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EXPERIMENTAL PAPER

Myocardial stunning following no flow ischaemia is diminished by levosimendan or cariporide, without benefits of combined administration☆,☆☆

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Summary
Aim of the study: Levosimendan, a calcium sensitiser, and cariporide, a blocker of the Na^+/H^+ exchanger, decrease necrosis and improve function following myocardial ischaemia. However, their role in myocardial stunning is unclear. We tested the hypothesis that levosimendan, cariporide, or their combination reduce stunning after global myocardial ischaemia.
Methods: In a prospective, controlled, randomised laboratory study isolated guinea pig hearts ($n = 48$) were perfused in a Langendorff apparatus. Stunning was induced by 20 min of global no-flow ischaemia. Levosimendan (0.1 μmol/l) or cariporide (1 μmol/l) were given either before or after ischaemia, and effects of both drugs combined were also assessed. Left ventricular developed pressure (LVdp) was assessed continuously before ischaemia and for 45 min after reperfusion.
Results: Levosimendan ($24 \pm 7\%$) and the combination of levosimendan and cariporide ($38.7 \pm 4\%$) increased LVdp from baseline values before ischaemia, without differences between groups. In contrast, cariporide alone decreased LVdp ($-11 \pm 2\%$) from baseline.

Ischaemia/reperfusion decreased LVdp by about 70% in vehicle treated hearts compared to baseline. Treatment with cariporide, levosimendan, or their combination both before and after ischaemia, and treatment with cariporide after ischaemia caused a 25% greater recovery of LVdp than in control hearts. There were no differences between these groups and no enhanced effect with levosimendan/cariporide combined.

In contrast, levosimendan only given after ischaemia did not improve LVdp.
Conclusions: Cariporide diminished stunning when given before or after ischaemia, while levosimendan was only effective if given before ischaemia. Thus, levosimendan or cariporide may be useful in settings where ischaemia/reperfusion is to be expected.

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Introduction

Myocardial stunning or post-ischaemic left ventricular (LV) dysfunction denotes dysfunction that persists after reperfusion in the absence of necrosis and is completely reversible.[1] Although reperfusion terminates ischaemia and is essential for restoring normal function, it also enhances reperfusion injury and cell damage. Stunning occurs in several conditions, e.g., after cardiac surgery[2] or after cardiac arrest and resuscitation.[3,4] One mechanism for stunning is believed to be the increase in intracellular Na^+ concentration activating the Na^+—Ca^{2+} exchanger to work in the reverse direction leading to an intracellular Ca^{2+}-overload.[5]

Cariporide (HOE-642) is a selective inhibitor of the Na^+/H^+-exchanger 1, and the primary isoform is expressed in cardiac myocyte sarcolemma, and shows cardioprotective effects in ischaemia and reperfusion,[6] and reduces infarct size in pigs and rats.[7,8] In studies on myocardial stunning, controversial results were obtained, with either improvement of myocardial function or no improvement.[9–11]

The calcium sensitiser levosimendan increases myocardial contractile force by enhancing the sensitivity to calcium of myofilaments[12] without increasing the intracellular calcium concentration.[13] However, levosimendan also opens mitochondrial K_{ATP} channels,[14] decreases myocardial infarct size, and improves myocardial function after left anterior descending coronary artery occlusion.[15] While the ability of levosimendan to improve function in stunning is well described,[16,17] there are no data addressing its potential ''cardioprotective'' effect.

Accordingly, following global ischaemia cariporide could decrease stunning by preventing the decrease of the intracellular Ca^{2+}-transient while levosimendan could improve LV function by an independent mechanism, in particular when given in combination, due to their different mechanisms of action and the potential cardioprotective effects of levosimendan. Therefore, we assessed their effect on myocardial performance in isolated hearts when given either before and after no flow ischaemia/reperfusion.

Method

This study was conducted in accordance with German governmental regulations complying with the European Community guidelines for the use of experimental animals.

