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Alanine minimises hepatocyte injury after ischemia-reperfusion in an *ex vivo* rat liver model

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ABSTRACT: Ischemia-reperfusion injury is a determinant in liver injury occurring during surgery, ischemic states and multiple organ failure. The pre-existing nutritional status of the liver, *i.e.*, fasting, might contribute to the extent of tissue injury. This study investigated whether alanine, an amino acid precursor of glucose, could protect *ex vivo* perfused livers of fasting rats from reperfusion injury. The portal vein was cannulated, the liver removed and perfused in a closed *ex vivo* system. Isolated livers were perfused either with glucose 1 g/L and 10 g/L, or with equal concentrations of alanine (n = 10 in each group). The experiment consisted of perfusion for 15 min, ischemia for 60 min, and reoxygenation during 60 min. Enzymes, glucose, lactate and bilirubin were analysed in perfusate samples. The proportion of glycogen as well as activation of caspase 3 was determined in biopsies. Alanine at a concentration of 10 g/L attenuated enzymes release in the perfusate during reoxygenation when compared to glucose-treated groups. Lactate level in the perfusate was lowest in alanine groups. Ischemia-reperfusion and mainly alanine activated apoptosis, specifically in Kupffer and endothelial cells. Alanine presents a protective effect on normothermic ischemia-reperfusion injury of the fasting rat liver when compared to glucose.

Introduction

For more than two decades, experimental studies have shown that livers from fed animals are more resistant to normothermic ischemic injury than livers from fasted animals (Bradford *et al.*, 1986; Caraceni *et al.* 1999; Gasbarrini *et al.,* 1993). Stadler *et al.,* (2005) showed that rat livers of fasting animals exposed to *ex viv*o prolonged perfusion or normothermic ischemia-reperfusion injury are much more sensitive than livers of fed animals. Specifically, in this experiment, preservation of the *ex vivo* continuously perfused rat liver was altered more severely by fasting than by warm ischemia.

The deleterious effect of fasting could be explained by the lack of energetic substrates such as glycogen or other glycogenic compounds (Caraceni *et al.,* 1999). Glycogen is essential to maintain the cellular integrity by supplying glucose for adenosine triphosphate (ATP) generation. Glycogen reserves, however, provide only a short-term supply of glucose and become especially critical beyond 18-24 h fasting (Klover *et al.,* 2004). Over the years, evidence has mounted that under physiological conditions glucose utilisation by liver is rather limited and that glucose is in fact a poor precursor for glycogen (Boyd *et al.,* 1981; Hue *et al.,* 1975). This finding has been defined as the 'glucose paradox'. Efficient glycogen synthesis required the presence of glucose plus gluconeogenic precursors like fructose, glycerol, lactate, but also amino acid as glutamine or alanine. Specifically, the effect of the amino acid is to divert the gluconeogenic flux of glucose-6-phosphate from glucose production into the formation of uracil-diphosphate glucose and glycogen (Katz *et al.,* 1984).

Theoretically, exogenous substrates can also provide energy to livers. Alanine is quantitatively the most important amino acid precursor of glucose in the liver. In the

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overnight and prolonged fasted state in humans, it has been demonstrated that ^{14}C-labeled alanine is incorporated into blood glucose (Felig 1975). In the so-called glucose-alanine cycle, alanine serves as a carrier of ammonia and of the carbon skeleton of pyruvate from muscle to liver. Arriving in the liver, pyruvate is incorporated into glucose and ammonia is converted into urea for excretion (Dölle 2000; Felig *et al.,* 1974). Pyruvate is an antioxidant and produces acetyl co-enzyme A as the immediate substrate of the Krebs cycle to generate ATP (Mallet 2000). Previous studies have shown that alanine presents a beneficial effect at reperfusion in *ex vivo* rat and mice livers after cold ischemia (Arnault *et al.,* 2002; Patry *et al.,* 1996; Patry *et al.,* 1999). Alanine improves significantly the recovery of ATP and gluconeogenic capacity of livers from fasted but not from fed animals (Arnault *et al.,* 2002).

It can be hypothesised that the beneficial effects of alanine may also operate during normothermic ischemia. Therefore, this study was designed to investigate whether this amino acid could protect an *ex vivo* perfused fasting rat liver from warm ischemia-reperfusion injury. We compared the effect of alanine to glucose on hepatic integrity using clinical biological markers like enzymes. We also have a special attention to histological features, *i.e.* necrosis and apoptosis.

