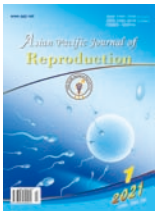

Original Article

Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

doi: 10.4103/2305−0500.306432

Testicular oxidative stress and apoptosis status in streptozotocin−induced diabetic rats after treatment with rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*), and sutherlandia (*Lessertia frutescens*) infusions

Temidayo S. Omolaoye[1,2], Shantal Windvogel[1,3], 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*

[3]*Centre for Cardio−metabolic Research in Africa, Stellenbosch University, South Africa*

ABSTRACT

Objective: To investigate the testicular oxidative stress and apoptosis status, as well as the sperm functional parameters in streptozotocin (STZ) induced diabetic rats following treatment with rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and sutherlandia (*Lessertia frutescens*) infusions.

Methods: Diabetes was induced by injecting fourteen-week-old adult male Wistar rats (250-300 g) with a single intraperitoneal injection of STZ (45 mg/kg body weight). Fifty rats were randomly divided into five groups: the vehicle group received 0.1 M citrate buffer, the diabetic control group received 45 mg/kg STZ, the diabetic+rooibos group received 45 mg/kg STZ + 2.0% rooibos, the diabetic+honeybush group received 45 mg/kg STZ + 4.0% honeybush, and the diabetic+sutherlandia group received 45 mg/kg STZ + 0.2% sutherlandia. Rats were sacrificed 7 weeks after induction of diabetes mellitus. The testes and epididymides were harvested and weighed after induction. Spermatozoa were retrieved from the cauda epididymis for motility, concentration, and morphology analysis, and the testis was used for all biochemical assays. Oxidative stress was determined by measuring malondialdehyde levels, catalase, and superoxide dismutase activities, while apoptotic biomarkers were evaluated by Western blotting assays.

Results: After induction of diabetes, rats in the diabetic control group, diabetic+rooibos group, diabetic+honeybush group, and diabetic+sutherlandia group presented with significantly elevated blood glucose levels as compared with the vehicle group (P<0.001). Rats in the diabetic control group had a reduction in sperm progressive motility, while rats in the diabetic+rooibos group and the diabetic+sutherlandia group displayed an increase in progressive motility as compared with the diabetic control group. The diabetic control animals showed a 40.0% decrease in sperm concentration when compared to the vehicle group, and there were no significant differences in sperm kinematic and speed parameters between the groups. In addition, the percentage of morphologically normal spermatozoa was increased by 13.0%, 16.0%, and 15.0% after treatment with rooibos, honeybush, and sutherlandia, respectively and the rats in the diabetic+infusion groups also displayed an increase in superoxide dismutase activity when compared to the diabetic control group.

Conclusions: Rooibos, honeybush and sutherlandia infusions may partly alleviate diabetes-induced sperm function impairment by reducing oxidative stress.

KEYWORDS: Diabetes; Sperm; Oxidative stress; Apoptosis; Rooibos; Honeybush; Sutherlandia

1. Introduction

Diabetes mellitus is a metabolic disorder that results either from a lack of insulin secretion or the insensitivity of the target tissue to the effect of insulin[1]. Diabetes mellitus is one of the non-communicable diseases that pose a threat to general health, including male reproductive health[2–4]. Studies have highlighted some of the negative impacts of diabetes mellitus on male fertility, including endocrine function deregulation, testicular dysfunction, spermatogenesis disruption, reduced sperm motility, and decreased normal sperm morphology[5,6]. One of the pathways through which diabetes mellitus exerts its effect on male fertility is through the development of oxidative stress and the subsequent induction of apoptosis[7]. Kanter *et al* showed that after diabetes mellitus induction in rats, there was an increase in testicular malondialdehyde (MDA) levels with reduced antioxidant enzyme activities in glutathione peroxidase and superoxide dismutase

✉To whom correspondance may be addressed. E-mail: Stefan.duplessis@mbru.ac.ae

This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial-Share Alike 4.0 License, which allows others to remix, tweak and buid upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

©2021 *Asian Pacific Journal of Reproduction* Produced by Wolters Kluwer- Medknow. All rights reserved.

How to cite this article: Omolaoye TS, Windvogel S, du Plessis SS. Testicular oxidative stress and apoptosis status in streptozotocin-induced diabetic rats after treatment with rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*), and sutherlandia (*Lessertia frutescens*) infusions. *Asian Pac J Reprod* 2021; 10(1): 11-20.

Article history: Received: 29 July 2020; Revision: 10 September 2020; Accepted: 26 October 2020; Available online: 15 January 2021

(SOD). This subsequently resulted in severe testicular damage as evidenced by disrupted seminiferous tubule structure. Also, the increased number of spermatozoa with fragmented DNA following apoptosis activation was observed[8]. Additionally, Chen *et al* reported a significant decrease in the testicular antioxidant activities (SOD, catalase) and an increase in MDA and reactive oxygen species (ROS) levels in diabetes mellitus rats. This was followed by an elevated Bax/Bcl-2 ratio, which is indicative of apoptosis[5]. This phenomenon has been described by several other authors[2,9]. Hence, it is evident that diabetes mellitus negatively impacts male fertility. Since it has been reported that diabetes mellitus affects 422 million people globally[10], including men of reproductive age[11], it is therefore essential to explore every possible strategy to combat diabetes mellitus and its associated male reproductive complications.

Rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*), and sutherlandia (*Lessertia frutescens*) are plants endemic to Southern Africa[12,13]. They are caffeine-free beverages derived from the leaves and stems of their respective plants. The infusions derived from rooibos contain diverse bioactive compounds, including dihydrochalcones, cyclic dihydrochalcone, phenylpropenoids, flavones, flavonols, and flavanones[14]. Honeybush contains compounds such as flavanones, flavones, isoflavonols, phenolic acid, inositols, and xanthones[15], while sutherlandia has phytochemicals such as flavonoids, non-protein amino acids, free amino acids, and cycloartanol glycosides[16].

Due to the presence of rich bioactive compounds in these teas, studies have explored their role in different systems and cells as antioxidants, anti-inflammatory agents, anti-carcinogens, and anti-diabetic agents[17,18]. Although there are several known health benefits of these infusions, not much is known about the role of rooibos, honeybush and sutherlandia on diabetes mellitus-impaired male reproductive function. Awoniyi *et al* reported that rooibos increased SOD and glutathione activities in rat sperm, and subsequently improved sperm function in oxidative stress-induced rats[17]. 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[19]. Since the reports on the effect of these infusions remain 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. Materials and methods

2.1. Animal care

Healthy adult male Wistar rats (14 weeks old) weighing 250-300 g were housed at the Animal Unit of the Faculty of Medicine and Health Sciences, Stellenbosch University [(18-23) ℃, 12:12 light/dark cycle] for seven weeks. Rats 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 Animal.

