Impact of levosimendan and ischaemia–reperfusion injury on myocardial subsarcolemmal mitochondrial respiratory chain, mitochondrial membrane potential, $\text{Ca}^{2+}$ cycling and ATP synthesis

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Abstract

OBJECTIVES: Levosimendan (LS) is increasingly used in case of myocardial failure after cardiac surgery. The impact of LS on myocardial mitochondrial functions, such as respiratory chain function (RCF), mitochondrial membrane potential ($\Delta \Psi_m$), $\text{Ca}^{2+}$- Handling, mitochondrial permeability transition pore (mPTP) opening and ATP during ongoing ischaemia/reperfusion (IR) injury, is not well understood. Depending on LS, I/R injury or the combination of both, we analysed myocardial functions in a retrograde Langendorff-model followed by the analysis of subsarcolemmal mitochondrial (SSM) functions.

METHODS: Rat hearts were divided into four study groups; two were subjected to 30 min of perfusion without (control) or with the application of 1.4 µmol/20 min LS (Levo). Experiments were repeated with hearts being subjected to 40 min of normothermic stop-flow ischaemia and 30 min of reperfusion without (IR) or with LS application (Levo-IR). Systolic left ventricular pressure (LVPsys), left ventricular function, mitochondrial functions, such as respiratory chain function (RCF), mitochondrial membrane potential ($\Delta \Psi_m$), Ca$^{2+}$-retention capacity (CRC), Ca$^{2+}$-induced swelling and Ca$^{2+}$-fluxes after (re)perfusion.

RESULTS: I/R injury suppressed LVPsys (1381 ± 927 vs 2464 ± 913 mmHg/s, $P = 0.01$ at 30 min (re)-perfusion time). IR revealed complex I-V state3 (19.1 ± 7.4 vs 27.6 ± 11.0 mmolO$_2$/min; $P < 0.044$) and II-V state3 (20.6 ± 6.8 vs 37.3 ± 9.10 mmolO$_2$/min; $P < 0.0001$) suppression and Levo limited I-V (14.8 ± 11.1 vs 27.6 ± 11.0 mmolO$_2$/min; $P < 0.001$) and II-V (24.1 ± 6.4 vs 37.3 ± 9.10 mmolO$_2$/min; $P < 0.0001$) function. After energizing, $\Delta \Psi_m$ hypopolarization was observed in Levo (0.76 ± 0.04 vs 0.84 ± 0.04; $P = 0.02$), IR (0.75 ± 0.06 vs 0.84 ± 0.04; $P = 0.007$) and Levo-IR (0.75 ± 0.06 vs 0.06 ± 0.04; $P = 0.01$). IR (AUC: 626 vs 292; $P = 0.003$) and Levo-IR (AUC: 683 vs 292, $P = 0.003$) increased $\text{Ca}^{2+}$-induced mPTP opening susceptibility. CRC declined in IR (6.4 ± 21 vs 105.2 ± 6; $P = 0.04$) or Levo (6.5 ± 20 vs 105.2 ± 6; $P = 0.023$). $\text{Ca}^{2+}$ uptake was delayed in IR and Levo-IR without LS impact ($P < 0.0001$). $\text{Ca}^{2+}$ liberation was increased in Levo-IR. ATP synthesis was reduced in Levo (0.49 ± 0.14 vs 0.74 ± 0.14; $P = 0.002$) and Levo-I/R (0.34 ± 0.18 vs 0.74 ± 0.14; $P < 0.002$).

CONCLUSION: LS limited RCF at complex IV and V with $\Delta \Psi_m$ hypopolarization suggesting a specific mPTP$^{\text{Ca}^{2+}}$-dependent pathway. $\text{Ca}^{2+}$ redistribution from SSM by LS during I/R injury possibly prevents from $\text{Ca}^{2+}$ overload due to mPTP opening. LS-induced mPTP flickering did not promote permanent $\text{Ca}^{2+}$-induced mPTP opening. LS-dependent inhibition of ATP generation presumably resulted from complex IV and V limitations and lowered $\Delta \Psi_m$. However, a resulting impact of limited ATP synthesis on myocardial recovery remains arguable.

Keywords: Myocardium · Mitochondria · Ischaemia/reperfusion injury · Levosimendan · $\Delta \Psi_m$ · $\text{Ca}^{2+}$

INTRODUCTION

Myocardial ischaemia–reperfusion (I/R) injury complicates cardiac surgery and increases mortality, especially in candidates with an impaired ventricular function.