Isolated heart preparation

Male Duncan Hartley guinea pigs weighing 300—750 g (Harlan-Winkelmann, Borken, Germany) were kept in accordance to animal welfare guidelines and supplied ad libitum with commercial guinea pig chow and tap water. Their hearts were excised during isoflurane anaesthesia 10 min after injection of sodium heparin (1000 IU i.p.). After thoracotomy, a cannula was inserted into the ascending aorta and cardiac perfusion was started in situ using a non-recirculating Langendorff technique and a freshly prepared, modified Krebs-Henseleit bicarbonate buffered solution (NaCl 120 mM; KCl 4.0 mM; $NaHCO_3$ 24 mM; $CaCl_2$ 2.0 mM; $MgSO_4$ 1.2 mM; KH_2PO_4 1.0 mM; glucose 10 mM), bubbled with 95% O_2 and 5% CO_2. This yielded a perfusate pH of 7.40, a P_{O_2} of 550 mmHg, and a P_{CO_2} of 38 mmHg. Aortic pressure was maintained at 60 mmHg. Hearts were suspended in a temperature-controlled chamber and temperature was maintained at $37 \pm 1\,^{\circ}C$.

For measurement of LV pressure a fluid-filled latex balloon (volume: 0.6 ml, Hugo Sachs, March/Hugstetten, Germany) attached to a pressure transducer by a stainless steel gauge needle was inserted into the LV via the left atrium. The balloon volume was set to maintain a LV diastolic pressure of 5—10 mmHg. The hearts were paced using right atrial electrodes with a frequency 10% higher than spontaneous heart rates or at least 240 min^{-1} throughout the experiments.

Measurements

Aortic pressure (electromanometry, PvB DPT-6003, Kircherson, Germany) and coronary flow (ultrasound transit time flow probe, Transonics, Ithaka, NY) were continuously measured. LV function was assessed by measuring LV developed pressure (LVdp), LV diastolic pressure, +LV dp/dt, and −LV dp/dt. Variables were continuously recorded on a thermoarray chart recorder (Astro-Med Dash 8U, West Warwick, RI) and stored on tape.

Stunning model

Since there is a wide range of duration of ischaemia (8—30 min) in global no flow experiments reported,[18,19] we established a stunning model before onset of the experiments assessing myocardial necrosis with 2,3,5-triphenyltetrazolium chloride (TTC) staining. After 5 min of stabilisation isolated guinea hearts ($n = 5$) were paced (300 min^{-1}) for 10 min. Afterwards they were exposed to either 20 or 60 min of global ischaemia by clamping aortic inflow and then reperfused for 60 min. For TTC staining, ventricles were cut into 6—7 mm thick transverse slices and immediately stained with 0.1 m TTC in KH_2PO_4-buffer (pH = 7.4, 38 °C) for 15 min. TTC staining showed red tissue (viable tissue[20]) in all hearts after 20 min of no flow ischaemia and reperfusion. In contrast, after 60 min of ischaemia and subsequent reperfusion slices of left ventricles showed multiple myocardial infarctions. Therefore, a 20-min duration of no flow ischaemia was used in subsequent experiments.

Experimental protocol

A 5-min equilibration period was used following isolation of the hearts. If a heart failed to develop a LV pressure of more than 60 mmHg at 10 min the experiment was terminated. If the LV pressure changed by more than 10% during minutes 10—15 myocardial function was considered unstable and these experiments were also terminated.

Following stabilisation baseline values were recorded. Hearts were then perfused continuously for 5 min with either vehicle, cariporide (1 μmol/l), levosimendan (0.1 μmol/l), or levosimendan (0.1 μmol/l) and cariporide (1 μmol/l) combined with eight guinea pigs in each group, and effects were assessed.

