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Effect of *Ginkgo biloba* extract on sperm quality, serum testosterone concentration and histometric analysis of testes from adult Wistar rats

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***Ginkgo biloba* extract is a widely consumed phytotherapic used mainly in the treatment of dementia and Alzheimer's disease, and it has also been employed to treat erectile dysfunction and as an aphrodisiac. This work was aimed at evaluating its effect on sperm quality, serum testosterone concentration and on the histometric analysis of the testes from adult male Wistar rats. Three-month-old Wistar rats were treated with distilled water (control group) and the aqueous extract of *Ginkgo biloba* at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0), once daily, for 56 consecutive days. Counting and morphological evaluation of sperm collected from cauda epidydimis were analyzed. Histometric measures of the testes were also taken. This work shows that adult Wistar rats exposed to *Ginkgo biloba* presented histometric alteration in the volume of the Leydig cells and this finding suggests a possible functional deficit in these cells.**

Key words: *Ginkgo biloba*, phytotherapy, rats, sperm, testes, toxicity.

INTRODUCTION

The extract of *Ginkgo biloba* (GBE) is one of the most consumed phytotherapics in the world, being used to treat dementia and Alzheimer's disease, and to ameliorate peripheral blood flow (Oken et al., 1998; Mar and Bent, 1999; Stromgaard and Nakanishi, 2004). GBE is a mixture of approximately 300 components extracted from the leaves, ranging between 22 and 27% of flavonoid glycosides (flavones: quercetin, kaempferol and isorhamnetin; biflavones: bilobetin, ginkgetin, isoginkgetin and sciadopitysine), 5 and 7% of *terpene trilactones* (ginkgolides: A, B, C e J) and less than 5 ppm of ginkgolic acids (WHO, 1999; Van Beek, 2002; Oh and Chung, 2004; Smith and Luo, 2004).

Other medicinal uses for GBE include treatment of erectile dysfunction as a result of chronic use of antidepressants (Kang et al., 2002; Mackay, 2004; Moyad et al., 2004; Wheatley, 2004; Tamler and Mechanick, 2007) and as an aphrodisiac (Malviya et al., 2011) which led to an increasing intake of the extract by men. Although, many plants are popularly used to regulate

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male fertility (Kamal et al., 2003), not many repots are specifically related to the effect of *G. biloba* extract. Al-Yahya et al. (2006), reported weight reduction of the cauda epidydimis and the prostate in addition to reduced levels of nucleic acids, low gestational index and increased preimplantation loss in *Swiss* mice treated with the extract. Additionally, elevated doses of GBE reduced sperm penetration in the zona pellucida of hamster oocytes (Ondrizek et al., 1999a) and inhibited human sperm motility (Ondrizek et al., 1999b).

However, there were experimental studies which showed protective effects of GBE against testicular damage of many substances and other injuries: doxorubicin (Yeh et al., 2009), cadmium (Predes et al., 2011), cisplatin (Amin et al., 2012), diethylstilbestrol (Wang et al., 2008), carbon tetrachloride (Chávez-Moralez et al., 2010), testicular torsion/detorsion (Kanter, 2011). In addition, Taepongsorat et al. (2008) showed that quercetin, one of the main components of GBE, ameliorated reproductive parameters such as motility, vitality and sperm concentration, and increased testicular weight in rats.

Many substances including environmental contaminants have been related to the occurrence of adverse effects on the male reproductive system for acting as endocrine dysregulators, for instance, the parabens (Hoberman et al., 2008; Tavares et al., 2009), organoclorine pesticides (Bretveld et al., 2007; Uzun et al., 2009; Victor-Costa et al., 2010), bisphenols (Sakaue et al., 2001) and phthalates (Hu et al., 2009; Saillenfait et al., 2009; Martino-Andrade and Chaoud, 2010) can interfere with the male reproductive system by mimetizing the endogenous estrogenic action or changing its metabolism. The estrogenic effects of GBE and its main isolated flavonoid components (quercetin, kaempferol and isorhamnetin) were reported by Oh and Chung (2004). They showed the affinity of these compounds to the human recombinant estrogenic receptors α (ER-α) and, especially to β (ER-β) *in vitro*.

Fransen et al. (2010), described the toxicological risks of GBE and alerted to the small number of animal assays so far developed. The authors mentioned the need for a multidisciplinary investigation in order to ensure a safe use of this phytotherapic. A recent study using GBE was developed by the National Toxicology Program in 2013 (NTP, 2013) using both F344/N rats and B6C3F1/N mice. The animals were treated five times a week during three months and two years in doses raging 100, 300 and 1,000 mg of GBE per kilogram of body weight in rats and 200, 600 and 2,000 mg of GBE per kilogram of body weight in mice. The animals showed no lesions on male reproductive system and sperm concentration in a general analysis, although this study led to the conclusion that GBE caused cancer of the thyroid gland in male and female rats and male mice, and liver cancer in male and female mice.

