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"α-Lipoic Acid Reduces Post-Reperfusion Syndrome in Human Liver Transplantation - a pilot study"

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Abbreviations: LT, Liver Transplantation; IRI, Ischemia Reperfusion Injury; ROS, Reactive Oxygen Species; PRS, Post-Reperfusion Syndrome; ALA, Alpha Lipoic Acid; MELD, Model for End-Stage Liver Disease; qPCR, Quantitative Polymerase Chain Reaction; SLPI, Secretory Leukocyte Peptidase Inhibitor; BIRC2, Baculoviral IAP Repeat Containing 2; HIF-1α, Alpha Subunit of Hypoxia-Inducible Factor-1; PHD1, Prolyl-Hydroxylase-1.

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ABSTRACT

A double-blind randomized controlled trial was performed to compare the safety and efficacy of α-lipoic acid(ALA) in liver transplantation. The grafts were randomized to receive ALA or placebo before the cold ischemia time. Furthermore, patients transplanted with the ALA-perfused graft received 600mg of intravenous ALA, while patients with the non-perfused graft received the placebo just before graft reperfusion. Hepatic biopsy was performed 2 hours post-reperfusion. Blood samples were collected before, during and 1 and 2 days after reperfusion. qPCR analysis was performed on biopsies to assess genes involved in the response to hypoxia, apoptosis, cell growth, survival and proliferation, cytokine production and tissue damage protection. Nine of 40 patients developed post-reperfusion syndrome(PRS),

but 7 of them belonged to the control group. There was a decrease in PHD2 and an increase in HIF-1α and Birc2 transcript levels in the biopsies from the ALA-treated vs the control group of patients. Additionally, plasma levels of alarmins were lower in ALA-treated patients than control patients, which suggests that ALA-treated grafts are less inflammatory than untreated grafts. These results showed that ALA is safe for use in liver transplantation, induces gene changes that protect against hypoxia and oxidative stress and reduces the appearance of PRS.

1. INTRODUCTION

In the last decade, there has been a shortage of organs, and therefore, the use of non-optimal donors has increased. Livers from marginal donors show increased susceptibility to ischemia reperfusion injury (IRI) [1, 2]. IRI is considered a major cause of primary graft non-function following liver transplantation and is associated with high rates of morbi-mortality and a high incidence of both acute and chronic rejection[3]. Moreover, severe cases of IRI can lead to multiorgan failure; in this context, one of the major organs affected is the kidney. Another manifestation of hepatic IRI is post-reperfusion syndrome (PRS), which is characterized by hemodynamic instability after reperfusion.

Liver transplantation ischemia is a type of cold ischemia, where the organ lacking blood flow is simultaneously cooled during ischemia. Reactive oxygen species (ROS) and oxidative stress are the most significant mediators of IRI[4]. The ROS generated by hepatocytes at the onset of IRI, in conjunction with cytokine release from Kupffer cells, attract neutrophils to the site of damage[5]. The immediate immune reaction is mediated by the production of proinflammatory cytokines and

chemokines, increased generation of ROS, enhanced adhesion molecule expression and infiltration of tissues by circulating lymphocytes and/or monocytes[5-7].

In animal models, several strategies have been assessed to protect the liver from IRI[8]. However, none of these strategies are currently used in humans to prevent the occurrence of IRI in liver transplantation. Because ROS are a central mediator in IRI, a putative strategy to minimize tissue damage is neutralization of ROS with the use of antioxidants.

α-Lipoic acid (ALA) is a powerful natural antioxidant, acts both intra- and extracellularly and has two reduced and oxidized isomeric forms; due to these pharmacokinetic properties, it has a high potential for pharmacological action[9, 10]. The antioxidant activities of ALA include direct inactivation of ROS, regeneration of endogenous antioxidants (glutathione and vitamin E and C), metal-chelating activity (Fe^{2+}, Cu^{2+} and Cd^{2+}) and repair of tissue damage generated by oxidative stress[9-11]. Additionally, ALA regulates the translational signals that are involved in the IRI process[12].

