EFFECTS OF ROOIBOS (*Aspalathus linearis*) ON THE TESTICULAR PARENCHYMA OF ADULT BALB/C MICE SUBMITTED TO A HYPERCALORIC DIET

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Abstract
Oxidative stress due to obesity plays a detrimental role in the testicular microenvironment and sperm parameters. We explored the impact of a hypercaloric diet in male BALB/c mice as a condition to trigger damage to the spermatogenic process and the antioxidant effect of *Aspalathus linearis* as well. We used a hypercaloric diet in animals divided into 3 groups: Control, Hypercaloric diet control (HC) and Hypercaloric diet and Rooibos infusion (HCR). Morphometric parameters, enzyme dosages, cell viability, and tubular histopathology were evaluated. Body weight increased in HCR animals at weeks 3, 4, and 8. We found a reduction in seminiferous epithelium height, with an increase in the tubular diameter of the HCR group. Catalase levels were lower in HC and HCR, while carbonyl protein was decreased in HC. We estimate that it induces oxidative stress (OS) capable of affecting the seminiferous epithelium and that the infusion of *A. linearis* does not demonstrate a potential benefit in cell preservation.

Keywords: High fat diet. Obesity. Oxidative stress. Reproduction. Testis.

1. Introduction

Several studies address the impairment of the male reproductive system due to obesity (Kefer 2009; Salas-Huetos 2017; Leisegang 2019), stating that the increase in infertility is as a result of the prevalence of obesity in male subjects (Leisegang 2019). Excess visceral adipose tissue in obese individuals causes changes in hormone levels and promotes a chronic inflammatory state in the male reproductive tract, physiologically harming sperm during both spermatogenesis and sperm maturation in the epididymis, reducing sperm quality and increasing the risk of male infertility (Liu 2017).

Several mechanisms relate the negative effects of obesity on fertility, such as the production of adipokines by adipose tissue, inducing severe inflammation and oxidative stress in the male reproductive tract. In particular, reactive oxygen species (ROS) play a fundamental role in sperm maturation, fertilization capacity and sperm parameters. Although indispensable, excess ROS production require antioxidant defense mechanisms to counteract this dysregulation. Oxidative stress is characterized by systemic imbalance with exacerbated production of ROS by the body. Evidence suggests that oxidative stress is

related, regardless of etiology, to male infertility. About 30 to 80% of infertile men have high levels of reactive species in the semen (Wagner 2017; Agarwal 2019; Dutta 2019).

The administration of antioxidants is one of the methods to counteract oxidative stress side effects in the reproductive tract. Recently, the interest in the performance of various natural and commercial products to prevent and treat several diseases has increased, due to the antioxidant characteristics of their main components. In this context, the species *Aspalathus linearis*, popularly known as Rooibos, stands out. Recent studies demonstrate the beneficial effects of using Rooibos tea, owing to its effectiveness in lowering the levels of blood lipid markers (Uličná et al. 2006; Awoniyi 2012; Sasaki 2019).

Therefore, considering that obesity generates metabolic and inflammatory impairment in the body and in the reproductive tract, especially in the testicular parenchyma, we seek to investigate how the harmful effects caused by obesity could be attenuated. This study aimed to confirm the harmful potential of the hypercaloric diet on the spermatogenic process, as well as to determine whether the administration of the species *A. linearis* is able to attenuate or minimize the effects of oxidative stress in the male reproductive tract.

2. Material and Methods

Experimental groups

Adult male BALB/c mice (5-7 weeks of age, 22 - 23 g) were kept in the animal facility of the Biochemistry Department of the Federal University of Triângulo Mineiro (UFTM) for eight weeks, with controlled temperature (22 ± 2°C) and light cycle (12/12h dark/light cycles). The animals were divided into three groups (n=8): Control (normocaloric diet and water), HC (hypercaloric diet and water) and HCR (hypercaloric diet and Rooibos) for 8 weeks. All groups had free access to food and water supply except the HCR group, which received the infusion as the only water source. The administered diet and the water were introduced together from the beginning to the end of the experiment. The normocaloric diet (4.0 kcal/g) contained 65.8% carbohydrates, 3.1% fat and 31.1% protein. The HC diet was composed of 45% condensed milk, 10% refined sugar, and 45% chow diet. The macronutrient composition of the chow diet (4.0 kcal/g) was 65.8% carbohydrate, 3.1% fat, and 31.1% protein, while the HC diet (4.4 kcal/g) was 74.2% carbohydrate, 5.8% fat, and 20% protein. It is important to note that the HC diet contained at least 30% refined sugars, mostly sucrose (Oliveira et al. 2013).

Body weight was measured every week, while water intake and food supply were daily measured. The commercial infusion of Rooibos was prepared following Ulična et al. (2006). All experimental procedures were evaluated and approved by the Ethics Committee for the Use of Animals at UFTM (CEUA-UFTM) (protocol 11/2018/CEUA/PROPPG - number: 23085.002424/2018-59).

Tissue harvesting and processing

The animals were euthanized and dissected after intramuscular anesthesia, applied to the left quadriceps with a mixture of xylazine (10 mg/kg) and ketamine (100 mg/kg) considering the weight of each animal. Left vas deferens were removed, frozen in a cryovial and stored at -20 ˚C for subsequent sperm analysis. Testicular weight was measured after animals were euthanized and the organ was removed.

The left testes were frozen in liquid nitrogen and cryopreserved at -80ºC, for further enzymatic analysis of oxidative stress. The right testes were fixed in 2.5% glutaraldehyde solution in 0.1mol/L sodium phosphate buffer, pH 7.2 for 24 h, for histological analysis. Briefly, the material was routinely dehydrated in ethanol and embedded in glycolmethacrylate (Historesina®), according to the manufacturer's recommendations. Histological sections with 3 µm of thickness were made in an automatic microtome (Leica® Jung RM2055), respecting the interval of 40 µm between sections. Histological sections were stained with toluidine blue/1% sodium borate for morphological and testicular morphometric analysis.

Testis biometry, morphometry and stereology

The gonadosomatic index (GSI%) was obtained by dividing the testicular weight by the body weight multiplied by 100 (Melo 2010). The mean seminiferous tubule diameter was obtained after measuring 10 cross-sections of random seminiferous tubules from each animal. The height of the seminiferous epithelium was measured in the same tubular sections as the tubule diameter was obtained (as the average of two diametrically opposite measurements). The areas of the seminiferous tubule (STAr), lumen (LAr) and epithelium (EAr) were estimated according to the formulas: $STAr = \pi TR^2$ (TR = tubular radius*); LAr* $= \pi LR^2$ (LR = luminal radius); *EAr = STAr - LAr*. The epithelial/tubule ratio (ETR) ratio was calculated using the formula: *RET = EpAr/STAr*.

The volumetric proportions (%) of the testicular parenchyma components were analyzed using an orthogonal grid with 357 intersection points placed on the testicular parenchyma digital images (100X magnification). The number of intersections located over the interstitium and the seminiferous tubules were counted, determining the proportion of each component in 10 images per animal. All morphometrical analysis were made using the ImageJ software.