Siegers (1999), mentioned that alquilfenols, represented by the ginkgolic acids, are toxic compounds that can cause allergies, and display mutagenic and carcinogenic properties. Koch et al. (2000), reported immunotoxicological effects of the extract in mice by causing lymphoproliferative reactions if applied parenterally on skin. For this reason, the extract should not have more than 5 ppm of alquilfenols (Siegers, 1999; WHO, 1999; Fransen et al., 2010). Sierpina et al. (2003) reported the inhibitory effect of the extract on the platelet activation factor and its potential anticoagulant effect.

Therefore, due to the fact that GBE intake by the population is not focused on its active constituents but on the extract, and owing to the possibility that the flavonoid compounds present in GBE may act as endocrine dysregulators and may alter the physiology of the organs of the male reproductive system, this work had the objective of evaluating the effect on the sperm quality, serum testosterone concentration and, specifically, on histometic analysis of the testes from adult male Wistar rats treated with GBE.

METHODOLOGY

The experimental protocol followed the international norms established in the manual about care and use of laboratory animals (National Research Council, 2003) and was approved by the Ethics Committee in Animal Experimentation of the Federal University of Juiz de Fora (UFJF) (protocol number 016/2010).

Ginkgo biloba extract

The extract of *G. biloba* was imported from China by JR Pharma pharmacy in Juiz de Fora City – Minas Gerais State, Brazil. The quality control carried out by Galena Laboratory showed that the extract is composed of 28.2% of ginkgoflavoglicosides (15% of quercetin, 10.9% of kaempferol and 2.3% of isorhamnetin), 8.3% of terpenolactones and less than 5 ppm of ginkgolic acids.

Animals

Eighty Wistar rats obtained from the vivarium of the Biology Center of Reproduction (CBR/UFJF) were used. The animals were three months old and weigh around 250 g. They were placed in polypropylene cages (49×34×16 cm) kept in acclimatized shelves (ALESCO®), with airflow, and under standard laboratory conditions, with a controlled temperature of 22 ± 2°C, and a 12 h light/dark photoperiod. They were fed on rat chow pellets and received water *ad libitum*. Each cage contained the maximum of five animals.

Experimental groups, doses of GBE, administration route and duration of treatment

The rats were randomly distributed into four groups: one control (n=20) and three treated groups (n=20/group). The control group received 1 ml of distilled water and the treated groups received the extract once daily at the concentrations of 3.5 mg/kg (GBE 3.5); 7.0 mg/kg (GBE 7.0) and 14.0 mg/kg (GBE 14.0). The lowest dose corresponded to the therapeutic dose used in the human being (WHO, 1999; Blumenthal et al., 2003; Sierpina et al., 2003; Smith and Luo, 2004). The doses of 7.0 mg/kg and 14.0 mg/kg were twice and four times higher than GBE 3.5, respectively. The extract was administered intragastrically for 56 consecutive days, which corresponds to the duration of spermatogenesis in the rat (Russell et al., 1990). One day after the end of treatment, the animals were

anesthetized with a combination of ketamine chloridrate (90 mg/kg) and xylazine chrloridrate (10 mg/kg), administered intraperitoneally (Wolfensohn and Lloyd, 1994), following death by rupture of the diaphragm.

Testes weight

After euthanasia, the animals underwent laparotomy for the removal and weighing of the testes.

Sperm count

Sperm were collected from the epididymal secretion of the right epididymis cauda (Seed et al., 1996). The secretion from the epididymis was immediately placed in 50 µL phosphate saline solution heated at 37°C and placed in a Petri dish. The sperm were counted in a hemocytometer with improved double Neubauer ruling and the total concentration was estimated according to the formula:

$x = a \times 300 \times 10^4$

where: x = final sperm concentration/mL
a = mean obtained from the eight lateral squares of the hemocytometer

Evaluation of sperm vitality

The sperm smear was stained by use of eosin-nigrosin staining technique (WHO, 2010). Only sperm with head and tail were considered. The data were expressed in percentage of live sperm.

Evaluation of the spermatic morphology

The sperm smear was stained by use of Shorr technique for counting and morphological classification in order to determine the abnormality index (WHO, 2010). The morphological alterations considered, according to Seed et al. (1996) and Perreault and Cancel (2001) were:

2.7.1 Amorphic head: loss of the typical hook shape of the rat sperm;
2.7.2 Cauda abnormality: coiled or broken.

Data were expressed in percentage of normal sperm for each animal.