Mitochondrial dysfunction is the driving force for myocardial I/R injury, since mitochondria are responsible for ATP synthesis, cellular $\text{Ca}^{2+}$ homeostasis and cell death regulation. Oxidative phosphorylation breakdown during ischaemia results in ATP depletion. Subsequent collapse of the cellular Na$^+$/K$^+$-ATPase, followed by cellular $\text{Ca}^{2+}$ influx, alters intracellular signalling and enzyme phosphorylation. $\text{Ca}^{2+}$ uptake via the mitochondrial $\text{Ca}^{2+}$ uniporter is driven by the negative mitochondrial
membrane potential (\(\Delta\Psi_m\)), established by the respiratory chain. Mitochondrial Ca\(^{2+}\) efflux is provided either by the Na\(^+\)/Ca\(^{2+}\) exchanger or by the mitochondrial permeability transition pore (mPTP), with Ca\(^{2+}\)-binding sites regulating pore activity, resulting in mPTP flickering between open and closed states [1]. \(\Delta\Psi_m\) breakdown, cellular Ca\(^{2+}\) overload and rapid pH increment during reperfusion promote permanent mPTP activation leading to mitochondrial loss and cell death. Hence, stabilization of the RCF, \(\Delta\Psi_m\) and mitochondrial Ca\(^{2+}\) homeostasis is pivotal for preserving biochemical processes during myocardial I/R injury.

Levosimendan (LS), a clinically approved Ca\(^{2+}\) sensitizer, is increasingly applied in case of myocardial failure after cardiac surgery. By binding to the hydrophobic pocket at the amino-terminal region of Troponin C (Trop C) at systolic intracellular Ca\(^{2+}\) concentrations, LS increases Ca\(^{2+}\) affinity to Trop C. LS stabilizes the Ca\(^{2+}\)-induced conformational change of Trop C, resulting in positive inotropic action without changing relaxation time. Additionally, LS is a K\(^+\) ATP channel opener, explaining smooth muscle relaxation and vasodilatation [2]. LS-dependent pre- and afterload reduction reduces myocardial wall tension and improves coronary perfusion, thus making mitoK\(^+\) ATP channel opening a cardioprotective mechanism. Kopustinskiene et al. [3] examined the mitoK\(^+\) ATP channel opening effect of LS on native myocardial mitochondria, suggesting a protective effect of LS in a preconditioning regime of I/R injury. Additionally, Hönisch et al. [4] postulated a reduction of infarct size in a post-conditioning model, suggesting an involvement of mitoK\(^+\) ATP channel opening as well.

Its potential of improving myocardial function and suppressing I/R injury makes LS an appealing drug in case of ventricular dysfunction after cardiac surgery. Clinically, LS improved survival and end-organ functions of patients suffering from myocardial dysfunction after cardiac surgery. Early application after the onset of low cardiac output is advisable to improve outcome [5].

While LS-dependent myocardial preconditioning is well analysed, limited research on its impact on mitochondria during ongoing I/R injury has been conducted.

Regarding mitochondrial morphology, two major populations, subsarcolemmal mitochondria (SSM) and interfibrillar myocardial mitochondria, have to be considered. Differences in their morphology and cellular localizations lead to the diversity of biochemical properties and susceptibility to ischaemia. SSM appear to be more susceptible to ischaemic damage and a protective diazoxide-dependent effect is more pronounced in SSM [6]. Therefore, this study elucidates the impact of LS on healthy state SSM after the onset of I/R injury with a focus on respiratory chain function (RCF), \(\Delta\Psi_m\) stability, Ca\(^{2+}\) handling, mPTP susceptibility and ATP generation.

**MATERIALS AND METHODS**

**Animals**

Animals received humane care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised in 1996 as well as in compliance with the European Convention on Animal Care. Local authorities approved all animal use. Male Wistar rats (280–320 g) were obtained from Janvier (St Berthevin Cedex, France).

**Experimental set-up**

All hearts received 5 min of Langendorff pre-perfusion for equilibration before experimentation and were randomly divided into four study groups: single perfusion for 30 min (control), 40 min of normothermic stop-flow ischaemia followed by 30 min of reperfusion (IR) and repetitive set-ups of both with a constant injection of 1.4 \(\mu\)mol LS over a period of 20 min (0.07 \(\mu\)mol/min), starting after 5 min of (re-)perfusion (Levo; Levo-IR). After (re-)perfusion, SSM were isolated and analysed (Fig. 1).

**Chemicals and buffers**

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich GmbH (Munich, Germany).

![Flowchart of the experimental design. Non-ischaemic groups (Control and Levo) were matched to ischaemic groups (IR and Levo-IR). At the beginning of Langendorff implantation (LD Implant), hearts received pre-perfusion for 5 min to allow equilibration and baseline values of cardiac marker enzymes were quantified. Then, hearts underwent either 40 min of stop-flow ischaemia followed by 30 min of re-perfusion (IR and Levo-IR) or direct perfusion for 30 min (Control and Levo). If applied (Levo and Levo-IR), levosimendan was directly infused into the aortic root from (re-) perfusion time 5–25 min. After 30 min (re-) perfusion, the cardiac marker enzymes were determined from eluate and the tissues were harvested for mitochondria isolation.](https://academic.oup.com/ejcts/article-abstract/49/2/e54/2755322)
Animal preparation and Langendorff perfusion

