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Full Length Research Paper

Long-term testicular toxicity caused by doxorubicin treatment during pre-pubertal phase

O. Brilhante[1], T. Stumpp[2]* and S. M Miraglia[2]

[1]Centre for Health and Rural Technology, Academic Unit of Veterinary Medicine, Federal University of Campina Grande, Patos – Paraíba, CEP 58700 – 970, Brazil.
[2]Department of Morphology and Genetics, Federal University of São Paulo. Rua Botucatu, 740. Edifício Leitão da Cunha, 2° Andar. Vila Clementino, São Paulo – SP, CEP: 04023-900, Brazil.

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Doxorubicin is a potent drug widely used against different types of cancer and can cause damage to healthy tissues. Some studies have shown that this drug causes apoptosis of male germ cells. However, a detailed and long-term morphological study about the damages caused by doxorubicin administration to early prepubertal testis and to future fertility of the rat is missing. Male rats were treated at 15 days and 22 days with the dose of 5 mg/kg of doxorubicin and sacrificed at 40, 64 and 127 days of age. The testes were submitted to morphometric and stereological analyses. The reproductive capacity of the adult rats was also tested. The doxorubicin-treated rats showed germ cell depletion, tubular vacuolization, multinucleated formations of spermatids and germ cell showing apoptotic characteristics. Reduction of seminiferous tubule volume was observed in all doxorubicin-treated subgroups but the volume densities decreased only in the adult subgroup. The frequency of tubular sections containing type A spermatogonia was progressively reduced in these rats, which showed null fertility. Taken together the results of the present study we conclude that the administration of doxorubicin during early prepubertal age can lead to severe injury of the adult fertility.

Key words: Spermatogenesis, doxorubicin, infertility, morphometry, stereology, testis.

INTRODUCTION

Doxorubicin (14-Hidroxydaunorubicin) is an antracyclic antibiotic drug widely used in the treatment of a variety of cancers (Di Marco and Arcamone, 1975; Schwartz, 1976). Doxorubicin has multiple mechanisms of action, including its interaction with the enzyme topoisomerase II, metal ion chelation and free radical generation (Gewirtz, 1999; Kiyomiya et al., 2001; Xu et al., 2005). More recently doxorubicin was found to reduce the viability of cancer cells via RNA damage (Fimognari et al., 2008).

Although doxorubicin is considered a very potent and efficient chemotherapeutic drug, it also kills healthy cells, especially those under rapid and constant proliferation, such as the male germ cells. It has been shown that doxorubicin causes germ cell apoptosis (Hou et al., 2005; Matsui and Takahashi, 1999; Shinoda et al., 1999; Suominen et al., 2003; Yeh et al., 2007), reduction of sperm counts (Kato et al., 2001; Yeh et al., 2007) and decrease in testicular weight (Yeh et al., 2007). Doxorubicin also affects testicular lipids (Zanetti et al., 2007). However, although some studies have addressed the effects of doxorubicin on the testes, a detailed and long term evaluation of the effects of doxorubicin on spermatogenesis and its impact on the fertility of rats treated during early prepubertal phase is missing.

In previous studies, our group observed that etoposide, another topoisomerase II poison, causes germ cell apoptosis, seminiferous epithelium depletion and irreversible Sertoli cell damage (Freitas et al., 2002; Stumpp et al., 2004, 2006 and 2008). These studies also showed that the testicular damages caused by the administration of this drug to prepubertal rats can be extended to adulthood, leading to serious fertility harm

*Corresponding author. E-mail: taiza.stumpp@gmail.com. Tel: 55 11 55764262. Fax: 55 11 55764263.

