Effect of Nutritional Status on Oxidative Stress in an Ex Vivo Perfused Rat Liver

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Background: Normothermic ischemia–reperfusion is a determinant in liver injury occurring during surgical procedures, ischemic state, and multiple organ failure. The preexisting nutritional status of the liver might contribute to the extent of tissue injury and primary nonfunction. The aim of this study was to determine the role of starvation on hepatic ischemia–reperfusion injury in normal rat livers.

Methods: Rats were randomly divided into two groups: one had free access to food, the other was fasted for 16 h. The portal vein was cannulated, and the liver was removed and perfused in a closed ex vivo system. Two modes of perfusion were applied in each series of rats, fed and fasting. In the ischemia–reperfusion mode, the experiment consisted of perfusion for 15 min, warm ischemia for 60 min, and reperfusion during 60 min. In the nonischemia mode, perfusion was maintained during the 135-min study period. Five rats were included in each experimental condition, yielding a total of 20 rats. Liver enzymes, potassium, glucose, lactate, free radicals, i.e., dienes and trienes, and cytochrome c were analyzed in perfusate samples. The proportion of glycogen in hepatocytes was determined in tissue biopsies.

Results: Transaminases, lactate dehydrogenase, potassium, and free radical concentrations were systematically higher in fasting rats in both conditions, with and without ischemia. Cytochrome c was higher after reperfusion in the fasting rats. Glucose and lactate concentrations were greater in the fed group. The glycogen content decreased in both groups during the experiment but was markedly lower in the fasting rats.

Conclusions: In fed rats, liver injury was moderate, whereas hepatocytes integrity was notably impaired both after continuous perfusion and warm ischemia in fasting animals. Reduced glycogen store in hepatocytes may explain reduced tolerance.

NORMOTHERMIC ischemia–reperfusion injury is an important determinant in the pathogenesis of the liver damage occurring during surgical procedures, such as hepatic resection and liver transplantation, and during clinical conditions such as ischemic hepatitis and multiple organ failure syndrome. It is well known that the presence of fatty degeneration reduces the tolerance of the liver to ischemia–reperfusion injury.1–9 A potentially important and manageable factor is the preexisting nutritional status of the liver. In clinical transplantation, starvation of the donor, due to prolonged intensive care unit hospitalization or lack of an adequate nutritional support, increases the incidence of hepatocellular injury and primary nonfunction.10 For more than two decades, experimental studies have shown that livers from fed animals are more resistant to the normothermic ischemic injury than livers from fasting animals. In animal models, in vitro and in vivo studies have shown that fasting exacerbates normothermic ischemic injury.9,11–15

A major factor that could relate the reduced tolerance of livers to warm ischemia–reperfusion injury to the nutritional status is the amount of glycogen stores available in the liver.9 In the absence of oxygen, glycogen is essential to maintain the cellular integrity by supplying glucose for adenosine triphosphate (ATP) generation.

The aim of this study was to determine the role of the nutritional status on the consequences of hepatic ischemia–reperfusion injury in normal rat livers. Specifically, to isolate the contribution of the ex vivo perfusion, the degree of liver injury was measured in two control groups, fed and fasting, in which no ischemia was induced. We evaluated the effect of fasting on glycogen content and on hepatic integrity using different clinical biologic markers.

Materials and Methods

Animals
Female Wistar rats purchased from Harlan Nederland (Horst, The Netherlands) with a body weight of 150–200 g were acclimatized for at least 5 days to a room temperature of 24°–26°C with a 12:12-h light:dark cycle. Standard laboratory Chow and water were provided ad libitum. Rats were randomly allocated to two groups, fed and fasting. In experiments using fasted animals, food was withdrawn ± 16 h with free access to tap water before starting the perfusion. All animals used in this study were cared for in our animal research facilities. The experiment was performed after receiving the approval of the Animal Care Committee of the Free University of Brussels (Brussels, Belgium).