Rats were anaesthetized by an isoflurane/oxygen composition (5%/95%). After decapitation, hearts were excised. The aorta was cannulated for retrograde perfusion with 10 ml/min iced Custodiol® (histidine, tryptophan, and ketoglutarate) solution for 2 min (Köhler Chemie, Germany) for transfer from the animal laboratory to the Langendorff apparatus (maximum transfer time due to facility restrictions: 3 min). Krebs-Henseleit bicarbonate (KHB) buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.25 mM CaCl2, 1.2 mM KH2PO4, 25 mM NaHCO3, and 11 mM glucose) gassed with carbogen was used for constant pressure Langendorff perfusion (aortic pressure = 73.6 mmHg). At the beginning of Langendorff perfusion, a balloon was placed in the left ventricle and left ventricular end-diastolic pressure (LVEDP) was adjusted to 18 mmHg. Before being subjected to group-dependent treatment, all hearts received 5 min of pre-perfusion for equilibration. IR and Levo-IR were subjected to 40 min of normothermic stop-flow ischaemia before 30 min of reperfusion. For stop-flow ischaemia, the intraventricular balloon remained deflated (LVEDP = 0 mmHg) to avoid inhomogeneous myocardial ischaemia patterns. Control and Levo received 30 min of perfusion without ischaemia after equilibration. During (re-)perfusion, Levo and Levo-IR received LS application between 5 and 25 min as described above (Fig. 1). During (re-)perfusion, left ventricular pressure (LVP) and its derivate (contractility, LVdp/dtmax) were determined by the intraventricular balloon. In this context, the balloon was re-inflated to the pre-ischaemic LVEDP of 18 mmHg. The LVEDP was maintained at stable values throughout the experiment by constant manual re-adjustments using a Hamilton syringe. After equilibration, eluate was collected at 0 and 30 min during (re-)perfusion and stored at −80°C. Afterwards, hearts were transferred into iced perfusion buffer for isolation of mitochondria. The cardiac apex was excised and stored at −80°C for later analysis.

Cardiac enzyme analysis

Troponin T, CK-MB isoenzyme, myoglobin and heart fatty acid-binding protein (hFABP) were quantified from eluate. Enzymes were determined according to the standard validated procedures of the local department for medical chemistry. Values were expressed as international units per millilitre (U/ml). hFABP was quantified from the eluate and cytosolic fraction of the SSM isolation according to the manufacturer's instructions (HK141 ELISA Kit, Hycult Biotech, Uden, Netherlands).

Isolation of myocardial mitochondria

SSM were isolated according to Palmer et al. [7] using a buffer containing 180 mM KCl, 10 mM EDTA, 0.5% albumin at pH 7.4. Briefly, tissues were dispersed by using the Potter technique followed by differential centrifugation. Washing and storage of the SSM pellet was performed in 225 mM mannitol, 75 mM sucrose and 20 mM Tris, pH 7.4. A bicinchoninic assay determined SSM protein concentrations (BCA Kit, Fisher Scientific, Germany).

Assay of mitochondrial oxygen consumption and analysis of respiratory chain complexes

According to a modified protocol by Zini et al. [8], a temperature-controlled Clarke-type oxygen electrode (Hansatech Instruments Ltd, Norfolk, UK) measured oxygen consumption of SSM in a buffer (300 mM mannitol, 10 mM KH2PO4, 10 mM KCl and 5 mM MgCl2, pH 7.2) at 25°C.

Analysis of mitochondrial membrane potential

Ten millimolar succinate energized 100 µg SSM suspended in a 200-µl buffer (20 mM HEPES, 250 mM sucrose, 10 mM MgCl2 and 12.5 mM KH2PO4, pH 7.2). Two micromolar rotenone inhibited e− backflow into complex I. SSM was labelled by 400 nM 5,5,6,6-tetrachloro-1,10,3,3-tetraethylbenzimidazolylcarbocyanine iodide (JC1, Enzo Life Sciences GmbH, Lorrach, Germany). JC1 green fluorescence (525 nm) reflected JC1 monomers. The formation of J-aggregates led to orange fluorescence (590 nm), detected by an Infinite® 200 Pro multimode reader. The ratio of J-aggregate to JC1+ mitochondria was determined in native mitochondria after JC1 staining and after energizing with succinate. ∆Ψm stability was assessed after carbonyl cyanide m-chlorophenyl hydrazine (CCCP)-induced (20 µM) SSM uncoupling. Decay of ∆Ψm resulted from maximum values before and minimum values after uncoupling with CCCP.

Determination of mitochondrial Ca2+ uptake

Pyruvate and malate (2.5 mM each) in a buffer (250 mM sucrose, 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) and 0.005 mM EGTA, pH 7.2) energized 200 µg SSM. Ca2+ uptake of SSM was determined by 0.5 µM Calcium Green-5N at 25°C, detecting free extra-mitochondrial Ca2+ ions. The injection of 50 µM Ca2+ initiated Ca2+ uptake by SSM. An Infinite® 200 Pro multimode reader acquired Ca2+ signals with excitation and emission set to 500 and 530 nm, respectively.