(Freitas et al., 2002; Stumpp et al., 2004). Thus, the aim of the current study was to perform a detailed morphometric and stereological study of the testes from rats treated with doxorubicin during the early prepubertal phase, when the hematotesticular barrier starts its development (Russell and Griswold, 1993); besides, we also investigated whether the spermatogenesis damage caused by this treatment is extended to adulthood and its impact on the fertility. The evaluation of the damage caused by doxorubicin to the prepubertal testis in this critical age can contribute to further studies aiming testicular protection against harmful side effect of doxorubicin. Okada et al. (2009) and Vendramini et al. (2010) have shown that cytoprotective drugs can reduce the side effects of chemotherapeutic drugs administered to pre-pubertal rats. Studies about fertility preservation are particularly important for the Paediatric Oncology, since sperm collection is not possible in child patients. Besides, although there are species-specific differences related to the cytotoxic responses to the chemotherapeutic-treatments, the damages and the recovery of spermatogenesis after this type of therapy are similar between men and rodents in many aspects (Meistrich, 1986). Thus, the analysis of morphometric and stereological testicular parameters can provide important information about the extension of the damages caused to spermatogenesis and about the dynamics of the testicular compartments during the post-natal development after the treatment at early prepubertal phase, contributing to the comprehension of fertility harm in adulthood. Therefore, although there are studies showing the testicular damage caused by doxorubicin administration to adult rats (Saalu et al., 2009), a detailed and long term morphological and morphometric study about the future fertility and the testicular damages caused by doxorubicin when administered to the early prepubertal rats is missing. This fact motivated the realization of this research since doxorubicin is largely used in pediatric oncology.

METHODS

Thirty-six 15-day old Wistar rats (*Rattus norvegicus albinus*) were distributed into two groups: control (C) and doxorubicin-treated (D). The rats of D group received the total dose of 5 mg/kg of doxorubicin (Rubidox®, Bergamo-São Paulo Brasil) via intraperitonial route. The flask of Rubidox® contains 300 mg of lyophilic powder constituted by 50 mg of the chloridrate of doxorubicin and 250 mg of lactose. The total dose was fractioned in two doses of 3 and 2 mg/kg administered when the rats were 15 and 22 days old, respectively. The animals of the sham control group (C) were treated with saline solution at the same ages adopted for the doxorubicin-treated group (D). After the treatment, the rats were allowed acclimation periods that lasted 18, 42 and 105 days. Then, at the end of these periods the rats were submitted to decapitation when they were 40, 64 and 127 days old, respectively.

Each group (C and D) was distributed into three subgroups, with 6 rats each. These subgroups were established according to the euthanasia ages: 40 (C_{40} and D_{40}), 64 (C_{64} and D_{64}) and 127 (C_{127} and D_{127}) days of age. The animals were maintained under controlled photoperiod (12 h light; 12 h dark) and temperature (22 to 23°C). Food and water were allowed "*ad libitum*". The experimental protocol followed the ethical principles adopted in animal researches by the Brazilian College of Animal Experimentation. The schedule concerning animal care and treatment was approved by the Institutional Ethical Committee of São Paulo University (Approval number 0559/08).

Testicular morphometry and stereology

Rats were anesthetized with thiopental (Cristalia, Brazil) and their testes were dissected and weighed. The volume of the testes (Vt) was obtained according to the Scherle's method (Scherle, 1970). The testicular axes (the minor and the major axes) were also measured using a micrometer caliper. The value of the minor testis axis was calculated through the mean obtained from the two minor axes since the testis is an ellipsoid (Freitas et al., 2002). The testes were Bouin's liquid-fixed for 48 h, sub-sampled and processed for paraplast-Plus embedding. Two 5 µm cross sections were obtained from each testis (left and right) and submitted to the Periodic Acid-Schiff histochemical method and counterstained with Harris' Hematoxylin (PAS+H). The parameters analyzed were: seminiferous tubule diameter, volume density (Vv) and volume (V) of the testicular compartments (tubular and interstitial compartments), as well as the frequencies (in percentage) of tubular sections containing each germ cell type.

Seminiferous tubule diameter and volume densities were performed using a special ocular lens coupled to a light binocular microscope (Gundersen et al., 1988). The volume density (Vv) was obtained using an integrating lens with 25 equidistant points, at x100 magnification (Weibel, 1963). One thousand two hundred and fifty points were randomly counted per testis in x100 magnification. The seminiferous tubule diameter was measured using a micrometer eyepiece at 80x magnification. When the sections were oblique, only the minor axis was considered (Miraglia and Hayashi, 1993; Stumpp et al., 2004). For all analyses, 50 fields were randomly scrutinized in each testicular cross section, totalizing 200 fields per rat.

Testicular histopathological analysis

The same testicular cross sections submitted to morphometric and stereological analyses were used for the histopathological analysis under light binocular microscope. Four testicular sections were randomly analyzed in each rat (two per testis) through the Leica QWin V3 (Cambridge, UK) image analysis system using x20 and x40 objective lenses. The whole testicular sections were analyzed and the alterations observed were described. Thus, from randomly sampled fields, we obtained the images using the referred analysis system, which were captured by a digital camera connected to the light microscope.

