Semmelweis University

Doctoral School of Clinical Medicine

Role of preoperative pharmacological pretreatments in reducing ischemia-reperfusion injury of the liver

Ph.D. Thesis Outline

Dr. Rita Stangl, MD

Supervisor:
Dr. Attila Szijártó MD, Ph.D

Official reviewers:
Dr. Gábor Jancsó MD, Ph.D
Dr. Balázs Hauser MD, Ph.D

Head of the Final Examination Committee:
Prof. Dr. György Wéber MD, D.Sc

Members of the Final Examination Committee:
Dr. Ákos Csomós MD, Ph.D
Dr. Csapó Zsolt MD, Ph.D

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INTRODUCTION

Main aspects of the hepatic ischemia-reperfusion injury

Playing a central part in systemic metabolism and homeostasis, the liver performs highly oxygen-dependant functions. This makes its cells rather susceptible to hypoxia and microcirculatory dysfunction of any origin. Many surgical interventions on the liver – resections of malignancies, traumatic emergency operations of the injured parenchyma and transplantations – require clamping of the portal vessels for the purpose of blood-loss minimalization, which causes additional ischemia-reperfusion (I-R) injury to the liver tissue. Cells subjected to ischemia switch to anaerob metabolism resulting in interstitial acidosis. After consumption of their high-energy phosphate supplies, loss of membrane ion gradients occurs along with a functional damage of intracellular enzyme-cascades. Revascularisation means an oxidative burst to the cells, which upsets the redox balance and leads to production of reactive oxygen species (ROS) causing lipid peroxidation and imbalance of transmembrane salt-water shifts. Cell injury leads to the rapid activation of the inhabitant tissue macrophages (Kupffer-cells) that play a key role in the local inflammatory response. The resulting cytokine and ROS production attracts and activates neutrophil granulocytes, which further deteriorate the hepatic microcirculation.

Protective strategies against ischemia-reperfusion injury

To counteract the described damaging cascade, has recently been a challenging topic of surgical research. Injury may be decreased either by taking direct measures against the known pathways of its pathomechanism, or via enhancement of the cellular ischemia-tolerance by inducing endogen defence mechanisms. By preconditioning, a targeted treatment applied prior to an ischemic insult, it is possible to activate these endogenous adaptive defence mechanisms of the cells. Murry et al. used short I-R episodes to trigger tis process, but with according to this concept, pharmacological interventions can also be applied to reach the same goal. A key event in I-R injury is the opening of mitochondrial permeability transition pores (MPTPs). Inhibition of these channels by different pharmacological substances (e.g. cyclosporine A), or by techniques that delay normalisation of postischemic acidosis (e.g. postconditioning) are the most promising achievements in this field. Also direct measures can be taken against the secondary effects of I-R injury and the postischemic inflammatory

response (antibodies against cytokines, inhibition of leukocyte-functions and reducing the oxidative stress reaction).

Glutamine

L-glutamine is a conditionally essential amino acid. Apart from its metabolic functions and the role it plays in protein synthesis, it is also involved in nucleotide- and DNA-synthesis, mitochondrial ATP-metabolism, cell differentiation and proliferation, and apoptosis. It also influences the expression of some important proteins involved in I-R injury. Among others, it inhibits the synthesis of the inducible nitrogen-monoxide synthase and induces the expression of the heat shock protein 70, both mechanisms being part of an antiinflammatory and indirect antioxidative effect. As the precursor molecule of the first-line cellular antioxidant glutathione, glutamine also plays a direct key role in the integrity of redox homeostasis. Sufficient intracellular glutamine concentration is therefore protective against external injuries. Recent studies claimed a positive effect for glutamine as part of parenteral nutrition in critically ill patients, sepsis, inflammatory bowel diseases, pancreatitis and myocardial ischemia. Experimental data suggests that it also promotes liver regeneration, and reduces the necroenzyme-release of the postischemic liver tissue. This was found along with the elevation of glutathione levels and a possible antiapoptotic effect.

Levosimendan

Levosimendan is a new inodilatator agent that has been proven effective in the treatment of acute and chronic heart failure – also after acute myocardial ischemia. It enhances myocardial contractility by binding to Troponin C in myocytes, stabilizing the Ca^{2+}-bound conformation of the Troponin C- Troponin I- complex, which is necessary to the actin-myosin interaction. Thus, in contrast to other positive inotropic substances, levosimendan does not increase the intracellular level of cyclic adenosine monophosphate (cAMP), therefore not leading to increased myocardial Ca^{2+}-concentration, energy-consumption and oxygen demand. In addition, levosimendan causes vasodilation through opening different potassium channels. As it opens both, sarcolemmal and mitochondrial K^{+}_{ATP}-channels, an antiischemic role was proposed and could be proven lately in cardiological settings. The opening of the mito-K^{+}_{ATP}-channels is one of the crucial steps in preconditioning via inhibition of MPTP-opening. Therefore levosimendan may also have direct antiischemic effects in other organs, like the liver.

