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Levosimendan protects against ischemia – reperfusion injury in the human heart muscle. A pilot study

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ABSTRACT

The consequences of myocardial infarction (MI) are an increasing problem worldwide. Despite spectacular progress in the invasive treatment of ischemic heart disease, the ability to limit the ischemia-reperfusion (I/R) injury remains largely unrealized. Recent studies have shown that stimulation of opioid receptors may confer a cardioprotective effect against I/R injury. Levosimendan, the inodilator, is indicated for the short-term treatment of acutely decompensated heart failure. We tested the hypothesis that levosimendan may provide cardioprotection in the opioid-like mechanism in the human myocardium.

INTRODUCTION

Ischemic heart disease remains the leading cause of morbidity and mortality in developed countries. Restoring coronary flow is mandatory to reduce ischemic myocardial damage and may at least in part save the post-ischemic heart muscle contractile function. However, reperfusion can potentially exacerbate lethal tissue injury in the mechanism known as ischemia-reperfusion (I/R) injury, which in clinical settings, manifests as the decrease of potential benefits of reperfusion [1]. Experimental and clinical evidence suggest that levosimendan may be part of the endogenous cardioprotective response to I/R injury [2]. Levosimendan, an inodilator, is indicated for the short-term treatment of acutely decompensated heart failure. The pharmacological effects of levosimendan consists in the improvement of heart muscle contractility through increased calcium sensitization of troponin C [3,4] and vasodilation mediated by the opening of potassium channels on the vessels' smooth muscle cells [5,6], as well as cardioprotection through the opening of mitochondrial potassium channels in cardiac muscle cells [7].

Levosimendan improves hemodynamics without an increase in the consumption of oxygen in heart failure patients. The drug is indicated for the short-term treatment of decompensated heart failure especially in cases where inotropic therapy is insufficient [8,9]. Levosimendan has shown a cardioprotective effect in cardiac surgery settings [10].

In the current study, the effect of levosimendan on the human ischemic myocardium under *in vitro* condition is assessed using the pathomorphological method to detect intensity of the early damage of the heart tissue.

MATERIALS AND METHODS

The experiments were performed on muscular trabeculae obtained from the appendages of the right atrium of 10 consecutive patients subjected to elective coronary artery bypass surgery. Specimens from patients diagnosed with diabetes, significant valvular heart disease, or severe heart failure therapy were excluded from the study. The tissue fragments were transported from the cardiac surgery room to the laboratory in an ice-cold Krebs-Henseleit solution ([M]: NaCl 118.0, KCl 4.70, $CaCl_2$ 1.52, $MgSO_4$ 1.64, $NaHCO_3$ 24.88, KH_2PO_4 1.18, glucose 11.0, and sodium pyruvate 2.0; pH 7.4). From each patient, two muscular trabeculae, each less than 1 mm in diameter, were incubated in 2 separate organ baths (Schuler Organbath, Hugo Sachs Elektronik,

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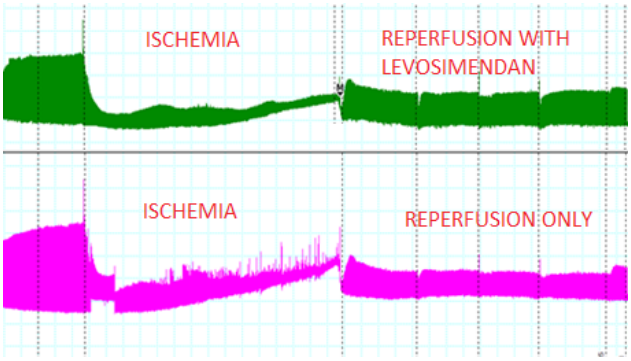
March-Hugstetten, Germany [HSE]), both filled with Krebs-Henseleit solution warmed to 37°C. Two trabeculae from each patient were studied simultaneously and exposed to a hypoxia protocol, including 60 min of hypoxia (incubation in Krebs-Henseleit buffer deprived of glucose and pyruvate and saturated with 95% argon and 5% carbon dioxide) with subsequent 60 min of re-oxygenation (incubation in Krebs-Henseleit buffer saturated with 95% oxygen and 5% carbon dioxide). The buffer was replaced every 15 mins., except at the time of hypoxia.