Determination of mitochondrial Ca2+ retention capacity

According to a modified protocol by Matas et al. [9], energized mitochondria (2.5 mM pyruvate/malate) in a buffer (250 mM sucrose, 10 mM MOPS and 0.005 mM EGTA, pH 7.2) were incubated with 0.5 µM Calcium Green-5N at 25°C. CaCl2 pulses (5 µmol) applied in 60 s intervals determined the Ca2+ amount necessary to trigger mPTP opening. A rapid fluorescence increment indicated SSM Ca2+ release by mPTP opening. In a parallel assay, additional application of 0.5 µM cyclosporine A (CSA) delayed mPTP opening.

Mitochondrial permeability transition pore susceptibility testing by Ca2+-induced swelling

Ca2+-induced swelling of energized SSM was determined according to a modified protocol by Simon et al. [10]. One hundred and fifty microgram of SSM was suspended in a 200-µl buffer (250 mM sucrose and 5 mM KH2PO4, pH 7.2). Complex I was blocked by 3 µM rotenone. The addition of 6 mM succinate energized SSM. Ca2+ (50 µM) induced swelling, determined by the absorption decline at 520 nm by an Ultrospect 3000 spectrophotometer (GE Healthcare, Munich, Germany). Ca2+-induced swelling in a parallel assay was inhibited by 50 mM pyruvate.

Analysis of mitochondrial ATP content and ATP production

For ATP content detection, 2.5 mM pyruvate and malate energized 100 µg SSM. The bioluminescence assay was performed.
according to the manufacturer’s instructions (Molecular Probes, Life Technologies, Darmstadt, Germany). ATP production was quantified in energized SSM (50 μg), incubated with increasing ADP (0.4, 0.5 and 0.6 μM) concentrations. The bioluminescence signal was detected by an Infinite® 200 Pro multimode reader (Tecan, Crailsheim, Germany).

**Statistical analysis**

Data acquisition was performed using Microsoft Excel. Data analysis was done with Graphpad Prism for Mac OS X, Version 6.0e. Results are expressed as means ± standard deviation (SD), unless otherwise stated. An ANOVA in combination with Bonferroni correction for multiple comparison tests was applied to test for intergroup differences. The analysis of continuous data was performed using a two-way ANOVA with Bonferroni correction for multiple comparison tests. A value of \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Myocardial function**

Figure 2 demonstrates myocardial functions. Compared with Control, IR induced loss of maximum systolic left ventricular pressure (LVP_{syst}, \( P = 0.04 \)) and left ventricular contractility (LVP_{d/dt}_{max}, \( P = 0.004 \)). Compared with Levo, loss of LVP_{syst} (\( P = 0.001 \)) and LVdp\_d/dt_{max} (\( P < 0.0001 \)) was observed in Levo-IR.

**Myocardial marker enzymes**

As determined from eluate, significant differences between I/R injury groups (IR and Levo-IR) and corresponding Control groups (Control and Levo) were evident at 30 min of reperfusion for TropT, CK/MB, myoglobin and hFABP. LS did not impact on the liberation of myocardial marker enzymes. Compared with Controls (1011 ± 924 pg/ml), baseline TropT values did not differ in IR (798 ± 625 pg/ml), Levo (1582 ± 1008 pg/ml) or Levo-IR (1023 ± 392 pg/ml). After 30 min of (re-)perfusion, differences between Control and IR (710 ± 589 vs 2541 ± 1503 pg/ml, \( P < 0.0001 \)) or Levo and Levo-IR (1427 ± 1122 vs 3719 ± 1765 pg/ml, \( P < 0.0001 \)) achieved significance. Similar results were obtained for CK/MB (\( P > 0.24 \)) with equal baseline values among Controls (25.4 ± 21.3 U/l), IR (19.0 ± 13.8), Levo (30.8 ± 15.1 U/l) and Levo-IR (29.7 ± 23.0 U/l). After 30 min of (re-)perfusion, significant differences between Control and IR (0.2 ± 0.3 vs 27 ± 27 U/l, \( P = 0.005 \)) or Levo and Levo-IR (6.0 ± 8.4 vs 33.9 ± 18.4 U/l, \( P < 0.0007 \)) were evident. Myoglobin and hFABP values were exclusively obtained after 30 min of reperfusion time. Regarding hFABP, significant differences were detected between Levo and Levo-IR (0.72 ± 0.88 vs 2.82 ± 1.21 μg/mg protein, \( P < 0.002 \)). Myoglobin differed between Controls and IR (12.2 ± 12.2 vs 224.5 ± 128.5 μg/ml, \( P < 0.0001 \)) and IR and Levo-IR (224.5 ± 128.5 vs 111.1 ± 105.9 μg/ml, \( P < 0.03 \)).

