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Impact of early levosimendan administration in patients with decompensated chronic heart failure on oxidative stress parameters — the rationale and study design

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ABSTRACT

Oxidative stress plays a key role in the development of heart failure. The study is a phase III, single-centre, randomized, double-blind, clinical trial that assesses the influence of early intravenous levosimendan administration in patients with decompensated CHF on oxidative stress parameters and the function of enzymatic antioxidative mechanisms. A total of 50 patients with diagnosed CHF with reduced ejection fraction, LVEF ≤ 35% are randomized in a 1:1 ratio to receive either levosimendan or dobutamine. The hypothesis is that levosimendan in patients with decompensated heart failure may reduce ROS production and/or enhance antioxidant protection. MDA and LPO concentrations and the activity of antioxidant enzymes SOD, GPX, GR, and CAT will be measured in plasma samples.

Keywords: oxidative stress, decompensated chronic heart failure, levosimendan, dobutamine

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Introduction

Excessive production of free radicals leads to increased oxidative stress on cellular structures and causes changes in molecular pathways [1]. Oxidative stress may promote myocardial and vascular dysfunction due to an imbalance between increased production of reactive oxygen species (ROS) and impairment of their neutralization by antioxidant defence systems involving enzymatic and nonenzymatic mechanisms [2, 3]. Chronic heart failure (CHF) is associated with increased local and systemic release of oxygen-derived free radicals [3]. Furthermore, antioxidant defence decreased in patients with CHF. There is some evidence that oxidative stress is a prognostic factor in CHF [3]. Overproduction of ROS is observed in heart failure patients and it results from several pathogenic mechanisms: recurrent ischemic episodes and post reperfusion microcirculatory damage, oxidation of catecholamine, changes in the mitochondrial proteome and activation of biologically active substances such as nitric oxide synthase, cytokines, angiotensin II and xanthine oxidase [4, 5, 6]. Oxidative stress leads to cellular death and apoptosis through damage to the cellular proteins and membranes by the initiation of lipid peroxidation and also by activating the matrix metalloproteinases, promoting fibrosis of the heart muscle and unfavourable remodelling [6, 7, 8]. Moreover, ROS contribute to exerting direct negative inotropic effects through the reduction of free calcium in intracellular cytosolic and reduce antioxidant capacity and activity of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) in CHF [3, 6, 9]. Malondialdehyde (MDA), is one of the products of polyunsaturated fatty acids peroxidation and MDA concentration was positively correlated with the presence of heart failure, and also with the NYHA functional class [3].

Inotropic agents like dobutamine or levosimendan are used for the treatment of patients with acutely decompensated heart failure in an effort to increase cardiac output and restore oxygen supply [10]. Levosimendan is a myocardial Ca^{2+} sensitizer and opener of ATP-dependent potassium channels with inotropic, vasodilating, and cardioprotective functions [11, 12]. Levosimendan is used primarily in the management of acute heart failure but there is evidence that its complex mechanism of action can exert several pleiotropic effects like organ protection and plays a role against endothelial cell death related to the anti-inflammatory, anti-oxidative and antiapoptotic pathways [13].

Material and methods

The study was designed to assess the impact of early intravenous levosimendan administration versus intravenous dobutamine administration in patients with decompensated CHF on oxidative stress parameters.

The study is a phase III, single-centre, randomized, double-blind clinical trial. A total of 50 patients (25 for each study arm) are randomized in a 1:1 ratio to receive either levosimendan or dobutamine, which will be administered as intravenously continuous 24-hour infusion (Fig. 1). All decompensated heart failure patients admitted to the hospital are screened for eligibility up to 36 hours. After the enrolment of 50 patients (25 per arm), statistical analysis will be performed (pilot phase).

Study population — inclusion and exclusion criteria

The study population will consist of patients (men and female) with diagnosed CHF with left ventricular ejection fraction (LVEF) $\leq$ 35% who are > 18 years old and meet all inclusion criteria and do not meet any exclusion criteria (Tab. 1). Each study participant will
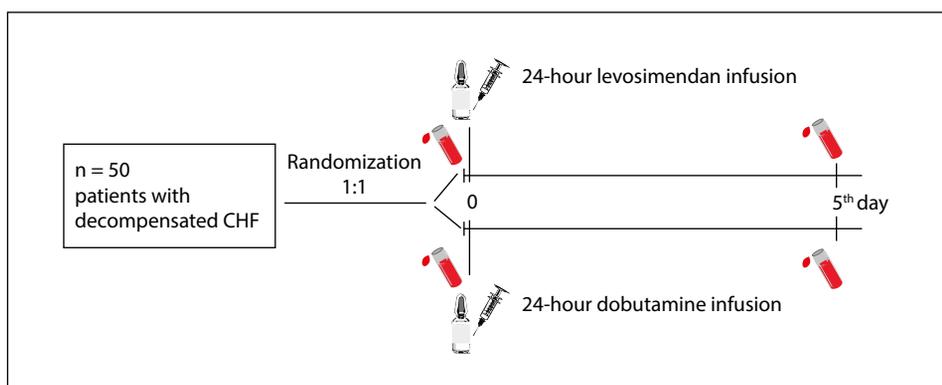

