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How bad is brazilian ginseng extract for reproductive parameters in mice?

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Summary. Properties attributed to the *Panax ginseng* are also attributed to the Brazilian ginseng, such as adaptogenic and aphrodisiac effects. There are studies demonstrating that the Brazilian ginseng (BGE) possibly increases the serum levels of testosterone and nitric oxide in mice and rats. The present study aimed to evaluate the effects of its extract on male fertility and sperm quality. Male Swiss mice (n=60) were divided into six groups. The control animals were provided 0.5 mL of water, and 0.5 mL of water containing 7 mg/kg per day (d) sildenafil citrate. Other animals were treated with BGE at 100 mg/kg/d, 200 mg/kg/d, and 400 mg/kg/d by gavage for 42 days. Finally, animals from the last group received 200 mg/kg BGE every 3 days (3-3d) by gavage for 42 days. The results showed a reduction in the number of resistant spermatids in the testis and damage to daily sperm production, culminating in a reduction in the number of epididymal spermatozoa. Although the sperm quality decreased in all experimental animals, only males treated with BGE 100 mg/kg/d showed pre and post implantation embryo losses. We concluded that BGE alters sperm viability compromising the embryonic development after implantation.

Key words: Embryonic loss, Phytotherapic, Reactive oxygen species, Sperm quality

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Introduction

Many Brazilian plants have shown promising pharmacological potential related to reproductive functions, acting positively on testis tissue and sperm cells (Farombi et al., 2013). Among them, the species *Pfaffia glomerata* (Amaranthaceae), commonly known as Brazilian ginseng, has medicinal importance, being used as an ornamental species and for culinary uses (Nicoloso et al., 2001; Silva Júnior and Osaida, 2005; Silva, 2010). Its tuberous roots are of great commercial interest due to the presence of a wide variety of triterpenic saponins, including the phytoecdysteroid 20-hydroxyecdysone (20E), considered to be its main active compound (Nicoloso et al., 1999; Vigo et al., 2004). In addition, Brazilian ginseng has been widely used in traditional medicine as an aphrodisiac (Lorenzi and Matos, 2002; Gomes et al., 2014; Corrêa et al., 2016).

There are studies demonstrating that *P. glomerata* possibly increases the serum levels of testosterone and nitric oxide in mice and rats (Freitas et al., 2004; Teixeira et al., 2006; Matta et al., 2020). The latter activates guanylate cyclase that, in turn, increases the concentrations of cyclic guanosine monophosphate (cGMP) causing relaxation, vasodilation (Cerqueira and Yoshida, 2002) and, consequently, penile erection (Subramoniam et al., 2013). Some commercial drugs, such as sildenafil citrate, also act to increase cGMP levels by inhibiting phosphodiesterases, which prolongs tissue relaxation and erection (Glenn et al., 2007). Nevertheless, sildenafil citrate has a negative effect on the spermatozoa, increasing sperm motility with early activation of the acrosome reaction decreasing

fertilization capacity (Glenn et al,. 2007).

To our knowledge, the effect of *P. glomerata* on male fertility remains elusive. Matta (2012) reported that mice treated with Brazilian ginseng extract (BGE) at the concentrations of 300 and 400 mg/kg/day showed increased serum testosterone levels and increased proportion of cavernous bodies within the penis, although with decreased daily sperm production. Indeed, several studies have shown negative effects of compounds derived from plants on male fertility, altering sperm viability (Aladakatti and Ahamed, 2005a,b; Gadelha et al., 2011; Kumari et al., 2011; Mahajan et al., 2012; Oshio et al., 2015). It is known that testicular modifications can lead to impaired fertility by interfering with sperm quality, which could impact embryonic development and offspring welfare (Wai-sun et al., 2006). In this sense, a study using BGE from the 1st to 3rd day of gestation (preimplantation period) showed increased embryo and foetal loss, as well as the occurrence of malformations, compared to BGE administration during implantation (4th-6th days of gestation) and post implantation periods (7th-9th days of gestation) (Barilli and Montanari, 2008).

Recent results have shown that the long term intake of *P. glomerata* hydroalcoholic extract alters the organization of the seminiferous tubules causing germ cell loss [24 in press]), however its effects on the epididymis, which is responsible for promoting maturation, transport and sperm storage events (Robaire et al., 2015), have not been described yet. Therefore, the present study aimed to evaluate the effects of its extract on sperm quality and male fertility, using different BGE concentrations. The epididymis was also evaluated for markers of oxidative stress and sperm quality in order to characterize the environment in which sperm were matured and stored during BGE treatment.

Materials and methods

Plant extract preparation

The diploid *P. glomerata* of accession 22 (Gomes et al., 2014) used in this study was taken from the gene bank owned by the Plant Tissue Culture Laboratory (LCT-BIOAGRO) of the Federal University of Viçosa (UFV) in Minas Gerais, Brazil. They were grown in experimental fields located in Vila Valério (18°58'55.5"S 40°15'17.8"W), Espírito Santo, Brazil, and harvested in November 2015. Samples were oven dried at 55°C, and ground using a knife mill (SL32, Solab). A voucher specimen was deposited in the Leopoldo Krieger Herbarium, Juiz de Fora, Minas Gerais, Brazil (number CESJ-63317).

The preparation of the hydroalcoholic extract of *P. glomerata* roots (BGE), as well as the phytochemical analyses, were performed in the Laboratory of Biodiversity of Department of Biochemistry and Molecular Biology of UFV. The extract was obtained from 700 g of crushed roots, left in maceration for 48h with ethyl alcohol (95%) then submitted to percolating with the same solvent. After exhaustive extraction, the extract was taken to the Rotary evaporator with reduced pressure and freeze-dried (lyophilizer VirTis BenchTop K) for complete removal of the solvent with a yield of 9.3%.

Animals

Male Swiss mice (n=60; 55 days old) were provided by the Central Animal Laboratory of the Center of Biological and Health Sciences of UFV. The animals were housed in polypropylene cages (n=5 animals/cage), under controlled temperature (22°C), humidity (60-70%), and photoperiod (12 h light/dark). Food and drinking water were provided *ad libitum*. This study was carried out in strict accordance with the recommendations in the ethical guidelines of the National Council for the Control of Animal Experimentation (CONCEA). All experimental procedures were approved by the Ethics Committee for Animal Use of UFV (protocol 044/2015).

Experimental design

The animals were randomly divided into six groups (n=10 animals/group). The control animals were provided 0.5 mL of water (control), and 0.5 mL of water containing 7 mg/kg/day of sildenafil citrate. Other animals were treated with BGE at 100, 200 and 400 mg/kg/day by gavage, every day for 42 days. Finally, animals from the last group received BGE 200 mg/kg every 3 days (3-3d) by gavage, for 42 days. The extract was resuspended in 0.5 mL water regardless of the concentration used. The experimental period was determined using the duration of spermatogenesis in mouse (Russell et al., 1990a). BGE concentrations were chosen based on previous studies using the same plant extract (Dias et al., 2019; Matta et al., 2020). Sildenafil citrate was used as positive control since it is a potent and selective inhibitor of phosphodiesterase-5 (PDE5), which increases nitric oxide levels (Fan Chung, 2006; Ückert et al., 2006), and induces acrosome reaction (Glenn et al., 2007).

On the 43rd day of experiment, males (n=4 animals/group) were mated with normal adult non-treated females to evaluate fertility indexes and sperm quality (Wise et al., 2008; Borges et al., 2015; Lima et al., 2018), whereas the remaining (n=6 animals/group) were euthanized for oxidative stress and epididymal histology analysis.

Natural mating and fertility

Natural mating was performed with non-treated females in a 1:2 ratio up to 72 h. Vaginal smears were done at 24, 48 and 72 h after mating. The detection of sperm in the vaginal smear of each female confirmed mating, and it was used for establishing Gestation Day 0

(GD0). Males and females were separated at the end of this period.

On GD 17, females were anesthetized with sodium thiopental (30 mg/kg, i.p.), and euthanized. Their reproductive tract was removed and dissected. Ovaries were evaluated for the presence and number of *corpora lutea*. To do so, histological sections (3 μm thick) were obtained with range of 60 μm, and analysed under light microscopy (Olympus, CX40, Tokyo, Japan). In addition, uterine horns were used for quantifying areas of implantation sites, and the number of foetuses.