Solution and Chemicals
An albumin-free Hank’s balanced salt solution was prepared using 0.4 g/l KCl, 0.06 g/l KH2PO4, 0.35 g/l NaHCO3, 0.048 g/l Na2HPO4, 0.14 g/l CaCl2, and 1 g/l glucose. Insulin, 35 U/l, and 2.38 g/l HEPES were added. All these chemicals were obtained from Sigma (Bornem, Belgium). The solution was saturated with 100% O2.
(0.5 l/min), pH adjusted to 7.35 ± 0.05 using 0.2 m NaOH, and supplemented with NaCl to achieve 300 mOsm. Perfusate gases (Rapid Lab; Chiron Diagnostics, Halstead, United Kingdom) were measured from the inlet and effluent to assure that the perfusate was adequately oxygenated.

**Hepatectomy and Perfusion**

The procedure of liver perfusion was essentially as described by Sugano et al.\(^1\)\(^4\) In short, the animals were anesthetized with pentobarbital sodium intraperitoneally (50 mg/kg), the abdomen was opened, and heparin (1,000 U/kg) was administered via the inferior vena cava. The portal vein was cannulated with a 22-gauge catheter, secured in place, and immediately perfused with the previously described hemoglobin-free solution. The liver was removed under continuous perfusion and transferred to the closed system 

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**Experimental Protocol**

**Control Livers of Fed and Fasting Rats.** The liver was connected to the perfusion apparatus and perfused at a normal perfusion flow rate of 5 ml/min for 135 min without disruption (n = 5/group) throughout the experimental procedure to maintain normoxia. Control livers were not subjected to ischemia–reperfusion.

**Warm Ischemia Livers of Fed and Fasting Rats.** The experiment consisted of three phases: (1) the liver was perfused for 15 min at the same flow rate, i.e., 5 ml/min; (2) perfusion was stopped for 60 min, and the liver stored at 37°C in the perfusate milieu; (3) perfusion resumed and lasted for 60 min more (n = 5/group).

At different time points (0, 10, 15, 75, 105, 120, and 135 min after the start of the perfusion) 1-ml samples were withdrawn from the perfusate leaving the organ. Thin liver biopsies were obtained from the median lobe at 0, 75, 105, and 135 min.

**Endpoints**

The aspartate aminotransferase (AST, U/l), alanine aminotransferase (ALT, U/l), lactate dehydrogenase (LDH, U/l), γ-glutamyltransferase (U/l), alkaline phosphatase (U/l), glucose (mg/dl), lactate (mg/dl), potassium (mEq/l), and bilirubin (mg/dl) concentrations were measured in the perfusate, at the different time points, on a Beckman LX20 (Beckman, Fullerton, CA). Reactive oxygen species (ROS), i.e., diene and triene concentrations expressed as a percentage of the oxidative index, were measured in the perfusate at the same times, using a spectrophotometric technique as described elsewhere.\(^1\)\(^5\)\(^6\) In short, lipid extracts of the samples were prepared in 2:1 chloroform:methanol. Aliquots of the lipid fraction were evaporated to dryness under a vacuum at room temperature and dissolved in hexane. Conjugated dienes and trienes were detected in the hexane solution by the simultaneous calculation of the second derivative of the absorbance spectrum between 200 and 300 nm. Dienes were measured at 233 nm, and trienes were measured at 270 nm (Beckman DU-70 Spectrophotometer; Beckman).

The release of cytochrome c in the perfusate was measured using ELISA Kit BMS 263 (Medical & Biologic Laboratories Co. Ltd., Nagoya, Japan). The rat cytochrome c ELISA is an enzyme-linked immunosorbent assay for quantitative detection (spectrophotometric absorbance at 450 nm, Sanofi Diagnostics Pasteur Pr2100; Sanofi Diagnostics, New York, NY). The assay uses two affinity-purified polyclonal antibodies against cytochrome c. The assay range of this kit is 0.78–50 ng/ml. The concentration of cytochrome c is calibrated from a standard curve based on reference standards.

The liver biopsies were immersed in 10% formalin, embedded in paraffin, and processed by standard techniques and submitted to histologic examination (light microscopy). The sections of all biopsies (0, 75, 105, 135 min) were stained with hematoxylin–eosin and blindly scrutinized by one observer for morphologic analysis. The biopsies of 0 and 135 min were also stained with periodic acid–Schiff (PAS).

