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ORIGINAL ARTICLE

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Effect of glycine on isolated, perfused rabbit livers following 48-hour preservation in University of Wisconsin solution without glutathione

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Abstract Glycine has been shown to decrease membrane injury in isolated cells due to hypoxia or cold ischemia. The mechanisms of action of glycine are not known, but glycine may be useful in organ preservation solutions or in treating recipients of liver transplantation. In this study the isolated, perfused rabbit liver was used to measure how glycine affected liver performance after 48-h preservation in University of Wisconsin (UW) solution without added glutathione. UW solution is less effective for 48-h liver preservation when glutathione is omitted. Rabbit livers stored for 48 h without glutathione show a large increase in enzyme release (LDH and AST) from the liver and a reduction in bile production. The addition of 15 mM glycine to UW solution, in place of glutathione, did not improve bile production or reduce enzyme release. However, infusion of 10 mM glycine into the reperfused liver lowered LDH release significantly (from 2383 ± 562 units/100 g to 1426 ± 286 units/100 g) during the initial reperfusion of the 48-h preserved liver. Hepatamine, a parenteral nutrition solution containing glycine, as well as other amino acids, was also effective in lowering LDH release from the preserved liver. Although glycine reduced LDH release, it did not decrease the amount of AST released from the liver, nor did it improve bile production. Thus, we conclude that glycine, either in UW solution or given to the liver upon reperfusion, has no significantly beneficial effect as tested in this model. Further testing of glycine, however, should be conducted in an orthotopic transplant model in the rat or dog.

Key words Liver, isolated perfusion, rabbit · Preservation, liver, rabbit · Glycine, isolated perfusion, rabbit liver · UW solution, liver, rabbit

Introduction

Methods used to cold store livers for transplantation can cause tissue injury, even in organs preserved for relatively short periods of time (2–10 h). The injury is usually characterized by damage to cellular membranes, resulting in a leakage of intracellular enzymes into the reperfusion medium. In livers preserved for short periods of time, the injury is relatively quickly reversed with a rapid return of normal liver functions. However, in longer term liver preservation (24 h or more) [1, 8, 18], the injury is more severe and can lead to delayed return of normal liver function (primary or initial dysfunction) or to failure of the graft (primary nonfunction). In livers preserved for long periods of time, the cellular membrane damage is greater than after short periods of preservation, as indicated by the large increase in serum enzymes that leak from the organ [6].

The causes of membrane damage are not known and have been suggested to be related to increased cytosolic

calcium and activation of phospholipases or proteases [14], removal of calcium from the organ through chelation by the anions in the preservation solution [3], or damage caused by oxygen free radicals [5]. Prevention of membrane injury may be a key to improving liver preservation. Stabilization of the plasma membrane would suppress the leakage of critical metabolites from the cell and decrease the leakage of ions through the membrane, thus reducing reperfusion tissue cell swelling and conserving energy required to pump electrolytes across the cell membrane. Stabilization of organelle membranes such as mitochondrial or endoplasmic reticulum could lead to increased energy generation, effective control of intracellular calcium concentration, and other subcellular functions. Furthermore, suppressing membrane injury could provide the damaged liver an opportunity to reverse other forms of preservation injury and facilitate regeneration of adenine nucleotide, metabolites such as glutathione and pyridine nucleotides, etc., and carry out biosynthetic reactions necessary to repair preservation/reperfusion-induced damage.

Recently, Weinberg et al. [20] have shown that glycine, the simplest amino acid, stabilizes renal tubule cell membranes and suppresses hypoxic-induced cell damage. Others [10] have shown that glycine may improve preservation of the kidney. Marsh et al. [11] have shown that glycine also stabilizes the plasma membrane of hypothermically preserved hepatocytes stored for up to 6 days. The primary beneficial effect of glycine was membrane stabilization and suppression of leakage of lactate dehydrogenase (LDH) from cells exposed to hypoxia or cold ischemia. The beneficial effects of glycine appear to be more prominent when present during the period of reperfusion. Glycine is also one of the amino acids that makes up glutathione. Glutathione is important in successful long-term storage of the liver [2], and the beneficial effects of glutathione could be related to hydrolysis of this compound to its constituent amino acids, including glycine.

