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Effects of bleomycin, etoposide and cisplatin treatment on Leydig cell structure and transcription of steroidogenic enzymes in rat testis

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ABSTRACT

Cytotoxic anticancer chemotherapy affects pituitary–testicular hormonal axis in humans and in animals. This study investigated the effects on Leydig cells of three cycles of bleomycin, etoposide and cisplatin (0.75, 7.5, and 1.5 mg/kg, respectively; BEP) chemotherapy in rat testis. The chemotherapy has induced hyperplasia of and degenerative changes in Leydig cells at the end of BEP exposure, which remained so even after a recovery time of 63 days. The increased testicular oxidative stress at the end of the chemotherapy returned to normal level after the recovery time. The chemotherapy has stimulated the transcription of scavenger receptor class type-B1 (*SCARB1*), steroidogenic acute-regulatory protein (*StAR*), cytochrome P450 cholesterol side-chain cleavage (*CYP11A1*), *CYP17A1*, and inhibited that of 17β-hydroxysteroid dehydrogenase (*HSD17B6*) and *CYP19A1* in association with increased cholesterol and decreased testosterone levels. Even after the recovery time, the chemotherapy still had inhibitory effects on the transcription of all of the above genes in addition to luteinizing hormone receptor and *HSD3B1*, but not on the StAR gene. The cholesterol and testosterone levels also did not show any significant differences with the control group. The decreased testosterone level at the end of chemotherapy was probably due to inhibition of *HSD3B1* and *HSD17B6* genes. In conclusion, clinically relevant dose-levels and treatment protocols of BEP chemotherapy adversely affect Leydig cell function. The BEP chemotherapy inhibits the transcription of steroidogenic enzymes and that these effects sustain over an extended period of time without returning to normal levels.

1. Introduction

Combination chemotherapy of bleomycin, etoposide and cisplatin (BEP) is very effective to combat several types of cancer, especially the testicular neoplasms. Most cytotoxic anticancer drugs disrupt testicular functions in both humans (Howell et al., 1999; Howell and Shalet, 2005; Kenney et al., 2014) and animals (Delbes et al., 2010; Kilarkaje et al., 2013; Marcon et al., 2008), which ultimately lead to infertility and associated endocrine disorders (Jahnukainen et al., 2011). In males, BEP chemotherapy destroys germ cell population and spermatogenesis and interrupts pituitary–testis hormonal axis. Three cycles of 21 days each of human therapeutic dose-levels of BEP induce germ cell death, single- and double-strand DNA breaks (Delbes et al., 2009; Narayana et al., 2012) and impair the fertility outcome in rats (Bieber et al., 2006). The BEP chemotherapy also up-regulates the expression of proto-oncogenes and oxidative stress-related genes (Delbes et al., 2009), induces both hypo- and hyper-methylation of DNA (Chan et al., 2012) and other epigenetic modifications in germ cells of male rats (Maselli et al., 2012, 2013). The BEP-treated male rats also become infertile (Kilarkaje et al., 2013), and even if they regain fertility, they still have significant effects of the drugs on fertility such as persistent pre-implantation loss and morphologically abnormal offspring (Marcon et al., 2008).

The endocrine effects of the BEP chemotherapy are not clearly known. The BEP chemotherapy in humans induces low- to low-normal testosterone levels (Tomita et al., 2007) and high levels of serum follicle-stimulating hormone and luteinizing hormone (Spermon et al., 2006; van Basten et al., 1999). These results indicate a persistent effect on both Leydig and Sertoli cells of the BEP chemotherapy in cancer patients (Berger et al., 1996; Brennemann et al., 1997; Palmieri et al., 1996). However, many other studies reveal no such significant effects of BEP chemotherapy on testosterone, luteinizing hormone, and steroid hormone binding globulin in cancer survivors (Lackner et al., 2005), except the occasionally elevated follicle stimulating hormone levels in some of them (Pectasides et al., 2004). In addition to the existing controversy regarding the effects of BEP chemotherapy on Leydig cell functions, it is not known whether or not these drugs alter testosterone synthesis pathway in

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Leydig cells. In view of this, the present study investigated the differential gene expression of proteins involved in testosterone synthesis pathway in three cycles of BEP treated rats.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (13–15 weeks-old) were housed in the Animal Resources Center at the Kuwait University Health Sciences Center. The animal room temperature was 23–25 °C, and humidity was 50–60%. All animals had free access to food and tap water. The animals were looked after, treated and killed in accordance with the international guidelines (NIH publication no. 86-23, revised 1985), and the guidelines of Animal Ethics Committee of Kuwait University.

