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Citation for published version (APA):
Heijnen, B. H. M. (2003). Assessment and preservation of liver function in hepatic ischemia and reperfusion

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Chapter 4

Pronounced effect of minor changes in body temperature on ischemia and reperfusion injury in rat liver.

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Abstract

This study examined the effects of 1°C hypo- or hyperthermia on in vivo liver I/R injury in fifteen fasted male Wistar rats. Rats were ventilated and rectal temperature was maintained at 36°C, 37°C (normothermic) or 38°C. In all rats 70% liver ischemia was induced by clamping the afferent vessels to the median and left lateral lobes for 60 min and reperfusion was allowed for 90 min. Changes in plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alpha-glutathione S-transferase (α-GST) levels were measured, hemodynamics and bile secretion were monitored and arterial blood gas analysis was performed. All ventilated rats showed a normal pH, paCO₂ and paO₂. AST, ALT and α-GST levels were significantly higher in the 38°C group when compared to the 36°C and 37°C groups after ischemia. No differences in bile secretion were found between all groups. Histopathological alterations were in agreement with AST, ALT and α-GST levels in plasma. We conclude that a decrease of only 1°C in body temperature significantly attenuates liver I/R injury, whereas an increase of 1°C significantly increases liver I/R injury.

Key words: Hypothermia, hyperthermia, alpha glutathione S-transferase.
Effect of minor changes in body temperature

Introduction
Liver injury due to normothermic ischemia and reperfusion (I/R) processes has been the focus of a large number of animal studies. Most of these studies have been performed in controlled models of ischemia and subsequent reperfusion. Temporary cross clamping of the pedicle to the left lateral and median lobes is the most commonly used in vivo model of liver I/R. Although it is considered a reliable model, little attention has been paid to the impact of body temperature on the results produced with this model. The influence of temperature on I/R injury is already well established in literature (2), however the influence of only 1°C fluctuation in body temperature on liver I/R injury has not been investigated. Based on previous studies performed in our laboratory (unpublished results; Heijnen BHM, Straatsburg IH, van Gulik TM), we hypothesized that even small fluctuations in body temperature could have a major impact on the extent of liver I/R injury. This study was undertaken to examine in detail the influence of 1°C body temperature fluctuation on the extent of liver I/R injury in an in vivo rat model of 60 min of normothermic liver ischemia, by clamping the afferent vessels to the median and left lateral lobes (70%), and 90 min of reperfusion.

Materials and methods
Animal preparation
This study was approved by the Animal Experiment Committee of the Academic Medical Center, University of Amsterdam, The Netherlands. Male Wistar rats (325-375 g; Broekman, Someren, The Netherlands) were allowed to acclimatize to the laboratory environment for 7 days with free access to water and standard laboratory chow (Hope Farms, Woerden, The Netherlands). Rats were housed under standard environmental conditions with a 12-hour light/dark cycle. Before use in experiments, rats were fasted overnight with free access to water.

Temperature control and anesthesia
Fifteen rats were divided into four groups. Three groups of 5 rats were anesthetised via inhalation of a mixture of O₂ : N₂O (1 : 1 V/V, 2 l/min) and isoflurane 2-3% (Florene®, Abbott Laboratories Ltd., Queensborough, United Kingdom). After endotracheal intubation (14G Venflon®), rats were ventilated (Zoovent ventilator, Instruvet, Amerongen, The Netherlands) and anesthesia was maintained with the same mixture. Adequate ventilation was verified by continuous monitoring of end-tidal CO₂, assuring physiological pH during the entire procedure. After manual removal of
fetal content, a temperature probe (HP temperature module M 1029A, Agilent Technologies Netherlands B.V., Amstelveen, The Netherlands) was inserted up to 1.5 cm in the rectum after induction of anesthesia. Rectal temperature of either 36°C, 37°C or 38°C was maintained during the procedure and controlled by keeping the animals in supine position on a heating pad with the additional use of a heating lamp.

Surgical procedure
In all animals a silicone catheter (diameter 0.9 mm) was inserted into the left carotid artery for assessment of hemodynamic parameters during operation and for collection of blood samples. Arterial blood pressure was maintained at the initial level by adjustment of the isoflurane concentration in the mixture.

After midline laparotomy, the common bile duct was cannulated with a polyethylene catheter (Ø 0.4 mm) for collection of bile. Bile was continuously collected before ischemia (15 min), during ischemia (60 min) and during reperfusion (90 min). After dissection of the falciform ligament, the afferent vessels to the median and left lateral lobes were exposed by evert the hepatic lobes upward. An atraumatic vascular clip was applied to these vessels to induce partial hepatic ischemia (70%) during 60 min. Complete cessation of blood flow was verified with a laser-Doppler perfusion monitor (Periflux System 4000, Perimed Ltd, Suffolk, United Kingdom) equipped with a fiberoptic probe (Probe 408). At the end of the ischemic period, the clip was removed and subsequent reperfusion was initiated. After 90 min of reperfusion liver biopsies were collected, fixed in 4% buffered formaldehyde and routinely processed for haematoxylin and eosin staining of paraffin sections (4μm). Per animal, biopsies of two I/R liver lobes and one non-I/R control lobe were examined for vascular congestion of erythrocytes, parenchymal cell swelling, cytoplasmic microvacuolisation, cytoplasmic fading due to protein leakage and appearance of pyknotic and fragmented nuclei.

