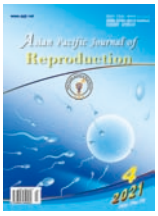

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Descriptive histomorphological evaluation of the testis and caudal epididymis following treatment with rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and sutherlandia (*Lessertia frutescens*) in healthy and streptozotocin-induced diabetic rats

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ABSTRACT

Objective: To evaluate the testis and the cauda epididymis after treating both healthy and diabetic rats with rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*), and sutherlandia (*Lessertia frutescens*), respectively.

Methods: Ninety male Wistar rats were randomly divided into nine groups (n=10 per group). Fifty rats were randomized to receive tap water, 0.1 M citrate buffer, 2.0% fermented rooibos, 4.0% fermented honeybush, or 0.2% unfermented sutherlandia. Diabetes was induced *via* a single intraperitoneal injection of streptozotocin (STZ) at 45 mg/kg in 40 rats. The diabetic rats were randomized to receive tap water, 2.0% fermented rooibos, 4.0% fermented honeybush, or 0.2% unfermented sutherlandia. Rats were sacrificed after seven weeks of diabetic induction. Blood was collected for hormonal assay, while the testes and caudal epididymis were retrieved for histomorphological evaluations.

Results: Diabetic rats treated with 2.0% fermented rooibos exhibited a significant increase in fasting plasma glucose (FPG) *versus* the vehicle group (P=0.002), and a 3.6% decrease compared to the diabetic control group (P>0.05). Diabetic rats treated with honeybush or sutherlandia showed 23.6% and 15.4% decrease in FPG when compared to the diabetic control group, respectively (both P>0.05). The diabetic control rats had a significant decrease in the percentage of histologically normal seminiferous tubules compared to the control group (P=0.049). There was 21.0%, 14.0%, and 5.4% rise in the percentage of normal seminiferous tubules in diabetic rats receiving rooibos, honeybush or sutherlandia, respectively, compared to the diabetic control group. The infusion control groups (rooibos, honeybush and sutherlandia) showed normal seminiferous tubules, presence of spermatozoa in the epididymal lumen, and had normal overall architecture. Both testicular and epididymal morphology were altered in the diabetic control group, but these disruptions were mildly ameliorated by rooibos, honeybush, and sutherlandia.

Conclusions: The detrimental effects of diabetes on the histomorphological architecture of the testis and epididymis are mildly ameliorated by the infusions (2.0% fermented rooibos, 4.0% fermented honeybush, and 0.2% unfermented sutherlandia).

KEYWORDS: Testis; Epididymis; Rooibos; Honeybush; Sutherlandia; Diabetes; Histomorphological evaluation; Streptozotocin.

Significance

Diabetes alters spermatogenesis and impairs male fertility. Development of diabetes resulted in increased percentage of altered seminiferous tubules of the testes with tubules showing atrophy and sloughing, and the caudal epididymal tubules displaying few spermatozoa and sometimes clear lumen. After the infusion (rooibos, honeybush and sutherlandia) treatment, these characteristics were mildly ameliorated. In diabetes, rooibos, honeybush, and sutherlandia may mildly improve the histomorphological architecture of the seminiferous tubule and caudal epididymal tubule, hence improving spermatogenesis.

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1. 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[2,3], while it takes 35-52 days in rodents[4]. 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[5]. 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 house early and late spermatids[6]. Following spermatozoa production in the seminiferous tubules of the testis, sperm are transferred into the epididymis and move through the caput, and corpus region and are stored in the caudal epididymis until ejaculation. The individual segment of the epididymis contributes specifically to the stability of the luminal microenvironment, which is vital for sperm maturation by the time they reach the caudal region. 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 a 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[7]. It is estimated that between 35%-51% of diabetic men have testicular and or erectile dysfunction[8]. 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[9]. Soudamani *et al*, on the other hand, reported a 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[10]. Several other authors have also reported abnormalities in the testis and epididymis of diabetic rats[11,12]. The adverse effects seen have been attributed to disrupted endocrine pathway, impaired insulin signalling pathway, excessive formation of reactive oxygen species and the development of oxidative stress, and increased apoptosis[13]. Refer to the publication of Omolaoye and Du Plessis (2018)[14] for a detailed review on the mechanism through which diabetes impairs spermatogenesis.

Interestingly, Ayuob *et al* reported that although the symptoms of DM were improved after treating diabetic rats with antidiabetic drugs (metformin, pioglitazone and sitagliptin), these drugs, however, induced histopathological changes on the testicular structures (testis, epididymis, and seminal vesicle)[15]. They further added that the testis of diabetic rats treated with metformin showed deformed primary spermatocytes, has few spermatozoa in the lumen and 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 managing 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 (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and sutherlandia (*Lessertia frutescens*) are indigenous Southern African plants. Rooibos originates from the Cederberg Mountains of the Western Cape region of South Africa[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]. Although it is well documented that unfermented rooibos has higher antioxidant properties than fermented rooibos, fermented rooibos is still preferrable in food because of the reddish-brown leaf color and woody-fynbos-floral honey flavor it has after fermentation[18]. 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[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 southeastern regions of Botswana and Lesotho[20]. The infusions from these plants contain 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]. Honeybush has been shown to offer photoprotection against ultraviolet B-induced skin damage in SKH-1 mice. This was evidenced by the reduction in erythema, the peeling and hardening

of the skin. It was suggested that honeybush enhanced cell proliferation, hence the observation[22]. Sutherlandia has 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[23].

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[24]. These authors in another manuscript also reported decreased testosterone production by Leydig cells treated with rooibos *in vitro*. This suggests that rooibos may have anti-androgenic activities[25]. Hence, the role of rooibos on male reproductive histomorphology remains controversial. Additionally, to the best of our knowledge, 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.