Germ and Sertoli cells count

Germ cell's populations that constitute the seminiferous tubules in Stage VII/VIII of the seminiferous epithelium cycle were counted according to Oliveira et al. (2013) and corrected for the mean nuclear/nucleolar diameter, as described by Amann and Almquist (1962). Populations of type A spermatogonia (GoA), primary spermatocytes in leptotene/pre-leptotene (SPTL) and pachytene (SPTP), round spermatids (SPDAr) and Sertoli cells (SC) were quantified in 10 cross sections of the tubule seminiferous per animal.

In order to evaluate the efficiency of the spermatogenic process and of the Sertoli cells, the ratios between the germ cell's populations, as well as between germ cells and Sertoli cells were estimated. The following ratios were used: spermatogonial mitosis efficiency coefficient (*SPTL/GoA*); general spermatogenesis yield (*SPDAr/GoA*); meiotic index (*SPDAr/SPTP*); Sertoli cell index (*SPDAr/SC*); Sertoli cell's total support capacity (*GoA + SPTL + SPTP + SPDAr/SC*).

Sperm morphology

Cryopreserved sperm, contained in one centimeter of the vas deferens adjacent to the epididymis, were collected and resuspended in a 0.1 M sodium phosphate buffer solution, pH=7, for 15 minutes. One drop of the solution was placed on a slide and covered with a cover slip. The slide was immediately observed under a light microscope, with phase contrast, in a 40X objective. Two hundred sperm from each animal were evaluated to verify the presence of abnormalities in the head and tail (Seed et al. 1996).

Oxidative stress analysis

Enzyme assays for oxidative stress were performed from testicular samples kept at -80°C. Each testis was weighed and homogenized in 1000 µL of a 20 mM potassium phosphate buffer solution (pH 7.4) and centrifuged at 12000 rpm, 4 ºC, for 10 minutes. After centrifuging the homogenate, this material was fractionated and the supernatant and pellet stored separately in cryovials at -20 ºC. The supernatant was used to measure nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST), malondialdehyde (MDA) and total protein levels. The pellet formed was resuspended in 10% trichloroacetic acid for subsequent dosage of carbonyl protein. Duplicates were made for all samples. All enzyme activities were determined in duplicate using a spectrophotometer (UV-Mini 1240, Shimadzu).

CAT activity was evaluated according to the method described by Aebi, where the decomposition rate of H_2O_2 was measured at 0, 30 and 60 seconds. SOD activity was determined by using the supernatants according to the method described by Del Mestro and McDonald (1984), while glutathione S-transferase (GST) levels were determined by the formation of conjugated glutathione-2,4-dinitrobenzene

(CDNB). Malondialdehyde (MDA) levels were determined using TBARS solution (15% trichloroacetic acid / 0.375% thiobarbituric acid / 0.25 M hydrochloric acid). The total MDA levels in each sample were determined by using a standard curve from known concentrations of 1,1,3,3-tetramethoxypropane (TMPO) (Rosengren and Shertzer 1993; Wallin 1993). The concentration of nitric oxide (NO) was indirectly determined through the content of nitrite/nitrate by the Griess reaction (Ricart-Jané et al. 2002). Total protein was measured using bovine serum albumin as standard curve (Lowry 1951) and used for homogenization of oxidative stress data.

Germ cell viability

Semi-serial histological sections (1 µm) of the seminiferous tubules were stained with acridine orange (AO) and propidium iodide (PI). Digital images were used to assess viability and cell morphology changes. Ten fields showing seminiferous tubules were analyzed per region and per animal (Giri and Roy 2016; Dias et al. 2019, 2020). The cell classification was based on the fragmentation and condensation of the cell nucleus, along with the integrity of the membrane, according to double fluorescent labeling by acridine orange and propidium iodide. The histomorphological criteria for classification of cell viability indicated that viable cells have a green nucleus, which makes their recognition easily noticeable. Dead cells show color ranging from yellow to orange in agreement with the estimate of loss of membrane integrity indicated by labeling with propidium iodide. Acridine orange demonstrates potential in staining both viable cells and cells dying, whereas propidium iodide reveals only cells that show loss of cell integrity (Dias et al. 2019; Giri and Roy 2019).

Histopathology analysis

The proportions of normal and pathological seminiferous tubules were estimated by counting 200 random tubules in histological preparations from the testes of each animal. According to Johnsen (1970), there are 10 levels of alteration in the seminiferous tubule. In our study, the scores were modified to eight levels: Level 1 - intact tubules with germ cells arranged in their normal location and few vacuoles; Level 2 - vacuoles at the base of the epithelium; Level 3 - vacuoles at the apex of the epithelium; Level 4 - vacuoles at the apex and base of the epithelium; Level 5 - spermatogenic cells in tubular lumen and cell degeneration; Level 6 - tubules with only basal cells; Level 7 - tubules with only Sertoli cells; Level 8 - tubules without Sertoli or germ cells, characterizing a state of irreversible degeneration. These levels were grouped into normal (Level 1), mild pathologies (Levels 2 and 3), moderate pathologies (Levels 4 and 5), and severe pathologies (Levels 6, 7 and 8), as described by Dias *et al.* (2019).

Statistics

Statistical analyzes were performed using GraphPad Prism Software version 5.00.288. Data were analysed using the Shapiro-Wilk t testo assess normality. For parametric data, we used ANOVA followed by Tukey test. For non-parametric data, the Kruskal-Wallis test was used. The significance level was defined as 5%.

3. Results

Food/water consumption and caloric intake

During the eight weeks of treatment, both food intake (Figure 1A) and energy intake (Figure 1B) were significantly higher among mice from groups C and HCR ($p<0.001$) and from groups HC and HCR ($p<0.05$). Food consumption in the HC group, however, was not different from that in the control group, although caloric intake was higher ($p<0.001$). Water consumption in the HCR group was higher than in the HC group ($p<0.001$), with the same diet, while water consumption of the HC group was lower than the control ($p<0.001$) (Figure 1C).