Frequency of tubular sections containing the main types of germ cells

The testicular sections submitted to PAS+H method were also examined under a light microscope, with the aim to evaluate the presence of the main germinal lineage cells (Hayashi and Cedenho, 1980; Miraglia and Hayashi, 1993). For this goal, 50 seminiferous tubule sections were analyzed in each testicular section (right and left), totalizing 200 sections per rat. The percentages of tubular sections containing the main germ cell types were calculated.

Table 1. Body weight and testicular morphometric data in the sham control (C) and doxorubicin-treated (D) rats.

Control subgroups	Body weight (g)	Testicular weight (g)	Middle minor axis (mm)	Major axis (mm)	Total testicular volume (mm^3)	Tubular diameter (μm)
C_{40}	124.43 ± 5.98	0.69 ± 0.04	8.56 ± 0.35	15.22 ± 0.55	616.67 ± 33.25	195.12 ± 5.89
C_{64}	277.95 ± 17.96	1.53 ± 0.09	10.57 ± 0.20	19.63 ± 0.54	1149.83 ± 47.37	225.44 ± 5.27
C_{127}	345.42 ± 16.48	1.6 ± 0.12	11.96 ± 0.30	20.90 ± 1.19	1567.15 ± 93.27	276.60 ± 5.53
Doxorubicin subgroups						
D_{40}	117.98 ± 6.99	0.38* ± 0.11	6.62* ± 1.10	12.46* ± 0.95	341.67* ± 113.38	155.92* ±18.88
D_{64}	220.50* ± 6.58	0.66*± 0.09	7.64* ± 0.36	15.85* ± 1.00	619.17* ± 95.02	191.06** ±16.68
D_{127}	302.65* ± 34.37	0.67* ± 0.12	7.62* ± 045	15.54* ± 0.96	637.50* ± 114.55	168.76** ±9.81

Values expressed as mean ± S.D. Significant differences: * ($p \leq 0.05$); ** ($p \leq 0.01$).

Fertility index

At 117 days of age, the animals of the C127 and D127 subgroups were mated with two primiparous females each, for two estrous cycles. The mating was performed to investigate the reproductive capacity of the doxorubicin-treated male rats. The fertility index in each group was obtained by the ratio between the number of born pups and the number of mated females (Miraglia and Hayashi, 1993). The mating of the 64 day old rats was not carried out because the aim of the current study was to evaluate the reproductive capacity only of the adult doxorubicin-treated rats. For this, when the rats reached 64 days of age, they were allowed to undergo a complete period of spermatogenesis (long-term evaluation), which in rats corresponds to approximately 53.2 days (Clermont, 1972); for this reason, the rats were mated when they were 117 days old.

Statistical analysis

The data obtained from control and doxorubicin-treated rats were compared using the Student parametric statistical test. The use of one and two asterisks respectively indicates the significant differences of the doxorubicin subgroups in comparison with the control subgroups when $0.01 \leq p \leq 0.05$ and $p < 0.01$.

RESULTS

Body weight

The doxorubicin treated rats from D_{64} and D_{127} subgroups showed a significant reduction of the body weight. The 40-day old rats did not show alteration of body weight when compared with the respective sham control subgroup (Table 1).

Testicular morphometry and stereology

At all ages studied, the doxorubicin-treated rats showed significant reduction of the testicular weight and volume, as well as of the testis axes and seminiferous tubule diameter (Table 1). Accentuated decrease of the seminiferous tubule volume was also observed in 40, 64 and 127 day old doxorubicin-treated rats (Table 2). On the other hand, the volume of the interstitial compartment was reduced in the D_{40} and D_{64} subgroups and the volume densities of the interstitial and tubular compartments showed accentuated reduction only in the D_{127} subgroup (Table 2).

Testicular histopathology

The control groups showed normal characteristics of the seminiferous epithelium. All the germ cell types typical of each age were present (Figures 1 to 3). At 40 days, the most differentiated germ cells in the control group were step 17 spermatids (Figure 1). At 64 and 127 days, mature spermatids were observed in the control group (Figures 2 and 3).

The doxorubicin-treated rats (Figures 4 to 12) showed accentuated damage of the seminiferous epithelium. They showed depletion and vacuolization of this epithelium (Figures 4, 7 and 11), as well as germ cells with condensed peripheral chromatin, suggesting apoptosis (Figures 4, 5 and 12). Multinucleated formations of round spermatids (Figures 4, 6 and 12) as well as cellular debris and detached germ cells in the tubular lumen (Figure 8) were frequently observed; some of them were Sertoli cell (Figure 9)

Table 2. Testicular stereology in the sham control (C) and doxorubicin-treated (D) rats.

