L-Alanyl-Glutamine Attenuates Oxidative Stress in Liver Transplantation Patients

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ABSTRACT

Background. Ischemia/reperfusion injury during liver transplantation can cause severe damage to the graft. The objective of this randomized, double-blind study was to evaluate the possible protective effects of L-alanyl-glutamine on the liver graft.

Methods. The sample included 33 patients from a liver transplantation service in Northeastern Brazil. Before cold ischemia, the patients received 50 g of L-alanyl-glutamine (treatment group) or saline (control group) through the portal vein. The graft was biopsied at the time of recovery, at the beginning of warm ischemia, and at the end of transplantation to determine malondialdehyde (MDA), heat-shock protein (Hsp)70, nuclear factor kappa-beta (NFkB), superoxide dismutase (SOD), and reduced glutathione (GSH) levels.

Results. The blood parameters were similar in the two groups. In the treatment group, MDA did not increase at the beginning of cold ischemia and decreased at the end of transplantation. This phenomenon was not observed in the control group. GSH, SOD, Hsp70, and NFkB levels were similar in the two groups.

Conclusions. Our findings suggest that preconditioning with L-alanyl-glutamine attenuates the effects of ischemia/reperfusion-related oxidative stress and reduces lipid peroxidation in the grafts of liver transplantation patients.

ISCHEMIA/REPERFUSION INJURY (IRI) has been associated with damage from reactive oxygen species (ROS) and may be directly responsible for primary liver graft dysfunction. Sinusoidal endothelial cell injury is central to the pathophysiology of graft injury [1,2].

ROS release is one of the first and most important components of tissue injury after reperfusion of ischemic organs and is the main cause of hepatocyte apoptosis. Evidence suggests that ROS are critical mediators of hepatic IRI associated with liver transplantation or with liver inflammation and/or infection [3].

Early graft dysfunction has a major impact on prognosis and survival in post-transplantation patients [4–6]. The incidence of graft dysfunction is up to 27% in recipients of liver grafts from deceased donors [7]. This is the most common cause of early re-transplantation [8], with an incidence of 4% to 8% in the literature [9–15].

Much effort has been invested in discovering how to attenuate the effects of injury from ischemia/reperfusion. The proposed methods include ischemic preconditioning, modified preservation solutions, and administration of antioxidants [16,17].

The use of antioxidants to neutralize hepatocytic ROS production constitutes an important adjuvant treatment for IRI and inflammatory disease [18]. Glutamine, a glutathione precursor and a potent natural antioxidant, has turned out to be a promising weapon in the fight against the harmful effects of oxidative stress [19].

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Several antioxidants have been used to reduce the effects of oxidative stress in the body and being extended to decrease ischemia/reperfusion. Antioxidants have been applied also to the organs to be transplanted. Glutathione is a potent antioxidant body that antagonizes the effects of oxidative stress. Glutamine is a precursor of glutathione and has been studied in nutraceutical doses to prevent such damage in the body.

The beneficial effects of glutamine have been demonstrated in laboratory animals, including organs used for transplantation such as the pancreas, lung, kidney, and liver [19,20,23,34].

No clinical study, however, examined the glutamine in humans undergoing liver transplantation.

Given the low proportion of donors, studies are needed to raise the quality of the grafts to be transplanted. This tends to contribute to a better use of organs and probably decreased morbidity and post-operative mortality rates.

The objective of the present study was to evaluate the possible protective effects of preconditioning with L-alanyl-glutamine on the grafts of liver transplantation patients.

METHODS

In this prospective, systematic, randomized, double-blind study, 33 patients were submitted to liver transplantation at a specialized service in Northeastern Brazil (Hospital Universitário Walter Cantidico/HUWC, Universidade Federal do Ceará/UFC) between May 22 and December 17, 2013, with the use of grafts from deceased donors.

This prospective study initially randomly assigned 40 patients into 2 groups. This clinical research and this sample were supported in the literature [20,21,33,34]. However, during the course of the work, we did not have enough supplies and logistics for all samples, and only 33 patients were included in this study.