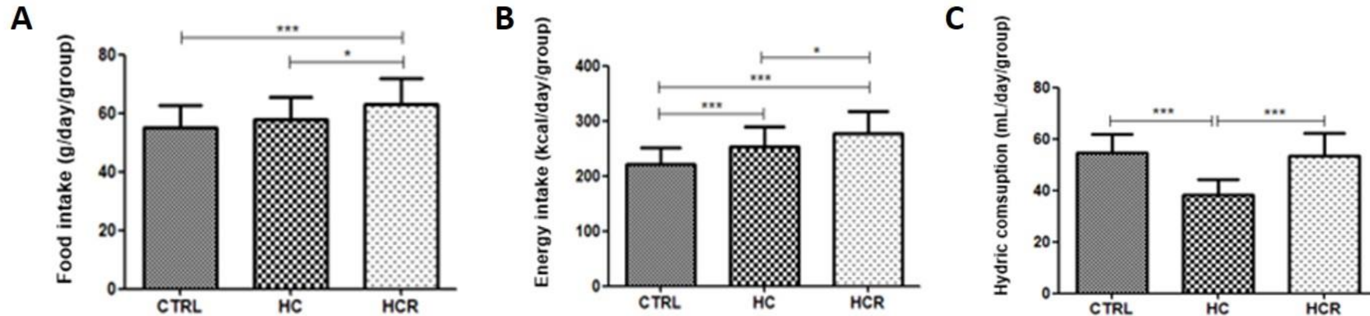

Figure 1. Food and water intake after the hypercaloric diet intake and after *Aspalathus linearis* treatment. A - Daily food intake (g/day); B - Daily energy intake (kcal/day); C - Daily hydric consumption (mL/day). Treatment time = eight weeks. Data are mean ± SD. *p<.05, ***p<.001. CTRL= control, HC= hypercaloric diet, HCR= hypercaloric diet+Rooibos.

Body and testicular weight

Animals fed hypercaloric diet and treated with Rooibos infusion gained weight compared to the other groups at weeks 3, 4 and 8, respectively (Figure 2A). Despite the increase in body weight, the testicular weight (Figure 2B) and the GSI (Figure 2C) were not altered.

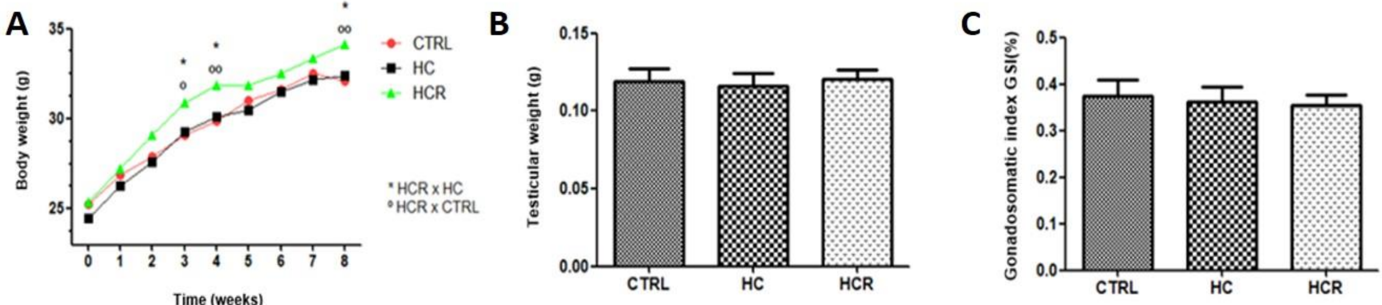

Figure 2. Body and testis weights. A - Weekly weight gain of animals treated with a normocaloric and hypercaloric diet, with or without Rooibos. B - Testicular weight. C - gonadosomatic index. Data are mean ± SD. *p<0.05. CTRL= control, HC= hypercaloric diet, HCR= hypercaloric diet+Rooibos. GSI= gonadosomatic index.

Testicular morphometry and stereology

No statistical differences were found in the morphometric and stereological measurements of the seminiferous tubules (Table 1, Fig. 3).

Table 1. Seminiferous tubules morphometry.

Seminiferous tubule	Control	HC	HCR
Tubule diameter (µm)	223.0 ± 11.56	226.2 ± 11.64	228.5 ± 0.78
Lumen diameter (µm)	63.57 ± 16.63	67.63 ± 23.46	81.59 ± 9.55
Epithelium height (µm)	79.70 ± 11.25	79.30 ± 17.14	74.75 ± 4.23
Tubule area (µm^2)	39136 ± 4045	40285 ± 4129	41024 ± 281.40
Lumen area (µm^2)	3364 ± 1711	3952 ± 2320	5286 ± 1249
Epithelium area (µm^2)	35772 ± 4688	36332 ± 6316	36678 ± 1894
ETR	0.92 ± 0.04	0.90 ± 0.07	0.87 ± 0.03
IVP (%)	9.29 ± 2.41	7.38 ± 0.94	7.94 ± 1.43
TVP (%)	90.71 ± 2.41	92.62 ± 0.94	92.06 ± 1.43

[1](ETR) Epithelium/tubule ratio; (IVP) Interstitial volumetric proportion; (TVP) Tubule volumetric proportion. Values are mean ± SD (ANOVA and t test, p<0.05). HC= hypercaloric diet, HCR= hypercaloric diet+Rooibos.