2. Materials and methods

2.1. 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.

The plants used in preparing the infusions were processed during the same season. The same batch of individual plant material was used throughout the study. That is, the plant batch that was prepared at the start of the experiment was used throughout the treatment period. Infusions were prepared according to previously established methods[26]. Briefly, 2% fermented rooibos was prepared by adding 20 g of dried rooibos in 1 L of boiled water and allowed to soak for 30 min. After 30 min, the mixture was initially filtered by using a cheesecloth and then filtered with a number 4 and a number 1 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[27], and were stored at 4 ℃.

Fermented honeybush (4.0%) 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 h). It is important to note that the herbal teas serve as the only drinking fluid for the animals in the infusion groups. The fluid intake of the animals was measured three times a week. Additionally, an initial 4.0% sutherlandia herbal tea was prepared but was further diluted to 0.2% due to the bitter taste.

2.2. Determination of the polyphenolic content in rooibos, honeybush and sutherlandia

Analyses were performed by using the concentrations that the animals were treated with. That was, 2% fermented rooibos, 4% fermented honeybush and 4% unfermented sutherlandia.

2.3. Soluble solid content

The soluble solid content of the infusions (rooibos, honeybush and sutherlandia) were assessed gravimetrically (6 repetitions) and analysed in triplicate at each time point[28]. In brief, the glass beakers were initially washed, placed in the oven overnight at 70 ℃ and cooled in a desiccator for another 24 h. For the assay, beakers were weighed before adding 1 mL of the respective infusions. The aliquots were dried in an oven for 24 h at 70 ℃, whereafter, were placed in a desiccator for another 24 h. Glass beakers with the dried content were weighed again. The soluble solid content in each herbal tea was determined by subtracting the initial weight from the final weight.

2.4. Total polyphenol content

The total polyphenol content was measured as described by Arthur *et al*, using the Folin-Ciocalteau method[29]. 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 and 80 µL of 7.5% (m/v) Na_2CO_3 were added respectively. The plate was prevented from light, gently vortexed and allow 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.

2.5. Animals and experimental design

Ninety adult (fourteen-week old) male Wistar rats of weight range 250-300 g were housed at the Stellenbosch University's Faculty of Medicine and Health Sciences Animal Unit (18-23 ℃, 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[30]. Rats were individually caged and had free access to both food and water/infusions.

Diabetes was induced by administering a single intraperitoneal injection of streptozotocin (STZ) at 45 mg/kg body weight from a stock solution of 30 mg/mL (dissolved in 0.1 M citrate buffer pH 4.5) (STZ S0130-IG, Sigma, South Africa). Rats were feed fasted

overnight before injecting with STZ. 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.

The 90 rats were divided into 9 groups, with 10 rats in each group, including: the normal control group , the vehicle group (receiving 0.1 M citrate buffer + water), the rooibos control group (receiving 2.0% fermented rooibos), the honeybush control group (receiving 4.0% fermented honeybush), the sutherlandia control group (receiving 0.2% unfermented sutherlandia), the diabetic control group (receiving STZ 45 mg/kg + water), the diabetic+rooibos group (receiving STZ 45 mg/kg + 2.0% fermented rooibos), the diabetic+ honeybush group (receiving STZ 45 mg/kg + 4.0% fermented honeybush), and the diabetic+sutherlandia group (receiving STZ 45 mg/kg + 0.2% unfermented sutherlandia). Food intake, fluid intake and body weights were measured thrice a week, and blood glucose levels were measured before morning feeding once weekly. After 7 weeks of DM induction, rats were sacrificed, and blood samples were immediately collected. The testes, epididymis and visceral fats were also harvested and weighed. The relative testicular and relative epididymal weights were expressed as a percentage of body weight. The reported fasting plasma glucose levels were recorded immediately before sacrifice and the average blood glucose which represents the mean of the weekly blood measurement taken over seven weeks treatment period was also reported. It is worth noting that the intra-peritoneal glucose tolerance was also performed, please refer to Omolaoye *et al*[31] for explicit details.

2.6. Measurement of testosterone

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

2.7. Histology

The right testis and the epididymis were fixed in a 10% formalin solution. Tissues were kept in formalin for a minimum of 48 h to allow for complete fixation. The fixed tissues were processed according to the previously described protocol[32]. 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 min, rinsed thoroughly in tap water for 2 min and further rinsed in distilled water for another 2 min. Sections were oxidized in 1% periodic acid (Sigma, South Africa) solution for 10 min 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 min and then washed in lukewarm tap water for 7 min 30 s. At this point, sections turned dark pink. Sections were counter stained with haematoxylin solution for 30 s, washed in tap water for 3 min, then dehydrated, and cleared with xylene. Sections were mounted and allowed to air dry.

2.8. Descriptive histomorphology

To properly analyse the morphology of the testis and cauda epididymis, an initial blinded overview of the sections stained with AB/PAS was performed. Thereafter, tubules were further analysed blindly and categorized as previously described[32]. Briefly, one hundred seminiferous tubules per animal were randomly counted across different fields, with field area ranging between 234.16 mm^2-348.97 mm^2. Seminiferous tubules were categorized into groups as previously described by Omolaoye *et al*[32]. Tubules were considered normal when it showed complete spermatogenic phases, regular cellular organization, typical 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 were characterized by having excessive number of immature cells in their lumen and there were absence of some or all of the spermatogenic phases (Supplementary Figure 1). The cauda epididymis was considered normal/intact if the following were absent (Supplementary Figure 2): 1) Cribriform changes (this represents a hyperplastic modification of the epithelium, *e.g.* infolding of the epithelium); 2) Inflammatory infiltrate (excessive accumulation of cells in the interstitium, appearing as a vesicle filled with cells or scattered); 3) Debris in the lumen (presence of cytoplasmic shedding in the lumen instead of spermatozoa); 4) Clear cells/tubules (clear cells occur as a consequence of debris in the lumen); 5) Sperm compaction (this occurs as a result of excessive fluid resorption due to lack in sperm production); 6) 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 234.16 mm^2-

348.97 mm^2. The epididymal tubules were then categorized as normal or abnormal (if one of the listed pathologies was 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.