The patients were randomly assigned to one of two groups. The first subject was randomly allocated into one group. The patients were then systematically assigned to one of two groups: 1) control group (n = 17): after laparotomy, 40 min before cold ischemia, 80 mL of saline solution was administered through the portal vein; 2) treatment group (n = 16): after laparotomy, 40 min before cold ischemia, 80 mL of solution containing 50 g L-alanyl-glutamine was administered through the portal vein.

The primary outcome was oxidative stress evaluation (malondialdehyde [MDA], reduced glutathione [GSH], and superoxide dismutase [SOD] activity). The secondary outcome was graft dysfunction (aspartate aminotransferase [AST], alanine aminotransferase [ALT], and bilirubin).

The exclusion criteria were age under 6 years or over 70 years, donors from other cities, re-transplantation, fulminant hepatitis, and liver grafts with >60% steatosis.

In both groups, the grafts were preserved in Custodiol solution. Before the transplantation (S1), at the beginning of warm ischemia (S2), at the end of the transplant procedure (S3), and 1st (S4), 3rd (S5), 7th (S6), and 30th postoperative days (S7), were collected in the peripheral blood of the transplanted patients’ samples for measurement of AST, ALT, total bilirubin, and international normalized ratio (INR) (Table 1).

Liver segment II was biopsied to determine baseline tissue parameters (B0).

The liver segment II was biopsied again at the end of warm ischemia (B1) and at the end of the transplantation procedure (B2). The biopsy liver tissue was used to determine the levels of MDA, GSH, SOD, heat-shock proteins (Hsp70), and nuclear factor kappa-beta (NFκB).

Liver segment II was chosen only to standardize the place of sample collection.

Lipid peroxidation was measured with the use of a QuantiChrom TBARS kit (BioAssay Systems). GSH levels were quantified by use of a QuantiChrom glutathione assay kit (BioAssay Systems) with the use of Ellman’s reagent (5,5′-dithiobis-[2-nitrobenzoic acid]). Free thiols react with this compound, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate, with a peak absorbance at 412 nm. In the SOD assay, the superoxide anion (O2•−), which is produced by xanthine oxidase–assisted catalysis, reacts with WST-1, forming a compound with a peak absorbance at 440 nm. In the presence of SOD, reaction with WST-1 is inhibited. The assay was conducted with the use of a QuantiChrom superoxide dismutase kit (BioAssay Systems). Hepatic Hsp70 and NFκB levels were measured by use of enzyme-linked immunosorbent assay kits S0873Hu and S0824Hu, respectively (Cloud-Clone Corp). Samples from both groups were used, blindly, to the protocol optimizations. Therefore, the resulting data do not contain 16 to 17 samples per group.

The study protocol was previously approved by the HUWC/UFC Research Ethics Committee and filed under No. 216.272 (March 11, 2013).