2.2. Infusion preparation

Fermented rooibos (2.0%; leaves and stem, Carmien SA PTY LTD, South Africa), fermented honeybush (4.0%; leaves and stem, Afrinaturals, South Africa), and unfermented sutherlandia (0.2%; leaves and stem, Afrinaturals, South Africa) were prepared according to standard protocols. Preparation methods conformed to the experimental established protocols for rooibos[19,20], honeybush[21,22], and sutherlandia[23]. Briefly, 2.0% rooibos was prepared by adding 20 g of dried rooibos in 1 L of boiling water, stirring, and allowed to rest for 30 min. The mixture was filtered three times by 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 dark plastic containers and stored at 4 ℃. Honeybush (4.0%; 40 g in 1 L) and sutherlandia (0.2%) were prepared following the same protocol. All infusions (rooibos, honeybush, and sutherlandia) were prepared every other day (48 h). The herbal infusions served as the only drinking fluid for these infusion groups. The fluid intake of the animals was measured three times a week.

2.3. Determination of the polyphenol content present in rooibos, honeybush, and sutherlandia

All analyses were performed by using the infusion concentrations that the animals were treated with 2.0% fermented rooibos, 4.0% fermented honeybush, and 0.2% unfermented sutherlandia.

2.3.1. Soluble solid content

The soluble solid content of the infusions (rooibos, honeybush, and sutherlandia) was evaluated gravimetrically (6 repetitions) and analysed in triplicate at each time point. Briefly, the glass beakers used were initially washed, placed in the oven overnight at 70 ℃, and cooled in a desiccator for another 24 h. For the assay, dried beakers were weighed prior to adding 1 mL of the respective infusions. The aliquots were dried in an oven for 24 h at 70 ℃, whereafter, they were placed in a desiccator for another 24 h. Glass beakers containing the dried content were weighed again. The soluble solid content in each herbal infusion was determined by subtracting the initial weight from the final weight.

2.3.2. Total polyphenol content

The total polyphenol content was measured as described by Arthur *et al*, using the Folin-Ciocalteau method[24]. Briefly, an initial 20 µL of blank (deionized water), standard (10-100 mg/L gallic acid) and the respective infusions were loaded into a 96-well plate. Thereafter, 100 µL of Folin-Ciocalteau reagent (G7384-100C, Sigma, Aldrich, USA) and 80 µL of 7.5% (m/v) Na_2CO_3 were added respectively.

The plate was prevented from light, gently vortexed, and allowed to stand for 2 h at room temperature. The absorbance was measured at 765 nm and expressed as mg gallic acid equivalents per mg soluble solids.

2.4. In vivo experiment

2.4.1. Diabetes induction

Diabetes was induced in the experimental animals with streptozotocin (STZ) (S0130-IG, Sigma, South Africa). A stock solution (30 mg/mL) was prepared by dissolving it in freshly prepared sodium citrate buffer (pH 4.5). Wistar rats were administered a single intraperitoneal injection of STZ (45 mg/kg body weight) from the stock solution. Animals were feed fasted overnight before injecting with STZ (45 mg/kg). The successful induction of diabetes was confirmed after one week, if animal showed a blood glucose level of ≥14 mmol/L, using a Glucoplus™ glucometer (Figure 1)[25].

2.4.2. Study design

Fifty male Wistar rats were randomly divided into five groups (n=10 in each group). The vehicle group received 0.1 M citrate buffer, the diabetic control group received 45 mg/kg STZ, the diabetic + rooibos group received 45 mg/kg STZ + 2.0% rooibos, the diabetic + honeybush group received 45 mg/kg STZ + 4.0% honeybush, and the diabetic + sutherlandia group received 45 mg/kg STZ + 0.2% sutherlandia. Animals were allowed to acclimatize for one week prior to the onset of the study. Treatment with the different infusions already started during acclimatization. Body weights were measured thrice weekly. To confirm the diabetic status of the animals, blood glucose was measured once a week. Seven weeks after diabetes mellitus induction animals were sacrificed by administering a lethal dose of sodium pentobarbital. The testes and the epididymides were harvested and weighed. The testis was used for all biochemical assays and spermatozoa were retrieved from the caudal epididymis for sperm 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.

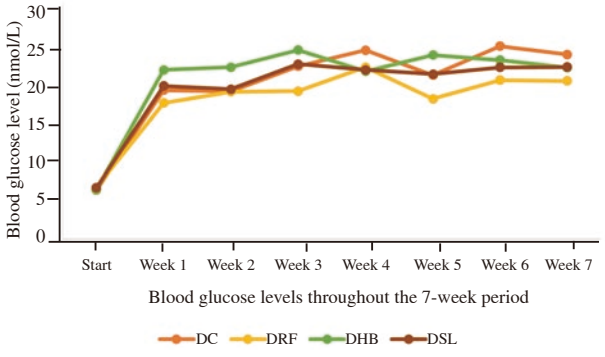

Figure 1. Blood glucose levels throughout the 7-week period. DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic+sutherlandia.

2.4.3. Sperm functional parameters

The harvested left epididymis was defatted and placed in a Petri dish that contained a 2 mL solution of Dulbecco's modified Eagle's medium-low glucose (Sigma Chemicals, St Louis, MO, USA), at 37 ℃. After rinsing, the cauda area of the epididymis was dissected into a separate dish containing 2 mL Hams solution and spermatozoa were allowed to swim out. After 30 s of sperm retrieval, sperm motility was analysed. Sperm solution for morphology and concentration analysis were obtained by dissecting the caudal area into smaller pieces after motility was analysed and left for further 5 min allowing a maximum number of spermatozoa to swim out.

At 30 s, 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*[26]. Sperm morphology was analysed using computer-aided sperm morphology analysis (SCA 5.4), and it conformed with the formerly established methods by van der Horst *et al*[27]. 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 2 mL Dulbecco's modified Eagle's medium and allowing sperm to swim out during a 5-minute period. The tissue pieces were removed after 5 min, and the sperm solution was mixed homogenously. Of the 2 mL solution, 10 µL was removed and diluted in 50 µL of Dulbecco's modified Eagle's medium, whereafter the sperm concentration was measured *via* CASA.

2.4.4. Oxidative stress parameters

2.4.4.1. Catalase

Frozen testicular tissue samples (n=7) were homogenized in cold lysis buffer (25 mM Na_3PO_4, 0.5% Triton X-100) and centrifuged at 25 200×g for 20 min at 4 ℃. Tissue homogenates were diluted 10 times in deionized water. From the diluted samples and standards, 5 µL were loaded in triplicate into UV microplate wells. Catalase assay buffer (170 µL) was added into each well, and lastly, 50 µL of H_2O_2 was added into the wells, and analysis was performed immediately on a plate reader (Multiskan spectrum) at 240 nm every 60 s over 5 min by using SkanIt RE for MSS 2.2 (ThermoScientific™ Inc.) software.