RESEARCH OBJECTIVES

The Experimental Surgery and Training Centre of the 1st Department of Surgery at Semmelweis University has been engaged in studying I-R injuries of different organs in praxis-oriented experimental models to find means to increase ischemic tolerance in these settings. The aim of the present study was to further investigate the effect of two pharmacological substrates given as pretreatment in a rat model of portal ligation. Both investigated molecules are already in clinical use and may therefore bare the chance of adding to their fields of indication.

1st Experiment: 'long-term' glutamine pretreatment

In a former study we demonstrated a protective role for 'short-term' glutamine pretreatment (see below) in a similar experimental setting. Continuing the research on this topic, we administered glutamine 24 hours before the 60 minutes' liver ischemia.

The asked questions were as follows:

1/ Is the current form of glutamine pretreatment able to reduce the definitive I-R injury of the liver, as evaluated after 24 hours of reperfusion?

2/ Is it able to improve hepatic microcirculation during the first hour of reperfusion?

3/ How does it influence the redox homeostasis of the liver tissue?

2nd Experiment: 'short-term' and 'long-term' levosimendan pretreatment

Similar to the two windows of glutamine pretreatment, levosimendan was administered 1, or 24 hours prior to liver I-R. As this drug also potentially has a direct hepatoprotective effect, the study protocol was designed to mimic the two protection windows of ischemic preconditioning.

Following questions were posed in the 2nd experiment:

1/ Can levosimendan reduce the severity of the liver lesion, detected after 24 hours of reperfusion?

2/ Is it able to improve the hepatic microcirculation at an early stage of reperfusion?

3/ Does levosimendan pretreatment have any effect on the liver's redox-homeostasis?

4/ Are there any hints of a direct hepatoprotective effect of levosimendan in the current experimental setting?

5/ Is there any difference between the drug's effects in the two pretreatment windows?

MATERIALS & METHODS

Study design

Male Wistar rats, weighing 250g were used in the experiments. They were kept on standard chow and water ad libitum in specific, pathogen-free conditions at 22-24ºC. In the last 12 hours before the operation, only water was provided. Each experiment started at the same time of the day to avoid the influence of circadian rhythm.

Anaesthesia was induced by an intraperitoneal bolus injection and maintained by the continuous intravenous administration of ketamine and xylasine. Invasive blood pressure monitoring was applied in the right carotid artery. Intraoperative normothermia was maintained by a heating pad. Measurement of body temperature was also made by laser Doppler flowmeter, parallel to the microcirculatory measurements.

A standardized surgical model of our laboratory was applied to generate the I-R injury of the liver. Following median laparotomy and mobilization of the liver lobes, 60 minutes of ischemia was induced to the lobes III., IV. and V. by clamping of the biliovascular trunk using an atraumatic microvascular clip. Splanchnic perfusion was maintained by the shunting small hepatic lobes (I., II., VI. and VII.), which were removed immediately before reperfusion. Thus reperfusion affected only the postischemic tissue (65-70% of the total hepatic mass).

Hepatic microcirculation was measured by laser Doppler flowmetry (LDF) on the V. liver lobe. After registering the baseline flow, monitoring was continued throughout the ischaemic period and the first hour of reperfusion.

Pharmacological pretreatment:

1st Experiment: 'long-term' glutamine pretreatment

As this was the pursuance of an earlier study, only one pretreatment window was investigated in the current study. 24 hours prior to ischemia 500 mg/kg Dipeptiven® was administered intravenously in the treatment group. After hydrolization equimolar amount of alanine and glutamine are freed in the serum from this solution. This dose equals the suggested therapeutic dose according to the manufacturer's instructions. However, due to the recent changes in the guidelines, it differs from the previously applied dose of glutamine pretreatment.

In both, the sham operated and the I-R control groups, mixed amino acid solution (Aminoven®) was administered 24 hours prior to ischemia.

2nd Experiment: 'short-term' and 'long-term' levosimendan pretreatment

Levosimendan (Simdax®) pretreatment was applied intravenously 1 or 24 hours prior to liver I-R at a total dose of 54 µg/kg. This was calculated from the highest possible human dose mentioned at the introduction of the drug: 24µg/kg bolus injection and 0,4µg/kg/h continuous infusion. The application form resembled the pattern of ischemic preconditioning: 5 cycles of infusion (5 minutes each), interrupted for 10 minutes every time. The two pretreatment windows may also equal the first and the second window of protection described in connection to preconditioning.

