REPRODUCTION
RESEARCH

Resveratrol improves reproductive parameters of adult rats varicocelized in peripuberty

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Abstract

The aim of this study was to investigate the protective action of resveratrol against the reproductive damage caused by left-sided experimental varicocele. There was a reduction of testicular major axis in the varicocele group when compared with the other groups; the testicular volume was reduced in varicocele group in comparison to the sham–control and resveratrol groups. The frequency of morphologically abnormal sperm was higher in varicocele and varicocele treated with resveratrol groups than in sham–control and resveratrol groups. The frequency of sperm with 100% of mitochondrial activity and normal acrosome integrity were lower in varicocele group than in varicocele treated with resveratrol, sham–control and resveratrol groups. Sperm motility was also reduced in varicocele group than in other groups. The sperm DNA fragmentation was higher in varicocele group than in other groups. Testicular levels of malondialdehyde were higher in varicocele and varicocele treated with resveratrol groups. The varicocele and varicocele treated with resveratrol groups had a significantly higher frequency of TUNEL-positive cells than sham–control and resveratrol groups; however, immunolabeling of the testes from varicocele treated with resveratrol group showed a lower number of apoptotic germ cells in comparison with the left testis of rats of the varicocele group. Reproductive alterations produced by varicocele from peripuberty were reduced by resveratrol in adulthood. Resveratrol should be better investigated as an adjuvant in the treatment of varicocele. Daily administration of resveratrol to rats with varicocele from peripuberty improves sperm quality in the adulthood.

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Introduction

Infertility affects about 15% of couples trying to conceive and the male factor contributes to about half of these cases (Shefi & Turek 2006). In fact, male infertility is a problem that is growing steadily, so that currently about 8% of men at reproductive age seek medical help (Miyaoka & Esteves 2012). In this context, varicocele plays an important role as it is the most common cause of male infertility, affecting from 15 to 20% of healthy adults and adolescents worldwide (Jarow *et al*. 1996, Shefi & Turek 2006, Agarwal *et al*. 2012), prevailing in 35% of cases of male primary infertility and in 80% of cases of secondary male infertility (Jarow *et al*. 1996). Studies have already suggested that it disturbs from 25 to 40% of the semen analyses (Dohle *et al*. 2005).

Varicocele is an abnormal dilatation of the pampiniform venous plexus in the scrotum (Casey & Misseri 2015). It provokes a deficient blood supply, which results in venous stasis, causing hyperthermia and chronic hypoxia. In normal conditions, high rates of cell division in the spermatogenic process imply high rates of mitochondrial oxygen consumption by the seminiferous epithelium. Therefore, the poor vascularization of the testicular tissue, typically occurred in varicocele, reduces oxygen rates, then compromising mitochondrial metabolism (Aitken & Roman 2008, Ferramosca *et al*. 2015). As a consequence, an increase in reactive oxygen species (ROS) production may occur, which directly affects spermatogenesis (Naughton *et al*. 2001, Reyes *et al*. 2012) and increases germ cell apoptosis, necrosis and degeneration (Nasr Esfahani & Tavalaee 2012, Reyes *et al*. 2012). Thus, in the characteristic pathological process of varicocele, male infertility and oxidative stress are closely linked (Hendin *et al*. 1999, Miyaoka & Esteves 2012). Oxidative stress takes place when ROS levels are greater than the intracellular antioxidants can neutralize (Kefer *et al*. 2009). Although low oxygen tensions characterize the testicular microenvironment, this tissue remains vulnerable to oxidative stress due to its abundance of polyunsaturated fatty acids (PUFA) and the presence of ROS-generating systems. Besides that, other metabolites may also have a negative impact on

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sperm quality when ROS production exceeds the body's antioxidant defenses, which represents a threat to the overall capacity of fertilization (Aitken & Roman 2008, Aitken *et al*. 2014). Considering that the germinal cell lineage is highly sensitive and easily compromised by high levels of ROS (Aprioku 2013), it is also expected that oxidative stress harms the DNA of germ cells (Aitken *et al*. 2014) as well as the sperm membrane integrity, which is very vulnerable to lipid peroxidation (Hall *et al*. 1991).

In this context, treatment with antioxidants can be an alternative to reduce this damage or an adjunctive therapy for male infertility, including varicocele-related infertility (Hamada *et al*. 2013). The intake of foods rich in antioxidants by men with high levels of sperm DNA fragmentation and increased levels of ROS in the semen can improve pregnancy rates (Gil-Villa *et al*. 2009).

Resveratrol, a natural phytoalexin present in more than 70 kinds of fruits and leaves of edible plants (Harikumar & Aggarwal 2008), has beneficial properties such as (a) antioxidant, by inhibiting lipid peroxidation (Gulcin 2010), (b) anti-inflammatory, (c) vasodilator, (d) anti-cancer (Jang *et al*. 1997), (e) estrogenic (Frémont 2000, Bhat KPL *et al*. 2001, de la Lastra & Villegas 2005) and (f) antiapoptotic (Jang & Surh 2001).

Indeed, treatment with resveratrol was able to reduce the histopathological damage and apoptosis occurrence in testicular tissue of rats with lesions caused by ischemia–reperfusion at the left side (Yulug *et al*. 2014). Some other studies also point to a reduction in the occurrence of apoptosis in germ cells and in the testicular lipid peroxidation in rats treated with doxorubicin and resveratrol (Türedi *et al*. 2015). In addition, a partial reversion of the testicular damage has been observed in resveratrol-treated rats in experimentally induced varicocele from adulthood (Abdel-Dayem 2009). Also the sperm production in healthy rats is increased by resveratrol due to its antioxidant potential (Juan *et al*. 2005).

The antioxidant action of resveratrol is related to the stimulation of the production of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, by modulating various signaling pathways that regulate different molecular targets (Gambini *et al*. 2015). Resveratrol also acts preventing the oxidation of low-density lipids and increasing the "sequestration" of free radicals; thus, it acts as a protector of cell membranes by reducing the deleterious effects of oxidative stress (Frémont 2000, Bhat KPL *et al*. 2001, Saiko *et al*. 2008, Gambini *et al*. 2015). Resveratrol also seems to interact with many different proteins, including cyclooxygenases, ribonucleotide reductase, kinases and DNA polymerases (Saiko *et al*. 2008), as well as to participate in many pathways and sets of intracellular events; therefore, it may constitute an interesting adjuvant therapy to the treatment of varicocele.

In the present research, we propose to investigate the antiapoptotic and antioxidant potential protective effects of RES against the spermatogenic damage caused by varicocele in rats, as well as its ability to improve sperm qualitative parameters such as sperm morphology, motility, mitochondrial activity, acrosome integrity and DNA integrity. The rat is a good model for varicocele induction as the insertion of its left spermatic vein in the left renal vein is positioned at a 90° angle, which is anatomically similar to humans. In consequence, a partial ligature of the left renal vein can be surgically performed, inducing an increase in the venous pressure, resulting in the venous reflux into the left vein; this is further reflected in the pampiniform plexus, simulating what happens in teenager patients with varicocele (Saypol *et al*. 1981, Turner 2001, Zhang *et al*. 2008). As the sprouting of varicocele usually coincides with the onset of puberty (Skoog *et al*. 1997), due to the physical growth and the increase in abdominal pressure (Delaney 2004), and as such effect can produce progressive complications on the fertility (Bong & Koo 2004), we induced varicocele in rats at peripuberty and studied the reproductive parameters at adulthood.

Materials and methods

Obtainment of the animals

Totally, 70 peripubertal male Wistar rats (*Rattus norvegicus albinus*) were used. They were obtained from mating animals belonging to the Center of Development of Experimental Models for Medicine and Biology (CEDEME) of the Federal University of Sao Paulo (UNIFESP) and were maintained in a local animal facility, where they were housed in polypropylene cages (40 × 30 × 15 cm) under shavings and appropriate conditions of hygiene, temperature (22°C ± 2°C), humidity (45–55%) and light (12 h light:12 h darkness). Rat chow (Nuvilab CR-1 feed, Nuvital) and water were provided *ad libitum*. This study followed the ethics principles adopted by the Brazilian College of Animal Experimentation and the experiment was approved by the Ethics Committee for Animal Research of the Federal University of Sao Paulo, Brazil (CEUA no. 419454).