Tissue preparation

After euthanasia, the animals underwent laparotomy for the removal and weighing of the testes. The gonadosomatic index that represents the testicular weight expressed as percentage of the body weight was determined using the formula below:

$x = (a \div b) \times 100$

where: x = gonadosomatic index (GSI); a = total weight of the testes (g); b = body weight (g)

The testes were fixed in Karnovsky modified fixative (4% paraformaldehyde: 4% glutaraldehyde a 0,1ml/L phosphate saline buffer, pH 7.4). Twenty four hours after the beginning of fixation, the tunica albuginea and the parenchyma of the right testicle were removed and weighed. The left testicle was embedded in histological resine and to conduct a light microscope examination, it was sectioned at 3 µm thickness for toluidine blue – 1% sodium borate staining. The histological sections were photographed for further analysis.

Testicular histometric analysis

Volumetric density of tubular and intertubular testicular compartments

2,660 points were counted with the program Image-Pro Plus® version 4.5.0.29 (Media Cybernectis – EUA) using a standardized grid of 266 points overlaying 10 images taken at random using 10× objective lens. The points were classified as belonging to the tubular or intertubular compartment. For the evaluation of the volumetric density of tubular and intertubular testicular compartments, the following formulas were used:

$x = (a \div 2{,}660) \times 100$
$y = (b \div 2{,}660) \times 100$

where: x = volumetric density of the tubular compartment; y = volumetric density of the intertubular compartment; a = total sum of the overlaying points in the tubular compartment; b = total sum of the overlaying points in the intertubular compartment

Volume of the tubular and intertubular testicular compartments and the tubulosomatic index

The specific testicular density was considered to be 1 (Johnson et al., 1981) therefore, the parenchyma weight (g) was considered equal to its volume (ml). For determination of the volume of the tubular and intertubular testicular compartments the formulas below were used, respectively:

$x = (a \times z) \div 100$
$y = (b \times z) \div 100$

where:x = volume of the tubular compartment (mL); y = volume of the intertubular compartment (mL); z = weight of the testicular parenchyma (g); a = volumetric density of the tubular compartment
b = volumetric density of the intertubular compartment

The tubulosomatic index (TSI) was also calculated using the formula below:

$x = (a \div y) \times 100$

where: x = tubulosomatic index (TSI); a = volume of the tubular compartment; y = body weight

Diameters of the seminiferous tubule and the tubular lumen, and the seminiferous epithelium height

Twenty transversal sections of the most circular seminiferous tubules were photographed (10× objective lens) for each animal and the diameter and radius were measured in each section. In addition, two measurements of the seminiferous epithelium height were taken at opposite positions and their mean value was considered (Figure 1). The luminal diameter was also determined considering the difference between the diameter of the tubule and the sum of the two heights of the seminiferous epithelium.

Total length of the seminiferous tubules per testicle and per testicle gram

After establishing the seminiferous tubules radius and volume occupied, the total length of the seminiferous tubules was taken by using the formula below (Attal and Courot, 1963):

