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Analysis of the Influences of Short-Term Levosimendan Exposure on Oxidant/Antioxidant Status and Trace-Element Levels in the Physiological Status of the Thoracic Aorta of Rats

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Abstract The objective of this study was to evaluate the effect of levosimendan (chemical formula $C_{14}H_{12}N_6O$) exposure on oxidant/antioxidant status and trace-element levels in the thoracic aorta of rats. Eighteen male Wistar albino rats were randomly divided into two groups of eight animals each. Group 1 was not exposed to levosimendan and served as a control. Levosimendan (12 μg/kg) diluted in 10 ml 0.5 % dextrose was administered intraperitoneally to group 2. Animals of both groups were killed after 3 days, and their thoracic aortae were harvested for determination of changes in tissue oxidant/antioxidant status and trace-element levels. The animals in both groups were killed 72 h after levosimendan exposure, and thoracic aortae were harvested for determination of the lipid peroxidation product MDA and antioxidant GSH levels and the activities of antioxidant enzymes such as SOD, GSH-Px and CAT. It was found that MDA, GSH and CAT enzyme levels increased in thoracic aortae of rats after levosimendan administration. SOD and CA enzyme activities and the level of antioxidant GSH decreased in thoracic aortae of rats after levosimendan treatment. Pb, Cd and Fe levels of thoracic aortae were significantly higher ($P < 0.001$) and Mg, Mn, Zn and Cu were significantly lower ($P < 0.001$) in the levosimendan group compared to the control group. These results suggest that short-term levosimendan treatment caused an increase in free radical production and a decrease in antioxidant enzyme activity in thoracic aortae of levosimendan-treated rats. It also causes a decrease or increase in many mineral levels of the thoracic aorta, which is an undesirable condition for normal pharmacological function.

Keywords Levosimendan · Oxidative stress · Free radical · Oxidant · Antioxidant · Thoracic aorta · SOD · GSH-Px · MDA · Rat

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Introduction

Congestive heart failure is a complex hemodynamic and metabolic syndrome in which both cardiac function and metabolism are impaired (Cohen-Solal et al. 1995). An important characteristic of this condition is an imbalance between energy production and utilization. Oxygen consumption of the heart is correlated with cardiac workload (Balaban 1990). Dilated cardiomyopathy refers to weakened cardiac muscle that is unable to pump strongly enough to empty the heart properly in each beat. In restrictive cardiomyopathy, the muscle becomes so stiffened and inextensible that the heart cannot fill properly during ejection. The degeneration of the myocardium

associated with muscular dystrophy is often accompanied by cardiomyopathy (Colledge et al. 2010; Basel et al. 2012). Levosimendan is a cardiovascular drug for the treatment of acute and decompensated heart failure. It has positive inotropic and anti stunning effects mediated by calcium sensitization of contractile proteins (Pollesello and Papp 2007; Jamali et al. 1997) and vasodilatory and anti-ischemic effects mediated by the opening of ATP-sensitive potassium (K_{ATP}) channels in vascular smooth muscle cells (Jamali et al. 1997; De Witt et al. 2002) The vasodilatory effect of levosimendan causes a reduction in both preload and afterload and an improvement in oxygen supply to the myocardium. Compared with other inodilators, it improves myocardial contractility without increasing oxygen requirements (Du Toit et al. 1999). Aerobic organisms are protected from oxygen toxicity by a natural antioxidant defense system involving enzymatic and nonenzymatic mechanisms (Basel et al. 2012). The increased formation of reactive oxygen species (ROS) and decreased antioxidant defense are defined as oxidative stress, which is widely recognized as an important feature of many diseases. Superoxide dismutase (SOD) and catalase (CAT) are cellular antioxidants which protect cells from oxidative stress. Lipid peroxidation (LPO) is one of the most important expressions of oxidative stress induced by ROS. Malondialdehyde (MDA) is an indicator of LPO and increases in various diseases (Nordberg and Arner 2001). CAT is mainly a heme-containing enzyme. The predominant subcellular localization of enzyme is in the peroxisomes, in which it catalyzes the dismutation of hydrogen peroxide to water and molecular oxygen. CAT activity is highest in the liver, relatively high in the kidney and very low in the heart (Nordberg and Arner 2001).