<p>| Table 1. AST, ALT, INR, and TB Levels at Different Times in Liver Transplantation |</p>
<table>
<thead>
<tr>
<th>Sample</th>
<th>Cont (n = 12)</th>
<th>Glut (n = 12)</th>
<th>Cont (n = 10)</th>
<th>Glut (n = 14)</th>
<th>Cont (n = 13)</th>
<th>Glut (n = 12)</th>
<th>Cont (n = 10)</th>
<th>Glut (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>96 ± 87</td>
<td>159 ± 230</td>
<td>63 ± 53</td>
<td>115 ± 151</td>
<td>1.61 ± 0.57</td>
<td>1.48 ± 0.38</td>
<td>4.89 ± 3.22</td>
<td>3.61 ± 3.20</td>
</tr>
<tr>
<td>S2</td>
<td>1268 ± 942</td>
<td>2080 ± 1709</td>
<td>915 ± 500</td>
<td>1319 ± 859</td>
<td>2.13 ± 0.54</td>
<td>4.72 ± 3.92</td>
<td>3.25 ± 1.11</td>
<td>3.19 ± 1.81</td>
</tr>
<tr>
<td>S3</td>
<td>1555 ± 910</td>
<td>2542 ± 1850</td>
<td>1034 ± 438</td>
<td>1403 ± 878</td>
<td>2.56 ± 0.98</td>
<td>2.77 ± 2.32</td>
<td>3.25 ± 1.37</td>
<td>3.79 ± 1.90</td>
</tr>
<tr>
<td>S4</td>
<td>1875 ± 2211†</td>
<td>1729 ± 2073</td>
<td>1369 ± 1010†</td>
<td>1070 ± 770†</td>
<td>2.30 ± 1.34</td>
<td>2.69 ± 2.36</td>
<td>6.03 ± 8.02</td>
<td>4.21 ± 2.98†</td>
</tr>
<tr>
<td>S5</td>
<td>909 ± 1511†</td>
<td>779 ± 1331</td>
<td>1024 ± 568†</td>
<td>872 ± 670†</td>
<td>1.40 ± 0.35</td>
<td>1.49 ± 0.89</td>
<td>5.93 ± 6.85</td>
<td>3.76 ± 3.47</td>
</tr>
<tr>
<td>S6</td>
<td>86 ± 72</td>
<td>104 ± 79</td>
<td>280 ± 93</td>
<td>262 ± 167</td>
<td>1.20 ± 0.19</td>
<td>1.17 ± 0.22</td>
<td>6.52 ± 6.08</td>
<td>5.43 ± 5.97</td>
</tr>
<tr>
<td>S7</td>
<td>39 ± 21</td>
<td>65 ± 79</td>
<td>60.7 ± 25</td>
<td>102 ± 98</td>
<td>—</td>
<td>—</td>
<td>1.61 ± 1.29†</td>
<td>1.32 ± 1.15†</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard error of the mean; Friedman test followed by Dunn’s post-test, P < .05.

Abbreviations: Cont, control group; Glut, treatment (L-alanyl-glutamine) group; AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, international normalized ratio; TB, total bilirubin.
† Versus S1 control group.
RESULTS

The characteristics of the donors and the graft recipients were similar in the two groups (Tables 2 and 3). Likewise, the groups did not differ significantly with regard to time of cold and warm ischemia ($P > .05$).

![Fig 1](image)

In the glutamine group, there was one death from severe sepsis, and another patient underwent re-transplantation for primary nonfunction and died. In the control group, there were also two deaths, one for primary nonfunction and another for sepsis. In this control group, there was a re-transplantation for primary nonfunction. The graft survival and mortality rates were similar in both groups.

Graft tissue concentrations of GSH were similar for the two groups at all times ($B_0$, $B_1$, $B_2$). However, in the control group, GSH tissue concentrations were significantly lower at $B_1$ than at $B_0$ ($P < .001$) and at $B_2$ than at $B_0$ ($P < .01$). Similarly, in the treatment group, concentrations were significantly lower at $B_1$ than at $B_0$ ($P < .01$) and at $B_2$ than at $B_0$ ($P < .001$).

Graft tissue concentrations of MDA were similar in the two groups at all times ($B_0$, $B_1$, $B_2$). However, in the control group, MDA concentrations were significantly higher at $B_1$ than at $B_0$ ($P < .01$), whereas $B_2$ and $B_0$ were statistically similar (Fig 1). In the treatment group, $B_1$ was statistically similar to $B_0$, but MDA levels were significantly lower in $B_2$ than in $B_0$ ($P < .001$) (Fig 2). Graft tissue concentrations of SOD were similar in the two groups at all times ($B_0$, $B_1$, $B_2$). The same was true for Hsp70 and NFKB (results not shown).

DISCUSSION

To our knowledge, this is the first study to evaluate the protective effect of preconditioning with L-alanyl-glutamine on liver grafts in humans.

The choice of L-alanyl-glutamine dose (50 g) was based on a study by Alves et al [20], who successfully minimized IRI in the lower limbs of patients submitted to revascularization. The dose is believed to be applicable to liver grafts regardless of donor weight.
The phenomena observed in the present study are still poorly understood because of the lack of research on glutamine administration to liver transplantation patients. According to Maring [25], graft dysfunction involves multiple factors and little-known mechanisms. Unlike in vitro and animal studies, clinical studies are difficult to control because recipients are subject to a constellation of complex circumstances, even during the surgical procedure, which may interfere with the protective effects of glutamine [25].