The study protocols are presented in Figure 1. Levosimendan (10^{-5}M) was administered at the time of re-oxygenation in the levosimendan protocol group. The second trabecula was subjected to I/R protocol only as a control group. Every trabecula was stretched to 90% of its optimal tension strength, according to the Frank-Starling relationship, and all trabeculae were driven throughout experiments with 1 Hz 50 ms square stimuli using platinum field electrodes and a stimulator (Type 215, HSE). The systolic function of every trabecula was recorded using an F30 isometric force transducer (Type 372, HSE). The signal was enhanced with a bridge amplifier (Type 336, HSE), recorded by a PowerLab/4SP system, and analyzed offline using chart software (AD Instruments, Chalgrove, Oxfordshire, UK). Each experimental protocol was completed with an application of 10 µM of norepinephrine (NE) to assess the viability of trabeculae.



An example of the recording obtained during the experiment.
The trabeculae's contraction amplitudes are shown for levosimendan (above) and the control (below)

Figure 1. Effects of hypoxia and reperfusion on the contractile function of cardiomyocytes (control group)

Next, the trabeculae were transported in a formaldehyde solution to the histology laboratory to reveal the early signs of ischemic myocardial tissue injury.

Paraffin blocks containing tissue fragments were cut into 4 µm slices with a rotary microtome and mounted on microscope slides. Slides were stained using hematoxylin, eosin and Masson's trichrome with Fast Green FCF. Tissue images were captured using a Nikon Eclipse microscope and a Canon EOS 500D digital camera at 100-fold magnification and 4762×3168 pixel resolution.

The obtained histologic analysis data were divided into three groups:

1. Preexisting lesions of muscular trabeculae: inflammatory infiltrations, enlarged myocytes, nuclear enlargement and % of fibrosis.
2. Recent ischemia without necrosis: edema, cell swelling, focal loss of myofibrils, intracellular vacuolization myocytolysis, pre-myocytolysis, and focal loss of cross-striations.
3. Markers of ischemia with subsequent early necrosis: contraction bands, hypereosinophilic fibers, waveform fibers, fuchsinophilia, pyknosis, and immunohistochemistry C4d.

We used the Mann-Whitney rank sum test to compare the groups. The p values <0.05 were considered statistically significant. Statistical analysis was performed using SigmaPlot software v. 10.0.1.2 (Systat Software Inc., San Jose, USA).

The study was conducted according to the Declaration of Helsinki guidelines and approved by the Bioethical Committee of the Medical University of Silesia (NN-6501-98/07). According to the decision, individual patient consent was waived.

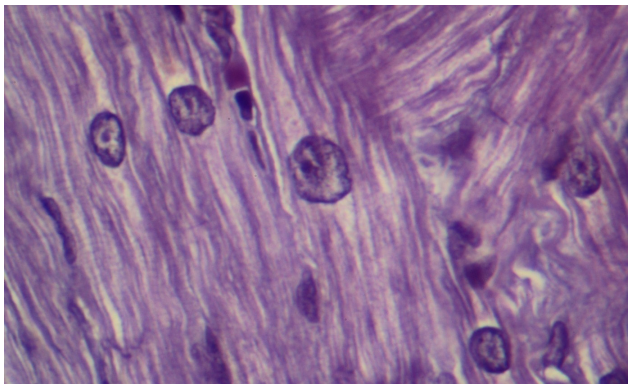


Figure 2. Atrial muscle – atrial cardiomyocytes normal size. H&E. 100-fold

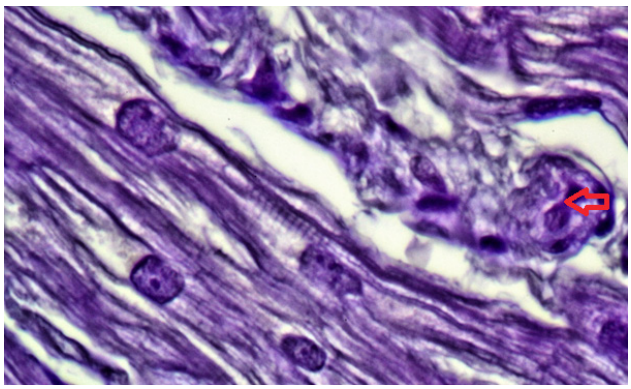


Figure 3. Atrial muscle – loss of cross-striations. H&E. 400-fold. Contrast limited adaptive histogram equalization (CLAHE)