2.4.4.2. SOD

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

2.4.4.3. Thiobarbituric acid reactive substance (TBARS) assay

Frozen testicular tissue samples (n=10) were homogenized in lysis buffer (0.1 M KPi, 1.15% KCl) by homogenising in a Bullet

blender®(Next Advance Inc.) at speed 9 for 3 min with a 1-minute rest interval in-between. To measure the levels of MDA in testicular tissue, 100 µL of standards and samples were pipetted into the corresponding 10 mL glass tubes, followed by adding 1 mL of sodium dodecyl sulfate and 2 mL of 10% trichloroacetic acid-butylated hydroxyl toluene buffer solution. Samples were vortexed, and after resting for 10 min, 2 mL of thiobarbituric acid 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 h at 100 ℃), whereafter it was cooled on ice for 15 min. The standards and samples were centrifuged at 1 008×*g* for 15 min at 4 ℃ and the supernatants were retrieved. From the supernatants, 250 µL of each standard and sample were loaded in triplicate into microplate wells and analysed on a plate reader (Multiskan spectrum) at a wavelength of 532 nm within 30 min after centrifugation.

2.5. Apoptotic markers

Apoptotic markers were measured by using Western blot procedures. Tissue homogenates and protein determination were obtained as previously described[28]. Tissue lysates were prepared by diluting sample in Laemmli sample buffer and lysis buffer, boiled for 5 min and 50 µg protein/µL was separated by electrophoresis on a 12% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis mini-protein gel. The running protocol consisted of an initial 10 min electrophoresis at 100 V and 200 mA followed by 30-40 min at 200 V and 200 mA. Gels were activated by using ChemiDoc (BioRad). Thereafter, the proteins were transferred onto a millipore Immobilon-P transfer membrane (0.45 µm) (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 1:1 000 ratio while the secondary antibody was diluted in TBS-Tween in a 1:4 000 ratio. All data points were from independent biological repeats (n=4-5). Measured apoptotic biomarkers were caspase 3 (Sigma-Aldrich), caspase 7 (Abcam, SA), poly (ADP-ribose) polymerase (PARP) (Cell Signalling Technology), p38 mitogen-activated protein kinase (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.

2.6. 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 by using the Shapiro-Wilk, Anderson-Darling, Kolmogorov-Smirnov and D'Agostino & Pearson, and normality tests. When data passed all normality tests, one-way analysis of variance with 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, Pearson's two-tailed correlation matrix was performed. A probability level of P<0.05 was considered statistically significant and results were expressed as mean±standard deviation (mean±SD).

2.7. Ethics statement

This study was approved by the Stellenbosch University Animal Ethics Committee (ethics approval number: SU-ACUD17-00016).

3. Results

3.1. Anthropometric data

The rats in the diabetic control group and the diabetic+honeybush group gained less body weight, while rats in the diabetic+rooibos group and the diabetic+sutherlandia group significantly decreased body weight as compared with the vehicle group (P<0.001). Rats in the diabetic+rooibos group and the diabetic+sutherlandia group presented with significantly increased relative testicular weight as compared with the vehicle group (P<0.05). After one week of diabetes induction, the rats in the diabetic control group, diabetic+rooibos group, diabetic+honeybush group, and diabetic+sutherlandia group significantly elevated blood glucose levels as compared with the vehicle group (P<0.001) (Table 1).

3.2. Total polyphenol contents

There was a significant difference in the amount of soluble solid contents between the herbal teas (P<0.001). The soluble solid content of the rooibos infusion was significantly lower than honeybush (P<0.001) and sutherlandia (P<0.001).
The polyphenol content of rooibos was significantly higher than honeybush (P<0.001) and sutherlandia (P<0.001), while honeybush was higher than sutherlandia (P<0.001) (Table 2).

3.3. Sperm functional parameters

Although not significant, rats in the diabetic control group had a nearly 7.0% decrease in total motility as compared with the vehicle group. Rats in the diabetic+rooibos group displayed a 4.3% increase in total motility as compared with the vehicle group, and a 12.0% increase compared with the diabetic control group (Figure 2A). Animals in the diabetic+honeybush, and diabetic+sutherlandia groups presented with a non-significant decrease in total motility when compared to the vehicle group. Additionally, there was a 15.17% decrease in the percentage of progressively motile spermatozoa of rats in the diabetic control group as compared with the vehicle group, while the diabetic+rooibos group displayed a 7.0% increase compared with the vehicle group and 26.2% increase compared with the diabetic control group. The diabetic+honeybush group displayed the largest decrease (17.0% decrease) in the progressive motility compared with the vehicle group and a mild reduction compared with the diabetic control group. On the other hand, the diabetic+sutherlandia group presented with a 15.0% increase in progressive motility as compared with the diabetic control group (Figure 2B). The diabetic control animals showed a 40.0% decrease in sperm concentration when compared to the vehicle group (Figure 2C). Animals in the diabetic+rooibos group showed a 25.0% increase in sperm concentration compared with the diabetic

Table 1. Basic anthropometric data.

Parameters	Vehicle	Diabetes control	Diabetes+rooibos	Diabetes+honeybush	Diabetes+sutherlandia	*P* value
Body weight at start of treatment (g)	273.50±18.23	278.30±13.11	282.90±15.80	284.70±22.30	274.20±14.20	0.951
Body weight after 8 weeks of treatment (g)	332.40±30.19	280.80±20.89†	266.70±32.95†	288.30±37.86*	247.40±30.60†‡	<0.001
Change in bodyweight (g)	58.94±20.78	2.51±21.58	-16.23±29.07†	3.65±30.48	-26.80±25.63†	<0.001
Testicular weight (g)	1.35±0.09	1.38±0.11	1.36±0.14	1.34±0.15	1.21±0.22	0.300
Relative testicular weight (%)	0.41±0.04	0.49±0.05	0.52±0.08*	0.47±0.03	0.49±0.08*	0.002
Blood glucose levels (average of 7 weeks) (mmol/L)	6.31±0.36	25.76±2.59†	24.73±5.01†	21.93±5.77†	24.71±3.07†	<0.001
Fasting blood glucose at sacrifice (mmol/L)	5.66±1.02	19.62±7.49†	18.91±9.14*	14.98±9.43*	16.60±8.92*	<0.001

$^{*}P$<0.05 *vs.* the vehicle group; $^{\dagger}P$<0.001 *vs*. the vehicle group; $^{\ddagger}P$<0.05 *vs*. the diabetes+honeybush group.

Table 2. Total polyphenol contents.

Parameters	Rooibos	Honeybush	Sutherlandia
Soluble solid contents (mg/mL)	5.180±0.003	13.400±0.003*	16.260±0.012*†
Total polyphenol content (mg gallic acid/mg soluble solids)	0.1730±0.0003†‡	0.1710±0.0003‡	0.0300±0.0003

$^{*}P$<0.001 *vs.* rooibos; $^{\dagger}P$<0.001 *vs.* honeybush, $^{\ddagger}P$<0.001 *vs.* sutherlandia.