2.9. Statistical analysis

GraphPad Prism™ software (GraphPad™ Software, Version 8.2, San Diego, CA, USA) was used for the statistics. Normal data distribution was measured by using the Anderson-Darling, Kolmogorov-Smirnov, Shapiro-Wilk and D'Agostino & Pearson, 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, Kruskal-Wallis test and Dunns *post-hoc* test were carried out. Significance was set at $P<0.05$ and data were expressed as mean±standard deviation (mean±SD).

2.10. Ethics statement

The study was approved by the Stellenbosch University Animal Ethics Committee (Grant No. SU-ACUD17-00016).

3. Results

3.1. Anthropometric parameters

Rats in the diabetic control group presented with a significant increase in fasting plasma glucose levels compared to the control ($P=0.020$) and vehicle ($P<0.001$) groups. After seven weeks of treatment, although diabetic rats receiving rooibos presented with a significant increase in fasting plasma glucose compared to the vehicle ($P=0.002$) group, a non-significant percentage decrease (3.6%; $P>0.05$) was observed when compared to the diabetic control group. On the other hand, diabetic rats receiving honeybush presented with a non-significant increase in fasting plasma glucose when compared to the vehicle group ($P=0.06$), but a percentage decrease of 23.6% ($P>0.05$) was observed when compared to the diabetic control group. While diabetic+sutherlandia animals presented with a significant increase in fasting plasma glucose compared to the vehicle group ($P<0.001$), but showed a non-significant decrease (15.4%; $P>0.05$) in the fasting plasma glucose level when compared to the diabetic control group (Table 1).

Additionally, diabetic+rooibos, diabetic+honeybush, and diabetic+sutherlandia rats presented with significantly increased average blood glucose when compared to the control group ($P<0.001$) and the vehicle group ($P<0.001$). No significant difference was observed in the average blood glucose of diabetic rats receiving the respective infusions when compared to the diabetic control rats (Table 1).

The diabetic control group gained less body weight, while rats in the diabetic+rooibos, diabetic+honeybush, and diabetic+sutherlandia groups were significantly lighter compared to the control ($P<0.001$) and vehicle ($P<0.001$) groups. While no significant difference was observed in the body weights of diabetic rats receiving the respective infusions when compared to the diabetic control rats (Table 1).

There was no statistically significant difference in testicular weights between these groups. However, the epididymal weight of the diabetic control rats was 10% less compared to the control rats. Diabetic + rooibos rats presented with a significant reduction in epididymal weight compared to the control group ($P=0.030$). Diabetic + honeybush epididymal weight was more than 10% less than the control group, while the diabetic+sutherlandia group displayed a significant decrease compared to the control group ($P=0.001$) (Table 1).

The diabetic group presented with a significant decrease in visceral fat weight compared to the control and vehicle groups ($P<0.001$). All diabetic animals receiving the respective infusions presented with a significant reduction in visceral fat weight compared to the control and vehicle groups ($P<0.05$), but showed a non-significant difference compared to the diabetic control group (Table 1).

Table 1. Anthropometric data.

Parameters	Control	Vehicle	RF	HB	SL	DC	DRF	DHB	DSL
Fasting plasma glucose, mmol/L	6.15±0.73	5.66±1.02	5.69±0.89	6.12±1.70	5.49±0.68	19.62±7.49^{a,b*}	18.91±9.15^{b}	14.98±9.44	16.60±8.93^{b*}
Average blood glucose, mmol/L	6.38±0.25	6.31±0.37	6.46±0.35	6.37±0.33	6.41±0.27	25.76±2.59^{a*,b*}	24.73±5.01^{a*,b*}	21.93±5.78^{a*,b*}	24.71±3.074^{a*,b*}
Body weight, g	344.60±19.04	332.40±30.19	349.40±21.41	345.30±25.55	352.60±19.37	275.10±11.38^{a*,b*}	266.70±32.95^{a*,b*}	288.30±37.86^{a*,b*}	247.40±30.60^{a*,b*}
Testicular weight, g	1.41±0.12	1.35±0.10	1.35±0.08	1.44±0.12	1.42±0.11	1.38±0.11	1.36±0.15	1.34±0.15	1.21±0.23
Epididymal weight, g	0.47±0.04	0.44±0.03	0.46±0.03	0.48±0.05	0.49±0.02	0.42±0.04	0.39±0.04^{a}	0.42±0.05	0.35±0.06^{a}
Visceral fat, g	13.22±2.94	12.40±3.02	12.25±2.87	12.78±3.78	12.58±3.34	3.07±1.36^{a*,b*}	2.48±1.54^{a*,b*}	4.21±2.51^{a,b}	2.38±1.20^{a*,b*}
Relative testicular weight, %	0.41±0.03	0.41±0.04	0.39±0.03	0.42±0.03	0.40±0.02	0.49±0.05	0.52±0.08^{a,b}	0.47±0.04	0.49±0.08
Relative epididymal weight, %	0.14±0.01	0.13±0.02	0.13±0.01	0.14±0.02	0.14±0.01	0.15±0.02	0.17±0.06	0.15±0.01	0.14±0.02

$^{a}P<0.05$ *vs.* the control group, $^{a*}P<0.001$ *vs* the control group; $^{b}P<0.05$ *vs.* the vehicle group; $^{b*}P<0.001$ *vs.* the vehicle group. RF=rooibos, HB=honeybush, SL=sutherlandia, DC=diabetic control, DRF=diabetic+rooibos, DHB=diabetic+honeybush, DSL=diabetic+sutherlandia. Fasting plasma glucose = mean of plasma glucose measured at sacrifice after 16 hours of fasting. Average blood glucose = mean of blood glucose measured over 7 weeks before morning feeding.