Fertility indexes (%) were determined: female mating index (number of females mated/ number of females x 100), male mating index (number of males mated/ number of males x 100), pregnancy index (number of females pregnant/ number of females mated x 100), male fertility index (number of males impregnating females/ number of males mated x 100), fertility potential (number of implants/ number of corpora lutea x 100), preimplantation loss (number of corpora lutea - number of implants/ number of corpora lutea x 100), and postimplantation loss (number of implants - number of viable foetuses/ number of implants x 100). Viable foetuses were those with no structural malformations and skeletal anomalies (Lima et al., 2018).

Collection of organs from male mice after mating period

Male mice were weighed and euthanized on the 3rd day after the mating period, when sperm reserves in the epididymis cauda are fully re-established (Lima et al., 2018). The mean body-weight gain for each group was calculated from the final weight minus the initial weight of the animals. Epididymides and testes were removed, dissected, and weights (absolute and relative to the body weights) were recorded. While the right epididymis and testis were stored at -20°C for sperm transit time analysis, the left epididymis was used for sperm analysis (morphology, motility and membrane integrity).

Daily sperm production per testis, sperm number, and transit time in the epididymis

Spermatids resistant to homogenization and sperm in the caput/corpus (C/C) epididymis and cauda (C) epididymis were counted as described by Robb et al. (Robb et al., 1978) and Fernandes (Fernandes et al., 2007). Briefly, the testis was decapsulated, weighed and homogenized in 5 mL 0.9% NaCl containing 0.05% Triton X-100. After a 5-fold dilution, the sample was transferred to Neubauer chambers (four fields per animal), and mature spermatids were counted. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 4.84 (the number of days that spermatids are present in the seminiferous tubules of mice). Following the same procedure applied for the testes, the caput/corpus and cauda epididymis portions were cut into small fragments (200 mg caput/corpus fragment; 100 mg cauda fragment) with scissors and homogenized in 1 mL saline-Triton solution for 3 min. Then, each homogenate was diluted (1:2), and sperm were counted as described for the testis. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion of epididymis by the DSP (Robb et al., 1978).

Sperm analyses

Freshly dissected portions of the cauda epididymis were cut three times and placed in a petri dish containing 500 μL tris-citric acid-fructose (Tris 3.025 g, citric acid 1.7 g, fructose 1.25 g, distilled water 100 mL), for 5 min at 37°C to enable sperm release. The fluid was collected for evaluation of sperm motility, morphology, as well as structural integrity of sperm membranes.

Sperm motility was assessed using 10 μL epididymal fluid placed between the slide and coverslip, previously heated to 37°C. Approximately 100 sperm were examined under the microscope (Bioval L-1000B, Brazil) at 400x magnification. Sperm were classified as either motile or immotile, and motility was expressed as percentage (Souza et al., 2016).

For sperm morphology analysis, the epididymal fluid (50 μL) was fixed in 100 μL 4% buffered formaldehyde. One hundred cells were examined under phase-contrast microscopy (1000x magnification; Bioval L-1000B, Brazil), being classified as defects in the head, midpiece, and tail. Results were expressed as percentages (Lima et al., 2018).

Finally, sperm samples (10 μL) were incubated in a solution of 4% buffered formaldehyde (10 μL) plus sodium citrate buffer (10 μL), carboxyfluorescein diacetate (CFDA; 20 μL), and propidium iodide (PI; 10 μL) for 8 min at 37°C (Harrison and Vickers 1990). Two hundred sperm were evaluated by epifluorescence microscope (610 nm, Olympus AX70 TRF, Tokyo, Japan) at 400x magnification, being classified into two categories: intact membranes ($CFDA^+/PI^-$), and non-intact membranes ($CFDA^-$; PI^+). Results were expressed as percentages.

Oxidative stress markers in the epididymis

The right epididymides were used for the assessment of oxidative stress markers. To that end, the activity of antioxidant enzymes was evaluated using aliquots of 100 mg of frozen epididymis homogenized in potassium phosphate-buffered saline 0.2 M pH 7.4, and centrifuged at 13.8 x g (4°C) for 10 min. The supernatant was used for the analysis of superoxide dismutase (SOD), catalase (CAT), and gluthatione S-transferase (GST) activities. SOD activity was estimated by the pyrogallol method based on the ability of this enzyme to catalyze the reaction of superoxide (O^{-2}) and hydrogen peroxide (H_2O_2) (Dieterich et al., 2000). CAT activity was evaluated by measuring the rate of H_2O_2 decomposition

according to Aebi (1984). Moreover, GST activity was estimated spectrophotometrically as described by Habig et al. (1974) and calculated from the rate of NADPH oxidation.

Lipid peroxidation was assessed in epididymis tissue analyzing tissue levels of malondialdehyde (MDA). Epididymis aliquots were homogenized in PBS and incubated with thiobarbituric acid to evaluate the levels of thiobarbituric acid-reactive substances (Wallin et al., 1993). Nitric oxide levels were indirectly determined by the quantification of nitrite/nitrate levels in the tissue according to the standard Griess reaction (Ricart-Jané et al., 2002). Finally, H_2O_2 production was determined through the Fenton reaction (Oliveira et al., 2018). The biochemical data were normalized in relation to total protein levels (Lowry et al., 1951) in the supernatant. The analyses were performed in duplicate. All enzyme activities were determined by duplicate using a spectrophotometer (UV-Mini 1240, Shimadzu) or an ELISA reader (Thermo Scientific, Waltham, MA, USA).

Cell viability

The left epididymides were fixed in Karnovsky solution (paraformaldehyde 4% : glutaraldehyde 4%, 1:1 in sodium cacodylate buffer 0.2 M, pH 7.4) (Karnovsky, 1965) for 24h, and routinely processed for 20-hydroxyethylmethacrylate embedding (Historesin®, Leica Microsystems, Nussloch, Germany). Histological sections (3 μm thick) were obtained using a rotary microtome (RM 2255, Leica Biosystems, Nussloch, Germany), and stained with toluidine blue-sodium borate 1% for qualitative analysis under light microscopy (Olympus CX40 optical microscope). Acridine orange (AO: green) and propidium iodide (PI: red) were used for evaluating cell viability, permeability and DNA denaturation (Mohammed et al., 2015; Ajina et al., 2017; Lima et al., 2018). This technique is used to evaluate DNA damage because it is a simple and inexpensive technique (Duran et al., 1998), being able to easily detect DNA - Protein binding faults (Tejada et al., 1984). Acridine is a metachromatic molecule, thus allowing to distinguish single strand from double strand DNA (Ichimura, 1975); its molecules intercalate the nitrogenous bases of an undenatured DNA, resulting in a green tint under fluorescence microscopy and results in an orange-red color when its molecules bind to the phosphate groups of the denatured DNA (Mello, 1982).Viable cells show a green nucleus with round intact structure, whereas non-viable cells exhibit a dense orange/red nucleus due to chromatin condensation, and the degree of loss of membrane integrity due to co-staining with PI (Giri and Roy, 2016). Digital images were captured by photomicroscope EVOS fl (Life Technologies, Carlsbad, Canada) and analysed with Image-Pro Plus 4.5® (Media Cybernetics, Silver Spring, MD) software according to Oberholzer et al. (1996). A total area of 30×10^4 μm^2 was used to calculate the number of PI positive cells of ductal epithelium, interstitium, and sperm cells located throughout the epididymis (initial segment, caput, and cauda regions).

Statistical analysis

The normal distribution of the results was tested by the Shapiro-Wilk test. Data with normal distribution were analysed by ANOVA followed by the post hoc Student-Newman-Keuls. The statistical analysis was

Table 1. Fertility indexes of adult Swiss mice treated with Brazilian Ginseng extract (BGE) at different concentrations daily (d) and every 3 days (3-3 d) and mated with non-treated females.

