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Original Article

Effects of levosimendan on myocardial ischaemia–reperfusion injury

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Summary

Background and objective: Levosimendan has a cardioprotective action by inducing coronary vasodilatation and preconditioning by opening K_{ATP} channels. The aim of this study was to determine whether levosimendan enhances myocardial damage during hypothermic ischaemia and reperfusion in isolated rat hearts. *Methods:* Twenty-one male Wistar rats were divided into three groups. After surgical preparation, coronary circulation was started by retrograde aortic perfusion using Krebs–Henseleit buffer solution and lasted 15 min. After perfusion Group 1 (control; $n = 7$) received no further treatment. In Group 2 (non-treated; $n = 7$), hearts were arrested with cold cardioplegic solution after perfusion and subjected to 60 min of hypothermic global ischaemia followed by 30 min reperfusion. In Group 3 (levosimendan treated; $n = 7$), levosimendan was added to the buffer solution during perfusion and the hearts were arrested with cold cardioplegic solution and subjected to 60 min of hypothermic global ischaemia followed by 30 min reperfusion. At the end of the reperfusion period, the hearts were prepared for biochemical assays and for histological analysis. *Results:* Tissue malondialdehyde levels were significantly lower in the levosimendan-treated group than in the non-treated group ($P = 0.019$). The tissue Na^+–K^+ ATPase activity was significantly decreased in the non-treated group than in the levosimendan-treated group ($P = 0.027$). Tissue myeloperoxidase (MPO) enzyme activity was significantly higher in the non-treated group than in the levosimendan-treated group ($P = 0.004$). Electron microscopic examination of the hearts showed cardiomyocytic degeneration at the myofibril, mitochondria and sarcoplasmic reticulum in both non-treated and levosimendan-treated groups. The severity of these findings was more extensive in the non-treated group. *Conclusions:* Treatment with levosimendan provided better cardioprotection with cold cardioplegic arrest followed by global hypothermic ischaemia in isolated rat hearts.

Keywords: HEART; DRUG, levosimendan; ISCHEMIC PRECONDITIONING, hypothermic, cardioplegic; RAT.

Introduction

Potassium cardioplegia has been routinely used for several decades to achieve cardiac arrest and to protect the myocardium during open-heart surgery. In clinical practice, in order to attenuate ischaemic injury of hearts during cardiac surgical operations, cold cardioplegia is usually applied to rapidly arrest the beating hearts and the ischaemic hearts are in a hypothermic environment. Protection by hypothermia occurs early during ischaemia [1]. Although hypothermia is an important factor for the preservation of ischaemic myocardium by lowering myocardial metabolism, it might also exert deleterious effect by itself, such as hyper-contracture

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due to elevated cytosolic Ca^{2+} [2] and coronary artery spasm leading to reduction in myocardial protection [3].

Levosimendan is currently in clinical use and it has been approved by several European countries and recommended in the European Society of Cardiology guidelines, as inotropic therapy for the short-term treatment of acute severe decompensated heart failure in adults [4]. It has a dual mechanism of action, increasing myocardial contractility and inducing coronary and systemic vasodilatation. Its inotropic effect is mediated by calcium sensitization of the contractile proteins [5] and the vasodilator effect is mediated by opening the adenosine triphosphate-regulated potassium channel (K_{ATP}) in vascular smooth muscle cells [6,7]. Stimulation of K_{ATP} channels improves coronary blood flow and protects the ischaemic myocardium. K_{ATP} channels have been observed also in rat ventricular myocytes, which could be related to the findings that levosimendan protects the ischaemic myocardium and decreases the infarct size in coronary-ligated animals [8–11]. Previous papers have demonstrated the advantage of levosimendan as an inotrope on the mechanical recovery of heart and as a cardioprotective agent on myocardium in normothermia. Beneficial effects of levosimendan on hypothermic ischaemia–reperfusion (I/R) have only been shown in small clinical and animal studies [12,13]. These studies have only focused on its cardiac contractile performance. There is a lack of data on its effects of hypothermic myocardial I/R injury manifested by biochemical and ultrastructural evidence. In addition, there is some doubt about the additive protective effect of preconditioning when used in combination with hypothermia or cardioplegia during extended periods of global I/R [13–15].