Figure 1. Trial schema, CHF — chronic heart failure

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
• Hospitalization due to decompensation of chronic heart failure within the last 12 months and requiring intravenous therapy • LVEF $\leq$ 35% revealed in the latest echocardiography as assessed by Simpson's two-dimensional method • NT-proBNP $\geq$ 1000 pg/mL • Optimal medical treatment at least one month before hospitalization • Clinically symptomatic patients with INTERMACS 3 or 4	• HF associated with uncorrected, severe structural valvular disease according to the ESC criteria • SBP < 90 mmHg • Exacerbation of HF associated with infection, acute coronary syndrome, pulmonary embolism • Hypertrophic cardiomyopathy • Severe chronic lung disease e.g. COPD or respiratory failure • Planned revascularization (PCI or CABG) • Severe arrhythmias or advanced atrioventricular blocks • End-stage HF, qualified for palliative treatment • eGFR < 30 ml/min/1,73 m^2 • History of hypersensitivity to levosimendan or dobutamine • Comorbidities affecting the patient's poor prognosis • Pregnancy

LVEF — left ventricular ejection fraction; NT-proBNP — N-terminal pro-B-type natriuretic peptide; HF — heart failure; ESC — European Society of Cardiology; SBP — systolic blood pressure; COPD — chronic obstructive pulmonary disease; PCI — percutaneous coronary interventions; CABG — coronary artery bypass grafting; eGFR — estimated glomerular filtration rate

be treated according to the latest European Society of Cardiology guidelines for the management of patients presenting with HF [10].

Study group — 24-hour i.v. continuous infusion of levosimendan — 0.1 μg/kg/min

Control group — 24-hour i.v. continuous infusion of dobutamine — 3.0 μg/kg/min

Blood samples will be collected at baseline, directly after randomization (time point 0, before drug administration) and after 5 days post-infusion of the drug.

The blood drawing will be performed by qualified medical personnel and blood will be collected in two different tubes with EDTA with a total capacity of 16 mL, using a venous catheter (18G) inserted into a forearm vein. Whole blood will be immediately centrifuged (3000 $\times$ g, for 10 min, at 4°C), and plasma samples will be transferred to Eppendorf tubes and frozen at — 80°C until analysis. MDA and LPO concentrations and the activity of antioxidant enzymes (SOD, GPX, glutathione reductase [GR] and catalase [CAT]) will be measured in plasma samples, using commercially available assay kits according to the manufacturer's specifications.

The activity of superoxide dismutase

Plasma SOD activity will be determined using a Superoxide Dismutase Assay Kit (Abnova Catalog Number KA0783). SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. The SOD assay kit utilizes water-soluble tetrazolium-1 (WST-1) that produces a water-soluble yellow-coloured formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is related linearly to the xanthine oxidase activity, and this process is inhibited by SOD. This inhibition activity of SOD is measured by the colourimetric method at OD 450 nm. The enzymatic and colourimetric reaction will be made at 96-well plates. According to the manufacturer's protocol 200 μl of WST-1 solution is added to the 20 μl of samples. The enzymatic reaction starts by adding 20 μl of xanthine oxidase solution to all wells. The plate is incubated at 37°C for 20 minutes and the absorbance at 450 nm is read using a microplate reader.

Activity of glutathione reductase

Plasma GR activity will be determined using a Glutathione Reductase Assay Kit (Abnova Catalog Number KA0881). In the assay, GR catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) which reacts with 5, 5'-Dithiobis (2-nitrobenzoic acid) (DTNB) to produce yellow colour 2-nitro-5-thiobenzoate anion (TNB2-) gives an intense yellow colour — λmax = 405 nm. The enzymatic and colourimetric reaction will be made at 96-well plates. According to the manufacturer's protocol, 50 μl of mixed solution of DTNP, NADPH and GSSG is added to the appropriate volume of samples and TNB standards. The absorbance at 405 nm is read at time (T1) and after 10 min incubation at room temperature at time (T2) using a microplate reader. The GR activity is read from the TNB standard curve.

Glutathione peroxidase activity

Plasma GPx activity will be determined using a Glutathione Peroxidase Assay Kit (Abnova Catalog Number KA0882). In the Glutathione Peroxidase Assay Kit, GPx reduces Cumene Hydroperoxide while oxidizing GSH to GSSG. The generated GSSG is reduced to GSH with the consumption of NADPH by GR. The decrease of NADPH (easily measured at 340 nm) is proportional to GPx activity. The enzymatic reaction will be made at a 96-well plate. According to the manufacturer's protocol, the 40 μl of mixed solution of GR, NADPH and GSH is added to the appropriate volume of samples and NADPH standards. The plate is incubated at room temperature and then 10 μl of Cumene Hydroperoxide solution is added to start the GPx reaction The absorbance at 340 nm is read at time (T1) and after 5 min incubation at room temperature at time (T2) using a microplate reader. The GPx activity is read from the NADPH standard curve.

