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Anti-apoptotic effect of spermatogonial stem cells on doxorubicin-induced testicular toxicity in rats

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ABSTRACT

The present study was designed to investigate whether spermatogonial stem cells (SSCs) have possible effect on doxorubicin (DOX)-induced testicular apoptosis and damaged oxidant/antioxidant balance in rats. Sixty male Albino rats were divided into 3 groups: the saline control group, the testicular toxicity group (2 mg/kg DOX once a week for 8 weeks) and the third group is a donor stem cells transplanted following pre-treatment with DOX. After the 8th week, the rats were sacrificed and tissues were collected and examined for CD95, CD95L, Caspase 3, and Caspase 8 gene expression using RT-PCR. While malondialdehyde (MDA), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) were determined using colorimetric kits. Biochemical, histopathological and PCR results showed improvement of the SSCs' group compared to the DOX-group. It was observed that spermatogonial stem cell affected DOX-induced activation of intrinsic apoptotic signaling pathway via preventing DOX-induced increases in CD95 and CD95L levels as well as cleaved Caspase-8 and Caspase-3 levels in testicular tissues, however, spermatogonial stem cell decreased Dox-induced NF-κB activation as well. It can be concluded that SSCs may be utilized to develop new cell-based therapies, and to advance germline gene therapy.

1. Introduction

Each year, an estimated 160,000 children are diagnosed with cancer worldwide, and it has been estimated that a male infant has a 1 in 300 chance of being diagnosed with a malignancy by the age of 20 (Ries et al., 2007). Fortunately, success rates in treating childhood cancer have increased dramatically over the past few decades, and now approximately 80% of children survive following treatment (Steliarova-Foucher et al., 2004; Howlader et al., 2011). Many therapies to treat cancer are gonadotoxic and can lead to infertility, and fertility potential has an important impact on quality of life according to cancer survivors (Carter et al., 2005; Lee et al., 2006). In fact, the American Society of Clinical Oncology now recommends that the reproductive risks of cancer therapies and fertility preservation options should be routinely discussed with patients' prepubertal boys, adolescents, and adult men' before beginning treatment (Lee et al., 2006; Keros et al., 2007). Spermatogonial stem cells (SSCs) maintain spermatogenesis throughout their lives, and they are defined by their ability to undergo both self-renewing cell divisions and differentiation, leading to the production of haploid sperm. Regeneration of spermatogenesis following SSC transplantation has now been established in several animal models, including rodents, goats, sheep, pigs, dogs, and monkeys (Mikkola et al., 2006; Herrid et al., 2009; Hermann et al., 2012).

Anthracycline antibiotics are widely used as chemotherapeutic drugs in the treatment of human hematological malignancies and solid tumors. Doxorubicin (DOX) has become one of the most prescribed anticancer drugs (Hannun, 1997). The clinical use of DOX is associated with testicular dysfunction characterized by altered sperm development, production, structural integrity and motility rates in association with increased cellular apoptosis (Kato et al., 2001; Prahalathan et al., 2005; Trivedi et al., 2011).

The present work aims to investigate whether spermatogonial stem cells (SSCs) has possible effect against DOX-induced testicular apoptosis and damaged oxidant/antioxidant balance in rats.

2. Materials and methods

2.1. Drugs and chemicals

Doxorubicin hydrochloride (50 mg) was purchased from Pfizer Company, Egypt (Adriblastina®). All other chemicals were purchased from Sigma Chemicals Co., (St. Louis, MO, USA).

Abbreviations: ANOVA, analysis of variance; CAT, catalase; DOX, doxorubicin; DMEM, Dulbecco's modified Eagle's medium; ELISA, high-sensitivity enzyme-linked immunosorbent assay; EDTA, ethylenediaminetetraacetic acid; GSH-Px, glutathione peroxidase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NBT, nitroblue tetrazolium; NF-κB, nuclear factor kappa B; qPCR, quantitative real-time PCR; C_q, quantification cycle; St, seminiferous tubules; SOD, superoxide dismutase; SSCs, spermatogonial stem cells; TBA, thiobarbituric acid.

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2.2. Experimental protocol

2.2.1. Donor cells preparation and transplantation analysis

Cells for transplantation were obtained from the testes of male white Albino rat between 5 and 10 days by a two-step enzymatic digestion protocol (Brinster and Avarbock, 1994). In this procedure, the tunica albuginea was first removed or peeled from the testis, thereby exposing the seminiferous tubules. The testes were then incubated in approximately 10 volumes of Hanks' balanced salt solution without calcium or magnesium (HBSS) containing 10 mg/ml collagenase (Type IV, Sigma) at 37 °C with gentle agitation for 15 min or until the tubules were separated. Dispersion of the tubules can be hastened by careful dissection, spreading of the tubules, and removal of intertubular cellular strands when visible. The addition of 300 μg/ml DNAse facilitates intertubular cell dispersion. The testis tubules were then washed 2 to 4 times in 3–4 ml of HBSS followed by incubation at 37 °C for 5 min in HBSS containing 1 mM EDTA and 0.25% trypsin. Separation and dispersion of the tubule cells can be hastened by pipetting and gentle agitation. When most of the cells were dispersed, the action of trypsin was terminated by adding a 10 to 20% volume of fetal bovine serum. The inclusion of 400 μg/ml of DNAse decreases stickiness and facilitates dispersion of the cells. Following digestion, any large pieces of undigested material were removed, and the cell suspension was filtered through a nylon mesh with 60 μm pore size to remove large clumps of cells. The filtrate was centrifuged at 600 ×*g* for 5 min at 16 °C and the supernatant was carefully removed from the pellet. The cells in the pellet were then resuspended in 400 μl Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at a concentration of (2–3) × 10^7 cells/ml. The cells were maintained at 5 °C until the time of loading into an injection pipette, usually 1 to 4 h (Ogawa et al., 1997).