In light of these reports, our study was designed to investigate whether levosimendan attenuates myocardial I/R injury in a clinically relevant model of cold cardioplegic arrest and hypothermic global ischaemia on isolated rat hearts.

Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the local Ethics Committee of animal experiments. Animal care experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No: 85–23, revised 1996). The animals were fasted overnight but allowed free access to water and maintained in a room kept at 24°C. Twenty-one Wistar rats (250–270 g) were randomly divided into three equal groups. Levosimendan was obtained from Orion Pharma, Espoo, Finland (Simdax™). It was diluted appropriately and added to Krebs–Henseleit buffer (KHB) solution used for stabilization and reperfusion period in Group 3.

The animals were anaesthetized with intramuscular ketamine 60 mg kg^{-1} and systemically heparinized by injecting 3.5 mg (3500 IU) of heparin into the peritoneal cavity. They were operated when unresponsive to noxious stimulation. Midline sternotomy was performed and each heart was rapidly excised and immersed in 37°C KHB solution. The aorta was cannulated distal to the aortic valve. The hearts were mounted on a modified Langendorff apparatus and coronary circulation was started by retrograde aortic perfusion using KHB solution (NaCl 118, $CaCl_2$ 3.0, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.2, Glucose 11.1 mmol L^{-1}) at a flow rate of 10 mL kg^{-1} min^{-1} at 37°C for a 15 min stabilization period while perfusion pressure in the aorta was maintained constant at a pressure of 40 mmHg in all groups. In this period, levosimendan (24 μg kg^{-1}) was added to KHB solution in Group 3.

Control hearts (Group 1) received no further treatment and the hearts were homogenously divided for biochemical assays and for histological analysis. Isolated hearts of study Groups 2 and 3 were arrested with hypothermic (+4°C) St Thomas' hospital cardioplegic solution (NaCl 110, KCl 16, MgCl 16, $CaCl_2$ 1.2, $NaHCO_3$ 10 mmol L^{-1}) at a flow rate of 15 mL kg^{-1} min^{-1} for 3 min. Throughout the ischaemic arrest period, the hearts were kept in a 20°C isotonic saline (0.9% NaCl) jacketed heart chamber. After cardioplegic arrest and 60 min of global ischaemic period, KHB solution was administered for reperfusion, 30 min at 37°C in Groups 2 and 3. In this period, levosimendan was added to KHB (0.2 μg kg^{-1} min^{-1}) used in Group 3. Figure 1 briefly illustrates the protocol.

At the end of the reperfusion period, the hearts were homogenously divided for biochemical assays and for histological analysis carried out using electron microscopy.

The malondialdehyde (MDA) levels, as an index of lipid peroxidation, were determined by thiobarbituric acid reaction according to the method of Yagi [16]. The principle of the method depends on measurement of the pink colour produced by interaction of barbituric acid with MDA, elaborated as a result of lipid peroxidation. The coloured reaction 1,1,3,3-tetraetoxypropane was used as the primary standard. MDA levels in heart tissue were expressed as nmol g^{-1} tissue.

Ten percent homogenate of the tissue was prepared in 0.3 mol sucrose containing 1 mmol magnesium by homogenizing for 90 s using a