Parameters	Control	Sildenafil citrate	BGE100	BGE200	BGE400	BGE 200/3-3d
Number of females	8	8	8	8	8	8
Number of females mated	8	8	8	8	8	8
Number of males	4	4	4	4	4	4
Number of males mated	4	4	4	4	4	4
Number of males impregnating females	3	3	3.5	3.5	4	3
Number of females pregnant	6	5	6	6	6	6
Female mating index (%)	100	100	100	100	100	100
Male mating index (%)	100	100	100	100	100	100
Pregnancy index (%)	71	63	75	75	71	67
Male fertility index (%)	75	75	87.5	87.5	100	75
Fertility potential (%)	65.28±50.67[a]	79.96±49.90[a]	82.15±33.66[a]	100.00±44.89[a]	84.44±54.20[a]	70.65±54.82[a]
Ovary weight (mg)	37.0±0.9[a]	33.0±4.0[a]	34.0±4.0[a]	30.00±9.0[a]	38.00±13.0[a]	41.00±6.0[a]
Number of corpora lutea	13.00±1.41[a]	11.75±0.5[a]	12.25±1.26[a]	9.75±1.89[a]	11.25±2.06[a]	13.00±0.82[a]
Number of viable fetuses	10.50±2.17[a]	9.83±1.83[a]	10.00±2.16[a]	10.50±1.76[a]	11.83±3.37[a]	12.83±1.17[a]
Fetal weight (g)	2.91±2.05[a]	2.55±1.06[a]	2.24 ±1.00[a]	3.39±1.31[a]	3.83±1.25[a]	3.60±0.92[a]
Number of implants	12.00±2.12[a]	12.25±0.5[a]	11.50±0.58[a]	11.50±1.73[a]	12.25±1.5[a]	13.75±1.26[a]
Pre-implantation losses (%)	4.35±5.03[a]	0±0[a]	10.70±2.65[b]	4.36±4.03[a]	0±0[a]	0±0[a]
Post-implantation losses (%)	7.82±0.99[a]	26.85±8.98[b]	23.61±8.56[b]	12.85±7.55[a]	12.69±3.10[a]	6.69±0.27[a]

Values are expressed as mean ± S.D. Different superscript letters (a,b,c) in the same row indicate differences between these parameters ($p\leq0.05$). ANOVA with post hoc Student Newman Keuls test. BGE100, BGE 100mg/kg/day; BGE200, BGE 200mg/kg/day; BGE400, BGE 400mg/kg/day; BGE200/3-3d, BGE 200mg/3-3 days.

performed using Statistica for Windows 3.11 software. Differences were considered significant when $p<0.05$. Results were expressed as mean ± standard deviation (S.D.).

The principal component analysis (PCA) was performed to verify possible clusters, eliminate redundancies and define the most important variables during the separation of groups under different BGE treatments. For that, data were transformed (ranging) for standardization due to different scale magnitudes. The level of importance of each variable was determined by eigenvector values (McGarigal et al., 2000), with substantial correlation values determined for each attribute in relation to principal components (PC) 1 and 2. The level of importance of each PC was determined by the Broken-stick method, where eigenvalues exceeding the expected values were kept for interpretation. Analyses were performed using the Fitopac 2.1.2.85 software (Shepherd, 2010).

Results

BGE impaired fertility indexes

Results from female mating index and male mating index showed that all animals presented the ability to mate (Table 1). Although sperm was observed in the vaginal smear of all females, the number of pregnant females was lower in mice from sildenafil citrate groups, which in turn decreased male fertility index in the same experimental group (Table 1). The pregnancy index was less than 70% in male mice treated with BGE 200

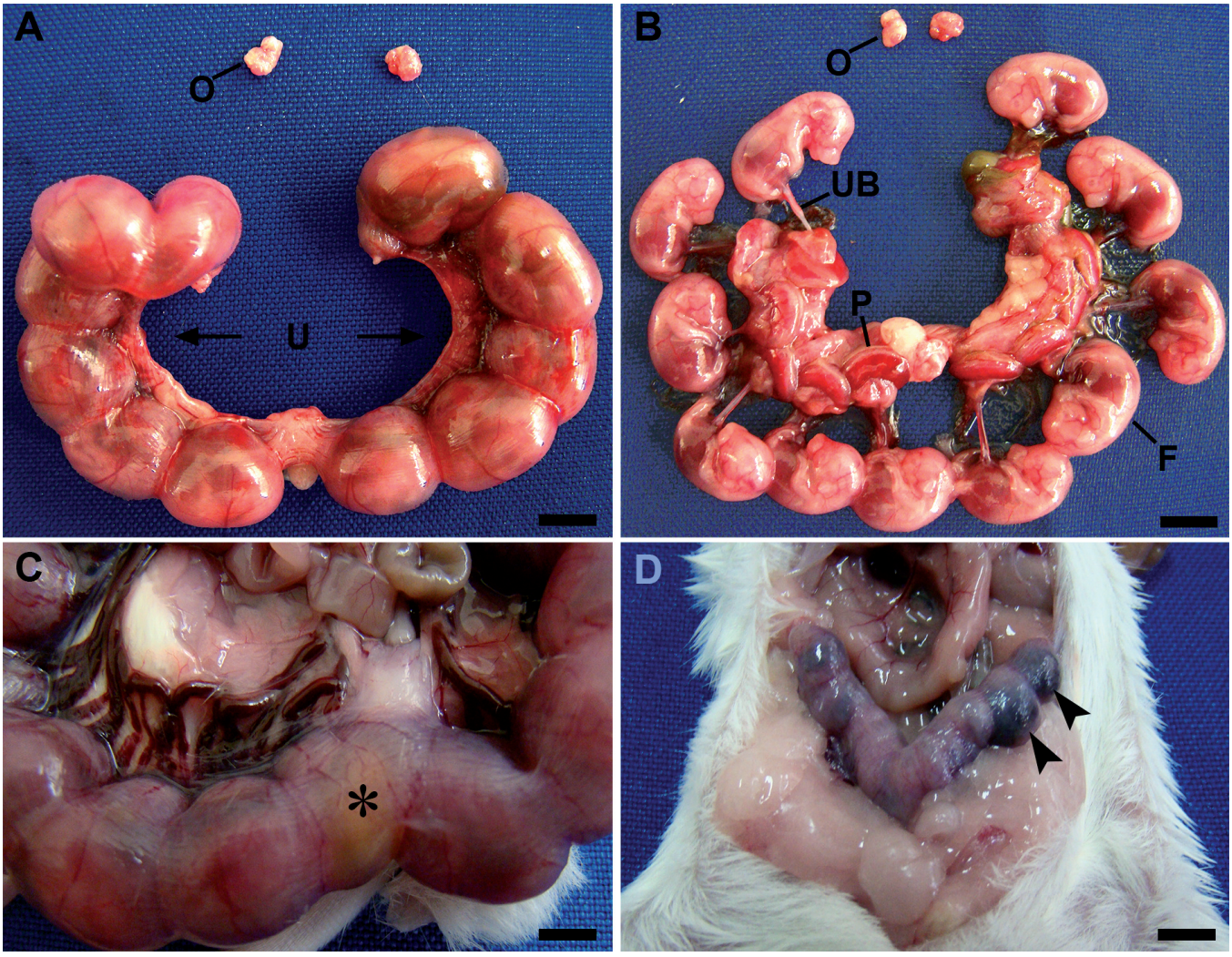


Fig. 1. Photos of pregnant uterus from non-treated females mated with Swiss mice treated with Brazilian ginseng extract (BGE). Uterus (U) and ovaries (O) from females mated with non-treated **(A)** and treated males **(B)**. Note that in **B** is possible to observe fetus (f), umbilical cord (UB), and placenta (f). **C.** Mummified fetus (arrow). **D.** Fetus resorption (head arrow). Scale bars: 50 μm.

mg/kg/3-3d and in animals from the sildenafil citrate group (Table 1). Nevertheless, the fertility potential was higher in BGE 100, 200 and 400 mg/kg, however such variation was not significant. (Table 1).

