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Original research article

The preventive role of levosimendan against bleomycin-induced pulmonary fibrosis in rats

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ABSTRACT

Background: In this study, the effects of levosimendan used in the treatment of acute congestive heart failure upon pulmonary fibrosis in rats induced with bleomycin (BL) were analyzed.
Methods: A total of 33 male Sprague-Dawley type rats were categorized into five groups randomly. About 2.5 U/kg BL was intratracheally administered to the rats in the BL, BL + L_1, BL + L_2, and BL + L_3 groups, and 0.9% saline was intratracheally administered at the same rate to the control group. 0.3, 1, and 3 mg/kg levosimendan was intraperitoneally administered to the BL + L_1, BL + L_2, and BL + L_3 groups, respectively. Blood and tissue samples were taken from the rats euthanized to determine the changes in erythrocyte enzyme activities and to conduct histopathological evaluations after 14 days. With values between 0 and 3, histopathological scoring damage was assessed by the presence of inflammation and fibrosis in a semiquantitative manner.
Results: Compared with those in the C group, glutathione reductase (GR) and Catalase (CAT) enzymes decreased in the BL group; compared with that in the BL group, GR increased in the BL + L_1 and BL + L_3 groups, 6-phosphogluconate dehydrogenase (6PGD) increased in the BL + L_3 group, and CAT increased in the BL + L_2 and BL + L_3 groups ($p < 0.05$). In the histopathological evaluation, fibrosis occurred in all rats in the BL group, and tissue damage was noticed to be generally less in the BL + L_1, BL + L_2, and BL + L_3 groups ($p < 0.001$).
Conclusions: The results obtained from biochemical and histopathological evaluations indicate that levosimendan had an anti-fibrotic effect without a dose-dependent response on pulmonary fibrosis.

Introduction

In recent years, levosimendan has been frequently used to treat acutely decompensated heart failure [1]. Unlike other cardiotonic agents, this agent is a calcium-sensitizing drug with unique properties. For example, it causes calcium to bind to troponin C in the myocardium, and it opens ATP-sensitive calcium channels in the vascular smooth muscle. These mechanisms enable levosimendan to exert positive inotropy and vasodilative properties [2,3]. Levosimendan has been reported to have antiapoptotic, anti-inflammatory, antioxidative, and neuroprotective properties in different studies. According to Trikas et al. [4], levosimendan has anti-inflammatory and possibly anti-apoptotic properties, yielding a decrease in the expression of proinflammatory cytokines, TNF-α receptors, and sFas, immediately after infusion in the patients with severe heart failure. Similar results were reported by the other researchers [5,6]. Karakus et al. [7] found that levosimendan has anti-inflammatory effects against inflammatory induction by carrageenan in the rat model. Levosimendan shows these effects due to the reduction of IL-1, IL-6, and TNF-α levels and decreases the oxidative stress. Similar results were obtained from the study of Avgeropoulou et al. [8]. In an *in vitro* study, levosimendan was shown to have neuroprotective properties in traumatic brain injury [9].

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Still without adequate treatment, idiopathic pulmonary fibrosis is a chronic interstitial pulmonary disease that is rapidly progressing and fatal, and is characterized by fibroblast proliferation, large amounts of extracellular matrix accumulation including collagen and fibronectin, cell injury, inflammation, and fibrosis in the alveolar structure [10–12]. Fibrosis is triggered by a chronic inflammatory process that causes pulmonary injury, modulated pulmonary fibrogenesis, and fibrotic scar in the end stage [11]. Bleomycin (BL) is a cytostatic antibiotic used as cancer chemotherapy because of its potent antitumoral activity [13,14]. BL is generally used to create experimental pulmonary fibrosis in different animal models as it causes alveolar cell damage and pulmonary inflammation [15]. The administration of BL causes pulmonary injury, which is characterized by an interstitial edema with an influx of inflammatory and immune cells. BL has a key role in the development of pulmonary fibrosis [13].

Oxidative stress has a triggering role in the pathogenesis of pulmonary fibrosis [16,17]. Studies have examined the antioxidative effects of various antioxidants on a BL-induced model [18–21]. In the current study, we aimed to investigate the potential antioxidative and anti-inflammatory effects of levosimendan in a BL-induced model of pulmonary fibrosis in rats.

Materials and methods

Chemicals

BL was purchased from Neon Laboratories (Tumocin, Mumbai, India) and thiopental sodium from Abbott (Pentothal Sodium, İstanbul, Turkey). Levosimendan, glucose 6-phosphate (G6P), 6-phosphogluconate (6PGA), glutathione disulfide (GSSG), nicotinamide adenine dinucleotide phosphate ($NADP^{+}$), nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediaminetetraacetic acid (EDTA), and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemical (St Louis, Missouri, USA). All other chemicals were also obtained from Sigma Chemical or MERCK (Darmstadt, Germany). Bleomycin was dissolved in 0.9% saline, and levosimendan was dissolved in 1% dimethyl sulfoxide (DMSO).