These results suggest that short-term levosimendan treatment on thoracic aorta MDA and GSH levels and SOD, CAT, glutathione peroxidase (GSH-Px) and carbonic anhydrase (CA) enzyme activities in rats. In other words, our aim was to investigate whether, in addition to its positive inotropic action, levosimendan could ameliorate the LPO-related disturbances in thoracic aortae of rats. With the assumption that levosimendan directed to the thoracic aorta also affects adjacent organs in rats, we designed our study to investigate the effect of levosimendan exposure of the thoracic aorta on oxidative stress and some trace-element levels in the thoracic aorta.

Materials 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 Yuzuncu Yil University (Van, Turkey). Rats were housed in specific cages. A 12 h light/dark cycle was maintained, and the rats were fed ad libitum. The study was approved by the local ethics committee of Yuzuncu Yil University.

Animals were randomly divided into two groups, each consisting of eight rats. The animals in group 1 were not treated with drug and served as a control. Levosimendan (12 μg/kg) diluted in 10 ml 0.5 % dextrose was administered intraperitoneally to group 2. After 3 days, animals in both groups were killed and their thoracic aortic segments harvested for the evaluation of tissue oxidant/antioxidant status and trace-element levels after 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 1,600×*g*, MDA levels in tissue homogenate supernatant were determined by thiobarbituric acid (TBA) reaction according to Yagi (1994). 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.

Measurements of SOD and GSH-Px Enzyme Activities

Tissues were homogenized in physiological saline (1 g in 5 ml) using a homogenizer (B. Braun, Melsungen, Germany) and centrifuged at 4,000×*g* for 20 min (Heraus Labofur 200; Thermo Scientific, Dreieich, Germany). GSH-Px activity was determined by monitoring the changes in NADPH absorbance at 340 nm and by measuring the decrease of H_2O_2 absorbance at 240 nm (Aebi 1974). 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 the NBT reduction rate (Durak et al. 1996).

Measurement of GSH Level

GSH levels were measured by the technique of Sedlak and Lindsay (1968) at 412 nm. Samples were precipitated with 50 % TBA and centrifuged at 1,000×*g* for 5 min. The reaction mixture contained 0.5 ml of supernatant, 2.0 ml of Tris–EDTA buffer (0.2 M, pH 8.9) and 0.1 ml of 10 mM dithiobis-2-nitrobenzoic acid (DTNB). The solution was kept at room temperature for 5 min, and subsequently absorbance was read at 412 nm.

Measurement of CAT Enzyme Activity

Erythrocyte CAT activity was measured by the method described by Aebi (1974). Briefly, the supernatant (0.1 ml) was added to a quartz cuvette containing 2.95 ml of 19 mM H_2O_2 prepared in potassium phosphate buffer (50 mM, pH 7.00). The change in absorbance was monitored at 240 nm for 5 min using a Shimadzu spectrophotometer (UV-1201; Kyoto, Japan).

Measurement of CA Activity

CA activity was assayed by hydration of CO_2 measured by the method of Rickli and Wilbur-Anderson (Topçuoglu et al. 2009) using bromothymol blue as indicator.

Measurements of Mineral Levels

Two milliliters of a 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 and stirred occasionally; 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 saturate was closed, and radiation was applied for 3 min at 450 W. After addition of 0.5 ml of the same acid mixture, radiation was repeated for 3 min. After cooling for 5 min, 2.0 ml of 0.1 M HNO_3 was added, and the solution was transferred to a Pyrex tube. After centrifugation, the clear solution was used for determination of Cu, Zn, Mg, Mn, Pb, Cd and Fe (Bush et al. 1995). Measurements were performed by atomic absorption spectrophotometry using a UNICAM-929 spectrophotometer (Unicam, Leeds, UK).

Statistical Analysis

Data are reported as means ± SD. Parameters were compared between two groups using the Mann–Whitney *U* test. All statistical analyses were carried out using the SPSS® statistical software package (version 13.0; SPSS, Inc., Chicago, IL), and $P \leq 0.05$ was considered statistically significant.

Results

The tissue MDA level, which is as an indicator of oxidative stress, was significantly increased in the levosimendan group (113.9981 ± 3.074 vs. 68.228 ± 7.805 mg/dl, $P < 0.001$).

The levels of protective enzymes such as SOD, which play important roles in cell defense against oxidative stress, were significantly decreased in the levosimendan group (39.56 ± 0.441 in control group and 15.305 ± 0.40802 in study group, $P < 0.001$).