Control subgroups	Seminiferous tubule volume (mm^3)	Interstitial compartment volume (mm^3)	Tubular volume density (%)	Interstitial compartment volume density (%)
C_{40}	494.35 ±21.57	122.32 ±15.43	80.21±1.61	19.79 ±1.61
C_{64}	796.02 ±23.10	353.81 ±41.23	69.30 ±2.51	30.70 ±2.51
C_{127}	1132.08 ±75.65	435.07 ±30.41	72.21 ±1.45	27.79 ±1.45
Doxorubicin subgroups				
D_{40}	266.82** ±95.35	74.84** ±21.00	77.51 ±4.16	22.49 ±4.16
D_{64}	441.83** ±90.34	177.34** ±22.27	70.14 ±5.69	29.19 ±4.90
D_{127}	326.90** ±42.10	310.60 ±137.19	52.71** ±10.50	47.29** ±10.50

Values expressed as mean ± S.D.; Significant differences: ** ($p \leq 0.01$).

Table 3. Germ cell frequencies (percentages of seminiferous tubules cross sections containing the specific germ cell types) in the control (C) and doxorubicin-treated (D) rats. (Means ± SD).

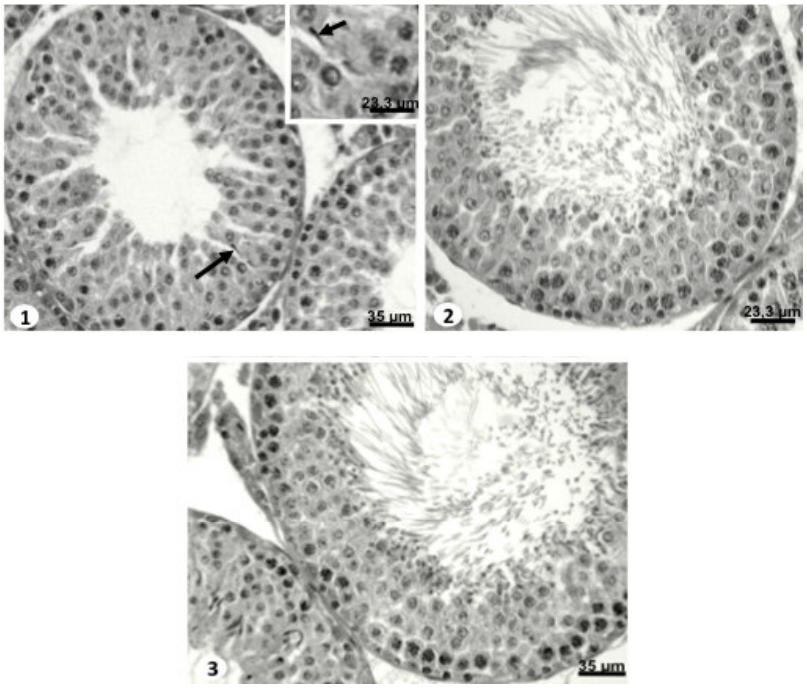
Control subgroups	A spermatogonia	B and Intermediary spermatogonia	Primary spermatocytes	Round spermatids	Elongated spermatids	Sertoli cells
C_{40}	76.67 ± 5.14	45.00 ± 5.95	100.00	65.00 ± 7.57	54.33 ± 4.99	100.00
C_{64}	93.33 ± 1.97	54.17 ± 3.34	100.00	67.17 ± 5.05	98.33 ± 0.47	100.00
C_{127}	96.00 ± 1.91	66.17 ± 2.41	100.00	100.00	100.00	100.00
Doxorubicin subgroups						
D_{40}	59.58* ± 8.59	35.42 ± 12.00	88.33 ± 10.34	72.50 ± 7.83	9.83** ± 6.59	100.00
D_{64}	55.42** ± 9.94	51.67 ± 8.50	98.00* ± 1.41	67.50 ± 8.04	41.17 ± 25.6	100.00
D_{127}	43.33** ± 4.00	57.50* ± 6.45	86.83** ± 15.13	56.17** ± 11.65	30.83** ± 17.78	100.00

Values expressed as mean ± S.D.; * and ** indicate significant differences at $p \leq 0.05$ and $p \leq 0.01$ respectively.

and elongated spermatids (Figure 10). In the rats of the D_{40} subgroup these alterations were less frequent than in the D_{64} and D_{127} subgroups. The most accentuated histological alterations were observed in the D_{127} subgroup, in which a large quantity of tubular sections contained only Sertoli cells.