Control and sham-operated animals received the vehicle (5% glucose) in the same volume and application pattern.

Experimental groups

1st Experiment: 'long-term' glutamine pretreatment

Except the induction of liver ischemia, ***Sham****-operated* animals (n=10) were subjected to the same surgical procedures as the other groups, also including resection of the liver lobes I., II., VI. and VII. The mixed amino acid solution was administered on the first day of the experiment. On the second day the operation without an ischemic episode followed and microcirculation was measured for altogether 120 minutes. 23 hours later tissue and blood samples were collected for further investigation.

In the *I-R control (****'I-R'****) group* (n=10) 60 minutes of liver ischemia was established 24 hours after the mixed amino acid infusion. Reperfusion was allowed for 24 hours, in the first hour of which the microcirculatory flow was monitored by LDF.

The *glutamine (****'Gln'****)* group (n=10) receiving L-alanyl-L-glutamine pretreatment differed from the I-R group only in the composition of the administered amino acid solution.

2nd Experiment: 'short-term' and 'long-term' levosimendan pretreatment

Two pretreatment windows were investigated for levosimendan in the same experiment. 55 animals were randomly separated into two main groups, or 'categories'. In the *'short-term' **(A)** category*, pretreatment was administered 1 hour before the operation, while animals of the *'long-term' **(B)** category* were pretreated 24 hours before the operation.

Simple sham-operated ***(1)*** animals (n=5), as an additional group, were applied to retrieve information about the surgical stress. They received 5% glucose infusion as pretreatment and were subjected to the whole operative procedure, except for the induction of liver ischemia, but including resection of the lobes I., II., VI. and VII. *Pretreated and sham-operated groups* ***(A/1, B/1)*** (n=5-5) were used to allow observations of the isolated haemodynamic effects of levosimendan according to the two pretreatment categories. The same surgical procedure was applied as in case of the simple sham-operated group, but the animals also received levosimendan according to the corresponding pretreatment category.

To investigate the effects of levosimendan pretreatment on the I-R injury of the liver, *I-R control* ***(A/2, B/2)*** and *levosimendan-pretreated I-R groups* ***(A/3, B/3)*** were compared in each pretreatment window, like described in the 1st experiment for glutamine above. (n=10/group) Animals of these groups underwent the entire surgical procedure, including the 60 minutes of partial liver ischemia and liver resection followed by 24 h of reperfusion. Pretreatment with or without levosimendan was preformed 1 hour or 24 hours prior to liver I-R, according to the 'short-term' or the 'long-term' pretreatment protocol.

Reperfusion and sampling

Liver microcirculation was monitored with LDF throughout the first hour of reperfusion, after which laparotomy was sutured and the ketamine/xylasine infusion was stopped. 23 hours later the animals were anaesthetized intraperitoneally again and samples (serum and liver tissue) were taken under standardized circumstances.

Investigation methods

Haemodynamic monitoring: Blood pressure and heart rate were monitored invasively through the right carotid artery with a blood pressure gauge.

Assessment of the hepatic microcirculation: Liver microcirculation was evaluated by laser Doppler flowmetry (Moor Instruments Ltd., London, UK). The measured flux is proportional to the total number of moving red blood cells and their mean speed in the investigated tissue volume (mm^3). Therefore, the LDF probe was carefully replaced in the same position on lobe V. after every manipulation requiring its removal. For the characterization of the individual flow graphs, a mathematical correction was made. A

relative scale was utilized at each animal, where 100% means the baseline flux registered before ischemia, and 0% means the "biological zero" flux registered throughout ischemia. (T_{flux}=*(flux-bz)/(baseline-bz) x 100*). To compare the flow graphs, the integral of the reperfusion segment of the graphs (RA: reperfusion area) and the maximal plateau, reached between the 50th and 60th minutes of reperfusion, (PM: plateau maximum) were used.

Histopathologic analysis: Samples from the excised III., IV. and V. lobes of the liver were fixed in 4% neutral-buffered formalin for 24 h, dehydrated and embedded in paraffin. 3-5 µm thick sections were stained with hematoxylin and eosin (H&E). The following alterations were evaluated by two experienced pathologists who were blind to the identities of the samples: (1) cellular swelling, (2) lipoid degeneration, (3) sinusoidal congestion, (4) tissue haemorrhage, (5) leukocyte infiltration, (6) necrosis and (7) signs of apoptosis. These pathological lesions were semiquantitatively scored as follows: 0: no alteration, +: <10% of affected cells, ++: <50% of affected cells, +++: >50% of affected cells. Thus, overall the maximum score was 21.