Table 3. Sperm kinematic parameters at 30 seconds.

Parameters	Vehicle	Diabetes control	Diabetes+rooibos	Diabetes+honeybush	Diabetes+sutherlandia	*P* value
VCL (μm/s)	161.60±66.59	163.30±42.49	170.00±27.87	155.70±35.00	152.20±50.79	0.924
VAP (μm/s)	70.48±34.04	71.00±20.83	78.12±14.57	70.25±15.30	67.90±26.78	0.686
VSL (μm/s)	41.89±22.31	42.54±15.69	48.13±10.46	42.79±9.328	44.26±19.43	0.774
STR (%)	54.65±7.99	54.30±9.63	58.49±6.48	56.64±3.40	60.32±9.33	0.303
LIN (%)	23.81±8.83	24.75±7.48	28.11±5.52	26.91±3.52	27.71±7.90	0.311
WOB (%)	40.81±10.88	42.31±5.13	45.19±7.48	44.36±5.08	42.91±7.55	0.760
ALH (μm)	6.43±2.21	6.47±1.41	6.53±0.96	5.89±1.16	5.71±1.53	0.492
BCF (Hz)	13.58±5.53	14.29±3.42	16.35±3.29	16.35±2.39	16.06±6.60	0.357

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).

Table 4. Sperm speed and progressive motilities.

Parameters	Vehicle	Diabetes control	Diabetes+rooibos	Diabetes+honeybush	Diabetes+sutherlandia	*P* value
Rapid speed (%)	42.87±30.40	45.78±22.76	51.75±18.66	41.93±20.40	42.56±24.97	0.811
Medium speed (%)	12.74±8.17	11.71±5.15	14.80±10.75	16.06±6.09	11.16±9.56	0.636
Slow speed (%)	17.15±8.03	20.02±7.52	13.32±5.14	18.33±5.28	15.86±6.58	0.268
Rapid progressive motility (%)	19.61±16.10	22.82±13.95	26.16±14.56	20.01±12.59	25.13±16.20	0.808
Medium progressive motility (%)	31.26±18.07	30.35±12.66	34.91±14.95	32.00±10.84	24.90±14.79	0.637
Non-progressive motility (%)	21.89±9.16	24.34±7.40	18.80±7.82	24.24±7.83	19.54±7.38	0.393

control group, while the diabetic+honeybush group presented with a 30.0% increase in sperm concentration compared with the diabetic control animals. Sperm concentration in the diabetic+sutherlandia group was significantly decreased as compared with the vehicle group (P<0.05) (Figure 2C). Although not significant, the diabetic control animals showed a 26.0% decrease in the percentage of normal sperm morphology compared with the vehicle group. Rats in the diabetic+rooibos group, diabetic+honeybush group, and diabetic+sutherlandia group also presented with a decrease in normal sperm morphology compared with the vehicle group (18.0% decrease, 14.0% decrease, 14.5% decrease, respectively). However, when compared with the diabetic control animals, they showed a mild improvement in the percentage of morphologically normal spermatozoa (13.0%, 16.0%, and 15.0%) respectively (Figure 2D). Morphological sperm defects observed in the diabetic control animals were mostly head and mid-piece defects with sperm displaying amorphous shapes. These characteristics were mildly improved in the diabetic+infusion treated groups (Figure 2E).

There were no significant differences in sperm kinematic and speed parameters between the groups (P>0.05) (Tables 3, 4).

3.4. Oxidative stress parameters

There was a significant difference in SOD activity as measured in the testicular tissue between the groups (P<0.05). The rats in the diabetic control group presented with a decrease of 13.5% in SOD activity as compared with the vehicle group [(142.30±24.57) μL/μg *vs*. (164.60±33.67) μL/μg], while the diabetic+rooibos group displayed an increase (18.8%) in SOD activity compared with the vehicle group [(195.60±66.09) μL/μg *vs*.(164.60±33.67) μL/μg] and 37.4% increase compared with the diabetic control group (195.60±66.09) μL/μg *vs*. (142.30±24.57) μL/μg]. The diabetic+honeybush group displayed an increase of 32.9%, 53.7% in SOD activity compared to the vehicle group and the diabetic control group, respectively (P=0.06). The diabetic+sutherlandia group also presented with 28.3%, 48.0% increase in SOD activity compared to the vehicle group and diabetic control group, respectively (Figure 3A). Additionally, the diabetic+honeybush group significantly increased catalase activity as compared with the vehicle group

[(133.40±42.81) µL/µg *vs*. (20.64±16.38) µL/µg, *P*=0.005] and 170% increase compared with the diabetic control group [(133.40±42.81) µL/µg *vs*. (49.24±21.59) µL/µg, *P*=0.300] (Figure 3B). Although not significant, the diabetic control group displayed more than a doubling increase (119.0%) in the MDA levels compared to the vehicle group (*P*=0.200), while it was reduced by 41.0%, 60.0%, and 27.0% respectively in the diabetic+rooibos group, diabetic+honeybush group, and diabetic+sutherlandia group group compared to the diabetic control group (Figure 3C).

3.5. Apoptotic markers

The expression of caspase 7 in the testicular tissues of diabetic control animals increased by 75.7% compared to the vehicle group (*P*=0.2), while the diabetic+rooibos group, diabetic+honeybush group, and diabetic+sutherlandia group showed 5%, 11%, -17% increase in caspase 7 compared with the diabetic control group (Figure 4A). Diabetic control animals displayed an increase (28.0%, 34.0%, 29.0%, 27.5%) in the expression of cleaved PARP, pP38 MAPK, JNK55kDa and JNK46kDa, respectively (Figure 4B-E). The testicular tissues of the diabetic+rooibos group, the diabetic+honeybush group, and the diabetic+sutherlandia group showed an increase in cleaved PARP (19.0%, 41.0%, and 15.0%, respectively) compared with the diabetic control group. The diabetic+honeybush group displayed a significant increase in the phosphorylation of pP38 MAPK compared with the vehicle group (3.630±2.576 *vs*. 0.459±0.375, *P*<0.05; interquartile range (IQR) = 4.927 *vs*. 0.6467). In addition, the diabetic+rooibos group, the diabetic+honeybush group, and the diabetic+sutherlandia group showed a 25.0%, 53.5%, 41.5% decrease in the expression of JNK55kDa compared to the diabetic control group, with the diabetic+honeybush group showing a significant difference (*P*<0.05). To further evaluate apoptotic activity, the total expression of XIAP was measured. Diabetic control animals presented with nearly a 20% decrease in the total expression of XIAP compared to the vehicle group (0.579±0.427 *vs*. 0.690±0.211; IQR=0.8503 *vs*. 0.4488), while the diabetic+rooibos group significantly decreased as compared with the vehicle group (0.294±0.227 *vs*. 0.690±0.211, *P*<0.05; IQR=0.259 *vs*. 0.4488). The diabetic+rooibos group, the diabetic+honeybush group, and the diabetic+sutherlandia group displayed a further decrease in the expression of total XIAP compared to the diabetic control group (52.6% decrease, 33.9% decrease, 50.6% decrease, respectively) (Figure 4F). Blots for the apoptotic markers were illustrated in Figure 4G-K.

