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Original article

Effect of short- and medium-term toxicity of doxorubicin on spermatogenesis in adult Wistar rats

Robson Campos Silva[a,*], Daniella Maria Coelho Britto[a], Wagner de Fátima Pereira[b], Gustavo Eustáquio Alvin Brito-Melo[b], Cristiane Tolentino Machado[a], Marcelo Mattos Pedreira[c]

[a] Laboratory of Cellular Biology, Department of Basic Sciences, Federal University of Jequitinhonha and Mucuri Valleys,Alto da Jacuba, Diamantina, Minas Gerais, Brazil
[b] Immunology Laboratory, Integrated Health Research Center, Federal University of Jequitinhonha and Mucuri Valleys, Alto da Jacuba, Diamantina, Minas Gerais, Brazil
[c] Department of Animal Sciences, Federal University of Jequitinhonha and Mucuri Valleys, Alto da Jacuba, Diamantina, Minas Gerais, Brazil

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ABSTRACT

Doxorubicin (DXR) is a widely used chemotherapeutic anticancer agent that has potent activity against several solid and non-solid human malignant tumors, including childhood malignancies. However, DXR has serious toxic effects on tissues with rapid cell cycles, such as myeloid and lymphatic tissues, intestinal mucosa, testes and ovaries. In the present study, the short- and medium-term toxic effects of DXR on the reproductive system of male Wistar rats were evaluated using morphometric and stereological tools to quantify damage to the seminiferous epithelium. Adult male Wistar rats were treated with dose of 7.5 mg/kg of DXR and were sacrificed at seven, 14, 21 and 28 days after treatment. The testes were fixed in glutaraldehyde solution, routinely processed and embedded in plastic for evaluation under a light microscope. A significant reduction in testis weight was found as a result of massive germ cell apoptosis. Differences in comparison to the control group were found in the relative frequency of all stages of the seminiferous epithelium cycle, with significant differences for stages VIII–XI. Apoptosis significantly decreased the number of pachytene spermatocytes in the stages evaluated (I, II–III and VIII) at seven and 14 days. At 21 and 28 days after treatment, the testes exhibited the massive loss of germ cells that resulted in a missing cell layer. Moreover, reductions in the height of seminiferous tubules, tubular diameter and tubular compartment as well as an increase in the intertubular compartment were found in the period studied.

1. Introduction

The chemotherapeutic agent doxorubicin (DXR) or Adriamycin^{TR} is a widely used in the treatment of cancer. DXR is a glycoside antibiotic belonging to anthracycline family obtained from *Streptomyces peucetius* var. *caesius* [1] with potent chemotherapeutic activity against several solid and non-solid human malignant tumors, including childhood malignancies [2,3]. The major mechanisms involved in DOX-induced testicular toxicity include oxidative stress resulting from lipid peroxidation and cellular apoptosis [4]. Although the antitumor action of DXR is mediated by a large number of mechanisms, oxidative stress and the generation of toxic reactive oxygen species are the main causes of its toxicity [5].

In rats and mice, DXR is rapidly removed from the plasma after injection and deposited in tissues. The drug is mainly excreted through bile and moderately excreted through urine [6]. In some rodents, DXR accumulates in the kidneys, liver, heart, and small intestine with greater intensity than daunorubicin, which is another member of anthracycline family [6]. Studies have indicated that DXR has the ability to induce mutations and chromosomal aberrations in normal and malignant cells [7–9]. In a similar manner to other chemotherapeutic agents, DXR has serious toxic effects on tissues with rapid cell cycles, such as myeloid and lymphatic tissues, intestinal mucosa, testes and ovaries [5].

The prognosis of childhood cancer has improved markedly in the last 20 years. However, the long life expectancy makes young survivors especially prone to the late onset consequences of successful cancer therapy, such as therapy-induced secondary malignancies, cardiac toxicity, and infertility [10]. The impact of chemotherapy on fertility and gamete quality is of concern to patients in the reproductive age [11]. Infertility is an unfortunate side effect of some cancer therapies that impacts the quality of life of survivors who are in their reproductive or pre-reproductive years [12].

The DXR-induced deterioration of sperm motion, sperm content and

* Corresponding author.
E-mail address: robson.silva@ufvjm.edu.br (R.C. Silva).

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sperm morphology, which were responsible for the adverse effect on male fertility [13] and the major mechanisms known to be involved in DOX-induced testicular toxicity include oxidative stress resulting from lipid peroxidation and cellular apoptosis [4].

Spermatogenesis is a highly coordinated cyclic process that begins with mitotic spermatogonial proliferation, proceeds through two meiotic divisions, and is followed by spermiogenesis, in which haploid spermatids develop into spermatozoa. Spermatogenesis involves different germ cell associations and is one of the most productive, self-renewing systems in the body, lasting from 30 to 75 days in mammals [14]. The stages of the cycle of seminiferous epithelium are characterized according to changes in the shape of the spermatid nucleus, the occurrence of meiotic divisions, and the arrangement of spermatids within the germinal epithelium [15]. The characterization of the stages of spermatogenesis and the estimation of the length of the spermatogenic are fundamental to determining spermatogenic efficiency and performing comparative studies among species [16]. Thus, the stages and cycle duration are the essential basis for understanding normal and abnormal spermatogenesis. In rats, this cycle of the seminiferous epithelium was divided into 14 stages [17], with the subsequent publication of additional guides for staging in rats [14,18].