For detection of lipoid degeneration, snap-frozen, 3 µm thick slices were stained with Sudan IV dye.

Immunohistochemical analysis: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to further assess the extension of necrotic areas. Commercially available kits were performed following the manufacturer's protocol. The histological slides were counterstained with haematoxylin. First the size of demarcated TUNEL-positive areas was evaluated as an indicator of irreversible tissue damage. Then a total of 1000 cells were counted in 10 non-overlapping fields to determine the ratio of TUNEL-positive cells in the areas without confluent positive staining. These separate TUNEL-positive cells may serve as a marker of apoptosis.

Poly (ADP-ribose) polymerase (PARP) activation was measured by the immunohistochemical detection of the enzyme's product, poly (ADP-ribose) (PAR), as described previously. On the liver paraffin slides 0.6 % hydrogen peroxide was used to block endogenous peroxidase. Antigen retrieval was performed in 0.2 M citrate buffer (pH 3). Nonspecific binding was quenched in 1.5% horse serum, and slides were incubated with a mouse monoclonal anti-poly (ADP-ribose) antibody (1:1000) overnight at 4 °C. After extensive washing with PBS, the sections were

incubated with a biotinylated horse anti-mouse secondary antibody (1:200) and avidin-biotin-peroxidase complex. The peroxidase activity was detected using DAB (3,3'-diaminobenzidine-tetrahydrochloride) as a chromogen and the counterstaining was performed with haematoxylin. Immunoreactivity was evaluated in the necrotic, demarcated areas as well as in the surrounding areas; the ratio of the PAR-positive part is shown as a percentile value.

Measurement of Serum ALT and AST: Blood samples were centrifuged (3000 rpm for 2x10 minutes, at room temperature) and the supernatant was collected. Serum samples were snap-frozen in liquid nitrogen and stored at -80°C. Alanine aminotransferase (ALT, a specific marker of liver injury) and aspartate aminotransferase (AST, a nonspecific marker of liver injury) were quantified by standard spectrophotometry using an automated clinical chemistry analyser.

Measurement of antioxidant state: Total scavenger capacity in plasma and liver homogenate samples were measured in $H_2O_2/OH^{\bullet}$ luminol microperoxidase system using Lumat LB 9051 luminometer. The chemiluminescence light intensity - given in relative light units (RLU) - is proportional to the concentration of free radicals, and reduced in the presence of free radical scavengers. Results were expressed as percentage compared to the background (RLU%). In the liver homogenate samples, protein content was measured using Lowry's method.

The samples' reducing power (RP) – also an indicator of overall antioxidant capacity – was assessed by the means of the Oyaizu's method. The change in absorbance, caused by the transformation of Fe^{3+} into Fe^{2+} was detected at 700 nm and was compared with that of ascorbic acid (AAE). Free SH-groups were detected by the Sedlack method based on the Ellmann's reaction. These results show the protein-related reducing power in mmol/L. The H-donating ability reflects the non-protein-bound antioxidant state of the samples. It was measured in the presence of a 1,1-diphenyl-2-picryl-hydrasyl radical at 517 nm using Blois' method as modified by Blázovics et al. The results were expressed in inhibition-percentage (inhib%). All spectrophotometric measurements were carried out with Jasco V-550.

Liver tissue viability: 5 mm thick cross-sections were made from snap-frozen parts of lobe No. V. The slices were incubated for 30 min at 37°C in nitroblue tetrazolium (NBT, 18 mg/l) and NADH (150 mg/l) reagents (Sigma-Aldrich Inc, St. Louis, MO, USA) diluted in 0.05M TRIS buffer (pH 7.6). Unused tetrazolium reagent was

removed by ascending (30%, 60% and 90%) and then descending concentrations of acetone. The amount of coloured reaction products is in direct proportion to the number of functioning mitochondrial NADH-dehydrogenase enzyme complex, therefore it can be used to determine mitochondrial integrity and cell viability. This was assessed by quantitative evaluation of the reaction. Ten random fields were microphotographed and the amount of the coloured reaction product was determined using Leica Qwin Pro image analysis software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). This area featuring intact functioning mitochondria, was compared to the total size of the microscopic field that were all evaluated separately. For each sample, the ratio was calculated as a ten-field-average and was expressed as a percentage of NBT positivity of non-operated control animals.

HSP72 expression: HSP72 expression of the liver was measured in tissue homogenate samples using Western blot analysis. The bands were visualized by chemiluminescence technique. The detection and quantitative analysis of the results was obtained by using ImageJ software.