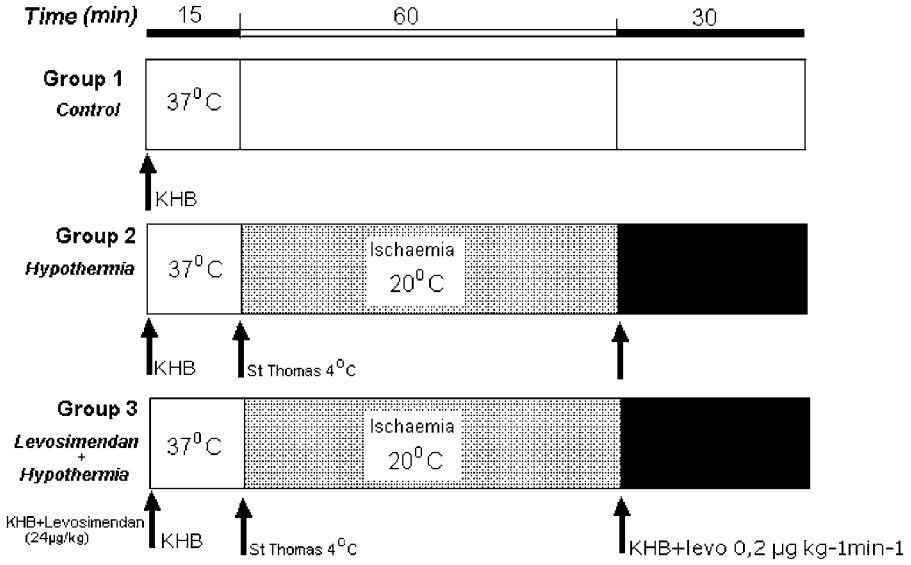

Figure 1.
Experimental protocol. KHB = Krebs–Henseleit solution; Levo = Levosimendan. Perfusion of the different solutions begins at the arrows.

Teflon pestle clearance 0.25–0.38 mm at 1000 rpm. To remove the debris, it was then centrifuged at 1000 rpm for 15 min. ATPase activities were determined on the resulting supernatants by measuring the rate of liberation of inorganic phosphate (Pi) from disodium ATP incubating media made as described previously [17,18]. Adenosine 5′ triphosphatases were as follows: Na^+–K^+ ATPase (mmol) – $MgCl_2$ 6, KCl 5, NaCl 100, EDTA 0.1, Tris–HCl buffer pH 7.4, 135 mM. Specific activities were calculated as nmol $Pi^{-1}\,h^{-1}$ protein. Protein content was determined according to the method described by Lowry and colleagues [19] and bovine serum albumin was used as a standard.

MPO is a haem-containing enzyme within the azurophil granules of neutrophils and MPO activity was measured as a simple quantitative method of detecting leuco-sequestration. For this, 300 mg tissue was homogenized in 0.02 mol EDTA (pH 4.7) in a Teflon Potter homogenizer. Homogenates were centrifuged 20 000*g* for 15 min at +4°C. After pellet was re-homogenized in 1.5 mL 0.5% hexadecyl-trimethyl-ammonium bromide prepared in 0.05 mol KPO_4 (pH 6) buffer, it was re-centrifuged at 20 000*g* for 15 min at +4°C. The determination of sera and supernatant tissue MPO activity depends on the fact that it reduces *o*-dianozidine and hence reduced *o*-dianozidine was measured at 410 nm by a spectrophotometer [20].

For transmission electron microscopic evaluation, the samples were fixed with 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated in graded alcohol series, cleared with propylene oxide and embedded in epon. Thin sections (50–70 nm) were cut by a microtome (Leica UCT-125; Leica, Wien, Austria) and contrasted with uranyl acetate and lead citrate. Sections were examined and photographed by an electron microscope (JEOL JEM-1011; JEOL Ltd, Tokyo, Japan) by a histologist blinded to the study groups.

Statistics

Previous studies documented that approximately 50% reduction in injury was found in the levosimendan-treated group [11,12]. According to these results, at least six rats were found to be sufficient for providing appropriate statistical power with α error of 5% and β error of 20%. In the planning period of our study, the sample size was arranged as seven rats in each arm to provide appropriate statistical power analyses.

Results are expressed as mean ± SD. All statistical analyses were carried out using SPSS statistical software (SPSS for windows version 10.0, Chicago, IL, USA). Data were analysed by using one-way ANOVA test. *Post hoc* comparisons were performed using least significant difference (LSD) test. *P*-values less than 0.05 were considered to be significant.

Results

A total of 21 hearts were studied in the protocol and 18 successful experiments were completed. One heart was excluded in each group because of aortic injury. The remaining hearts were randomly divided into three equal groups. All hearts started beating within a few minutes after reperfusion.

