BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH
www.bjournal.com.br

ISSN 0100-879X
Volume 45 (8) 681-791 August 2012

BIOMEDICAL SCIENCES

Braz J Med Biol Res, August 2012, Volume 45(8) 716-720

doi: 10.1590/S0100-879X2012007500075

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Brazilian Journal of Medical and Biological Research (2012) 45: 716-720
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Short-term levosimendan treatment protects rat testes against oxidative stress

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Abstract

The objective of this study was to evaluate the effect of short-term levosimendan exposure on oxidant/antioxidant status and trace element levels in the testes of rats under physiological conditions. Twenty male Wistar albino rats were randomly divided into two groups of 10 animals each. Group 1 was not exposed to levosimendan and served as control. Levosimendan (12 µg/kg) diluted in 10 mL 0.9% NaCl was administered intraperitoneally to group 2. Animals of both groups were sacrificed after 3 days and their testes were harvested for the determination of changes in tissue oxidant/antioxidant status and trace element levels. Tissue malondialdehyde (MDA) was significantly lower in the levosimendan group ($P < 0.001$) than in the untreated control group and superoxide dismutase and glutathione peroxidase (GSH-Px) levels were significantly higher in the levosimendan group ($P < 0.001$). Carbonic anhydrase, catalase and GSH levels were not significantly different from controls. Mg and Zn levels of testes were significantly higher ($P < 0.001$) and Co, Pb, Cd, Mn, and Cu were significantly lower ($P < 0.001$) in group 2 compared to group 1. Fe levels were similar for the two groups ($P = 0.94$). These results suggest that 3-day exposure to levosimendan induced a significant decrease in tissue MDA level, which is a lipid peroxidation product and an indicator of oxidative stress, and a significant increase in the activity of an important number of the enzymes that protect against oxidative stress in rat testes.

Key words: Levosimendan; Oxidative stress; Reactive oxygen species; Rat testes; SOD; GSH-Px; MDA

Introduction

Oxidative stress triggers a cascade that leads to the production of reactive oxygen species (ROS), accumulation of lipid peroxidation products such as malondialdehyde (MDA), massive secretion of systemic inflammatory mediators that can result in the development of systemic inflammatory response syndromes, impaired cell function, and multiple organ dysfunctions (1-4), while the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) play important roles in cell defense against oxidative stress. Antioxidants in cells, such as SOD, catalase (CAT), glutathione (GSH), and GSH-Px protect the organism against the damages of oxidative stress (5).

Under physiological conditions, the testes are vulnerable to oxidative stress. The testes have a poor vascularization, which means that oxygen tension in this tissue is low (6). Spermatogenesis is an extremely active replicate process. The high rates of cell division inherent in this process imply correspondingly high rates of mitochondrial oxygen consumption by the germinal epithelium. The competition for this element within the testes is extremely intense. Despite the low oxygen tensions that characterize the testicular microenvironment, this tissue remains vulnerable to oxidative stress due to the abundance of highly unsaturated fatty acids and the presence of potential ROS-producing systems. Since both spermatogenesis and Leydig cell steroidogenesis are vulnerable to oxidative stress, the low oxygen tension may be an important component of the

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Received December 23, 2011. Accepted May 4, 2012. Available online May 18, 2012. Published August 3, 2012.

mechanisms by which the testes protect themselves from free radical-mediated damage (7-9). Furthermore, the testes have an elaborate array of antioxidant enzymes and free radical scavengers to ensure that the twin spermatogenic and steroidogenic functions of this organ are not impacted by oxidative stress.

Levosimendan is a recently synthesized positive inotropic drug that improves myocardial contraction by the calcium sensitization of the contractile protein troponin C without increasing calcium concentration in myocardial cells, and also has a vasodilating effect by opening adenosine triphosphate-sensitive potassium channels (10). It was reported that a single dose of levosimendan seems to have anti-inflammatory and anti-apoptotic properties, reducing circulating proinflammatory cytokines and soluble apoptosis mediators (11). We thought that this agent, with its anti-inflammatory and anti-apoptotic properties, might affect the related mechanisms of oxidative stress in the testicular tissues that are vulnerable to oxidative stress under physiological conditions.

The aim of this experimental study was to evaluate the possible influences of short-term levosimendan exposure on oxidant/antioxidant status and trace element levels in the rat testes under physiological conditions.

Material and Methods

Treatment of animals

Twenty male Wistar albino rats, approximately 6 months of age, with an average body weight of 250-300 g were obtained from the Animal Laboratory of Yüzüncü Yil University, Van, Turkey. Rats were housed in cages with 5 rats per cage. A 12-h light/dark cycle was maintained and the rats were fed *ad libitum*. The study was approved by the Ethics Committee of he study was approved by the Ethics Committee of Yüzüncü Yil University.

