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ORIGINAL ARTICLE

Carnitine partially improves oxidative stress, acrosome integrity, and reproductive competence in doxorubicin-treated rats

Correspondence:
Sandra Maria Miraglia, Laboratory of Developmental Biology, Department of Morphology and Genetics – Federal University of Sao Paulo (UNIFESP), Botucatu Street, 740. Leitao da Cunha Building, 2nd floor. CEP: 04023-900, Sao Paulo – SP, Brazil.
E-mail: miraglia.sm@gmail.com

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R. E. L. Cabral, T. B. Mendes, V. Vendramini and S. M. Miraglia

Laboratory of Developmental Biology, Department of Morphology and Genetics, Federal University of Sao Paulo (UNIFESP), Sao Paulo, Brazil

SUMMARY

Doxorubicin has been largely used in anticancer therapy in adults, adolescents, and children. The efficacy of L-carnitine as an antioxidant substance has been confirmed both in humans and rats. Carnitine, present in testis and epididymis, is involved in sperm maturation. It is also effective in infertility treatment. As a continuation of a previous study, we evaluated whether some spermatic qualitative parameters, DNA integrity, chromatin structure, and fertility status, could be ameliorated by the carnitine treatment in adult rats, which were subsequently exposed to doxorubicin at pre-puberty. Pre-pubertal male rats were distributed into four groups: Sham Control; Doxorubicin; L-carnitine; L-carnitine + Doxorubicin (L-carnitine injected 1 h before doxorubicin). At 100 days of age, all groups were reassigned into two sets: One set was submitted to the evaluation of sperm motility, acrosome integrity, mitochondrial activity, sperm chromatin structure analysis (SCSA), and evaluation of the oxidative stress. The other set of rats was destined to the evaluation of reproductive competence. The percentage of spermatozoa with intact acrosome integrity was higher in the Carnitine+Doxorubicin group when compared with the Doxorubicin group. However, sperm motility and mitochondrial activity were not improved by carnitine pre-treatment. Both values of malondialdehyde and nitrite (indirect measurement of nitric oxide) concentrations were statistically higher in the only doxorubicin-treated group when compared to the Carnitine + Doxorubicin group. Fertility index and implantation rate were lower in Doxorubicin group, when compared to Carnitine + Doxorubicin group. Moreover, the percentage of spermatozoa with damaged DNA was higher in the Doxorubicin-treated group when compared to the Carnitine+Doxorubicin group. L-carnitine, when administered before doxorubicin, partially preserved the acrosome integrity, an important feature related to sperm fertilization ability that positively correlated with the reproductive competence and sperm DNA integrity at adulthood. In conclusion, L-carnitine attenuated the long-term alterations caused by doxorubicin in the germ cells and improved male reproductive capacity in adulthood.

INTRODUCTION

Doxorubicin has been largely used in anticancer therapy in adults, adolescents, and children (Müller *et al.*, 2011). Acute lymphocytic leukemia (ALL), lymphoma (Hodgkin and non-Hodgkin), and neuroblastoma are the commonest childhood cancers against which doxorubicin is proven to be effective (Hopkins-Donaldson *et al.*, 2002; Cazé *et al.*, 2010; Müller *et al.*, 2011). Many mechanisms of action have been proposed to justify the efficacy of doxorubicin; among them, the inhibition of topoisomerase II is known to be a key mechanism of its antiproliferative action (Tewey *et al.*, 1984). DNA intercalation and generation of free radicals are also cytotoxic effects of doxorubicin that result in the cell proliferation control and death (Tritton *et al.*, 1978; Hida *et al.*, 1995; Stevens *et al.*, 2007; Patel & Kaufmann, 2012). However, doxorubicin does not affect exclusively neoplastic cells, but also healthy dividing cells, for instance the spermatogonia (Jahnukainen *et al.*, 2000; Vendramini *et al.*, 2010).

According to the data compiled by the Childhood Cancer Survival Study (CCSS), a cohort study funded by the National Cancer Institute and other organizations, the survivor population who undertook chemotherapy during childhood or adolescence can be at increased risk of a broad spectrum of adverse outcomes, such as heart failure, nerve damage, second cancers,

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pregnancy loss, giving birth to offspring with low birthweights, and decreased educational attainment. Indeed, patients that had a diagnosis of Hodgkin's lymphoma enrolled in the survey, which probably received doxorubicin as part of their therapy, were least likely to sire a pregnancy than their siblings (Green *et al.*, 2010).

In an attempt to help those patients to overcome a certain or an imminent infertility in the future, techniques such as pre-pubertal testicular tissue cryopreservation, spermatogonium culture, and cytoprotection of the seminiferous epithelium should be considered (Wyns *et al.*, 2010).

Various studies have demonstrated that the use of cytoprotectors can contribute to reducing or attenuating the unwanted side effects of anticancer drugs aiming to promote a better quality of life of the survivors (Khan *et al.*, 2010; Chao *et al.*, 2011; Khositseth *et al.*, 2011; Lamas *et al.*, 2015). In the case of doxorubicin, preventive strategies are generally directed to the prevention of cardiotoxicity (Octavia *et al.*, 2012), but as infertility is also one of the aspects to measure quality of life (Monga *et al.*, 2004), several studies have been addressing new perspectives for fertility preservation.