CA levels, which play important roles in cells, were significantly decreased in the levosimendan group (0.415 ± 0.078 EU/gHb in control group and 0.208 ± 0.026 EU/gHb in study group, $P < 0.001$).

Levels of protective enzymes such as GSH and CAT, which play important roles in cell defense against oxidative stress, were 92.628 ± 8.783 and 68.942 ± 3.587 EU/gHb in the control group and 140.872 ± 3.54201 EU/gHb and 98.617 ± 5.905 EU/gHb in the levosimendan group, respectively, significantly higher in group 2 ($P < 0.001$). These data are presented in detail and compared in Table 1.

Trace-element levels were also analyzed in the two groups. Levels of Pb, Cd and Fe levels of thoracic aorta were significantly higher ($P < 0.001$) and Mg, Mn, Zn and Cu were significantly decreased ($P < 0.001$) in the study group compared to the control group (Table 2).

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	Levosimendan group	
	(mean ± SD)	(mean ± SD)	*P*
SOD (U/mg)	39.56 ± 6.1	15.305 ± 0.40802*	0.001
MDA (mg/dl)	68.228 ± 7.805	113.9981 ± 3.074*	0.001
GSH-Px (EU/gHb)$^{-1}$	63.41 ± 6.5	62.852 ± 1.184	0.000
GSH (EU/gHb)$^{-1}$	92.628 ± 8.783	140.872 ± 3.54201*	0.001
CA (EU/gHb)$^{-1}$	0.415 ± 0.078	0.208 ± 0.026*	0.001
CAT (EU/gHb)$^{-1}$	68.942 ± 3.587	98.617 ± 5.905*	0.001

SOD superoxide dismutase, *GSH-Px* glutathione peroxidase, *CA* carbonic anhydrase, *CAT* catalase, *GSH* antioxidant glutathione, *MDA* malondialdehyde

Data are reported as mean ± SD for $n = 8$/group. Rats were treated with levosimendan (12 μg/kg day, ip) for 3 days

* $P < 0.001$ compared to control

Discussion

This study was carried out to investigate the effect of intraperitoneal injection of levosimendan on oxidative stress of the thoracic aorta in rats. The pharmacokinetics of levosimendan underpins the prolonged beneficial hemodynamic effects that result from a single-dose regime. It is an appealing and promising alternative adjunct to current therapies for cardiac failure. Levosimendan is also of benefit in the setting of pulmonary vasoconstriction and right ventricular dysfunction (Kamath et al. 2009). The clinical documentation of the safety and efficacy of levosimendan in acute heart failure syndromes is one of the

Table 2 Effect of levosimendan treatment on thoracic aorta tissue trace-element levels

Trace element	Control group	Levosimendan group	
	(mean ± SD)	(mean ± SD)	*P*
Co (μg/dl)	0.585 ± 0.015	0.584 ± 0.034	0.907
Pb (μg/dl)	0.087 ± 0.022	0.178 ± 0.03701*	0.001
Cd (μg/dl)	0.072 ± 0.003	0.102 ± 0.011*	0.001
Mg (μg/dl)	24.637 ± 1.192	17.804 ± 2.12801*	0.001
Mn (μg/dl)	0.057 ± 0.014	0.026 ± 0.004*	0.001
Fe (μg/dl)	1.310 ± 0.384	3.5851 ± 11.2141*	0.001
Cu (μg/dl)	0.119 ± 0.043	0.027 ± 0.010*	0.001
Zn (μg/dl)	2.456 ± 0.627	1.276 ± 0.217*	0.001

Data are reported as mean ± SD for $n = 10$/group. Rats were treated with levosimendan (12 μg/kg day, ip) for 3 days

* $P < 0.001$ compared to control

most extensive databases of its type (De Luca et al. 2006), and the drug is currently approved in more than 40 countries for the treatment of acute decompensated heart failure. Recent experiences from small-scale studies and randomized clinical trials have generated interest in the additional use of this drug for the support of impaired cardiac function in patients with ischemic heart disease and cardiogenic shock. Levosimendan also has been evaluated as a bridge therapy for the perioperative phase of cardiac surgery (Pollesello and Papp 2007). Many compounds that open K_{ATP} channels in the cell plasma membrane also have been shown to act on K_{ATP} channels in mitochondria (Grover and Garlid 2000; Gross 2000). There is a consensus that the opening of mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels (Inoue et al. 1991) protects the heart against ischemia–reperfusion damage. The increased potassium influx associated with mitoK_{ATP} channel opening is sufficient to protect and preserve mitochondrial function, most probably via the normalization of matrix and intermembrane space volumes, in situations of distress such as ischemia and/or reperfusion (Garlid et al. 2003).