2.2. BEP treatment protocols

The dose-levels of BEP chemotherapy protocol for cancer patients are bleomycin, 30 mg/week; etoposide, 100 mg/m^2; and cisplatin, 20 mg/m^2 (Marcon et al., 2008). The body surface area adjusted doses to the rat are- bleomycin, 1.5 mg/kg; etoposide, 15 mg/kg; and cisplatin 3 mg/kg (Bieber et al., 2006; Delbes et al., 2009; Marcon et al., 2008). In patients, usually three-four cycles of BEP chemotherapy is given based on the severity of cancers. For the evaluation of the effects of the three cycles of the chemotherapy, the animals were randomly divided into four groups ($n=10$/group) as follows. Two control groups received only water, and the remaining two groups received (i.p.) three cycles of 0.5× dose-levels of bleomycin (0.75 mg/kg), etoposide (7.5 mg/kg) and cisplatin (1.5 mg/kg), as 1× dose-levels resulted in 100% animal mortality probably due to renal failure (Kilarkaje et al., 2013). Each cycle of the treatment period was for 21 days. Bleomycin was treated on days 2, 9 and 16 of each cycle. The other two drugs were given during the first five days of each cycle. A time gap of 30 min was maintained between the successive injections. One control group and one BEP group of rats were sacrificed the next day after the third cycle of the chemotherapy. The remaining two groups of rats were sacrificed 63 days (recovery time) after the third cycle; this period allowed the animals to recover from the effects of the chemotherapy. The animals in BEP group showed significant decreases in body and testicular weights and docile nature without much of activity. The amount of food and water intake was also significantly decreased as compared to the control group (data not shown). Two animals belonging to the BEP group died during the chemotherapy, which were replaced by new recruits.

2.3. Animal euthanization and tissue collection

The animals were euthanized by CO_2 inhalation; the heart was exposed and the venous blood was collected by cardiac puncture. Heparinized phosphate buffered saline (PBS; pH 7.4) was injected into the left ventricle to clear the blood from the testes. The testes and other reproductive organs were removed and cleaned. One testis was snap frozen in dry ice and stored in −70 °C, until use. The other testis was immersion fixed in paraformaldehyde for histopathological studies.

2.4. Evaluation of oxidative stress status in the testes

2.4.1. Total antioxidant status

The total antioxidant status (TAS) in the testes was estimated by using a kit (Cat# RL0017; Rel Assay Diagnostics; Turkey) as described before (Kilarkaje et al., 2014). First, the testes were homogenized at high speed with a diluent [50 mM potassium phosphate buffer + 1 mM ethylenediamine-tetraacetic acid (EDTA; pH 7.0; 1:9, w/v)] and centrifuged at 4 °C. The supernatant was used for the assay. A 96-well-plate was used to read the absorbance of the samples. A volume of 125 μl of the assay buffer (reagent 1) was added to all the wells. For the blank wells (standard 1), 7.5 μl of water was added. For the sample wells, 7.5 μl of the homogenate was added. For the standard wells, 0.01 mmol Trolox equiv./l solution (standard 2) was added. The first absorbance was read (660 nm wavelength) in a microplate reader. After reading the absorbance, 18.75 μl of 2,20-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS; reagent 2) was added to each well and the second absorbance (660 nm wavelength) was read after 10 min. Differences (Δ absorbance) between the first and second absorbances of the sample and the standard were calculated. The difference between the ΔA of the sample and the ΔA of the standard 1 (A1), and the difference between the ΔAs of the standards 1 and 2 (A2) were derived. The ratio of A1 to A2 was equal to TAS in the testes, which was expressed in mmol/g tissue.

2.4.2. Total oxidant status

The total oxidant status (TOS) was quantified in the testes by using a commercially available assay kit (Cat#RL0024; Rel Assay Diagnostics; Turkey) as described previously (Kilarkaje et al., 2014). In a 96-well-plate, 18.75 μl of either standard, or the sample or the blank was added. After this step, to each well, 125 μl of the assay buffer (reagent 1) was added, and the first absorbance was read (530 nm wavelength) in a microplate reader. To this mixture, 6.25 μl of a pro-chromogen solution (reagent 2) was added and kept at room temperature for 10 min, and the second absorbance was read at 660 nm wavelength. Differences between the first and second absorbances were calculated for the samples and standard concentrations (Δ absorbance for each category). The TOS in the sample was equal to the ratio of Δ absorbance of the sample and that of the standard X 20. The TOS was expressed as mmol units of hydrogen peroxide equivalent.

2.5. Histopathological evaluation of Leydig cells

The testes were perfused with heparinized PBS followed by infusion of 4% paraformaldehyde and 0.1% glutaraldehyde. The organ was removed, immersed in paraformaldehyde, washed with Millonig's phosphate buffer, post-fixed in osmium tetroxide and washed with the buffer. The tissue was dehydrated in ascending grades of alcohol and treated with propylene oxide. The tissue blocks were penetrated with resin, encapsulated, polymerized and decapsulated. The semi thin section (1 μm) was taken by using glass knives and stained with toluidine blue (Narayana and Al-Bader, 2011). The sections were observed under 40× and 100× objectives, and structural changes in Leydig cells were observed. Representative images were photographed and processed in Adobe Photoshop software.

2.6. Estimation of cholesterol level in the testis

The total cholesterol level in the testes samples was quantified by Zak's method (Zak, 1977). The samples were homogenized in PBS and proteins in the homogenate were precipitated by treating with ferric chloride-acetic acid reagent (1:99 v/v), and filtered. In a 96 well-plate, 5 μl each of ferric chloride-acetic acid solution (blank) and cholesterol standard was loaded into two wells. The remaining wells were filled with 5 μl each of the samples. For all wells, 3 μl of concentrated sulfuric acid was added, mixed well and incubated at room temperature for 30 min. The optical densities of all mixtures were read at 540 nm in a microplate reader. The blank values were subtracted from test and standard values. The ratio of test to standard values (A) was calculated. The ratio of