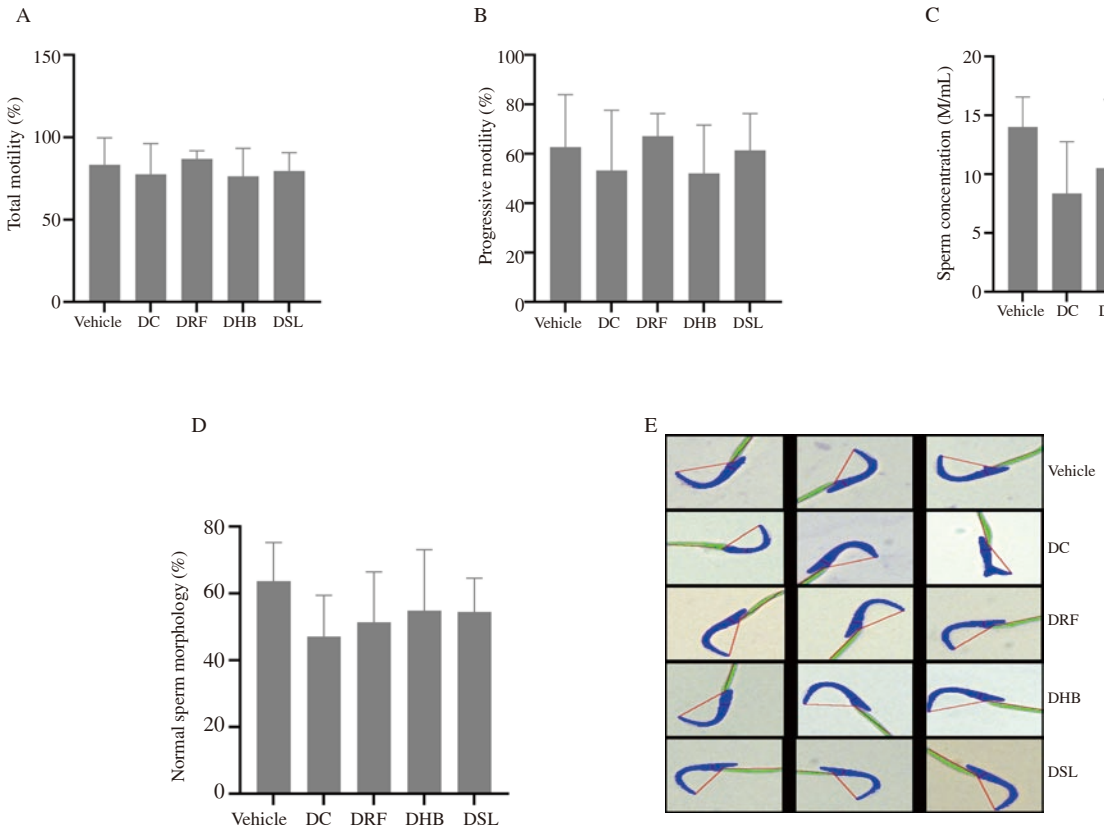

Figure 2. The effects of diabetes mellitus and rooibos, honeybush, and sutherlandia infusions on sperm functional parameters. A: total sperm motility (%), B: progressive motility (%), C: sperm concentration (M/mL), D: normal sperm morphology (%), E: Micrographs of sperm morphology (morphological sperm defects observed in the diabetic control animals are mostly head and mid-piece defects with sperm displaying amorphous shapes. These characteristics are mildly improved in the diabetic+infusion treated groups). *P<0.05 *vs.* the vehicle group; *P* value for total motility=0.500, progressive motility=0.300, sperm concentration=0.018, sperm morphology=0.200. DC=diabetes control, DRF=diabetes+rooibos, DHB=diabetes+honeybush, DSL=diabetes+sutherlandia.

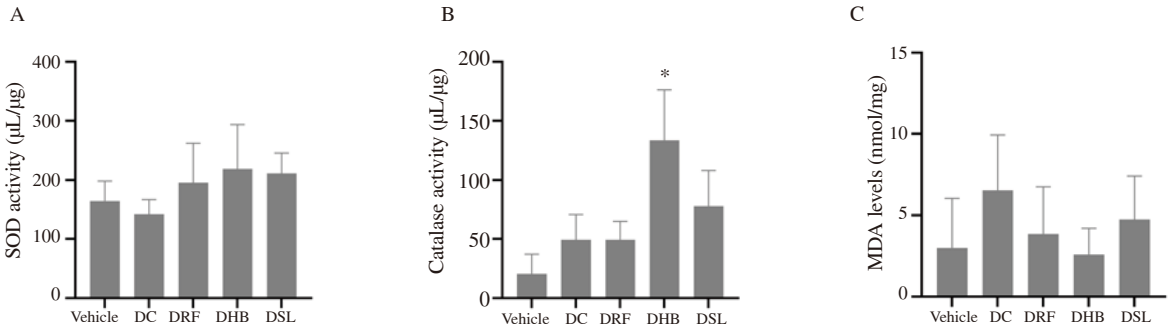

Figure 3. The effect of diabetes mellitus and rooibos, honeybush, and sutherlandia infusions on testicular oxidative stress. A: superoxide dismutase (SOD) activity, B: catalase activity, C.: malondialdehyde (MDA) levels. $^{*}P<0.001$ *vs.* the vehicle group. Global *P* value for SOD=0.040, catalase=0.008, MDA levels=0.070. DC=diabetes control, DRF=diabetes+rooibos, DHB=diabetes+honeybush, DSL=diabetes+sutherlandia.