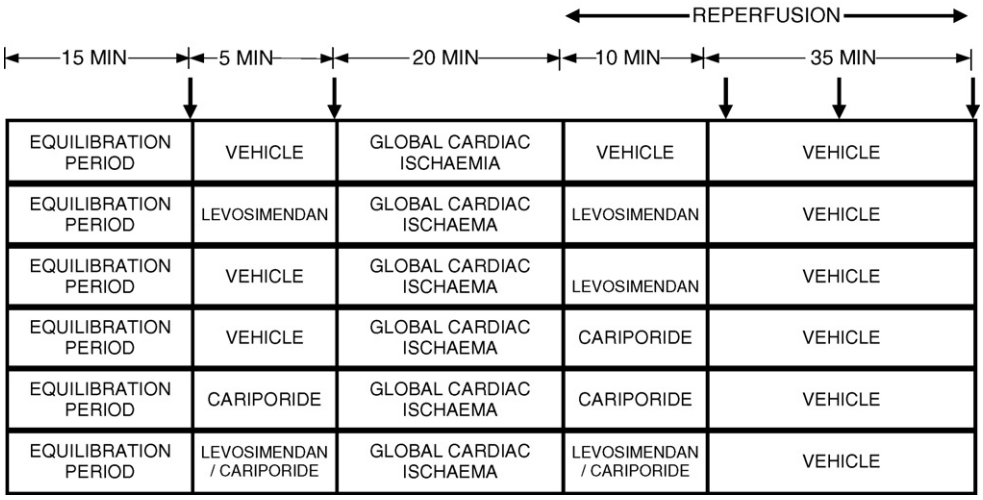

Figure 1 Schematic of experimental protocol. Following baseline measurements after an equilibration period subsequent measurements were made after pre-treatment, after global cardiac ischaemia, and 15, 30, and 45 min after reperfusion and post-treatment, respectively. Time of measurements is indicated by arrows.

Subsequently, all hearts were subjected to 20 min of global no flow ischaemia by clamping aortic inflow. Reperfusion was induced by allowing aortic inflow and treatments (vehicle, cariporide, levosimendan, or levosimendan and cariporide combined) were continued for 10 min after reperfusion. After 3 min of reperfusion cardiac pacing was restarted. Aortic perfusion pressure, LV pressure, and coronary flow were recorded 15, 30, and 45 min after reperfusion.

Effects of interventions were tested when made either before ischaemia (pre-treatment) or after ischaemia (post-treatment). The different treatment schemes are shown in Figure 1. Cariporide was kindly provided by Aventis Pharma GmbH (Frankfurt am Main, Germany), and levosimendan (Simdax®) was obtained from Orion Corporation (Espoo, Finland). Ingredients for perfusion buffers were purchased from Sigma—Aldrich (Taufkirchen, Germany). Levosimendan and cariporide were diluted in 0.9% saline and added to the perfusion buffer. The volume of the added test drugs was 0.1% of the modified Krebs-Henseleit solution.

Statistical analysis

Data are expressed as means ± standard deviation (S.D.). Statistical analysis was performed with SPPS 13.0 (SPSS, Chicago, IL).

Potential between group differences in values of variables at baseline were tested by one-way analysis of variance (ANOVA). Changes of values of variables from baseline over time were tested with the general linear model for repeated measurements and pairwise comparisons. The α-error was adjusted according to the Bonferroni method. Differences in mean values of changes from baseline between groups were determined with the general linear model (SPSS) using univariate analysis, analysis of covariance, and pairwise comparisons, with factors for treatment group and baseline value as covariate (ANCOVA). The following a priori null hypotheses were tested: there are no differences in variables (1) at baseline between groups, (2) in each treatment group between baseline and treatment before ischaemia, and at 15, 30, and 45 min of reperfusion, and (3) between the control group and treatment groups in LV variables over time.

Results

Baseline values were not different between groups (Table 1).