Animals and their distribution into the groups

Peripubertal 41-day-old rats were randomly divided into four groups: varicocele (V; $n = 21$), varicocele–resveratrol (V-RES; $n = 21$), resveratrol (RES; $n = 14$) and sham–control (SC; $n = 14$). The groups V and V-RES were composed by animals submitted to experimental left varicocele (ELV), which was induced by a standard surgical procedure (Turner 2001) at the age of 41 days, under anesthesia (ketamine–xylazine 4:1) (Dopalen–Anasedan - Ceva, Sao Paulo, Brazil).

Resveratrol was given in a daily dose (300 mg/kg body weight; by gavage) to the animals of the groups V-RES and RES, from 42 to 100 days of age. The phytoalexin was obtained in its most stable form, trans-resveratrol (Domínguez *et al*. 2001, Chen *et al*. 2007, Das *et al*. 2008), which is extracted from the

roots of *Polygonum cuspidatum* (Sieb-Xi'an Pharmpro Union Co., Ltd., Xi'an, China). Trans-resveratrol is stable for at least 42 h (Trela & Waterhouse 1996). The animals of the sham–control group were submitted to the same surgical procedure, at 41 days of age; however, they were not submitted to the partial ligature of the left renal vein. Carboxymethyl cellulose (resveratrol's diluent) was also given by gavage to these rats from 42 to 100 days of age. All animals that were submitted to a surgical procedure (V, V-RES and SC) were carefully monitored and orally treated with Ibuprofen at a dose of 20 mg/kg body weight (gavage) (Hawk *et al*. 2005) for five subsequent days after the surgery. Only animals that showed significant dilatation of the left spermatic vein on the day of killing (100 days of age) were used to compose the V and V-RES groups. According to Turner (2001), this technique has about 10% failure in the formation of varicocele. The rats that presented no dilatation of the left spermatic vein or that had renal atrophy were excluded from the experiment (about 14%).

Biometric and histological analyses

The testes, epididymides, prostate and seminal vesicles (empty and full) were collected and weighed using a semi-analytical electronic scale (Marte-AS1000). The testicular volume was acquired according to the Scherle's method (Scherle 1970). The relative weight of the testes, prostate and seminal vesicles were calculated (organ weight/100 g body weight). As testis is an ellipsoid of revolution organ, the measurements of the major and minor axes of both testes (Miraglia & Hayashi 1993) were also obtained using a digital caliper (INSIZE-1112-150). In the groups submitted to ELV (V and V-RES groups), left (side of the varicocele surgery, i.e., ipsilateral side) and right (contralateral side to the surgical procedure) testes were separately analyzed and respectively denominated in the results using the acronyms Vl, Vr, Vl-RES and Vr-RES

The testes were fixed by 24h immersion in Bouin's fixative. In sequence, testicular fragments were processed for paraffin-embedding. For histopathological analysis, two isotropic 4 µm-thick testicular sections were obtained and submitted to the periodic acid–Schiff histochemical method and counterstained with hematoxylin (PAS+H). Two sections were analyzed per animal using a Leica Qwin-V3 image analysis system (Leica - Cambridge, UK) under either ×40 or ×100 objective lenses; the testicular sections were completely scrutinized and the images captured using a digital camera connected to a light microscope. In addition, 7 µm-thick testicular sections were obtained and submitted to the TUNEL *(Terminal deoxynucleotidyl transferase-mediated dUPT Nick-End Labeling)* immunoenzymatic method for labeling of apoptotic cells and a subsequent stereological analysis as described previously by Stumpp *et al*. (2004).

Sperm morphology

To evaluate the sperm morphology, samples of 3 µL fresh fluid were collected from the epididymal cauda and homogenized in 2 mL distilled water. From this homogenate, smears were obtained on glass slides and stained by Schorr/hematoxylin method (Shorr 1941). The morphological analysis of the sperms contained in these smears was processed under light microscope at 500× final magnification. Two-hundred sperms were randomly evaluated and the morphological abnormalities (head shape, isolated heads and tail defects) were performed according to the description of Filler *et al*. (1993) and Miranda-Spooner *et al*. (2016).

Sperm mitochondrial activity

The mitochondrial activity was assessed using the 3,3′-diaminobenzidine (DAB) staining method proposed by Hrudka (1987). This technique is based on the oxidation of 3,3′-diaminobenzidine by the cytochrome *c* oxidase enzyme, when the reactant is polymerized and deposited on mitochondrial sheath along the sperm midpiece (Hrudka 1987). Initially, a sample of 50 mL epididymal cauda fluid was added to a solution containing 1 mg/mL DAB in phosphate buffer solution (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH = 7.4) in a ratio of 1:2 and incubated for 1 h, in the dark and in a water bath at 37°C. Negative controls were done to avoid nonspecific staining of the intermediate piece; for this goal, an aliquot of epididymal fluid was previously inactivated at 70°C, for 5 min. After incubation, 10 µL of the aliquots were smeared on glass slides (Precision Glass Line, Shanghai, China) and air dried; after drying, the smears containing the sperms were fixed in 10% formaldehyde for 10 min, washed with distilled water and allowed to dry again at room temperature. Subsequently, microscope coverslips were placed on a synthetic resin (Novo Entellan - Merck KGaA, Darmstadt, Germany).

Two-hundred sperms were analyzed under a phase-contrast microscope (Olympus BX-51 – Tokyo, Japan). The evaluation was based on the classification proposed by Hrudka (1987), as formerly commented: class I (intermediate piece with 100% of staining), class II (over 50% of staining in the intermediate piece), class III (less than 50% of staining in the intermediate piece) and class IV (absence of staining in the intermediate piece).

Acrosome integrity

Soon after the withdrawal of the epididymis, its caudal portion was dissected, isolated and cleaned. Sperm samples were collected and stored as described previously by Vendramini *et al*. (2012).

The acrosome integrity was assessed based on the methodology described by Varisli *et al*. (2009), with some minor modifications. To determine the acrosome integrity, we carried out the staining using 'PNA Lectin from *Arachis hypogaea* (peanut), Alexa Fluor 488 Conjugate' (Molecular Probes).

On the day of analysis, samples were thawed and transferred (50 mL each) to a catalogued Eppendorf dark tubes containing Alexa Fluor 488 100 mL PNA (20 ng/mL). The samples with dye were incubated at 37°C for 30 min; 20 µL samples were seeded on glass microscope slides and analyzed in an epifluorescence microscope Nikon Eclipse Cl (Nikon) with appropriate filter. Two-hundred sperms were quantified per slide and were classified as: (a) intact (sperm exhibiting intense and moderately bright fluorescence in the acrosome region) and (b) altered (sperm showing weak fluorescence, irregular or absent in the acrosome region).

Table 1 Testicular and epididymal biometric analysis obtained from control and experimental groups (SC, RES, V and V-RES) when they were 100 days old, considering each side (contralateral, r; ipsilateral, l) in the ELV induction (Vr and Vr-RES; Vl and Vl-RES). Data are presented as mean ± s.d. or as median (IQR: Q1–Q3).