Table 2. Total polyphenolic contents.

Parameters	RF	HB	SL
Soluble solid contents, mg/mL	5.18±0.00	13.40±0.00[a]	16.26±0.01[ab]
Total polyphenol content, mg gallic acid/mg soluble solids	0.17±0.00[c]	0.17±0.00[c]	0.03±0.00
Total polyphenol content per day, mg gallic acid/mg soluble solids/day	42.31±7.59	132.50±29.76[ac]	22.30±4.03
Daily phenolic intake, mg gallic acid equivalents/day/100 g body weight	12.12±2.07	38.78±10.66[c]	6.35±1.23

[a]P<0.001 *vs* the RF group, [b]P<0.001 *vs* the HB group, [c]P<0.001 *vs* the SL group. Following gravimetric analysis, the soluble solid content in each herbal infusion is determined by subtracting the initial weight from the final weight. Using GraphPad prism software, statistical difference between the groups is measured by using an ordinary one-way ANOVA and a Tukey's *post–hoc* comparison test.

Although not significant, diabetic control rats presented with an increase of just more than 20% in the relative testicular weight compared to the control and vehicle groups, respectively. Diabetic + rooibos rats displayed a significant increase in relative testicular weight compared to the control (P=0.020) and vehicle (P=0.014) groups. There was no statistically significant difference in relative epididymal weight between the groups (Table 1).

As for the above mentioned parameters (relative testicular weight, relative epididymal weight), the rooibos, honeybush, and sutherlandia groups were non-significantly different compared to the control group. In addition, there was no significant difference between the control group and the vehicle group (Table 1).

3.2. Soluble solid and phenolic contents

There was a significant difference in the amount of soluble solid contents between the herbal teas (rooibos, honeybush, and sutherlandia) (P<0.001). The soluble solid content of fermented rooibos was significantly lower than honeybush (P<0.001) and sutherlandia (P<0.001). The polyphenol content of fermented rooibos and honeybush was significantly higher than sutherlandia (both P<0.001). The daily consumption of total polyphenol was significantly elevated in the honeybush group when compared to the rooibos group and the sutherladia group, respectively (both P<0.001). Relative to the bodyweight per 100 g, rats treated with honeybush had significantly higher daily phenolic intake compared to the sutherladia group (P<0.001) (Table 2).

3.3. Testosterone

Although not significant, the diabetic control rats presented with a percentage decrease in plasma testosterone concentration (-9.78%, -16.5%) when compared to the control and vehicle groups, respectively. The diabetic+rooibos rats displayed a significant decrease in plasma testosterone concentration compared to the vehicle group [(811.2±126.5) nmol/L *vs*. (1085.00±95.66) nmol/L, P=0.04]. The diabetic+rooibos, diabetic+honeybush and diabetic+sutherlandia groups presented with a non-significant difference in testosterone concentration when compared to the diabetic control group. On other hand, the animals receiving rooibos, honeybush and sutherlandia, showed a non-significant percentage increase (5.4%, -3.2%, 4.3%) in the level of plasma testosterone when compared to the control group respectively (Figure 1).

Additionally, plasma testosterone concentration was correlated 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=0.020), epididymal weight (r=0.4188, P<0.001) and visceral fat weight (r=0.4719, P<0.001) (Supplementary Figure 3).

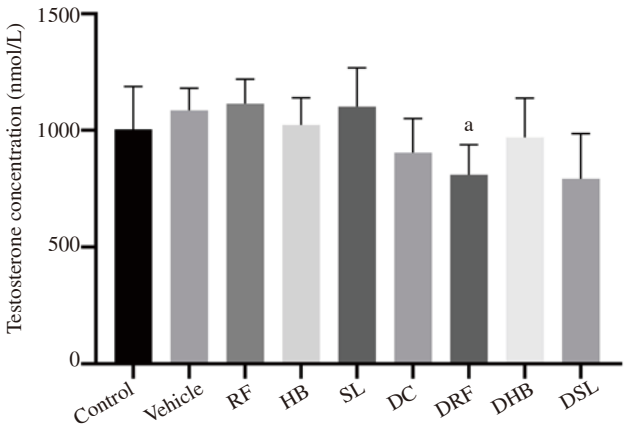

Figure 1. Plasma testosterone concentration. [a]P<0.05 *vs.* the vehicle group. RF: rooibos; HB: honeybush; SL: sutherlandia; DC: diabetic control; DRF: diabetic+rooibos; DHB: diabetic+honeybush; DSL: diabetic+sutherlandia.