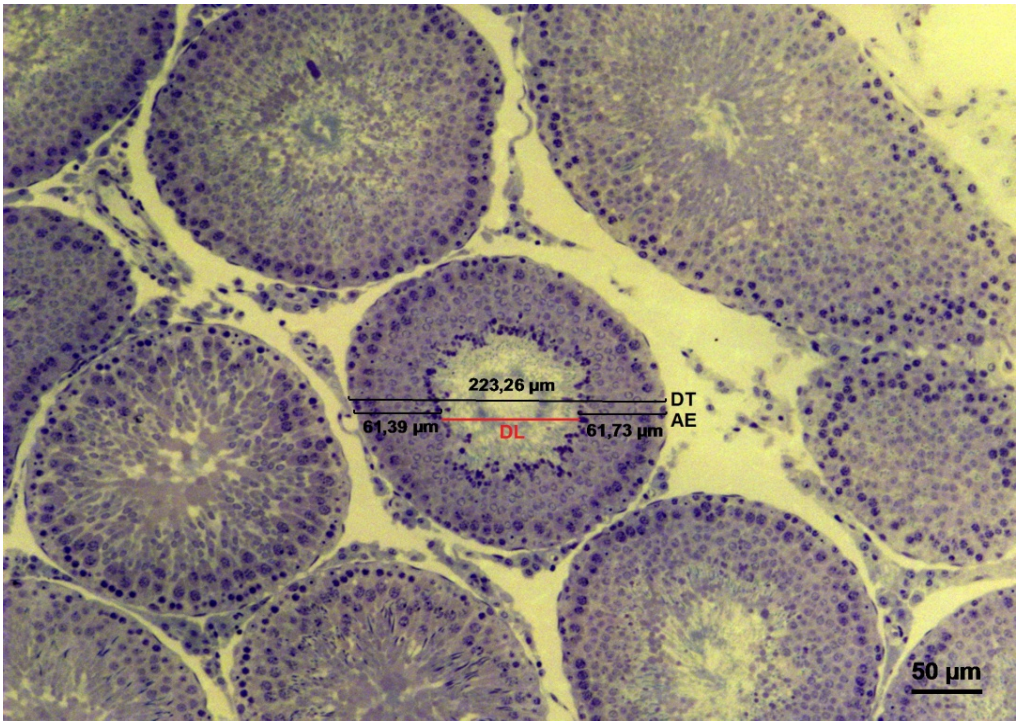

Figure 1. Determination of the histometric measures of seminiferous tubules from Wistar rat testicle. TD – Total tubular diameter; LD – Luminal tubular diameter determined by the difference between the total diameter and the sum of both measures of the epithelium height (EH1+EH2); EH – Height of the seminiferous epithelium determined by mean of two diametrically opposed measures (EH1 and EH2). Toluidine blue – 1% sodium borate staining. Thickness: 3 µm.

$x = a \div (\pi \times r^2)$

where: x = total length of the seminiferous tubules per testicle; a = volume tubular compartment; π = *pi value* (= 3.14); r = seminiferous tubules radius.

The total length of the seminiferous tubules was divided by the total weight of the testes to obtain the length of the tubules per gram of testicle.

Volumetric density and volume of the elements from the intertubular tissue

One thousand points overlaying the images captured from the intertubular region in different histological sections from each animal testicle were counted with 40× objective lens and using a standardized grid of 609 points. The points were classified and quantified when found in the Leydig cells, blood vessels, lymphatic spaces, connective tissue and macrophages (Figure 2). The volumetric density of the elements was calculated according to the formula below:

$x = (a \div 1{,}000) \times 100$

where: x = volumetric density of the elements from the intertubular compartment; a = total sum of the overlaying points on each element of the intertubular compartment

Volume of the intertubular elements was calculated with the formula below:

$x = (a \times z) \div 100$

where: x = volume of the intertubular element

Hormonal concentration of serum testosterone

Blood was collected under anesthesia by cardiac puncture before the euthanasia and the serum was stored at the temperature of -80°C for posterior analysis of serum testosterone concentration. The serum from nine animals of each group were randomly collected to be used at analysis of testosterone concentration using Microreader ELISA plate ASYS HITECH GMBH® (Austria) and the kit Testosterone EIA Kit (Caymman Chemicals®).

Statistical analysis

The data were analyzed using the Levene test for evaluation of data distribution. Data showing normal distribution were analyzed with ANOVA followed by the Tukey test. Data without normal distribution were analyzed with the Kruskal-Wallis test, followed by the Mann-Whitney test. The level of significance considered was $\alpha = 0.05$. For multiple comparisons with the Mann-Whitney test, the significance level was 1%. The tests were performed using Statistical Package for the Social Sciences program (SPSS), version 13.

RESULTS

The mean weight of the left and right testes, the testicular structures such as tunica albuginea and parenchyma, and the gonadosomatic index (GSI) did not significantly

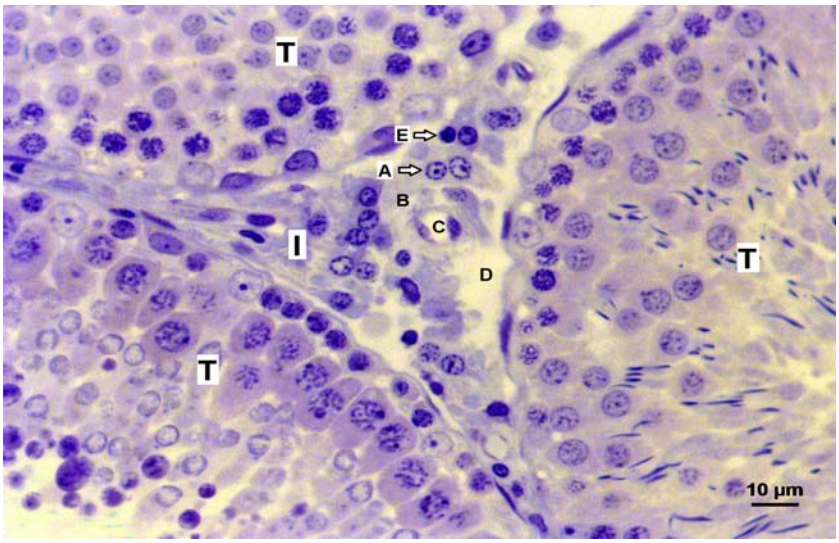

Figure 2. Photomicrography of histological sections of Wistar rat testicle. Identification of components from the intertubular testicular compartment I – Intertubular compartment composed of: A –Leydig Cells (arrow); B – Connective tissue; C – Blood vessel; D – Lymphatic spaces and E – Macrophage (arrow). T – Tubular compartment with seminiferous tubules. Toluidine blue – 1% sodium borate staining. Thickness: 3 µm.