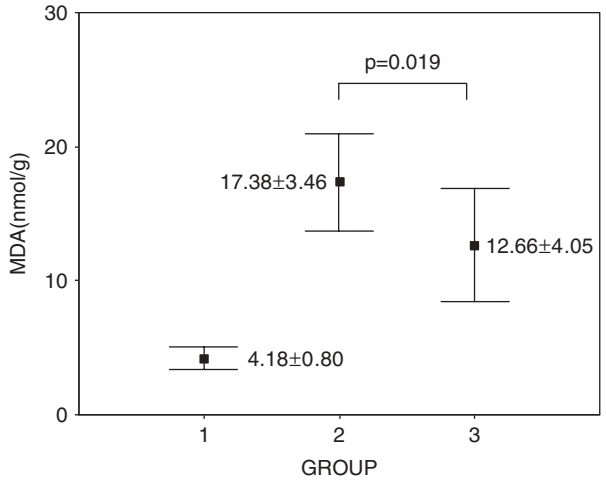

Figure 2.
Malondialdehyde (MDA) concentrations. Data are mean ± SD.

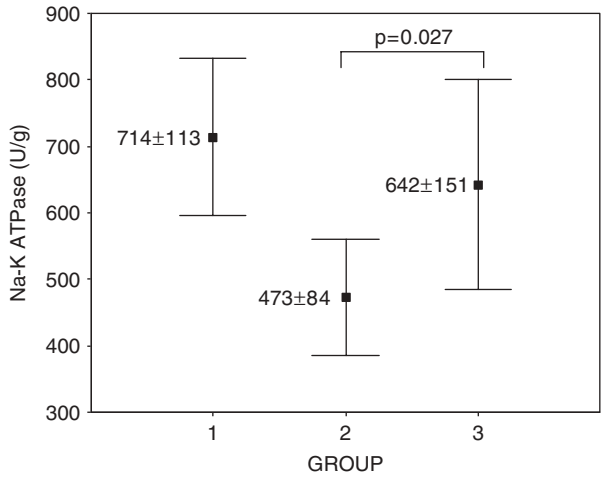

Figure 3.
Na–K ATPase enzyme activity. Data are mean ± SD.

The tissue MDA levels in the non-treated group (Group 2) were significantly increased when compared to the control group (Group 1) ($P = 0.0001$). Levosimendan treatment significantly decreased the I/R-induced elevation in tissue MDA levels in comparison with the non-treated group ($P = 0.019$) (Fig. 2). The tissue Na^+–K^+ ATPase enzyme activity was significantly decreased in the non-treated group when compared to the control group ($P = 0.003$). Na^+–K^+ ATPase was significantly protected in the levosimendan-treated group (Group 3) when compared to the non-treated group ($P = 0.027$). There was no significant difference between the levosimendan-treated group and the control group in Na^+–K^+ ATPase enzyme activity ($P = 0.317$) (Fig. 3).

Tissue MPO enzyme activity was significantly higher in the non-treated group in comparison with

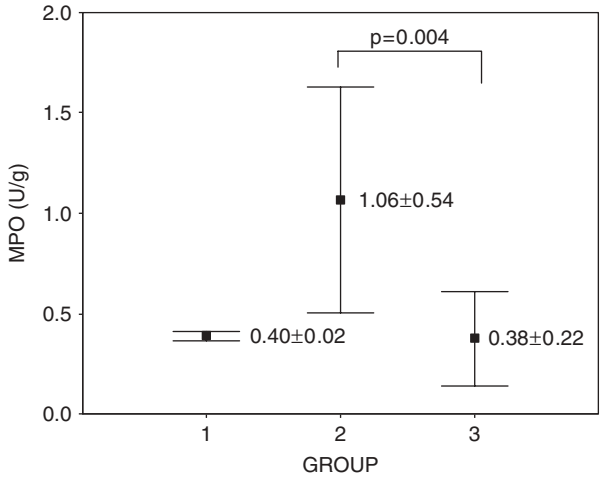

Figure 4.
Myeloperoxidase (MPO) enzyme activity. Data are mean ± SD.

the control group ($P = 0.005$). On the other hand, levosimendan treatment significantly decreased the tissue MPO level when compared to the non-treated group ($P = 0.004$). There was no significant difference between the control group and the levosimendan-treated group in MPO enzyme activity ($P = 0.923$) (Fig. 4).