3.4. Histology

Histomorphological overview of the testis is presented in Figure 2 (A-E). Diabetic control rats presented with a significant decrease in the percentage of histologically normal seminiferous tubules compared to the control group [(49.00±13.85) *vs*. (68.00±7.54), P=0.049]. While diabetic + rooibos, diabetic + honeybush and diabetic + sutherlandia rats displayed an upregulation (21.0%, 14.0%, 5.4%, respectively) in the percentage of normal seminiferous tubule compared to the diabetic control group. The infusion control groups (rooibos, honeybush, and sutherlandia) on the other hand showed no significant difference in the percentage of normal seminiferous tubules when compared to the control group, respectively [(66.33±8.078)%; (67.13±12.54)%; (66.40±15.62)% *vs*. (68.00±7.54)%] (Figure 2C). Diabetic control rats displayed a percentage increase of 27.12% in the number of atrophic tubules compared to the vehicle group [(38.90±10.84)% *vs.* (30.60±4.81%)] (Figure 2D), while no significant difference was observed in the percentage of sloughing tubules between the groups (Figure 2E).

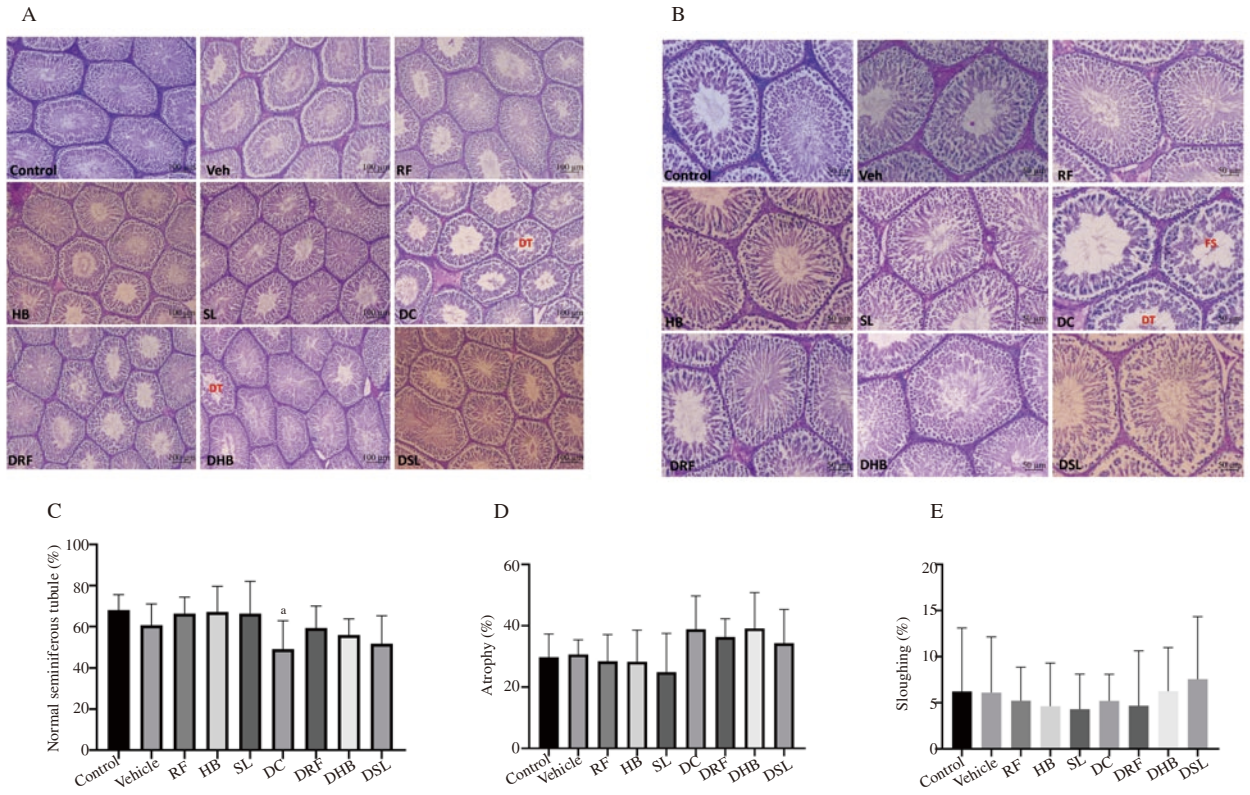

Figure 2. Histology of the testis. A and B: Histomorphological overview of the testis (AB/PAS stain). The seminiferous tubules of the control and infusion control groups (RF, HB, SL) show complete spermatogenic phases, regular cellular organization, typical cellular association and regular interstitial spaces with few tubules displaying atrophy or sloughing. The tubules of the diabetic control (DC) rats show frequent disorganization and the lumen having fewer spermatozoa. Tubules also show epithelium shrinkage, few or absence of germ cells and cellular disorganization. These characteristics are also observed in the seminiferous tubules of diabetic rats receiving the infusions (DRF, DHB, DSL), but are mildly reduced. DT=disorganized tubule, FS=few spermatozoa. A: Micrographs scale bar=100 μm. B: Micrographs scale bar=100 μm; C: The percentage of normal seminiferous tubule; D: The percentage of atrophic tubules; E: The percentage of tubules undergoing sloughing. [a]$P<0.05$ *vs*. the control group.

Furthermore, there was a significant difference in the percentage of histomorphologically normal caudal epididymis between groups (P=0.01). A decrease was observed in the percentage of normal epididymal tubules of the diabetic control group, and they showed a reduction of -23% and -20% in the diabetic control group when compared to the control and vehicle groups, respectively. Although not significant, the percentage of normal epididymal tubules was higher in the diabetic + rooibos, diabetic + honeybush, diabetic + sutherlandia groups when compared to the diabetic control group (23%, 20%, and 10%), respectively (Figure 3A-C). While no significant difference was observed in the percentage of intact caudal epididymis of rats receiving the infusions (rooibos, honeybush and sutherlandia) when compared to the control rats, respectively [(86.25±4.787)%; (91.67±2.887)%; (89.50±10.95)% *vs*. (88.33±1.443)%)].