L-carnitine is very widely considered as an effective substance to treat infertility (Lenzi *et al.*, 2003; Cavallini *et al.*, 2004) and is also a promising candidate for adjuvant therapy with the aim to reduce multiple organ toxicity after anticancer treatments (Sayed-Ahmed, 2010). It is naturally found in our body due to its physiological function and its efficiency in promoting the oxidation of long-chain fatty acids in the mitochondria (Bremer, 1962). More recently, additional mechanisms of carnitine were described, as the removal of excess acyl groups in various tissues and the modulation of intracellular coenzyme A (CoA) homeostasis (Ramsay & Arduini, 1993; Hoppel, 2003). In the testis and epididymis, it is found in large amounts, but is in a higher concentration in this last one, which is an expected feature considering the involvement of the epididymis in sperm maturation and motility acquisition (Jeulin & Lewin, 1996; Ng *et al.*, 2004).

The efficacy of L-carnitine as an antioxidant has been observed in both humans (Waldner *et al.*, 2006) and rats (Vicari *et al.*, 2002; Alshabanah *et al.*, 2010). However, there are no reports relating the protection of carnitine against the oxidative stress produced by doxorubicin in the testis.

According to Gülçin (2006), carnitine is a potent free radical scavenger, especially to superoxide anion, which is involved in the formation of hydrogen peroxide and hydroxyl radicals, known inducers of oxidative damage in the germ cells and/or sperm membrane, as well as in the DNA (Aitken & Roman, 2008).

The protective action of carnitine on male germ cells depletion, in rats, was already described as effective against the damage caused by gamma radiation (Topcu-Tarladacalisir *et al.*, 2009; Kanter *et al.*, 2010), cyclophosphamide (Cao *et al.*, 2016), busulfan (Dehghani *et al.*, 2013), etoposide (Okada *et al.*, 2009), and doxorubicin (Cabral *et al.*, 2014). Moreover, in a testicular ischemia/reperfusion model using rats, Dokmeci *et al.* (2007) reported that the pre-treatment with carnitine (500 mg/kg) prevented the increase in malondialdehyde levels caused by detorsion (reperfusion).

In a previous report (Cabral *et al.*, 2014), we showed some protection promoted by L-carnitine against the damage caused by the exposure to doxorubicin in pre-pubertal rats, when we could observe the improvement of the following: (i) the late effects on spermatogenesis; (ii) the quantitative sperm parameters, and, more importantly, (iii) the quality of sperm DNA. Using the same protocol applied here, the benefits of L-carnitine were observed both in a mid- and in a long-term analyses. These results stimulated the continuity of the study aiming to evaluate the extension of its beneficial action on reproduction against doxorubicin cytotoxicity. Also, considering that the integrity of sperm chromatin is essential for the transmission of paternal genetic information and that it can influence the embryonic development, we also decided to investigate whether the integrity of sperm chromatin would correlate with the fertility status.

MATERIALS AND METHODS

Animals and groups

Pre-pubertal male Wistar rats (*Rattus norvegicus albinus*; $n = 48$) were purchased from a local animal facility (CEDEME/Unifesp, Sao Paulo, Brazil) and transferred to the Developmental and Reproductive Biology animal facility one week before the beginning of the experiments. The rats were housed in polypropylene cages (40 × 30 × 15 cm), under standard controlled conditions: hygiene, temperature (≈23 °C), humidity (45–55%), and photoperiod (12-h/12-h light/dark). Food (Nuvilab CR-1 feed, Nuvital, Sao Paulo, Brazil) and distilled water were provided *ad libitum*. This study followed the ethics principles adopted by the Brazilian College of Animal Experimentation. The experiment was approved by the Ethics Committee for Animal Research of the Federal University of Sao Paulo, Brazil (CEUA no.0181/12).

At 30 days of age, rats were weighted and distributed into four different groups ($n = 15$ per group), according to the type of treatment: Sham control (SC), treated with 0.9% saline solution; Carnitine (CA), treated with a single dose of 250 mg/kg of body weight (b.w.) of L-carnitine (Sigma C0283, Milan, Italy); Doxorubicin (Doxo), treated with a single dose of 5 mg/kg b.w. of doxorubicin hydrochloride (Adriblastina RD; Pfizer, Guarulhos, SP, Brazil); and Carnitine + Doxorubicin (CAD), treated with a single dose of L-carnitine (250 mg/kg b.w.) 1 h before doxorubicin treatment (5 mg/kg b.w.). L-carnitine was diluted in bidistilled water in a concentration of 50 mg/mL. Doxorubicin was diluted in 0.9% saline solution in a concentration of 1 mg/mL. Both drugs were diluted immediately before treatment and administered using intraperitoneal route (Sayed-Ahmed *et al.*, 2001).

Rats aging 100 days were reassigned into two different sets, according to the reproductive outputs: (i) reproductive capacity and fertility index ($n = 24$; 6 per group); and (ii) sperm qualitative parameters and oxidative stress measurements ($n = 36$; 9 per group). At this age, the rats are sexually mature and sperm concentration in the epididymal cauda reaches its maximum (Robb *et al.*, 1978).

Collection of testes and sperm samples

The rats used for this purpose ($n = 9$/group) were submitted to euthanasia using carbon dioxide inhalation (Hackbarth *et al.*, 2000). Subsequently, the testes were carefully removed from the scrota. They were decapsulated, and 0.1 g of samples of each organ was frozen at −20 °C. On the day of the analysis, the frozen testes were thawed and homogenized in 1.15% KCl solution. The homogenized samples were used for measuring testicular

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malondialdehyde concentration (MDA) and indirect determination of nitrite concentration (NO).

The left epididymis cauda was used for sperm collection according to Vendramini *et al.* (2012). Right after sperm collection, a sample of 50 μL was withdrawn for mitochondrial activity analysis. Aliquots containing 1 mL of 4×10^6 spermatozoa/mL were stored at −80 °C for the Sperm Chromatin Structure Assay (SCSA™) and acrosome integrity. The right epididymis cauda was used to assess sperm motility right after its collection.