	Groups ($n = 7$)					
Parameters	SC	RES	Vr	Vl	Vr-RES	Vl-RES
Epididymis						
Absolute weight (mg)[a]	644.29 ± 48.94	631.43 ± 60.12	650.00 ± 78.95	684.29 ± 31.55	612.86 ± 77.83	610.26 ± 82.26
Relative weight (mg%)[a]	0.16 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.18 ± 0.02	0.16 ± 0.02	0.16 ± 0.01
Testicular						
Absolute weight (g)[b]	1.70 (1.66–1.80)	1.72 (1.66–1.8)	1.62 (1.50–1.73)	1.68 (1.57–1.75)	1.70 (1.64–1.75)	1.76 (1.71–1.80)
Relative weight (g%)[b]	0.43 (0.42–0.46)	0.46 (0.43–0.46)	0.40 (0.38–0.46)	0.43 (0.39–0.50)	0.43(0.42–0.47)	0.45 (0.44–0.49)
Volume (cm^3)[b]	1.63 (1.51–1.70)	1.58 (1.52–1.65)	1.43 (1.39–1.56)	1.50 (1.38–1.55)	1.54 (1.51–1.60)	1.55 (1.50–1.60)
Major axis (mm)[a]	20.68 ± 0.55	21.03 ± 0.86	19.53 ± 0.91[c]	19.85 ± 0.63[d]	20.55 ± 0.57	21.15 ± 0.61
Minor axis 1 (mm)[a]	11.55 ± 0.37	11.21 ± 0.59	10.96 ± 0.46	11.13 ± 0.76	11.13 ± 0.30	11.18 ± 0.30
Minor axis 2 (mm)[a]	12.19 ± 0.48	12.35 ± 0.25	11.98 ± 0.54	12.09 ± 0.70	11.97 ± 0.25	12.08 ± 0.22

[a]ANOVA and SNK. [b]Analysis of Kruskal–Wallis and Dunn's test. [c]Vr < SC, RES, Vr-RES and Vl-RES; [d]Vl < RES and Vl-RES ($P < 0.05$).

Sperm motility

The sperm motility was evaluated using a computerized sperm analysis system (computer-assisted sperm analysis – CASA) and following the methodology described by Klinefelter *et al.* (1991), with some modifications (Miranda-Spooner *et al.* 2016). For this goal, after the dissection, the epididymal cauda was removed and subsequently transferred to 35 mm culture plates, containing Hank's balanced salt solution (HBSS - 136,8 mM NaCl, 5,4 mM KCl, 0,3 mM Na2HPO4, 0,5 mM KH2PO4, 12 mM NaHCO3, pH 7,3), which was supplemented with 2 mg/mL BSA. The plates were left for 30 min at 37°C in order that the spermatozoa could move into the culture medium. Then, an aliquot of this medium containing sperms was drawn by capillary action into a pre-warmed chamber slide with a depth of 100 µm (LEJA Standard Count, Nieuw-Vennep, The Netherlands). The slides containing the sperms were placed into a Hamilton Thorne Integrated Visual Optical System (HTM-IVOS; Hamilton-Thorne Research, Beverly, MA, USA) with specific software for rat sperm analyses (Toxicology System, version 12, Hamilton-Thorn Biosciences). The motion parameters investigated were percentage of motile sperm, curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN) and straightness (STR).

Sperm DNA fragmentation (comet assay)

The sperm DNA fragmentation was evaluated using the Comet assay as described previously (Codrington *et al.* 2004, Vendramini *et al.* 2012) and with minor changes (Miranda-Spooner *et al.* 2016).

Samples containing sperm, from the same pool used for the acrosome reaction, were thawed and diluted in 0.5% low melting point agarose (LGC Laboratories, Sao Paulo, SP, Brazil), pre-warmed (37°C) at a concentration of 4×10^6 cells/mL. An aliquot (125 µL) of the solution was placed on slides pre-coated with 1% normal melting point agarose (LGC Laboratories, Sao Paulo, SP, Brazil). The second lysis buffer (37°C), which contained proteinase K (0.1 mg/mL), was used to cover the slides for 2 h and 30 min at 37°C. The horizontal electrophoresis was performed using TBE buffer at 30 V (1.3 v/cm) (Vendramini *et al.* 2013). Finally, the slides were fixed with pre-chilled

Table 2 Biometric analysis of testis, epididymis, seminal vesicle and prostate obtained from control and experimental groups (SC, RES, V and V-RES) when they were 100 days old, considering the both testes in ELV induction (V and V-RES groups). Data are presented as mean ± s.d. or as median (IQR: Q1–Q3).

	Groups ($n = 7$)			
Parameters	SC	RES	V	V-RES
Body weight (g)[a]	404.29 ± 31.47	388.43 ± 37.10	383.43 ± 35.35	374.86 ± 36.34
Absolute epididymis weight (mg)[a]	644.29 ± 48.94	631.43 ± 60.12	667.14 ± 30.39	611.43 ± 76.90
Relative epididymis weight (mg%)[a]	0.16 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.01
Absolute ventral prostate weight (mg)[a]	588.57 ± 49.81	577.14 ± 134.37	648.57 ± 164.66	528.57 ± 115.82
Relative ventral prostate weight (mg%)[a]	0.15 ± 0.02	0.15 ± 0.03	0.17 ± 0.03	0.14 ± 0.03
Absolute full seminal vesicle (g)[b]	1.15 (1.05–1.22)	1.31 (1.14–1.46)	1.25 (1.06–1.50)	1.26 (1.22–1.29)
Relative full seminal vesicle (g%)[a]	0.29 ± 0.04	0.35 ± 0.08	0.34 ± 0.07	0.33 ± 0.03
Absolute empty seminal vesicle (mg)[b]	540 (450–580)	520 (480–550)	550 (470–570)	480 (440–570)
Relative empty seminal vesicle (mg%)[b]	0.14 (0.11–0.15)	0.13 (0.12–0.15)	0.14 (0.12–0.16)	0.13 (0.11–0.16)
Testicular				
Absolute weight (g)[a]	1.75 ± 0.12	1.75 ± 0.12	1.63 ± 0.13	1.72 ± 0.06
Relative weight (g%)[b]	0.43 (0.42–0.46)	0.46 (0.43–0.46)	0.42 (0.39–0.47)	0.44 (0.43–0.48)
Volume (cm^3)[a]	1.62 ± 0.11	1.62 ± 0.13	1.44 ± 0.12[c]	1.55 ± 0.07
Major axis (mm)[a]	20.68 ± 0.55	21.03 ± 0.86	19.69 ± 0.60[d]	20.84 ± 0.53
Minor axis 1 (mm)[a]	11.55 ± 0.37	11.21 ± 0.59	11.05 ± 0.48	11.16 ± 0.27
Minor axis 2 (mm)[a]	12.19 ± 0.48	12.35 ± 0.25	12.04 ± 0.53	12.02 ± 0.21

[a]ANOVA and SNK. [b]Analysis of Kruskal–Wallis and Dunn's test. [c]V < SC and RES; [d]V < SC, RES and V-RES ($P < 0.05$).