4. Discussion

Studies have reported alteration in the cellular arrangement and ultimately disruption of the seminiferous tubule in DM[10]. The absence of spermatozoa in the luminal epididymis has likewise been shown after DM induction or when toxins were administered to rodents[33]. The use of antidiabetic drugs such as metformin, pioglitazone and sitagliptin have been shown to confer histopathological changes in the testis and epididymis of rats[15], hence it is pertinent to develop natural strategies in treating DM and its male reproductive complications.

The infusion control groups (rooibos, rooibos and sutherlandia) of the current study presented with normal fasting plasma glucose compared to the control group. The diabetic control rats showed elevated fasting plasma glucose, reduced visceral fat and body weight. Although there was no statistically significant difference in the testicular weight of the diabetic control rats, 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[9,10,34]. After treating diabetic rats with the respective infusions for seven weeks, diabetic + rooibos rats presented with a significant difference in fasting plasma glucose compared to the

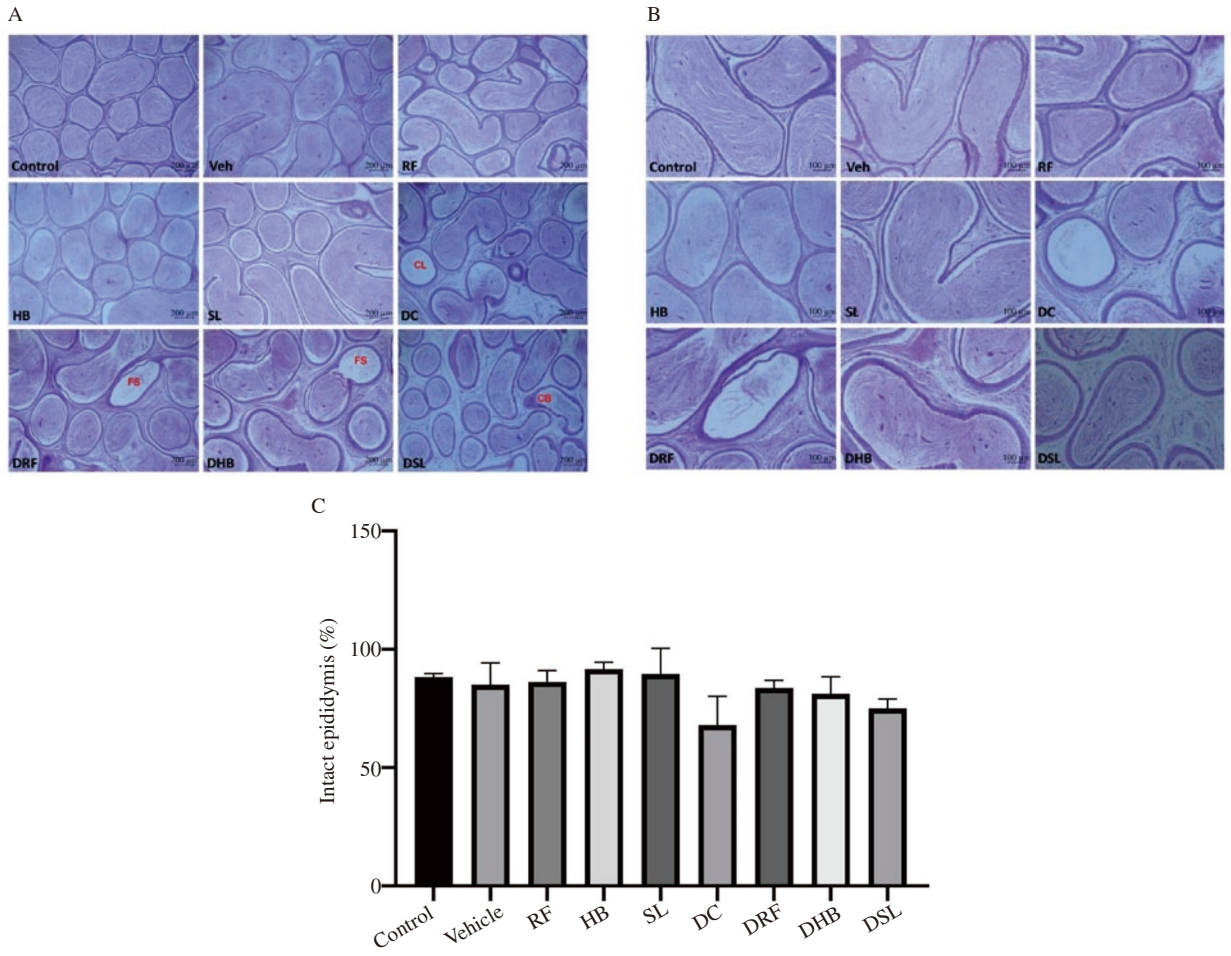

Figure 3. Histology of the cauda epididymis. A and B: Histomorphological overview of the cauda epididymis (AB/PAS stain). The caudal epididymides of the control and infusion control groups (RF, HB and SL) show intactness, as there are limited or no presence of tubules showing hyperplastic modification, excessive accumulation of cells in the interstitium, cytoplasmic shedding in the lumen instead of spermatozoa and alteration of the epithelial organization. The tubules of diabetic control (DC) rats are frequently void of spermatozoa and have cribriform changes. These characteristics are slightly improved in the diabetic rats receiving the infusions (DRF, DHB and DSL). A: Micrographs scale bar=200 µm; B: Micrographs scale bar=100 µm; C: The percentage of normal/intact epididymis. CL=clear lumen, CB=cribriform change, FS=few spermatozoa.