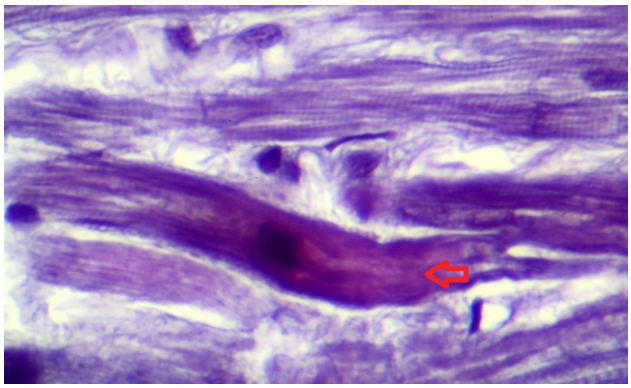


Figure 4. Atrial muscle – hypereosinphilic fibre without cross-striations shows waviness type 2. H&E 400-fold

RESULTS

The obtained results are presented in tables with a head-to-head comparison of trabeculae pairs.

We observed significantly more frequent presentation of focal loss of cross-striations (8 *vs.* 2 points, Tab. 2, p=0,012) and the wavyform fibres (4 *vs.* 0 points Tab. 3, p=0,027) in the control *vs.* levosimendan group. Of note, the number of cell lesions in the levosimendan group was lower than in the control group, both for the signs of recent ischemia and early necrosis.

The preexisting lesions in examined tissue fragments are shown in Table 1. Signs of recent ischemia without necrosis and markers of ischemia with subsequent early necrosis are shown in Tables 2 and 3, respectively.

Table 1. Preexisting lesions of muscular trabeculae

	Fibrosis [af%]	Inflammatory infiltrations	Enlarged myocytes	Nuclear enlargement only	Σ
Levo 25	5	0	1	0	6
Levo 25	3	1	0	0	4
Levo 25	5	0	0	0	5
Levo 25	4	1	0	1	6
Levo 25	8	0	0	1	9
Levo 25	4	0	0	0	4
Levo 25	3	0	0	1	4
Levo 25	2	0	0	0	2
Levo 25	4	1	1	0	6
Levo 25	3	1	0	0	4
					Σ 50

Control	3	0	0	0	3
Control	3	0	1	0	4
Control	3	1	1	0	5
Control	7	0	1	0	8
Control	6	0	0	0	6
Control	7	0	0	0	7
Control	6	0	0	1	7
Control	6	0	1	0	7
Control	9	0	1	0	10
Control	4	0	0	0	4
					Σ 61

Described lesions: 0 - none, 1 - present in a few cells, 2 - present in multiple cells

Table 2. Signs of recent ischemia without necrosis

	Edema	Cell swelling	Focal loss of myofibrills	Intracellular vacuolation myocytolysis	Premyocytolysis	Focal loss of cross-striations	Σ
Levo 25	0	1	1	1	1	0	4
Levo 25	0	1	1	1	1	1	5
Levo 25	0	0	1	0	0	0	1
Levo 25	0	0	0	0	0	0	0
Levo 25	0	1	1	1	0	0	3
Levo 25	1	0	0	1	1	0	3
Levo 25	0	0	1	0	0	0	1
Levo 25	0	0	1	1	0	0	2
Levo 25	0	0	1	1	1	0	3
Levo 25	0	1	1	0	1	1	4
							Σ26

Control	0	0	1	1	1	0	3
Control	0	0	1	1	1	0	3
Control	0	0	1	1	2	2	6
Control	0	0	2	2	1	1	6
Control	1	1	1	1	2	2	8
Control	0	0	1	2	2	0	5
Control	0	0	1	2	1	1	5
Control	1	1	1	1	2	0	6
Control	0	1	2	2	2	1	8
Control	1	1	1	1	1	1	6
							Σ56

The signs include the following: 0 - none, 1 - present in a few cells, 2 - present in multiple cells