Mating

Sexually mature rats (n = 6/ group) were mated with normal females weighting 200–250 g for fertility and reproductive capacity evaluation. Each rat was mated overnight with two females in proestrus. At the following morning (considered day 1 of gestation), females were checked for pregnancy; this test was considered positive when there were spermatozoa in the vaginal smears (Vendramini *et al.*, 2012).

Acrosome integrity

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).

Samples of epididymal fluid were thawed and transferred (50 μL each) to a catalogued Eppendorf amber 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 under an epifluorescence microscope Nikon Eclipse Cl (NIkon, Melville, NY, USA) with appropriate filter. Two hundred spermatozoa were quantified per slide and were classified as (i) intact (spermatozoa exhibiting intense and moderate bright fluorescence in the acrosome region) and (ii) altered (spermatozoa showing weak fluorescence, irregular or absent in the acrosome region).

Sperm Chromatin Structure Assay

The analysis of sperm chromatin integrity using acridine orange was performed as described by Evenson *et al.* (2002) and Vendramini *et al.* (2012), with minor modifications. The samples containing acridine orange-stained (AO; Merck/Calbiochem-USA, Cotia. Brazil) spermatozoa were immediately analyzed using a FACSCANTO II flow cytometer (BD Biosciences, Sao Paulo, Brazil). The AO-stained spermatozoon DNA analyzed produced green fluorescence (detected by a 515–530 nm band-pass filter) and red fluorescence (detected by a 630–650-nm long-pass filter). Ten thousand events were analyzed for each sample. The raw data were processed using WINLIST 3D 8.0 Software (Verity Software House, Topsham, ME, USA) where the following parameters relating to the susceptibility of sperm DNA fragmentation were assessed, according to Evenson & Wixon (2005): the mean DNA fragmentation index (DFI) and the percentage of cells outside the main population (% DFI).

Testicular malondialdehyde concentration

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. Immediately after, the tubes were cooled and centrifuged at 1878 ***g*** for 10 min. The samples were placed into 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).

Indirect measurement of nitric oxide by evaluation of nitrite concentration

Testicular nitric oxide (NO) was measured as nitrite as described by Miranda *et al.* (2001) and Abd-Allah *et al.* (2009), with following minor changes. From the previously prepared testis homogenate, 0.5 mL was added to 0.5 mL of absolute ethanol and then centrifuged at 1878 ***g*** for 10 min. In sequence, 300 μL of vanadium chloride (VCl3, 0.8% in 1M HCl) was added to 300 μL of the supernatant. Three hundred μL of a mixture 1 : 1 of Griess 1 (*N*-(1-naphthyl) -ethylenediamine) and Griess 2 reagents (sulfanilamide, 2% in 5% HCl.) were also added. Immediately after, the solution was left at room temperature for 30–35 min. Following the incubation, the samples were placed in plates for analysis using the enzyme-linked immunosorbent assay (ELISA) and the absorbance was measured at 540 nm. Concentrations of NO (nmol/g tissue) were determined from a standard curve of different concentrations of sodium nitrite.

Sperm motility

The motion parameters of spermatozoa collected from epididymal fluid were analyzed using the CASA System (computer-assisted sperm analysis) with the standard and specific program Hamilton Thorne Integrated Visual Optical System (HTM-IVOS Toxicology System, version 12, Hamilton-Thorn Biosciences, Beverly, MA, USA) for rat sperm motility analysis, according to the following method described by Klinefelter *et al.* (1991), with some modifications.

After the collection, the right epididymis cauda was transferred to a petri dish containing 3 mL of Hank's balanced salt solution (HBSS—KCl, KH_2PO_4, NaCl, Na_2HPO_4, and $NaHCO_3$; pH 7.2), which was supplemented with 2 mg/mL BSA. The plates were left for 30 min at 37 °C, so the spermatozoa could move into solution. Then, an aliquot was transferred into a pre-warmed chamber slide with a depth of 100 μm (LEJA Standard Count, Nieuw-Vennep, the Netherlands). The slides containing spermatozoa were placed into a HTM-IVOS Toxicology System with specific software for rat sperm analysis.

The motility parameters measured were as follows: percentage of motile spermatozoa, 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 mitochondrial activity

The mitochondrial activity evaluation was carried out according to the methodology of Hrudka (1987). This technique is based on the oxidation of DAB by the cytochrome c oxidase enzyme, when the reactant is polymerized and deposited on the mitochondrial sheath along the sperm midpiece. Thus, the classification of spermatozoa concerning the mitochondrial activity followed the subsequent scale: Class I (100% of the intermediate piece stained, indicating 100% of mitochondrial activity), Class II

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(over 50% of the intermediate piece stained, indicating low reduction of mitochondrial activity), Class III (less than 50% of stained intermediate piece, indicating high reduction of mitochondrial activity), and Class IV (no staining in the intermediate piece, indicating absence of mitochondrial activity). Two sperm smears were obtained per animal. Two hundred spermatozoa were analyzed under a phase-contrast microscope (Olympus BX-51 – Japan) at a final magnification of 1000×.

Stock solutions of 1 mg/mL 3,3′-diaminobenzidine (DAB) in PBS were prepared with 137 mM NaCl, 2.7 mM KCl, Na_2HPO_4 4.3 mM, 1.4 mM KH_2PO_4, pH = 7.4 and maintained at – 4 °C shielded from light until use. A 100 mL aliquot was added to each 50 mL sample (a ratio of 1 : 2), and samples were incubated 1 h in the dark and in a water bath at 37 °C. Negative controls were done to avoid non-specific 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 aliquot was smeared on glass slides (Precision Glass Line, China) and air-dried; after drying, the smears containing the spermatozoa 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 (Entellan—Merck).