Effects on LV function of treatment before ischaemia

Animals treated with levosimendan before ischaemia showed an increase of $+dp/dt$ by 29% ($p < 0.05$). LVdp and $-dp/dt$ although changed from baseline by approximately 20—30% (Figures 2—4), but differences did not attain statistical significance (Figures 2—4). Of note, levosimendan resulted in an increase of LVdp by about 15% ($p < 0.05$) compared to vehicle. Treatment with levosimendan and

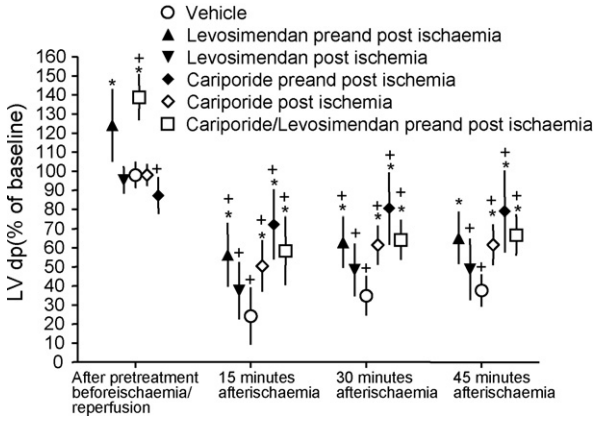

Figure 2 Effects of treatments on LV developed pressure before and after ischaemia/reperfusion. *$p < 0.05$ compared to vehicle; +$p < 0.05$ compared to baseline (within group effect).

Table 1 Cardiac variables at baseline for all treatment groups

Variable	Vehicle	Levosimendan pre- and post-ischaemia	Levosimendan post-ischaemia	Cariporide pre- and post-ischaemia	Cariporide post-ischaemia	Cariporide/levosimendan combined pre- and post-ischaemia	*p*
LVdp (mmHg)	78 ± 8	73 ± 15	73 ± 18	76 ± 11	75 ± 18	71 ± 7	0.914
+d*p*/d*t* (mmHg/s)	1603 ± 304	1550 ± 313	1540 ± 513	1679 ± 276	1473 ± 398	1406 ± 177	0.691
−d*p*/d*t* (mmHg/s)	−1068 ± 191	−1060 ± 371	−1015 ± 427	−1149 ± 385	−953 ± 299	−930 ± 130	0.766
LVEDP (mmHg)	7.4 ± 1.6	7.4 ± 2.3	7.4 ± 1.1	7.7 ± 1.7	6.9 ± 2.1	8.8 ± 1.6	0.446

Means ± S.D. *p*-Values refer to comparison to vehicle.

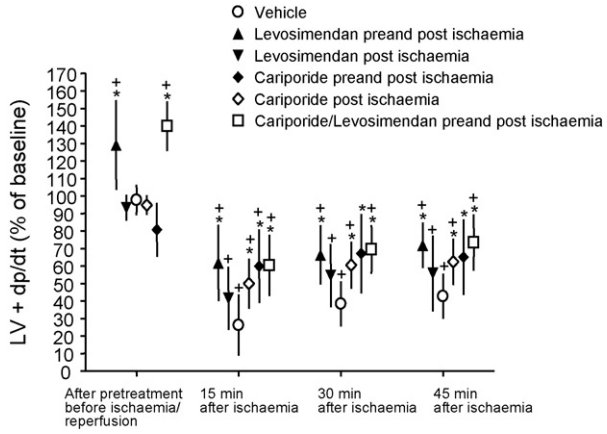

Figure 3 Effects of treatments on LV d*p*/d*t* before and after ischaemia/reperfusion. *$p < 0.05$ compared to vehicle; +$p < 0.05$ compared to baseline (within group effect).

cariporide combined improved LV function as indicated by increased LVdp (38%, $p < 0.001$), +d*p*/d*t* (39%, $p = 0.001$), and −d*p*/d*t* (21%, $p = 0.035$) after 5 min versus baseline values, and increased LVdp in comparison to vehicle treated hearts ($p < 0.05$). In contrast, hearts treated with cariporide alone showed a significant decrease in LVdp by about −11% ($p < 0.005$) compared to baseline. Hearts treated with vehicle showed no changes from baseline.