The animals were randomly divided into two groups, each consisting of 10 rats. The animals in group 1 were not treated with the drug and served as control. In group 2, levosimendan (12 $\mu g \cdot kg^{-1} \cdot day^{-1}$, diluted in 10 mL 0.9% NaCl, *ip*, for 3 days) was injected intraperitoneally. After 3 days, animals in both groups were sacrificed and their testes were harvested for the evaluation of tissue oxidant/antioxidant status and trace element levels after short-term levosimendan exposure.

Biochemical analysis

Measurement of MDA level. A 50-mg tissue specimen was homogenized in 0.15 M KCl. After centrifugation of the homogenate at 1600 *g*, MDA levels in tissue homogenate supernatant were determined by thiobarbituric acid (TBA) reaction according to Kavak et al. (12,13). The principle of this method is based on measuring absorbance of the pink color produced by the interaction of TBA with MDA at 530 nm. MDA levels are reported as mg/dL.

Measurements of SOD and GSH-Px enzyme activities. The tissues were homogenized in physiological saline (1 g in 5 mL) using a homogenizer (B. Braun Melsungen AG 853202, Germany) and centrifuged at 4000 *g* for 20 min (Heraus Labofur 200, Germany). GSH-Px activity was determined by monitoring the changes in NADPH absorbance at 340 nm (14) and by measuring the decrease of H_2O_2 absorbance at 240 nm (15). SOD activity was measured by the method based on nitroblue tetrazolium (NBT) reduction rate. One unit of SOD activity is the amount of enzyme protein causing 50% inhibition of NBT reduction rate (16). SOD and GSH-Px activities are reported as mIU/mg and EU/g Hb, respectively.

Measurement of GSH level. GSH levels were measured by the technique of Sedlak and Lindsay (17) at 412 nm. The samples were precipitated with 50% TCA and centrifuged at 1000 *g* for 5 min. The reaction mixture contained 0.5 mL supernatant, 2.0 mL Tris-EDTA buffer (0.2 M, pH 8.9) and 0.1 mL 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid; DTNB). The solution was kept at room temperature for 5 min and subsequently absorbance was read at 412 nm. GSH levels are reported as mIU/mg.

Measurement of CAT enzyme activity. Erythrocyte CAT activity was measured by the method described by Aebi (18). Briefly, the supernatant (0.1 mL) was added to a quartz cuvette containing 2.95 mL 19 mM H_2O_2 prepared in potassium phosphate buffer (50 mM, pH 7.0). The change in absorbance was monitored at 240 nm for 5 min using a Shimadzu spectrophotometer (UV-1201, Japan). CAT activity is reported as EU/g Hb.

Measurement of carbonic anhydrase (CA) activity. CA activity was assayed by hydration of CO_2 measured by the method of Rickli and Wilbur-Anderson (19) using bromothymol blue as indicator. CA activity is reported as EU/g Hb.

Measurements of mineral levels. Two milliliters of the mixture of HNO_3/H_2O_2 (2:1) was added to 0.7 g of the tissue sample. The mixture was placed in a water bath at 70°C for 30 min, stirred occasionally, and subsequently, 1.0 mL of the same acid mixture was added, and the suspension was transferred to a Teflon vessel for digestion in a microwave oven. The bomb was closed and radiation was applied for 3 min at 450 W. After the addition of 0.5 mL of the same acid mixture, radiation was repeated for 3 min. After cooling for 5 min, 2.0 mL 0.1 M HNO_3 was added, and the solution was transferred to a Pyrex tube. After centrifugation, the clear solution was used for the determination of Cu, Zn, Mg, Mn, Pb, Cd, and Fe (20,21). Measurements were performed by atomic absorption spectrophotometry using a UNICAM-929 spectrophotometer (Unicam Ltd., UK). Trace element levels are reported in µg/dL.

Statistical analysis

Data are reported as means ± SD. The parameters were compared between the two groups using the Mann-Whitney

U-test. All statistical analyses were carried out using the SPSS® statistical software package (SPSS for Windows version 13.0, SPSS Inc., USA) and a P value of ≤0.05 was considered to be significant.