Reproductive competence

The rats were submitted to euthanasia by carbon dioxide inhalation at the 20th day of pregnancy. In sequence, they were laparotomized and their uterine horns removed. The implantation rate, resorptions, and pre- and post-implantation loss rates were obtained according to the method proposed by Costa-Silva *et al.* (2007). The fertility index was calculated as the ratio between the number of live fetuses and the number of mated females (Vendramini *et al.*, 2010).

Statistical analysis

The results were analyzed using the SIGMA PLOT 12.0 software (Systat Software Inc., San Jose, CA, USA). The values with normal distribution were analyzed according to the parametric test 'one-way analysis of variance' (ANOVA), aiming to compare the independent different groups: SC, CA, Doxo, and CAD. When the differences were significant, the multiple comparison Student–Newman–Keuls test (SNK) was used. However, when the values did not show normal distribution, they were submitted to the nonparametric test of analysis of variance, the Kruskal–Wallis test; in sequence, if significant, the data were submitted to the multiple comparison post hoc Student–Newman–Keuls test. Differences were considered significant when $p \leq 0.05$.

RESULTS

Acrosome integrity

As shown in the Fig. 1, the number of spermatozoa with intact acrosome was significantly higher in CAD, CA, and SC groups than in the Doxo group, demonstrating an improvement of this parameter in animals previously treated with carnitine. In addition, rats exposed to carnitine, either alone or before doxorubicin, had the acrosome integrity statistically similar to the control group.

Sperm Chromatin Structure Assay

The DFI spermatozoa %, which infers the percentage of damaged cells, was significantly higher in the Doxo group in comparison with the CAD, CA, and SC groups (Fig. 2). The mean DFI did not present significant differences among the groups (Fig. 3).

Testicular malondialdehyde concentration

The concentration of malondialdehyde in the testis, a marker of lipid peroxidation, was significantly lower in the CAD, CA, and SC groups when in comparison with the Doxo group. In addition, animals from the CAD group presented significantly higher levels of this marker, in comparison with those observed in SC group (Table 1).

Indirect measurement of nitric oxide by evaluation of nitrite concentration

Similarly to the malondialdehyde concentration, the nitrite concentration in the testis was significantly lower in the CAD, CA, and SC groups in comparison with the Doxo group (Table 1).

Sperm motility

Doxorubicin treatment led to a significant decrease in two progression parameters of sperm motility: VAP (average path velocity) and VSL (straight-line velocity) but only when compared to the SC group. However, there was no evident improvement of these parameters in doxorubicin-treated rats that were previously treated with L-carnitine. In addition, the other parameters related to motility did not differ among the groups (Table 2).

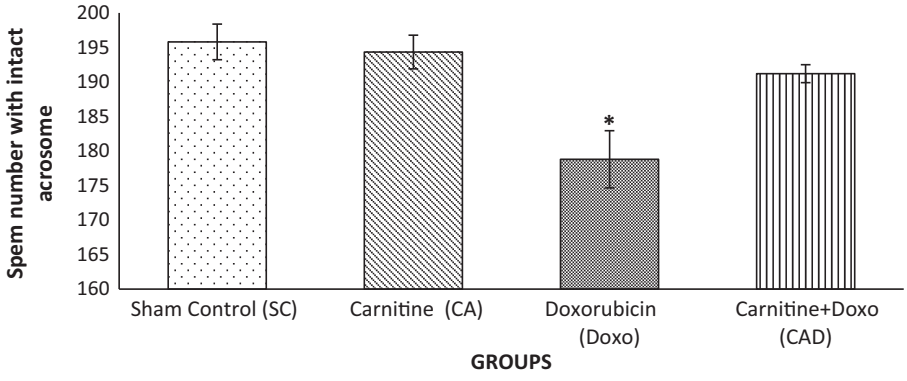

Figure 1 Sperm number with acrosome integrity in SC, CA, CAD, and Doxo groups. Data are reported as mean ± SD. ANOVA and SNK post-test ($n = 6$). *Significant ($p \leq 0.05$). *Doxo < CAD, CA, and SC.

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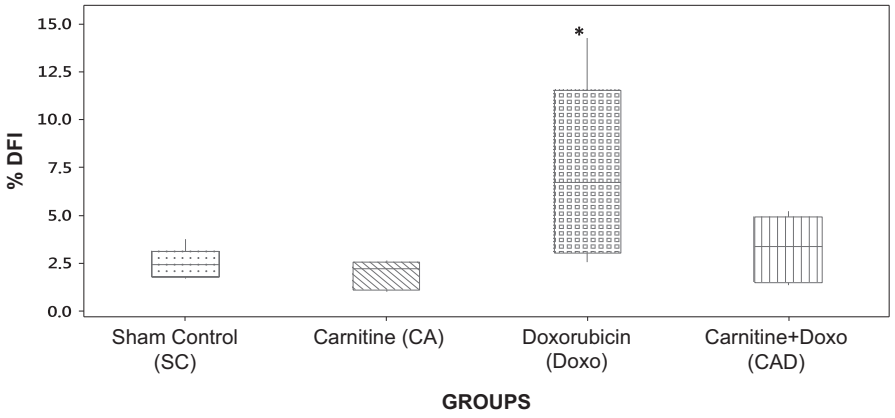

Figure 2 Percentage of DFI observed by sperm chromatin structure assay (SCSA) from control (Sham Control) and treated rats (Carnitine—CA, Doxorubicin—DOXO, and Carnitine + Doxo—CAD). Data are reported as median and interquartile range (Q1-Q3). ANOVA of Kruskal–Wallis and SNK post-test; ($n = 6$). *Significant ($p \leq 0.05$). a: Doxo> CAD, CA and SC.