Blood sampling
Blood samples (500 μl) were collected from the carotid artery prior to induction of ischemia (T0), at the end of the ischemic period (T1) and after 90 min of reperfusion (T2) in microtainer tubes containing lithium heparin (Becton Dickinson and Company, Franklin Lakes, New Jersey). Samples were centrifuged (10 min, 3000 rpm, 4°C) and plasma was collected for the assessment of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and α-glutathione-S-transferase (α-GST) as parameters for hepatocellular injury. Activity of AST and ALT in plasma
was determined by routine laboratory testing. Protein levels of α-GST were analyzed by ELISA (HEPKIT™-Alpha, Biotrin, Dublin, Ireland) according to the instructions provided. After 90 min of reperfusion, routine arterial blood gas analysis was performed (ABL™ 505 and OSM 3 hemoximeter®, Radiometer, Copenhagen, Denmark).

**Statistical analysis**
Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis (Student t-test) was performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA). A p-value <0.05 was considered significant.

**Results**

**Hemodynamics**
In all groups 70% liver occlusion produced minor alterations in systemic hemodynamics (mean arterial blood pressure (MAP) and heart rate) immediately after occlusion. Hemodynamic parameters returned to pre-ischemic values within five to ten minutes and splanchnic congestion was not observed. All rats remained hemodynamically stable during the entire procedure.

**Blood gas analysis**
After 90 min of reperfusion, rats kept at 36°C, 37°C and 38°C showed a physiological blood pH (pH=7.35-7.45), normal blood values of paCO₂ (38±5, 34±2, 31±3 mmHg, resp.) and paO₂ (184±32, 189±12, 204±23 mmHg, resp.), indicating adequate ventilation.

**Hepatocellular injury**
Hepatocellular injury was assessed by measuring the activity of AST and ALT and protein levels of α-GST in plasma. AST and ALT levels at the end of ischemia (T1) and after 90 min of reperfusion (T2) were elevated in rats of all groups when compared to pre-ischemic (T0) values (figures 1 and 2; T1 vs T0, all data p<0.05; T2 vs T0, all data p<0.05). After 90 min of reperfusion at 38°C body temperature, plasma AST and ALT levels were elevated when compared to plasma levels obtained at 36°C and 37°C, indicating increased hepatocellular injury during I/R at 38°C. In addition, ALT levels in ventilated rats at 37°C were elevated when compared to rats at 36°C body temperature after 90 min of reperfusion, suggesting a direct relation between body temperature and extent of hepatocellular I/R injury (figures 1 and 2).
Figure 1 and 2
Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) measured pre-ischemia (T0), at the end of ischemia (T1) and after 90 min of reperfusion (T2) in rats with rectal temperatures of either 36°C (dashed bar), 37°C (grey bar) or 38°C (open bar). Bars represent means ± SEM.
* Significantly different from rats at 36°C (p<0.05).
# Significantly different from rats at 37°C (p<0.05).

α-GST levels after 90 min of reperfusion (T2) were elevated in rats of all groups when compared to pre-ischemic (T0) values (figure 3; T2 vs T0, all data p<0.05). In contrast to AST and ALT levels, plasma α-GST levels at the end of ischemia (T1) were only different from pre-ischemic levels in rats kept at 37°C (p<0.05). α-GST levels in rats at 38°C were elevated when compared to rats at 36°C but not when compared to rats at 37°C (p=0.08) after 90 min of reperfusion (figure 3).

Bile secretion was measured during the entire procedure as parameter of hepatocyte function. Overall, bile secretion was reduced during liver ischemia and during 90 min reperfusion when compared to pre-ischemic levels in all rats. Only rats kept at 36°C showed a recovery of bile
secretion after 90 min of reperfusion (figure 4). However, bile secretion was never different between rats kept at 36°C, 37°C or 38°C.

**Figure 4**
Bile secretion as percentage of pre-ischemic values. Bile secretion during ischemia (T1) and during 90 min of reperfusion (T2) in rats with rectal temperatures of either 36°C (dashed bar), 37°C (grey bar) or 38°C (open bar). Bars represent means ± SEM. All groups showed significant reduction in bile secretion at T1 and T2 when compared to T0 (significance not shown).
* Significantly different from rats at 36°C at T1 (p<0.05).

Histopathology
Light microscopic evaluation of liver I/R injury was performed on H&E stained sections of liver biopsies after 60 min of ischemia and 90 min reperfusion in all groups. After liver I/R at 36°C only mild vascular congestion in the major vessels and vacuolisation in the I/R lobes was observed, while the non-I/R control lobes showed almost no alterations. More pronounced vacuolisation and protein leakage was observed in I/R lobes of ventilated rats at 37°C. Severe histopathological alterations were observed in I/R liver lobe sections at 38°C (figure 5) as compared to the non-I/R control lobes.
Discussion

The results of this study show that minor changes in body temperature have substantial impact on the extent of liver I/R injury.