Currently, there are several preclinical studies that support the use of ALA in IRI models and have shown beneficial effects[13, 14]. In addition, two pilot studies investigating the use of ALA in IRI settings have been reported. The first study examined hepatic reduction, while the second one was carried out in simultaneous kidney-pancreas transplant patients[15, 16]. Both studies showed the beneficial effect of ALA.

Therefore, the aim of the present study was to determine the effect of ALA in patients undergoing liver transplantation by evaluating the several early clinical endpoints such as the appearance of PRS, patient survival and rejection episodes and changes in liver tissue transcripts, plasma alarmins and biochemical liver function parameters. Here, we report the results of a 2 year randomized controlled trial in liver transplant patients.

2. MATERIALS AND METHODS

2.1 Patients

This is a randomized, double-blind, placebo-controlled clinical trial performed to evaluate the use of ALA in IRI in liver transplantation. The study included 40 liver transplant patients (25 men and 15 women; 59.3±10.8 years old). Transplants were performed between September 2015 and December 2016 at the Hospital Italiano of Buenos Aires, Argentina.

The research activities being reported are consistent with the Principles of the Declarations of Helsinki and Istanbul, as outlined in the “Declaration of Istanbul on Organ Trafficking and Transplant Tourism.” The institutional research committee from Hospital Italiano approved this prospective study (n°2394) and the informed consent for the administration of ALA. The use of ALA is not approved in Argentina in liver transplantation; therefore, this study required and obtained the approval of the ANMAT (National Administration of Food, Technology and Medicines).

The recipient selection criteria were as follows: (1) aged 18 to 69 years; and (2) received a liver transplant. The exclusion criteria were as follows: (1) fulminant hepatic failure; (2) reduced liver (split and living donor); (3) liver retransplant. Donors were analyzed according to whether they were optimal donors or expanded criteria donors [ECD, non-heart beating donors, donor >65 years, sodium >155 mEq/L, steatosis >30%, prolonged cold ischemia time (>16 hours), prolonged warm ischemia time (>90 min)].

The patients received induction therapy consisting of methylprednisolone (1 g) and basiliximab (20 mg on days 0 and 4), and they were started on a triple immunosuppressive protocol (tacrolimus with a target level of 8 to 12 ng/ml, prednisone tapered to 4 mg/day and mycophenolate mofetil at 2 g/day).

Samples of liver tissue (wedge biopsy) were obtained from the donor liver, and a second sample was taken from the patient 2 hours after reperfusion because previous studies have shown that IRI in humans becomes detectable after 30 min of ischemia[17]. The tissue was stored in RNAlater at -70°C for quantitative polymerase chain reaction (qPCR) analysis.

Blood samples were obtained and biochemical parameters of liver function were measured before and at the end of surgery (2 hours after the unclamping procedure) on days 1, 2, 3, 7, and 30 after liver transplantation.

2.2 Experimental design

In our previous study, with simultaneous kidney and pancreas transplant patients, we observed better outcomes when ALA was given to the donor and the recipient rather than the recipient alone. This suggested us that an early intervention with ALA, could be more effective to reduce the effect of IRI. However, in this study the Ethical Committee did not authorize to give ALA to the donors, but to the graft. Therefore, and in order to treat the graft as early as possible, we decided to administer ALA directly to the liver by portal vein, before the cold ischemia and before the graft reperfusion. The justification of this regimen of treatment was based on that graft cold ischemia and reperfusion are noxius factors that trigger ROS. Twenty-two patients were recruited and preconditioned with 600 mg of ALA (in 100 ml NaCl) administered to the donor portal vein immediately before the cold ischemia time and another 600 mg of ALA 15 min prior to the reperfusion (ALA-treated group). Eighteen patients received placebo in identical conditions (untreated control group). The dose of ALA chosen for the current study was based on our previous clinical trial, that showed to be safe for patients and grafts [16].