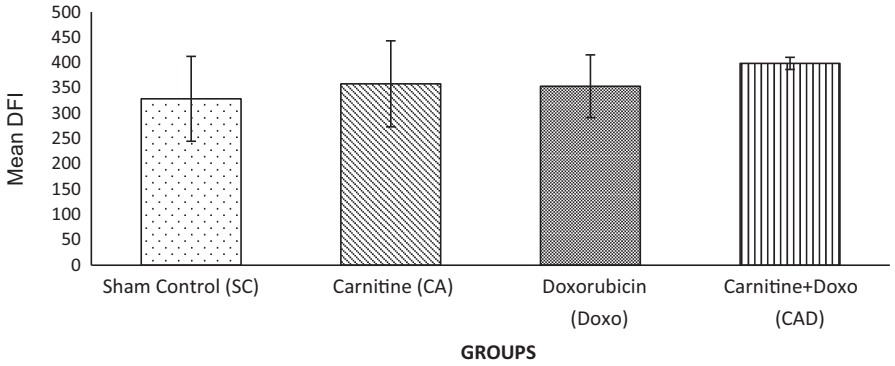

Figure 3 Mean DFI observed by sperm chromatin structure assay (SCSA) from control (Sham Control) and treated rats (Carnitine—CA, Doxorubicin—DOXO, and Carnitine + Doxo—CAD). Data are reported as mean ± SD; ANOVA ($n = 6$). Not significant.

Table 1 Data on testicular malondialdehyde concentration and indirect measurement of nitric oxide by evaluation of nitrite concentration in the control (SC) and experimental groups (Carnitine—CA, Carnitine+Doxo—CAD, and Doxorubicin—Doxo)

Groups				
Concentration (nM/g)	SC	CA	Doxo	CAD
Malondialdehyde concentration	161 (95–218)	178 (166–228)	307 (247–351)*[a]	230 (185–313)*[b]
Oxide nitric concentration	12 (11–18)	15 (3–17)	28 (15–36)*[a]	15 (3–36)

Data are reported as median and interquartile range (Q1-Q3). ANOVA of Kruskal–Wallis and Dunn's post-test ($n = 6$). *Significant ($p \leq 0.05$). [a]Doxo > CAD, CA and SC. [b]CAD > SC.

Table 2 Sperm motility (CASA HTM-IVOS) of animals in the Sham Control (SC) and experimental groups (Carnitine—CA, Carnitine+Doxo—CAD, and Doxorubicin—Doxo)

Groups				
Parameters	SC	CA	Doxo	CAD
Motility (%)	99 ± 1.4	98 ± 3.7	97 ± 1.2	99 ± 1.9
VAP (μm/s)	268 ± 15.5	236 ± 24.8	228 ± 23.2*	246 ± 23.7
VSL (μm/s)	220 ± 32.6	179 ± 19.9	169 ± 21.3*	184 ± 14.1
VCL (μm/s)	372 ± 71.3	354 ± 32.7	346 ± 44.4	368 ± 39.9
ALH (μm)	14 ± 0.79	14 ± 0.77	13 ± 1.2	13 ± 1.3
BCF (Hz)	17 ± 2.0	16 ± 3.9	13 ± 1.0	15 ± 1.7
STR (%)	75 ± 2.5	73 ± 0.8	73 ± 2.3	72 ± 2.1
LIN (%)	53 ± 2.3	51 ± 1.4	50 ± 2.5	51 ± 2.2

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. Data are reported as mean ±SD. ANOVA and SNK post-test ($n = 6$). Doxo < SC. *Significant ($p \leq 0.05$).

Sperm mitochondrial activity

A significant decrease in sperm number with active mitochondria (Class I) was observed in the Doxo and CAD groups in comparison with SC and CA groups. In addition, this analysis revealed an increased frequency of Class II spermatozoa (more than 50% of the midpiece was stained) in Doxo and CAD groups when compared with CA and SC groups On the contrary, significant differences regarding the Classes III and IV were not observed among all groups. Thus, in the condition of this experiment, the previous treatment with carnitine did not improve the sperm mitochondrial activity of adult rats exposed to a single dose of doxorubicin at the pre-puberty. In addition, the pre-pubertal rats solely treated with carnitine (CA group) did not show evident changes in this parameter in any of the classes I to IV at adulthood in comparison with the control rats (Table 3).

Reproductive capacity

At the 20th day of pregnancy, we observed one dead fetus and another with abnormal development, in the Doxo group. Rats from the Doxo group showed a worse fertility index in comparison to those from the CAD, CA, and SC groups (Table 4). The implantation rate was also significantly lower in the Doxo group than in the CAD, CA, and SC groups. On the contrary, the rate of resorptions was significantly higher in the Doxo group when compared to SC group, although you can check a much higher

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Table 3 Analysis of mitochondrial activity in 200 spermatozoa of animals of the control (SC) and the experimental groups (Carnitine—CA, Carnitine + Doxo—CAD, and Doxorubicin—Doxo) groups

	Number of spermatozoa			
Groups	Class I (100% of activity)	Class II (activity: >50%)	Class III (activity: <50%)	Class IV (100% of no activity)
SC	197 (188–200)	3.5 (0.75–7.75)	0 (0–0.5)	0 (0–0)
CA	193 (187–200)	6.5 (0.75–8)	0.5 (0–1.75)	0 (0–0.25)
Doxo	165 (163–196)[a]*	18 (4–25)[b]*	4 (1–15)	0 (0–3)
CAD	178 (175–190)[a]*	16.5 (13–19.25)[b]*	3 (0–3.25)	1.5 (0.75–2)

Data are reported as median and interquartile range (Q1–Q3). ANOVA of Kruskal–Wallis and Dunn's post hoc test ($n = 6$). *Significant ($p \leq 0.05$). [a]CAD and Doxo < SC and CA. [b]CAD and Doxo > SC and CA.

value on Doxo group in comparison with the CAD, CA groups. Likewise, the Doxo group also showed a higher percentage of post-implantation loss rate but only when compared to the SC control group. We did not observe significant differences among the groups related to the rate of pre-implantation loss (Table 4).