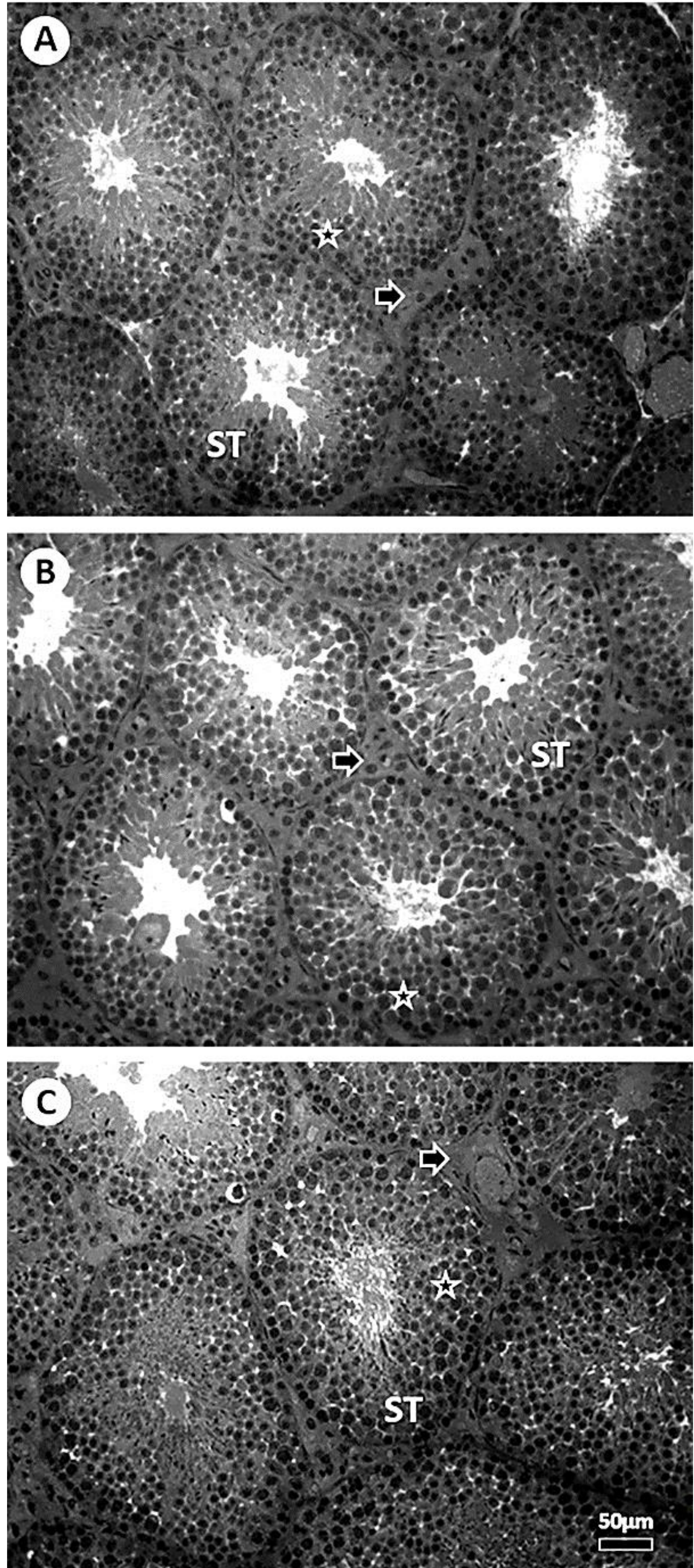

Figure 3. Testicular parenchyma showing the seminiferous tubules (ST), the interstitium (arrow) and the germinal epithelium of all experimental groups. A – control; B - hypercaloric diet, and C - hypercaloric diet + roiboos. No significant changes were noticed in the seminiferous tubules and interstitium. Bar = 50µm.

Germ and Sertoli cell counts

The number of spermatocytes in pre-leptotene/leptotene (SPTL) was increased in the HC and HCR groups ($p<0.01$). The mitotic index was significantly higher in the HC ($p<0.05$) and HCR ($p<0.01$) groups (Table 2).

Table 2. Germ and Sertoli cells count by seminiferous tubules cross-sections in stage VII/VIII of the seminiferous epithelium, as well as the ratios between cell populations.

Parameters	Control	HC	HCR
Spermatogonia	0.85 ± 0.14	0.68 ± 0.12	0.62 ± 0.21
Spermatocyte in pre-leptotene/leptotene	11.49 ± 0.89 [b]	13.98 ± 1.26 [a]	12.99 ± 1.53 [ab]
Spermatocyte in pachytene	8.25 ± 0.83	9.15 ± 1.71	8.26 ± 1.42
Round spermatid	18.38 ± 2.08	20.16 ± 3.26	18.60 ± 2.52
Sertoli Cell	2.60 ± 0.36	3.05 ± 0.53	2.45 ± 0.52
Spermatogenic yield	21.83 ± 2.82	30.21 ± 6.88	32.81 ± 12.73
Mitotic index	13.70 ± 1.93 [a]	21.17 ± 5.12 [b]	22.19 ± 4.81 [b]
Meiotic index	2.24 ± 0.32	2.23 ± 0.30	2.33 ± 0.60
Sertoli cell index	7.19 ± 1.46	6.86 ± 1.99	8.01 ± 2.62
Sertoli support capacity	15.21± 2.60	14.94 ± 3.83	17.18 ± 3.92

[1] Values are mean ± SD. Different superscripts ([a,b]) are $p<0.05$ (ANOVA and t test, n=6) HC= hypercaloric diet, HCR= hypercaloric diet+Rooibos.

Sperm morphology

There were no significant changes in sperm morphology (Table 3).

Table 3. Sperm morphology.

Sperm parameters (%)	Control	HC	HCR
Normal	97.25 ± 0.68	97.92 ± 1.28	97.66 ± 1.47
Tail defects	2.58 ± 0.58	1.75 ± 1.33	1.67 ± 0.87
Head defects	0.17 ± 0.25	0.33 ± 0.60	0.67 ± 0.75

[1] Values are mean ± SD. HC= hypercaloric diet, HCR= hypercaloric diet+Rooibos.

Oxidative stress assay

The levels of carbonylated protein were reduced in HC ($p<0.05$). Furthermore, catalase levels decreased in the HC and HCR groups ($p<0.01$) when compared to the control, as well as a decrease in the HCR group compared to the HC group. MDA, ON, SOD and GST levels were not changed (Figure 4).

Germ cell viability

Figure 5 illustrates the assessment of cell viability (%) in histological sections of seminiferous epithelium stained by acridine orange/propidium iodide. No changes on germ cells viability were observed between groups.

Histopathology

No significant pathological alterations were observed in the seminiferous tubules of HC and HCR groups (Figure 6).

4. Discussion

In the present study, we explored the potential impact of the induction of a hypercaloric diet in male BALB/c mice as a condition triggering damage to the testicular seminiferous tubules in order to test the attenuating effect of the plant species *Aspalathus linearis* on testicular cells.

The adoption of controlled hypercaloric diets has been widely used in animal models for inducing the state of obesity as a consequence of higher caloric intake, whether from lipids or carbohydrates (Nascimento et al. 2008; Pereira 2018). In this sense, we observed a relevant acceptance of the hypercaloric refined carbohydrate diet by the treated mice, which might be due to the increase in food consumption between the groups. The inclusion of condensed milk in diets could help to increase food intake (Chu et al. 2014). The consumption of palatable and high-calorie foods is among the most important predisposing factors for obesity in rodents (Velloso 2009; Rosini 2012). On the other hand, there are

studies that reveal that the lower food intake of hypercaloric diets, both lipids and carbohydrates, but with high caloric content, still express important body weight gain (Nascimento et al. 2008; White 2013; Chu et al. 2014; Fan 2015; Funes et al. 2019; Gómez-Elías et al. 2019).