**Analysis of respiratory chain function**

Comparing complex I–V (C I–V) of IR with Control (Fig. 3) yielded a decelerated state3-respiration (\( P = 0.044 \)). LS applications (Levo and Levo-IR) limited state3-respirations compared with corresponding groups, namely control (\( P < 0.0001 \)) and IR (\( P = 0.06 \)). In this context, effective C I–V inhibition by the combination of I/R injury and LS in Levo-IR summarized when compared with control (\( P < 0.0001 \)). State3-respiration of complex II–V showed similar results. Compared with Control, declines of state3-respiration in IR (\( P < 0.0001 \)) or Levo were highly significant (\( P < 0.0001 \)). Complex IV function of Levo compared with Control also showed a limited respiration (\( P = 0.04 \)).
Mitochondrial membrane potential determination

$\Delta \Psi_m$ and its stability in the presence of the uncoupler CCCP were determined from JC1-labelled SSM (Fig. 4). Regarding global-curve analysis, significant $\Delta \Psi_m$ hypopolarization of SSM subjected to IR ($P = 0.04$) or Levo ($P = 0.02$) was evident when compared with Control. Before energizing (a), Control SSM demonstrated highest $\Delta \Psi_m$ generation. Compared with Control, differences regarding $\Delta \Psi_m$ were observed in Levo ($P = 0.02$), IR ($P = 0.05$) or Levo-IR ($P = 0.006$). During CCCP-induced $\Delta \Psi_m$ breakdown (d), Controls showed slow $\Delta \Psi_m$ decay, which was significantly accelerated in IR ($P = 0.08$) or Levo ($P = 0.045$). Levo-IR resulted in limited $\Delta \Psi_m$ decay after 20 min assay time, when compared with IR ($P = 0.04–0.03$). Values are expressed as means; indication of standard errors was waived for readability.

Mitochondrial Ca$^{2+}$ uptake and Ca$^{2+}$ liberation

Ca$^{2+}$ cycling was determined from SSM stained by Ca$^{2+}$ green and challenged by 50 µM Ca$^{2+}$ green and challenged by 50 µM Ca$^{2+}$ (Fig. 5). According to non-linear

Figure 3: Respiratory chain analysis of myocardial SSM. SSM were analysed regarding complex I–V (A), complex II–V (B) and complex IV (C) functions. Values are expressed as means ± SD. SSM: subsarcolemmal mitochondria.

Figure 4: Analysis of $\Delta \Psi_m$ generation of JC1-labelled SSM: (a) represents SSM $\Delta \Psi_m$ generation determined from basal J-aggregate+/JC1+ ratios before and maximum ratios after the addition of rotenone/succinate (b → c). (d) Represents maximum loss of $\Delta \Psi_m$, determined from maximum J-aggregate+/JC1+ ratios before and minimum ratios after the addition of CCCP. Compared with Control, baseline $\Delta \Psi_m$ differences were evident in Levo ($* P = 0.02$), IR ($** P = 0.05$) or Levo-IR ($*** P = 0.05$). After energizing with rotenone/succinate (b → c), differences to Controls occurred in IR ($** P = 0.01$), Levo ($* P = 0.02$) or Levo-IR ($*** P = 0.006$). During CCCP-induced $\Delta \Psi_m$ breakdown (d), Controls showed slow $\Delta \Psi_m$ decay, which was significantly accelerated in IR ($* P = 0.08$) or Levo ($P = 0.045$). Levo-IR resulted in limited $\Delta \Psi_m$ decay after 20 min assay time, when compared with IR ($P = 0.04–0.03$). Values are expressed as means; indication of standard errors was waived for readability. $\Delta \Psi_m$: mitochondrial membrane potential; SSM: subsarcolemmal mitochondria.

Figure 5: Ca$^{2+}$ green curve of SSM challenged by Ca$^{2+}$. (A) Represents an overview of mitochondrial Ca$^{2+}$ and subsequent liberation. (B) Analysis of Ca$^{2+}$ uptake by SSM and (C) Ca$^{2+}$ redistribution from SSM. Values are expressed as means. Indication of errors was waived. Control demonstrated a high Ca$^{2+}$ uptake K-value (time coefficient: 0.043 ± 0.016). IR demonstrated a decelerated uptake ($K = 0.038 ± 0.095$). Compared with IR, Levo-IR revealed no impact on uptake ($K = 0.027 ± 0.041$). Ca$^{2+}$ liberation from loaded SSM was slow in control (slope: 1.77 ± 0.17 RFU/s). Levo did not alter Ca$^{2+}$ release (1.62 ± 0.59 RFU/s). Ca$^{2+}$ release was accelerated in **IR (4.87 ± 0.56 RFU/s). ***Levo-IR displayed a boosted Ca$^{2+}$ liberation (slope: 7.38 ± 0.75 RFU/s). Slopes between groups differed significantly ($P < 0.0001$, each). SSM: subsarcolemmal mitochondria.
regression analyses, controls exhibited a high Ca²⁺ uptake with a K-value (time coefficient) of 0.043 ± 0.016. IR demonstrated a decelerated uptake of K = 0.030 ± 0.044. In Levo uptake remained unchanged with K = 0.038 ± 0.095. Compared with IR, Levo-IR revealed no additional impact on uptake with K = 0.027 ± 0.041.