between control and treated groups and between the treated groups (Table 1). The treatment with GBE at all dose levels for 56 days did not significantly alter the number, vitality and morphology of cauda epidydimus sperm (Table 2). The differential analysis of the tubular and intertubular testicular compartments with respect to volumetric density, volume in the organ and the tubulosomatic index (TSI) were not significantly different between the groups (Table 3).

The total and luminal diameters, the height of the seminiferous epithelium, the total length of the tubule per testicle and length of the seminiferous tubule per testicle gram were not statistically different when comparing control and treated groups and between the treated groups (Table 4). There was no significant difference in the volumetric proportions of the Leydig cells, blood vessels, lymphatic spaces, connective tissue, macrophages, and blood vessel volume. However, there was a significant difference in the volume of the Leydig cells in which the groups GBE 3.5 and 7.0 had lower statistical means than the control and the GBE 14.0 groups (Table 5). Photomicrographys of cross histological sections of Wistar rat testicle showing Leydig cells are presented on Figure 4.

The testosterone serum concentrations exhibited great variations (Figure 3). However, there was no significant difference between the groups.

DISCUSSION

The toxicological evaluation of a substance in animal models is primordial for determining the potential risk for human life and is a necessary step to ensure the safety of a medication (Asare et al., 2011). To this end, the international protocols provide useful guidelines and recommendations of tests to be employed (OECD, 2009). The assessment of the weight of the reproductive organs is one of the most sensitive parameters for detection of a substance influence on the male reproductive system (Mangelsdorf et al., 2003). Alterations in the testicular weight can indicate modifications in the seminiferous tubules or interstitial edema and consequently in the sperm production (Sellers et al., 2007).

There is a growing indication that the phytotherapics act as modulators of the estrogenic receptors (Patisaul and Adewale, 2009). Assinder et al. (2007), showed that a diet rich in genistein, glicitein and daidzein, flavonoids with estrogenic activity, increased the apoptotic rate of the testicular germinative cells and was capable of interfering with the spermatogenic process in the rat. In addition, Das et al. (2004) showed that the metanolic extract of *Vitex negundo*, a plant rich in flavonoids, had a negative action in the spermatic quality by reducing the number and motility of sperm. Studies with *G. biloba* extract evidenced reduced capacity of the sperm to penetrate the *zona pellucida* in the hamster oocyte (Ondrizek et al., 1999) and inhibition of motility of human sperm (Ondrizek et al., 1999). However, alterations in the gamete concentration, vitality and morphology were not observed in this study, suggesting that the extract did not exert a direct effect on the production and maturation of sperm. In this analysis, this study is in agreement with

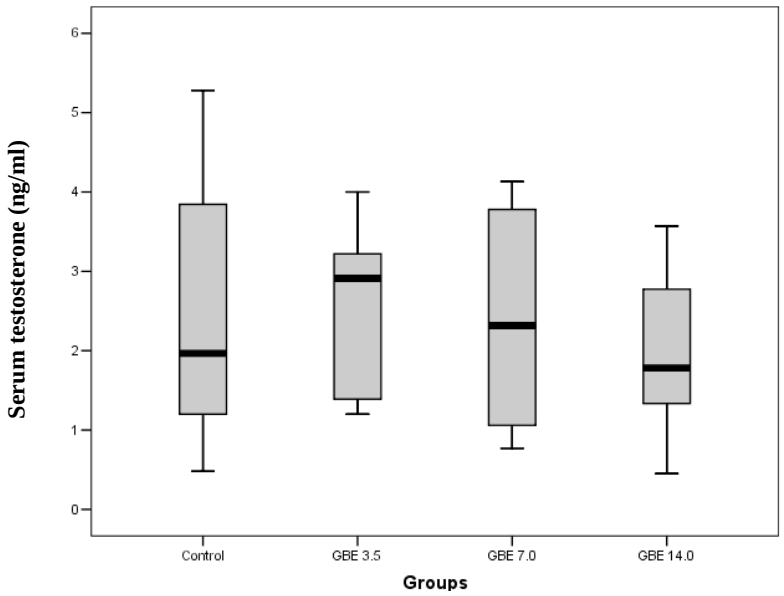

Figure 3. Total serum testosterone concentration (ng/ml) of Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data analyzed by ANOVA. *p=0.838*.