Ovary weight and the number of corpora lutea were not altered by the treatments (Table 1). The ovaries and uterus from all pregnant females presented normal appearance (Fig. 1A), as well as the placenta and umbilical cord of normal foetuses (Fig. 1B). In addition, the number of implants and viable foetuses did not differ among groups, as well as fetal weight (Table 1). Finally, the percentage of pre-implantation losses was significantly higher for animals treated with BGE 100 mg/kg/d, whereas the percentage of post-implantation losses was higher for the groups treated with sildenafil citrate and BGE 100 mg/kg/d (Table 1). The percentage of post-implantation losses was mainly characterized by fetal mummification (Fig. 1C) and fetal reabsorption (Fig. 1D).

BGE reduced the number of mature spermatids and daily sperm production in the testis

The number of mature spermatids within the seminiferous epithelium and the daily sperm production was diminished due to BGE and sildenafil citrate intake along with daily sperm production, mainly at the highest BGE concentration (Table 2). Thus, sperm count in the caput/corpus epididymis was lower in mice treated with BGE and sildenafil citrate (Table 2). Although sperm count was decreased after BGE intake, sperm transit time in epididymis remained unaltered (Table 2).

BGE affected sperm quality

Mice treated with BGE at 100 mg/kg/d and 200 mg/kg/3-3 d showed lowest sperm motility values, while sildenafil citrate increased sperm motility ($p<0.05$; Table 3).

BGE and Sildenafil citrate intake induced significant alterations in sperm morphology, including modifications in the acrosomal and plasma membranes ($p<0.05$; Fig. 2, Table 3). Animals treated with sildenafil citrate and BGE 100 mg/kg/d presented the highest proportions of tail defects ($p<0.05$). Moreover, animals treated with BGE at 100, 200 and 400 mg/kg/d showed the higher percentages of sperm with cytoplasmic droplets ($p<0.05$; Table 3).

Table 2. Sperm count parameters in testis and epididymis of Swiss mice treated with Brazilian Ginseng extract (BGE) at different concentrations daily (d) and every 3 days (3-3d).

Parameters	Control	Sildenafil citrate	BGE100	BGE200	BGE400	BGE 200/3-3 d
Spermatid number (x10^6 testis)	20.73±0.32^a	6.38±0.76^b	6.91±0.78^b	7.51±1.62^b	4.50±0.26^c	6.19±0.76^b
Spermatid number (x10^6/g testis)	229.16±32.29^a	64.63±5.49^b	89.39±11.08^b	88.24±27.67^b	53.37±13.56^b	60.89±14.15^b
Daily sperm production (x10^6/testis)	4.28±0.067^a	1.32±0.157^b	1.43±0.16^b	1.55±0.33^b	0.93±0.054^c	1.28±0.16^b
Daily sperm production (x10^6/g testis)	42.01±4.56^a	12.37±1.21^b	14.34± 1.82^b	14.69±2.22^b	9.98±2.11^b	11.35±2.65^b
Caput/corpus epididymis sperm number (x10^6)	10.30±1.71^a	2.82±0.62^b	3.98±1.08^b	3.99±0.82^b	3.08±1.11^b	5.03±1.53^b
Caput/corpus epididymis sperm number (x10^6/g)	367.81±21.61^a	125.31±21.15^b	161.25±30.99^b	162.19±12.64^b	152.81±14.70^b	173.44±45.87^b
Sperm transit time in the caput/corpus epididymis (days)	2.40±0.36^a	2.16±0.50^a	2.77±0.54^a	2.67±0.86^a	3.33±1.25^a	3.87±0.81^a
Cauda epididymis sperm number (x10^6)	13.58±1.91^a	3.62±0.34^b	5.65±1.13^b	5.54±1.26^b	0.76±0.34^b	6.35±0.94^b
Cauda epididymis sperm number (x10^6/g)	812.34±33.35^a	245.31±9.37^b	407.75±30.87^b	364.69±9.26^b	296.87±56.74^b	418.21±45.71^b
Sperm transit time in the cauda epididymis (days)	3.17±0.43^a	2.76±0.20^a	3.97±0.68^a	3.80±1.53^a	3.48±0.70^a	5.09±1.39^a

N=4 males/ group. Values are expressed as mean ± S.D. Different superscript letters (a,b) in the same row indicate differences between these parameters ($p\leq0.05$). ANOVA with post hoc Student Newman Keuls test. BGE100, BGE 100mg/kg/day; BGE200, BGE 200mg/kg/day; BGE400, BGE 400mg/kg/day; BGE200/3-3d, BGE 200mg/3-3 days.

Table 3. Sperm motility and sperm morphology of Swiss mice treated with Brazilian Ginseng extract (BGE) at different concentrations daily (d) and every 3 days (3-3d).

Parameters	Control	Sildenafil citrate	BGE100	BGE200	BGE400	BGE200/3-3 d
Total motility (%)	66.67±9.12^a	86.67±18.92^b	48.33±19.74^c	66.67±9.57^a	76.67±9.57^{ab}	0.00±0^d
Normal sperm morphology (%)	70.00±3.46^a	42.00±8.88^b	28.33±10.00^b	44.33±12.34^b	38.00±5.29^b	57.33±5.03^b
Abnormal sperm morphology (%)	30.00±3.46^a	58.00±8.89^b	71.67±10.21^b	55.67±12.34^b	62.00±5.29^b	42.67±5.03^b
Defects midpiece (%)	0.33±0.58^a	3.33±1.53^a	4.33±3.21^a	1.67±1.53^a	3.00±1.73^a	4.67±3.06^a
Defect tail (%)	8.0±1.0^a	17.00±2.65^b	19.33±5.13^b	11.67±3.21^{ab}	12.00±2.00^{ab}	15.33±4.04^{ab}
Cytoplasm droplet (%)	21.67±4.16^a	37.67±7.09^a	48.00±10.15^b	42.33±12.50^b	47.00±3.00^b	22.67±7.77^a

N=4 males/ group. Values are expressed as mean ± S.D. Different superscript letters (a,b,c,d) in the same row indicate differences between these parameters ($p\leq0.05$). ANOVA with post hoc Student Newman Keuls test. BGE100, BGE 100mg/kg/day; BGE200, BGE 200mg/kg/day; BGE400, BGE 400mg/kg/day; BGE200/3-3d, BGE 200mg/3-3 days.

BGE altered the activity of CAT and GST in the epididymis via H_2O_2 production

Hydrogen peroxide production was higher in all treated mice compared to control (Fig. 3). Although SOD activity remained unchanged in all experiment groups ($p>0.05$), CAT activity was reduced in the epididymis of animals treated with BGE and sildenafil citrate ($p<0.05$; Fig. 3). Inversely, GST activity was increased in the epididymis after BGE intake compared to control and BGE 200 mg/kg/d ($p<0.05$; Fig. 3). The latter, in turn, was the only group presenting high levels of nitric oxide ($p<0.05$; Fig. 3), whereas animals receiving BGE 200 mg/kg/3-3d showed the lowest level of MDA ($p<0.05$; Fig. 3).

Sperm cells in the epididymal lumen

The epididymis was divided into 3 portions in order to facilitate cell analysis. In the epididymis initial segment, sperm, interstitial cells, and epithelial cells were highly permeable to PI after sildenafil citrate and BGE 100 and 200 mg/kg/d intake ($p<0.05$; Fig. 4). The same pattern was observed in the caput and cauda regions ($p<0.05$; Fig. 4). However, the opposite pattern was observed in the higher BGE concentrations ($p<0.05$; Fig. 4). In the cauda region, the reduction in the percentage of this cell type (permeable to PI) was markedly observed in animals receiving BGE 200 mg/kg either daily or administered every 3 days ($p<0.05$; Fig. 4).

Overall, epididymal cells were positively stained with AO as shown in Fig. 4. However, sperm cells within the lumen, as well as some epithelial cells, were co-stained with PI due to alterations of plasma membrane permeability (Fig. 4). The high amount of sperm cells permeable to PI detected in the initial segment of mice receiving BGE 200 and 400 mg/kg/d, as well as 200 mg/kg/3-3d was reduced in the caput and cauda regions (Fig. 5A). Interestingly, while sperm cells nuclei were labelled in red, as shown in the epididymal lumen from initial segment and cauda regions (Fig. 5), the epithelial cells showed red cytoplasm and a green nucleus. The epithelium, when observed under light microscopy, presented high amounts of vesicles with different sizes and vacuoles in its apical and basal portions, especially in the cauda region (Fig. 5). Furthermore, animals treated with BGE 200 mg/kg/3-3d presented no sperm permeable to PI in the caput and cauda region (Fig. 5), as observed in control.