Discussion

The results of the present study showed that treatment with levosimendan provided better protection on myocardium compared with cold cardioplegic arrest alone in hypothermic global I/R injury, as manifested by tissue MDA, MPO and Na–K ATPase levels and electron microscopic examination.

Myocardial cooling is considered as effective method for protecting the myocardium by lowering myocardial metabolism during ischaemia. Hypothermic protection of ischaemic myocardium is associated with the preservation of high-energy phosphates, which may facilitate the maintenance of membrane integrity during ischaemia [21,22]. However, rapid cooling might adversely influence cardioplegic myocardial protection [3]. Contractile dysfunction, oedema and calcium dyshomeostasis have been reported after re-warming from profound hypothermia [23,24]. It has been reported that deep hypothermia could be responsible for phospholipidic bilayer disruptions and changes in enzymatic activities [25] and re-warming could cause cellular alterations. Knerr and Lieberman [26] also showed that deep hypothermia could alter ionic pump activities. In addition, it has been reported that use of local cooling and infusion of cold cardioplegic solutions cause coronary artery spasm, resulting in the unequal supply of cardioplegic solution and

a subsequent reduction in myocardial protection [27]. Our results demonstrated the injured state of myocardium based on biochemical and ultrastructural evidence. Rapid cooling and prolonged ischaemia with St Thomas' cardioplegic solution alone might be responsible for cardiomyocytic injuries in the non-treated group.

Levosimendan increases myofilament sensitivity to calcium [28]. It has been shown that levosimendan attenuates contraction of hearts via this mechanism [29]. Cardiac myocytes contain two distinct K_{ATP} channels: one in sarcolemmal membrane and other in mitochondrial inner membrane [30]. Levosimendan has vasodilating properties that reduce cardiac preload and afterload. Vasodilatation is attributed to the opening of K_{ATP} channels in the sarcolemmal membrane of vascular smooth muscle. This effect produces both coronary and systemic vasodilatation and coronary blood flow is enhanced [6,7,31]. It also activates K_{ATP} channels on the mitochondrial inner membrane of rat cardiac myocytes [8,9]. Recent evidence demonstrated that levosimendan can mimic ischaemic preconditioning, improve cardiac function and decrease infarct size in animal models through its K_{ATP}-channel opening properties in normothermic conditions [11,32]. However, it has been reported that protective effects of preconditioning might be failed when used in combination with hypothermia or cardioplegia during extended periods of global I/R [14,15].

In the present study, St Thomas' cardioplegic solution was used to achieve rapid cold arrest and the hearts were subjected to 60 min of hypothermic global ischaemia. With this solution, myocardial cooling was provided rapidly and metabolic demand was reduced. To attenuate the side-effects of rapid cooling, levosimendan was administered in recommended clinical concentration ($24\,\mu g\,kg^{-1}$ bolus over 10 min) before cardioplegic arrest and followed by an infusion of $0.2\,\mu g\,kg^{-1}min^{-1}$ during reperfusion. We found that treatment with levosimendan has profitable effects on myocardial protection beyond that obtained by cold cardioplegia alone. The reduction of myocardial injury seen in this model could be explained by the action of levosimendan as a coronary vasodilator by opening the K_{ATP} channel. In addition, the I/R injury of myocardium might be attenuated by the preconditioning effect of levosimendan.

Previous studies showed that administration of levosimendan protects the myocardium against I/R injury, but the impact of this effect on myocardial protection in low temperature had not been evaluated. To our knowledge, no previous studies have dealt with the influence of levosimendan on the cardiac muscle cell in deep hypothermia which hampers the comparative analysis of our data. Tritapepe and colleagues [12], in a pilot study published recently, reported that infusion of levosimendan before commencing cardiopulmonary bypass, in patients undergoing coronary revascularization, resulted in improvements in hemodynamic performance and reduction in troponin I release. In that study, cold-blood cardioplegia was used at 6–8°C and agreed that those data were consistent with a preconditioning effect in humans.