2.2.2. Transplantation of spermatogonial stem cells

In order to inject donor cells directly into the rete testis, the testis was removed from the body cavity and reflected laterally. The rete lies primarily under the vascular pedicle where it contacts the surface. The most obvious vessels are the large veins that lie under the tunica and drain the testis. Insert the loaded micropipette into the area cranial to the vessels and adjacent to the efferent ducts. The micropipette should be almost parallel to the surface of the testis. After entering the rete, increase the pressure very slowly in the injection tube until the cell suspension fills the rete and flows into the tubules. About 10–20 μl of cell suspension was required to fill one testis. Filling of the tubules can be monitored by observing the movement of the cell suspension, which was facilitated by adding a small amount of trypan blue to the injection medium. After the tubules were completely filled, withdraw the micropipette. Return the testis to the abdomen and repeat the injection on the other testis. After replacing the testis in the abdominal cavity, suture the skin with one or two clips (Ogawa et al., 1997).

2.3. Animals and experimental design

The recipient rats were 15–30 days of age. The animals were maintained under standard conditions of humidity, temperature (25 ± 2 °C) and light (12 h light/12 h dark). They were fed with a standard rat diet and had free access to water. The study was conducted in accordance with the Guidelines for Ethical Care of Experimental Animals and was approved by the Animal Care and Use Committee of Biochemistry Department, Faculty of Pharmacy, Zagazig University.

The animals were randomly divided into three groups, each of 20 rats. The first group was administered sterile saline and left as a control group. The second group was injected intraperitoneally (i.p.) with DOX; which already reconstituted in sterile saline; as a weekly single dose of 2 mg/kg body weight for 8 weeks (eribaşl et al., 2012). The third group was injected with both DOX and spermatogonial stem cells, and in this group DOX was injected (i.p.) at a dose of 2 mg/kg body weight/week for 8 weeks while spermatogonial stem cells were administered as only a single dose after 4 weeks from DOX injections. Owing to the fact that rats need a period of 48–52 days for the exact spermatogenic cycle including spermatocytogenesis, meiosis and spermiogenesis (Türk et al., 2010; Çeribaşl et al., 2010), the administration period was set at 8 weeks.

At the end of the experimental work, animals were sacrificed by decapitation. Blood samples were collected into tubes containing EDTA and centrifuged at 3000 rpm for 10 min to obtain plasma. One of the testes was prepared for light and electron microscope examination of seminiferous tubules (st). The other testes and plasma samples were stored at −20 °C until biochemical analyses.

2.4. Epididymal sperm concentration, motility, and abnormal sperm rate

The epididymal sperm concentration in the right cauda epididymal tissue was determined with a hemocytometer using a modified method of Yokoi et al. (2003). Freshly isolated left cauda epididymal tissue was used for the analysis of sperm motility. The percentage sperm motility was evaluated using a light microscope with heated stage described by Sönmez et al. (2005). To determine the percentage of morphologically abnormal spermatozoa, the slides stained with eosin–nigrosin (1.67% eosin, 10% nigrosin and 0.1 M sodium citrate) were prepared. The slides were then viewed under a light microscope at 400× magnification. A total of 300 spermatozoa were examined on each slide, whereas the head, tail and total abnormality rates of spermatozoa were expressed as percentage (Ateşşahin et al., 2006).

2.5. Biochemical analysis

2.5.1. Preparation of tissue homogenates

Tissue samples were homogenized in phosphate buffer (0.5 M, pH 7.0) (1/10, w/v) and then centrifuged for 5 min at 16,000 ×*g* at 4 °C to sediment unbroken cells and cellular debris. In the supernatant, the determination of lipid peroxidation, glutathione peroxidase activities, catalase, as well as superoxide dismutase content was determined.

2.5.2. Assay of oxidative stress

2.5.2.1. Lipid peroxidation. Lipid peroxide was estimated in testis homogenate by measurement of malondialdehyde (MDA) levels spectrophotometrically whereas the testis samples were homogenized in an ice-cold 50 mM potassium phosphate buffer (pH 7.5), centrifuged for 15 min at 12,000 ×*g* at 4 °C and then the supernatant was collected. MDA in the supernatant can react with freshly prepared thiobarbituric acid (TBA) to form a colored complex which has maximum absorbance at 535 nm. The nmol MDA/g wet tissue was calculated from the plotted standard curve prepared from 1,1,3,3-tetraethoxypropane (Buege and Aust, 1978).

2.5.3. Assay of antioxidant enzymes

2.5.3.1. Glutathione peroxidase (GSH-Px) activity. Glutathione peroxidase (GSH-Px) activity was spectrophotometrically determined according to the method of Lawrence and Burk (1976). The reaction mixture contains 1 ml of sodium phosphate buffer (pH 7.0) and 0.1 ml of NADPH reagent. Homogenate (0.01 ml) was added to the reaction mixture and then it was initiated by adding 0.1 ml of H_2O_2. The decrease in absorbance at 340 nm was recorded over a period of 3 min. Protein concentrations were determined using the method of Lowry et al. (1951). The GSH-Px activity was expressed as IU/g protein.