Principal component analysis

The total data variation was 42.51%, with the most important attributes of the group having correlation values >0.6 (Fig. 6). For PC1 (horizontal axis) the most relevant attributes and their correlation values were daily sperm production (-0.9895), spermatozoa per organ per head / body (-0.8905), sperm per organ per tail

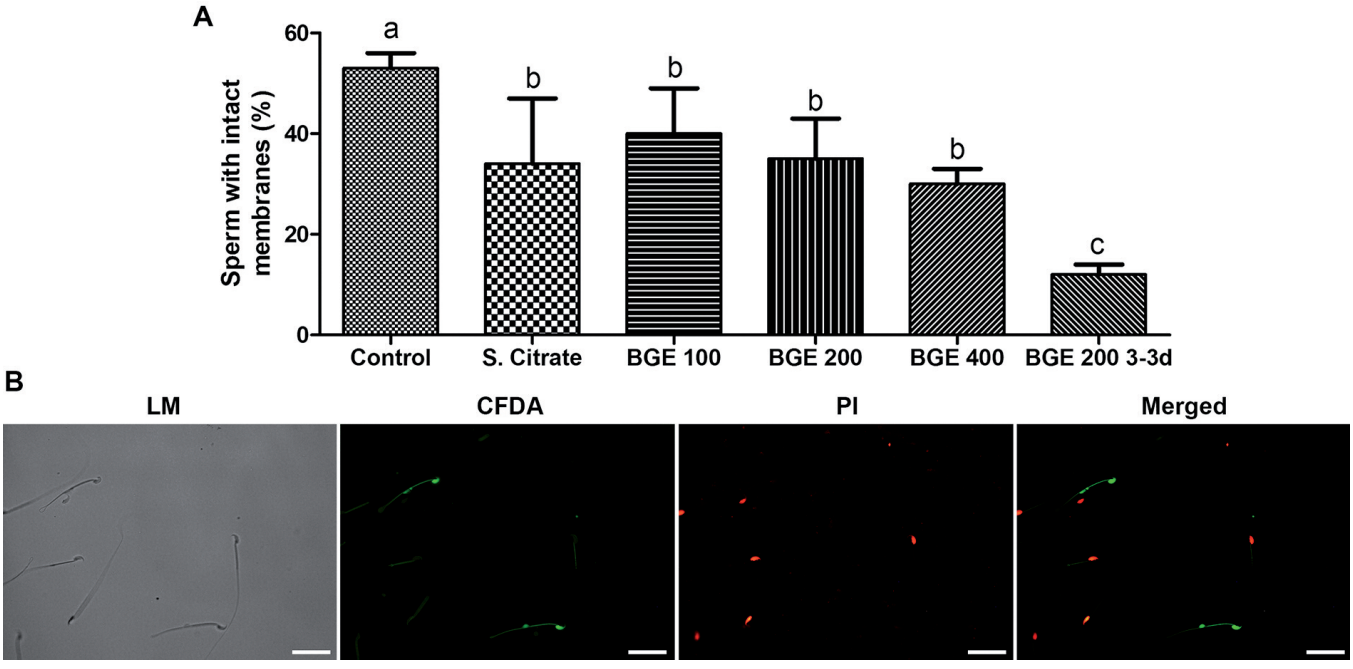


Fig. 2. A. Percentage of sperm with intact plasma and acrosomal membranes from Swiss mice treated with Brazilian ginseng extract (BGE) at different concentrations daily (d) and every 3 days (3-3 d) (n=6/group). Mean±S.D. Different letters (a,b,c) denote statistical difference among the groups ($p\leq0.05$) by Student Newman Keuls test. **B.** Representative images of sperm with intact membranes labeled with carboxifluorescein diacetate (CFDA; green) and sperm with non-intact membranes labeled with propidium iodide (PI; red). LM: Spermatozoa observed at light microscopy. Merged: CFDA and PI stained sperm. Scale bars: 20 µm.

(-0.9182), spermatozoon per gram of body per head (-0.9419), spermatozoa per gram of organ per tail (-0.9411), number of spermatids per testicle (-0.9895), number of spermatids per gram of testis (-0.9667), daily sperm production per gram (-0.9718), normal spermatozoa (-0.7275), spermatozoa with pathology (0.7275) and fertility potential (0.3988). The separation of treatments from the control was evidenced mainly by the reduction in the number of sperm, sperm production and sperm morphology.

In the PC2 (vertical axis), the parameters responsible for the separation of the treatments were Transit in CC days (0.5376), Transit in days C (0.5775), Transit total (0.6978), Site of implantation (0.5671) the attributes shown with their respective values correlation (Fig. 6). The separation of the discontinuous group was evident in the graph, being this difference due to the number of spermatozoa in the epididymis, spermatids in the testis, daily sperm production, reduction in the number of sperm with pathologies in relation to the treatments.

Discussion

Herein, we describe the first evidence of the effect of Brazilian ginseng on sperm viability and male fertility in mice, as well as the conditions of the epididymal environment in which sperm were matured and stored. Overall, daily administration with BGE for 42 days promoted a reduction in the number of resistant spermatids in the testis and a decrease of the daily sperm production, culminating in a reduction of the number of epididymal sperm. Although sperm quality decreased in all groups, only males treated with BGE 100 mg/kg/d showed pre and post implantation embryo losses.

The damage caused by BGE consumption on sperm and epididymis cells leading to the decrease of the daily sperm production may have been caused by some compounds contained in the extract. Among flavonoids, saponins, coumarins and alkaloids (Dias et al., 2019) is the 20-hydroxyecysdone (20E), the major component found in BGE (Vigo, 2004; Dias et al., 2019).