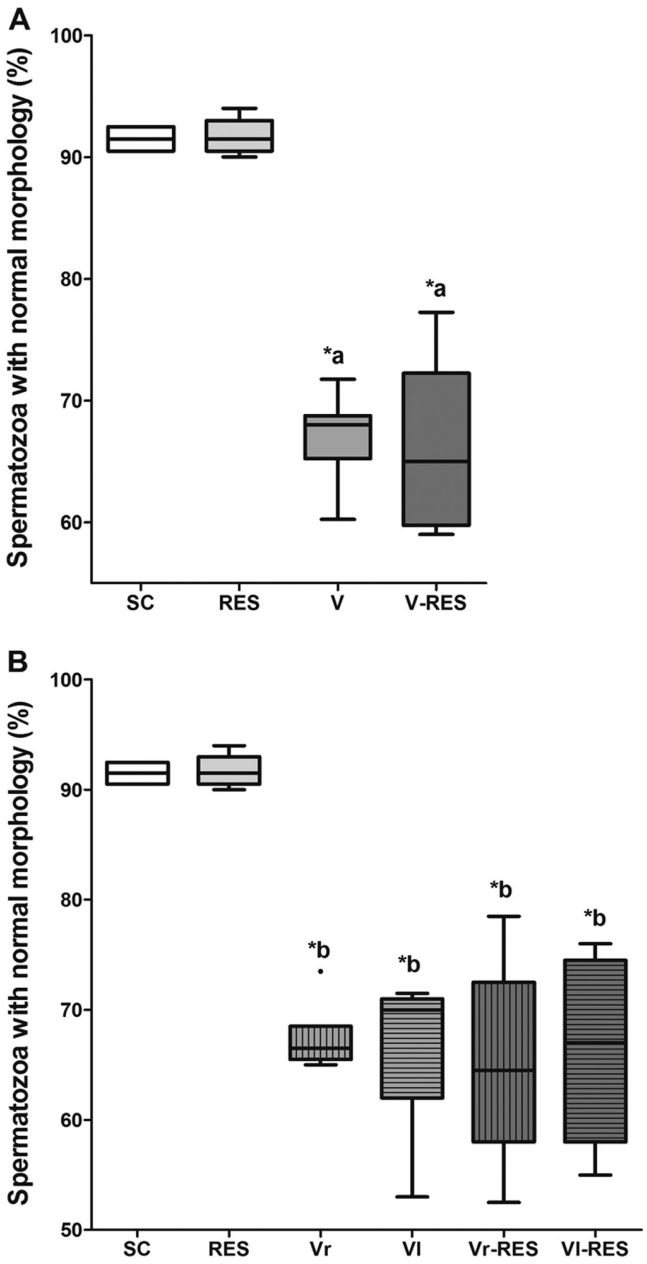

Figure 1 Percentage of spermatozoa with normal morphology in SC, RES, V, and V-RES groups (A) and in SC, RES, Vr, Vl (sides in the V group), Vr-RES, and Vl-RES (sides in the V-RES group) (B) ($n = 7$). Values are expressed as median and interquartile range (Q1 – Q3). Analysis of Kruskal–Wallis and Dunn's test ($P < 0.05$). *a SC and RES > V and V-RES; *b SC and RES > Vr, Vl, Vr-RES and Vl-RES.

70% ethanol, air dried and stored in the dark until analysis (Haines *et al*. 2002).

On the day of analysis, the samples were stained with ethidium bromide (2 µL/1.5 mL PBS/slide) and analyzed under a epifluorescence microscope Nikon Eclipse Cl (Nikon - Tokyo, Japan); 50 cells were analyzed per slide (two slides per animal), using the specific image analysis system for Comet assay (Comet Assay LUCIA, v.7.02.00, Praha, Czech Republic). The following parameters were analyzed: DNA in the cauda (%), tail length and tail extent moment (tail length × cauda DNA%/100) and olive tail moment (cauda DNA% × a fraction of the tail length/100).

Analysis of the lipid peroxidation, at the testicular level, by dosage of malondialdehyde (MDA)

On the day of killing, the testes were removed and decapsulated and 0.1 g samples were frozen at −20°C. These samples were thawed and homogenized in 1.15% KCl solution.

A sample of 0.1 mL of the testicular homogenate was added to a tube containing 1.5 mL acetic acid (20%, pH 3.5), 0.2 mL sodium dodecyl sulfate (SDS: 8.1%), 1.5 mL 2-thiobarbituric acid (TBA: 0.8%) and 0.7 mL water. The tubes were shaken and incubated in a water bath at 95°C for 60 min. Subsequently, the tubes were cooled and centrifuged at 1878 g for 10 min. The samples were placed in plates for analysis using the Enzyme-Linked Immunosorbent Assay (ELISA) and the absorbance was measured at 532 nm. The concentrations are expressed as malondialdehyde nmol/g tissue according to the methodology described by Abd-Allah *et al*. (2009).

Statistical method

The results were statistically analyzed using the Sigma Plot 12.0 software. The values with normal distribution were analyzed according to the parametric test one-way analysis of variance (ANOVA) aiming to compare the different groups (SC, RES, V and V-RES). When the differences were significant, the Student–Newman–Keuls test (SNK) was also used. However, when the values did not show normal distribution and similarity of variance, they were submitted to the non-parametric test of analysis of variance, the Kruskal–Wallis test; in this case, when the results were significant, a post-test for multiple comparisons was applied (Dunn's test or the SNK test). Differences were considered significant when $P < 0.05$.

Results

Resveratrol promoted some improvement of testis volume in varicocelized rats

Tables 1 and 2 show the body weight and the biometric data of testes, epididymides, seminal vesicles (full and empty) and prostate obtained from the control and experimental groups. In Table 1, the testicular and epididymal data were presented considering the ipsilateral (l) and contralateral (r) sides to the surgery of varicocele induction.

Our results showed a significant reduction of testicular volume in varicocelized rats (V group) in comparison with sham–control (SC) and resveratrol (RES) groups. The treatment with resveratrol given to varicocelized rats (V-RES group) showed a trend of improvement of this parameter; although not significant ($P = 0.078$), the mean testicular volume was higher than in V group. In addition, it did not differ from the SC and RES groups (Table 2).

The reduction of testicular volume in the V group, in comparison to the SC and RES groups, was a result of the

Table 3 Analysis of mitochondrial activity in control and experimental groups (SC, RES, V, and V-RES) when they were 100 days old, considering the both testes in ELV induction (V and V-RES). Data are presented as mean ± S.D. or as median (IQR: Q1–Q3).

Groups ($n=7$)	**Class I** (%)[a]	**Class II** (%)[a]	**Class III** (%)[b]	**Class IV** (%)[b]
SC	89.93 ± 4.77	8.86 ± 4.0	0.5 (0.0–3.0)	0.0 (0.0–0.0)
RES	93.86 ± 3.35	5.36 ± 2.85	1.0 (0.0–1.5)	0.0 (0.0–0.0)
V	38.18 ± 8.48[c]	44.79 ± 4.79[d]	15.5 (9.25–19.75)[d]	1.5 (0.25–2.0)[e]
V-RES	63.57 ± 9.17[c]	29.21 ± 6.04[d]	5.75 (3.5–7.25)[d]	0.25 (0.0–1.0)

[a]ANOVA and SNK. [b]Analysis of Kruskal–Wallis and Dunn's test. [c]V < V-RES < SC and RES; [d]V > V-RES > SC and RES; [e]V > SC and RES ($P<0.05$).

reduction occurred in its major axis (Table 2), as the testis is an ellipsoid model. This change also occurred when each side of the testis was individually analyzed (Table 1).

The incidence of sperm anomalous forms found in varicocelized rats was not improved by resveratrol

The frequency of sperms with normal morphology was lower in the varicocelized rats (V and V-RES groups) than in the SC and RES groups (Fig. 1A). When each epididymis side (ipsilateral and contralateral) was individually considered, differences were still evident in both epididymides in comparison to the SC and RES groups (Fig. 1B). Many types of abnormalities of head (banana-shaped, detached, backward bent) and of tail (coiled, bent, broken) were observed in the spermatozoa of varicocelized rats both in V and V-RES groups. Besides, spermatozoa with multiple defects were also noted in these groups but very rarely observed in the SC and RES groups. The most frequent changes that occurred in V and V-RES groups were bent tail, sperm head detachment from its tail and banana-shaped head sperm.

Resveratrol improved sperm mitochondrial activity in varicocelized rats

The rats of the V and V-RES groups showed alterations in the mitochondrial activity when compared with the SC and RES groups such as (i) increased frequencies of class II (more than 50% of the midpiece was stained) and class III sperm (less than 50% of the midpiece was stained) and (ii) reduced frequency of class I sperm (100% of the midpiece was stained). These results showed that varicocelized rats of both groups had higher number of sperms with inactive mitochondria in comparison to SC and RES groups. However, in the varicocelized rats treated with resveratrol (V-RES group), the quantity of sperms with active mitochondria (class I) was higher than in the V group, whereas the frequencies of class II and III sperms were lower than in rats that did not receive the treatment (Table 3).

However, the frequency of class IV sperms (absence of mitochondrial activity) was just altered in the V group.