Statistical analysis: Values were expressed as means ± SD. T-test (Statisoft software) was used for statistical analysis. A $p<0.05$ confidence interval was considered statistically significant and a $p<0.01$ confidence interval was considered as strong statistical significance.

RESULTS

Previous experimental data: 'short-term' glutamine pretreatment

It was mentioned before, that the current study of 'long term' glutamine pretreatment is directly linked to our former experimental data on this topic. In the first investigation on the protective effects of preoperative glutamine administration against liver I-R, parenteral pretreatment was applied just before the operation. Changes in study design allow only limited comparison of the two investigations, but the results from the first pretreatment window are essential to give a complete picture of our findings on this topic. Therefore the summary of this first experiment shall follow.

Duration and induction of liver I-R was similar to the method described above. Glutamine pretreatment was administered in form of Dipentiven® solution in a dose of 400 mg/kg, being recommended by the manufacturer at that time. Animals of the

sham-operated and the I-R control group received the same fluid load in form of 0.9% saline solution during the 3 hours of pretreatment. 60 minutes of partial liver ischemia was followed by 6 hours of reperfusion, in the first hour of which hepatic microcirculation was monitored using LDF. Anaesthesia was maintained during the whole experiment that ended with the collection of blood and liver tissue samples.

Evaluating the complete 60 minutes of microcirculation measured by LDF, the glutamine flow graph demonstrated significant improvement, which was attributed to a slow increase in flow during the second half of the investigated period. According to the histopathological analysis, there was less periportal PMN (polymorphonuclear leukocyte) infiltration and destruction in the Gln-pretreated group. In the damaged areas, apoptotic bodies could be identified, but necrosis was not observed. Further evaluation of these findings was done via the TUNEL-assay that resulted in increased positive staining after I-R injury in the Gln-pretreated animals, when only non-necrotic areas were considered. Immunohistochemical analysis of active caspase-3, as a specific marker of apoptosis, showed an even more remarkable increase of positive staining in the Gln group. The reduced plasma TNF-α level is in concordance with this finding and refer to a lower rate of inflammatory response. Evaluating the changes of antioxidant state, an overall positive effect of the pretreatment could be concluded. Liver homogenate samples showed significantly lower RLU% values in the Gln group compared to the I-R control group, while the free SH-groups' concentration showed significant increase. A slight improvement of antioxidant parameters was observed in the serum samples, too. These are altogether encouraging findings for further investigations on this topic.

1st Experiment: 'long-term' glutamine pretreatment

Haemodynamics: In the sham-operated group's blood pressure and heart rate proved stable throughout the registered period. In both, the I-R control and the Gln group, haemodynamic parameters were similar to the sham-operated group at baseline and during ischemia. Significant drop in blood pressure was registered at the onset of reperfusion ($p_{I\text{-}R}$=0.035; p_{Gln}=0.039). After about 5-10 min, mean arterial pressure started to increase slowly and constantly, reaching the baseline blood pressure within the first hour of reperfusion. We found no significant difference between the two groups.

Microcirculatory changes: No significant baseline differences in microcirculation values (flux) were measured between the groups; however, there was a drop in the flux during the ischemic period in both groups subjected to ischemia-reperfusion, while the flow of the Sham group remained constant during the entire experiment. Comparing the reperfusion segment of the Gln and the I-R group, both the integral of the reperfusion curve (RA_{Gln}: 30.8%; RA_{I-R}: 19.3%) and the plateau maximum (PM_{Gln}: 40.9%; PM_{I-R}: 23.2%) of the flow graph showed improving tendency after glutamine pretreatment. Full recovery of hepatic microcirculation was not achieved during the measured time period: by the end of the first hour of reperfusion, the flow of the two I-R injured groups was still significantly lower than that of the Sham group.

Histopathological analysis: According to the applied classification, in the I-R group, 60 min of ischemia and 24 h of reperfusion induced significant degeneration, vacuolization (a phenomenon known as 'ballooning degeneration'), periportal neutrophil infiltration, and tissue haemorrhage. Histologic evidence for necrosis was observed, while in less damaged areas, signs of lipoid degeneration could be seen in the same centrolobular localization (added together: 12.4 points, moderate damage). Gln group demonstrated less periportal neutrophil infiltration and less tissue destruction. Tissue haemorrhage could not be observed (added up: 10.2 points; moderate damage, significant difference: $p=0.047$). According to the scores, reduction of the liver lesions occurred in the Gln group. In the H&E-stained samples apoptotic cell death was quite rare and not characteristic for either of the groups, while demarcation of necrotic areas could be seen in both groups subjected to I-R.