Table 3. Markers of ischemia with subsequent early necrosis

	Contraction bands	Hypereosinophilic fibres	Wavyform fibres	Fuchsinophilia	Pyknosis	Σ
Levo 25	1	1	0	1	0	3
Levo 25	1	0	0	1	0	2
Levo 25	1	1	0	0	0	2
Levo 25	1	0	0	0	0	1
Levo 25	1	0	0	1	0	2
Levo 25	1	0	0	1	0	2
Levo 25	0	1	0	0	0	1
Levo 25	1	1	0	0	0	2
Levo 25	0	0	0	1	0	1
Levo 25	1	1	0	0	0	2
						Σ18

Control	1	1	0	0	0	2
Control	1	1	0	0	0	2
Control	0	1	1	0	1	2
Control	1	1	1	1	0	4
Control	0	1	0	1	0	2
Control	1	1	1	0	0	3
Control	1	0	0	0	1	2
Control	0	1	1	1	0	3
Control	1	1	0	0	0	2
Control	0	0	0	1	1	2
						Σ24

The markers include the following: 0 - none, 1 - present in a few cells, 2 - present in multiple cells

DISCUSSION

Levosimendan is documented as a calcium sensitizer and a positive inotropic and vasodilating factor, and its properties are mainly useful in severe low-output heart failure. In addition, levosimendan has been demonstrated to have a protective effect against I/R injury. The first anti-ischemic effects of levosimendan were shown in 1994 in isolated rabbit hearts by Rump *et al.* [11]. The authors revealed that left ventricular systolic pressure and coronary blood flow significantly increased following levosimendan administration after the occlusion of the first postero-lateral branch of the circumflex artery for 120 minutes. The end-diastolic pressure remained unchanged [12]. Moreover, *in vitro* work by Maytin *et al.* [13] showed that levosimendan protected cardiomyocytes from apoptosis induced by hydrogen peroxide by activating mitochondrial ATP-dependent K+ channels. This effect was abolished by the K+ channel inhibitor, 5-hydroxydecanoic acid. A solid hypothesis of how levosimendan might influence I/R-induced cardiac apoptosis was provided via the prevention of mitochondrial calcium overload and the opening of potassium ATP-dependent channels in the inner membrane of the mitochondria [14]. Thus, levosimendan may confer cardioprotection in an opioid-dependent manner [15].

Levosimendan cardioprotection can be divided into short-term and long-term effects. Short-term cardioprotection includes preconditioning, postconditioning, anti-stunning and anti-ischemic effects. Long-term cardioprotection assumes anti-remodelling, anti-apoptotic and anti-inflammatory effects [16]. According to *ex-vivo* research, levosimendan preconditioning may reduce infarct size by 90% in the animal model of I/R injury [17]. Anti-stunning effect of levosimendan in patients with acute coronary syndrome has also been shown. In addition, the total number of hypokinetic segments was significantly decreased with levosimendan treatment [18], and the anti-ischemic effect of levosimendan was shown in a rodent model of healed MI [19]. Other studies have revealed that the preoperative use of levosimendan in high-risk patients with severe left ventricular dysfunction reduced mortality [20]. Also, levosimendan confers significant benefits in the postoperative period with improvements in hemodynamic parameters. Herein, postoperative mortality in patients with severe left ventricular dysfunction was lower in a levosimendan group than in a dobutamine group [21]. The beneficial effect of levosimendan can be achieved intraoperatively with its inodilatory effects. However, levosimendan is also used in the preoperative setting to benefit patients through its cardioprotective properties that come into effect later, during the intraoperative period [16].

To the best of our knowledge, the study we undertook was the first to elicit levosimendan's protective effect against I/R injury based on observations of histological changes in the human myocardium. Our preliminary results suggest the levosimendan group tended towards less tissue damage than the control group. Hematoxylin-eosin staining under light microscopy reveals detectable morphological differences between the analyzed groups. More significant damage features in the control group are marked in focal loss of cross-striations and wavyform fibers. Wavy myocardial fibers, associated with focal edema, are the characteristic sign of acute myocardial ischemia, especially within the first 1 to 3 hours [22].

The beneficial effect of levosimendan protection from I/R-induced apoptosis may become a practical therapeutic goal. The existing clinical and experimental data is promising, but it must be confirmed in larger cohorts. Moreover, identifying the underlying molecular mechanisms for the protective effects of levosimendan in I/R injury and apoptosis remains a challenge for the future.

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