Materials and Methods

Animals

Female Wistar rats purchased from Harlan Nederland (Horst, The Netherlands) with a body weight of 150-200 g were acclimated for at least five days to the room temperature of 24-26°C with a 12:12-h light-dark cycle. Standard laboratory chow and water were provided *ad libitu*m. Food was withdrawn 18 hours with free access to tap water before starting the perfusion. All animals used in this study were cared for in our animal research facilities, and the experiment was performed after receiving the approval of the Animal Care Committee of the Free University of Brussels.

Solution and Chemicals

An albumin-free Hank's Balanced Salt Solution (HBSS) was prepared using 0.4 g/L KCl, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$, 0.048 g/L Na2HPO$_4$, and 0.14 g/L $CaCl_2$. Insulin (35 IU/L) and HEPES 2.38 g/L were added. All these chemicals and alanine were obtained from Sigma (Bornem, Belgium). The solution was saturated with 100% O_2 (0.5 L/min), pH adjusted to 7.35 ± 0.05 using NaOH 1.0 M 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 adequate oxygenation.

Hepatectomy and perfusion

The procedure of liver perfusion is described elsewhere (Stadler *et al.,* 2005). The animals were anaesthetised with pentobarbital sodium (NEMBUTAL, Abbott Laboratories, North Chicago, IL, USA) intraperitoneally (50 mg/kg), the abdomen was opened and heparin (1000 IU/kg) 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 solution. The liver was removed under continuous perfusion and transferred to the closed system *ex vivo* arrangement. The system (circuit volume 125 mL) was maintained at 37°C. Liver temperature was monitored with a temperature probe placed under the liver. The perfusate, *i.e.,* enriched HBSS solution, passed sequentially through a peristaltic pump (ISMATEC REGLO, Fisher Bioblock Scientific, Tournai, Belgium) at a flow rate of 5 mL/min to obtain a perfusion pressure ±12 cm H_20 through the portal vein.

Experimental protocol

The experiment consisted of three phases: (1) the liver was perfused for 15 min; (2) perfusion was stopped for 60 min, the liver stored at 37°C in the perfusate milieu; (3) reperfusion started for further 60 min. At different time-points (0, 10, 15, 75, 90, 105, 120, 135 min after the start of the infusion), 1 mL-samples were withdrawn from the perfusate leaving the organ. Thin liver biopsies were obtained at 0 and 135 min.

The rats were randomly divided into four groups (n = 10 in each group): livers were perfused with either two concentrations of glucose, *i.e.,* 1 and 10 g/L, or alanine 1 and 10 g/L. Glucose and alanine were added to the perfusate before the start of the experiment.

End points

The aspartate aminotransferase (AST, IU/L), alanine aminotransferase (ALT, IU/L), lactate dehydrogenase (LDH, IU/L), γ-glutamyltransferase (IU/L), alkaline phosphatase (IU/L), glucose (mg/dL), lactate (mg/dL) and bilirubin (mg/L) concentrations were measured in the perfusate, at the different time-points, on a BECKMAN LX20 (Beckman, Fullerton, CA, USA).

The liver biopsies were immersed in 10% formalin, embedded in paraffin and processed by standard techniques and submitted to histological examination. The sections were stained with haematoxylin-eosin, and blindly scrutinised for morphological analysis. Other sections were assayed with a Periodic Acid Schiff (PAS) stain.

Caspase 3 activity in liver tissue

Apoptosis was evaluated by light microscopy using an *in situ* immunostaining technique in a blinded fashion. Caspase 3 activity was measured on deparaffinised tissue sections.

Immunohistochemistry using an antibody specific to cleaved and activated caspase 3 (CASPASE 3 ASP 175, Cell Signalling Technology, Danvers, MA, USA) was performed after pre-treatment with methanol/H_2O_2 to inhibit peroxydases and using a routine avidine-biotin-immunoperoxydase kit reagents (AVIDINE-BIOTINE, Vector, Burlingame, Ca, USA). The number of apoptotic cells was reported on the number of hepatocytes in the selected region and expressed as a percentage. Five fields of each stained slide were analysed.