Ca²⁺ liberation from loaded SSM was slow in control with a slope of 1.77 ± 0.17 RFU/s. Compared with Control, Levo did not present an altered Ca²⁺ release (1.62 ± 0.59 RFU/s). In IR, Ca²⁺ release was accelerated up to 4.85 ± 0.65 RFU/s. Levo-IR displayed a boosted Ca²⁺ liberation to 7.38 ± 0.75 RFU/s when compared with IR. Slopes between groups differed significantly (P < 0.0001).

Mitochondrial Ca²⁺ retention capacity

Mitochondrial Ca²⁺ retention capacity (CRC; Fig. 6) was determined in the absence or presence of CsA. In the absence of CsA, control SSM resisted 10.5 ± 2.6 Ca²⁺ pulses before mPTP opening. Compared with control, IR resulted in an impaired CRC of 6.4 ± 2.1 pulses (P = 0.04). Levo demonstrated a similar limitation to 6.5 ± 2.0 pulses (P = 0.023) and in Levo-IR, CRC was reduced down to 4.9 ± 1.7 (P = 0.0008).

After CsA-induced mPTP blockade, control SSM again showed the highest CRC of 18.5 ± 4.7 pulses. Compared with Control, IR resisted 10.1 ± 2.3 pulses (P < 0.0001), Levo 13.1 ± 4.5 pulses (P < 0.001), and Levo-IR 9.8 ± 3.9 (P < 0.0001).

Mitochondrial membrane transition pore susceptibility towards Ca²⁺

In the presence of 50 µM Ca²⁺, mitochondrial membrane transition pore (mPTP) opening caused mitochondrial swelling (Fig. 7). Compared with Controls, swelling acceleration was observed in IR (P = 0.023) and Levo-IR (P = 0.003).

The application of 0.1 M pyruvate delayed Ca²⁺-induced mPTP opening in all four study groups resulting in vanishing differences between the groups (P = 0.2).

When compared with experiments without pyruvate supplement resulted in delayed swelling in Control (P = 0.003) or Levo-IR (P = 0.002) and IR (P = 0.0007).

Mitochondrial ATP and ATP production capacity

ATP content of SSM (Fig. 8) was determined in the absence of ADP. Compared with Control, Levo-IR caused a significant reduction in ATP content (P = 0.04).

ATP production capacity was determined from SSM supplemented with increasing ADP concentrations. Levo resulted in a limited ATP production when compared with control (P = 0.0006). When compared with IR, ATP production was also reduced in Levo-IR (P = 0.006). In case of Levo, these results were accentuated in the presence of 0.5 µM (P = 0.04) and 0.6 µM ADP (P = 0.007). Compared with IR, Levo-IR revealed impaired ATP production at any ADP concentration (P = 0.02-0.0002).

Relative ATP production capacity was computed from absolute ATP production values divided by mean ATP contents determined at baseline in the absence of ADP. Compared with control, relative ATP production capacity was accelerated in IR (P = 0.003), but Levo-IR compared with IR presented an inhibited acceleration of relative ATP production capacity (P = 0.0005).

![Figure 6: SSM Ca²⁺ retention capacity of (A) native SSM and (B) after mPTP blockades by CSA. Values are expressed as means ± SD. SSM: subsarcolemmal mitochondria; mPTP: mitochondrial permeability transition pore; CSA: cyclosporine A.](https://academic.oup.com/ejcts/article-abstract/49/2/e54/2755322/)

![Figure 7: Ca²⁺-induced swelling of (A) native SSM and (B) after pyruvate-dependent acidification. Values are expressed as means and error indication was waived. Compared with Controls, swelling was accelerated in **IR (P = 0.023) and ***Levo-IR (P = 0.003). Application of 0.1 M pyruvate delayed Ca²⁺-induced mPTP opening in all groups, resulting in vanishing differences between groups (P = 0.2). When compared with experiments without pyruvate supplement resulted in delayed swelling in Control (P = 0.003) or Levo-IR (P = 0.002) and IR (P = 0.0007). SSM: subsarcolemmal mitochondria; mPTP: mitochondrial permeability transition pore; CSA: cyclosporine A.](https://academic.oup.com/ejcts/article-abstract/49/2/e54/2755322/)
DISCUSSION

LS improves the failing myocardium in case of low cardiac output syndrome after cardiac surgery. It is also believed to protect the myocardium from mitochondrial dysfunction when used for pharmacological preconditioning before inducing I/R injury. MitoK\textsuperscript{ATP} channel opening appears to be the underlying mechanism [3, 11].