Despite the contrary laboratory evidence published by Lin et al [26] and Sozen et al [23], in our study, the administration of L-alanyl-glutamine before graft perfusion had no influence on inflammation, as indicated by the lack of a significant difference between the treatment group and the control group with regard to NFkB levels. Alternatively, glutamine may influence inflammatory parameters other than NFkB. More recently, Lin et al [26] reported that glutamine appears to have a protective effect on steatotic liver tissue. The absence of steatosis from most of the grafts included in our study may explain why NFkB levels remained unaffected by treatment with L-alanyl-glutamine.

IRI did not affect graft tissue concentrations of NFkB in either group of patients. This disagrees with a study by Peralta et al [27], who demonstrated that ROS-related IRI can activate NFkB. Conceivably, in human liver grafts, the mechanism responsible for ROS-related damage does not necessarily involve NFkB activation.

As in a study by Schuster et al [28], SOD levels remained unchanged in both the treatment group and the control group. The administration of L-alanyl-glutamine was not associated with increased SOD concentrations in our patients. In contrast, Xu et al [29] found glutamine to protect the bowels of rats submitted to IRI. It would be reasonable to assume that glutamine produces a stronger effect when combined with other antioxidants [28].

In both groups, GSH concentrations were depleted after the graft had been submitted to ischemia and reperfusion, probably as a consequence of the IRI mechanism itself and subsequent damage from oxidative stress, as described in a review by Eltzschig and Carmeliet [30]. However, unlike in Xu et al [29], in our study, L-alanyl-glutamine induced no changes in GSH levels.

Likewise, and in contrast with recent results published by Hwang et al [31], preconditioning with L-alanyl-glutamine did not affect the concentration of Hsp70 (which is believed to protect graft tissue), possibly because these proteins are not activated by ischemia and reperfusion in human liver grafts. In addition, no association was observed between Hsp70 levels and time of cold ischemia or reperfusion.

One might speculate that a longer period of preconditioning with L-alanyl-glutamine would have affected our results, but L-alanyl-glutamine was delivered in bolus directly into the portal vein, favoring absorption by the liver. This route of administration differs from the route used by Mondello et al [32] and Pires et al [33] (peripheral intravenous) and by Xu et al [29] (peritoneal).

The present study allows us to infer that L-alanyl-glutamine protects the liver graft against lipid peroxidation, as shown by the finding that MDA concentrations remained unchanged after cold ischemia, whereas one would expect them to increase because of IRI, especially by the end of the procedure [21]. In our study, MDA concentrations in the treatment group decreased after reperfusion, suggesting a potential protective effect of preconditioning with L-alanyl-glutamine against oxidative stress and, consequently, IRI. This phenomenon was not observed in the control group.

MDA concentrations also decreased in pancreatic islets from human subjects studied by Avila et al [34] and in lower limbs submitted to IRI in work by Alves et al [20], matching our findings. Similar results were reported for animal models by Zhang et al [19] and Xu et al [29].

However, the present study was conducted on human liver recipients, and measurements were taken at different moments. Our sample of 33 patients is relatively large, considering the difficulty of conducting clinical studies. Only 14 patients were included in the sample of Tsai et al [21], and Avila et al [34] evaluated 6 human pancreases. Even animal studies have been conducted on mostly small samples, as in Shuster et al [28] and Lin et al [26], both of whom evaluated 6 rats. It is hoped the present study will contribute to better establish the effects of preconditioning with L-alanyl-glutamine.

CONCLUSIONS

This randomized, double-blind study of liver transplantation patients was designed to help clarify the mechanisms of ischemia/reperfusion injury in humans. On the basis of MDA concentrations, preconditioning with L-alanyl-glutamine...
reduced IRI-induced lipid peroxidation in the grafts of patients submitted to liver transplantation.

The complexity of the biochemical and metabolic factors involved in IRI makes it a major challenge for clinical research. Consequently, the positive effects of preconditioning with L-alanyl-glutamine on graft recipients, as observed in this study, open up important lines of investigation. Because of the lack of similar studies, we believe that the present model may serve to validate a range of hypotheses regarding graft protection against ischemia/reperfusion injury.

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REFERENCES