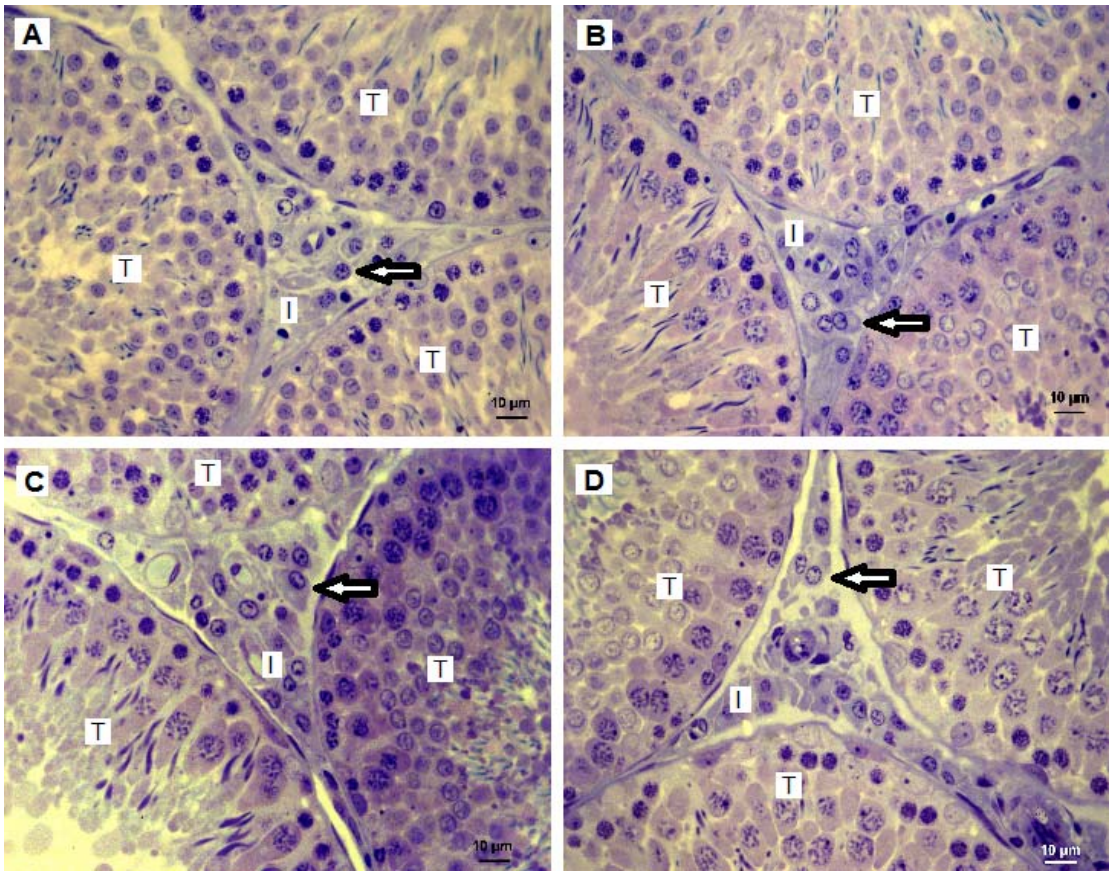

Figure 4. Photomicrographys of histological sections of Wistar rat testicle. A – Control group; B – GBE 3.5; C – GBE 7.0 and D – GBE 14.0. I – Intertubular compartment. T – Tubular compartment. Leydig Cells (arrows). Toluidine blue – 1% sodium borate staining. Thickness: 3 µm.

Table 1. Mean weight of testes, right testicular tunica albuginea and parenchyma, and the gonadosomatic index (GSI) of Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data expressed in mean±standard deviation.

Parameter	Control (n=20)	GBE 3.5 (n=20)	GBE7.0 (n=20)	GBE 14.0 (n=20)	*p*
Testes (g)	1.39±0.10	1.33±0.11	1.38±0.15	1.40±0.11	0.253
Albuginea (mg)	42.85±3.50	40.15±4.11	42.75±4.38	41.35±4.45	0.132
Parenchyma (g)	1.21±0.11	1.16±0.12	1.21±0.11	1.21±0.13	0.406
GSI	0.84±0.09	0.81±0.07	0.88±0.07	0.84±0.08	0.075

Data analyzed by ANOVA. *p* = p-value. GSI - Gonadosomatic index

Table 2. Concentration, vitality and morphology of spermatids from Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data expressed in mean±standard deviation.