Glycogen content

The glycogen content in hepatocytes was established at two time-points, *i.e.,* 0 and 135 min, using the NIH-IMAGE software (National Institutes of Health, Bethesda, MD, USA) (Stadler *et al.,* 2005; Stadler *et al.,* 2007; Sato 2004). Using a measuring apparatus consisting of a personal computer and a video-microscope (LEITZ DIALUX 20ES, Leitz, Wetzlar, Germany), the PAS stained slides were viewed through a ×100-oil immersion objective. The glycogen content in hepatocytes was established as follow (Stadler *et al.,* 2005). A periportal and a perivenular region were first identified microscopically based on the presence of the terminal hepatic venule. Forty hepatocytes in the midzone of the acinus were analysed. For the evaluation of PAS slides using NIH-IMAGE, pictures were captured onto the hard drive of the workstation computer. Thereafter, captured images can be opened in NIH-IMAGE program for evaluating indices of positivity on PAS slides. NIH-IMAGE provides the average gray value within the selected regions of interest and this value is the sum of the gray values of all pixels in the selection divided by the number of pixels. Two fields of each

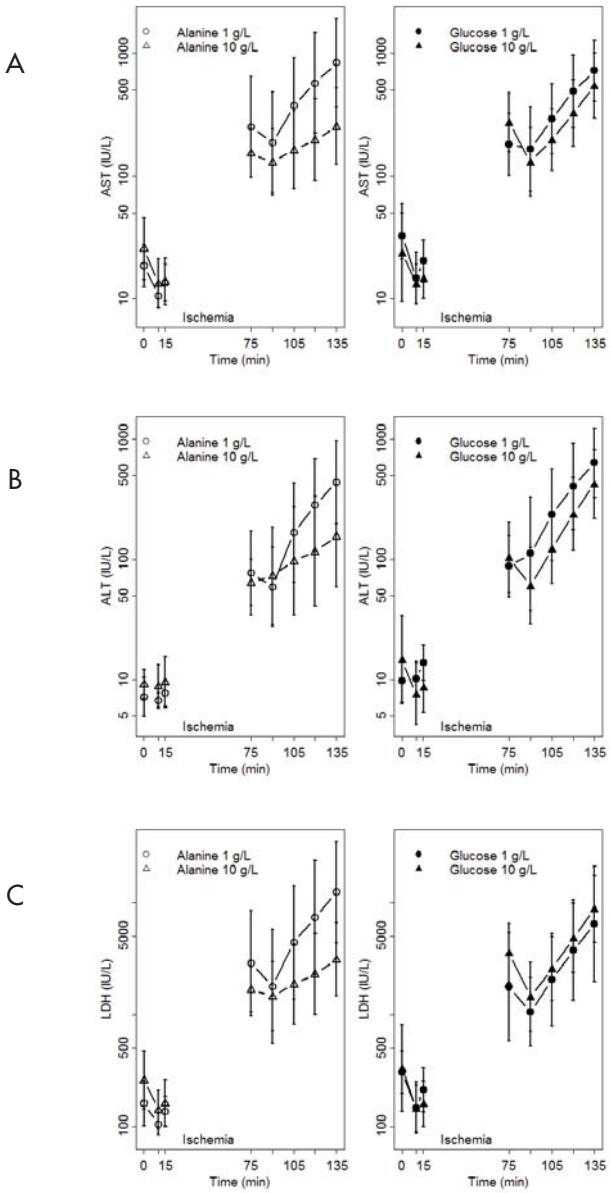

FIGURE 1. A) concentration of aspartate aminotransferase (AST). B) concentration of alanine aminotransferase (ALT). C, concentration of lactate dehydrogenase (LDH) in the medium during the different phases of the experiment (mean ± SD). During reoxygenation of the liver (75-135 min), perfusate enzymes levels were greater in the two glucose and 1 g/L alanine groups compared to the 10 g/L alanine-treated rat livers ($P < 0.05$). Concentrations are presented on a log-scale. IU = International Unit (n = 10 in each group).

PAS stained slide were analysed. From these area data, the glycogen index for the image was calculated, and expressed as a percentage.