Lipoid degeneration is also an indicator of the severe hepatocyte lesion. In the Gln-pretreated group, it could be verified to a notably lower extent according to Sudan IV staining, than in the I-R group. The Sham group showed no signs of this alteration.

Immunohistochemical analysis: *TUNEL-positivity* appears as a result of DNA-damage in I-R injury, especially in the demarcated areas, where a mixed form of apoptotic and necrotic cell destruction is likely to occur. The size of the demarcations was expressed as a percentage of the whole liver section: Sham group: 0%, I-R group: 14.9%, Gln group: 4.2% ($p=0.023$). Isolated TUNEL-positive cells usually represent apoptotic DNA-damage, although the assay's specificity for apoptosis is quite low after I-R injury. The quantitative analysis of this point wise TUNEL-positive staining showed

no difference between the three groups (mean values: Sham group: 1.8‰, I-R group: 2.9‰, Gln group 2.55‰).

Strong positive staining for *Poly-ADP-ribose-polymerase (PARP) activity* is a marker of over-activated DNA-repair in the tissue, which is known to cause further tissue damage via ATP-depletion. This phenomenon is characteristic for excessive DNA-damage and therefore also likely to appear in the wake of I-R injury. We evaluated PAR-positivity of the demarcated areas and in isolated cells using the technique described earlier. The size of the demarcated areas shows good correlation with the TUNEL-staining: Sham group: 0%. I-R group: 16.9%, and Gln group: 4.8% (p=0.027). As for the Sham group, PAR-positivity in isolated cells was also very rare. There was no significant difference between the two I-R-injured groups in this aspect, but there was a lower rate in the Gln group (p=0.166). Overall tissue PARP-activity of the Gln group, expressed as a percentage of the whole liver section, decreased significantly in comparison to the I-R group (p=0.013).

Necroenzyme levels: Serum ALT levels of the Gln and the I-R group were significantly higher compared to the Sham group (p_{Gln}=0.0004, p_{I-R}=0.00009), but the elevation was significantly lower in the Gln-pretreated group, than in the I-R control group (p=0.042). Serum AST level appeared to be also significantly lower in the Gln group (p=0.044), while no significant difference could be detected, when comparing the Gln and the Sham group (p=0,112).

Antioxidant measurements: *Chemiluminescent intensity* was significantly decreased in both liver homogenate and plasma samples of the glutamine pretreated animals. After 24 h of reperfusion plasma samples showed significantly lower RLU values in the Gln group than in the I-R group (p= 0.0496). The difference between liver homogenate samples was even more outstanding and RLU vales of the Gln group showed strongly significant improvement compared to the I-R group (p=0,0003).

According to the detailed *spectrophotometric analysis* of the liver homogenate samples, glutamine pretreatment led to significant improvement of the tissue's redox homeostasis. Compared to the I-R group, a strongly significant increase in the *free SH-groups' concentration* appeared in the Gln group (p=0.0001). The *H-donating ability* in the samples of the Gln group was restored to the level of the Sham group (p=0.7675) and significantly increased in comparison to the I-R group (p=0.0205). *Tissue reducing power* showed no significant improvement according to the I-R group

(p=0.0878), but it was restored to the level of the Sham group (p=0.9082), while in the I-R group it decreased.

2nd Experiment: 'short-term' and 'long-term' levosimendan pretreatment

Haemodynamics: In the simple sham operated (1) group, mean blood pressure did not change throughout the whole experiment. Compared to the groups that received glucose 5% only, there was a significant reduction of the mean arterial blood pressure (p=0.044) and a significant elevation in the heart rate (p=0.049) of the levosimendan groups by the end of the pretreatment period. These changes also occurred in the sham-operated pretreatment groups and sustained in the A/1 group even during laparotomy. Haemodynamic parameters did not change significantly throughout the 60 minutes of ischemia in any of the experimental groups. After induction of reperfusion tachycardia and a significant reduction in the MAP (p_A=0.047, p_B=0.033) occurred in the I-R control animals (A/2, B/2), which slowly started to normalize during the first hour of reperfusion. The haemodynamic parameters of group B/2 were slightly worse than those of the A/2 group and none of them completely reached the baseline values during the monitored interval. In the levosimendan-pretreated groups dropping of the blood pressure was not significant upon reperfusion and was also restored to the baseline MAP level by the end of the first hour of reperfusion. The character of these changes was similar in the two pretreatment windows, and the difference among the groups was also non-significant. Even though, it is important to note, that MAP values registered at the beginning of ischemia were higher in group B/3 than in A/3 (p=0,11). This correlates with the difference between the sham-operated animals of the 'short-term' and 'long-term' pretreatment category.

