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ORIGINAL ARTICLE

# Possibility of conditioning predamaged grafts after cold storage: influences of oxygen and nutritive stimulation

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apoptosis, endoplasmic reticulum, endoplasmic stress, machine perfusion, oxygen, preservation, resuscitation, substrate, transplantation.

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## Summary

The potential of short-term oxygenated perfusion *after* cold storage (CS) to reverse deleterious priming of nonheart beating donors grafts should be investigated, addressing the respective role of oxygenation and nutrients or metabolic charge. Livers were retrieved 30 min after cardiac arrest of male Wistar rats and preserved with histidine tryptophan ketoglutarate (HTK)-solution for 18 h by CS. After 16 h, some livers were put on an oxygenated machine-preservation-circuit for the last 2 h and conditioned by cold perfusion with either HTK (conHTK), HTK supplemented with adenosine, phosphate and glucose (conHTK+) or Williams-E solution (conWE). Upon warm reperfusion, postconditioning with any of the solutions led to a significant (three- to fivefold) reduction of parenchymal damage (ALT, GLDH-release) compared with CS. Metabolic recovery (bile production) was also significantly enhanced compared with CS, with best results found after conHTK. The beneficial effect of postconditioning with HTK was associated with a significantly mitigated cleavage of caspase 12 and 3. We conclude from these data that conditioning of predamaged livers is possible even after CS by short-term oxygenated perfusion in the cold and, under these conditions, not depending on energetic support or nutritive stimulation.

## Introduction

The increasing shortage of donor organs from 'brain dead' donors with intact circulation is responsible for an increasing clinical interest in the suitability of organs from nonheart beating donors (NHBD), i.e. organs, retrieved after cardiac arrest of the donor [1,2]. Organ grafts from NHBD principally suffer from inevitable warm ischaemia, which produces an urgent need for optimized organ preservation subsequent to retrieval of the graft.

There is a growing body of evidence, indicating that aerobic preservation of marginal donor livers is superior to conventional cold storage [3]. The continuous supply of oxygen by either gaseous insufflation [4] or oxygenated machine perfusion [3,5] has been shown to allow for restoration of structural integrity and energetic homeostasis during ischaemic preservation of predamaged livers, and life sustaining graft function was obtained after transplantation of those grafts, where primary nonfunction was observed after anoxic cold storage [6,7].

The major drawback of continuous oxygenated machine preservation lies in its comparatively cumbersome applicability, the necessity of being timely available at the place of organ retrieval and the need of continuous surveillance thereafter. The elegant alternative for aerobic liver preservation using gaseous oxygen insufflation via the venous vascular system [8] is substantially less cumbersome but still meant to be present already upon harvest of the graft.

Under simple cold storage conditions, energetic homeostasis is seriously compromised at the end of preservation, but cellular adenosine triphosphate (ATP) depletion is particularly accentuated by preceding warm ischaemia in NHBD. Parenchymal susceptibility to ischaemia/repefusion injury is likely to increase as energy-dependent ion pumps fail to rapidly re-equilibrate ion gradients across cell

 

membrane and to restore adequate cell volume regulation upon reperfusion [9]. Energetic homeostasis is furthermore necessary to promote cellular repair mechanisms and counteract proteolytic tissue degradation [10,11].

It was thus the aim of the present study to evaluate the potential of a terminal aerobic resuscitation perfusion of initially cold stored liver grafts to improve cellular homeostasis and thereby enhance tissue resistance to subsequent warm reperfusion injury.

In this context, it was of particular interest to delineate the respective role of oxygenation, energetic substrates and nutrients or metabolic charge. Therefore, we have set up an experimental protocol comparing simple cold storage with or without end ischaemic short-term oxygenated machine perfusion just prior to warm reperfusion of NHBD-grafts. Different perfusion media were used for this purpose as to address the isolated provision of oxygen while the liver is still exposed to an intracellular-type preservation medium, the putative additional impact of energetic substrates and the potential of a fully nutritive perfusion with extracellular type medium.

## Materials and methods

All experiments were performed in accordance with the federal law regarding the protection of animals. The principles of laboratory animal care (NIH Publication No. 85-23, revised 1985) were followed.