Statistical analysis

Biological results were expressed in terms of means and standard deviations (SD). Response curves in the different experimental conditions were obtained by simple linear interpolation between consecutive time-points (0-135 min). For enzymes, a log transform was applied to normalise the distributions and stabilise the variances. All statistical calculations were then carried out on the transformed values. Response curves were compared by the method developed by Zerbe (1979). This approach, which does not make any assumption about the type of evolution, allows comparing response curves not only over the entire study period but also for pre-specified time intervals, *i.e.,* before ischemia (0-15 min), during ischemia (15-75 min) and after ischemia (75-135 min), respectively.

Glycogen levels and caspase 3 activated-cells at baseline and after 135 min were compared by a paired Student's t test. Comparison of values between groups was done by one-way analysis of variance. All results were considered to be significant at the 5% critical level ($P < 0.05$). Statistical analyses were carried out by using SAS version 9.1 for Windows (SAS Institute, Cary, NC, USA) and S-PLUS version 6.2 for Windows (MathSoft Inc, Seattle, WA, USA) packages.

Results

Livers appeared normal and uniform in colour throughout the study period in all groups.

Enzymes

As seen in Fig. 1A-C, enzymatic profiles were similar in each 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. During the reoxygenation phase, perfusate AST levels (see Fig. 1A) were greater in the glucose and in the 1 g/L alanine groups than in the 10 g/L alanine-treated group ($P < 0.05$). The profile of the ALT and LDH curves was similar to that of AST as shown in Figs. 1B and C. Whereas reoxygenation enhanced the release of ALT and LDH in the four groups, perfusate ALT and LDH levels were greater in the glucose groups and in the 1 g/L alanine-treated livers than in the 10 g/L alanine group ($P < 0.05$). The values of AST, ALT and LDH recorded at the end of the experiment (135 min) are displayed in Table 1.

The concentration of γ-glutamyltransferase and alkaline phosphatase in the perfusate was low in all groups (inferior to 5 IU/L) without any significant difference between glucose and alanine-treated rat livers (data not shown).

Glucose, lactate and bilirubin

The levels of glucose in the perfusate in the different groups are displayed in Fig. 2A. Globally, the concentrations of glucose were the highest in the glucose 10 g/L when compared to glucose 1 g/L and alanine-treated livers ($P < 0.01$).

Ischemia caused an elevation in lactate at the time of reperfusion, mainly in the fed 10 g/L glucose-treated groups ($P < 0.01$ when compared to alanine groups) (see Fig. 2B). Concentrations of lactate at the end of the experiments are given in Table 1.

Bilirubin levels remained low in all groups and no significant difference was observed between experimental conditions.

Histology

No differences were observed in haematoxylin-eosin stained slides of liver specimens between the four groups. Hepatocytes and sinusoidal cells exhibited a normal morphology. No necrotic hepatocytes were found in the different groups. No evidence of inflammation was present.

TABLE 1

Biological variables measured at 135 min in the perfusate (n = 10 in each group)

Variables	Glucose 1 g/L	Glucose 10 g/L	Alanine 1 g/L	Alanine 10 g/L
AST (IU/L)	826 ±478	626 ± 500	1,102 ± 886	315 ± 214 *
ALT (IU/L)	787 ±612	510 ± 302	557 ± 361	230 ± 187 *
LDH (IU/L)	8,879 ±4,545	10,630 ± 6,319	18,060 ± 14,862	3,957 ± 2,775 *
Lactate (mg/dL)	3.9 ±3.6	17.1 ± 7.9 †	2.5 ± 5.2	5.4 ± 4.5

* $P < 0.05$ vs. glucose and alanine 1 g/L groups; † $P < 0.01$ *vs.* other groups; AST = aspartate aminotransferase; ALT = alanine aminotransferase; LDH = lactate dehydrogenase; IU = International Unit