Microcirculatory changes: Compared to the corresponding I-R group, both 'short-term' and 'long-term' levosimendan pretreatment lead to strongly significant improvement of the hepatic microcirculation (RA: p_A=0.0012, p_B=0.0010; PM: p_A=0.0019, p_B=0.0007).

The flow of the 'long-term' sham-operated animals decreased in comparison to the sham-operated group of the 'short-term' category, or the simple sham-operated group, where no relevant changes could be observed. Even if the reduction of the flow in the B/1 group was not significant, it still might resemble the greater surgical stress of the 'long term' pretreatment. This correlates with the fact that a similar difference

between the two I-R control groups could also be observed, the B/2 group featuring lower RA and PM level as the A/2 group. According to its effect on the microcirculation, application of levosimendan 24 hours prior to the operation seems to be even more efficient.

Histopathological analysis: In the 'short-term' sham-operated (A/1) group, pathological alterations were not detectable, except for occasional mild sinusoidal dilatation. In the I-R control group of this pretreatment category (A/2), histological evidence of large necrotic areas could be observed. This was accompanied by significant periportal lymphocyte infiltration and tissue haemorrhage (11.6 points). In the levosimendan-pretreated (A/3) group, necrotic areas appeared to be dramatically smaller and more of focal character. Tissue haemorrhage was not typical and the lymphocyte infiltration was less extensive (7.8 points).

In the 'long-term' sham-operated (B/1) group sinusoidal dilatation was observed along with even some perivascular oedema (4.8 points). This group showed an increase of tissue damage in comparison to the two other sham-operated groups. In the I-R control (B/2) group extensive, frequently panlobular necrosis was observed accompanied by significant lymphocyte infiltration and tissue haemorrhage (12.3 points). The levosimendan pretreated (B/3) group was characterized by focal necrosis, milder tissue haemorrhage and less severe inflammatory infiltration (7.9 points).

Semiquantitatively there was a moderate-to-severe injury in every group subjected to ischemia and reperfusion. In the I-R control groups, however, the degree of histological damage was higher. Significant difference in favour of the levosimendan-pretreatment could only be observed between the 'long-term' groups ($p=0.021$), while in case of the 'short-term' pretreatment protocol an improving tendency appeared ($p=0.089$).

Immunohistochemical analysis: After *TUNEL-staining* diffuse positive areas could be observed in every I-R-injured group, while in the Sham groups, *demarcated* areas were absent. After levosimendan pretreatment, significant reduction of the demarcated necrotic areas was observed compared to the corresponding I-R groups in both, the 'short-term' and the 'long-term' pretreatment category ($p_A=0.034$, $p_B=0.05$). *Isolated TUNEL-positive cells* appeared more frequently in the B/1 group than in the other sham-operated groups, while there was a significant rise in their number in every group subjected to I-R. This point wise TUNEL-positivity also occurred to be

different in the levosimendan-pretreated and the I-R control groups, appearing to increase in the former ones. Moreover this difference showed also statistical significance in the 'long-term' pretreatment category (p=0.04).

PARP-activity was only assessed in the groups with liver I-R since the occurrence of positivity in the sham-operated groups is not very likely (see 1st experiment). In both levosimendan-pretreated groups, a significant reduction of total PAR-positivity was observed compared to the corresponding I-R groups (p_A=0.02, p_B=0.04). After 'short-term' levosimendan pretreatment (A/3), the point wise PAR-positive staining decreased significantly (p_A=0.02; p_B=0.15), while in the 'long-term' pretreatment category significant reduction was seen in the size of the demarcated PAR-positive areas (p_A=0.06; p_B=0.04). The explanation for this could be the overall more severe tissue injury observed in the groups of the 'long-term' pretreatment protocol, which could also be reduced by levosimendan. While the proportion of the demarcated areas appeared to be similar with both TUNEL- and PAR- staining, the point wise positivity differed. This might be due to the higher sensitivity of the PAR-staining in detecting on going cellular injury, while TUNEL-positivity refers to irreversible cellular damage. Isolated positivity, however, announces lower extent of tissue injury and is therefore a marker of favourable outcome.

Necroenzyme levels: *Serum ALT levels* of the I-R control groups were significantly higher compared to the corresponding levosimendan-pretreated groups (p_A=0.02; p_B=0.005). *Serum AST levels* exceeded the normal value in all groups, showing a more pronounced increase in the 'long-term' pretreatment category, than in the corresponding 'short-term' groups. The increase in the B/2 group was strongly significant in comparison to the A/2 group (p_2=0.009), suggesting a higher degree of systemic damage in the 'long-term' pretreatment category. This was further supported by a similar significant difference seen between the A/3 and B/3 groups (p_3=0.02). However, serum AST level could be significantly reduced by levosimendan in the 'long-term' pretreatment category (p_B=0.04).