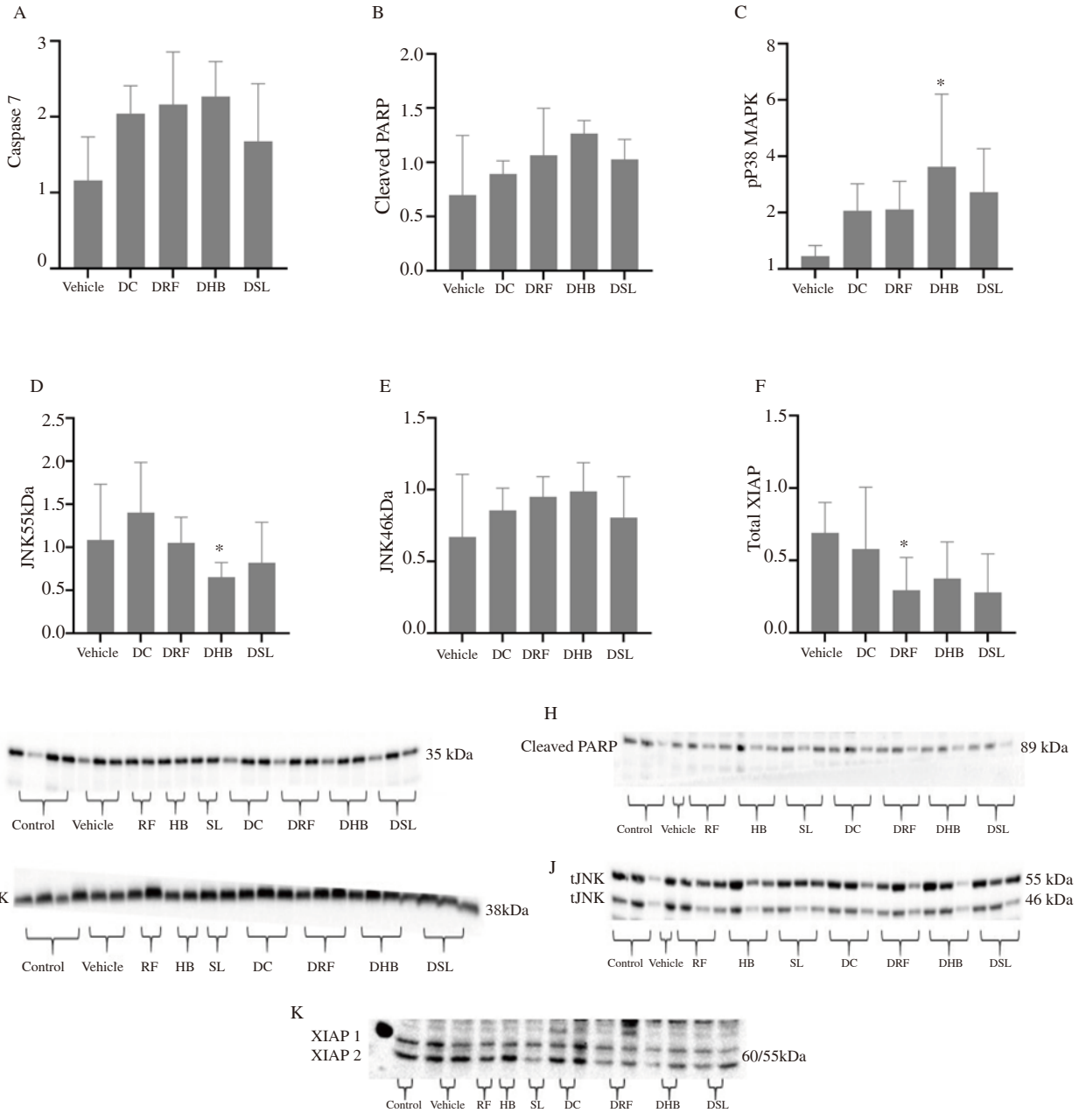

Figure 4. The effects of diabetes mellitus and rooibos, honeybush, and sutherlandia infusions on testicular apoptotic biomarkers. A: caspase 7, B: cleaved PARP, C: pP38MAPK, D: JNK55kDa, E: JNK46kDa, F: total XIAP (XIAP1+XIAP2), G: blot for caspase 7, H: blot for cleaved PARP, I: blot for pP38 MAPK, J: blot for JNK, K: blot for XIAP. $^{*}P<0.05$ *vs.* the vehicle group. Global *P* value for caspase 7=0.054, cleaved PARP=0.080, pP38MAPK=0.020, JNK55kDa=0.100, JNK46kDa=0.400, XIAP=0.020. DC=diabetes control, DRF=diabetes+rooibos, DHB=diabetes+honeybush, DSL=diabetes+sutherlandia, RF=rooibos, HB=honeybush, SL=sutherlandia.

4. Discussion

The use of phytochemical containing natural products has been a crucial part of traditional medicine. The presence of the diverse bioactive compounds in these products has initiated new insights on how they can be beneficial to humanity, either by extracting the bioactive compounds[29] or by using the whole plant[20,30]. Rooibos, honeybush, and sutherlandia are plants containing diverse bioactive compounds, with associated health benefits[17,30]. Several studies have highlighted their role in preventing the development of cell toxicity[31], alleviating oxidative stress in different organ systems including the brain[30], skin[32], and many more. However, little is known about the effects of these infusions in diabetes mellitus-induced male reproductive function impairment. Hence, the current study investigated the plausible ameliorative role of rooibos, honeybush, and sutherlandia on diabetes mellitus-induced sperm function impairment and also set out to determine testicular oxidative stress and apoptosis status after treating diabetic rats with these infusions.

As expected, following diabetes mellitus induction, rats in the diabetic control group, diabetic+rooibos group, diabetic+honeybush group, and diabetic+sutherlandia group presented with elevated blood glucose levels, and they gained less body weight or even lost weight during the treatment period. These are known characteristics of type 1 diabetes mellitus, as shown by several studies[33]. Hence, animals became diabetic. The soluble solid content (mg/mL) of rooibos was lower than that of honeybush and sutherlandia, while the total polyphenol content of rooibos was higher than honeybush and sutherlandia., Awoniyi *et al* in the same vein, reported that the soluble solid content of 2% fermented rooibos used in their study was (5.0±1.2) mg/mL[17]. The soluble solid content of honeybush differed from the report of North *et al*[22]. The difference in the soluble solid content of honeybush, and the total polyphenol content of rooibos, honeybush, and sutherlandia may in part be due to the difference in harvest times, production process, and the part of the plant material used, amongst others.

The diabetic control animals of the current study presented with a non-significant decrease in the percentage of total and progressive motilities. This is partly in agreement with several studies that showed reduced sperm motility in both diabetic men[3] and diabetic rats[33]. Additionally, diabetic control animals presented with a decrease in the percentage of sperm with normal morphology. This concurs with other studies that reported reductions in the number of spermatozoa with normal morphology after inducing diabetes in rodents[34]. Interestingly, in the current study, the diabetic+rooibos group presented with an increase in progressive motility when compared to the diabetic control group. However, what is more, interesting is that the diabetic+rooibos group had a mild increase in progressive motility compared to 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 oxidative stress-induced rats with both fermented and green rooibos[17]. Regarding the effect of honeybush and sutherlandia on sperm functional parameters of diabetic rats, the result of the current study showed that the rats in the diabetic+honeybush group presented with a decrease in sperm motility. However, there was a mild increase in sperm motility of the diabetic+sutherlandia group, and this represents the first study reporting the effect of honeybush and sutherlandia on sperm motility. Although not significant, and not in the normal range of the vehicle rats, the diabetic+rooibos group, diabetic+honeybush group, and diabetic+sutherlandia group had increased number of spermatozoa with normal sperm morphology.