Previous studies involving rats have revealed that cytostatic drugs are able to induce apoptosis in the spermatogenic epithelium and the cell types most sensitive to this phenomenon are spermatogonia, zygotene spermatocyte, early pachytene spermatocytes, primary and secondary spermatocytes [19,20]. This apoptotic pathway has been associated with increased levels of caspase 9, 3, 8, 12, Fas and Bid as well as a disturbance in the Bcl-2 family protein balance [21].

The aim of the present study was to investigate the short- and medium-term effects of exposure to DXR on male reproduction in rats, emphasizing the impact of DRX on the apoptosis of germ cells, absence of the germ cell layer and the frequency of the stages of the seminiferous epithelium cycle.

2. Materials and methods

2.1. Animals

Twenty male Wistar rats aged six to eight weeks (body weight: 250–300 g) were obtained from the animal housing facilities of the Federal University of Minas Gerais (Brazil) and kept under controlled environmental conditions with free access to food and water. The experimental protocol was approved by the local animal experimentation ethics committee (CETEA-UFMG: 99/2009).

2.2. Experimental design

The animals were divided into two groups: an experimental group ($n = 16$) that received a single dose (7.5 mg/kg) of doxorubicin (Eurofarma, Brazil) in the tail vein of non-anesthetized animals and a control group ($n = 4$) that received phosphate-buffered saline (PBS: 0.15 mol/L sodium chloride and 0.01 mol/L phosphate buffer, pH 7.4) under the same conditions. Animals were euthanized with pentobarbital at seven, 14, 21, and 28 days after treatment with DXR (experimental group) and at 28 days after PBS injection (control group).

2.3. Preparation of tissue for microscopy

The animals were euthanized with pentobarbital during the perfusion procedure used for fixation of the testis (n = 20). The animals were subsequently perfusion-fixed through whole body perfusion [22]. Briefly, after the intraperitoneal injection of heparin, the rats were anesthetized and the abdomen and thoracic cavity were opened to expose the heart. A needle was inserted into the left ventricle and 0.9% saline solution was used to clear the blood vessels. After clearance, a two-way valve apparatus was used to introduce 4% buffered glutaraldehyde into the vessels without removing the needle. The animals were perfused for 25–30 min, immediately after which the testes were removed and weighed, then cut longitudinally by hand with a razor blade into small fragments. These fragments were immersed in 4% buffered glutaraldehyde for 12 h. Tissue samples measuring 2–3 mm in thickness were routinely processed and embedded in plastic (glycol methacrylate) for histological and stereological analyses.

2.4. Biometric data and testis stereology

Sections of approximately 5 µm in thickness were obtained, placed on glass slides, and stained with hematoxylin and eosin. Counts (frequency of stages) were made with light microscopy (X 1000), based on previous studies [14,18]. Five hundred randomly selected tubules from each animal were classified in the fourteen stages at seven and 14 days after treatment with DXR. The percentage frequency of the stages was calculated by the ratio between the number of sections in each stage and the total number of sections analyzed and multiplying by 100 [23]. As pachytene spermatocytes are found in all stages of the spermatogenic cycle in rats [18], pachytene counts were performed in fifty randomly selected tubules (X 400) from each animal for comparisons between the control and experimental groups. Among the fourteen stages classified, cells were counted in the three stages with the highest frequencies (I, II–III and VIII) and consequently more reliable for statistical analysis. As it was not possible to classify the experimental group at 21 and 28 days after the injection of DXR in stages, an estimate of damaged tubules was performed by evaluating 500 randomly selected tubules (X 100). Tubular diameter and height of the seminiferous tubule epithelium were measured (X 100) using an ocular micrometer calibrated with a stage micrometer. At least thirty tubular profiles that were round or nearly round were chosen randomly and measured. The volume densities of testis tissue components were determined using a 447-intersection grid placed on the ocular of the light microscope. Fifteen randomly chosen fields (6705 points) were scored (X 400). Artifacts were rarely seen and were not included in the data. The points were classified as tubular compartment (comprising tunica propria, epithelium, and lumen) and intertubular compartment (including Leydig cells, connective tissue, blood, and lymphatic vessels).

2.5. Statistical analysis

All data were submitted to normality and homoscedasticity tests. Depending on the results of the normality test, either analysis of variance ANOVA and subsequent regression or one-way ANOVA and Tukey's test were performed using the Sigma Stat 3.5 program (Systat Software, Inc.). A p-value < 0.05 was considered indicative of statistical significance.

3. Results

3.1. Biometric data

Fig. 1 shows the results referring to body weight, testicular weight, and the gonadosomatic index (GSI) (testis mass divided by body weight) in the control and DRX-treated groups. No significant differences in body weight were found between the groups. However, significantly lower testis weight and GSI were found in the DXR-treated rats.

3.2. Testis stereology

3.2.1. Frequency of the stages of the seminiferous epithelium cycle

The frequencies of all stages of the seminiferous epithelium cycle were altered in DXR-treated rats at seven and 14 days compared to the control group (Fig. 2). Significant differences between groups were found for stages VIII–XI. A significant increase in stage VIII was found