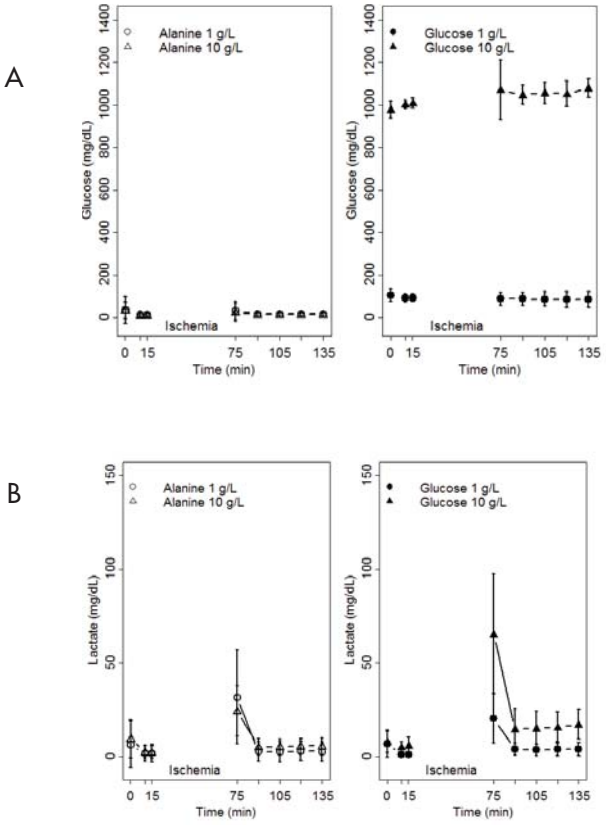

FIGURE 2. A) concentration of glucose. B) concentration of lactate in the medium during the different phases of the experiment (mean ± SD). Ischemia caused an elevation in lactate at the time of reperfusion, mainly in the 10 g/dL glucose group ($P < 0.01$) (n = 10 in each group).

Minimal changes, consisting mainly of occasional hepatocytes with vacuolisation or ballooning, were observed in the livers of the four rat groups at the end of the experiment. Areas of hepatocellular vacuolar change, detachment of the endothelial lining, and mild sinusoidal congestion were observed in the four groups. However, the acinar architecture was always maintained (data not shown).

Caspase 3 activation

Results are displayed in Fig. 3. Ischemia-reperfusion increases the number of caspase 3-activated cells in all groups ($P < 0.05$). The percentage of apoptotic cells was greater in the 10 g/L alanine-treated livers when compared to the other groups ($P < 0.05$) (see Fig. 4). Alanine increases caspase 3 activity mainly in Kupffer and endothelial cells.

Glycogen content

Liver glycogen contents from the different experimental conditions are given in Table 2. Glycogen decreased significantly in the glucose-treated groups between 0 and 135 min ($P < 0.05$). Glycogen was significantly higher in the glucose 10 g/L when compared to glucose 1 g/L and alanine-treated groups at the start of the experiment ($P < 0.01$). At the end of reperfusion, the concentration of glycogen was the lowest in the glucose 1 g/L ($P < 0.01$).

TABLE 2

Evolution of hepatic glycogen content, expressed in percentage (%), in the four groups at baseline and the end of the experiment (n = 10 in each group)

Group	Glycogen 0 min	Glycogen 135 min	P-value
Glucose 1 g/L	10.8 ± 7.6	5.2 ± 2.7	0.05
Glucose 10 g/L	21.8 ± 3.2	17.9 ± 4.0	0.03
Alanine 1 g/L	16.4 ± 8.8	14.2 ± 9.6	0.64
Alanine 10 g/L	12.2 ± 3.5	10.5 ± 5.3	0.46

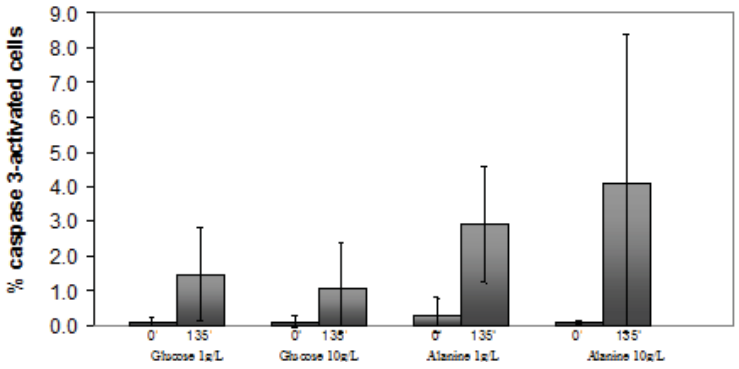

FIGURE 3. Histogram showing percentages of caspase 3-activated cells at the start and at the end of experiment. Ischemia-reperfusion increases the number of apoptotic cells in all experimental conditions ($P < 0.05$). The percentage of apoptotic cells was greater in alanine-treated livers when compared to the glucose treated livers ($P < 0.05$) ($n = 10$ in each group).