The organs were flushed with 2 L of aortic flush with University of Wisconsin solution, and the organs were then packed and stored in ice until transplantation. The grafts were randomized to receive drug or placebo according to simple random numbers enclosed in numbered and sealed envelopes, which were opened in the pharmacy after the donor organ was accepted.

2.3 SLPI and Reg3a/PAP sandwich ELISA

SLPI was measured by sandwich ELISAs (lower limit of detection: 0.31 ng/ml) as previously described[18]. Reg3/PAP was determined by sandwich ELISA following the manufacturer's instructions (Pancreas PAP, Dynabio, France). Absorbance was read in a plate reader (Rayto, China) at 450 nm with subtraction of 630 nm.

2.4 qPCR analysis

The determination of various transcripts in patient biopsies was performed through qPCR assays. The biopsies were kept at -70°C in RNAlater solution (Ambion, US) until processing. Twenty-seven biopsies were processed (13 controls and 14 of the ALA-treated group). Initially, mRNA purification was performed using the RNeasy Mini Kit (QIAGEN). A dry homogenate was generated with the biopsies using liquid nitrogen. This sample was resuspended in RLT Plus lysis buffer with needles of different caliber for better degradation. A 3 min centrifugation was performed at 10,000 rpm. The supernatant was passed through a gDNA eliminator column (30 sec at 10,000 rpm). The eluate was combined with 70% ethanol, and 0.7 ml was transferred to the RNeasy column. After centrifugation (15 sec at 10000 rpm), the eluate was discarded, and the column was washed with RW1 buffer. RPE buffer was added to the column and washed twice (15 sec and 2 min at 10000 rpm). Dry centrifugation was performed to remove all possible buffer. Finally, 50 µl of RNase-free water was added to the column and centrifuged for 1 min at 10,000 rpm, and the eluate was recovered. Quantification was performed using Nanodrop equipment, and purity was determined by analyzing the relationship between the absorbance at 260 and 280 nm. cDNA was then obtained with an RT2 First Strand kit (QIAGEN). For this, 50 µl of the RNA was centrifuged for 15 sec. The gDNA elimination mixture was

prepared for each sample, and 700 ng of RNA was added to a final volume of 10 µl. The sample was centrifuged and incubated at 42°C for 5 min, immediately switched to ice and incubated for 1 min. The RT cocktail was prepared (according to the protocol). The gDNA elimination mixture was combined with 10 µl of the Cocktail RT and incubated for 15 min at 42°C, and the reaction was stopped by heating at 95°C for 5 min. Finally, 91 µl of distilled water was added.

Amplification and detection of cDNA by real-time quantitative PCR was performed using the GoTaq® qPCR Master Mix reagent in a 96-well format on a Stratagene Mx 3000P kit. The cycling program was as follows: 95°C for 2 min, 40 cycles at 95°C for 15 sec and at 60°C for 45 sec. Finally, a cycle of 1 min at 95°C, 30 sec at 55°C and 30 sec at 95°C was performed. The sequence of primers used were designed and assessed by the research group (Supplementary Table 1). The specificity was evaluated by melting curve analysis and agarose gel analysis after qPCR amplification. The analysis of the results was carried out by the $2^{-\Delta\Delta Ct}$ method, using 28S as the control gene.

2.5 Clinical endpoints

Several early clinical endpoints were assessed in order to evaluate the safety and the effect of ALA administration in liver transplantation. Among them, we determined the occurrence of severe PRS, rejections, graft and patient survival, the levels of plasma alarmins (REG3a/PAP and SLPI) and blood biochemical data that indicated liver and kidney function. PRS was defined as a decrease in the mean arterial pressure of more than 30% of the baseline value for more than one minute during the first five minutes after graft reperfusion. For the analysis, patients were classified