DISCUSSION

Peroxidative damage has been considered the most significant cause of testicular functional impairment, as consequence of a variety of conditions involving ischemia, metabolic diseases as diabetes and xenobiotic exposure (Aitken & Roman, 2008). Doxorubicin treatment is associated with damage mediated by a complex cascade involving production of ROS (Hida *et al.*, 1995; Jahnukainen *et al.*, 2000) and lipid peroxidation (Suominen *et al.*, 2003). Resveratrol, ginkgo biloba, nano-zinc oxide, and DL-alpha-lipoic acid seem to be effective to reduce the reproductive damage caused by doxorubicin in the testis (Prahalathan *et al.*, 2006; Yeh *et al.*, 2009; Badkoobeh *et al.*, 2013; Türedi *et al.*, 2015).

This is the second study we undertake using a single treatment of carnitine 1 h before doxorubicin during pre-pubertal phase; thus, we confirmed the previous assumption that the early germ cells were damaged by doxorubicin (Au & Hsu, 1980) and partially protected by carnitine in an extent that guaranteed an improved reproductive capacity and outcome.

The reduction of the oxidative stress in carnitine/doxorubicin rats in comparison with doxorubicin rats (evidenced in the measurements of malondialdehyde and nitric oxide) seemed to be the principal mechanism for carnitine protection. Carnitine is a good scavenger of ROS acting as a protector to the cell membrane (Lenzi *et al.*, 2003) in adverse situations. Although only two markers of oxidative stress have been investigated, we suppose that carnitine supplementation improves the oxidative status and of antioxidant defenses, as observed in hepatic injury (Alshabanah *et al.*, 2010).

Doxorubicin disturbs the absorption, synthesis, and excretion of carnitine in non-tumoral tissues (Sayed-Ahmed, 2010). Importantly, the reduced concentration of carnitine caused by doxorubicin treatment observed in rat liver is reversed after exogenous supplementation with carnitine (Alshabanah *et al.*, 2010). In rats, carnitine is mainly synthesized in the liver and then distributed to other organs (Vaz & Wanders, 2002). When given alone, doxorubicin can compromise the carnitine transporters (possibly inhibiting OCTN2—organic cation/carnitine transporter 2), as already observed in mice by Hu *et al.* (2012); in a secondary moment, doxorubicin can cause impairment of carnitine endogenous biosynthesis in the tissue (Alshabanah *et al.*, 2010) as a result of the cellular lesion. Therefore, adequate carnitine exogenous supplementation before the exposure to doxorubicin could reestablish carnitine homeostasis altered by the anticancer drug (Sayed-Ahmed, 2010), thus restoring the protective effect of carnitine, in at least some extent. The possible involvement of doxorubicin as a potential substrate also transported by OCTN2, and then competing with carnitine, deserves a future investigation. However, as the protocols of treatment and/or models used by Alshabanah *et al.* (2010) and Hu *et al.* (2012) were different than ours, a pharmacokinetic study of the supplementation of carnitine before the administration of doxorubicin in rats should be better scrutinized.

In addition, L-carnitine pre-treatment was reported to be a significant inhibitor of doxorubicin-induced NADPH oxidase activation, reducing the damage caused by this antineoplastic agent in cardiomyocytes (Chao *et al.*, 2011). In the testis, NADPH oxidase activation is an important ROS-producing source (Li *et al.*, 2013), feeding the metabolic need of spermatogonial cells for moderate levels of ROS to facilitate the stem cell self-renewal that is in parallel regulated by a sequential activation of different Nox genes (Morimoto *et al.*, 2015). Doxorubicin-induced DNA damage, besides the role of the topoisomerase II inhibition, can be caused by direct hydrogen peroxide generation, and it involves NADPH oxidase activation, which is considered to be a critical apoptotic trigger (Mizutani *et al.*, 2005). Therefore, the regulation of the NADPH oxidase activity could indirectly contribute for a decrease of the lesion in spermatozoa arisen from these cells, then providing more stable chromatin present in the gametes.

Another alteration caused by the treatment with doxorubicin is the evident modification of the content and fatty acid composition of testicular lipids, which probably affects both long- and

Table 4 Data of analysis of the reproductive competence and fertility index of control (SC) and experimental rats (CA, CAD, Doxo) mated with primiparous females

Groups	Parameters				
	Implantation rate	Resorption rate	Pre-implantation loss	Post-implantation loss	Fertility index
SC	99.40 ± 0.59	0 (0–0.89)	0 (0–0.89)	0 (0–0.89)	13.9 ± 0.65
CA	93.95 ± 2.19	9.09 (0–11.11)	0 (0–0)	9.09 (0–11.11)	13.8 ± 1.30
Doxo	86.82 ± 2.69*[a]	13.88 (6.73–18.57)*[b]	3.33 (0–9.98)	14.81 (6.73–23.75)*[b]	9.9 ± 1.02*[a]
CAD	93.63 ± 1.19	7.14 (4.54–8.33)	0 (0–0)	7.73 (6.49–8.52)	12.37 ± 0.94

Data are reported as median and interquartile range (Q1–Q3) and as mean ± SE. ANOVA and SNK. ANOVA of Kruskal–Wallis and Dunn's post hoc test ($n = 6$; 2 females/male). *Significant ($p \leq 0.05$). [a]Doxo < CAD, CA and SC. [b]Doxo > SC.