Discussion

Results from the present study show that alanine at concentrations of 10 g/L decreases the extent of ischemia-reperfusion injury in the fasting rat liver. Under the present experimental conditions, alanine had a protective effect as suggested by the lowest efflux of enzymes (AST, ALT and LDH). These measures have been used previously as indicators of hepatocellular damage (Stadler *et al.,* 2005; Stadler *et al.,* 2007; Gores *et al.,* 1986).

Glucose at concentrations of 1 or 10 g/L seems less efficacious to protect livers of fasting animals when compared to alanine. Previous studies with perfused livers showed that glucose uptake was very low (Boyd *et al.,* 1981; Huang *et al.,* 1988; Plauth *et al.,* 1991; Storer *et al.,* 1981). This fact explains the limited utilisation of glucose by liver. In rats, there is evidence that the capacity of glucose utilisation is limited and glycogen deposition in liver is predominantly a gluconeogenic process (Katz *et al.,* 1984). This could explain the low protective effect of glucose observed in our study. Le Couteur *et al.* (1994) showed that reperfusion of *ex vivo* perfused liver impairs cell membrane transport of glucose in fasted rats. The use of an *ex vivo* blood-free medium in our liver perfused model implies some limitations, *e.g.* the role of erythrocytes, when compared to *in vivo* experiment. When the perfusion media contained erythrocytes, glucose uptake was markedly increased (Katz *et al.,* 1984). This fact makes a comparison with our results difficult. Production of lactate at the time of reperfusion is higher in glucose groups when compared to alanine-treated livers probably consequence of anaerobic glycolysis.

Our results are in line with previous experiments, as that of Arnault *et al.* (2002) where alanine, added at reperfusion in an *ex vivo* perfused rat liver after 24 hours of cold

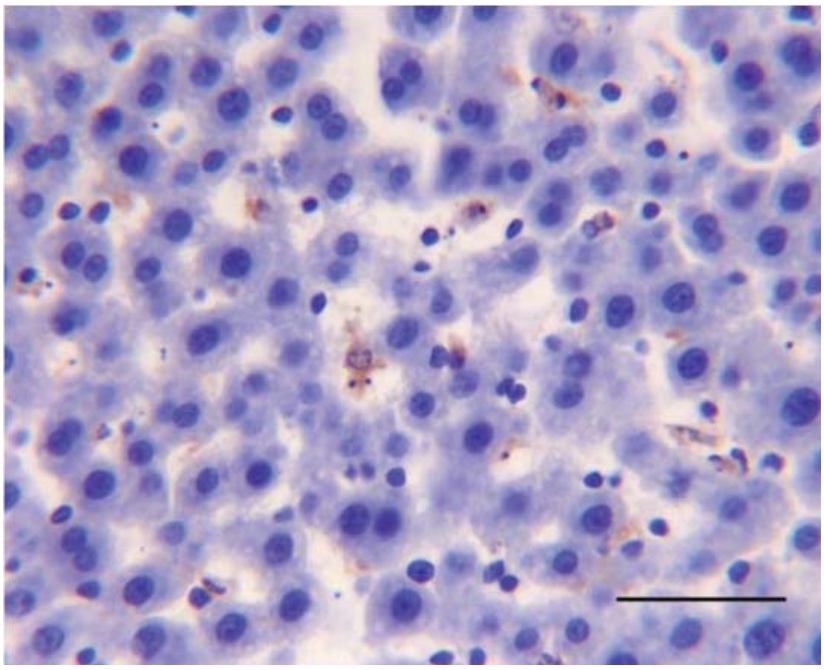

FIGURE 4. Light micrograph of *ex vivo* alanine-treated rat liver slide stained with a caspase 3 antibody at the end of the experiment, *i.e.* 135 min. Staining is observed predominantly in Kupffer and endothelial cells. Scale bar 50 microns.