Effects on LV function following no flow ischaemia

In all groups ischaemia/reperfusion evoked a decrease of LVdp over the complete observation period when compared to baseline ($p < 0.05$) (Figure 2) ranging from a 70% decrease in vehicle treated hearts to 30% in hearts treated with cariporide before and after ischaemia. LV d*p*/d*t* was significantly diminished from baseline in all groups at 15, 30, and 45 min after reperfusion, except for the hearts treated with cariporide before and after ischaemia (Figure 3). In this group, +d*p*/d*t* recovered so much, that there was no significant difference to baseline anymore at 30 and 45 min of reperfusion. LV relaxation as measured by −d*p*/d*t* was significantly impaired after 20 min of no flow ischaemia in all groups at 15, 30, and 45 min of reperfusion ($p < 0.05$).

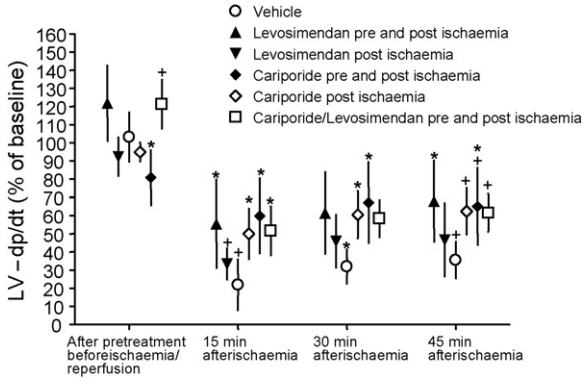

Figure 4 Effects of treatments on LV −d*p*/d*t* before and after ischaemia/reperfusion. *$p < 0.05$ compared to vehicle; +$p < 0.05$ compared to baseline (within group effect).

Effect on LV performance of pre- and post-treatments

Hearts treated with cariporide, levosimendan, or the combination before and after ischaemia, and hearts treated with cariporide after ischaemia showed a 25% greater recovery of LVdp and +dp/dt than vehicle treated hearts at 15, 30 and 45 min of reperfusion ($p < 0.05$, Figures 2 and 3). However, there where no differences in myocardial function between different treatments.

Levosimendan treatment started with reperfusion caused no significant improvement of myocardial function compared to vehicle.

LV relaxation measured by $-$dp/dt improved when compared to vehicle at 15 min of reperfusion in all treatment groups except for levosimendan treatment given at reperfusion ($p < 0.05$). After 45 min of reperfusion only hearts treated before and after ischaemia with levosimendan or cariporide showed a greater improvement of diastolic function (Figure 4) than vehicle treated hearts.

Discussion

Levosimendan, cariporide, and their combination improved LV function in comparison to vehicle, if treatment was started before ischaemia/reperfusion. This anti-stunning effect was seen as well when cariporide started directly after ischaemia, but not when levosimendan treatment was started after ischaemia. The combination of cariporide and levosimendan did not result in further improvement of myocardial function.

Limitation of methods

Crystalloid perfused guinea pig heart preparations have been used for studies of ischaemia/reperfusion. However, the use of such preparations neglects the influence of leukocytes/thrombocytes[21] as well as neurohumoral inputs for myocardial stunning.[22,23] However, its advantage is control of loading conditions. Furthermore, although other investigators have used rats for the examination of myocardial stunning we believe like others,[24] that the guinea pig heart is a model for ischaemia/reperfusion closer to humans. Nevertheless, our data may not predict the effects of interventions in humans.