Studies have shown that diabetes mellitus induces subtle molecular changes that are essential for sperm quality and function through the development of oxidative stress which was caused by the imbalance in the ratio of ROS production and the antioxidant activities[3,35]. In the diabetic control animals of the study, there was a decrease in the activity of SOD, accompanied by an increased level of MDA. This is supported by studies that reported a significant increase in the plasma level of TBARS in diabetic rats[25]. This also concurs with the findings of Uličná *et al* who showed increased levels of MDA in the plasma, liver, kidney, and lens of diabetic rats[36]. However, the rats in the diabetic+rooibos group, diabetic+honeybush group, and diabetic+sutherlandia group presented with increased SOD activity. Interestingly, not only did their SOD activity surpass the diabetic control group, there was an increase in SOD activity compared to the vehicle group. Additionally, the rats in the diabetic+honeybush group presented with significantly 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[23]. In oxidative stress, sperm motility is affected because of the increased ROS production. The sperm plasma membrane has a very high percentage of polyunsaturated fatty acids[37] which are essential for sperm motility. These polyunsaturated fatty acids are vulnerable to ROS and hence the invasion of the sperm plasma membrane leads to lipid peroxidation[38]. Lipid peroxidation occurs when ROS reacts 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 oxidative stress-induced damage, it is necessary to treat the underlying cause[35] and then suppress pro-oxidants by antioxidants[39].

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 diabetes mellitus on sperm motility. Although the rats in the diabetic+honeybush group 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 diabetes mellitus.

The induction of apoptosis as a result of oxidative stress has been shown in several pathologies, including diabetes mellitus[40]. High levels of ROS alter the integrity of mitochondrial membrane[41], which results in mitochondria DNA damage and subsequently affects sperm functions negatively. In the current study, the diabetic control animals displayed increased caspase 7, cleaved PARP, p38MAPK, and JNK46kDa, which are all biomarkers of apoptosis. This is partly in agreement with Roessner *et al* who reported an increase in the cleavage of caspase 3, elevated ROS production, and disrupted mitochondrial potential in diabetic men[2]. 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 was 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 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[42]. In lieu of this, XIAP was quantified. The expression of XIAP in the testicular tissue of diabetic control rats was decreased, while diabetic rats 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 the diabetic control rats may indicate that caspases were not inhibited and thus apoptosis ensued. 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.

The current study has highlighted the negative impact of diabetes mellitus on sperm functional parameters through increased lipid peroxidation and reduced antioxidant activity. Increased antioxidant enzyme activity in the infusion treatment groups is observed, which may be partly responsible for the observed improvement in sperm motility and morphology in the diabetic+rooibos group and diabetic+sutherlandia group. Additionally, the current study has shown the increased expression of apoptotic biomarkers in diabetes mellitus, which are not alleviated by the infusions. This suggests that these infusions play a role in alleviating diabetes mellitus-induced sperm function impairment through suppression of oxidative stress, but their role in apoptosis is still unclear. Therefore, based on the results of the current study, it can be speculated that disease-related male reproductive impairment such as diabetes mellitus, rooibos, and sutherlandia may be beneficial in ameliorating male fertility complications.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgements

The authors would like to thank Dr Michelle Smit-van Schalkwyk for the generous donation of tissue samples as well as the Harry Crossley Foundation for the research grant provided.

Authors′ contributions

Temidayo S Omolaoye carried out conceptualization, study design, data analysis, data interpretation, drafting of manuscript, and editing. Shantal Windvogel made data interpretation, review and editing. Stefan S du Plessis was responsible for study design, data interpretation, review and editing, and supervision.

References

[1] American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2014; **37**(Suppl 1): 1355.

[2] Roessner C, Paasch U, Kratzsch J, Glander HJ, Grunewald S. Sperm apoptosis signalling in diabetic men. *Reprod Biomed Online* 2012; **25**(3): 292-299.

[3] Bhattacharya SM, Ghosh M, Nandi N. Diabetes mellitus and abnormalities in semen analysis. *J Obstet Gynaecol Res* 2014; **40**(1): 167-171.

[4] Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res* 2010; **107**(9): 1058-1070.

[5] Chen Y, Wu Y, Gan X, Liu K, Lv X, Shen H, et al. Iridoid glycoside from *Cornus officinalis* ameliorated diabetes mellitus-induced testicular damage in male rats: Involvement of suppression of the AGEs/RAGE/p38 MAPK signaling pathway. *J Ethnopharmacol* 2016; **194**: 850-860.

[6] Khaki A, Fathiazad F, Nouri M, Khaki AA, Maleki NA, Khamnei HJ, et al. Beneficial effects of quercetin on sperm parameters in streptozotocin-induced diabetic male rats. *Phyther Res* 2010; **24**(9): 1285-1291.

[7] Allan DJ, Harmon BV, Roberts SA. Spermatogonial apoptosis has three morphologically recognizable phases and shows no circadian rhythm during normal spermatogenesis in the rat. *Cell Prolif* 1992; **25**(3): 241-250.

[8] Kanter M, Aktas C, Erboga M. Protective effects of quercetin against apoptosis and oxidative stress in streptozotocin-induced diabetic rat testis. *Food Chem Toxicol* 2012; **50**(3-4): 719-725.

[9] Guneli E, Tugyan K, Ozturk H, Gumustekin M, Cilaker S, Uysal N. Effect of melatonin on testicular damage in streptozotocin-induced diabetes rats. *Eur Surg Res* 2008; **40**(4): 354-360.

[10]Sugawara E, Nikaido H. Properties of AdeABC and AdeIJK efflux systems of *Acinetobacter baumannii* compared with those of the AcrAB-TolC system of *Escherichia coli*. *Antimicrob Agents Chemother* 2014; **58**(12): 7250-7257.

[11]López-Alvarenga JC, Zariñán T, Olivares A, González-Barranco J, Veldhuis JD, Ulloa-Aguirre A. Poorly controlled type Ⅰ diabetes mellitus in young men selectively suppresses luteinizing hormone secretory burst

mass. *J Clin Endocrinol Metab* 2002; **87**(12): 5507-5515.

[12]Joubert E, Gelderblom WCA, Louw A, de Beer D. South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides*-A review. *J Ethnopharmacol* 2008; **119**(3): 376-412.

[13]van Wyk BE, Albrecht C. A review of the taxonomy, ethnobotany, chemistry and pharmacology of *Sutherlandia frutescens* (Fabaceae). *J Ethnopharmacol* 2008; **119**(3): 620-629.

[14]Beelders T, Kalili KM, Joubert E, De Beer D, De Villiers A. Comprehensive two-dimensional liquid chromatographic analysis of rooibos (*Aspalathus linearis*) phenolics. *J Sep Sci* 2012; **35**(14): 1808-1820.