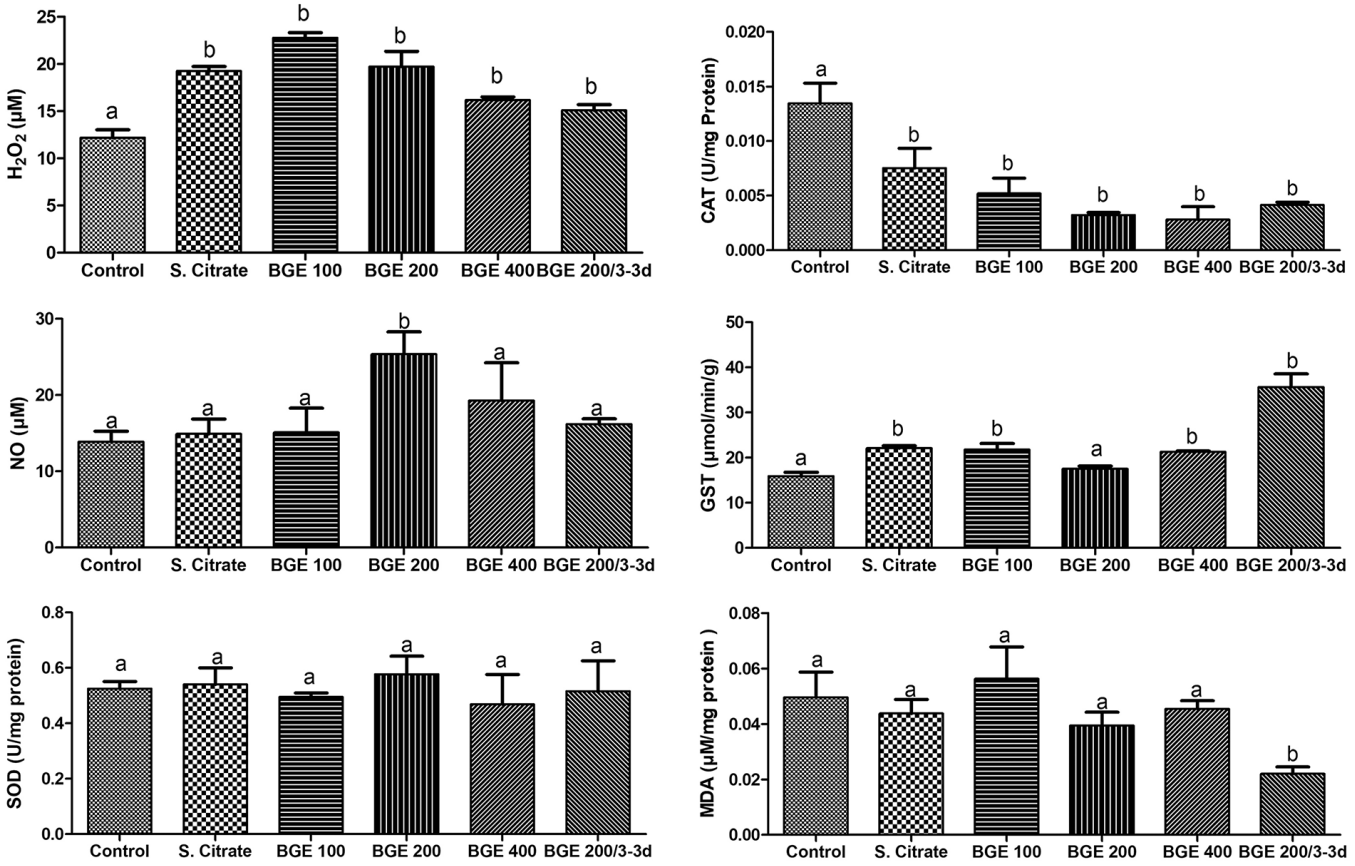


Fig. 3. Activities of antioxidant enzymes and metabolites from lipid peroxidation and nitrosative stress in the epididymis of Swiss mice treated with Brazilian ginseng extract (BGE) at different concentrations daily and every 3 days (3-3 d). H_2O_2: Peroxide hydrogen; NO: Nitric oxide; SOD: superoxide dismutase; CAT: catalase; GST: Glutathione S-transferase; MDA: Malondialdehyde. Mean±S.D. Different superscript letters (a,b) denote statistical difference among the groups ($p \leq 0.05$) by Student Newman Keuls test.

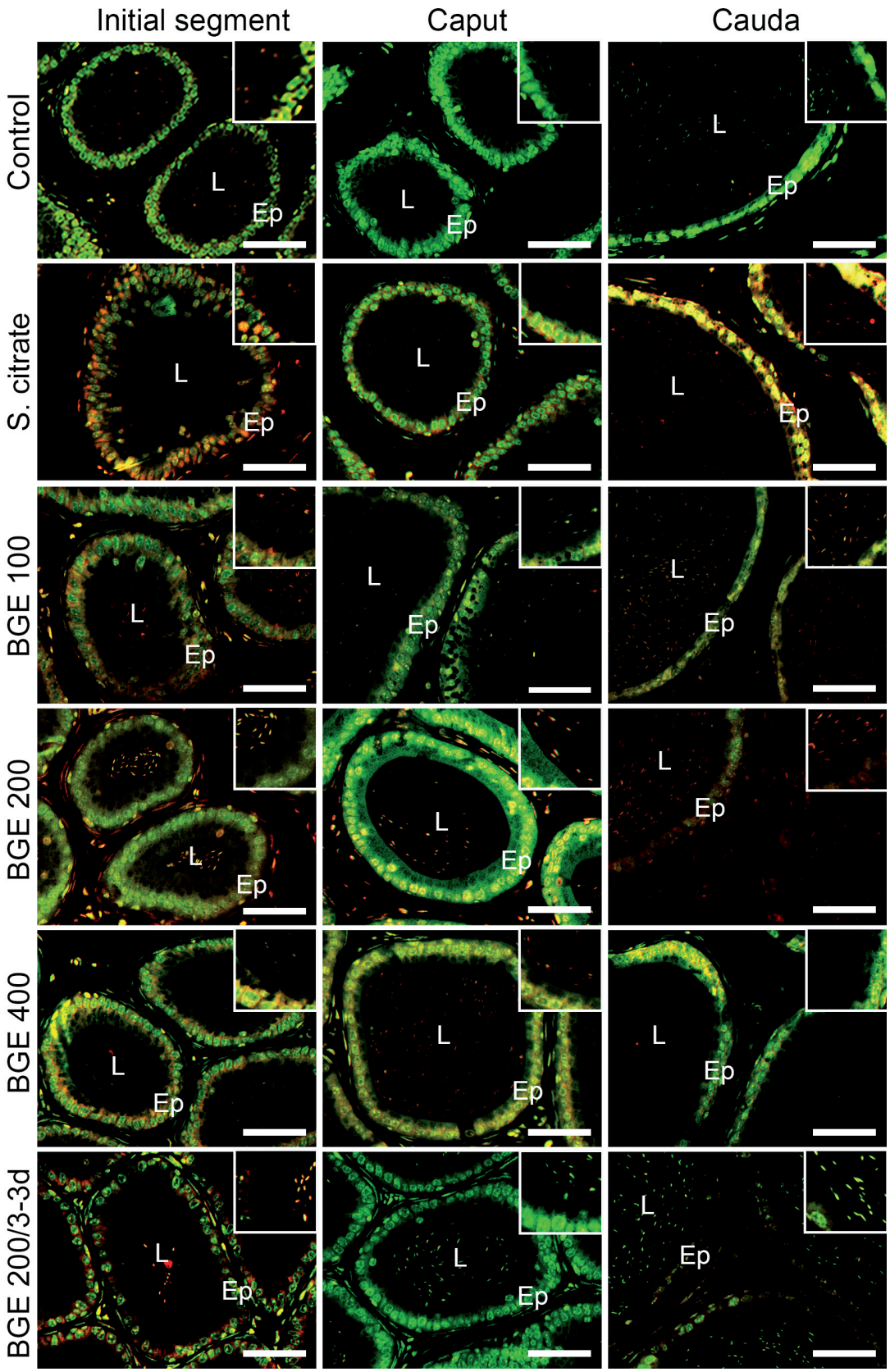


Fig. 4. Histological sections of the initial segment, head and tail of epididymis of mice treated with Brazilian Ginseng Extract (N=5/Group) analyzed with epifluorescence microscopy using fluorochromes orange acridine (AO, green) and propidium iodide (PI, red). The sections show viable cells (green) and apoptotic cells (orange/red). Control (Water); BGE 100, 200 and 400 mg/kg (Daily doses of BGE), BGE 200 mg/kg D (discontinuous doses of BGE 3-3d). L-Lume; Ep-Epithelium. The details in the photo show the spermatozoa on the fire. Scale bars: 50 µm.

Altogether, such compounds were mostly related to the increased ROS levels and consequent alteration in the activity of CAT and GST antioxidant enzymes, while SOD activity remained unaltered. It is known that SOD is considered the first line of defense against deleterious effects of oxyradicals in the cell by the catalysis of the radical O^{-2} in H_2O_2, which is readily degraded by CAT and GST (Aitken and Roman, 2008). In this study, high H_2O_2 levels probably increased CAT activity to its maximum, reaching exhaustion, with subsequent decrease of its activities and abilities to eliminate H_2O_2 (Aitken and Roman, 2008; Lima et al., 2018). The molecule H_2O_2 is extremely deleterious to the cell. It can cross lipid layers and promote damage to macromolecules such as lipids, proteins and DNA, culminating in cell death (Garcimartína et al., 2017). However, in the current study, a significant increase in the production of MDA, a by-product of lipid peroxidation, in the epididymis of mice treated with BGE was not observed. This fact may be related to the maintenance of the high activity of GST enzyme that was able to prevent the deleterious effect of H_2O_2, avoiding the intense production of MDA in these animals, as well as NO. Therefore, it is suggested that the administration with BGE stimulated ROS production without triggering the lipid peroxidation process, during the 42-day administration time.

The NO metabolite, however, had its concentration increased only in males treated with BGE 200 mg/kg/d. This group was the only group that presented reduced GST activity, probably due to exhaustion of its activity, as occurred with CAT. It is known that GST is the main antioxidant enzyme in the epididymis. It is involved in the phase II reactions of the antioxidant activity, reducing the production of lipid peroxidation through the reduction of hydroperoxides (Hayes et al., 2005). Thus, it is responsible for cellular detoxification through glutathione conjugates with xenobiotics, and aldehyde products produced in lipid peroxidation, making them more soluble in water (Habig et al., 1974). Its activity, as well as that of CAT, can be inhibited directly by high concentrations of NO (Kostic et al., 2000; Wong et al., 2001).