vehicle control group. However, they showed a very mild decrease in fasting plasma glucose compared to the diabetic control group. The extremely mild decrease in the fasting plasma glucose of the diabetic + rooibos rats of the current study is partly supported by studies that reported the hypoglycaemic effect of rooibos, both in *in vivo* and *in vitro* experiments[35,36]. Opuwari and Monsees reported that rooibos had no significant effect on the testicular and epididymal weight of control rats treated for fifty-two days[24]. 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[37]. The diabetic + rooibos rats of the current study showed a reduction in the testicular, epididymal and visceral fat weights. The diabetic + honeybush rats, on the other hand, showed no significant difference in the fasting plasma glucose levels when compared to vehicle groups. When compared to the diabetic control 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[38,39]. Despite the moderate improvement seen in fasting plasma glucose, they still presented with a decrease in body and epididymal weights. Regarding sutherlandia, diabetic + sutherlandia animals showed improved fasting plasma glucose, but their tissue weight remained on the lower side. Several studies have highlighted the hypoglycaemic potential of sutherlandia[40,41], 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[41]. Cumulatively, these infusions displayed a very mild hypoglycaemic potential, but the animals (diabetic + rooibos, diabetic + honeybush and diabetic + sutheralndia) showed reduction in the testicular, epididymal and visceral fat weights. It can be speculated that 1) there is an increased usage of fat for energy and for the formation of cholesterol; 2) there

is a breakdown of the body's protein and amino acid oxidation. Since alteration in the testicular and epididymal morphology observed in DM has been associated with a reduction in hormone levels[42-44], the plasma testosterone concentration of the current study was positively correlated to the testicular weight, epididymal weight and visceral fat weight. These findings further show that these rats had less fat, and plausibly had less cholesterol and thus leading to altered steroidogenesis.

The diabetic control rats 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 diabetic control rats 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 diabetic control rats showed a reduced percentage of normal/intact epididymides. The histopathological changes observed in both the testis and epididymis of the diabetic control rats of the current study are in agreement with Arikawe *et al* who reported altered seminiferous tubule morphology in diabetic rats[45]. 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[10].

Coming to the infusion treated groups, the diabetic + rooibos and diabetic + sutherlandia rats 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 diabetic + honeybush rats presented with a mild increase (6%) in plasma testosterone concentration with improved histo-morphology of the seminiferous tubules and epididymis. Although mechanism 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.

In conclusion, the current study evaluates the histomorphology of the testis and cauda epididymis after treating both healthy and diabetic rats with rooibos, honeybush, and sutherlandia. The infusion control groups (rooibos, honeybush and sutherlandia) show normal seminiferous tubule cellular association, presence of spermatozoa in the epididymal lumen, and have normal overall architecture. Both the testicular and epididymal morphology are altered in DM, but these disruptions are mildly ameliorated by rooibos, honeybush, and sutherlandia even at varying concentrations. Hence, it can be concluded that 1) DM does have detrimental effects on the histomorphological architecture of the testis and epididymis, 2) the individual control infusions have no obvious adverse effect on the architecture of the testis and epididymis, and 3) the infusions mildly improve the histomorphology of the testis and epididymis in diabetes. Since this represents one of the few studies reporting these effects, further investigations are required regarding the mechanisms involved in this process.

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 be advised to investigate the effects during longer studies. 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, to 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 components of the herbal teas should be carried out in future studies. Since people tend to consume diverse teas simultaneously, a group mimicking this phenomenon maybe included in future studies.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Authors' contributions

Temidayo S. Omolaoye conceptualized, curated, and analyzed the data, interpreted the results, drafted, and edited the manuscript. Stefan S. du Plessis supervised, and conceptualized the project, interpretated the results, edited and reviewed the manuscript.

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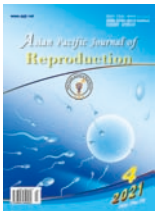

Original Article

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Descriptive histomorphological evaluation of the testis and caudal epididymis following treatment with rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) and sutherlandia (*Lessertia frutescens*) in healthy and streptozotocin-induced diabetic rats