MDA is one of the many products of lipid peroxidation [33]. I/R peroxides polyunsaturated membrane lipids and damage their structure and functions [34]. Na–K ATPase is a membrane-bound enzyme that performs cellular functions in ionic and osmotic balance. Lipid peroxidation changes membrane fluidity and enzyme activity [35]. I/R of isolated guinea pig hearts reduced the Na–K ATPase activity and this effect of injury was prevented by oxygen free radical scavengers [36]. Higher levels of ATPase activity might have protected the myocardium from more severe injury following I/R. In the present investigation, we observed that myocardial lipid peroxidation increased significantly after 60 min of global hypothermic ischaemia and a few minutes of reperfusion in both study groups compared with the control group. Although these results demonstrated the injured state of myocardium, treatment with levosimendan before cold cardioplegic arrest was effective in reducing myocardial lipid peroxidation compared to hypothermic cardioplegic arrest alone. This was correlated with the enzyme activity of Na–K ATPase, which may play a key role in the prevention of I/R injury.

Mullane and colleagues [37] found that MPO enzyme activity can be used for the quantitative assessment of neutrophil infiltration into ischaemic myocardium and myocardial neutrophil accumulation may reflect the degree of myocardial injury. However, the heart is similar to most other organs in that it has a resident population of leucocytes located within the interstitium [38,39]. Keller and colleagues [39] found numerous resident cardiac leucocytes in their isolated rat heart model and these leucocytes were found to be approximately equally distributed between mast cells and macrophages. Hence, the leucocyte population should be considered in isolated blood-free models and high levels of MPO may be ascribed to degranulation of the resident leucocyte population. In our study, MPO enzyme activity was significantly lower in the levosimendan-treated group. This result can be commented in favour of the effect of levosimendan, which might occur with less neutrophil accumulation and subsequent injury after I/R.

Electron microscopic examination revealed ultrastructural changes. There was focal cardiomyocytic degeneration at the myofibril, mitochondria and sarcoplazmic reticulum in both study groups. There was mitochondrial damage as evidenced by the loss of cristae and dense mitochondria and intracellular sytoplasmic vacuolization at perinuclear localization.

Histological analyses were performed by a histologist blinded to the treatment groups, and reported that the severity of these findings was more extensive in the non-treated group than in the levosimendan-treated group. Although electron microscopic examination cannot show the degree of cell injury quantitatively, in this study, levosimendan treatment had better protection with this model and this result is correlated with the other biochemical determinations.

Moderation of ischaemic injury by the K_{ATP}-channel opener, levosimendan, resulted in lesser myocardial cell damage. Minimizing ischaemic injury of myocardium, like preconditioning, is possibly explained by the mitochondrial K_{ATP}-channel agonist effects of levosimendan. In addition, the reduction of myocardial injury seen in this model could be explained, in part, by the action of the drug as a coronary vasodilator by opening the K_{ATP} channel on the sarcolemmal membrane of vascular smooth muscle cells, since the drug was administered before the ischaemic event.

We recognize limitations in the interpretation of our findings. First of all, a model was constituted similar to the clinical practice and it is therefore far from explaining the exact mechanism responsible for cardioprotection. Second, the study was performed on animals without cardiovascular disease. Accordingly, direct applicability to human patients with cardiovascular disease remains to be proven.

In conclusion, our results suggest that treatment with levosimendan provided better cardioprotection with cold cardioplegic arrest followed by 60 min global hypothermic ischaemia in the isolated rat hearts. In this model, cardioprotective effects of levosimendan might be explained in two ways: first, its pharmacological preconditioning activity by mitochondrial K_{ATP}-channel opening; second, indirectly vasodilator action (enhancing the rapid cooling contraction and increase in coronary blood flow) by sarcolemmal K_{ATP}-channel opening, all of which improve myocardial protection. Thus, it might be concluded that the use of levosimendan may be advantageous in the setting of stressed hearts with hypothermic ischaemic injury where conventional cold cardioplegic arrest cannot provide adequate myocardial protection. Further studies are in progress to identify the exact mechanisms of levosimendan on rapid cooling and hypothermic I/R injury.

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