In our model of ongoing I/R injury, we applied an increased LVEDP (18 mmHg) to mimic congestive myocardial insufficiency. In analogy to the results of Kolseth obtained from a porcine model of acute ischaemia, LS failed to improve ventricular function [12]. Instead, he described the impact on chronotropy and vasodilatation, resulting in an improved cardiac output at high-dose LS application (80 µg/kg). Failure to detect the impact of LS presumably results from limitations of our model: a short perfusion time and the disability to monitor vascular relaxation or improvement of ventricular filling are to be addressed. During I/R injury, mitochondrial energy supply determines organ recovery. SSM are highly susceptible to ischaemic damage. RCF plays a decisive role for maintaining energy supply with its regulation being linked to RCF plays a decisive role for maintaining energy supply with its regulation being linked to RC.

In the presence of LS, limited ATP production was evident. This explains a disparity between our results and the results of other workgroups [11].

Figure 8: Analysis for (A) absolute ATP content and production capacity of SSM and (B) relative production capacity with absolute values divided by content values set to 100%. Values are expressed as means ± standard error of the mean. Compared with Control, Levo-IR caused ATP content loss (\(P = 0.04\)). Levo resulted in limited ATP productions when compared with control (\(P = 0.0006\)). Differences in ATP production between IR and Levo-IR were also significant (\(P = 0.006\)). In case of Levo, these results were accentuated in the presence of 0.5 µM (\(P = 0.04\)) and 0.6 µM ADP (\(P = 0.007\)). Compared with IR, Levo-IR revealed impaired ATP production at any ADP concentration (\(P = 0.02–0.0002\)). Asterisks indicate statistical significances of the rows (\(*P < 0.05, **P < 0.01, ***P < 0.001\)). SSM: subsarcolemmal mitochondria.

The respiratory chain complexes I, III and IV generate the \(\Delta\Psi_m\) as a result of energy transfer through the electron transport chain. Hence, \(\Delta\Psi_m\) generation depends on an intact RCF [21]. However, the \(\Delta\Psi_m\) hypopolarization we observed is unlikely a consequence of RCF suppression, since group-dependent impact on state2 function remained insignificant. In case of I/R injury, presumably inner mitochondrial membrane damage results in \(H^+\) leakage promoting \(\Delta\Psi_m\) loss. LS-dependent \(\Delta\Psi_m\) hypopolarization on the other hand most likely results from mitoK\textsuperscript{ATP} opening, as advocated by Kopustinskiene et al. [11]. MitoK\textsuperscript{ATP} channel opens lower \(\Delta\Psi_m\) due to direct \(H^+/K^+\) exchange.

Besides ATP synthesis, \(\Delta\Psi_m\) is the driving force for mitochondrial Ca\textsuperscript{2+} uptake. Racay et al. [22] demonstrated mitochondrial Ca\textsuperscript{2+} uptake to be proportional to \(\Delta\Psi_m\).

Depending on I/R injury, we observed limited mitochondrial Ca\textsuperscript{2+} uptake following an impaired \(\Delta\Psi_m\). This was accompanied by an accelerated Ca\textsuperscript{2+} release, reinforced in Levo-IR. We consider detected alteration of Ca\textsuperscript{2+} handling a result from mitoK\textsuperscript{ATP} activation and transient Ca\textsuperscript{2+}-induced mPTP opening [23, 24]. In this context, we demonstrated a maximum CRC reduction in Levo-IR, suggesting an early temporary Ca\textsuperscript{2+}-induced mPTP opening. Huser and Blatter [25] described brief intermittent mPTP openings as a rescue phenomenon to alleviate Ca\textsuperscript{2+} overload in the presence of cellular stress. We observed CRC reduction in Levo or Levo-IR; in review of an unaffected Ca\textsuperscript{2+} uptake or Ca\textsuperscript{2+}-induced swelling by LS, our data also suggest an early temporary Ca\textsuperscript{2+}-induced mPTP opening.

In contrast to CRC, Ca\textsuperscript{2+}-induced mitochondrial swelling indicated permanent mPTP opening. mPTP opening was promoted by I/R injury but not by LS. Pyruvate supplement inhibited Ca\textsuperscript{2+}-induced mPTP opening of SSM presumably due to acidification and complex I activation, increasing \(\Delta\Psi_m\). In the presence of pyruvate, group-dependent differences of mPTP responses vanished, also suggesting no adverse effect of LS on mitochondrial Ca\textsuperscript{2+} handling. Therefore, our data indicate I/R injury being the relevant factor resulting in permanent mPTP opening and LS did not have relevant impact on mPTP.

ATP synthesis reflects the common final path of RCF and \(\Delta\Psi_m\). In the presence of LS, limited ATP production was evident. This
effect allegedly depends on the observed complex IV and V inhibition. Complex IV inhibition together with mitoK\textsuperscript{\text{ATP}} opening resulted in low \(\Delta \Psi\)m, consequently depressing the driving force for ATP synthesis at complex V. A similar effect has been reported earlier for the mitoK\textsuperscript{\text{ATP}} opener pinacidil, also resulting in delayed mitochondrial ATP synthesis from increased K\textsuperscript+ flux [24]. The impact of a restricted ATP synthesis on mitochondrial energy supply remains vague. An adverse LS-dependent energy balance during I/R injury is imaginable.