In our investigation, the LVdp was used as the main variable of contractile function. Other investigators have used the measurement of absolute intracardiac pressure (the sum of developed pressure and end-diastolic pressure). However, in settings where a significant decrease in LV diastolic function or even a contracture like state can occur, changes in total pressure would not reflect closely alterations in systolic contractile state. Furthermore, the recovery of LVdp has often been used previously to assess ischaemic/reperfusion damage and has been shown to correlate with other markers of myocardial cellular damage.[25] Thus, LVdp is an appropriate variable for the investigation of myocardial function post-ischaemia in isolated guinea pigs hearts.

Effects of levosimendan

Levosimendan perfusate concentration was 0.1 μmol/l, similar to concentrations used by other investigators, reflects free levosimendan plasma concentrations in levosimendan treated patients,[26] and does not decrease ATP concentration or phosphorylation potential of ischaemic myocardium.[27,28] Treatment before ischaemia increased +LV dp/dt by 29% and developed LV pressure by 24%. This is in line with data from other experiments in guinea pigs.[27] In humans, levosimendan improves contractility of stunned[16] or infarcted myocardium[29] after percutaneous coronary interventions. However, in all these studies levosimendan was infused after reperfusion, and, therefore, ''cardioprotective'' effects of levosimendan were not assessed. Nevertheless, levosimendan is used in situations with myocardial stunning.[30,31] Thus, a clinically relevant objective not yet addressed is to compare the cardioprotective effects of levosimendan when given before ischaemia with the effects of levosimendan treatment started after ischaemia.

Our results suggest that short-term levosimendan treatment, when started before ischaemia and continued into the early reperfusion phase, results in a reduction of stunning which outlives the treatment period. In contrast, treatment with levosimendan for 10 min starting only after reperfusion did not improve myocardial stunning following global myocardial ischaemia. This contrasts to observations in patients after percutaneous coronary interventions where a significant increase of cardiac index[29] and global myocardial function[16] were noted after levosimendan infusion. In patients, levosimendan has an elimination half-life of $\sim$1 h, and its active metabolite OR-1896 has an elimination half life of 70–80 h.[32] Therefore, infusion of levosimendan in already stunned myocardium can yield both inotropic and cardioprotective effects. However, in isolated rabbit hearts the increase in LV systolic pressure following 5 min of levosimendan perfusion declines to initial values after a 5-min washout.[33] Accordingly, due to the short washout period of levosimendan in isolated hearts the inotropic effects of levosimendan vanishes after 5-min reperfusion period. Thus, our results reflect effects of levosimendan independent of its inotropic abilities. This suggests that treatment with levosimendan before ischaemia results in a cardioprotective effect decreasing subsequent myocardial stunning.

In animals, a cardioprotective effect of levosimendan mediated by activation of mitochondrial K_{ATP} channels has been described.[15] When levosimendan was started 15 min before evoking regional myocardial ischaemia and was continued for 60 min during coronary artery occlusion, infarct size was reduced by about 50%. Furthermore, in low flow ischaemia stunning model in isolated guinea pig hearts, levosimendan treatment during ischaemia improved LVdp by around 28%.[34] In isolated rabbit hearts two periods of 5 min perfusion with levosimendan before ischaemia also improved myocardial function and decreased myocardial infarct size.[33] Also, in a rat model of cardiopulmonary resuscitation after 6 min of cardiac arrest, levosimendan, when given after 2 min of ventricular fibrillation, decreased myocardial injury and improved myocardial contractility.[35] These effects were abolished by the K_{ATP}-channel blocker Glibenclamide. Thus, levosimendan's effects appear similar

to those of other K_{ATP}-channel openers, whose effectiveness depends of application timing (before or during ischaemia).[36,37]

In our experiments, treatment with levosimendan before ischaemia was necessary to diminish subsequent myocardial stunning. This is in line with data in patients undergoing coronary artery bypass grafting showing decreased post-operative troponin I concentrations and a higher cardiac index when levosimendan was infused before aortic crossclamping.[38] In any case, our findings show an ''anti-stunning'' effect of short-term levosimendan treatment in a no-flow ischaemia model, that, similar to other ''cardioprotective'' drugs, depends on timing of treatment. Thus, clinical investigations are required to test in patients, e.g., in those undergoing percutaneous coronary interventions to test whether the ''cardioprotective'' effect of levosimendan depends on the timing of treatment before ischaemia.

