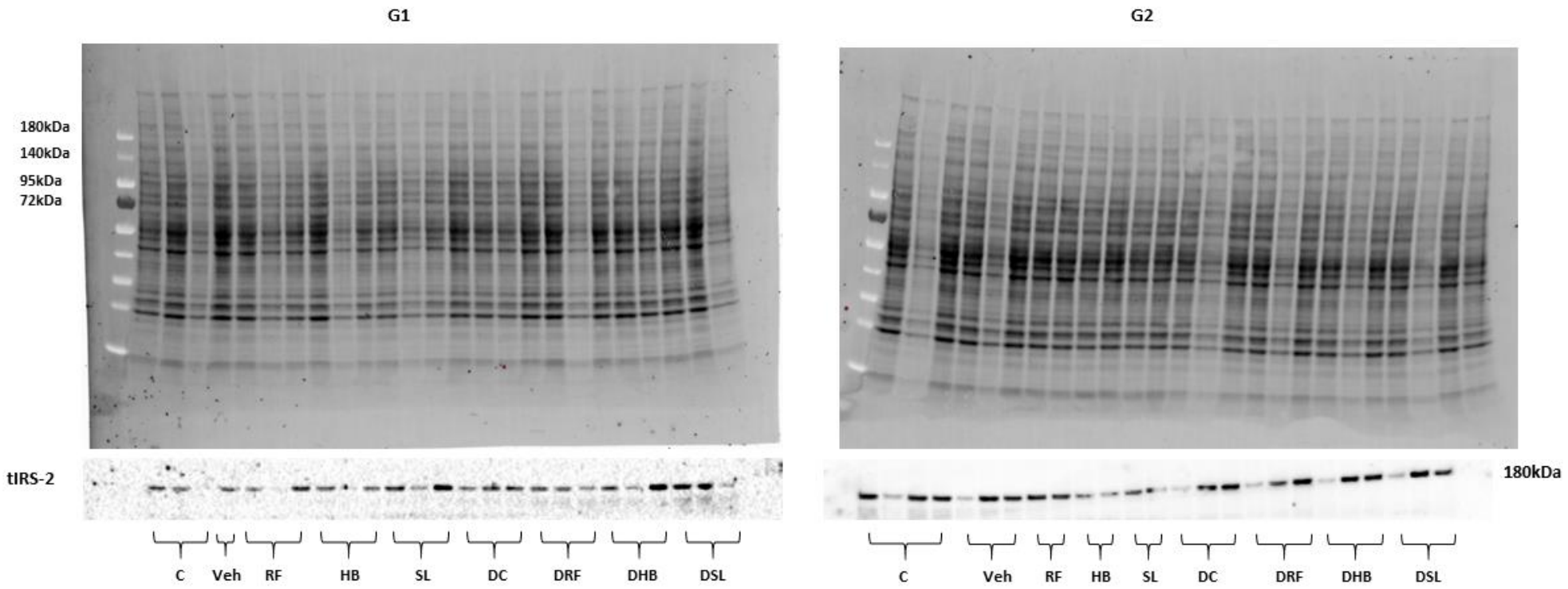

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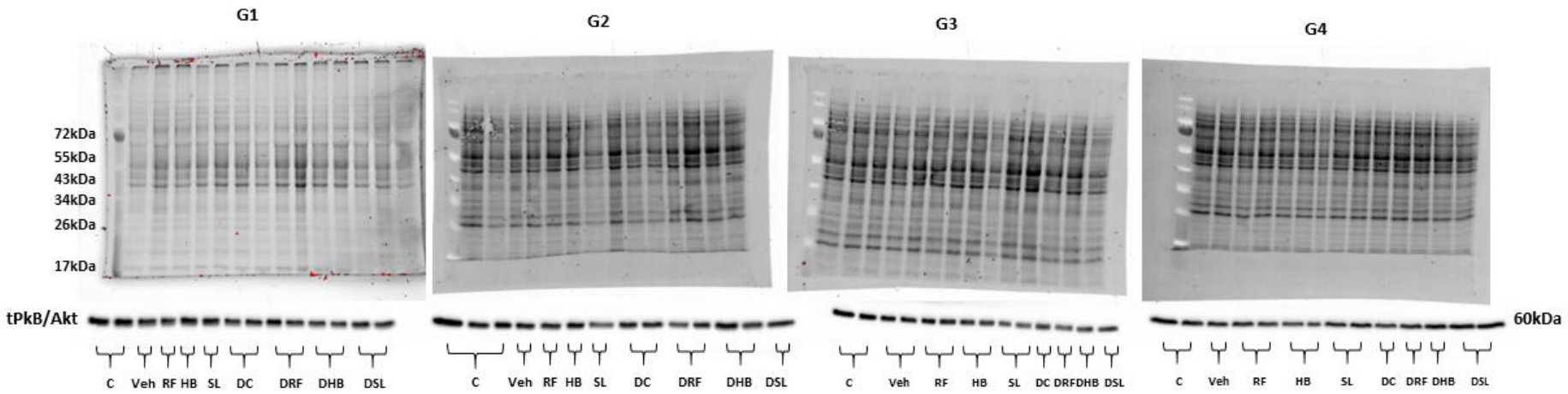