Animals

A total of 33 ten-week-old Sprague-Dawley male rats weighing 180–240 g were obtained from the Laboratory Animal Unit of Fırat University (Elazığ, Turkey). Animal care and application processes were performed considering the ethical rules of the national laboratory animals. The study was approved by the Ethics Committee for the use of experimental animals of Fırat University. The rats housed in plastic breeding cages were maintained in an air-conditioned room in 12-h light and 12-h dark cycles at a constant temperature of 21 ± 1 °C. Food and water were provided *ad libitum* with access to standard chow.

Animal model of pulmonary fibrosis induced by BL

Rats were divided into the following five groups:

C: Control group
BL: Bleomycin (2.5 U/kg)
BL + L_1: Bleomycin (2.5 U/kg) + levosimendan (0.3 mg/kg)
BL + L_2: Bleomycin (2.5 U/kg) + levosimendan (1 mg/kg)
BL + L_3: Bleomycin (2.5 U/kg) + levosimendan (3 mg/kg)

Before starting the animal applications, the rats were anesthetized by intravenous injection of thiopental sodium (25 mg/kg). A single sublethal dose of BL (2.5 U/kg) was intratracheally administered to the animals, and 0.9% saline at the same volume was intratracheally administered to the animals in the control group. This dose was determined according to previous studies [22,23]. Levosimendan was injected intraperitoneally a day after the intratracheal injection of BL. Fourteen days after the BL injection [24], sodium pentobarbital (75 mg/kg) was administered to the rats at a high dose, and then the animals were euthanized through exsanguination. The tissue samples obtained from the rats were fixed in a solution of 10% formaldehyde. The blood samples obtained from these animals were maintained at −80 °C.

Preparation of blood samples

The blood samples placed into EDTA-containing tubes were centrifuged in 2500 × *g* at 4 °C for 15 min. The buffy coat, leukocytes, and plasm were taken out of the tubes. The erythrocytes were isolated. After washing them with 0.9% NaCl solution for three times, 1 ml of erythrocytes were hemolyzed with 1.5 volume of ice-cold water. Subsequently, intact cells and erythrocyte ghost membranes were centrifuged in 14,500 × *g* at 4 °C for 30 min and then eliminated. The pH of the hemolysis was adjusted to 8.7 using solid tris.

Measurement of the erythrocyte enzyme activity

To measure the *in vivo* enzyme activity of glutathione reductase (GR) spectrophotometrically, the method before described by Carlberg and Mannervik [25] was used. One unit of enzyme activity (U) was considered as the oxidation of 1 mM NADPH per minute under the assay conditions at 25 °C and at pH of 8.0.

Glucose 6-phosphate (G6PD) enzyme activity was determined by monitoring the NADPH production in 340 nM absorbance at 25 °C. Under experimental conditions, one unit (U) of enzyme activity was defined as the total enzyme amount necessary for decreasing 1 μmol/min of $NADP^{+}$ in the presence of G6PD [26].

In vivo 6PGD enzyme activity was determined by monitoring the NADPH production in 340 nM absorbance at 25 °C. Under experimental conditions, one unit (U) of enzyme activity was defined as the total amount of enzyme needed for decreasing 1 μmol/min of $NADP^{+}$ in the presence of 6-phosphogluconate (6PGA) [26].

Catalase enzyme activity was measured according to the method described by Bergmeyer [27] and through the disappearance of hydrogen peroxide (H_2O_2) spectrophotometrically at 25 °C and at 240 nM. One unit of enzyme activity (U) was adopted as the amount of enzyme to decompose 1 μmol of H_2O_2 per min at pH 7.0.

Evaluation of histopathology

The samples tissues obtained from the rats were instantly collected for histopathological examination. The tissues were fixed in 10% neutral-buffered formaldehyde solution for 48 h. The tissues were washed in running water and dehydrated with ethanol. After dehydration, the tissues were placed in xylene to obtain transparency and then embedded in paraffin. The paraffin-blocked tissues were cut in 5 μm-thick sections using a microtome (Leica RM2235; Leica Instruments, Nussloch, Germany) and stained with hematoxylin–eosin. In addition, the slides were stained with Masson's trichrome to determine fibrosis. The histopathological semiquantitative evaluation was conducted by a pathologist who was blinded to the study groups. It was performed using a light microscope (Olympus BX53, Tokyo, Japan) supported with a digital camera and an imaging system (CellSens