in two groups: i) without or with mild PRS; and ii) with severe PRS. The definition of mild and severe PRS was based on Hilmi et al. study [19]. Patients with mild PRS were those that showed a short-lived (≤ 5 minutes) of a decrease in blood pressure and/or heart rate less than 30% of the anhepatic levels and respond to the i.v. administration of 1 g CaCl2 and/or i.v. boluses of ≤ 100 µg epinephrine without requiring continuous infusion of vasopressor agents. On the contrary, severe PRS was defined as patients with persistent hypotension (more than 30% of the anhepatic level), asystole, or hemodynamically significant arrhythmias; patients who required a vasopressor infusion during the intraoperative period and infusion continued through the postoperative period. Other presentations of sever PRS included prolonged (defined as greater than 30 minutes) or recurrent (defined as reappearing within 30 minutes after resolution) fibrinolysis that required treatment with antifibrinolytic agents.

Furthermore, several transcripts were analyzed in liver biopsies derived from genes involved in the response to hypoxia (HIF-1α, HIF-2α, PHD1 and PHD2), apoptosis (Birc2), cell growth, survival and proliferation (Sestrin2, mTOR, Reg3a), cytokine production (IκBα) and tissue damage protection (SLPI).

2.6 Statistical analysis

All data are expressed as the mean ± standard deviation or median (interquartile range) as indicated in each figure or table. Statistical differences between experimental groups were calculated by GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA, 1994-1999). Mann-Whitney test, Wilcoxon matched-pairs signed rank test and Fisher's exact test, were used as indicated in each Figure and Table; $p<0.05$ was considered significant.

3. RESULTS

Based on previous work with ALA administration in simultaneous kidney-pancreas transplant patients[16], we evaluated the effects of ALA administration in liver transplantation. For this single center placebo-controlled clinical trial, 40 patients were recruited and randomly divided into a control group and a group treated with ALA as described in the Materials and Methods. The clinical and demographic characteristics of the patients are shown in Table 1. There were no statistically significant differences between the ALA-treated and untreated groups in recipient and donor age and gender, Model for End-Stage Liver Disease (MELD), Child-Pugh score, pre-transplant serum creatinine, bilirubin, alanine aminotransferase (ALT) and warm and cold ischemia time. Of 40 grafts used in this study, 19 organs belonged to ECD; 9 and 10 in the untreated and ALA-treated group, respectively. The clinical and demographic characteristics of the patients´ that received ECD organs are shown in Table 2. For these groups of patients, there were no statistically significant differences between the ALA-treated and untreated groups, except for pre-transplant total serum bilirubin and MELD, which were higher in ALA-treated than untreated group of patients (Table 2).

3.1 RT-qPCR analysis of liver biopsies

To assess the impact of ALA treatment directly on the graft, we first analyzed mRNAs related to the response to hypoxia in liver biopsies obtained at the end of the reperfusion by qPCR. Figure 1 shows that ALA-treated patients had significantly higher and lower levels of HIF-1α and PHD2, respectively, than the untreated group, suggesting that the perfusion of the organ with ALA neutralizes the ROS produced under hypoxia and protects the liver parenchyma from further damage. However, no

difference was observed between the ALA-treated group and untreated group in other hypoxia-related genes, such as HIF-2α and PHD1 (Figure 1). The effect of the treatment was further analyzed by studying other transcripts related to apoptosis (Birc2), cell growth, survival and proliferation (Sestrin2, mTOR, Reg3a), cytokine production (IκBα) and tissue damage protection (SLPI). Figure 2 shows that the Sestrin2, mTOR, REG3a, IκBα and SLPI transcript levels were similar in both groups of patients. However, transcript levels of Birc2 were significantly higher in the ALA-treated group than the control group (Figure 2).