**Glycogen Content**

The proportion of glycogen in hepatocytes was determined with NIH-Image software (National Institutes of Health, Bethesda, MD; available on the Internet\(^*\)) at two time points, i.e., 0 and 135 min.\(^1\)\(^7\) NIH-Image is widely used in biologic research, for ultrastructural and morphometric studies on hepatocytes, specifically for glycogen content.\(^1\)\(^8\)–\(^2\)\(^0\)

Using a measuring apparatus consisting of a personal computer and a video-microscope (Leitz Dialux 20Es; Leitz, Westlar, Germany), the PAS-stained slides were viewed through a ×100 oil immersion objective. The glycogen content in hepatocytes was established as follows. First, a periportal or pericentral region was identified microscopically based on the presence or absence of a hepatic artery and bile duct near a portal venous lumen. Forty hepatocytes in the midzone of the lobule were analyzed. For the evaluation of PAS slides using NIH-Image, pictures are captured onto the hard drive of the workstation computer. Thereafter, captured images can be opened in NIH-Image program for evaluating

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indices of positivity on PAS slides. NIH-Image provides the average gray value within the selected regions of interest and this value, expressed as a percentage, is the sum of the gray values of all pixels in the selection divided by the number of pixels. Two fields of each PAS-stained slide were analyzed. From these area data, the glycogen index for the image was calculated and expressed as a percentage.

Statistical Analysis

Results were expressed in terms of means and SDs. Response curves in the various experimental conditions were obtained by simple linear interpolation between consecutive time points (0–135 min). For enzymes, a log transform was applied to normalize the distributions and stabilize the variances. All statistical calculations were then conducted on the transformed values.

Response curves were compared by the nonparametric method developed by Zerbe.21 This approach offers the advantage of comparing response curves not only globally but also for prespecified time intervals. Given the experimental protocol, three separate analyses were performed: before ischemia (0–15 min), during ischemia (15–75 min), and after ischemia (75–135 min). Curves were also compared over the entire study period (0–135 min). In some cases, outlying observations or response curves (departing by more than 4 SDs from the mean) were discarded from the analysis because they could impair the validity of the statistical analysis. To compare analytical levels between the beginning (15 min) and the end of ischemia (75 min) in each group, a paired Student t test was applied. To assess the immediate effect of reperfusion on each analyte, except cytochrome c and glycogen content, results obtained at 75 min and 90 min were also compared using the paired t test.

All results were considered to be significant at the 5% critical level (P < 0.05). Statistical analyses were performed by using the SAS version 9.1 for Windows (SAS Institute, Cary, NC) and S-PLUS version 6.2 for Windows (MathSoft Inc., Seattle, WA) packages.

Results

The livers maintained a normal, uniform, bright color without any sign of perfusion defects throughout the perfusion studies in all experimental conditions.

Enzymes

As seen in figures 1A–C, enzymatic profiles were similar in each nutritional status group, with a slight decrease immediately after the beginning of the experiment, followed by an overall increase of activities until the end of the experiment. The evolution of AST (fig. 1A) was more influenced by the nutritional status (fed or fasting) than by the presence or absence of ischemia.

Fig. 1. Evolution of (A) aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT), and (C) lactate dehydrogenase (LDH) in the medium during the different phases of the experiment (mean ± SD). Concentrations are presented on a log scale. For LDH, the response curve of rat R070 was excluded because its activities were systematically 4 or more SDs away from the mean of the other rats during the period 75–135 min. n = 5/group.
The increase of AST activity between 75 and 135 min was significantly higher in fasting rats than in fed rats (P < 0.01). No effect of ischemia on AST evolution was observed throughout the 135-min experimental period in fasting rats (P = 0.29). In fed rats, however, AST activities were significantly higher from upon reperfusion in livers subject to 60 min ischemia as compared with control livers (P = 0.02).