Parameter	Control (n=20)	GBE 3.5 (n=20)	GBE 7.0 (n=20)	GBE 14.0 (n=20)	*p*
SC (10^6/ml)	232.20±59.60	224.06±53.38	263.31±84.49	234.93±61.81	0.266
SV (% live)	84.58±5.24	83.55±8.30	85.71±7.18	84.50±7.89	0.838
SM (% normal)	98.71±0.73	98.82±0.99	98.18±1.04	98.63±0.83	0.158

Data analyzed by ANOVA. *p* = p-value. Data analyzed by ANOVA. SC – sperm concentration (10^6/mL); SV – spermatic vitality (% of live sperm); SM – spermatic morphology (% of normal sperm).

Table 3. Volumetric density, volume of testicular compartments and tubulosomatic index (TSI) of Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data expressed in mean ±standard deviation.

Parameter	Control (n=5)	GBE 3.5 (n=5)	GBE 7.0 (n=5)	GBE 14.0 (n=5)	*p*
VDT (%)	89.10±2.66	90.31±4.04	90.47±2.64	88.56±1.85	0.680
VDI (%)	10.90±2.66	9.69±4.04	9.53±2.64	11.44±1.85	0.680
VTT (ml)	1.07±0.03	1.04±0.05	1.08±0.03	1.07±0.02	0.376
VTI (ml)	0.13±0.03	0.11±0.05	0.11±0.03	0.14±0.02	0.582
TSI	0.32±0.01	0.32±0.00	0.33±0.01	0.32±0.01	0.058

Data analyzed by ANOVA. *p* = p-value. VDT – Volumetric density of tubular testicular compartment; VDI – Volumetric density of intertubular testicular compartment; VTT – volume of tubular testicular compartment (mL); VTI – volume of intertubular testicular compartment (mL); TSI – Tubulosomatic index.

Table 4. Histometric data of seminiferous tubules from Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data expressed in mean ±standard deviation.

Parameter	Control (n=5)	GBE 3.5 (n=5)	GBE 7.0 (n=5)	GBE 14.0 (n=5)	*p*
TD (µm)	222.59±8.64	221.52±7.17	226.62±7.74	230.39±5.47	0.247
LD (µm)	122.62±5.31	122.10±3.69	125.90±4.01	124.92±3.04	0.427
EH (µm)	49.99±2.56	49.71±2.82	49.01±1.14	52.74±1.58	0.066
TL (m)	27.70±1.93	27.21±2.43	26.91±2.25	25.73±1.36	0.487
LS (m/g)	10.00±0.70	10.23±0.92	9.72±0.81	9.19±0.49	0.186

Data analyzed by ANOVA. *p* = p-value. TD –Total diameter (µm); LD –Luminal diameter (µm); EH – Epithelium height (µm); TL – Total length of seminiferous tubules per testicle (m); LS – Length of seminiferous tubules per testicle gram (m/g).

Table 5. Volumetric density and volume of components of the intertubular testicular compartment from Wistar rats treated intragastrically with distilled water (Control group) and with *G. biloba* extract at the dose levels of 3.5 (GBE 3.5); 7.0 (GBE 7.0) and 14.0 mg/kg (GBE 14.0) for 56 days. Data expressed in mean ±standard deviation.

Parameter	Control (n=5)	GBE 3.5 (n=5)	GBE 7.0 (n=5)	GBE 14.0 (n=5)	*p*
LEY (%)	44.30±3.91	49.52±7.44	50.08±2.69	45.32±3.53	0.173
BLV (%)	5.30±0.73	5.98±0.95	6.32±0.44	6.10±1.07	0.281
LYM (%)	20.18±1.60	17.48±1.72	19.38±4.79	20.24±4.13	0.550
COT (%)*	29.18±0.70	23.38±9.89	23.72±4.70	24.82±6.68	0.362
MAC (%)	2.36±0.54	1.98±0.27	1.90±0.42	1.88±0.29	0.228
VLEY (µL)	61.00±6.78[e]	46.00±6.93[a,f]	46.40±1.82[b]	64.40±6.88[c,d]	**
VBLV (µL)	7.00±1.58	6.00±2.12	7.60±1.67	8.20±1.30	0.245
VLYM (µL)	24.80±6.06	16.60±3.72	22.40±9.76	28.60±9.74	0.140
VCOT (µL)	33.80±6.69	25.60±11.52	26.20±6.54	34.60±10.67	0.283
VMAC (µL)*	2.60±0.55	2.40±1.14	2.00±0.00	2.4±0.55	0.416