In this study, we have measured the effects of glycine on the preserved and reperfused rabbit liver. Glycine was studied as an agent to replace glutathione in University of Wisconsin (UW) solution and as an additive to the reperfusion medium. Glycine, present during reperfusion of the rabbit liver, suppressed membrane injury (leakage of LDH from the liver), but did not suppress the loss of another critical liver function, bile production.

Materials and methods

New Zealand white rabbits weighing 1.5–2.0 kg were used in these experiments and were allowed free access to food and water. The methods for liver procurement, cold storage, and isolated perfusion have been published previously [7]. Briefly, under barbiturate anesthesia, the abdomen was opened and the bile duct cannulated. The portal vein was dissected free and the hepatic artery ligated. A cannula was inserted into the portal vein and after distal portal ligation and dividing the infrahepatic caval vein, the livers were flushed out in situ with 150–200 ml of UW solution at 4 °C and delivered from a height of 50 cm. The liver was excised and the gallbladder removed and the cystic duct ligated on the back table while keeping the liver cold.

Livers were either reperfused immediately (0 h preservation group) or cold stored (4 °C) for up to 48 h. Prior to reperfusion the livers were flushed out with Ringer's lactate (100 ml at room temperature, 20 °–22 °C). Reperfusion was done using Krebs-Henseleit bicarbonate (KHB) containing 10 mM glucose and 4 g% bovine serum albumin free of fatty acids for oncotic pressure (Sigma Chemical, St Louis, Mo., USA, fraction V), and 50 mg/l of both streptomycin sulfate and penicillin G (Sigma Chemical). A roller pump was used to continuously deliver perfusate at a rate of 3 ml/min per gram to the liver at a temperature of 37 °C. The perfusate was gassed with a mixture of O_2:CO_2 (95 %:5 %) via a membrane oxygenator (Sci Med, Minneapolis, Minn., USA, Model 0800-2A). The rate of gas flow was adjusted to keep the pH in the range of 7.4 ± 0.1 (100–200 ml 02:CO_2 per min). The oxygen tension was 450–500 torr. Reperfusion was done by recirculation of the perfusate (total volume about 1 l).

Bile was collected at 15-min intervals and bile production is reported as total bile collected per 2 h of reperfusion. Perfusate samples at the end of reperfusion (2 h) were used for measurement of enzyme content [i. e., aspartate amino transferase (AST) and LDH]. Enzymes were analyzed by colorimetric methods using assay kits supplied by Sigma Chemical (AST, Kit # 500; LDH, Kit # 500). At the end of reperfusion, liver tissue was removed for measurement of total tissue water (TTW) by oven drying [9] and the dried tissue was used for extraction of Na and K, which were measured by flame photometry. Results are reported as means with standard error of the mean for six or more livers per group. Statistical analysis was performed using Student's *t*-test with differences in means considered significant when P was less than 0.05.

The groups studied included livers preserved for 0 h, 24 h, and 48 h with UW solution or UW solution with glutathione (GSH) omitted. Livers preserved for 48 h in the UW solution, which contained 15 mM glycine in place of GSH. Livers preserved in UW solution without GSH but reperfused with KHB, which contained 10 mM glycine, livers preserved in UW solution without GSH and reperfused with KHB, which contained 50 ml/l hepatamine, and livers preserved for 48 h in the UW solution with GSH and reperfused with KHB, which contained 10 mM glycine or 50 ml/l hepatamine. Hepatamine is a solution containing essential and nonessential amino acids and used clinically as an infusion solution. Hepatamine contained (mg/100 ml): isoleucine (900), leucine (1100), lysine (110), methionine (100), phenylalanine (100), threonine (460), tryptophan (66), valine (840), alanine (770), arginine (600), histidine (240), proline (800), serine (500), glycine (900), cystine (< 20), and phosphate (10 mM) and acetate (62 mEq/l). The addition of 50 ml hepatamine to 1 l KHB gave a final glycine concentration of 6 mM. The compositions of UW solution and KHB have been given elsewhere [7].