ROS production may have negatively influenced the number and quality of sperm present in the epididymal lumen of mice treated with BGE. The number is directly related to the amount of viable spermatids. BGE intake caused germ cell death due to oxidative stress, impairing spermatogenesis, as previously observed (Dias et al., 2019), reflecting on the low number of late spermatids here observed. Spermatids play a crucial role in the quality of sperm, since they give rise to sperm during the cell differentiation process, undergoing morphological changes until they acquire their final shape (Russell et al., 1990b). In the testis, the main damage that ROS can cause in sperm is inadequate compaction of chromatin, which consequently increases the susceptibility of this cell to oxidative stress, culminating in DNA fragmentation (Kodama et al., 1997; De Iuliis et al., 2009).

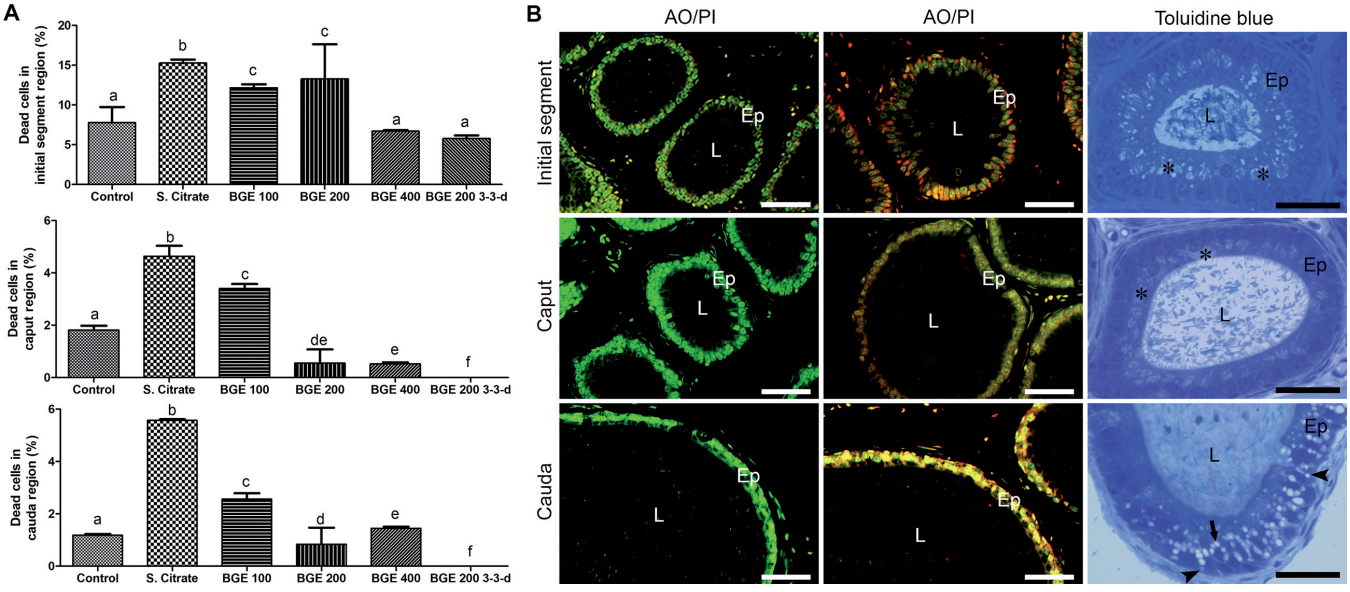


Fig. 5. A. Quantification of autophagy, dead cells in the lumen and interstitium. **B.** Histological sections of the epididymis of mice (n=5/group) analyzed under light microscopy with toluidine blue staining and epifluorescence microscopy using the fluorochromes orange acridine (AO, green) and propidium iodide (PI, red) . Control (Water); BGE 100, 200 and 400mg/kg (Daily doses of BGE), BGE 200mg/kg D (discontinuous doses of BGE 3-3d). L - Lume; Ep - Epithelium; C- Light cells; Arrowhead - Infranuclear lipids; (*) - Pale bodies; Seta - Supranuclear vacuoles. Scale bars: toluidine blue: 20 µm; orange acridine: 50 µm.

Regarding sperm quality, all animals treated with the extract presented alterations in sperm morphology and their structural damage in the acrosomal and plasma membranes. The presence of the so-called cytoplasmic droplet, mainly observed after daily BGE intake made sperm more susceptible to oxidative stress. This sperm pathology is an excess of residual cytoplasm that contains high levels of ROS-producing enzymes (Gomez et al., 1996). High levels of ROS damage the membranes and affect sperm motility and morphology (O'Bryan et al., 1998). Membrane damage and intense ROS production can trigger sperm apoptosis, which involves activation of caspase, phosphatidylserine exteriorization and DNA damage (Schuffner et al., 2002; Muratori et al., 2003). All these factors together can affect the fecundity potential of this cell (Ergur et al., 2002).

Moreover, ROS production observed in the epididymis after BGE intake may have culminated in sperm DNA damage, based on the detection of non-viable sperm cells in the lumen. In mice treated with

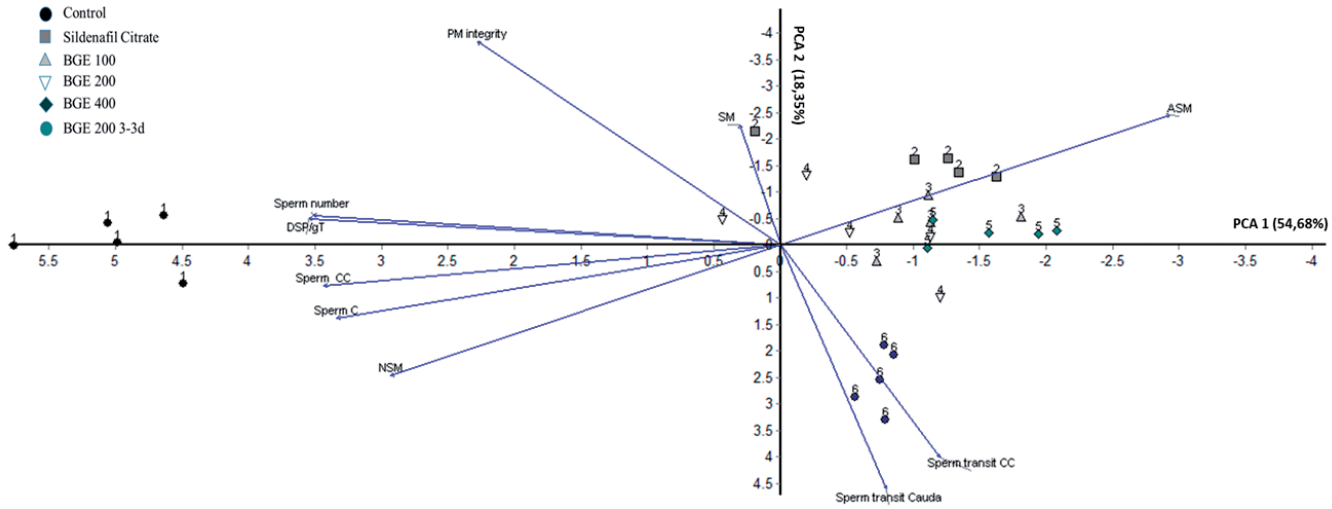
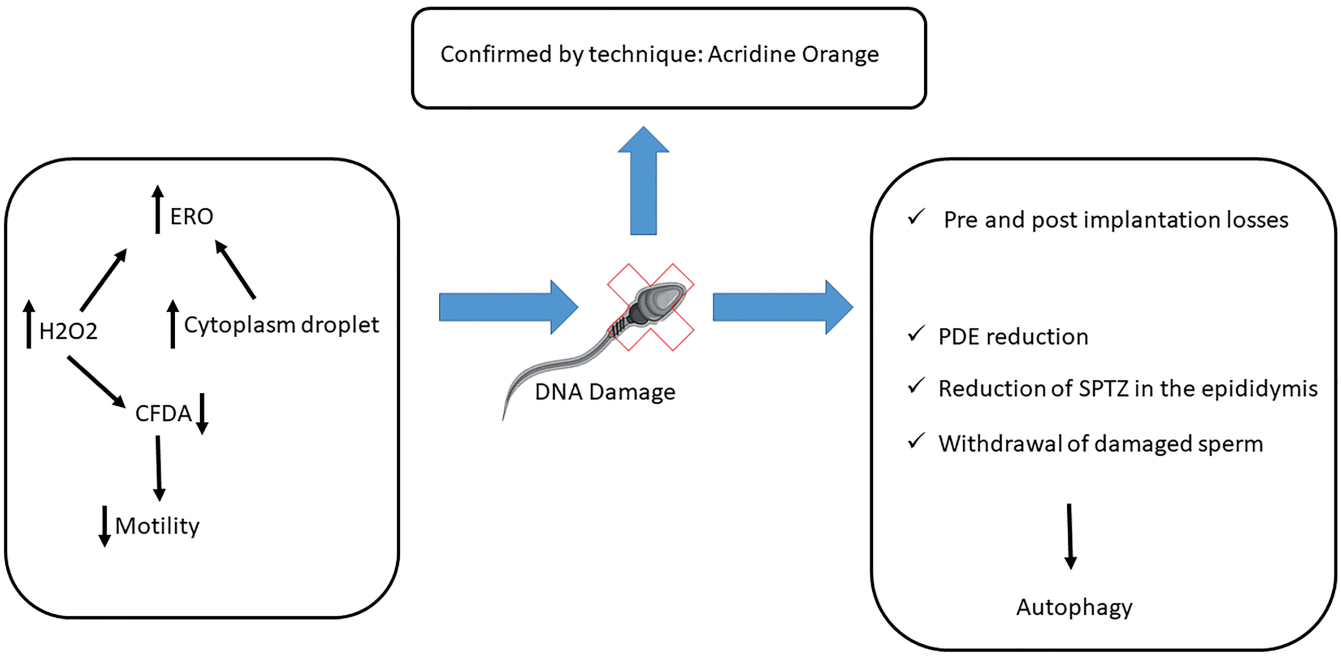