ischemia, exerts a beneficial effects as significantly improving the recovery of ATP and gluconeogenic capacity of livers from fasted animals. The longer the fasting period, the more deleterious are its effects and the more alanine compensates for them. Alanine may exert its specific protective effect on livers from fasted animals by acting metabolically as a substitute for lacking endogenous substrates, such as glycogen. Alanine is a key amino acid for ATP production through pyruvate and the Krebs cycle, thus improving the liver recovery of energy and function (Patry *et al.,* 1996; Astarcioglu *et al.,* 1995). In contrast, the addition of pyruvate did not improve the recovery of livers submitted to a 72-h fasting period before 24 hours preservation arguing against a single metabolic effect of alanine (Arnault *et al.,* 2002). The presence of an amine function (–NH_2 group) in alanine represents one of the main biochemical differences as compared to pyruvate. Alanine provides both calories and nitrogen, while pyruvate only provides calories. Lopez *et al.* (1998) have quantified the importance of alanine to hepatic nitrogen metabolism and showed that it is the principal amino acid utilised by the liver. With respect to the overall contribution of amino acids to glucose production in the post absorptive overnight fasting state, 75 % of glucose output is due to glycogenolysis rather than gluconeogenesis. It is primarily when glycogen stores are depleted and glucose output is dependent on gluconeogenesis that alanine availability assumed clinical importance in the regulation of blood glucose (Felig, 1975).

In a previous study using the same *ex vivo* perfused rat liver model, Stadler *et al.* (2007) showed that Intralipid®, a solution containing soybean oil, egg phospholipids and glycerol, decreased the extent of reperfusion injury in fasting animals when compared to glucose-treated groups. The authors concluded that beta oxydation of fatty acids and ketogenesis rather than glucose oxydation are regarded as a major source of energy for hepatocytes. These results are in agreement with the present hypothesis on the mechanism of action of alanine.

Nonparenchymal cells are important mediators of ischemic preservation-reperfusion injury. It is commonly stated that the initial injury phase during reperfusion is characterised by endothelial and Kupffer cell-induced oxidant stress (Caldwell-Kenkel *et al.,* 1991). In *in vivo* livers activated Kupffer cells release reactive oxygen species, cytokines, chemokines and other factors, which then leads to infiltration and activation of cells of the innate immune system, including neutrophils and CD4+ T lymphocytes (Jaeschke *et al.,* 2003; Zwaka *et al.,* 1997; Caldwell *et al.,* 2005; Jaeschke *et al*., 1991). These cytotoxic agents could diffuse into endothelial cells or hepatocytes and cause microvascular or metabolic injury to the liver (Alison *et al.,* 1994). The suppression of Kupffer cells ameliorates ischemia-reperfusion injury (Vanhorebeek *et al.,* 2005). In our experiment, we observed that ischemia-reperfusion increases activation of caspase 3 specifically in Kupffer and endothelial cells, not in hepatocytes. Activation of caspase 3 leads to apoptosis and to the orderly resorption of Kupffer cells that minimises inflammatory responses and leakage of cellular components into the extracellular space (Alison *et al.,* 1994). The absence of inflammation differentiates apoptosis from necrosis. As the integrity of the membrane of the apoptotic cell is maintained, there is no leakage of lysosomal enzymes that can damage nearby cells or elicit immune responses (Malhi *et al.,* 2006) while necrosis induces enzymes release (Gores *et al.,* 1986). In our experiment, we observed the greatest concentration of LDH, a marker of cell death, in glucose-treated groups.

Cells need ATP for maintaining their vital functions and paradoxically, for undergoing apoptosis (Jaeschke *et al.,* 2003). The percentage of Kupffer cells displaying caspase 3 activation is greater in alanine-treated livers when compared to the other groups. Execution of the apoptotic program includes energy-dependent steps, as sustained ATP production by alanine. This increase in Kupffer cells apoptosis induced by alanine when compared to the other groups might partially explain reduced reperfusion injury.

The protective effect of alanine against liver oxidative stress needs to be studied further before any solid conclusions can be drawn. Our study is mainly descriptive and not mechanistic. Although the effects observed in enzymatic levels are clear-cut, further studies are required to confirm our hypothesis on the energetic mechanisms involved in the protective effect of the amino acid against liver reperfusion injury in fasted animals, *e.g.* glycogen utilisation, hepatic ATP level and alanine oxydation.

In conclusion, this experiment shows that alanine at a concentration of 10 g/L in the perfusate attenuates hepatocellular injury in fasting rat livers after *ex vivo* normothermic ischemia-reperfusion when compared to glucose. The mechanisms involved in the protective effect of alanine need to be elucidated.

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