**LIMITATIONS**

Our study suffers from some limitations. We examined a variety of mitochondrial processes depending on LS, I/R injury or the combination of both. Although we were able to suggest underlying pathways, we did not prove our results in more specific systems of isolated mitochondria in the presence of specific inhibitors or channel openers. The present experimental set-up might also bias results. Future studies will have to focus on the effects of LS on ion currents through the inner mitochondrial membrane using specific inhibitors of e.g. H\textsuperscript+ and K\textsuperscript+ channels. Additionally, we performed material demanding analyses. The amount of SSM available from myocardium was less effective than calculated, resulting in varying replicates of the performed tests. Yet, the presented studies are quite robust and provide a reliable overview of LS impact on SSM under the circumstances of ongoing I/R injury.

**CONCLUSION**

I/R injury induced RCF dysfunction and \(\Delta \Psi\)m hypopolarization. LS limited RCF in a specific mitoK\textsuperscript{\text{ATP}}-dependent manner, possibly ameliorating I/R injury [18]. Succeeding hypopolarization depending on LS was evident. During I/R injury, LS increased Ca\textsuperscript{2+} redistribution from SSM due to mPTP flickering. Ca\textsuperscript{2+} extrusion might protect mitochondria from Ca\textsuperscript{2+} overload during I/R injury. In this context, LS did not promote permanent Ca\textsuperscript{2+}-induced mPTP opening.

On the other hand, impaired mitochondrial ATP generation in the presence of LS was observed, presumably due to complex IV and V limitations. The relevance of suppressed ATP synthesis on myocardial recovery is arguable [17].

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**REFERENCES**


APPENDIX. CONFERENCE DISCUSSION

Dr D. Chambers (London, UK): I have a question about your model. How do you measure function, with a balloon, intraventricular balloon?

Dr Sommer: We did.

Dr Chambers: And you do all your studies at the end of your 30 minutes of reperfusion?

Dr Sommer: In this case, after 30 minutes of reperfusion, quite a short reperfusion time because we don’t want to have interfering effects between Langendorff perfusion and the mitochondria, we want to focus on the early reperfusion phase.

Dr Chambers: So your levosimendan is there for the initial 20 minutes of those 30 minutes, is that correct?

Dr Sommer: We started reperfusion. After 5 minutes we start treatment with levosimendan and discontinue levosimendan 5 minutes before end. So we have a washout of levosimendan before we do a mitochondrial analysis.

Dr Chambers: So why did you start 5 minutes after the initiation of reperfusion?

Dr Sommer: We didn’t want to focus on a preconditioning or post-conditioning model, we wanted to analyse for therapeutic effect of this agent during existing injury.

Dr A. Diegeler (Bad Neustadt, Germany): Do you see any clinical impact? As you know for cardiac surgery we sometimes perform a precondition in patients with levosimendan before surgery.

Dr Sommer: Yes, that’s a hard question to answer. Various clinics try to do pre or post-conditioning by pharmacological means using PDE3 inhibitors or levosimendan, for instance. We wanted to have an insight of what happens.

Dr Diegeler: To be honest, we don’t actually know what happens, is there a benefit or not?

Dr Sommer: We don’t know.

Dr A. Franco-Cereceda (Stockholm, Sweden): I appreciate your model because it’s very interesting that you give the levosimendan after ischaemia has been there for half an hour and that actually resembles the clinical situation. Sometimes we have to do an acute coronary bypass surgery in patients developing myocardial infarctions. Do you see a role for levosimendan in that? Should we load the patients with levosimendan when they come to us, or is it detrimental and should be avoided? Because that’s a question we sometimes get in the operating room.

Dr Sommer: In the clinical focus, it’s an excellent therapeutic agent. If you take a look at our results, our results are not 100% conclusive. I cannot suggest levosimendan treatment and I cannot suggest avoiding it. The thing is we see downregulation of respiratory chain function. This might be beneficial because of reducing ROS production. On the other hand, we see limitation of ATP production that might be harmful. We don’t know. We see alterations of calcium cycling, this might be a rescue phenomenon of mitochondria getting rid of excessive calcium, and this might be helpful or not. At this moment we have just the information I demonstrated.

Dr Franco-Cereceda: You have only used 30 minutes of ischaemia. I mean, it’s global ischaemia, but it’s an experimental model which is fairly easy to extend the ischaemic time if you want to.

Dr Sommer: Of course.

Dr Franco-Cereceda: But you have only tried 30 minutes so far, so you don’t know if extended ischaemia will give you the same results.

Dr Sommer: Not at this moment. But if we change ischaemia time, we have different models. If you shorten the ischaemic times, you have more hyperactive ischaemic injury of mitochondria with up regulation of respiratory chain. If you prolong the model, you have hypoxic injury of mitochondria.

Dr Franco-Cereceda: I’m just trying to get the model to be more clinically relevant with a longer time of ischaemia, because half an hour is very short.

Dr Sommer: It is quite short.