3.2 Plasma alarmin levels in liver transplant patients

After the liver transplant, an acute stress systemic response was expected and was detected by measuring plasma alarmins, such as SLPI and PAP. Both proteins were assessed in the plasma of transplanted patients before reperfusion, during reperfusion and on the 1st and 2nd days after the surgery by sandwich ELISA. Figure 3 shows that the levels of both proteins significantly increased on day 1 after surgery in control patients compared to the values found during reperfusion. However, the increase in ALA-treated patients was milder for SLPI, while REG3a/PAP was not changed compared to the values found in reperfusion plasma samples (Figure 3). Plasma sample values were quite different for each patient. Therefore, the data are shown compared to the values found for each patient before the surgery (Figure 3).

3.3 Clinical follow up of patients

The appearance of severe PRS was recorded during the surgery, as was described in Materials and Methods. Notably, 9 of 40 patients developed severe PRS, but only two of them belonged to the ALA-treated group (Figure 4 and Table 3). Furthermore,

patient health was followed by monitoring the serum levels of total bilirubin, ALT and creatinine on the 1st, 3rd, 7th and 30th days after the surgery. Table 4 shows that there was no difference between the untreated group and ALA-treated group in these parameters.

Other variables analyzed were rejection episodes, the need for dialysis and patient survival. Three patients needed dialysis during the first week of transplantation in each group (Table 3). Severe rejection episodes were observed in 4 of 40 patients at 30 days, but only 1 belonged to the ALA-treated group (Table 3). Three patients died at six months post-transplant; two from the untreated and one from ALA-treated group of patient (Table 3).

As we mentioned above, 19 patients were transplanted with organs belonged to ECD. The analysis on those patients revealed that from a total of 6 patients that suffered PRS, 5 belonged to untreated group and only 1 to the ALA-treated group (Table 5). Furthermore, in this ECD group of patients, the total serum bilirubin measured at 30^{th} day post-transplant was lower in ALA-treated group than untreated. Three patients needed dialysis in untreated and only 1 in ALA-treated patients (Table 5). Severe rejections episodes were observed in 2 untreated and none in ALA-treated patients at 30 days post-transplant. Furthermore, the only 1 patient that died within the ECD group, belonged to the untreated group (Table 5).

4. DISCUSION

IRI in liver transplantation is a complex process that has an impact on short- and long-term graft function. One of the major factors involved in this phenomenon is the generation of ROS[20]. In the present clinical trial, we explored antioxidant therapeutic intervention to reduce early complications induce by ROS stress during liver transplantations. The antioxidant drug chosen was ALA, and its administration was done twice through the portal vein, one before cold ischemia and another before reperfusion. This experimental design was done in order to precondition the graft and alleviate ROS stress induced by cold ischemia and reperfusion injury. As main result, we found that ALA-treated group had a lower incidence of PRS than control patients.

The use of ALA in a transplantation setting is not a new strategy. In a previous work with simultaneous kidney-pancreas transplant patients, we showed that ALA treatment had a positive influence on early clinical outcomes and even on mRNA expression of various genes in the graft biopsies. Here, we also found that neutralizing ROS with ALA modified the expression of several transcripts related to hypoxia. We observed decreased levels of PHD2, but not the PHD1 isoform, in liver biopsies obtained after reperfusion. These enzymes post-translationally modulate the activity of the α-subunit of HIF-1. The hydroxylation of HIF-1α by PHD promotes its degradation. As the presence of oxygen is a requirement for this activity, this process is suppressed in hypoxia, allowing HIF-1α to escape degradation and activate the transcription of target genes. In fact, in parallel to the decrease in PHD2, we found an increase in HIF-1α (Figure 1), which was expected. The lack of changes in PHD1 and HIF-2α is not a surprise since PHD1 shows a preference for HIF-2α,

while PHD2 has a stronger substrate preference for HIF-1α[21]. Furthermore, in line with these results, Bernhardt W. *et al*. determined that reduction of PHD with a PHD inhibitor improved short- and long-term results after allogeneic transplantation in an animal model[22].