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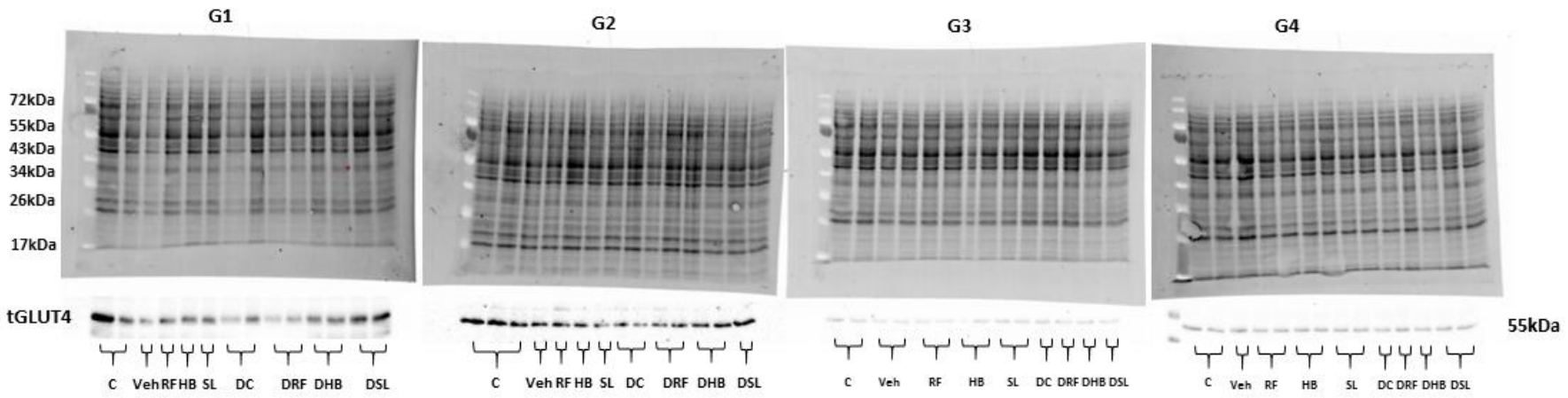

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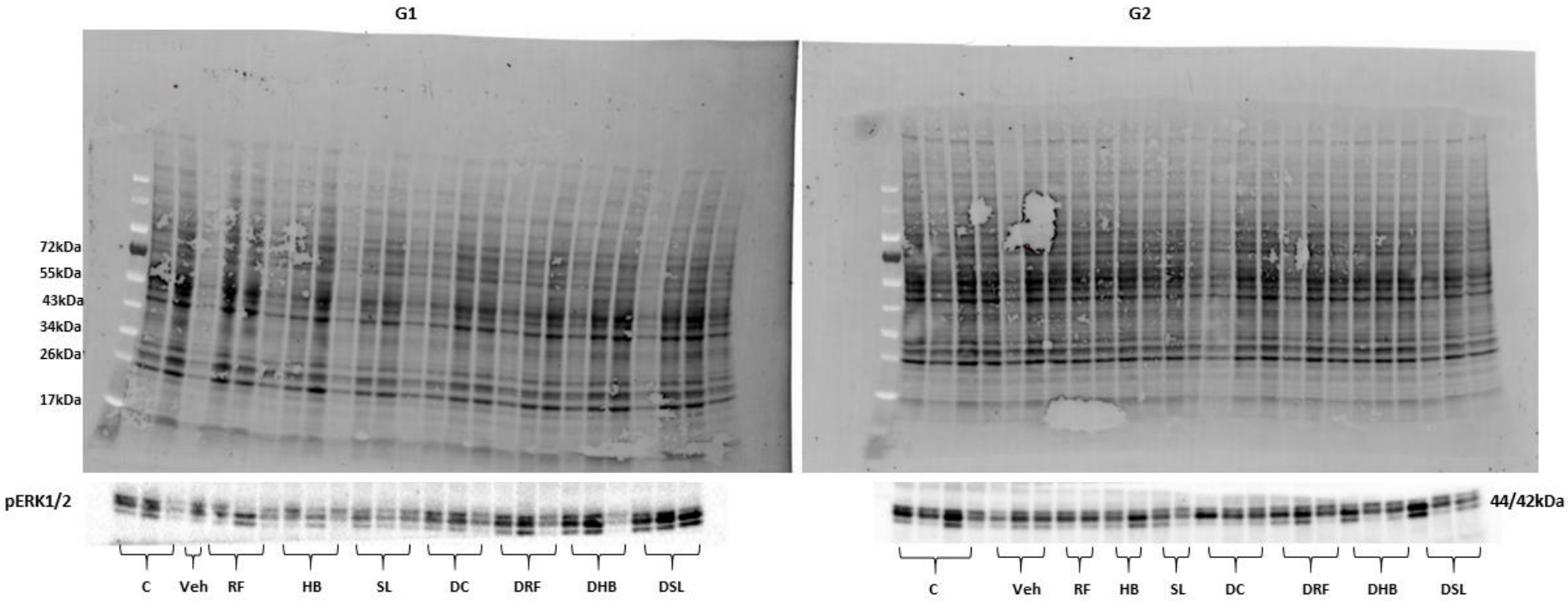