Livers were harvested from fed male Wistar rats (250–300 g bw) under general anaesthesia with isoflurane. The abdomen was opened by midline incision, the liver skeletonized and freed from all ligamentous attachments. The common bile duct was cannulated with a 27 G polyethylene tubing, allowing for collection of total bile outflow during reperfusion.

Cardiac arrest was induced by phrenotomy; 30 min later the portal vein was cannulated and the livers were rinsed *in situ* with 60 ml of histidine tryptophan ketoglutarate (HTK), as an established preservation solution for liver transplantation [12], but being void of the substrates investigated in this study. The organs were excised and randomly assigned to one of the following groups:

**1** Control livers were subjected to ischaemic storage in 125 ml of HTK kept at 4 °C in a thermostatically controlled water bath (group 1).

**2** Other livers were initially stored in the same manner for 16 h, but during the last 120 min of the preservation period they were put on an oxygenated machine perfusion circuit and conditioned by cold perfusion with either HTK (group 2a), HTK supplemented with 5 mM adenosine, 20 mM phosphate and 1 g/l glucose (group 2b) or with the fully nutritive Williams-E solution (group 2c).

Viability of all livers was evaluated thereafter upon reperfusion *in vitro* according to previously described techniques [13] in a recirculating system for 45 min at 37 °C with oxygenated (95% $O_2$-5% $CO_2$; $pO_2 >$ 500 mmHg) Krebs-Henseleit buffer at a constant flow of 3 ml/g × min. To simulate the period of slow rewarming of the organ during surgical implantation *in vivo* [14], all livers were exposed to room temperature on a Petri dish for 30 min prior to reperfusion.

Portal venous pressure was measured during isolated reperfusion by means of a water column connected to the portal inflow line and precalibrated to the calculated flow of 3 ml/g/min using polyethylene (PE)-catheters of length and size identical to the one used for the perfusion of the livers.

### Tissue preparation and biochemical analysis

Induction of apoptosis was looked for in tissue homogenates by Western analysis for cleavage of caspase 3, which was monitored by immunological detection with a polyclonal antibody recognizing the cleaved fragment of rat caspase 3 (Calbiochem, San Diego, CA, USA). At the end of the experiment, liver tissue was snap frozen between precooled steel tongs and immersed in liquid nitrogen. Specimens were then homogenized in the cold (4 °C) with 30 volumes of lysis buffer by means of an Ultra-Turrax (Janke&Kunkel KG, Staufen, Germany). The whole cell extracts were centrifuged in the cold and supernatants used for Western analysis as described previously [15].

In brief, equal amounts of protein (20 μg) were suspended in Laemmli buffer and loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After gel electrophoresis, proteins were blotted onto nitrocellulose membrane (0.2 μm) and homogeneous transfer confirmed by staining with Ponceau S. Membranes were then blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-Buffered Saline Tween-20 (TBST) and subsequently incubated overnight with the primary antibody. Proteins were then visualized on X-ray film via chemiluminescence after exposure to horseradish peroxidase conjugated secondary antibody (Phototope®; New England Biolabs, Inc., Schwalbach/Taunus, Germany).

### Gene expression of GADD153 and GRP78

Most recently, it has been postulated that IR-induced cellular damage might cause alterations of the secretory pathway, particularly at the level of endoplasmic reticulum (ER) function [16–18].

Involvement of endoplasmic stress was thus looked at by analysing the postischaemic increases in the expression of the ER-resident chaperone BiP and the proapoptotic



 

transcription factor GADD153 (**G**rowth **A**rrest and **D**NA **D**amage inducible protein) [19] by reverse transcriptase PCR (RT-PCR) analysis:

Total RNA was isolated from snap frozen samples using RNeasy kit (Quiagen, Hilden, Germany). RNA isolation was followed by DNase treatment. Eaqual amounts of total RNA were quantified by a spectrophotometer at 260 nm and processed for complementary DNA by incubation with d(T)15 primer (Roche Diagnostics, Mannheim, Germany) and SuperScript II RT (Life Technologies, Grand Island, NY, USA). A Perkin Elmer Biosystems PRISM 7700 Sequence Detection System (TaqMan) was used for mRNA quantification. The PCR reaction mix was prepared by using SYBR Green PCR reaction mix (Appplied Biosystems, Foster City, CA, USA). The amount of specific mRNA in the tissue was expressed in arbitrary units after normalization for the respective individual quantities of transcripts of glyceraldehyde phosphate dehydrogenase (GAPDH), which was analysed as house-keeping gene. Sequences of the PCR primers, purchased from Metabion (Martinsried, Germany), were as follows:

Glyceraldehyde phosphate dehydrogenase sense 5′-CGC TCC TGG AAG ATG GTG AT-3′ and antisense 5′-CTG GCA CAG TCA AGG CTG AGA-3′.

GRP78: sense 5′-AGG AAG CTG GGA AGG AAG AG-3′ antisense 5′-CCA GCC AGA CCC AGT TAT GT-3′.

GADD153: sense 5′-AGC AGA GGT CAC AAG CAC CT-3′ antisense 5′-CTG CTC CTT CTC CTT CAT GC-3′.

Enzyme activities of alanine aminotransferase (ALT) and glutamate dehydrogenase (GLDH) in the effluate were assessed photometrically using commercialized standard kits (Fa. Boehringer, Mannheim, Germany).

### Statistics

All values were expressed as means ± SEM. After proving the assumption of normality and equal variance across groups, differences among groups were tested by analysis of variance (ANOVA) followed by the appropriate *post hoc* comparison test. Statistical significance was set at $P < 0.05$.

## Results

Enzyme releases of ALT and Glutamat Dehydrogenase (GLDH) during reperfusion were taken as general parameters of hepatocellular injury of the liver.

Perfusate activities of ALT were significantly higher after simple cold storage (group 1) compared with the results obtained in groups 2 a–c (cf. Fig. 1). Moreover, a progressive rise in enzyme leakage was noted in group 1, documenting an ongoing reperfusion injury to the hepatic parenchyma. This reperfusion injury, however, was largely

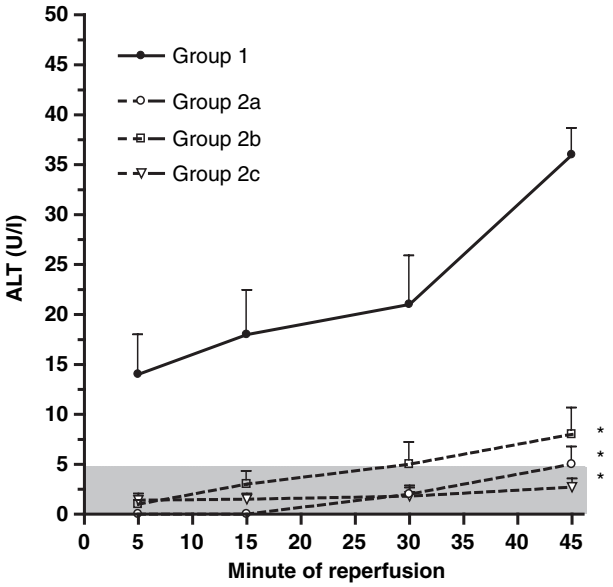


**Figure 1** Release of alanine aminotransferase (ALT) during normothermic reperfusion after 18 h of ischaemic preservation using different protocols. Values are given as mean ± SE; *$P < 0.05$ vs. group 1. For comparison, the normal range of ALT release, obtained from perfusion of nonischaemic livers with this model, is represented by the grey area.

abrogated by postconditioning the livers as carried out in group 2, and the obtained benefit was apparently independent from the medium used for this purpose.

A similar pattern was also found with respect to the intramitochondrial enzyme GLDH, the activities of which are shown in Fig. 2.

Functional recovery of the livers was in accord with the data on hepatic enzyme leakage. Hepatic bile production during reperfusion increased about more than 50% after postconditioning of the livers by oxygenated machine perfusion (Fig. 3) with respect to group 1 although statistical significance was only reached in group 2a.