In contrast to the clear effect of ALA treatment on hypoxia-related genes, we did not observe changes in transcripts related to cell growth, survival and proliferation (Sestrin2, mTOR, Reg3a), a negative regulator of the NF-κB pathway (IκBα) and SLPI, which has a role in wound healing. However, we observed higher levels of Birc-2 transcripts in biopsies from ALA-treated patients than untreated control patients. Since Birc2 encodes the protein c-IAP1, which is a caspase inhibitor and co-factor in the TNF signaling pathway[23], we hypothesized that the perfusion of the graft with ALA reduced the cell apoptosis in the graft. This hypothesis is supported by the fact that ALA-treated grafts were less likely to release alarmins, such as SLPI and REG3a/PAP, than control grafts, at least on the 1st day post-transplantation (Figure 3). On the 2nd day post-transplant, plasma levels of alarmins were similar for both group of patients, suggesting that the major effect of ALA is on the graft, probably by reducing the apoptosis and the inflammatory activity of the grafts. These findings are consistent with our previous work[16]. We showed that ALA treatment to the donor and the recipient have better short-term outcomes than those where only the transplant recipient was treated with ALA[16].

The relevance of the molecular and biochemical changes induced by ALA was observed in immediate clinical events, such as PRS. This syndrome, firstly described as a unique event characterized by a decrease in mean arterial pressure greater

than 30 % below the baseline value that last for at least 1 min and occurring during the first 5 min after reperfusion of the graft [24], then, it was classified by Hilmi et al in two groups according to the severity of the PRS[19]. The most severe event was named significant PRS and comprises to those patients with greater hemodynamic instability, a drop in mean arterial pressure and/or heart rate exceeding 30% of baseline, asystole or hemodynamically significant arrhythmias; or the need to start the infusion of vasopressors during the intraoperative period and to continue throughout the postoperative period. Other presentations of significant PRS included a prolonged (defined as lasting more than 30 min) or recurrent (defined as reappearing within 30 min after resolution) fibrinolysis that requires treatment with antifibrinolytic agents. On the contrary, those patients that presented a decrease of mean arterial pressure and/or heart rate that did not reach the 30% of baseline value, lasting for less than 5 min, and responsive to an intravenous bolus dose of CaCl2 (1 g) and/or epinephrine (≤ 100 µg) without the need to start a continuous infusion of vasopressors were classified as mild PRS. Similar to the Hilmi study, herein, patients with mild or no PRS were grouped together in order to compare with those that suffered severe PRS. Regardless the severity of the event, the incidence of this syndrome varies greatly between different studies, ranging from 12%[25, 26] to 77%[27]. In our trial we had a total of 22% severe PRS (9 patients from a total of 40), with a rate of 39% in the untreated control group and only 9% in the ALA-treated group of patients. Notably, this was not related to the presence of ECD in the recruited patients since 50% (9 of 18) and 45% (10 of 22) of patients in the control and ALA-treated groups, respectively, received grafts derived from ECD. In fact, less PRS was observed in ECD group of patients that were treated with ALA-treatment. Furthermore, the 22 % of severe PRS does not seem to be related to donor age, CIT

and WIT since liver transplantation has been done with young donors, short CIT and WIT. Several risk factors have been associated with the appearance and severity of PRS besides donor age, the CIT and WIT. Among them, it is recognized the recipient age, the Donor Risk Index, the presence of macrovescicular steatosis, the mismatch in size between the recipient and the grafted organ, the MELD score, the kidney function, the hemoglobin level, the impaired sympathetic activity and some aspects of the surgical technique. We do not know for sure, which of these risk factors influence more on the incidence of PRS in our study. However, we can speculate that the high recipient age could have been a relevant factor.