Results

The results in Table 1 show how preservation time and the omission of reduced glutathione (GSH) from UW solution (in 48-h preserved livers) affect various measures of liver viability. Increased preservation time caused a decrease in bile production and at 48 h (4.7 ± 0.5 ml/2 h per 100 g) the amount of bile produced was significantly less than in freshly reperfused livers (12.6 ± 1.3 ml/2 h per

Table 1 Effect of omitting glutathione (GSH) from UW solution on the isolated, perfused rabbit liver (*LDH* lactate dehydrogenase, *AST* aspartate amino transferase, *TTW* total tissue water). * $P < 0.05$ vs 0-h preservation, ** $P < 0.05$ vs 48 h + GSH preservation

Preservation conditions		Bile (ml/2 h per 100 g)	LDH (U/100 g)	AST (U/100 g)	TTW (kg water/ kg dry weight)	K/Na
UW + GSH	(0 h, $n = 6$)	12.6 ± 1.3	154 ± 24	31.0 ± 3.6	2.29 ± 0.12	11.0 ± 1.1
UW + GSH	(24 h, $n = 9$)	7.9 ± 0.9	375 ± 38	55.7 ± 8.6	2.59 ± 0.1	7.7 ± 0.8
UW + GSH	(48 h, $n = 9$)	4.7 ± 0.5*	881 ± 136*	117 ± 18*	2.55 ± 0.16	6.5 ± 0.9*
UW − GSH	(48 h, $n = 6$)	5.0 ± 0.7	2383 ± 562**	175 ± 36**	2.96 ± 0.11	5.8 ± 0.6

Table 2 Effect of glycine and hepatamine on rabbit livers preserved for 48 h in UW solution without glutathione (GSH) tested by isolated perfusion. Results are expressed as means with SEM (*TTW* total tissue water)

Treatment		Bile (ml/2 h per 100 g)	TTW (kg/kg dry weight)	K/Na
UW − GSH	($n = 6$)	5.0 ± 0.7	2.96 ± 0.11	5.8 ± 0.6
Glycine 10 mM in KHB	($n = 8$)	3.2 ± 0.7	2.88 ± 0.11	6.5 ± 0.9
Glycine 15 mM in UW	($n = 6$)	5.7 ± 1.1	3.15 ± 0.23	3.4 ± 1.0
Hepatamine in KHB	($n = 4$)	3.9 ± 1.4	2.18 ± 0.2	2.6 ± 0.7

Table 3 Effect of glycine and hepatamine on rabbit livers preserved for 48 h in UW solution with glutathione (GSH) tested by isolated perfusion. Results are expressed means as with SEM (*TTW* total tissue water)

Treatment		Bile (ml/2 h per 100 g)	TTW (kg/kg dry weight)	K/Na
UW + GSH	($n = 9$)	4.7 ± 0.5	2.55 ± 0.16	6.5 ± 0.9
Glycine 10 mM in KHB	($n = 4$)	4.8 ± 0.4	2.78 ± 0.11	6.6 ± 0.6
Hepatamine in KHB	($n = 4$)	2.5 ± 0.3	3.18 ± 0.2	4.4 ± 0.9

100 g). Omission of GSH from livers preserved for 48 h in UW solution did not affect bile production (5.0 ± 0.7 ml/2 h per 100 g), which was similar to the amount produced with GSH after 48-h preservation. There was evidence of increased hepatocellular damage in the preserved and reperfused rabbit livers, as shown by the increased release of LDH and AST. After 48-h preservation with GSH, the amounts of LDH and AST released from the liver were significantly greater than from livers preserved for 0 or 24 h. Livers preserved for 48 h without GSH showed an even greater release of LDH and AST than livers preserved for 48 h with GSH, and the differences were highly significant. There were no differences in TTW between any of the groups, but the ratio of K/Na was significantly lower after 48-h preservation, with or without GSH, than in 0-h preserved and reperfused livers. The omission of GSH, however, did not significantly affect the K/Na ratio.