Generation of ROS is a part of normal life, and their interaction with host antioxidant defense systems appears to exert a significant influence on cellular chemistry in health and disease. We found that the LPO product MDA increases in the thoracic aorta after levosimendan treatment. Because excess production of ROS is very harmful to tissues, ROS are expected to induce tissue damage in the thoracic aorta. Our results show that neighboring tissues might also be affected by levosimendan treatment of the thoracic aorta. Proteins, membrane lipids and nucleic acids are targets of free radical–mediated injury. Free radicals may act by peroxidation of membrane lipids, inactivation of enzymes by oxidation of sulfhydryl groups, depolymerization of polysaccharides and disruption of nucleic acids (Chandra et al. 1994).

In the present study, we found that the LPO products decreased in the thoracic aorta after treatment of levosimendan in rats. These results suggest that levosimendan administration may increase or decrease oxidative stress by decreasing free radical production in the thoracic aorta. Similar to our result, Hasslacher et al. (2011) suggested that levosimendan inhibits the release of ROS in polymorphonuclear leukocytes in patients with heart failure and septic shock. They concluded that levosimendan exerts distinct immunomodulatory effects by decreasing oxidative burst activity of polymorphonuclear leukocytes. In another study Parissis et al. (2007) investigated the effects of levosimendan on circulating markers of oxidative in patients with advanced heart failure. Consistent with our result, they suggested that levosimendan does not increase the levels of markers of oxidative and nitrosative stress, thus exerting cardioprotective effects in advanced cardiovascular diseases.

The antioxidant defense systems exist to prevent the formation of these increased reactive and free radicals. These include SOD, CA and other free radical scavengers, such as GSH. In our study, GSH levels increased and the activities of antioxidant enzymes, such as SOD and CA, decreased in aortic tissues of levosimendan-treated rats. The increased formation of ROS and decreased antioxidant defense are defined as oxidative stress, which is widely recognized as an important feature of many diseases. The decrease in antioxidant enzyme activities might be due to their use against free radical destruction or their inhibition by free radical species (Percy et al. 1990). DNA and proteins caused a loss of cell integrity, enzyme function and genomic stability (Paglia and Valentine 1967). Because glucose-6-phosphate dehydrogenase (G6PD) catalyzes the first step of the pentose phosphate pathway, which provides the NADPH necessary for conversion of oxidized glutathione to GSH, the increased tissue GSH level was probably due to the increased G6PD activity that caused the increased production of GSH.

Healthy life depends on the existence of appropriate amounts of various heavy elements. Deficiency in any of these trace elements leads to undesirable pathological conditions that can be prevented or reversed by adequate supplementation (Fraga 2005). We found that levels of Pb, Cd and Fe in the thoracic aorta were significantly higher and levels of Mg, Mn, Zn and Cu were significantly lower in the levosimendan group compared to the control group. Although iron is an essential nutritional mineral for all life forms, it is known that excess iron and iron deficiency also lead to oxidative DNA damage (Ames 2001). Copper deficiency affects various physiological functions that may be important in immunological defense against pathogenic challenge (Stabel et al. 1993). Iron increases oxidative stress, affects endothelial function, promotes inflammation,

downregulates nitric oxide production and induces renal dysfunction (Lustberg and Silbergeld 2002). Manganese is essential for normal physiological functioning in humans and other animals (Bureau et al. 2002). It also plays a role in the free radical scavenging activity of SOD. Cadmium is a ubiquitous toxic heavy metal and, unlike organic compounds, is not biodegradable and has a very long biological half-life (Greger 1999). Mg is the fourth most abundant cation in the body and plays a pivotal role as an enzyme cofactor in the biosynthesis of proteins and mineral administration (Rahnama 2002).

These results suggest that short-term levosimendan treatment causes an increase in free radical production and a decrease in antioxidant enzyme activity in the thoracic aorta of levosimendan-treated rats. It also causes a decrease or increase in many mineral levels of the thoracic aorta, which is an undesirable condition for normal pharmacologic function. However, more studies are needed to verify and clarify the roles of oxidative stress, antioxidant enzyme activities and trace-element levels in the pathogenesis of levosimendan.

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