The portal vascular perfusion pressure during normothermic reperfusion amounted to 5.5 ± 0.9 mmHg in group 1 and was found to be completely unaffected by the postconditioning protocol yielding values of 5.5 ± 0.6, 5.4 ± 0.8 and 5.5 ± 0.6 in groups 2a, 2b and 2c, respectively.

Dysregulation of intracellular homeostasis may result in adverse repercussions at the ER, which were investigated by determining the impact of organ preservation modalities on the expression of two mRNAs, known to be specifically induced upon ER stress: the ER resident stress response protein GRP78 and the pro-apoptotic transcription factor GADD153.

As depicted in Fig. 4, gene expression of GADD153 was found increased after preservation/reperfusion in group 1

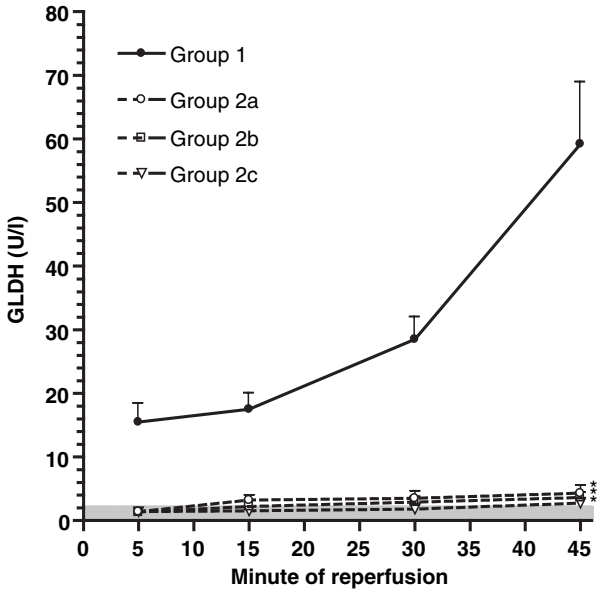


**Figure 2** Release of glutamate dehydrogenase (GLDH) during normothermic reperfusion after 18 h of ischaemic preservation using different protocols. Values are given as mean ± SE; *$P < 0.05$ vs. group 1. For comparison, the normal range of GLDH release, obtained from perfusion of nonischaemic livers with this model, is represented by the grey area.

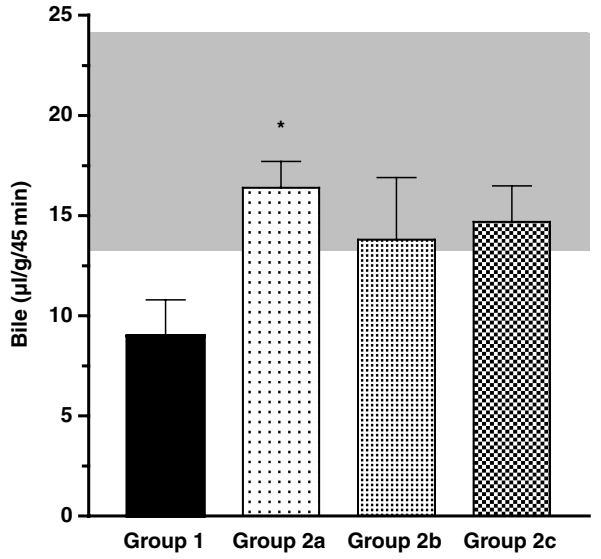


**Figure 3** Cumulative bile production during normothermic reperfusion after 18 h of ischaemic preservation using different protocols. Values are given as mean ± SE; *$P < 0.05$ vs. group 1. For comparison, the normal range of bile production, obtained from perfusion of nonischaemic livers with this model, is represented by the grey area.

to more than 10-fold the values obtained from nonischaemic control livers. This upregulation proved to be significantly prevented in group 2a but not in groups 2b and 2c.