Frequency of tubular sections containing the main types of germ cells

The frequency of tubular sections containing type A spermatogonia was reduced in all doxorubicin-treated rats while those comprising elongated spermatids were decreased when the rats were 40 and 127 days old (Table 3). In the D_{127} subgroup, the frequencies of type B/Intermediary spermatogonia and round spermatids were also reduced, whereas in the other subgroups (D_{40} and D_{64}) these reductions were not significant. Reduction of the frequency of primary spermatocyte was observed in the D_{64} and D_{127}



Figures 1 to 3. Testicular cross sections of 40, 64 and 127 day old control rats submitted to PAS+H method. At the three ages the seminiferous epithelium shows normal characteristics, typical of each age. At 40 days (Figure 1), spermiogenesis is not complete and step 17 spermatids (arrow and inset) are the most differentiated germ cell. At 64 (Figure 2) and 127 (Figure 3) days, step 19 spermatids are present, indicating that spermiogenesis is complete. Observe the integrity of the seminiferous epithelium at all ages.

subgroups; like the frequency of type A spermatogonia, the reduction of primary spermatocyte frequency was more accentuated in the D_{127} subgroup (Table 3). The D_{40} subgroup did not show alteration of this parameter.

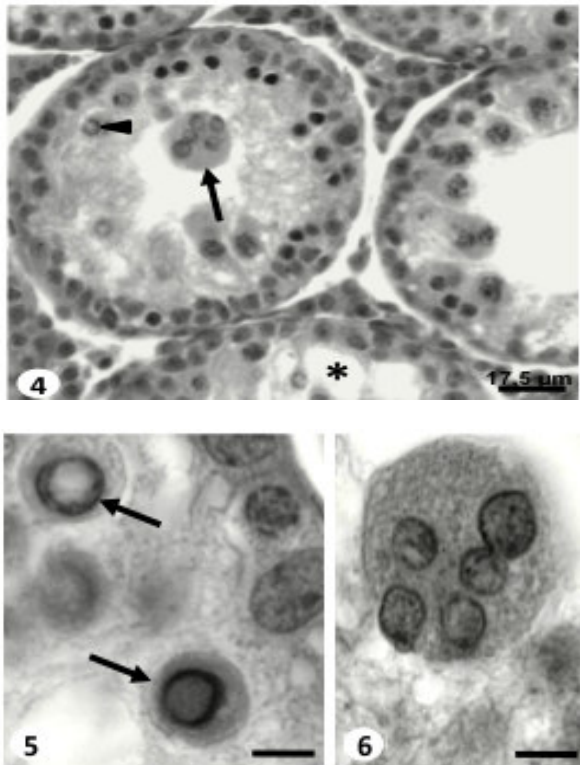
Fertility index

The fertility index was null in doxorubicin treated rats, that is, the matting of adult doxorubicin-treated rats with normal primiparous female rats did not yield offspring. The average fertility index observed in sham control rats was 10.5. One hundred and twenty six rats were born from the mating of 6 control rats with 12 normal female rats.

DISCUSSION

Doxorubicin is a very potent chemotherapeutic drug which has been used against a variety of cancers. Despite its efficiency, doxorubicin has been shown to cause death of healthy cells, especially those undergoing rapid proliferation. It has been shown that doxorubicin causes germ cell death and seminal alterations (Kang et al., 2002; Kato et al., 2001). However, the long term effects of the early prepubertal administration of doxorubicin on the reproduction have not been explored. The present study shows that doxorubicin produces serious damage to the testis of adult rats treated during the early prepubertal phase, suggesting severe harm to the fertility and possible future sterility.