Effects of cariporide

Treatment with cariporide 1 μmol/l before ischaemia decreased LVdp, consistent with data in isolated rat hearts.[39] On the other hand, 10 μmol/l cariporide for 15 min did not evoke changes in isolated rat hearts,[40,41] and cariporide (1 mg/kg body weight) did not decrease myocardial function in pigs.[9] Possibly there may be species-specific effects and dose effects as well.

Treatment with cariporide before global ischaemia and at reperfusion resulted in decreased myocardial stunning, if treatment started with reperfusion. In models of myocardial ischaemia/reperfusion injury various effects of cariporide have been reported.

When started before coronary artery occlusion cariporide resulted in improved post-ischaemic segment shortening and stroke work in pigs.[9] On the other hand, cariporide given 15 min before ischaemia did not improve myocardial function following two episodes (10 min) of coronary occlusion in rats but reduced ischaemia-induced microvascular dysfunction.[11] In contrast, in exercise induced stunning in dogs, cariporide failed to improve myocardial function.[10] In models of myocardial infarction, different effects were observed, depending on the timing of treatment. Treatment before ischaemia showed more beneficial effects than treatment following ischaemia in rabbits.[39,42] In pigs with coronary artery ligation and low residual blood flow, cariporide was protective when given after 15 min of myocardial ischaemia but not after 45 min.[43]

In animals undergoing cardiac arrest, resuscitability is improved by cariporide because of a decreased rate of ventricular fibrillation, lessened post-resuscitation myocardial dysfunction, and enhanced efficiency of closed-chest resuscitation.[44,45]

Clinical trials with cariporide have shown equivocal results. In patients with unstable angina or non-ST-elevation myocardial infarction, or undergoing high-risk percutaneous or surgical revasculation randomised to receive placebo cariporide, only the highest dose (120 mg every 8 h) decreased death or myocardial infarction.[46] In high risk patients undergoing coronary artery bypass grafting and receiving cariporide there was a 4% lesser incidence of myocardial infarction in the cariporide group while the mortality was 1% greater than in the placebo group.[47]

We could not see a significant difference with regard to timing of giving cariporide. In any case, our results indicate that cariporide attenuates myocardial stunning in guinea pigs undergoing global no flow ischaemia, i.e., in a setting similar to that after cardiac surgery or cardiopulmonary resuscitation. Thus, clinical investigations are necessary to test whether cariporide is helpful in no flow ischaemia and cardiopulmonary arrest.

Effects of levosimendan and cariporide combined

The combination of levosimendan and cariporide given before ischaemia improved myocardial function in comparison both to baseline and to placebo, although it did not result in differences from treatment with levosimendan alone. Cariporide given before ischaemia alone decreased LV myocardial function. This cardio-depressive effect of cariporide likely was abolished by levosimendan resulting in a net improvement of cardiac function.

Of note, the combination of cariporide with diazoxide, a mitochondrial K_{ATP}-channel opener, reduces ischaemia reperfusion injury more effectively than cariporide alone.[48–50] However, there are no reports on interactions of cariporide and levosimendan. Although both levosimendan and cariporide alone mitigated stunning in our study we could not find additive effects of combined treatment. The reasons for this finding are unclear, in particular with regard to the additive effects of diazoxide and cariporide.

In conclusion, levosimendan and cariporide when given before no-flow ischaemia each decreased myocardial stunning. Cariporide treatment with reperfusion also diminished stunning, but not treatment with levosimendan. Accordingly, levosimendan may be a useful option in situations where ischaemia/reperfusion is anticipated.

Conflict of interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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