The results in Fig. 1 show how glycine or hepatamine affected enzyme release from rabbit livers preserved for 48 h in UW solution without GSH ($n = 6$). The addition of glycine (10 mM) to the reperfusion medium (KHB) decreased hepatocellular injury ($n = 8$), and LDH was reduced from 2383 ± 562 U/100 g liver wet weight to 1426 ± 280 U/100 g (not significant, $P = 0.08$, Student's *t*-test). The addition of glycine (15 mM) to the UW solution ($n = 6$) had no effect on LDH release when rabbit livers were reperfused with KHB. The addition of hepatamine to the reperfusion medium ($n = 4$) significantly reduced hepatocellular injury after 48-h preservation of the liver and there was a significant reduction in the amount of LDH that leaked from the liver (848 ± 228 U/100 g liver wet weight, $P < 0.05$). There were no differences in the amount of AST that leaked from the livers in any of these groups.

The results in Table 2 show how glycine and hepatamine affected bile production, TTW, and the Na/K ratio. The addition of glycine to the reperfusion medium reduced bile production after 48-h preservation of the liver; however, the difference was not significant from livers preserved similarly and reperfused without glycine. The addition of glycine to UW solution or the addition of hepatamine to the reperfusion medium also had no significant effect on bile production, which remained low compared to 24-h or 0-h preserved livers. The differences in TTW and K/Na ratio were not significant between these groups.

The results in Table 3 show how glycine and hepatamine affected bile production, TTW, and the K/Na ratio in UW solution with GSH. The addition of hepatamine to the reperfusion medium reduced bile production after 48-h preservation of the liver, but the difference was not significant when compared to livers similarly preserved in UW solution with GSH and reperfused with 10 mM glycine or without (i.e., controls). TTW and K/Na ratio were not significantly different between these groups.

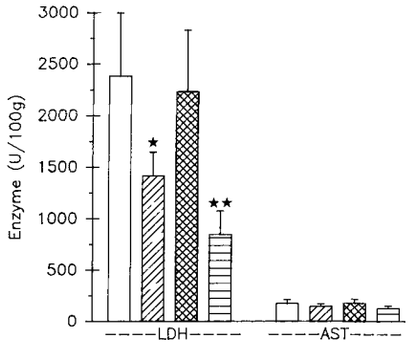

Fig. 1 Effect of 48 h of preservation of rabbit livers in UW solution without GSH on enzyme release during isolated reperfusion for 2 h. Values represent means and SEM [□ control (48-h preserved liver in UW solution without GSH, $n = 6$); ▨ glycine (10 mM) in KHB, $n = 8$; ▩ glycine (15 mM) in UW solution, $n = 6$; ▤ hepatamine in KHB, $n = 4$] * $P = 0.08$ (NS), ** $P < 0.05$ vs control

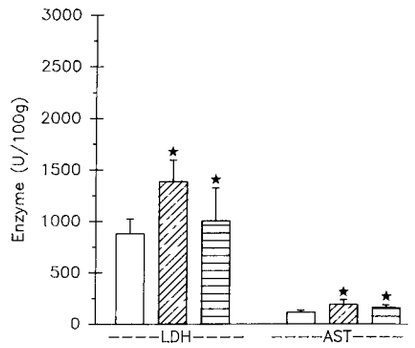

Fig. 2 Effect of 48 h of preservation of rabbit livers in UW solution with GSH on enzyme release during isolated reperfusion for 2 h. Values represent means and SEM [□ control (48-h preserved liver in UW solution with GSH, $n = 9$); ▨ glycine (10 mM) in KHB, $n = 4$; ▤ hepatamine in KHB, $n = 4$]. * P = NS vs control

Figure 2 shows the effects of LDH and AST release of glycine and hepatamine in KHB on livers preserved for 48 h in UW solution with GSH. There was no beneficial effect of these additives compared to the standard UW solution.

Discussion

Previous studies from our laboratory [2] as well as from others [13, 16] have shown that GSH is important in maintaining viability of the liver as tested in orthotopic transplantation in the dog and rat. Glycine is one of the constituent amino acids of GSH that is catabolized to its constituent amino acids in the liver. Thus, the cytoprotective effects of GSH in liver preservation could be derived from one of its breakdown products, glycine. The fact that glycine is cytoprotective in models of hypoxia [20] and cold ischemia [11, 12] has been well documented. In this study we measured the effects of replacing GSH in the UW solution with glycine on rabbit liver functions and hepatocellular injury following preservation. We also measured the effect of glycine in the reperfusion medium on the liver. Finally, we measured the effects of hepatamine, added to the reperfusion medium, on liver viability after 48-h preservation. Hepatamine is an FDA-approved parenteral nutritional supplementation solution containing essential and nonessential amino acids, including glycine. We chose this solution because we thought that if glycine administration to the reperfused liver proved to be beneficial, a solution that contained glycine and that could be administered to patients without excessive costs for FDA approval could be important in liver transplantation. One such solution would be hepatamine.