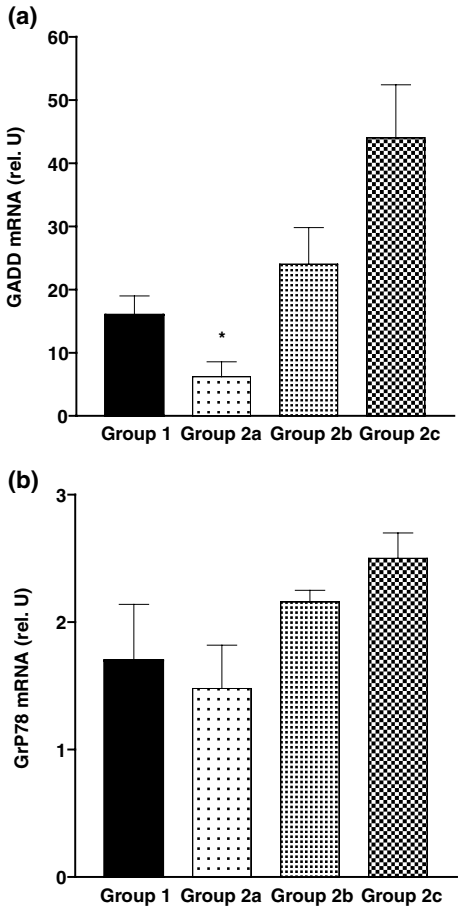


**Figure 4** Molecular gene upregulation related to endoplasmic stress response after ischaemic liver preservation: (a) induction of GADD153 mRNA in liver tissue upon normothermic reperfusion after 18 h of ischaemic preservation using different protocols. Values are given as mean ± SE; *$P < 0.05$ vs. group 1. (b) Induction of GRP 78 mRNA in liver tissue upon normothermic reperfusion after 18 h of ischaemic preservation using different protocols. Values are given as mean ± SE.

Regarding GRP78, these changes were far less pronounced in every group and differenced among the groups were not statistically different.

Further evidence of ER stress in the livers comes from the activation of the ER-specific caspase 12, recently found operative in initiating a novel pathway of cellular apoptosis [20].

Western blot analyses demonstrated significant cleavage/activation of caspase 12, evidenced by a 40% loss of the proenzyme in group 1 (Fig. 5). Postconditioning of the livers resulted in a significant alleviation of caspase 12 cleavage, with equal results in group 2 a, b or c respectively.

Induction of apoptosis was looked for by analysing cleavage of caspase 3 in tissue homogenates (Fig. 6). It was found that the appearance of cleaved caspase 3 was prominent in group 1 but significantly attenuated by



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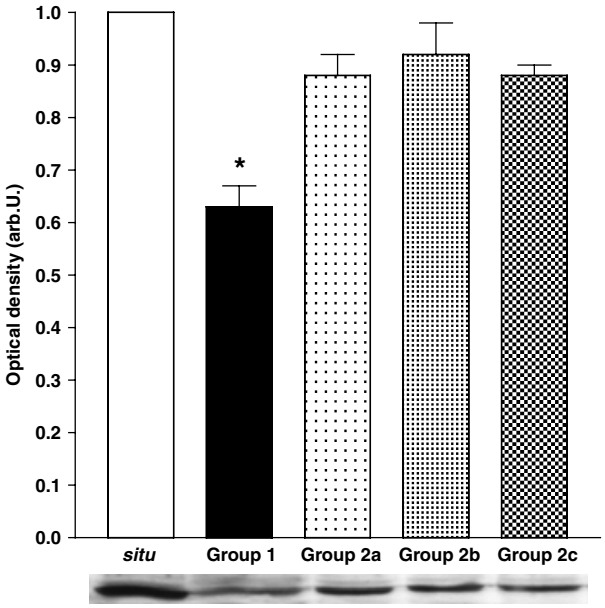


**Figure 5** Cleavage of caspase 12, shown by disappearance of the pro-zymogen of caspase 12 by immunoblotting. Numerical evaluation is based on densitometric analysis of 3 independent experiments. As a reference, procaspase 12 reactivity was also detected in nonischaemic livers, harvested *ex situ* and calculated as 1. Values are given as mean ± SE; $*P < 0.05$ vs. group 1.