The profiles of ALT and LDH were similar to those of AST in both conditions, as shown in figures 1B and C. Perfusion of fasting rats livers in a continuous or an ischemia-reperfusion fashion were superimposable but resulted in greater enzyme efflux rate than for fed animals (P < 0.01). For ALT, no difference was observed between control and ischemia-reperfused livers in both fasting (P = 0.51) and fed conditions (P = 0.12). The release of LDH in fasting rats livers was unrelated to ischemia (P = 0.09) but was significantly higher in ischemia-reperfused livers than in continuously perfused livers of fed rats (P = 0.007).

The values of AST, ALT, and LDH recorded at the end of the experiment (135 min) are displayed in table 1. Mean activities of γ-glutamyltransferase and of alkaline phosphatase were lower than 5 U/ml throughout the study period, and no difference was observed among the four experimental conditions.

**Glucose, Lactate, Potassium, and Bilirubin**

The concentrations of glucose and lactate in the perfusate were globally higher in fed rats than in fasting animals regardless of ischemia (P < 0.01; figs. 2A and B). In fasting rats, the glucose concentration was lower in the ischemia-reperfusion group (P = 0.038), whereas no difference was observed in fed rats (P = 0.13). At the beginning of reperfusion (75 min), a burst increase in lactate was observed in the two nutritional conditions, but only in rats with ischemia. Lactate concentrations differed significantly before and after reperfusion in fasting rats, depending on ischemia (P = 0.034). Overall, potassium efflux in fasting rats was greater than in the fed rats during the 75- to 135-min experimental period (P = 0.0012; fig. 2C). A marked peak of potassium release followed by a rapid decrease was observed at the beginning of reperfusion in both fasting and fed rats.

Concentrations of glucose, lactate, and potassium at the end of the experiments are given in table 1.

The concentrations of bilirubin remained low in all groups, and no significant difference was observed between experimental conditions (P = 0.81).

**Conjugated Dienes, Trienes, and Cytochrome c**

Concentrations of ROS, i.e., dienes and trienes, were globally higher in fasting rats than in fed rats. Figures 3A and B display the evolution of the dienes and trienes during the experiment. They increased throughout the perfusion period (P < 0.001). In fasting rats, ROS did not differ between rats with or without ischemia (P = 0.17 for dienes and 0.18 for trienes). In fed rats, ROS were significantly higher in the ischemia group. Dienes and trienes values at the end of the experiment are given in table 1.

Cytochrome c concentrations at the beginning and end of the experiment are displayed in table 2. In fasting rats, cytochrome c tended to increase in the ischemia-reperfusion group (P = 0.06) and became significantly higher than in the control condition (P = 0.03). In fed rats, cytochrome c decreased significantly in the ischemia-reperfusion condition and became significantly lower than for fasting rats in the same condition (P < 0.001).

**Histology**

Analysis of hematoxylin-eosin-stained slides revealed no differences in liver specimens between fed and fasting rats in both conditions. Hepatocytes and sinusoidal cells exhibited a normal morphology. Single-cell necrosis was rarely observed. No evidence of inflammation or fibrosis was present. Minimal changes, consisting mainly of occasional hepatocytes with vacuolization or ballooning, were observed at end of the ischemic period in the livers of fed and fasting rats. During the early period of reperfusion, areas of hepatocellular vacuolar change,
detachment of the endothelial lining, and mild sinusoidal congestion were observed in the two groups. However, the lobular architecture was always maintained.

Glycogen Content

The glycogen contents of livers from the different experimental conditions are given in table 2. The glycogen content decreased in all groups during the experiment but was significantly affected by the nutritional status (P < 0.001), being markedly decreased in fasting rats. An example of light micrographs of rat liver slides stained with PAS is shown in figure 4.

Discussion

Results from the current experiment confirm that the nutritional status is an important determinant for the extent of rat liver deterioration during continuous perfusion and after warm ischemia. Under the current experimental conditions, fasting causes liver cell injury. In livers in which perfusion was continued throughout the

Fig. 2. Evolution of (A) glucose, (B) lactate, and (C) potassium (K⁺) in the medium during the different phases of the experiment (mean ± SD). n = 5/group.