Catalase activity

Plasma CAT activity will be determined using an Abnova Assay Kit (Catalog Number KA0884). In the assay, catalase first reacts with H_2O_2 to produce water and oxygen, the unconverted H_2O_2 reacts with OxiRed probe to produce a product, which can be measured at 570 nm. Catalase activity is reversely proportional to the signal. The enzymatic and colourimetric reaction will be made at 96-well plates. According to the manufacturer's protocol, the 12 μL fresh 1 mM H_2O_2 is added to the appropriate volume of samples, high control samples (with inhibited catalase activity). The plate is incubated at 25°C for 30 min. Then, 50 μL of the Develop Mix (OxiRed Probe and HRP solution) is added to all samples and H_2O_2 standards. The plate is incubated at 25°C for 10 min and the absorbance at 570 nm is read using a microplate reader. The CAT activity is read from the H_2O_2 standard curve.

Analysis of MDA concentration

MDA concentration, indicating the intensity of lipid peroxidation processes. Plasma MDA concentration will be determined using a SunRed Assay Kit (Catalog Number 201-12-1372). The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). According to the manufacturer's protocol, the plasma samples and MDA standards are added to wells which are pre-coated with human MDA monoclonal antibodies, and then MDA antibodies labelled with biotin will be added and combined with Streptavidin-HRP to form an immune complex. Incubation will then be performed and the enzyme-linked immunosorbent assay will be washed again to remove free components. After adding the chromogen solution the colour of the liquid will change to blue. And at the effect of acid, the colour finally becomes yellow. The optical density is measured at 450 nm using a microplate reader. The OD value is proportional to the concentration of human MDA. The MDA concentration is read from the MDA standard curve. The Intra-assay precision CV is below 10% and the Inter-assay precision CV is below 12%.

Analysis of LPO concentration

Plasma LPO concentration will be determined using a SunRed Assay Kit (Catalog Number 201-12-0727). This kit uses a double-antibody sandwich enzyme-linked immunosorbent assay. The plate provided in the kit is pre-coated with an antibody specific to Human LPO. Standards or samples are added to the wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human LPO and Streptavidin-HRP conjugate are added successively to each plate well and incubated. The uncombined enzymes are washed away. Then chromogen solution is added and the colour of the liquid changes to blue. The enzyme-substrate reaction is completed by the addition of a stop solution and the color finally becomes yellow. The optical density (OD) is measured at a wavelength of 450 nm. The OD value is proportional to the concentration of human LPO. The LPO concentration is read from the LPO standard curve.

The additional tests and clinical data are being carried out in accordance with the previously published ELEPHANT study protocol (Early administration of LEvosimendan in Patients witH decompensAted chroNic hearT failure) [14].

Discussion

Oxidative stress plays a key role in the development and evolution of heart disease [7]. Heart failure is linked to ROS production and leads to the destabilization of endogenous oxidant-antioxidant pathways [7]. Reducing oxidative stress remains an attractive goal for the prevention and therapy of cardiovascular diseases. Prospective randomized trials need to be designed to evaluate the role of ROS in the pathology of cardiovascular diseases. It can enable the development therapeutic strategies that reduce ROS overproduction or increase ROS degradation resulting in protective effects of cardiovascular diseases [6]. This study is a phase III, single-centre, randomized, double-blind, clinical trial that assesses the influence of early intravenous levosimendan administration in patients with decompensated CHF on oxidative stress parameters. MDA and LPO concentrations and the activity of antioxidant enzymes SOD, GPX, GR, and CAT will be measured in the plasma samples. Results obtained in animal models (in vitro and in vivo) revealed that levosimendan could improve endothelial and mitochondrial function, and protect against peroxidation [15, 16]. It was noticed that the antioxidant glutathione and MDA levels decreased in levosimendan-injected rats. SOD, GPX and CAT activities were lower in the levosimendan injected group of rats compared to the control group. It was concluded that levosimendan caused lower free radical levels in the myocardium of rats and led to a lower synthesis of antioxidant enzymes and decreased activity of antioxidant enzymes [17]. Levosimendan was superior to dobutamine in terms of reduction of proinflammatory cytokine levels (IL-6 and TNF-α), BNP levels and oxidative stress parameters in patients with decompensated CHF observed within 48 h and up to five days post-infusion. MDA concentration was significantly lower after 5 days in levosimendan-treated patients compared to baseline values (2.3 ± 0.2 vs. 3 ± 0.3 microM, $p = 0.01$) and did not change in the dobutamine group [18]. Moreover, the MDA value was lower in the levosimendan group vs. in the dobutamine group after 5 days post-infusion ($-21.8 \pm 5.1\%$ vs. $14.9 \pm 8.5\%$, $p = 0.001$)[18].

Predicted results

Considering results of previous studies conducted mainly in animal models, there are premises that this project will demonstrate that levosimendan may reduce oxidative stress in patients with decompensated heart failure. The lower free radical level caused by levosimendan may lead to lower activity of antioxidant enzymes after five days post-infusion of levosimendan in patients with decompensated heart failure compared to patients treated with dobutamine.

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