Fig. 6. Principal Component Analysis (PCA) based on the control animal data set, Sildenafil Citrate, BGE 100, BGE 200, BGE 400 and BGE200 3-3d.



sildenafil citrate and BGE at different concentrations, mainly 200 mg/kg/d and 200 mg/kg/3-3d, damaged sperm were removed from the epididymal lumen by epithelial cells, from the occurrence of autophagy identified by both epifluorescence and light microscopy. Autophagy in the epididymis has already been described in the literature (Robaire et al., 2015) and is considered difficult to detect. This process should be efficient in removing non-viable cells without promoting oxidative stress or production of pro-inflammatory cytokines (Kurosaka et al., 2003).

Several mechanisms have been proposed for the removal of abnormal sperm (Jrad-Lamine et al., 2011) and of apoptotic cells (Raymond and Shur, 2009; Raymond et al., 2010). Quality control for sperm is believed to exist and the initial segment has been cited as the region where this activity takes place, since it has a narrow lumen. allowing close interaction between each sperm cell and the epithelium (Sutovsky, 2003; Guiton et al., 2013; Jrad-Lamine et al., 2013). Thus, diluted sperm have the opportunity to interact directly with an epithelium strongly infiltrated by immune cells, with phagocytic functions and presenting potent antigens (Smith et al., 2014). The main cell type present in the early segment that would perform this function is mononuclear phagocyte (MP). This cell emits projections that reach the lumen and can thus perform its function. Thus it has been proposed that such cells can clear defective epithelial cells and abnormal sperm in the epididymis, as well as luminal pathogens after an infection (Smith et al., 2014; Da Silva and Smith, 2015; Breton et al., 2019).

Although the precise role of MPs in the epididymis is unclear, they probably have macrophage-like functions contributing to epithelial homeostasis. Studies show that abundant peritubular MPs perform this function very efficiently, even under extreme, non-physiological conditions (Shum et al., 2014). This indicates that the proximal epididymis contains a highly efficient maintenance mechanism (Yeung et al., 2012).

Histological characteristics of autophagy, such as the presence of large numbers of vesicles and vacuoles of different shapes and sizes, as well as numerous pale bodies similar to large vacuoles, were seen distributed in all cytoplasmic levels of epididymal epithelial cells in gerbil (Domeniconi et al., 2007). Under light microscopy, the poorly stained supranuclear area observed in our study corresponds to the Golgi complex area in the principal cells (Domeniconi et al., 2007), and possibly supranuclear phospholipidose (Kempinas and Klinefelter, 2014). The basal portion of the epithelium, on the other hand, probably corresponds to infranuclear lipids previously described in Kempinas and Klinefelter (2014).

Autophagy led to a greater withdrawal of non-viable sperm from the epididymal lumen of mice treated with BGE 400 mg/kg/d and BGE 200 mg/kg/3-3d. It is known that, in addition to promoting oxidative stress, the 20E present in BGE can induce cell death (Li et al., 2016) via increasing intracellular calcium and activate caspase 3. The latter, in turn, promotes cell death (Li et al., 2016) by externalizing phosphatidylserine, which is fundamental for the recognition of damaged sperm cells by the epithelium (Kurosaka et al. 2003).

Sperm cell were highly affected after BGE 100 mg/kg/d intake, which influenced the high percentages of pre and post-implantation losses observed in this study. In fact, sperm alterations lead to fertilization failures (Vernet et al., 2004), evidencing the paternal effect on embryo development, as well as offspring normality and welfare (Wai-sun et al., 2006). Post-implantation losses were characterized by the occurrence of mummified and reabsorbed foetuses. Taking these findings account, it is suggested that embryonic losses were associated with poor sperm quality and DNA damage (Wai-sun et al., 2006; Garcia et al., 2012; Borges et al., 2015). DNA-damaged sperm are able to fertilize oocytes (O et al., 1988), causing a high incidence of structural abnormalities in post-implanted embryos (Ying et al., 1999; Chen et al., 2001). Although animals treated with BGE 200 and 400 mg/kg/d and 200 mg/kg/3-3d produced low quality sperm cells, the embryonic losses were not significant. This was probably due to the efficient sperm removal by the epididymal epithelium.

Importantly, BGE 100mg/kg/d acted similarly to the phosphodiesterase inhibitor sildenafil citrate, mainly on post-implantation loss. In the same way, both treatments acted similarly on changes in oxidative stress markers and sperm parameters, except motility. A previous study has reported increased sperm motility with the use of sildenafil citrate, generating early activation of the acrosome reactions (Glenn et al., 2007). Furthermore, several studies concluded that phosphodiesterase inhibitors may impair both pre- and post-implantation as well as embryonic development (Lacham-Kaplan and Trounson, 1994; Scott and Smith, 1995; Francis, 2005).

Although the findings observed after BGE 200 mg/kg/3-3d intake were similar to the control that found in control animals, such BGE concentration caused the worst sperm alterations among treatments. In addition, males from this group showed the highest percentage of sperm with non-intact acrosomal and plasma membranes, which may have influenced the motility outcomes and pregnancy index. Indeed, acrosome is critical for mammalian fertilization, and damage in its structure strongly impairs the interaction between sperm and zona pellucida (Lima et al., 2018).

Conclusion

BGE intake alters sperm morphology, motility and viability compromising the embryonic development after implantation. The BGE 200 mg/kg/3-3d group showed the most severe sperm damage, however with no fertility alterations, since the sperm with severe alterations were removed. However, the group receiving 100mg/kg showed the most important fertility alterations. Such

concentration acted similarly to sildenafil citrate, showing that both substances have potential deleterious effects on fertility. The principle of action of the extract is based on the production of ROS, culminating in sperm DNA damage. Further studies should be conducted to investigate the conditions of the sperm production environment.

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Conflict of interest. There are no conflicts of interest.

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