Fig. 3. Evolution of (A) dienes and (B) trienes in the medium during the different phases of the experiment (mean ± SD). For dienes, observation of rat R104 at 10 min was discarded from the analysis because it was outlying (more than 4 SDs from the mean value of the other rats). OI = oxidative index. n = 5/group.

Glycogen Content

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Discussion

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experiment, enzyme and potassium release was significantly higher in the fasting group when compared with the fed group. This could be interpreted as an indication that fasting plays a major role in liver integrity in ischemic and nonischemic states.

The determination of liver injury was accomplished using perfusate AST, ALT, LDH, and potassium concentrations. These measures have been used previously as indicators of cell injury in the perfused liver.22–25 The pattern of release of hepatocellular enzymes was similar between continuous perfusion and ischemia–reperfusion in fasting rats. These variables increased significantly in both control and ischemia–reperfusion conditions. This occurred more importantly in fasting than in fed animals. However, in fed rats, the release of potassium and cytosolic enzymes was higher after ischemia compared with the control group, indicating a greater injury of hepatocytes. Therefore, preservation of ex vivo continuously perfused rat liver seems to be altered more severely by fasting than by warm ischemia. Although it is assumed that loss of liver function is correlated with enzyme and potassium release, our findings provide no information about the functional integrity of the liver, in the absence of more sensitive tests, e.g., antipyrene clearance.26

Our results are in accord with previous experiments. For more than two decades, extensive experimental studies have shown that livers from fed animals are more resistant to the normothermic ischemic injury than livers from fasting animals.12,27–29 Bradford et al.12 and Le Cousture et al.30 showed that nutritional status is an important determinant of damage to hepatocytes due to hypoxia in a perfused rat liver. It has also been reported that survival after transplantation in rats is improved if donor livers of fed animals are used.31 Apart from that, our results did demonstrate that fasting itself leads to the same deleterious effect on hepatocytes as warm ischemia.

A major factor that could relate the reduced tolerance of normal rat livers to warm ischemia–reperfusion injury to the nutritional status is represented by the amount of glycogen stores available in the liver.25,31 Under normothermic conditions, the high hepatic glycogen content and sustained glycolytic ATP formation are thought to explain the increased resistance of livers from fed rats to ischemic injury compared with those from fasted rats.12,27,32 In absence of oxygen, glycogen is essential to maintain the cellular integrity by supplying glucose for ATP generation.33 When glycogen is consumed, ATP depletion rapidly develops, leading to a series of events that eventually cause irreversible cell injury and necrosis.34 Caraceni et al.35 showed that after 18 h of fasting, the hepatic glycogen was almost completely depleted. As a consequence of ischemia and reperfusion, the midzonal hepatocytes at the border between the anoxic pericentral and normoxic periportal hepatocytes are the first to undergo lethal cell injury.36,37 Cherid et al.36 also found that glycogen loss has a varying intensity and is predominant in pericentral and secondary in periportal regions during cold ischemia and reperfusion. Considering these results, we focused on the midzone of the lobule to determine glycogen content. In our study, glycogen content was significantly reduced in the fasting rats between the two time points, i.e., 0 and 135 min, when compared with the fed rats.

Some other parameters were affected by nutritional status and liver oxidative injury.

### Table 2. Evolution of Cytochrome c and of Hepatic Glycogen Content in the Fed and Fasting Rats at 0 and 135 min (n = 5/Group)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fasting Rats</th>
<th>Fed Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ischemia-Reperfusion</td>
</tr>
<tr>
<td>Cytochrome c, 0 min, ng/ml</td>
<td>11.5 ± 4.8</td>
<td>14.1 ± 5.1</td>
</tr>
<tr>
<td>Cytochrome c, 135 min, ng/ml</td>
<td>18.0 ± 11.8</td>
<td>50.8 ± 32.7*</td>
</tr>
<tr>
<td>Glycogen, 0 min, %</td>
<td>12.6 ± 4.8</td>
<td>12.6 ± 8.6</td>
</tr>
<tr>
<td>Glycogen, 135 min, %</td>
<td>10.8 ± 3.3</td>
<td>6.0 ± 2.6</td>
</tr>
</tbody>
</table>

* P < 0.05 ischemia–reperfusion vs. control in the fed or fasting groups. † P < 0.001 between ischemia–reperfusion groups. ‡ P < 0.001 fed vs. fasting.