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very long-chain polyunsaturated fatty acids (Zanetti *et al.*, 2007). As carnitine acts as a transporter of long-chain fatty acid from cytoplasmic compartment into mitochondria, where enzymatic beta-oxidation and energy production occur, it may have participated in the reduction in any derangement of the testicular lipid metabolism that occurred in doxorubicin-treated rats, contributing to the reduction of the damage in germ cells. Reinforcing this idea, in non-obese hereditary hypertriglyceridemic rats carnitine improves the lipid metabolism and has potential protective action against oxidative stress produced in some metabolic syndrome-related disorders (Cahova *et al.*, 2015).

Another assumptive primary target of doxorubicin is the Sertoli cell, an orchestrating component of the spermatogenic process (Brilhante *et al.*, 2012). OCTN2, a carnitine transporter, is present in the Sertoli cell membrane (Kobayashi *et al.*, 2005). In this matter, an in vitro study has already shown that carnitine acts directly on Sertoli cells improving their physiology and maturation (Palmero *et al.*, 2000). This could ameliorate the microenvironment within the seminiferous epithelium and consequently, reflecting in the improved quality of spermatozoa.

The main mechanism of the cytotoxic action caused by doxorubicin is blockage of the activities of DNA and RNA polymerases as well as of the topoisomerase II, being spermatogonia the preferably damaged cell present in the seminiferous epithelium. Among those cells, doxorubicin is known to especially target intermediate and type B spermatogonia (Meistrich, 1986; Vendramini *et al.*, 2010), although spermatogonia A can also be affected at all phases of spermatogenesis cycle (Matsui *et al.*, 1993). In addition, doxorubicin also inhibits the DNA methyltransferase 1—DNMT1, which is an important enzyme for chromosome stability and transcription (Yokochi & Robertson, 2004; Vendramini *et al.*, 2012). Indeed, it is a mutagenic and genotoxic drug (Au *et al.*, 1980; Hacker-Klom *et al.*, 1986) that alters the expression of genes involved in DNA repair and apoptosis (Wang *et al.*, 2012). Vendramini *et al.* (2012) showed that the alterations produced by a single dose of doxorubicin at pre-pubertal phase have implications in the paternal inheritance, decreasing early embryo quality. In the present research, we did not assess the effect of doxorubicin and/or carnitine on the early embryonic phase, but we observed an impairment of the reproductive competence in doxorubicin-treated pre-pubertal rats, which were mated with primiparous females at adulthood. Regarding the reproductive capacity, Kato *et al.* (2001) and Imahie *et al.* (1995) also reported a significant decrease, despite their report was developed using a different protocol of treatment with doxorubicin in adult rats. Considering that mechanisms of action presented by doxorubicin can produce DNA double-strand breaks as well as a blockage of cell cycle, they can signalize cascades of events conducing the germ cells to undergo apoptosis. Indeed, germ cell apoptosis is a well-known fate induced on doxorubicin-exposed dividing cells (Hou *et al.*, 2005; Cabral *et al.*, 2014; Jia *et al.*, 2015). Injured and abnormal germ cells can be removed from seminiferous epithelium through the apoptotic mechanisms. This event can represent an efficient defensive mechanism aiming to preserve the genomic quality of male gametes as it has been observed in different adverse situations (Aitken & Baker, 2013). Thus, it has also been suggested that protamine deficiency, reactive oxygen species, and abortive apoptosis may be responsible for sperm DNA damage (reviewed by Zini *et al.*, 2014). However, according to Vendramini *et al.* (2010 and 2012), if abortive apoptosis occurs at pre-puberty and injured spermatogonia survive, they may develop abnormal spermatozoa that will compete with the healthy ones to fertilize the oocytes.

As previously cited, other studies have been showing the benefits of different substances against the damage caused by the doxorubicin such as reduction in testicular apoptosis (Türedi *et al.*, 2015), and improvement of the integrity of seminiferous epithelium (Vendramini *et al.*, 2010) and the testosterone level (Hozayen, 2012) as well as of sperm quantitative parameters (Jalali & Hasanzadeh, 2013). Experiments in vivo have also shown that carnitine decreased the reproductive damage caused by busulfan (Dehghani *et al.*, 2013) and cyclophosphamide (Zhu *et al.*, 2015; Cao *et al.*, 2016). In the present study, alteration of the frequency of motile spermatozoa in doxorubicin-treated rats (treated or not with carnitine) was not observed, although other sperm quality parameters were.

It must be considered that the most common chemotherapeutic treatment includes not only a single antineoplastic drug, but also some others, in a combination of different agents that will certainly cause a distinctive effect on sperm quality and production. The negative impact of CHOP chemotherapy (including doxorubicin) was observed on the quality of male germ cells, genome integrity, and progeny outcome using the rat as a model (Vaisheva *et al.*, 2007). However, there are very few experimental studies that combine analyses of genome integrity and reproductive competence of doxorubicin-treated animals that have also been exposed to some protective substances (Vendramini *et al.*, 2010, 2012). This is a gap in clinical and experimental studies that deserve to be widespread and circumvented.