Temporary cross clamping of the pedicle to the left lateral and median lobes of the liver is the most commonly applied in vivo model of partial (70%) liver ischemia and reperfusion (I/R) in the rat. A major advantage of this model is the absence of severe splanchnic congestion. Splanchnic congestion, when prolonged for more than 20 min, will result in circulatory shock with intestinal ischemia, unless a concomitant decompressing portal venous-systemic shunt is created (5). Because venous return to the heart is not compromised in this model, only minor temporary changes in blood pressure are observed directly after selective vascular inflow occlusion. Despite its widespread use, little attention has been paid to standardisation and validation of this model.

A literature search on in vivo liver I/R studies in the rat (ca. 360 articles) learns that measurement and control of body temperature are not commonly practiced in experiments dealing with in situ models of liver I/R. The results of our study emphasize that only minor fluctuations in body temperature may have substantial impact on the extent of liver I/R injury. Our findings suggest that comparison between published results using a 70% partial liver I/R rat model should be made with caution.

The upper \( \text{paO}_2 \) limit in the ventilated rats was 204 mmHg, which is only marginally higher than normal \( \text{O}_2 \) pressures (ca. 160 mmHg). It has been reported that hyperoxia (100% oxygen, under normobaric conditions) can increase reactive oxygen species formation in different organs after at least 24 hours of exposition (1;13;16). Because in the ventilated rats exposure to high \( \text{O}_2 \) pressures lasted for a maximum of 3 hours, hyperoxia is not thought to contribute to the extent of liver I/R injury.

In this study plasma levels of AST, ALT and \( \alpha \)-GST were used as parameters of hepatocellular injury. Whereas AST and ALT are commonly used as parameters of hepatocellular injury, \( \alpha \)-GST is a relatively new plasma marker, which has not been frequently used. It has been reported that \( \alpha \)-GST is a sensitive parameter for assessment of hepatocellular injury (14), due to its distribution (80% of all \( \alpha \)-GST is present in hepatocytes) (4), high cytosolic concentration (5%) and shorter half-life (60 min). The present study confirms the sensitivity of \( \alpha \)-GST, however \( \alpha \)-GST did not discriminate in hepatocellular injury between rats kept at 38°C and 37°C after 90
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min of reperfusion whereas AST and ALT did, rendering α-GST less discriminative than conventional parameters.

The reported physiological temperature of rats varies between 37°C and 38°C, but male rats show a lower mean body temperature than female rats (37°C) (11). Therefore, a body temperature of 37°C is considered to be physiological in this study. In the present study, up to a fourfold difference in plasma levels of liver enzymes after liver I/R at 37°C and 38°C was observed, which was confirmed by the histopathological evaluation. These findings are in agreement with a detrimental cerebral outcome after complete cerebral ischemia in canines with an elevation of body temperature of only 1°C or 2°C (17). In order to elucidate the mechanism underlying the substantial influence of body temperature on the outcome of liver I/R injury further study is required.

The use of isoflurane is not likely to be of importance in this type of experiments. Some volatile anesthetics (halothane and enflurane) are known to impair hepatocellular integrity while others (isoflurane) preserve hepatic blood flow and oxygen delivery and thereby facilitate recovery from ischemia (6-10;12;15). In this study, no relation was found between the extent of liver I/R injury and the concentration of isoflurane used in the anesthetic mixture during the experiment (data not shown).

As parameter of hepatocyte function, bile secretion was measured during the entire procedure. Bile secretion is considered to be a good indicator of hepatocyte function (3). During I/R in this study however, bile secretion between rats with body temperatures of 36°C, 37°C or 38°C was not significantly different, although large differences in hepatocellular I/R injury were observed. Only rats kept at 36°C showed some recovery in bile secretion within 90 min of reperfusion, but bile secretion was still significantly lower when compared to pre-ischemic values. Although prolonged observation may reveal larger differences between the groups, previous studies in our laboratory showed incomplete recovery of bile secretion also after 24 hours of reperfusion (data not shown). Analysis of bile composition might show differences between groups but was not investigated in this study.

The results of this study suggest a significant role of body temperature in in situ models of liver ischemia and reperfusion and therefore body temperature should be controlled during the entire procedure. A drop of as little as one degree Celsius in body temperature gave rise to a 75% decrease in liver injury as reflected by the release of liver transaminases and α-GST in the blood. We suggest that standardisation of methods used in I/R studies is beneficial for comparison and
validation of animal models. This should include, as is our conclusion from the data presented here, conscientious maintenance of animal body temperature.

**Acknowledgements**

We are grateful for the expert biotechnical assistance of Ms. G.M. Huijzer and Dr. S.L. Abrahamse for valuable editorial comments.
References