The same alterations were observed when each epididymis cauda side was individually analyzed (Table 4), producing the same relationships among the groups as described above. When the mitochondrial activity was measured in the sperms from the ipsilateral side and it was compared with the contralateral side, no significant alterations of these parameters (class I–IV sperms) were observed in the varicocelized animals of the same group (Vl-RES × Vr-RES and Vl × Vr).

Resveratrol raised sperm total motility to control levels in varicocelized rats

As shown in Table 5, the treatment with resveratrol improved the sperm motility of the varicocelized (V-RES group) as it was higher than the one noted in the V group and raised sperm total motility to control levels (Table 5).

When each epididymis was individually analyzed, sperms collected from the contralateral epididymal cauda of the varicocelized rats treated with resveratrol (Vr-RES group) showed higher motility when compared with the sperms from both ipsilateral (Vl) and contralateral (Vr) sides of the varicocelized rats, which were not treated with resveratrol (V group). Also, there was an improvement in the sperm motility from the ipsilateral epididymal cauda in the group treated with resveratrol (Vl-RES) in comparison to the ipsilateral side of the V group (Vl) (Table 6).

Table 4 Analysis of mitochondrial activity from control and experimental groups (SC, RES, V and V-RES) when they were 100 days old, considering each side (contralateral, r; ipsilateral, l) in the ELV induction (Vr and Vr-RES; Vl and Vl-RES). Data are presented as mean ± S.D. or as median (IQR: Q1–Q3).

Groups ($n=7$)	**Class I** (%)[a]	**Class II** (%)[b]	**Class III** (%)[b]	**Class IV** (%)[b]
SC	89.93 ± 4.77	10.0 (4.5–12.5)	0.5 (0.0–3.0)	0.0 (0.0–0.0)
RES	93.86 ± 3.35	5.0 (2.5–7.5)	1.0 (0.0–1.5)	0.0 (0.0–0.0)
Vr	39.71 ± 8.28[c]	45.0 (40.5–46.5)[d]	10.5 (9–19.5)[d]	1.5 (0.5–3.0)[e]
Vl	36.64 ± 10.72[c]	44.0 (41–48.5)[d]	17.0 (11.5–21.5)[d]	1.5 (0.5–2.0)[e]
Vr-RES	64.00 ± 10.21[c]	28.0 (25.0–30.0)[d]	5.0 (2.5–8.0)[d]	0.0 (0.0–1.5)
Vl-RES	63.14 ± 10.80[c]	32.0 (30.5–37.5)[d]	6.0 (4.5–9.0)[d]	0.5 (0.0–0.5)

[a]ANOVA and SNK. [b]Analysis of Kruskal–Wallis and Dunn's test. [c]Vr and Vl < Vr-RES and Vl-RES < SC and RES; [d]Vr and Vl > Vr-RES and Vl-RES > SC and RES; [e]Vr and Vl > SC and RES ($P<0.05$).

Table 5 Data on sperm motility (CASA HTM-IVOS) of animals in the control and experimental groups when they were 100 days old.

	Groups ($n=7$)			
Parameters	SC	RES	V	V-RES
Motility (%)[a]	97 (94–97)	98 (94–98)	87 (78–89)[c]	96 (94–97)
VAP (μm/s)[b]	247.9 (238.8–265.9)	247.8 (215.4–259.3)	234.8 (222.4–250.2)	255.1 (244.5–264.9)
VSL (μm/s)[b]	198.5 (180.1–203.7)	186.6 (167.3–193.9)	175.5 (167.7–184.3)	192.8 (184.3–201.1)
VCL (μm/s)[a]	377.3 ± 40.4	368.3 ± 35.74	340.1 ± 27.8	383.8 ± 20.22
ALH (μm)[b]	13.9 (11.4–15.2)	13.4 (12.5–14.9)	11.5 (11.2–11.9)	14.2 (12.8–14.6)
BCF (Hz)[a]	13.33 ± 2.2	13.9 ± 2.52	13.65 ± 2.61	13.32 ± 1.08
STR (%)[a]	73.29 ± 2.43	72.87 ± 2.03	75.0 ± 2.14	72.91 ± 0.86
LIN (%)[a]	51.29 ± 2.14	50.12 ± 2.64	53.64 ± 2.37	50.68 ± 1.33

[a]ANOVA and SNK. [b]Analysis of Kruskal–Wallis and Dunn's test. [c]SC, RES and V-RES > V ($P<0.05$). VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity.

Resveratrol improved acrosome integrity in rats with varicocele

Using the staining with PNA, our data show that treatment with resveratrol in varicocelized rats (V-RES group) promoted an improvement of acrosome integrity compared with the V group (Fig. 2A). However, both the varicocelized rats (V and V-RES groups) had a lower frequency of sperms with acrosome integrity than the sham–control and resveratrol groups. Significant differences relating to this parameter were not observed when sperms originated from both ipsilateral and contralateral epididymal cauda of the V and V-RES groups were compared (Fig. 2B).

Resveratrol reduced sperm DNA fragmentation caused by varicocele

The Comet assay showed that the V group had an increase in all parameters relating to the DNA fragmentation (tail DNA percentage, comet tail length, comet tail extent moment and Olive tail moment) when compared with the control groups (SC and RES) and with the varicocelized rats treated with resveratrol (V-RES group), showing that resveratrol reduced sperm DNA fragmentation produced by experimental varicocele (Table 7).

In a similar way, sperms collected from the ipsilateral and contralateral epididymides in varicocelized rats (Vl and Vr respectively) had higher values of tail DNA percentage, comet tail length and comet tail extent moment when compared with those collected from the both sides (right and left) of the epididymis of the V-RES group (Vr-RES and Vl-RES) and to the control groups (SC and RES) (Table 8).

No significant reduction in testicular concentration of malondialdehyde was provided by resveratrol in varicocelized rats

The malondialdehyde, a marker for lipid peroxidation, had increased levels in both of the varicocelized groups (V and V-RES) when compared with SC and RES groups (Fig. 3A). Data analyzed in each testis individually had a similar relationship among the varicocelized and control groups, as described above (Fig. 3B).

Resveratrol reduced germ cell apoptosis in rats with varicocele

TUNEL-positive cells were found in all control and experimental groups. The brown labeling and distribution of these TUNEL-positive cells in the testis of the rats can be observed in Figs 4 and 5 respectively.

As demonstrated in these figures, the varicocelized (V group – Figs 4C, D and 5) and varicocelized treated with resveratrol (V-RES group – Figs 4E, F and 5) groups had a significantly higher number of TUNEL-positive cells than the SC (Figs 4A and 5) and RES (Figs 4B and 5) groups. The ipsilateral testis of rats from the varicocele

Table 6 Data on sperm motility (CASA HTM-IVOS) of animals in the control and experimental groups, also considering the laterality in varicocelized groups (Vr, Vl, Vr-RES, and Vl-RES). Data are presented as mean ± S.D. or as median (IQR: Q1–Q3).