At all ages studied, accentuated seminiferous epithelium depletion was observed. This probably happened due to an increase of apoptosis rate, since previous studies have demonstrated that this drug causes male germ cell apoptosis (Shinoda et al., 1999). The increase of germ cell death usually leads to a reduction of the morphometric and stereological parameters. Morphometric and stereological analyses are important tools in the investigation of drug targets. They can reveal subtle differences, which cannot be noticed in simple histopathological analysis. In addition, stereological analysis is particularly important to indicate the dynamics of the different compartments of an organ such as the testes in face of adverse conditions. The present study shows that the volume of the seminiferous epithelium reduced in all doxorubicin-treated rats as a result of germ cell death. On the other hand, the volume of the interstitial compartment was reduced only in the D_{40} and D_{64} subgroups, but not in the D_{127} subgroup, whereas the volume densities of both seminiferous

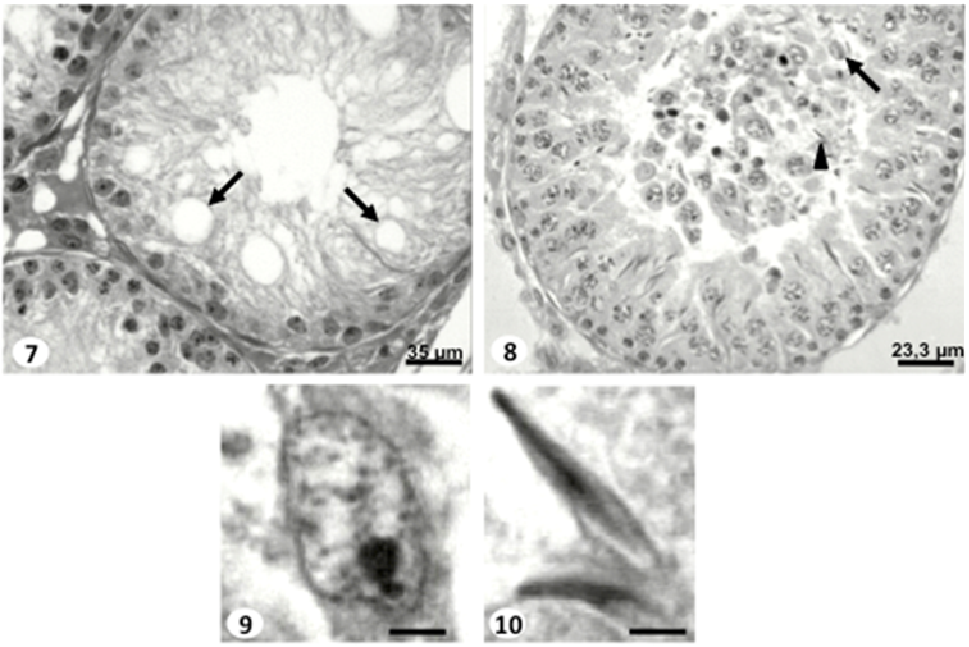


Figures 4 to 6. Testicular cross sections of 40 day old doxorubicin-treated rats submitted to PAS+H method. In Figure 4 a seminiferous tubule section showing accentuated germ cell depletion. This tubular section contains a multinucleated formation of round spermatids (arrow), intraepithelial vacuoles (asterisk) and cells with condensed and delineated chromatin (arrowhead), suggesting apoptosis. Figures 5 and 6 show, in detail, the apoptotic cells and the multinucleated formation, respectively.