Antioxidant measurements: Total scavenger capacity detected by the *chemiluminescence* technique, showed lower RLU% values in the serum of the levosimendan groups of both pretreatment windows, when compared to the corresponding I-R control groups (p_A=0,06; p_B=0,07). According to the liver homogenate samples, the A/3 group showed significant improvement in comparison

to the A/2 group (p_A=0.03), while the B/3 group featured an improving tendency (p_B=0.06).

Both pretreatment windows of levosimendan led to a significant improvement of the *reducing power* in the serum compared to the I-R control groups (p_A=0.01, p_B=0.03). In case of the liver homogenate samples, the improvement was only significant after 'short-term' levosimendan pretreatment (p_A=0.01; p_B=0.06). Regarding the *free SH-groups'* concentration, significant increase could be observed in the liver homogenate samples of both levosimendan-pretreated groups, according to their I-R control groups (p_A=0.02, p_B=0.03), while in serum samples, only tendencial improvement was detected (p_A=0.06, p_B=0.07). The *H-donating ability*, indicative of the non-protein-bound antioxidant capacity, showed significant improvement only in serum samples after levosimendan application according to the 'short-term' pretreatment category (p_A=0.04, p_B=0.08), while 'long-term' serum and liver homogenate samples showed improving tendency (p_A=0.06, p_B=0.07).

The liver tissue viability: The simple sham-operated group's viability, calculated from the positive NBT-staining, did not differ from the results of healthy animals. In the sham-operated group of the 'long-term' pretreatment category (B/1) however, significantly lower viability was detected (p=0.024), while positive staining of the 'short-term' sham-operated (A/1) group showed only non-significant decrease (p=0.104). Furthermore, NBT-positive staining in the I-R control group of the 'long-term' category (B/2) appeared to be significantly lower (p_2=0.0001), than that detected in the same group of the 'short-term' pretreatment category (A/2). Levosimendan pretreatment applied 24 hours before surgery (B/3) resulted in significant increase in tissue viability compared to the corresponding I-R control group, while the treatment of the A/3 group had only tendentious effect (p_B=0.003, p_A=0.14).

HSP72 expression: In every group subjected to ischemia-reperfusion, a significant (2-3 fold) increase was observed in the HSP72 expression compared to the sham-operated groups. Neither the 'short-term' nor the 'long-term' levosimendan pretreatment resulted in a change of the HSP72 expression pattern, compared to the I-R control groups.

CONCLUSION

According to the results above, following answers can be given to our questions:

1st Experiment: 'long-term' glutamine pretreatment

1/ Based on findings from the histological samples, L-alanyl-L-glutamine pretreatment administered intravenously 24 hours prior to the operation, is able the reduce the degree of liver ischemia-reperfusion injury.

2/ The 'long-term' glutamine pretreatment has no significant effect on the hepatic microcirculation during the early reperfusion period.

3/ Liver tissue antioxidant state can be significantly improved via glutamine administration, which might be the reason for the observed reduction of tissue injury.

2nd Experiment: 'short-' and 'long-term' levosimendan pretreatment

1/ both levosimendan pretreatment protocols are able to reduce the degree of hepatic injury after 24 hours of reperfusion.

2/ Liver microcirculation of the first 60 minutes of reperfusion can be improved significantly by both pretreatment protocols.

3/ According to tissue antioxidant state of the liver, improving tendency can be observed in all pretreatment groups.

4/ Direct hepatoprotective effect of levosimendan is not likely to play an important role in the current experimental setting, the positive impact of the pretreatment is more likely of haemodynamic origin.

5/ Levosimendan pretreatment applied 24 hours prior to surgery is able to induce more pronounced protective effects against hepatic ischemia-reperfusion injury than pretreatment applied immediately before the procedure.

6/ As an observation of rather methodical significance, pretreatment applied 24 hours prior surgery may be source of altogether greater operative stress in rodents.

Our results suggest that both glutamine and levosimendan pretreatment might have a place in the clinical practice, when preparing patients for elective major liver surgeries. In case of glutamine, protection might be due to its positive effect on redox homeostasis, while levosimendan acts through the influence of macrohaemodynamics. Their difference in the way of action might even allow a combination of the two pretreatment protocols (e.g. 'short-term' glutamine and 'long-term' levosimendan pretreatment).

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