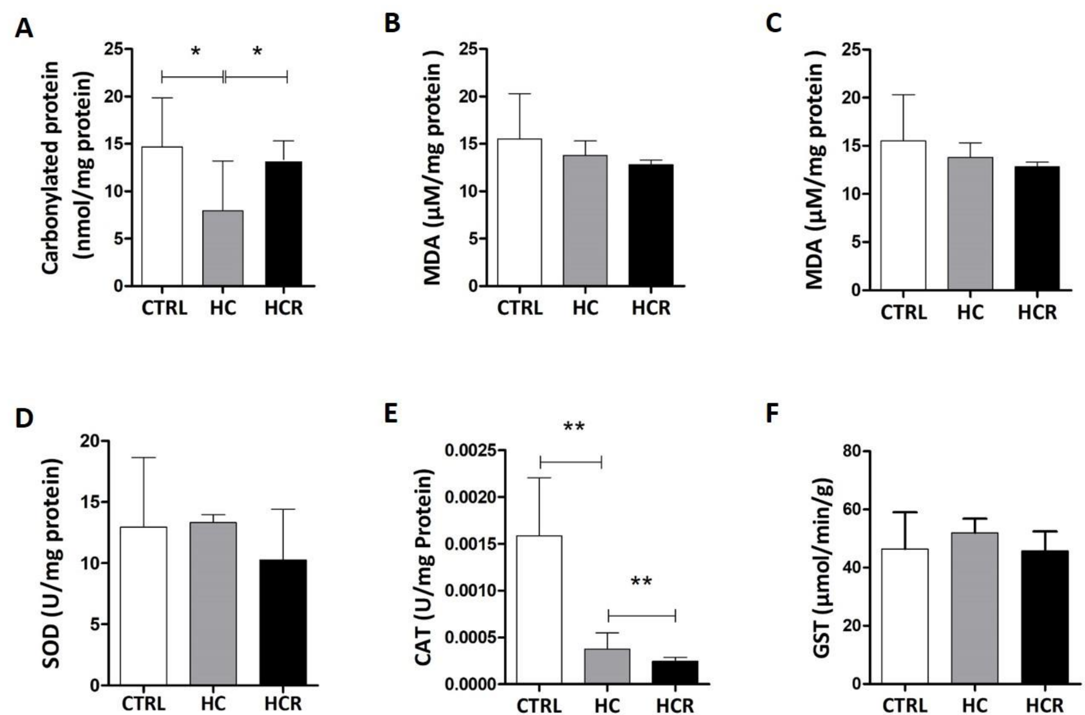

Figure 4. Levels of A - Carbonylated protein; B - Malondialdhyde (MDA); C - Nitric oxide (NO); D - Superoxide dismutase (SOD); E - Catalase (CAT) and F - Glutathione-S-transferase (GST). (Date are mean ± SD, ANOVA and t test, *p<0.05; **p<0.01, n=4) CTRL = Control, HC= hypercaloric diet, HCR= hypercaloric diet+Rooibos.

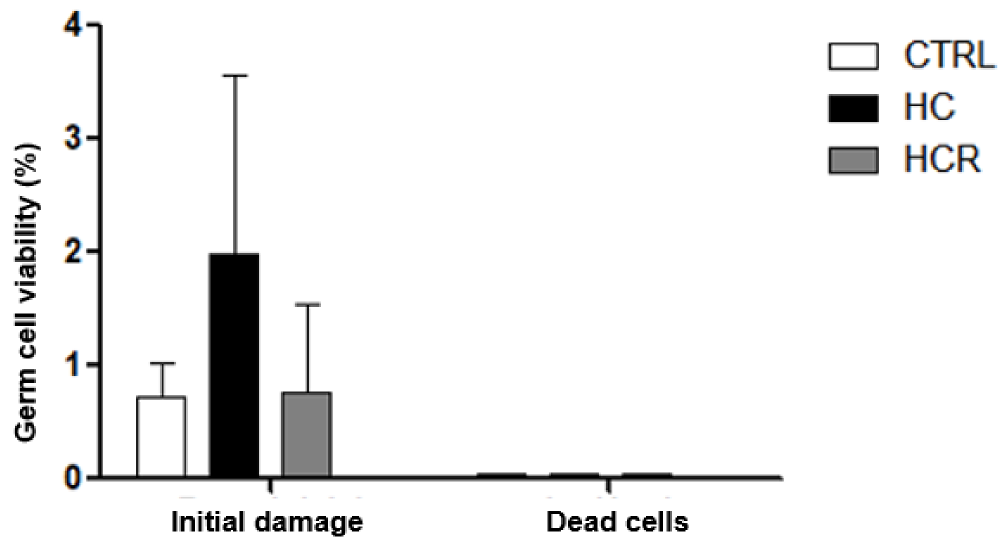

Figure 5. Germ cell viability (%) in histological sections of seminiferous tubule epithelium of BALB/c mice labeled with acridine orange and AO/IP propidium iodide (Date are reported as mean ± standard deviation of the mean, ANOVA and t test) CTRL = Control, HC= hypercaloric diet, HCR= hypercaloric diet+Rooibos.

It is worth mentioning that the energy composition of the diet is also a factor for weight gain in attempts to induce obesity in animal models. Pereira *et al*. (2018) report that the dietary intake of the high-fat diet (42% lipids) was lower than the normocaloric diet consumed by the control group. However, despite the low food intake, the treated animals showed weight gain because the energy density of the high-fat diet was higher. The animal's age at the beginning of the experimental protocol can also interfere with body mass gain, since young animals would convert energy gain into lean mass and not exclusively

into weight gain (Rosini 2012). Gómez-Elías et al. (2019) assume that different strains of mice chosen for the experimental design could influence the response to treatments for inducing obesity and fertility. Questions such as time and energy efficiency of treatments must be analyzed, so that the induction of obesity in rodents is efficient and reflects possible changes. Thus, Funes et al. (2019) indicate that the longer is the treatment period for inducing obesity by diet in rodents, the more the data tend to show significant changes in body weight and cause changes in testicular health. In the present study, there was a significant increase in food consumption and caloric intake. Although food consumption of the HC group was similar to that of the control group, their energy intake was higher, which demonstrates that the diet, by itself, has sufficient caloric intake. The HCR group also showed increased energy intake when compared to the control and HC group. Despite the record of energy efficiency by diet, the body weight of the animals was significantly higher, especially at the end of the treatment for the HCR group. Although such increase in energy intake and body weight has been recorded, testicular weight was not altered over the experimental time, as also described previously by Ribeiro et al. (2017), Asadi et al. (2017) and Gómez-Elías et al. (2019). On the other hand, Oliveira et al. (2013) did not record changes in body weight and food consumption, although the diet was able to expand retroperitoneal adipose tissue.

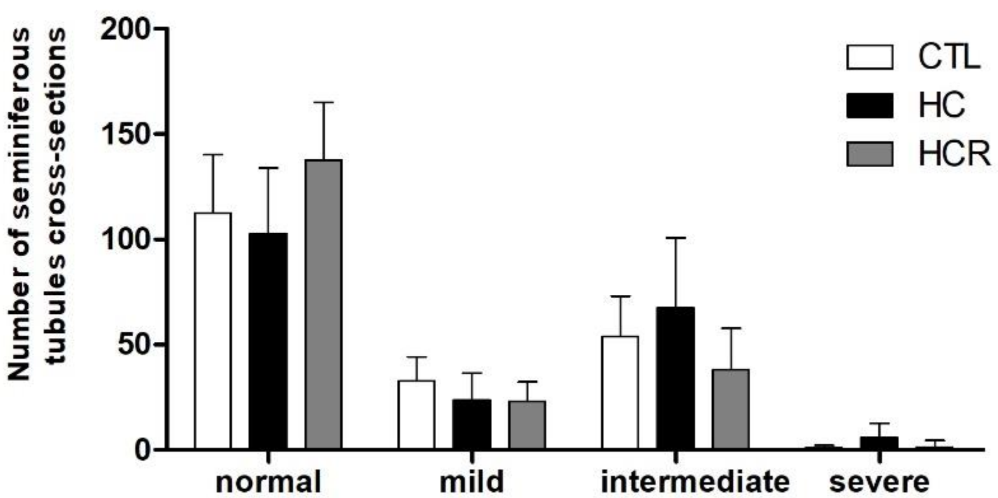

Figure 6. Histopathological evaluation of seminiferous tubules cross-sections, considering alterations within the seminiferous epithelium (mean ± SD, ANOVA and t test). CTRL = Control, HC= hypercaloric diet, HCR= hypercaloric diet+Rooibos.