	Groups ($n=7$)					
Parameters	SC	RES	Vr	Vl	Vr-RES	Vl-RES
Motility (%)[a]	97 (94–97)	98 (94–98)	88 (82–96)[c]	86 (61–89)[c,d]	97 (94–99)	97 (97–97.75)[d]
VAP (μm/s)[b]	247.9 (238.8–265.9)	247.8 (215.4–259.3)	236.3 (223.6–243.5)	234.1 (211.6–265.8)	245.9 (225.8–261.3)	273 (253.9–278.1)
VSL (μm/s)[b]	198.5 (180.1–203.7)	186.6 (167.3–193.9)	182.8 (170.5–186.2)	173.7 (160.5–184.1)	193.4 (168.9–196.8)	205 (192.4–207.3)
VCL (μm/s)[a]	377.3 ± 40.4	368.3 ± 35.74	344.8 ± 28.35	335.3 ± 38.10	367.5 ± 26.91	400.10 ± 44.01
ALH (μm)[b]	13.09 ± 2.80	13.67 ± 1.73	11.72 ± 1.05	11.40 ± 1.05	13.42 ± 1.40	14.37 ± 1.40
BCF (Hz)[a]	13.33 ± 2.2	13.9 ± 2.52	13.19 ± 3.52	14.13 ± 2.7	13.43 ± 1.67	13.21 ± 1.77
STR (%)[a]	73 (72–74)	72 (72–73)	74 (74–77)	75 (72–79)	73 (71–75)	72 (70–73)
LIN (%)[a]	51.29 ± 2.14	50.12 ± 2.64	53.28 ± 1.98	54.0 ± 3.83	51.0 ± 2.28	50.33 ± 2.5

[a]ANOVA and SNK. [b]Analysis of Kruskal–Wallis and Dunn's test. [c]SC, RES and Vr-RES > Vr and Vl; [d]Vl < Vl-RES ($P<0.05$). VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity.

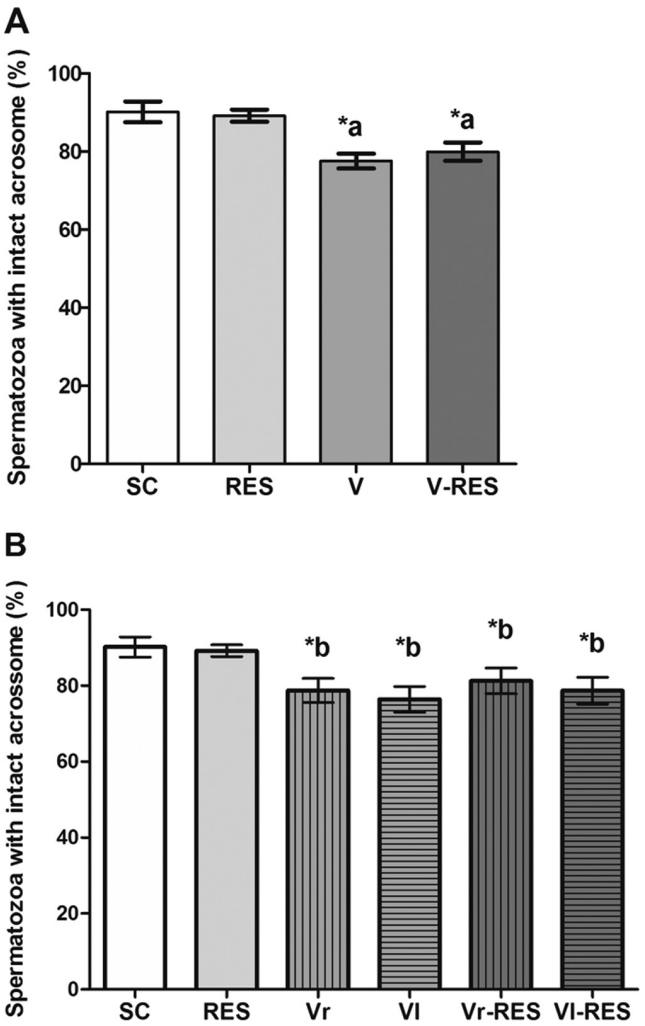

Figure 2 Percentage of spermatozoa with acrosome integrity from animals of the SC, RES, V and V-RES groups (A) and in SC, RES, Vr, Vl (sides in V group), Vr-RES and Vl-RES (sides in V-RES group) (B) ($n=7$). Values are expressed as mean and standard deviation. ANOVA and SNK. *a SC and RES > V-RES > V; *b SC and RES > Vr, Vl, Vr-RES and Vl-RES;. ($P<0.05$)

group (Vl – Figs 4C and 5) showed a higher number of TUNEL-positive cells when compared with the contralateral testis within the same group (Vr – Figs 4D and 5), as well to both testes of the V-RES group (Vr-RES and Vl-RES – Figs 4E, F and 5) and also to the SC (Figs 4A and 5) and RES (Figs 4B and 5) groups. Data obtained from each testis individually had a similar relationship among the groups varicocelized and controls as described above (Fig. 5).

Discussion

The physiopathological mechanisms of varicocele that cause infertility are still not clear (Aitken *et al*. 2014). However, oxidative stress is widely believed to be the principal underlying pathology linking varicocele with male infertility (Tremellen *et al*. 2008, Aitken *et al*. 2014).

Alterations regarding the numerical density of germ cell apoptosis and various sperm quality parameters such as mitochondrial activity, acrosome integrity, motility, frequency of morphologically normal sperms, sperm DNA integrity, as well as testicular lipid peroxidation level were noted in the varicocelized group (V group) used in this study. In addition, such alterations occurred in both ipsilateral and contralateral sides to the varicocele surgery. No disparity was observed between the results from the ipsilateral and contralateral sides of a same varicocelized group regarding almost all parameters studied, evidencing that the left-sided experimental varicocele had a comparable bilateral reproductive damage, except for the number of apoptotic germ cells, which was higher in the left testis. Thus, our data corroborate previous studies regarding

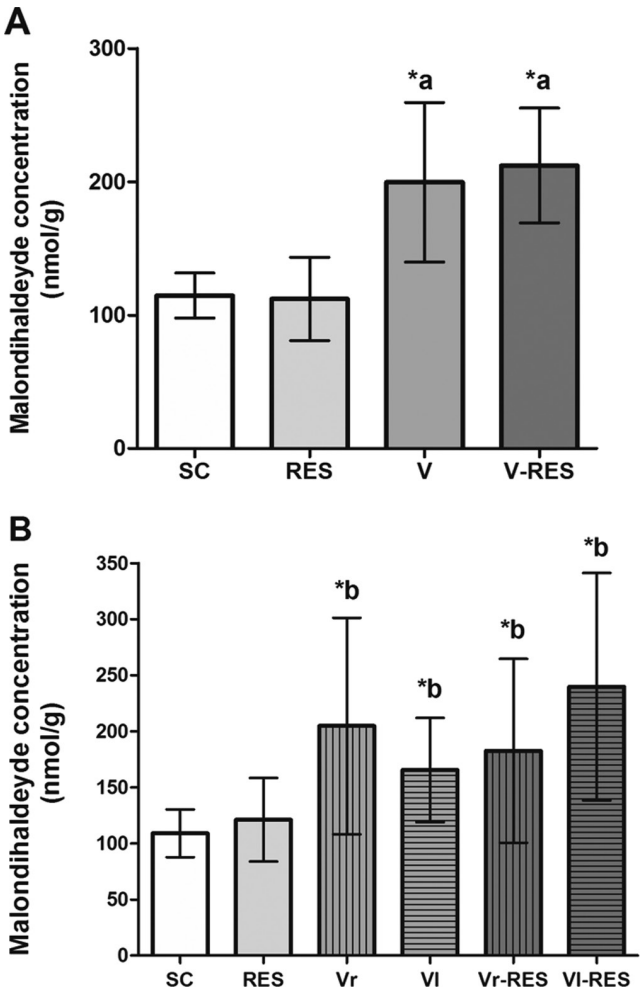

Figure 3 Testicular malondialdehyde concentration in animals of the SC, RES, V and V-RES groups (A) and in the same groups but considering the laterality in the varicocelized rats (Vr, Vl, Vr-RES and Vl-RES groups) (B) ($n=7$). Values are expressed as mean and standard deviation. ANOVA and SNK tests. *a V and V-RES > SC and RES; *b SC and RES <Vr, Vl, Vr-RES and Vl-RES ($P<0.05$).

Table 7 Sperm DNA fragmentation (Comet assay) of animals in the control and experimental groups. Data are presented as mean ± s.d. or as median (IQR: Q1–Q3).