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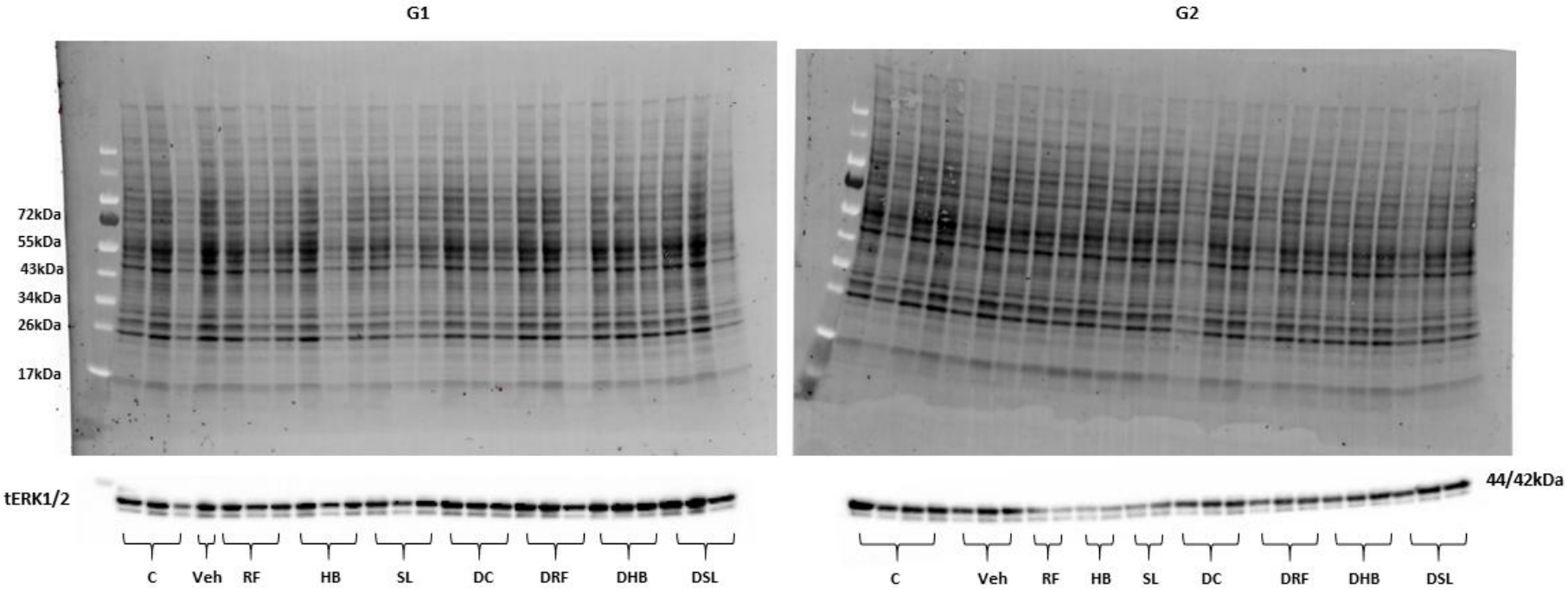

Appendix B: G1=gel 1, G2=gel 2. (G1 and G2 of A-F are 4-20% stain free precast gels (26 lanes)), while G1, G2, G3 and G4 of C and D are 12% stain free gel (15 lanes). Cumulatively, n=4-6, as each lane represent an animal.

Attached are the original copies of the published articles.