Results

All measurements were performed using appropriate analyses that demonstrated only the values of the parameters evaluated in the testicular tissues of rats. The tissue MDA level, which is as an indicator of oxidative stress, was 125.168 ± 3.702 mg/dL in group 1 and 99.667 ± 1.657 mg/dL in group 2 being significantly lower in the levosimendan group (P < 0.001). The levels of protective enzyme such as SOD and GSH-Px, which play important roles in cell defense against oxidative stress (5), were 11.465 ± 0.441 mIU/mg and 80.926 ± 1.466 EU/g Hb in group 1, and 22.945 ± 4.405 mIU/mg and 111.108 ± 5.470 EU/g Hb in group 2, respectively, being significantly higher in group 2 (P < 0.001). Nevertheless, there was no significant difference in CA, CAT, or GSH levels between group 1 (0.072 ± 0.005 EU/g Hb, 72.609 ± 2.649 EU/g Hb, 76.418 ± 1.807 mIU/mg) and group 2 (0.080 ± 0.026 EU/g Hb, 76.098 ± 6.338 EU/g Hb, 79.199 ± 3.440 mIU/mg, respectively). These data are presented in detail (minimum, maximum and mean ± SD values) and compared in Table 1.

Trace element levels were also analyzed in the two groups. Mg and Zn were 15.82 ± 0.73 and 1.694 ± 0.273 µg/dL in group 1, 24.11 ± 1.19 and 3.98 ± 0.432 µg/dL in group 2, respectively, with their values being significantly higher in the levosimendan group (P < 0.001). Nevertheless, Co, Pb, Cd, Mn, and Cu, with values of 0.6 ± 0.04, 0.16 ± 0.01, 0.08 ± 0.002, 0.03 ± 0.008, and 17.64 ± 0.69 µg/dL in group 1, and 0.44 ± 0.02, 0.05 ± 0.006, 0.04 ± 0.004, 0.01 ± 0.003, and 11.41 ± 1.25 µg/dL in group 2, respectively, were significantly lower in group 2 (P < 0.001). Fe level was found to be 3.67 ± 0.53 µg/dL in group 1 and 3.64 ± 0.51 µg/dL in group 2, with similar values for the two groups (P = 0.94). These data and their comparisons are presented in Table 2.

Discussion

The testes have an extremely active replication period and therefore imply correspondingly high rates of mitochondrial oxygen consumption by the germinal epithelium. In addition, low oxygen tensions in this tissue and high requirement of oxygen within the testes cause an intense competition for this vital element (6). The testes contain antioxidant enzymes and free radical scavengers presumably to ensure that spermatogenic and steroidogenic functions are not impacted by oxidative stress (7-9). Peroxidative damage is currently regarded as the single most important cause of impaired testicular function underpinning the pathological consequences of a wide range of conditions from testicular torsion to diabetes and xenobiotic exposure (7-9). Thus, these antioxidant defense systems are of major importance (7-9).

The testes have developed antioxidant defense systems comprising both enzymatic and non-enzymatic constituents. Concerning the enzymatic constituents of this defense system, the induction of oxidative stress in the testes precipitates a response characterized by the nuclear factor κB (NFκB)-mediated induction of mRNA species for SOD,

Table 1. Effect of levosimendan administration on tissue levels of MDA, an indicator of oxidative stress, and the levels of enzymes that act in cell defense against oxidative stress.

Enzyme activity	Control group (group 1)	Levosimendan group (group 2)
SOD (mIU/mg)	11.4 ± 0.44 (10.6-11.8)	22.9 ± 4.4* (19.06-30.8)
MDA (mg/dL)	125.1 ± 3.7 (121.3-129.5)	99.6 ± 1.6* (97.6-101.4)
GSH-Px (EU/g Hb)	80.9 ± 1.4 (80.01-83.8)	111.1 ± 5.4* (104.3-115.7)
GSH (mIU/mg)	76.4 ± 1.8 (74.1-78.3)	79.1 ± 3.4 (76.02-85.6)
CA (EU/g Hb)	0.07 ± 0.005 (0.07-0.08)	0.08 ± 0.02 (0.03-0.1)
CAT (EU/g Hb)	72.6 ± 2.6 (70.8-76.04)	76.1 ± 6.3 (66.2-82.05)

Data are reported as means ± SD and with range in parentheses for N = 10/group. Rats were treated with levosimendan (12 µg·kg^{-1}·day^{-1}, *ip*) for 3 days. SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; CA = carbonic anhydrase; CAT = catalase; GSH = antioxidant glutathione; MDA = malondialdehyde. *P < 0.001 compared to control.

Table 2. Effect of levosimendan treatment on testicular tissue trace element levels.