	Groups ($n = 7$)			
Parameters	SC	RES	V	V-RES
% Tail DNA[a]	1.84 ± 0.76	1.67 ± 0.65	3.88 ± 1,06[c]	2.33 ± 0.34
Tail length (μm)[b]	8.03 (6.63–15.35)	11.66 (6.09–14.89)	22.06 (18.44–40.92)[c]	14.65 (12.58–15.47)
Tail moment[b]	0.20 (0.12–0.56)	0.22 (0.12–0.51)	1.03 (0.84–2.51)[c]	0.49 (0.39–0.53)
Olive moment[b]	0.25 (0.15–0.58)	0.20 (0.15–0.48)	0.66 (0.62–1.47)[c]	0.40 (0.29–0.47)

[a]ANOVA and SNK. [b]Analysis of Kruskal–Wallis and SNK. [c]V > SC, RES and V-RES ($P < 0.05$).

the testicular contralateral injury in rats submitted to left-sided experimental varicocele (Wang *et al*. 2010, Liu & Ding 2014).

Although oxidative stress (OS) is an important factor in the sperm lesion caused by varicocele (Aitken *et al*. 2014) and in spite of the known efficacy of resveratrol as an antioxidant (Pinto *et al*. 1999, Saiko *et al*. 2008), its capability to decrease lipid peroxidation caused by varicocele in the testicular environment and in the epididymal level were not yet investigated. In the present research, we observed a favorable action of resveratrol on reproduction of rats submitted to a left-sided surgical varicocele. Indeed, in the results presented here, resveratrol significantly improved sperm qualitative parameters in these rats. However, the present data indicated that the relatively high dose of resveratrol used here did not relieve the lipid peroxidation caused by varicocele at the testicular level. To the best of our knowledge, other biomarkers of OS should also be investigated to better clarify its antioxidant potential in this circumstance. We cannot exclude the possibility that different mechanisms of antioxidant activity may have occurred. Besides, both levels of ROS and antioxidant mechanisms vary between testicular and epididymal environments. In fact, a reduction in glutathione peroxidase activity and an elevation in the malondialdehyde levels in the epididymis of varicocelized rats was already reported (QU *et al*. 2011). However, in the study presented here, we did not assess the OS in the epididymis.

Regarding the OS imbalance and its consequences to male fertility, some remarks have been mentioned in the literature: (i) plasma membrane lipid peroxidation, which damages fluidity and permeability of the membrane and inactivates membrane-bound receptors (Tremellen 2008); (ii) reduction in hyperactivation, motility and acrosome reaction through the change in the cyclic adenosine 3′,5′-monophosphate (cAMP) (reviewed by Agarwal *et al*. 2014); (iii) direct damage on sperm DNA such as strand breaks, crosslinks, chromosomal deletions, rearrangements and polymorphism (Hosen *et al*. 2015, reviewed by Tremellen 2008, Makker *et al*. 2009).

Lipid peroxidation of the sperm cellular membrane may also affect sperm structure in many different ways – by disrupting the acrosome, the intermediate piece and the axoneme – which will interfere with the capacitation process, the acrosome reaction and sperm viability. Besides, sperms have little cytoplasm and low antioxidant status, what makes them more susceptible to the effects of direct oxidative stress (Shefi & Turek 2006). The abundant concentration of PUFA and cholesterol present in the cellular membrane of mature sperms (Ko *et al*. 2014, Mendeluk *et al*. 2015) provides the necessary fluidity for the occurrence of the fusogenic events associated with the fertilization process (Nordberg & Arnér 2001); however, it also makes them more vulnerable to oxidative stress (Lenzi *et al*. 2000). A high ROS production along with a reduction in the total antioxidant capacity (Tramer *et al*. 1998) can impair the fluidity of the plasma membrane of the sperm and raise sperm DNA damage (Miyaoka & Esteves 2012).

In varicocelized rats, all of the aforementioned membrane structures are vulnerable to the process of lipid peroxidation occurred from the reaction between ROS and PUFA, or even to autocatalytic processes that produce toxic substances such as malondialdehyde (Slater 1984, Sharma & Agarwal 1996, Benedetti *et al*. 2012). Also, an imbalance between the production of ROS and the antioxidant capacity can result in the acidification of the seminal plasma in men with varicocele, which impairs sperm motility and inhibits the activity of antioxidant enzymes (Ghabili *et al*. 2009).

Table 8 Sperm DNA fragmentation (Comet assay) of animals in the control and experimental groups. Values are expressed as median (IQR: Q1–Q3)

	Groups ($n = 7$)					
Parameters	SC	RES	Vr	Vl	Vr-RES	Vl-RES
% Tail DNA	1.62 (1.15–2.63)	1.5 (1.17–2.24)	3.74 (2.57–3.95)	4.08 (3.15–5.81)	2.08 (2.04–2.60)	2.32 (2.13–2.75)
Tail length (μm)	8.03 (6.63–5.35)	11.66 (6.09–4.89)	18.79 (16.58–41.82)	20.33 (18.67–44.16)	15.89 (12.59–16.84)	12.88 (12.57–13.87)
Tail moment	0.20 (0.12–0.56)	0.22 (0.12–0.51)	0.93 (0.65–1.87)	1.15 (0.74–3.43)[a]	0.47 (0.35–0.50)	0.51 (0.36–0.52)
Olive moment	0.25 (0.15–0.48)	0.20 (0.15–0.48)	0.87 (0.51–0.91)	0.82 (0.52–2.06)	0.43 (0.36–0.49)	0.42 (0.31–0.46)

Analysis of Kruskal–Wallis and SNK. Values are expressed as median and interquartile range (Q1–Q3); [a]Vr and Vl > SC, RES, Vr-RES and Vl-RES ($P < 0.05$).

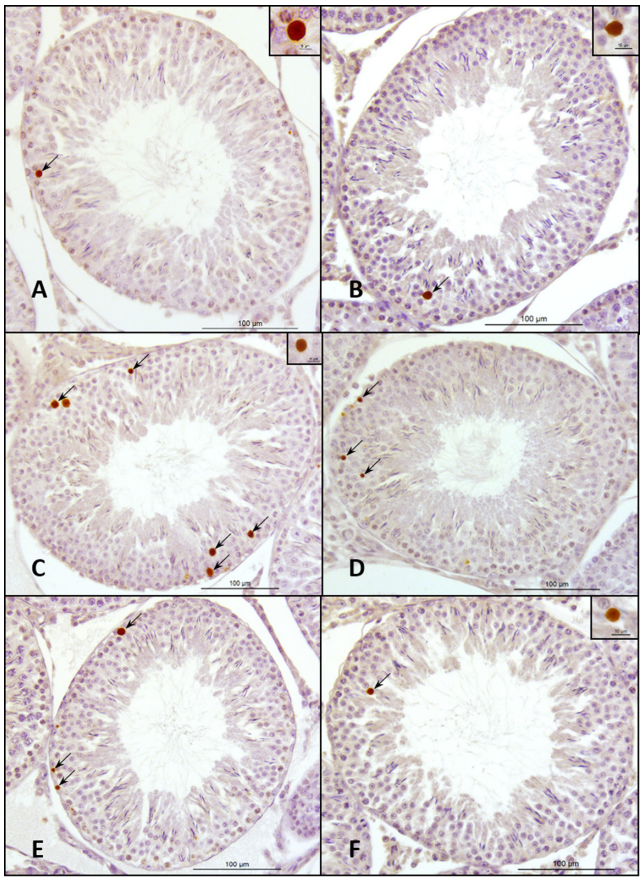

Figure 4 Photomicrographs of testicular cross sections of rats submitted to the TUNEL method, evidencing apoptotic germ cells (long arrows). Observe the seminiferous tubule sections of 100-day-old rats from the SC (A), RES (B), Vl (C), Vr (D), Vl-RES (E) and Vr-RES (F) groups, showing the TUNEL-positive cells brown-labeling; see the details. In the figures referring to the varicocele group (C and D) and varicocele treated with resveratrol (E and F), there was a greater number of labeled cells compared with sham–control group (A) and resveratrol (B). Bar 100 µm on photomicrographs and 10 µm on details.