Considering water consumption, Morton (1983) describes the Rooibos tea as a beverage with a pleasant aroma and a non-astringent taste. We consider that the differences in the amount of volume ingested by the control and HC groups, and between the HC and HCR groups may have been influenced by this characteristic. However, some studies demonstrate that water consumption by animals on normocaloric diets is equivalent to those on a hypercaloric diet (Nascimento et al. 2008; Lin 2016). Even though the increase in water consumption body weight gain was not altered, following the results from Awoniyi et al. (2012). Canda et al (2014) in order to verify the potential attenuating effect of Rooibos in Wistar rats induced to a framework of oxidative stress, they verified that the water consumption of Rooibos was lower than the group that received water as the sole water source. Despite the lower water consumption of Rooibos, the animals' body weight was not different between groups. Thus, we assumed that Rooibos intake would not imply to increase body mass.

Testicular weight and volume are often used to assess testicular development. As in our study Opuwari and Monsees (2014) did not find significant changes in testis weight and body weight after treatment with Rooibos (fermented) at a concentration of 2% and 5%. However, the authors believe that prolonged consumption of Rooibos could generate subtle structural changes in the male reproductive system. Thus, despite the increase in food and caloric intakes, the extension of the treatment period would possibly induce considerable increases in body and testicular weights. In addition, there is great variation

in the literature regarding the period that best describes an obesity condition and the type of diet administered (Rosini 2012; Mathijs 2014; Pereira 2018). Yuan et al. (2014) report that the decrease in testicular weight and volume is accompanied by hyperleptinemia, which is harmful to the testicular structure. In their study, they assume that the exposure time to the diet was not long enough to cause changes in body weight, nor in testicle weight. Kenagy and Trombulak (2016) and Lozi (2019) relate the size of the testes to the reproductive physiology of males and attest that testicular biometry can provide information that inferred changes in the rate of sperm production.

The GSI showed no significant changes between groups, which can be explained by the maintenance of testicular weight, because even with the increase in body weight, this proportionally did not reflect the increase in testicular weight. Mu *et al.* (2016) demonstrated that male Sprague-Dawley rats maintained on high-calorie diets for a period of eight weeks presented a reduction in GSI and testicular weight, indicating alterations in spermatogenesis. In addition, the authors cite atrophy in the diameter of the seminiferous tubule, predictive of the loss of sperm production capacity. Species whose testes have a high proportion of seminiferous tubules produce more sperm per mass unit (Hess and De Franca 2008).

The stereology of the seminiferous tubules shows us the existence of two distinct populations of cells that make up the seminiferous epithelium: germ cells and Sertoli cells (Karl and Capel 1998). It is known that obesity can cause damage to testicular cells causing changes, mainly, in the structure of the seminiferous epithelium in human and animal models (McPherson and Lane 2015). Our results indicate a decreasing trend in epithelial height and ETR (epithelium/tubule ratio) in the HCR group, with an increase in the lumen diameter. Although germ cell quantity and tubular diameter are related parameters, the decrease in the number of cells would not necessarily result in a decrease in the height of the epithelium, which would be possible due to cell rearrangement and increased intercellular distance (Gholami and Ansari 2015; De Oliveira 2020).

The determination of spermatogenesis efficiency can be assessed by counting the germ cell population. The number of primary spermatocytes in pre-leptotene was increased due to the hypercaloric diet intake, thus increasing the mitotic index. However, such increase was not reflected in an increase of the spermatogenic yield, nor in the numbers of other cells present in the adluminal epithelial space, including Sertoli cells. Sertoli cells regulate sperm production and testicle size as they support limited numbers of germ cells (Holsberger 2005; Mäkelä et al. 2019). According to Opuwari and Monsees (2014), long-term Rooibos intake would be harmful to sperm, which was not observed in the present study.

Even though the morphological and morphometrical analyses did not show important testicular alterations, the oxidative stress analyses showed reduction in carbonyl protein levels due to the hypercaloric diet intake. The dosage of carbonylated protein is often used as a marker of protein oxidation, being an indicator of the oxidant/antioxidant balance within the tissues (Gonçalves et al. 2012). On the other hand, MDA levels did not indicate changes between groups, which points to the absence of lipid damage to germ cell membranes, indicating that the treatments did not induce the generation of a sufficient amount of lipid peroxides capable of changing the structure of the plasma membranes of such cells. Awoniyi et al. (2012) reveal that the polyphenols present in Rooibos bind to lipid peroxides and, in this way, prevent the lipid peroxidation cascade. With the increased production of reactive oxygen species, there is activation of antioxidant enzymes (SOD, CAT and GSH, in that order) to reduce the amount of reactive oxygen species. SOD catalyzes the reaction of converting the superoxide radical to H_2O_2 (Barbosa 2010), while CAT converts H_2O_2 to oxygen and water (Bansal 2011). Both groups treated with the hypercaloric diet showed significant reductions of CAT levels, which may indicate that this enzyme reached exhaustion and its activity maintained normal to low levels of MDA and carbonyl protein. Thus, Rooibos was not able to prevent CAT exhaustion induced by the hypercaloric diet.

GST, in turn, is responsible for cell detoxification (Habig and Jakoby 1974) and activated after CAT depletion. GST activity was not changed between experimental groups. Thus, we believe there is an increase in ROS and that the first line of antioxidant defense was not enough to contain the increases in reactive oxygen species. The activation of the second line of defense naturally occurs to contain ROS increase, however, CAT, even reaching exhaustion, was capable of preventing lipid and protein damage in sperm membranes and in the testicular microenvironment, explainable by GST levels. Such assumption corroborates the fact that germ cell viability was not altered due to hypercaloric and/or Rooibos intake. In

addition, no significant histopathological alterations were noticed due to Rooibos and/or the hypercaloric intake, corroborating the germ cells analysis previously discussed.

5. Conclusions

The hypercaloric diet proposed for eight weeks was able to induce changes in the levels of carbonylated protein and catalase, indicating the beginning of the testicular oxidative stress process. However, such alterations were not able to induce significant changes in the testicular parenchyma, or even in the viability of cell germ cells. Furthermore, despite not causing testicular damage, the infusion of Rooibos at the concentration used in this work, failed to reverse the action of the hypercaloric diet on the exhaustion of the catalase enzyme. Further studies with a long-term intake of both Rooibos and the hypercaloric diet might be performed to verify the possible harmful effects of the hypercaloric diet over the testicular germ cells.