	Control group (group 1)	Levosimendan group (group 2)
Co	0.6 ± 0.04 (0.5-0.6)	0.44 ± 0.02* (0.4 - 0.4)
Pb	0.16 ± 0.01 (0.1-0.2)	0.05 ± 0.006* (0.05 - 0.06)
Cd	0.08 ± 0.002 (0.08-0.08)	0.04 ± 0.004* (0.03 - 0.04)
Mg	15.8 ± 0.7 (15.03-17.2)	24.1 ± 1.2* (22.7 - 25.8)
Mn	0.03 ± 0.008 (0.02-0.04)	0.01 ± 0.003* (0.01 - 0.02)
Fe	3.67 ± 0.5 (3.3-4.7)	3.64 ± 0.5 (3.1 - 4.6)
Cu	17.6 ± 0.7 (17.03-18.9)	11.4 ± 1.2* (9.1 - 12.4)
Zn	1.7 ± 0.2 (1.1-1.8)	3.9 ± 0.4* (3.2 - 4.4)

Data are reported in µg/dL as means ± SD with range in parentheses for N = 10/group. Rats were treated with levosimendan (12 µg·kg^{-1}·day^{-1}, *ip*) for 3 days. Co = cobalt; Cd = cadmium; Mg = magnesium; Mn = manganese; Zn = zinc; Co = copper; Fe = iron; Pb = lead. *P < 0.001 compared to control.

GSH-Px and glutathione-S-transferase (GSH-S-T) activities (22). SOD, a family of enzymes that catalyze the dismutation of two superoxide anions ($O_2^{-}\cdot$) to hydrogen peroxide (H_2O_2) and molecular oxygen, reduce tissue concentrations of superoxide radicals in order to prevent the induction of oxidative damage to lipids, proteins and DNA (23,24). The elimination of H_2O_2 is effected by catalase or glutathione peroxidase, with the latter predominating in the case of the testes (25,26). CAT is mainly a heme-containing enzyme. The predominant subcellular localization of the enzyme is in peroxisomes, in which it catalyzes the dismutation of hydrogen peroxide to water and molecular oxygen (24,27).

In the present study, we evaluated the effect of short-term exposure of levosimendan on oxidant/antioxidant status and trace element levels in testes of healthy rats. To our knowledge, this is the first study that evaluated the alterations in oxidant-antioxidant status and some trace element levels in the testes of a living organism after short-term levosimendan exposure. We have demonstrated that the tissue MDA level, which is a lipid peroxidation product and an indicator of oxidative stress, was significantly lower in the levosimendan-treated group ($P < 0.001$). These data indicated that 3-day short-term levosimendan treatment presented a significant decrease in MDA levels in the testes of rats, thus appearing to indirectly protect the testicular tissues against oxidative stress by decreasing the lipid peroxidation product. However, an important part of the protective enzyme activities including SOD and GSH-Px, which play important roles in cell defense against oxidative stress (5), was significantly higher in group 2 ($P < 0.001$). These data could also be interpreted to mean that this agent improved the protective mechanisms against the damages of oxidative stress by increasing some enzymatic defense mechanisms in the testicular tissues of rats. Even though the functions of antioxidant trace elements is not well known, our study showed that, while Mg and Zn levels were significantly higher ($P < 0.001$), Co, Pb, Cd, Mn, and Cu levels were significantly lower ($P < 0.001$) in group 2. Furthermore, Fe level was similar for the two groups ($P = 0.94$). A number of studies suggested that Zn deficiency can be the major cause of the increase in oxidative damage to tissues (28). Therefore, we may hypothesize that Zn might be an important factor for the response of the antioxidant system in germ cells. It has been suggested that Zn may be involved in several components of the oxidant defense including Cu/Zn SOD, an essential component of the antioxidant system (29), and that the formation of spermatids is affected by Mg deficiency. It has also been reported that the increase of MDA (lipid peroxidation) in the testes depends on Mg deficiency (30).

Although the antioxidant defense systems protect the testes against oxidative damage in order to support its dual functions of steroidogenesis and sperm production, a wide spectrum of endogenous and exogenous factors are known to disturb these defenses and to generate a state of oxidative stress. These pathological factors that damage the testes and their twin functions with their own mechanisms containing oxidative stress include cryptorchidism (31,32), testicular torsion (33-36), varicocele (37), infection, and diabetes mellitus (38). However, some analyses have shown significant protection against oxidative stress by some agents such as garlic extract (33), caffeic acid phenethyl ester (36), N-acetyl cysteine (39), pentoxifylline (40), resveratrol (34), and L-carnitine (35).

In conclusion, the present study revealed that short-term levosimendan exposure induced a significant decrease in tissue levels of MDA, which is a lipid peroxidation product and an indicator of oxidative stress, and a significant increase in an important part of protective enzyme activities against oxidative stress including SOD and GSH-Px, which play important roles in cell defense against oxidative stress. These data were interpreted to indicate that short-term levosimendan exposure supported the protection of rat testes under physiological conditions against oxidative stress by preventing lipid peroxidation, and also overactivating and/or increasing the protective antioxidant enzymes in the testes of rats. However, this requires more detailed and comprehensive studies.

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