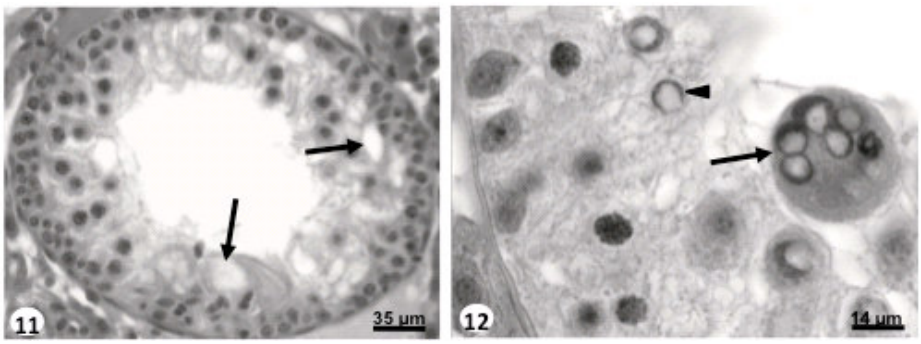
tubules and interstitial compartment were altered only in the adult rats, indicating that the treatment with doxorubicin during early prepubertal phase caused an imbalance in the proportion of these two testicular compartments. In a previous study realized by our group (Vendramini et al., 2010), a considerable reduction of the seminiferous epithelium height and volume density was observed in pubertal and adult rats that were treated with doxorubicin when they were 30 days old, that is, at a later stage of pre-puberty than that utilized in the current study (15 and 22 days). In the first study, a significant reduction of interstitial tissue volume density and an increase of lymphatic space volume density were observed in the doxorubicin-treated rats. The increase of lymphatic space volume density indicates the occurrence of testicular edema (Vendramini et al., 2010). Indeed, doxorubicin causes endothelial dysfunction and edema, as secondary effects of oxidative stress in the vascular wall. The vascular endothelium plays a fundamental role in the maintenance of organ function by forming a barrier regulating water and solute distribution between blood and tissues; however, this fluid control can be deregulated by oxidative stress (Wolf and Baynes, 2007; Wolf and Baynes, 2006), resulting in movement of water and proteins from the vascular system into tissues and compromising organ function. On the other hand, in the current study, adult rats, which started the treatment when they were 15 days, did not show alterations of the interstitial compartment volume, as a whole, but they showed differences in the seminiferous tubule compartment. These data indicate that the interstitial compartment volume of the adult rats recovered from the deleterious effect of the treatment with doxorubicin in the early prepuberty, whereas the seminiferous epithelium and spermatogenesis did not. Thus, although the main target of our study was the seminiferous epithelium, we cannot disregard a possible damage of Leydig cell and probable morphological alterations of the interstitial tissue. In rats, the morphological differentiation of Leydig cells starts between 15 and 16.5 days post-partum (dpp). An increase of the numerical density of these cells occurs in the second post-natal week indicating that there is cellular proliferation during this period (Haider, 2004; Huhtaniemi et al., 1984). So, in doxorubicin-treated rats, the possibility of Leydig cell damage during this period seems to be plausible and, in consequence, the reduction of its frequency and perhaps of other interstitial cell types could result in the decrease of the volume of the interstitial compartment in the 40 and 64 day old rats, counterbalancing the testicular volume alterations that probably occurred due to interstitial edema. It has been reported that anticancer agents can affect Leydig cells (Barcellona and Brinkley, 1973). Indeed, abnormal maturation of Leydig cells and interstitial fibrosis were observed in the testes of children submitted to doxorubicin treatment against lymphoblastic leukemia (Kobayashi et al., 1996). Thus, further studies must be carried out to better investigate this aspect.

The morphometric and stereological data concerning the seminiferous epithelium agree with the histopathological analysis since, in adulthood, germ cell depletion was more accentuated in D_{127} subgroup than in the other doxorubicin-treated subgroups.

It is known that doxorubicin causes death of intermediary and type B spermatogonia through its interaction with topoisomerase II, an enzyme present in high quantity in intermediary and type B spermatogonia (Hou et al., 2005; Jahnukainen et al., 2000). Although type B and intermediary spermatogonia are not the only cell types of the rat seminiferous epithelium under continuous division, their localization in the base of the seminiferous tubules makes them more vulnerable to the action of cytotoxic drugs (Lu and Meistrich, 1979; Stumpp et al., 2004). In addition, the frequency of tubular sections containing primary spermatocytes was also significantly reduced in doxorubicin-treated rats, mainly in the adulthood. Although in pre-meiotic DNA synthesis the



Figures 7 to 10. Testicular cross sections of 64 day old doxorubicin-treated rats submitted to PAS+H method. Figure 7 depicts a seminiferous tubule section showing accentuated germ cell depletion and intraepithelial vacuoles (arrows). In Figure 8, detached germ cells are present in the tubular lumen, although the seminiferous epithelium is partially recovered. Among the detached cells it is possible to observe some spermatid heads (arrowhead) and sertoli cell nuclei (arrow). Figures 9 and 10 show a sertoli cell nucleus and spermatid heads, respectively, observed in the tubular lumen.