[15]Kamara BI, Brandt EV, Ferreira D, Joubert E. Polyphenols from honeybush tea (*Cyclopia intermedia*). *J Agric Food Chem* 2003; **51**(13): 3874-3879.

[16]Avula B, Wang YH, Smillie TJ, Fu X, Li XC, Mabusela W, et al. Quantitative determination of flavonoids and cycloartanol glycosides from aerial parts of *Sutherlandia frutescens* (L.) R. BR. by using LC-UV/ELSD methods and confirmation by using LC-MS method. *J Pharm Biomed Anal* 2010; **52**(2): 173-180.

[17]Awoniyi DO, Aboua YG, Marnewick J, Brooks N. The effects of rooibos (*Aspalathus linearis*), green te (*Camelli sinensis*) and commercial rooibos and green te supplements on epididymal sperm in oxidative stress-induced rats. *Phyther Res* 2012; **26**(8): 1231-1239.

[18]Ojewole JAO. Analgesic, antiinflammatory and hypoglycemic effects of *Sutherlandia frutescens* R. BR. (variety incana E. MEY.) [Fabaceae] shoot aqueous extract. *Methods Find Exp Clin Pharmacol* 2004; **26**(6): 409-416.

[19]Opuwari CS, Monsees TK. *In vivo* effects of *Aspalathus linearis* (rooibos) on male rat reproductive functions. *Andrologia* 2014; **46**(8): 867-877.

[20]Marnewick JL, Rautenbach F, Venter I, Neethling H, Blackhurst DM, Wolmarans P, et al. Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. *J Ethnopharmacol* 2011; **133**(1): 46-52.

[21]Du Toit J, Joubert E. Optimization of the fermentation parameters of honeybush tea (*Cyclopia*). *J Food Qual* 1999; **22**(3): 241-256.

[22]North MS, Joubert E, de Beer D, de Kock K, Joubert ME. Effect of harvest date on growth, production and quality of honeybush (*Cyclopia genistoides* and *C. subternata*). *South African J Bot* 2017; **110**: 132-137.

[23]Tobwala S, Fan W, Hines CJ, Folk WR, Ercal N. Antioxidant potential of Sutherlandia frutescens and its protective effects against oxidative stress in various cell cultures. *BMC Complement Altern Med* 2014; **14**: 1-11.

[24]Arthur H, Joubert E, De Beer D, Malherbe CJ, Witthuhn RC. Phenylethanoid glycosides as major antioxidants in *Lippia multiflora* herbal infusion and their stability during steam pasteurisation of plant material. *Food Chem* 2011; **127**(2): 581-588.

[25]Ayeleso A, Brooks N, Oguntibeju O. Modulation of antioxidant status in streptozotocin-induced diabetic male wistar rats following intake of red palm oil and/or rooibos. *Asian Pac J Trop Med* 2014; **7**(7): 536-544.

[26]Omolaoye TS, Skosana BT, du Plessis SS. Diabetes mellitus-induction: Effect of different streptozotocin doses on male reproductive parameters. *Acta Histochem* 2018; **120**(2): 103-109.

[27]van der Horst G, Skosana B, Legendre A, Oyeyipo P, du Plessis SS. Cut-off values for normal sperm morphology and toxicology for automated analysis of rat sperm morphology and morphometry. *Biotech Histochem* 2018; **93**(1): 49-58.

[28]Marais E, Genade S, Huisamen B, Strijdom JG, Moolman JA, Lochner A. Activation of p38 MAPK induced by a multi-cycle ischaemic preconditioning protocol is associated with attenuated p38 MAPK activity during sustained ischaemia and reperfusion. *J Mol Cell Cardiol* 2001; **33**(4): 769-778.

[29]Dludla PV, Gabuza KB, Muller CJF, Joubert E, Louw J, Johnson R. Aspalathin, a *C*-glucosyl dihydrochalcone from rooibos improves the hypoglycemic potential of metformin in type 2 diabetic (db/db) mice. *Physiol Res* 2018; **67**(5): 813-818.

[30]Hong IS, Lee HY, Kim HP. Anti-oxidative effects of rooibos tea (*Aspalathus linearis*) on immobilization-induced oxidative stress in rat brain. *PLoS One* 2014; **9**(1): 1-9.

[31]Magcwebeba TU, Riedel S, Swanevelder S, Swart P, De Beer D, Joubert E, et al. The potential role of polyphenols in the modulation of skin cell viability by *Aspalathus linearis* and *Cyclopia* spp. herbal tea extracts *in vitro*. *J Pharm Pharmacol* 2016; **68**(11): 1440-1453.

[32]Tiedtke J, Marks O. Rooibos – the new 'white tea'; for hair and skin care. *Euro Cosmet* 2002; **6**: 16-19.

[33]Singh S, Malini T, Rengarajan S, Balasubramanian K. Impact of experimental diabetes and insulin replacement on epididymal secretory products and sperm maturation in albino rats. *J Cell Biochem* 2009; **108**(5): 1094-1101.

[34]Mangoli E, Talebi AR, Anvari M, Pourentezari M. Effects of experimentally-induced diabetes on sperm parameters and chromatin quality in mice. *Iran J Reprod Med* 2013; **11**(1): 53-60.

[35]Agarwal A, Virk G, Ong C, du Plessis SS. Effect of oxidative stress on male reproduction. *World J Mens Health* 2014; **32**(1): 1-17.

[36]Uličná O, Vančová O, Božek P, Čársky J, Šebeková K, Boor P, et al. Rooibos tea (*Aspalathus linearis*) partially prevents oxidative stress in streptozotocin-induced diabetic rats. *Physiol Res* 2006; **55**(2): 157-164.

[37]Lenzi A, Gandini L, Picardo M, Tramer F, Sandri G, Panfili E. Lipoperoxidation damage of spermatozoa polyunsaturated fatty acids (PUFA): Scavenger mechanisms and possible scavenger therapies. *Front Biosci* 2000; **5**(1): 1-5.

[38]Ding GL, Liu Y, Liu ME, Pan JX, Guo MX, Sheng JZ, et al. The effects of diabetes on male fertility and epigenetic regulation during spermatogenesis. *Asian J Androl* 2015; **17**(6): 948-953.

[39]Tremellen K. Oxidative stress and male infertility: A clinical perspective. *Stud Men's Heal Fertil* 2012; **14**(3): 325-353.

[40]Mahfouz RZ, du Plessis SS, Aziz N, Sharma R, Sabanegh E, Agarwal A. Sperm viability, apoptosis, and intracellular reactive oxygen species levels in human spermatozoa before and after induction of oxidative stress. *Fertil Steril* 2010; **93**(3): 814-821.

[41]Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 2000; **5**(5): 415-418.

[42]Scott FL, Denault JB, Riedl SJ, Shin H, Renatus M, Salvesen GS. XIAP inhibits caspase-3 and -7 using two binding sites: Evolutionary conserved mechanism of IAPs. *EMBO J* 2005; **24**(3): 645-655.