* Volumetric density of connective tissue and volume of macrophages analyzed by Kruskal-Wallis test. ** [a] Control group differs from GBE 3.5 (*p*=0.006 – *post hoc* de Tukey test); [b] Control group differs from GBE 7.0 (*p=0.007* – post *hoc* de Tukey test); [c] Group GBE 3.5 differs from GBE 14.0 (*p=0.001* – *post hoc* de Tukey test); [d] Group GBE 7.0 differs from GBE 14.0 (*p=0.001* – post *hoc* de Tukey test); [e] Control group does not differ from GBE 14.0 (*p*=0.808 – *post hoc* de Tukey test); [f] Group GBE 3.5 does not differ from GBE 7.0 (*p=1.000* – *post hoc* de Tukey test). LEY – Volumetric density of Leydig cells; BLV – Volumetric density of blood vessels; LYM – Volumetric density of lymphatic spaces; COT – Volumetric density of connective tissue; MAC – Volumetric density of macrophages; VLEY – volume Leydig cells (µL); VBLV – volume blood vessels (µL); VLYM – volume lymphatic spaces (µL); VCOT – volume connective tissue (µL); VMAC – volume of macrophages (µL).

NTP (2013) which did not demonstrate any effect on sperm analysis and reproductive organs weight.

According to Sharpe (1998), the male reproductive system possesses estrogenic receptors α (ER-α) and β (ER-β). Oh and Chung (2004), reported the affinity of the main flavonoid constituents isolated from GBE (quercetin, kaempferol and isorhamnetin) with the human recombinant estrogenic receptors α (ER-α), particularly the β (ER-β) *in vitro*. Hence, an estrogenic overload would be expected to occur as a consequence of the binding of GBE constituents to the estrogenic receptors, resulting in alterations in the spermatogenic process as caused by other disruptors such as the parabens (Hoberman et al., 2008; Tavares et al., 2009), organoclorine pesticides (Bretveld et al., 2007; Uzun et al., 2009; Victor-Costa, et al., 2010), bisphenols (Sakaue et al., 2001) and phthalates (Hu et al., 2009; Saillenfait et al., 2009; Martino et al., 2010). However, the data observed in the testes of GBE treated-rats did not show any statistical difference in the mean gonadal weight, total and luminal diameters, and the height of the epithelium of the seminiferous tubules when compared to the control group and between the GBE treated groups. Testicular weight exhibits a strong correlation with the number of germinative cells present in the gonads (Russell et al., 1990) and, according to França and Russell (1998), there is also a positive correlation between the spermatogenic activity and the diameter of the seminiferous tubules and the height of the epithelium. The findings obtained in this study indicate that GBE did not produce any modification in the morphology of the seminiferous tubules.

Substances exhibiting estrogenic effect can cause damage to the male reproductive system (Pflieger et al., 2004; Safe, 2004) by acting directly in the activity of the hormones of the hypothalamic-pituitary-gonadal axis or in the testicular structures. Shimomura et al. (2005), showed that the synthetic estrogens such as ethinylestradiol, used as female contraceptives and in the treatment of prostate hypertrophy and cancer, can bind to estrogenic receptors in the pituitary gland and in the hypothalamus, and reduce the secretion of the gonadotropin-releasing hormone (GnRH), the follicle stimulating hormone (FSH) and the luteinizing hormone (LH), and consequently reduce the levels of testosterone.

The reduction of the volume of the Leydig cells in the GBE treated-groups (GBE 3.5 and GBE 7.0), although not related to reduction of the serum testosterone level, could suggest some functional deficit of these cells. Johnson et al. (1992), have already demonstrated a reduction in Leydig cells volume and function in adult rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) - a toxic halogenated aromatic of industrial components - without a significant effect on spermatogenesis. In another study, Johnson et al. (1994) showed that the rats that received TCDD had a reduction in total volume of both Leydig cell smooth endoplasmic reticulum and mitochondria. Reduction in content of organelles that are responsible for various steps in steroidogenesis, could explain TCDD-reduced production of testosterone in rats.

Although, rats has had a decrease in serum testosterone concentration after being exposed to TCDD, these studies also further illustrate the reserve capacity of Leydig cell function to maintain spermatogenesis when the volume of these cells is significantly reduced.

In our study, other organelles could have reduced its volume but could have not influenced the testosterone production. This feature of reserve capacity of Leydig cell could explain why a decrease of serum testosterone concentration did not happen. This issue needs to be further investigated.

Conclusion

This study shows that adult Wistar rats treated with GBE displayed histometric alterations in the volume of the Leydig cells, although they did not exhibit macroscopic alterations in the organs of the male reproductive system or in the serum testosterone level, which could indicate some functional deficit of these cells.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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