Figures 11 and 12. Testicular cross sections of 127 day old doxorubicin-treated rats submitted to PAS+H method. In Figure 11 a seminiferous tubule section with accentuated germ cell depletion and intraepithelial vacuoles (arrows) is shown. Figure 12 shows a multinucleated formation of round spermatids (arrow) and cells with condensed and delineated chromatin (arrowhead), suggesting apoptosis. Note that in the multinucleated formation (arrow) the nuclei also show morphological characteristics of apoptosis.

role of topoisomerase II can be less pronounced, it is possible that other topoisomerases can be involved in the process, as observed for etoposide (Freitas et al., 2002; Hakovirta et al., 1993). The histomorphometric alterations noted in the D_{127} subgroup indicate that the lesion of type B spermatogonia was not the unique mechanism of action by which this drug damaged spermatogenesis. Severe germ cell depletion was observed in this subgroup, with some tubular sections showing only Sertoli cells. This fact suggests that type A spermatogonia were damaged, hampering the repopulation of the seminiferous epithelium. Indeed, in

rats, besides effective pre-mitotic DNA synthesis at stages II-III and IV-VI of the seminiferous epithelium cycle, in which B and intermediary spermatogonia are formed, there are other pre-mitotic peaks of DNA synthesis at stages IX, XII, XIV and I, involving A1, A2, A3 and A4 spermatogonia, respectively (Hakovirta et al., 1993). This fact corroborates with the data observed in this report, which show evident reduction of the frequency of seminiferous tubule sections containing type A spermatogonia in doxorubicin-treated rats at all ages studied. The harm of spermatogonia could be a possible cause of the depletion of spermatocytes and spermatids, although it could be caused by a direct effect of doxorubicin on pre-meiotic DNA synthesis. Indeed, a drastic depletion of seminiferous epithelium and the large quantity of tubular sections containing only Sertoli cells can indicate that reserve and renewing spermatogonia were harmed. In the current experiment, the frequency of type A spermatogonia was significantly decreased in all doxorubicin-treated groups. In addition, rats of D_{127} subgroup, when matted with fertile females, did not produce pups, indicating that these rats were infertile and reinforcing the hypothesis that doxorubicin can cause damage of stem spermatogonia. Although the doxorubicin-treated adult rats showed a significant loss of weight, they did not show ascites, alteration of locomotion and signal of physical debility during the mating period. This information is important, since the basic health condition is the minimal requisite necessary to successful reproduction and for a normal spermatogenic process. However, although the adult rats seemed to be healthy, we must consider the possibility of some doxorubicin-treated rats did not mate for other reasons. The analysis of the reproductive and sexual behaviour, such as mount and intromission latencies, intromission frequency, ejaculation latency, number of ejaculations among other behavioural parameters (Agmo, 1997; Ahlenius and Larsson, 1984) could provide more data about doxorubicin action on the reproductive performance of adult rats that were treated in the early pre-puberty. On the other hand, although a complete analysis of the reproductive performance and behaviour has not been carried out, the magnitude of the spermatogenic damages observed in the doxorubicin rats matches with their reproductive capacity, since all adult doxorubicin-treated rats showed null fertility index, an accentuated reduction of the seminiferous epithelium volume and tubular diameter and a decrease of the percentage of seminiferous tubules cross-sections containing the cycle-specific germ cell types, specially type A spermatogonia.

It is also important to consider that the first dose of doxorubicin was administered when the rats were 15 days old. At this age, the testes are still located in the abdomen and the seminiferous epithelium is undergoing important modifications. Moreover, Sertoli cells are ceasing their proliferation and starting to form the hematotesticular barrier (HTB) (Russell and Griswold, 1993). HTB is responsible for separation of seminiferous epithelium in two compartments: the basal compartment, where spermatogonia and pre-leptotene spermatocytes are located; and the adluminal compartment, in which the other gem cell types are protected from possible harmful agents. Thus, the administration of harmful agents before HTB is established can lead to the death of all germ cells. In addition, Sertoli cells are still under maturation and are more sensitive to adverse conditions than the adult Sertoli cells. It has been shown that Sertoli cells from prepubertal rats are irreversibly harmed by etoposide, a chemotherapeutic drug which action is similar to doxorubicin (Stumpp et al., 2006, 2008). Thus, it is also possible that, to some extent, the damages caused by doxorubicin have been mediated by Sertoli cells. This hypothesis is under investigation by our group.

CONCLUSION

In conclusion, the present study shows that the administration of doxorubicin to early prepubertal rats causes reversible stereological alterations of the interstitial compartment in adult phase, as well as severe damage of the adult spermatogenesis, leading to temporary infertility or even to complete sterility. These injuries are probably age and dose dependent and could be a result of type A spermatogonia and/or sertoli cell damage. However, although the histomorphometric data and the fertility index analysed altogether suggest the occurrence of definitive loss of fertility, the reversibility or not of the reproductive capacity of these animals must be better investigated.

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