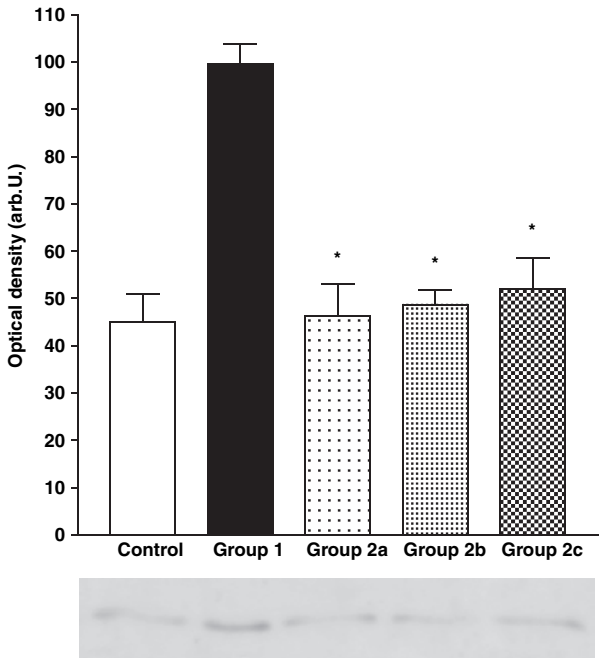


**Figure 6** Cleavage of caspase 3, shown by Western detection of the p18 cleavage product of caspase 3. Numerical evaluation is based on densitometric analysis of three independent experiments. Values are given as mean ± SE; $*P < 0.05$ vs. group 1. As a reference, the cleaved fragment of caspase three was also probed in nonischaemic controls.

postconditioning the livers prior to reperfusion in group 2. In analogy to the data on caspase 12, no differences in caspase 3 activation could be evidenced with respect to the perfusion medium (a, b or c) used in group 2.

## Discussion

This study demonstrates that conditioning of less than optimal donor livers is possible after ischaemic preservation of the graft by terminal short-term oxygenated machine perfusion with cristalloid solution.

Oxygenated machine perfusion has already been proposed as a tool to evaluate the viability of donor livers after prolonged times of ischaemic preservation [21] as well as a possible means to improve on tissue integrity immediately prior to transplantation [9]. It was consistently found that energetic status of the tissue could be significantly increased and improved viability of the graft was derived from that. Subsequent studies including postischaemic viability testing by isolated reperfusion *in vitro* actually confirmed that short-term oxygenated machine perfusion [22] or vascular oxygen persufflation [23] did improve hepatic bile production and lower parenchymal enzyme loss of rat livers after extended preservation times.

The originality of the present study lies in the reconditioning of livers, which were retrieved after cardiac standstill of the donor and hence subjected to an ischaemic challenge before organ preservation was initiated. Moreover, the respective impact of addition of precursors for adenine nucleotide synthesis or nutritional stimulation of the livers was addressed.

Although a direct role of adenine nucleotides in maintaining functional integrity of hepatocytes has been assumed [24], other results suggest endischaemic tissue levels of ATP to be of only relative importance for postischaemic liver viability [25–28]. Oxygenation of livers preserved in University of Wisconsin (UW) solution promotes an increase in tissue levels of ATP up to twice as high as after substitution of UW with HTK [29], probably because of its content of adenosine and phosphate, as possible precursors for adenine nucleotides, both of which are lacking in HTK [30]. Nonetheless, functional recovery after preservation using identical protocols did not show to be different, whether HTK or UW had been used [31]. Moreover, oxygenation of the liver in HTK or UW equally prevented the decline of the cellular cAMP (cyclic AMP; AMP is adenosine monophosphate) second messenger signal, and the functional benefit of aerobic preservation on postreperfusion viability could in large part be supplanted by pharmacological enhancement of cAMP [32].

Taken all together, it is tempting to conjecture that, under given experimental conditions, cellular ATP con-

centrations prior to reperfusion represent rather a readout of an over all satisfactory energy metabolism than a direct and proportional denominator of ulterior viability.

Accordingly, it was found in this study that neither addition of energetic substrates nor the use of a fully nutritional perfusion solution as medium for short-term hypothermic resuscitation perfusion did positively influence any of the parameters of graft viability upon reperfusion.

By contrast, the induction of gene expression for the proapoptotic GADD153 was significantly reduced by perfusion with unsupplemented HTK solution.