Although hepatamine contains a mixture of other amino acids that may be effective in their own right, Marsh et al. [11] showed that other amino acids (e.g., isoleucine) were not as effective as glycine in preventing the release of LDH from cold-stored hepatocytes. Histidine, one of the key components of a new abdominal organ preservation solution, histidine-tryptophane-ketoglutarate (HTK), has been suggested to be effective for liver preservation [4] because of its hydrogen ion buffer capacity. Sumimoto et al. [17] demonstrated, however, that histidine added to UW solution could easily be replaced by isoleucine with relatively poor hydrogen ion buffer capacity and, therefore, that histidine may act as an impermeant for suppression of hepatocellular-induced cell swelling. Thus, histidine may not be the important component in hepatamine that prevents LDH release.

The results of this study show that during hypothermic preservation there is a time-dependent loss of bile production and hepatocellular damage, as indicated by the release of LDH into the reperfusion medium. A statistically significant depression of bile flow and increased LDH release occur after 48 h of preservation and reperfusion. Similar results have been reported by others [7]. The omission of GSH from the UW solution used to preserve livers for 48 h resulted in greater injury than when GSH was present.

Therefore, in order to determine whether glycine had a beneficial effect in liver preservation or reperfusion, we used the 48-h preservation model, without GSH. The addition of 15 mM glycine to the UW solution in place of GSH was without beneficial effect on bile production, enzyme release, total tissue water, or ratio of tissue K/Na. However, reperfusion of 48-h preserved livers with glycine (10 mM) or hepatamine reduced the degree of hepatocellular injury, as indicated by the (marginally) significant reduction in release of LDH from the liver during reperfusion. This was the only parameter measured in this study that was slightly improved by glycine; bile production and other measures of liver viability were not affected

by the presence of glycine or hepatamine in the reperfusion medium.

The amount of LDH release in the perfusion group cold stored in UW solution without GSH and reperfused with 10 mM glycine in the reperfusion medium was, however, still higher than in the control UW with GSH group. Since livers cold stored in UW solution with GSH for 48 h and reperfused with glycine or hepatamine in KHB did not result in a decrease in LDH release compared to controls (i.e., UW solution with GSH), glycine as a constituent amino acid of GSH might contribute to the importance of GSH in liver preservation by its action during reperfusion to prevent oxygen free radical damage [15].

The mechanism of action of glycine in cytoprotection of cells exposed to hypoxia or cold ischemia has been suggested due to stabilization of the plasma membrane. Glycine suppressed the release of intracellular enzymes for damaged cells. However, glycine had no protective effects on other cellular functions including protein synthesis [19], mitochondrial functions [21], GSH regeneration [19], or ATP production [12, 21]. This membrane effect of glycine and the relationship between analogues of glycine and cytoprotection of hypoxic renal tubules have lead some investigators to suggest that glycine functions through specific membrane-bound receptors [22]. In this study the primary beneficial effect of glycine was the reduction in membrane damage as indicated by the decreased release of LDH from the issue. However, another function of the liver, bile production, was not affected by glycine and remained depressed. Thus, glycine may be beneficial in that it stabilizes the cellular membrane during reperfusion of the cold-stored liver, but it may not have a dramatic effect on other functions that are lost during hypothermic preservation.

In conclusion, the omission of GSH from UW solution reduces its preservation efficacy, and glycine does not (at least not in the rabbit model used here) appear to be a suitable replacement for GSH. The use of glycine or hepatamine in reperfusion of the liver is also of questionable value in improving liver viability. Although these treatments did suppress hepatocellular damage, they did not improve another function of the liver that may be more critically related to viability, bile production. The remarkable beneficial effects of glycine demonstrated by Weinberg et al. [20] and others, however, may be useful in future developments of preservation solutions, and the effects of glycine should be tested in the orthotopic transplant model of liver preservation in the dog or rat.

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