Therefore, as a consequence of excess of ROS, reduction of sperm viability, capacitation and acrosome reaction can occur and lead to infertility.

An increase in DNA oxidative damage within spermatogonia and spermatocytes was also noted in testicular biopsies from men with varicocele-associated oxidative stress (Tremellen 2008). We believe that the general low sperm quality observed in our ELV study could be a consequence of the damage caused by varicocele-induced ROS on not only the germ cells, during spermatogenesis in the testis, but also during the maturation process, in the epididymis (Tramer *et al*. 1998).

Along with the increase in ROS level, there is an augmented rate of apoptosis both in testis and in semen of men presenting varicocele (Simşek *et al*. 1998, Hendin *et al*. 1999). Indeed, the elevation of scrotal temperature produced in this condition produces heat stress within the testicular tissue, elevates oxidative stress, impairs spermatogenesis progression, and increases germ cell apoptosis (Shiraishi *et al*. 2010). The disruption of mitochondrial membranes and the release of cytochrome *c* can also take place as a response to the increase in the oxidative stress, then activating caspases, which are effector proteases engaged in the apoptotic process (Agarwal & Saleh 2002, Saleh & Agarwal 2002, Makker *et al*. 2009). Despite the different hypotheses proposed to explain the reproductive damage caused by varicocele on male reproduction and the physiopathological mechanisms involved, they all seem to culminate in a common point: apoptosis (Wang *et al*. 2010).

In this study, an increase in the number of TUNEL-positive cells occurred in both testes, but it was higher in the ipsilateral testis. Concerning the rate of germ cell apoptosis, our data are in accordance with those of Benoff *et al*. (2004), gathered from varicocele patients,

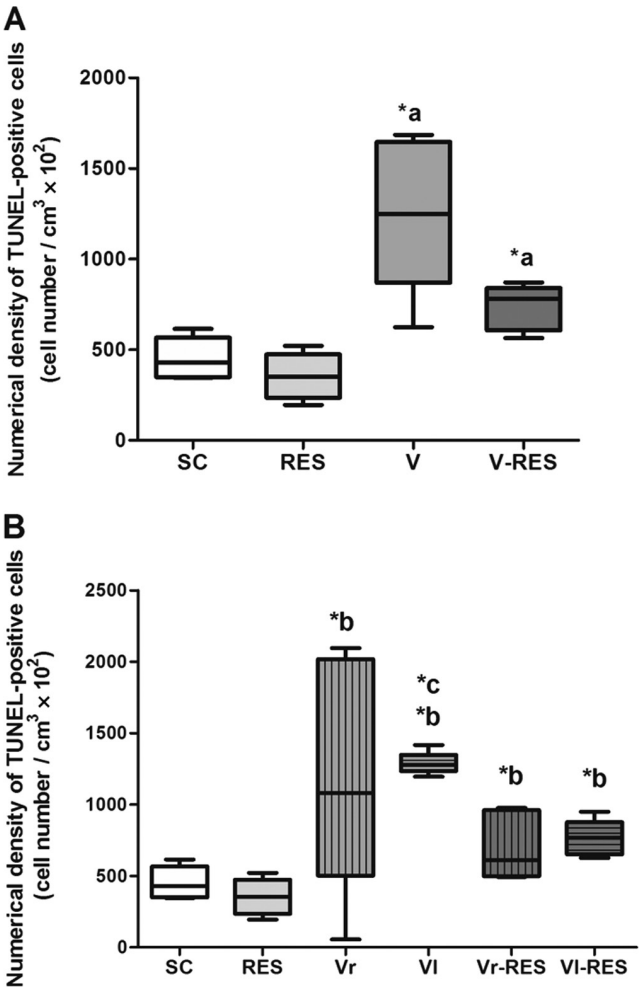

Figure 5 Box plot illustrating the distribution of data on the numerical density of TUNEL-positive cells of SC, RES, V and V-RES groups (A) and SC, RES, Vr, Vl, Vr-RES and Vl-RES groups (B) ($n = 5$). Values are expressed as median and interquartile ranges (Q1–Q3), Kruskal–Wallis analysis and SNK. *a V and V-RES > SC and RES; *b Vr, Vl, Vr-RES and Vl-RES > SC and RES; *c Vl > Vr, Vr-RES and Vl-RES.

and of Wang *et al*. (2010), who used adult varicocelized rats. However, Barqawi *et al*. (2004) observed an important increase in germ cell apoptosis only in the ipsilateral testis of varicocelized rats, in comparison to the control rats. Conversely, Lee *et al*. (2009), after examining the testes of adult varicocelized rats, found a higher frequency of apoptotic germ cells in both sides, although in the contralateral testis it happened later. Either the intervals of killing used to assess the effects of varicocele or the age when the ELV was induced could explain some divergences among the results reported by different authors, using the rat model.

In the results presented here, resveratrol reduced apoptosis rate in ipsilateral testis of varicocelized rats. Abdel-Dayem (2009) also described benefits of resveratrol to the spermatogenesis, in a histological analysis, in both testes of ELV in rats, although the extension of the effects to the germ cell death was not addressed in this study.

As germinal lineage is the major testicular component that implies in normal testicular volume, increased germ cell loss due to apoptosis leads to hypotrophy of the seminiferous epithelium, which may reflect in changes in the testicular morphometric parameters. Thus, the other important and indirect evidence linked to male infertility in varicocele patients is the reduction of the testicular volume. Indeed, testicular asymmetry is the first evidence of the presence of varicocele, especially in adolescents (Paduch & Skoog 2001). In addition, reduction of testicular size is generally an indicative of reduced spermatogenesis (Jarow 2001). Abdel-Dayem (2009) also showed that germ cell proliferation is protected by resveratrol under ELV in adult rats. Therefore, the current research corroborates the previous data, showing bilateral alterations of the testicular size in varicocelized rats and suggesting that resveratrol has potential to prevent the damage associated with the condition.

In our research, RES partially reduced testicular apoptosis and reverted alterations in sperm motility, acrosome integrity, mitochondrial activity and DNA fragmentation in the ipsilateral side to the varicocele surgery. In the contralateral side, there was also improvement of some parameters such as sperm motility, mitochondrial activity and DNA integrity. Regarding the motility and the DNA fragmentation, the sham–control, resveratrol and resveratrol/varicocelized results were close. In summary, we consider that the present results support the observations made by other authors concerning some of the most common reproductive changes in varicocele reported so far, regarding the following parameters: (i) sperm DNA integrity (Blumer *et al*. 2012, La Vignera *et al*. 2012, Tavalaee *et al*. 2014), (ii) mitochondrial function (Blumer *et al*. 2012, La Vignera *et al*. 2012), (iii) sperm chromatin condensation (La Vignera *et al*. 2012, Tavalaee *et al*. 2014), (iv) sperm normal morphology (Blumer *et al*. 2012, Tavalaee *et al*. 2014), (v) acrosome integrity (Blumer *et al*. 2012, Tavalaee *et al*. 2014) and (vi) DNA protamination and apoptosis index (La Vignera *et al*. 2012, Wang *et al*. 2010).

As mentioned previously, resveratrol improved most of the sperm quality parameters studied and acted reducing the apoptosis rate in the ipsilateral testis. Ultimately, treatment with antioxidants has been studied as an alternative or adjunctive therapy to avoid male infertility, even when related to varicocele (Hamada *et al*. 2013). Based on the present results and respecting the interspecies differences, we believe that resveratrol may represent, in the future, a valuable adjunctive compound in the treatment of patients with varicocele, which reinforces previous observations (Abdel-Dayem 2009). The reproductive performance and capacity of the animals, the epididymal oxidative stress, the DNA protamination and the PUFA level as a marker of sperm pathology are current objects of study of our group, aiming to better understand the protective role of the resveratrol and its mechanisms to prevent varicocele-related infertility.

Declaration of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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