Apparently, there is a predominant need for oxygen rather than metabolic substrates in ischaemically damaged livers, which may take a specific benefit from being able to restore mitochondrial phosphorylation under conditions of metabolic hibernation. Mitchell *et al.* [33] have demonstrated that brief periods of temporary, substrate-free hypothermic oxygenation during long-term storage of rat livers significantly improved postischaemic recovery of metabolism, while addition of adenine precursors did not. It is thus conjectured that the positive results obtained in this study relate to simple restoration/improvement of mitochondrial redox state and not to a replenishment of adenine nucleotides. Besides, it was also found that perfusion *per se* did not bring up the described protection, as conditioning the grafts simply with nonoxygenated HTK (bypassing the oxygenator) failed to provide functional improvements upon reperfusion, e.g. bile production 9.5 ± 0.9 µl/g/45 min vs. 16.4 ± 1.3 µl/g/45 min, observed after oxygenated perfusion.

However, it has to be kept in mind that the liver might be considered a privileged organ with respect to glucose metabolism, readily utilizing genuine glycogen stores, which is not the case in other organs, e.g. the kidneys. Hence, we have to be careful, when extrapolating our results to other organs. On the other hand, the significantly positive effects reported after mere gaseous oxygenation by vascular persufflation, being operative without any substrate in livers as well as in kidneys, do strengthen the pivotal role of aeration in this context [34].

It might, however, be argued that metabolic support would only be operative, if reconditioning perfusion will be carried out at higher temperatures. We cannot fully rule out this possibility; but in our hands, an increase of the reconditioning temperature up to 22 °C did not bring up any improvement over the respective results obtained at 4 °C (data not shown).

A prominent feature of liver reperfusion injury in both experimental and clinical transplantation is programmed cell death or apoptosis of liver sinusoidal cells and hepatocytes [35]. In this regard, a pathway for caspase activation and apoptosis has been linked to stress in the ER [36]. The involvement of ER-stress in the pathophysiology of liver preservation has recently been proposed by several groups [17,18] and activation of the ER-bound caspase 12 [16] as well as the upregulation of genes belonging to the growth arrest and DNA damage family (GADD) [17] is described after hepatic storage. Activated caspase 12 may, in turn, activate downstream executor caspases like caspase 9 and 3 [18,20,37], eventually leading to cellular apoptosis.

Overexpression of GADD153 protein induces apoptosis in response to ER-stress through mechanisms not yet fully elucidated but involving interaction with Bcl-2/Bax [38] and perturbations of the cellular redox state [39].

In our study, GADD153 mRNA was markedly upregulated by ischaemic preservation and reperfusion, as has previously been shown by Emadali *et al.* [17] during human liver transplantation, and we observed a significant cleavage of procaspase 12.

Postconditioning of the livers with either medium was able to attenuate enzymatic cleavage and thus activation of caspase 12 while only the use of unsupplemented HTK was able to significantly reduce gene upregulation of GADD153. Notwithstanding that, early apoptotic changes as judged from cleavage of caspase 3 were significantly reduced in all of the groups receiving postconditioning by oxygenated machine perfusion and thus, cleavage of caspase 3 was in close correlation with the activation of caspase 12. It is conjectured that the limited period of reperfusion inherent to our model may not have been sufficient for the gene expression of GADD153 to induce obvious consequences at the protein level. By contrast, it becomes clear already from here that neither energy substrates nor further nutrients did improve on the resuscitative potential of a terminal short-term oxygenated perfusion of liver grafts from NHBDs.

Finally, the question arises, if we really need 2 h of oxygenation in order to obtain a resuscitative effect in the graft. The time has been chosen arbitrarily, striving for a most reliable pilot effect, while still respecting a clinically feasible time window from arrival of the graft until surgical implantation. Further studies will show, if and how the resuscitative effect depends on the time of oxygenated perfusion.

However, we conclude from the data presented that endoplasmic stress responses as well parenchymal graft alterations are elicited upon reperfusion of unconditioned NHBD-livers, which are both subject to significant alleviation by even end-ischaemic reconditioning through oxygenated machine perfusion with cold preservation solution.

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