Fig. 4. Example of light micrographs of ex vivo rat liver slides (ischemia–reperfusion) stained with PAS at ×100 magnification. Top panels (A and B) refer to fed rats; A at 0 min and B at the end of the experiment, i.e., 135 min. Bottom panels (C and D) refer to fasting animals, C at 0 min and D at 135 min. Box panels display the glycogen content analyzed using the NIH-Image software (National Institutes of Health, Bethesda, MD, USA) at the same time-points.
status. The livers of fed rats had a greater release of glucose. Glucose release is an indicator of the presence of glycogen store in the livers of fed rats.\textsuperscript{37} The larger quantities of lactic acid that are generated in the fed state are probably a result of glycolysis and were paralleled by the greater glucose release from glycogenolysis to satisfy ATP demands. Intracellular acidosis is known to be protective against ischemia–reperfusion.\textsuperscript{38} This might contribute to the protection afforded by the fed state. In contrast, the livers of fasting rats had lower glucose and negligible lactate release. Le Couteur \textit{et al.}\textsuperscript{29} showed that reperfusion of \textit{ex vivo} perfused liver impairs cell membrane transport of glucose in fasted rats only.

Lipid peroxidation, the oxidation of unsaturated double bonds in cellular lipids, is implicated as one key mechanism of ROS-mediated tissue injury.\textsuperscript{15,39} Fasting is involved in series of complex effects on free radical defense mechanisms and causes alteration in tissue antioxidant defenses. A large body of evidence has been accumulated for the impact of ROS on reperfusion injury of the liver.\textsuperscript{40,41} Results of the current experiment confirm that food deprivation increases lipid peroxidation, as demonstrated by the increase in dienes and trienes in livers exposed either to continuous perfusion or to warm ischemia. This release of ROS occurs concomitantly with the release of other hepatocellular enzymes. This is in accord with a previously published experiment.\textsuperscript{39}

Because ROS are produced as part of normal cellular metabolism, biologic systems have evolved endogenous antioxidant defenses against ROS. Antioxidant defenses are balanced against pro-oxidant–generating systems.\textsuperscript{42,43} This balance can be challenged by fasting/starvation as demonstrated by Tanigawa \textit{et al.}\textsuperscript{13} Overnight fasting accelerates the conversion of xanthine dehydrogenase to xanthine oxidase, a ROS-generating enzyme system, during hypoxia in the rat liver.\textsuperscript{43}

Mitochondria are usually the main source of ROS in the cell. They are also equipped with proapoptotic protein, like cytochrome \textit{c}.\textsuperscript{44} In our study, cytochrome \textit{c} increased significantly only in the fasting ischemia–reperfusion group. A similar peak of cytochrome \textit{c} during reoxygenation has been observed by Takei \textit{et al.}\textsuperscript{45} after cold ischemia–reperfusion. Cytochrome \textit{c} was identified as a component required for a crucial step in apoptosis, caspase-3 activation and DNA fragmentation.\textsuperscript{46} Using electron microscopy, Caraceni \textit{et al.}\textsuperscript{7} showed that mitochondrial damage is greatly enhanced by fasting. Although the exact mechanisms remain uncertain, fasting may facilitate mitochondrial injury by decreasing the hepatic content of antioxidants and therefore sensitizing the mitochondria to the noxious action of ROS.\textsuperscript{44} Vendemiale \textit{et al.}\textsuperscript{47} demonstrated that mitochondria isolated from normal fasted livers presented a greater concentration of oxidized lipids and a lower content of ATP synthesis complex as compared with their fed counterpart.

Previous experiments have demonstrated that apoptosis of hepatocytes also occurs during ischemia–reperfusion.\textsuperscript{38,49} It could be interesting to further investigate whether the high concentration of free radicals and cytochrome \textit{c} affects apoptosis in this experimental setting. Also, further studies should focus on the effect of nutritional support on apoptosis in the liver.