The investigation of the impact of antioxidants on the chromatin structure is a very delicate issue and must be deeply investigated. The inheritable consequences of the exposure of the male gamete to cytoprotectors do not necessarily produce the expected outcome, as already seen in some reports. In two preceding experimental studies performed in vivo and using the same protocol of doxorubicin treatment utilized here, we observed that amifostine, a cellular protector still applied as a protective treatment, when previously administered to doxorubicin in pre-pubertal rats, reduced germ cell loss, improved sperm morphology and motility at adulthood (Vendramini *et al.*, 2010), and seemed to be positively protecting the germ cells. However, the reproductive outcome signalized that something was not working well in that drug relationship. After performing two different techniques to evaluate sperm DNA integrity, we found that actually, amifostine–doxorubicin association is very detrimental to the sperm genome and causes an evident DNA damage and the impairment of the conceptus development (Vendramini *et al.*, 2012), which justified the low fertility rate observed in the first investigation (Vendramini *et al.*, 2010).

On the contrary, the dosage of L-carnitine used in the present study does not seem to have any detrimental effect on sperm DNA, fertility index, or pregnancy outcome rate when administered with doxorubicin. In a former study, we showed that carnitine given to pre-pubertal rats, at the same conditions applied in the current study, prevented the germ cell apoptosis caused by doxorubicin and improved many testicular parameters and sperm morphology (Cabral *et al.*, 2014). We are showing here that carnitine also preserved the chromatin structure stability

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assessed by SCSA, confirming our previous data obtained using the Comet Assay (Cabral *et al.*, 2014).

Thus, the alterations in the sperm motility and mitochondrial activity observed in the doxorubicin groups seem to have not caused a noteworthy impact on the fertilization process, as no significant changes were observed in the frequency of motile spermatozoa in Doxo and CAD rats. On the other hand, decreases of sperm motility in cyclophosphamide-treated (Cao *et al.*, 2016) and in diabetic rats (Kang *et al.*, 2011) were enhanced when they were L-carnitine-treated. However, we treated the rats at pre-puberty (at 30 days of age) while they did at adulthood. Also, L-carnitine did not improve the mitochondrial activity altered by doxorubicin, as presented in our results. Although optimal mitochondrial function is essential for fertility, as an energy source for sperm survival and motile capability (Ballinger *et al.*, 1996), mitochondria-derived ATP seems not to be crucial for sperm motility; glycosylation has been considered the main mechanism of obtainment of energy and motility (Piomboni *et al.*, 2012). Conversely, the reduction in the sperm DNA damage and the improvement of acrosome integrity and of the sperm chromatin structure observed in the carnitine/doxorubicin group presented a positive correlation with the fertility index and pregnancy outcome. As the design of the current study involves techniques that are commonly used in the clinical practice for evaluating male reproduction, it is possible to presume upon the sperm fertilization capacity and the risks concerning embryonic loss, information that has been supporting decisions related to assisted reproductive technology—ART (Virro *et al.*, 2004).

In addition, there was a positive correlation between the percentage of intact acrosome and fertility (Saacke & White, 1972; Correa *et al.*, 1997). Integrity of acrosome is an important parameter for the success of sperm penetration into the zona pellucida (Makhluf *et al.*, 2006). Therefore, morphological abnormalities in the acrosome or in its size can make spermatozoa less able to bind to the zona pellucida, thus compromising the fertilization of the oocyte (Menkveld *et al.*, 1996; Garrett *et al.*, 1997). Carnitine plays important roles in sperm motility, maturation, and capacity of fertilization (Lenzi *et al.*, 1992; Shang *et al.*, 2006).

Carnitine is an indispensable substance for energy metabolism. In mammals, a balance among its endogenous synthesis, absorption from dietary sources, and its effective reabsorption by kidneys preserves the carnitine homeostasis (Vaz & Wanders, 2002). Cancer chemotherapy-induced multiple organ toxicity has shown to be alleviated by carnitine supplementation, without its interference with the therapeutic antitumoral properties of these drugs. Thus, the ineffectiveness of carnitine on the antitumor efficacy of doxorubicin was already demonstrated (reviewed by Sayed-Ahmed, 2010). There is not much information about the trinomial: carnitine/chemotherapy/sperm DNA damage and its involvement in the improvement of the reproductive competence and embryonic viability. Nevertheless, reduction in the DNA damage caused by actinomycin-D and hydrogen peroxide in vitro and improvement in the blastocyst development rate were already shown by the supplementation of culture media with carnitine (Abdelrazik *et al.*, 2009), as well as an increase in the quality of embryos obtained by intracytoplasmic sperm injection (Abdelrazik *et al.*, 2009) and the nuclear maturation of oocytes (Phongnimitr *et al.*, 2013).

Finally, there are two subjects that must be emphasized here: (i) The exposure to chemotherapeutic drugs may cause adverse effects on the progeny (Hales *et al.*, 2005); and (ii) an intact paternal genome is essential for successful fertilization and embryogenesis (Maselli *et al.*, 2014). In the present research, the quality and viability of early embryos were not evaluated, but the results of reproductive capacity showed that doxorubicin increased the embryonic loss in comparison to control rats; nevertheless, carnitine administered alone or previously to doxorubicin did not significantly cause any harmful alteration in the reproductive parameters covered by our studies, but it improved some of them. These data altogether have a relevant meaning as early embryo loss can be a sensitive marker of the adverse effects of chemotherapeutics on quality of spermatozoa and on integrity of male genome (Vaisheva *et al.*, 2007). However, even if most of the findings indicate the clinical benefits of L-carnitine, a few studies have already warned about its toxicity when administered in high doses (reviewed by Mongioi *et al.*, 2016). Further studies must be developed to better scrutinize the referred trinomial. Similar experimental studies are being developed but using protocols comparable to those used in the clinical practice.

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AUTHORS' CONTRIBUTION

RELC performed all the experimental work and contributed to the analysis of the results and writing of this manuscript. SMM executed the experimental design and supervised all the work, analysis of results, and writing and reviewing the manuscript. TBM contributed to the experimental work and to the analysis. VV contributed to the results analysis and revision of the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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