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References

AGARWAl, A., et al. Male oxidative stress infertility (MOSI): Proposed terminology and clinical practice guidelines for management of idiopathic male infertility. World Journal of Men's Health. *Korean Society for Sexual Medicine and Andrology.* 2019, **37**, 296–312. https://doi.org/10.5534/wjmh.190055

ASADI, N., et al. The impact of oxidative stress on testicular function and the role of antioxidants in improving it: A review. *Journal of Clinical Diagnostic Research*. 2017, **11**(5), 1-5. https://doi.org/10.7860/jcdr/2017/23927.9886

AWONIYI, D.O., et al. The effects of Rooibos (*Aspalathus linearis*), green te (*Camelli sinensis*) and commercial Rooibos and green tea supplements on epididymal sperm in oxidative stress-induced rats. *Phytotherapy Research*. 2012, **26**(8), 1231–1239. https://doi.org/10.1002/ptr.3717

BANSAL, A.K. and BILASPURI, G.S. Impacts of Oxidative Stress and Antioxidants on Semen Functions. *Veterinary Medicine International*. 2011, **2011,** 1–7. https://doi.org/10.4061/2011/686137

BARBOSA, K.B.F., et al. Estresse oxidativo: Conceito, implicações e fatores modulatórios. *Revista de Nutrição*. 2010, **23**(4), 629–643. https://doi.org/10.1590/S1415-52732010000400013

CANDA, B.D., OGUNTIBEJU, O.O. and MARNEWICK, J.L. Effects of consumption of Rooibos (Aspalathus linearis) and a Rooibos-derived commercial supplement on hepatic tissue injury by tert -butyl hydroperoxide in wistar rats. *Oxidative Medicine and Cell Longevity*. 2014, **2014**, 9. https://doi.org/10.1155/2014/716832

CHU, H.F., et al. Variations in the efficacy of resistant maltodextrin on body fat reduction in rats fed different high-fat models. *Journal of Agriculture and Food Chemistry*. 2014, **62**(1), 192–197. https://doi.org/10.1021/jf404809v

DE OLIVEIRA, C.F.A., et al. Comparative testis structure and function in three representative mice strains. *Cell and Tissue Research*. 2020, **382**(2), 391–404. https://doi.org/10.1007/s00441-020-03239-0

DIAS, F.C.R., et al. Hydroalcoholic extract of *Pfaffia glomerata* alters the organization of the seminiferous tubules by modulating the oxidative state and the microstructural reorganization of the mice testes. *Journal of Ethnopharmacology*. 2019, **233**, 179–189. https://doi.org/10.1016/j.jep.2018.12.047

DIAS, F.C.R., et al. How bad is brazilian ginseng extract for reproductive parameters in mice? *Histology and Histopathology*. 2020, **35**(10), 1135–1149. https://doi.org/10.14670/hh-18-244.

DUTTA, S., MAJZOUB, A. and AGARWAL, A. Oxidative stress and sperm function: A systematic review on evaluation and management. *Arabian Journal of Urology*. 2019, **17**(2), 87–97. https://doi.org/10.1080/2090598X.2019.1599624

FAN, Y., et al. Diet-induced obesity in male C57BL/6 mice decreases fertility as a consequence of disrupted blood-testis barrier. *PLoS One*. 2015, **10**(4), 1–15. https://doi.org/10.1371/journal.pone.0120775

FUNES, A., et al. A chronic high-fat diet causes sperm head alterations in C57BL/6J mice. *Heliyon*. 2019, **5**(11). https://doi.org/10.1016/j.heliyon.2019.e02868

GHOLAMI, S., ANSARI-LARI, M. and KHALILI, L. Histologic and histomorphometric changes of testis following oral exposure to methyl tertiary-butyl ether in adult rat. *Iranian Journal of Veterinary Research*. 2015, **16**(3), 288–292.

GIRI, B.R. and ROY, B. *Cysticercus fasciolaris* infection induced oxidative stress and apoptosis in rat liver: a strategy for host-parasite cross talk. *Parasitology Research*. 2016, **115**(7), 2617–2624. Available from: http://dx.doi.org/10.1007/s00436-016-5008-3

GÓMEZ-ELÍAS, M.D., et al. Association between high-fat diet feeding and male fertility in high reproductive performance mice. *Scientific Reports*. 2019, **9**(1), 1–9. https://doi.org/10.1038/s41598-019-54799-3

GONÇALVES, R.V., et al. Hepatoprotective effect of *Bathysa cuspidata* in a murine model of severe toxic liver injury. *International Journal of Experimental Pathology*. 2012, **93**(5), 370–376. https://doi.org/10.1111/j.1365-2613.2012.00835.x

GREENWALD, R.A. CRC Handbook of Methods for Oxygen Radical. by Thornalley PJ, Bannister JV, CRC Press, New York. 1985, 133-136. https://doi.org/10.1016/s0076-6879(84)05016-3

HABIG, W.H., PABST, M.J. and JAKOBY, W.B. Glutathione S transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*. 1974, **249**(22), 7130–7139. http://dx.doi.org/10.1016/S0021-9258(19)42083-8

HESS, R.A. and FRANÇA, L.R. Spermatogenesis and cycle of the seminiferous epithelium. *Advances in Experimental Medicine and Biology*. 2008, **636,** 1–15. https://doi.org/10.1007/978-0-387-09597-4_1

HOLSBERGER, D.R., et al. Cell-cycle inhibitors p27Kip1 and p21cip1 regulate murine sertoli cell proliferation. *Biology of Reproduction*. 2005, **72**(6), 1429–1436. https://doi.org/10.1080/15384101.2016.1207835

JOHNSEN, S.G. Testicular biopsy score count--a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Hormones*. 1970, **1**(1), 2–25. https://doi.org/10.1159/000178170

KARL, J. and CAPEL, B. Sertoli cells of the mouse testis originate from the coelomic epithelium. *Developmental Biology*. 1998, **203**(2), 323–333. https://doi.org/10.1006/dbio.1998.9068

KEFER, J.C., AGARWAL, A. and SABANEGH, E. Role of antioxidants in the treatment of male infertility. *International Journal of Urology*. 2009, **16**(5), 449–457. https://doi.org/10.1111/j.1442-2042.2009.02280.x

KENAGY, G.J. and TROMBULAK, S.C. Size and Function of Mammalian Testes in Relation to Body Size. *Journal of Mammalogy*. 1986, **67**(1), 1–22. https://doi.org/10.2307/1380997

LEISEGANG, K. HENKEL, R. and AGARWAL, A. Obesity and metabolic syndrome associated with systemic inflammation and the impact on the male reproductive system. *American Journal of Reproductive Immunology*. 2019, 82(5), 13178, 2019. https://doi.org/10.1111/aji.13178

LIN, B., et al. High-Fat-Diet Intake Enhances Cerebral Amyloid Angiopathy And Cognitive Impairment In A Mouse Model Of Alzheimer's Disease, Independently Of Metabolic Disorders. *Journal of the American Heart Association*. 2016, 5(6), 1–16. https://doi.org/10.1161/jaha.115.003154

LIU, Y. and DING, Z. Obesity, A Serious Etiologic Factor For Male Subfertility In Modern Society. *Reproduction*. 2017, 154(4), 123–131. https://doi.org/10.1530/rep-17-0161

LOWRY, O.H., et al. Protein Measurement With The Folin Phenol Reagent. *Journal of Biological Chemistry*. 1951, 193(1), 265–275. http://dx.doi.Org/10.1016/S0021-9258(19)52451-6