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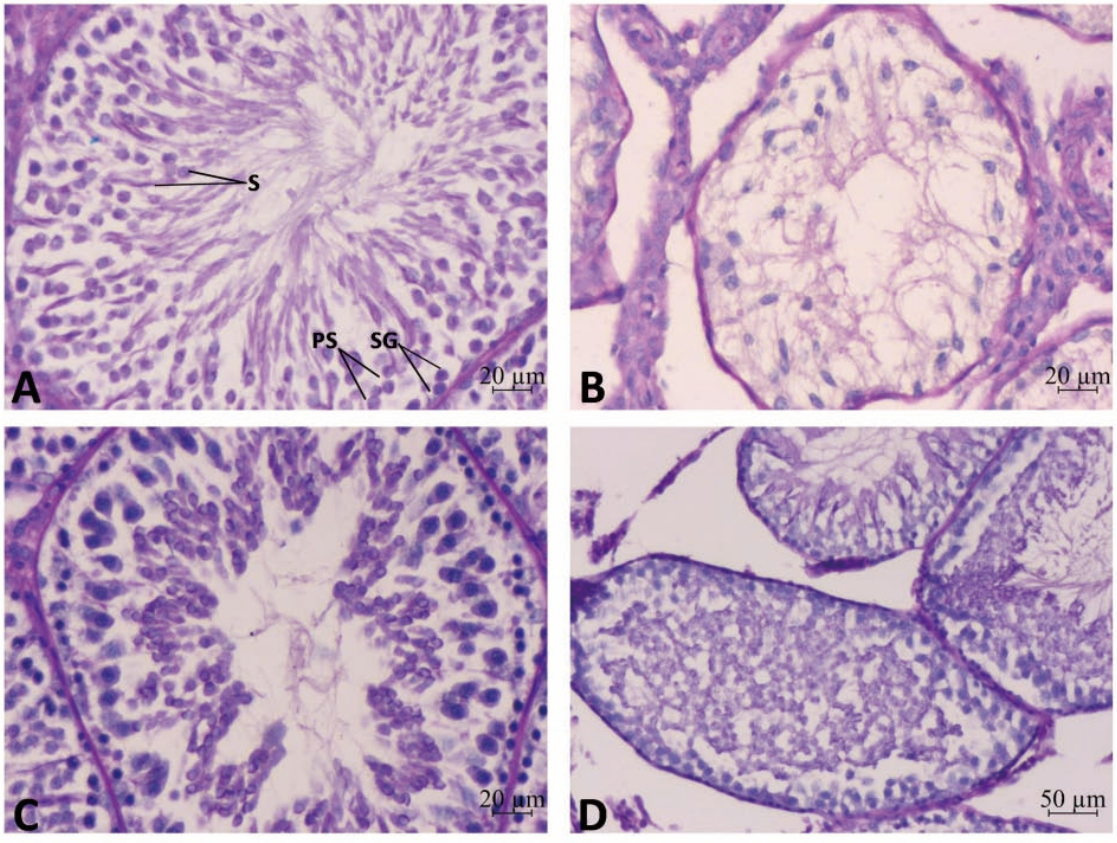
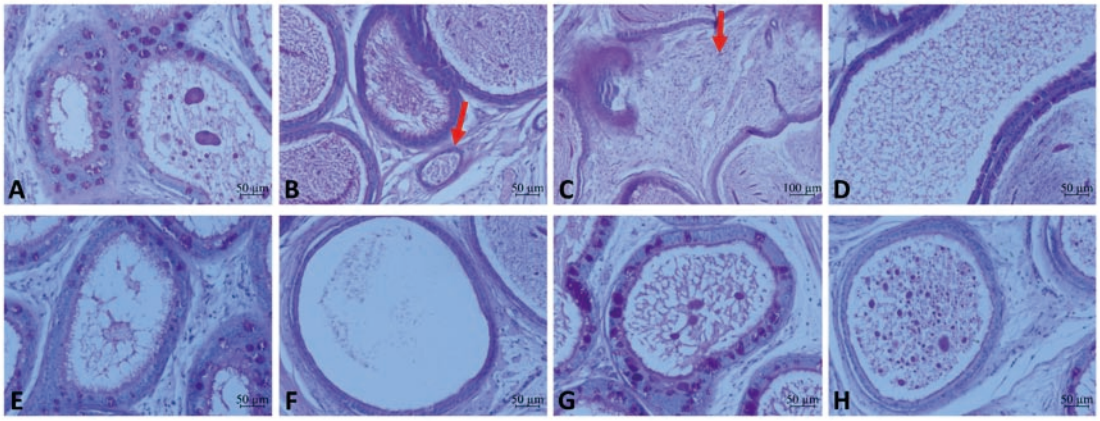
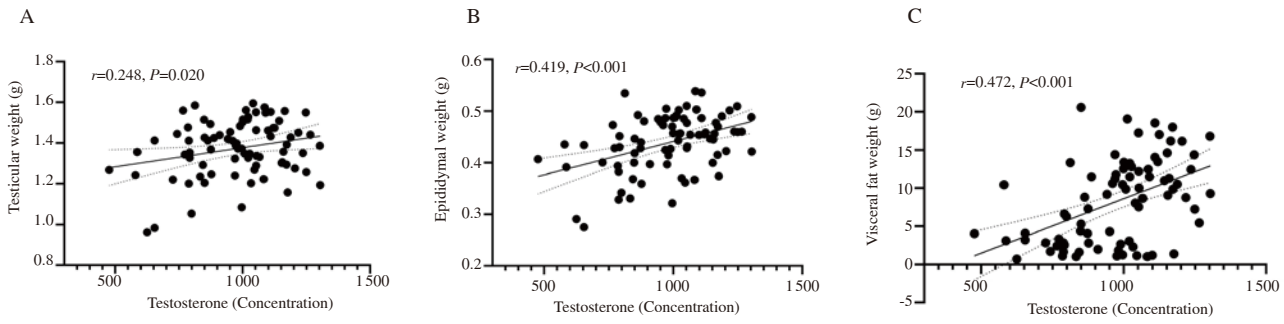
Supplementary Materials



Supplementary Figure 1. Classification for the seminiferous tubules (AB/PAS stain; A-C: the scale bar 20 μm; D: the scale bar 50 μm). A: Normal (complete spermatogenic phases, regular cellular organization, normal cellular association and regular interstitial spaces); B: Atrophic (epithelium shrinkage, few or absence of germ cells and cellular disorganization); C and D: Sloughy (presence of immature accumulation of cells in the lumen and absence of some or all of the spermatogenic phases). SG: spermatogonia, PS: primary spermatocytes, S: spermatids.



Supplementary Figure 2. Illustration of the cauda epididymal abnormalities (AB/PAS stain; the scale bar: 50 μm). A: Cribiform changes (hyperplastic modification of the epithelium, *e.g.* infolding of the epithelium); B and C: Inflammatory infiltrate (excessive accumulation of cells in the interstitium); D and E: Debris in the lumen (presence of cytoplasmic shedding in the lumen instead of spermatozoa); F: Clear lumen (no spermatozoa in the lumen); G: Epithelium disruption (altered epithelium organization); H: Cellular accumulation in the lume.



Supplementary Figure 3. Correlations between plasma testosterone concentration and testicular weight (A), epididymal weight (B), and visceral fat weight (C).