Various types of \textit{in vivo} and \textit{in vitro} experimental animal models have been used to study mechanisms of hepatic ischemia and reperfusion injury, but none has been used as extensively as the \textit{in vitro} isolated perfused rat liver. This model is simple, inexpensive, and useful for the study of liver function.\textsuperscript{50,51} Advantages of this model include maintenance of the three-dimensional anatomical structure of the liver, localization of zonal-specific injury, preservation of the liver sinusoid oxygen gradient, retention of all liver cell types, and evaluation of liver-specific damage because all other organs are absent.\textsuperscript{50} The model allows maintenance of the liver in a viable condition for variable periods of time. Using an oxygenated perfusion medium, these livers maintained grossly normal histology at the end of perfusion. In our experiment, a slight increase of potassium and enzymes was observed during prolonged continuous perfusion in fed rat livers. Although we used physiologic perfusion pressure to fully oxygenate and maintain normoxic conditions, it can be suggested that physical stress during normal flow perfusion could cause mild but progressive nonlocalized liver damage. This has been demonstrated by Bailey and Reinke.\textsuperscript{26}

We fulfilled the purpose of this study using an \textit{ex vivo} perfused organ to isolate the oxidative component of hepatic injury. With \textit{in vivo} models, direct markers of oxidation or enzyme release may be complicated by nonspecific contributions from systemic compartments. Our study demonstrated that 60 min of no flow normothermic ischemia produced remarkable oxidative stress, which was easily detected, using dienes and trienes as endpoints. This course of development of damage was based on other previous experiments.\textsuperscript{12,41,52} Using an isolated reperfused rat liver, Kato \textit{et al.}\textsuperscript{55} reported that the extent of oxidative stress and cell death was higher after 60 min of ischemia than after 30 min of ischemia.

Nevertheless, the use of an \textit{ex vivo} experimental model implies some methodologic limitations when compared with \textit{in vivo} experiments. The blood-free isolated liver did not evaluate the role of circulating neutrophils and platelets, which represents an important component of liver injury associated with ischemia–reperfusion.\textsuperscript{53} However, accumulated evidence has shown that neutrophils are not involved in the early phase (1 h after reperfusion) but in the severe inflammatory phase (24 h after reperfusion) of liver ischemia–reperfusion injury.\textsuperscript{54} Therefore, the reperfusion injury observed after the 60-min reperfusion period in the current study was consistent with the early reperfusion phase that is independent.
of neutrophils. Straatsburg and Frederiks\textsuperscript{55} showed that the presence of blood should be held responsible for mainly multifocal damage, whereas in a blood-free system, periporal and midzonal injuries predominate.

Overnight fasting has been one of the most common preparation techniques to reduce the risk of aspiration of gastric contents during anesthesia for patients undergoing surgery. Ischemia–reperfusion is involved in many clinical situations, such as shock, liver transplantation, and liver resection. Most healthy adults may have sufficient endogenous antioxidant reserves to withstand short periods of fasting. However, this duration of nutritional stress may produce significant disturbance in the pro-oxidant versus antioxidant balance, particularly in patients with poor nutritional status, such as candidates for transplantation or debilitated intensive care unit patients. The protective effect of nutrition on ischemic injury to the liver is further supported by Bortenschlager \textit{et al.}\textsuperscript{56} They demonstrated that increases in AST and ALT were significantly less in the fed rats than in the fasted animals after hemorrhagic shock, an ischemic injury. They suggested the antioxidant effect of nutrition as one of the protective mechanisms.

In conclusion, this experiment, using clinical biochemical markers, shows that rats of fasting livers exposed to \textit{ex vivo} prolonged perfusion and normothermic ischemia–reperfusion injury are much more sensitive than livers of fed animals. Regarding the oxidative stress to the tissues, overnight fasting increases liver injury during subsequent hypoxic perfusion. Reduced glycogen stores in hepatocytes may explain reduced tolerance. The results, however, might indicate the possibility that nutritional support could form part of a treatment strategy in clinical conditions where livers are exposed to a temporary stress situation.

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