MÄKELÄ J., et al. Testis Development. *Endocrinology Reviews*. 2019, 40(4), 857–905. https://doi.org/10.1210/er.2018-00140

MATHIJS, I., et al. Phenylpropenoic Acid Glucoside Augments Pancreatic Beta Cell Mass In High-Fat Diet-Fed Mice And Protects Beta Cells From Er Stress-Induced Apoptosis. *Molecular Nutrition and Food Research*. 2014, 58(10), 1980–1990. https://doi.org/10.1371/journal.pone.0157604

MCPHERSON, N.O. and LANE, M. Male Obesity And Subfertility, Is It Really About Increased Adiposity? Asian Journal of *Andrology*. 2015, 17(3), 450–458. https://doi.org/10.4103/1008-682x.148076

MELO, F.C.S.A., et al. The Effects Of Tynnanthus Fasciculatus (Bignoniaceae) Infusion On Testicular Parenchyma Of Adult Wistar Rats. *Biological Research*. 2010, 43(4), 445–450. http://dx.doi.org/10.4067/S0716-97602010000400009

MORTON, J.F. Rooibos Tea, *Aspalathus Linearis*, A Caffeineless, Low-Tannin Beverage. *Economic Botany*. 1983, **37**(2), 164–173. https://doi.Org/10.1016/J.Sajb.2011.07.004

MU, Y., et al. Curcumin Ameliorates High-Fat Diet-Induced Spermatogenesis Dysfunction. *Molecular Medicine Reports*. 2016, **14**(4), 3588–3594. https://doi.org/10.3892/mmr.2016.5712

NASCIMENTO, A.F., et al. A Hypercaloric Pellet-Diet Cycle Induces Obesity And Co-Morbidities In Wistar Rats. *Arquivos Brasileiros de Endocrinologia e Metabolismo*. 2008, **52**(6), 968–974. Https://doi.Org/10.1590/S0004-27302008000600007

OAKBERG, E.F. Duration of Spermatogenesis In The Mouse And Timing Of Stages Of The Cycle Of The Seminiferous Epithelium. *American Journal of Anatomy*. 1956, **99**(3). 507–516. https://doi.org/10.1002/aja.1000990307

OLIVEIRA, M.C., et al. Acute and Sustained Inflammation And Metabolic Dysfunction Induced By High Refined Carbohydrate-Containing Diet In Mice. *Obesity*. 2013, **21**(9). https://doi.org/10.1002/oby.20230

OLIVEIRA, C.F., et al. Foxn1 And Prkdc Genes Are Important For Testis Function: Evidence From Nude And Scid Adult Mice. *Cell and Tissue Research*. 2020, **380**(3), 615–625. https://doi.org/10.1007/s00441-019-03165-w

OPUWARI, C.S. and MONSEES, T.K. In Vivo Effects Of Aspalathus Linearis (Rooibos) On Male Rat Reproductive Functions. *Andrologia*. 2014, **46**(8), 867–77. https://doi.org/10.1111/and.12158

PEREIRA, J., et al. Ganho De Peso E Alterações Metabólicas Em Camundongos Submetidos À Dieta Hiperlipídica. *Ciência & Saúde*. 2018, **11**(1), 51. https://doi.Org/10.15448/1983-652x.2018.1.27672

RIBEIRO, S., et al. Sericin Exerts Antioxidation On Testis And Epididyms Of C57bl/6 Mice Fed High Fat Diet. International *Journal of Developmental Research*. 2017, **7**(10), 15813–15820. https://doi.org/10.1371/journal.pone.0120775

RICART-JANÉ, D., LLOBERA, M. and LÓPEZ-TEJERO, M.D. Anticoagulants And Other Preanalytical Factors Interfere In Plasma Nitrate/Nitrite Quantification By The Griess Method. *Nitric Oxide - Biological Chemistry*. 2002, **6**(2), 178–185. https://doi.org/10.1006/niox.2001.0392

ROSINI, T.C., DA SILVA, A.S.R. and MORAES, C. Obesidade Induzida Por Consumo De Dieta: Modelo Em Roedores Para O Estudo Dos Distúrbios Relacionados Com A Obesidade. *Revista da Associação Médica Brasileira*. 2012, **58**(3), 383–387. https://doi.org/10.1590/S0104-42302012000300021

SALAS-HUETOS, A., BULLÓ, M. and SALAS-SALVADÓ, J. Dietary Patterns, Foods And Nutrients In Male Fertility Parameters And Fecundability: A Systematic Review Of Observational Studies. Human Reproduction Update. 2017. 23(4), 371–389. https://doi.org/10.1093/humupd/dmx006

SASAKI, M., NISHIDA, N. and SHIMADA, M. A Beneficial Role Of Rooibos In Diabetes Mellitus: A Systematic Review And Meta-Analysis. *Molecules*. 2019, **23**(4), 1–15. https://doi.org/10.3390/molecules23040839

SEED, J., et al. Methods For Assessing Sperm Motility, Morphology, And Counts In The Rat, Rabbit, And Dog: A Consensus Report. *Reproductive Toxicology*. 1996, **10**(3), 237–244. https://doi.org/10.1016/0890-6238(96)00028-7

ULIČNÁ, O., et al. Rooibos Tea (Aspalathus linearis) Partially Prevents Oxidative Stress In Streptozotocin-Induced Diabetic Rats. *Physiological Research*. 2006, **55**(2), 157–64.

VELLOSO, L.A. The Brain Is The Conductor: Diet-Induced Inflammation Overlapping Physiological Control Of Body Mass And Metabolism. *Arquivo Brasileiro de Endocrinologia e Metabolismo*. 2009, **53**(2), 151–158. https://doi.org/10.1590/S0004-27302009000200006

WAGNER, H., CHENG, J.W. and KO, E.Y. Role Of Reactive Oxygen Species In Male Infertility: An Updated Review Of Literature. *Arabian Journal of Urology*. 2017, **16**(1), 35–43. https://doi.org/10.1016/J.Aju.2017.11.001

WALLIN, B., et al. Lipoprotein Oxidation and Measurement Of Thiobarbituric Acid Reacting Substances Formation In A Single Microtiter Plate: Its Use For Evaluation Of Antioxidants. *Analytical Biochemistry*. 1993, 108, 10–5. https://doi.org/10.1006/abio.1993.1002

WHITE, P.A.S., et al. Modelo De Obesidade Induzida Por Dieta Hiperlipídica E Associada À Resistência À Ação Da Insulina E Intolerância À Glicose. *Arquivos Brasileiros de Endocrinologia e Metabolismo*. 2013, **57**(5), 339–345. https://doi.org/10.1590/S0004-27302013000500002

YUAN, M., et al. Hyperleptinemia Directly Affects Testicular Maturation at Different Sexual Stages In Mice, And Suppressor Of Cytokine Signaling 3 Is Involved In This Process. *Reproduction Biology and Endocrinology*. 2014, **12**(1), 1–13. https://doi.org/10.1186/1477-7827-12-15

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