There is a consensus about the negative impact of PRS on patient survival measured by intraoperative and hospital mortality. However, it is not clear if PRS would specifically affect the levels of liver and kidney function markers. This could be due to differences in the patient population recruited and the definition of PRS. In fact, Fukazawa *et al.* did not observe early graft dysfunction while Nanashima *et al.* and Chung *et al.* showed an early increase in bilirubin[28-30]. In our study, although the clear effect of ALA on the lower incidence of the PRS, this was not associated with lower levels of plasma bilirubin and ALT but with plasma alarmins. These facts appear contradictory, but it might reflect that serum alarmins levels could be better markers for graft and patients´ survival than bilirubin or ALT. The overall rejection episodes at 30 days post-transplant were very low (10%), being 16.7% in the control and 4.5% in the ALA-treated group of patients. If we considered patients transplanted with ECD, the rejection episodes were 22.2 % and 0 % in the control and ALA-treated group, respectively. Moreover, in these liver transplanted patients with ECD, we observed lower values of total serum bilirubin in ALA-treated vs

untreated patients at 30th day post-transplant (Table 5). This suggest that ALA treatment could benefit and be more relevant in liver transplant patients with ECD. However, this issue required further investigation.

As patient death and graft rejections were low in the overall trial, it was not possible to definitively conclude a statistically significant effect of ALA on those parameters. Nevertheless, the beneficial effect of ALA on the incidence of PRS is very relevant considering the high perioperative mortality associated with this condition. For this reason, it is important to highlight the substantial difference in the incidence of PRS in our study between the treated group and the control group, particularly in patients receiving an organ from ECD (Table 5). Interestingly, by chance ALA treated group showed a higher MELD and bilirubin than untreated group, when the analysis was restricted to the ECD group (Table 2). This reinforce the idea that ALA-treatment could improve, at least the intraoperative clinical adverse events, even in unfavorable conditions.

Some of the limitations of our studies are related to the number of patients recruited and the high variability observed inside each group for each of variables analyzed. Although a higher number of patients would be desirable, our study showed that ALA administration in liver transplant patients is safe and reduces the appearance of PRS. The safety of the drug is manifested by the lack of a higher incidence of death in the treated group, as well as the lack of appearance of greater metabolic dysfunctions or other clinical complications. Currently, a second trial will be undertaken to recruit more patients, mainly with ECD, confirm the beneficial clinical

events of the current trial and further elucidate the mechanisms of action of ALA in the liver biopsies.

Our findings confirm the deleterious consequences of ROS as one of the major mechanisms responsible for graft injury in solid organ transplantation. Additionally, our results emphasize the necessity of an early intervention to achieve appropriate results, probably in the donor, before ablation. Preconditioning to reduce IRI is not a new concept[31, 32]. In an animal model, treatment with steroids or administration of the soluble ligand of P-selectin to donors with brain death increased the survival of the recipient compared to that of the untreated group[33]. However, it is possible that administration of ALA after transplantation or even at another dose could also help improve the outcomes. In fact, preclinical studies with animals have shown better results with high doses of ALA (100 mg/kg) and that lower doses (10 mg/kg), such as that used in our study, are less effective[34].

In general, ALA is a well-tolerated drug that may have minor side effects; however, in our study, these effects were not detected. The lack of intervention strategies at early time points in solid organ transplantation to improve short-term clinical outcomes, plus the safety and good results obtained with ALA, prompted us to propose ALA treatment as a strategy to neutralize the harmful effects of ROS induced by IRI.

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FIGURE LEGENDS

Figure 1. Relative mRNA expression of hypoxia-related genes in liver biopsies of transplanted patients. RT-qPCR was performed on biopsies of liver transplant patients from the treated and untreated patients. Data represent the median with the interquartile range of the relative expression of HIF-1α and 2α, PHD1 and PHD2 mRNA (- ΔCt) in the biopsies of the ALA-treated versus untreated control groups. *p<0.05. Mann-Whitney test.

Figure 2. Relative mRNA expression of genes involved in cell growth, survival and proliferation, and tissue damage protection in liver biopsies of transplanted patients. qPCR was performed on the same biopsies as in Figure 1. Data represent the median with the interquartile range of the relative expression of Birc2, Sens2, mTOR, Reg3a, IκBα and SLPI of the ALA-treated versus untreated control groups. *p<0.05. Mann-Whitney test.

Figure 3. Plasma levels of SLPI and Reg3a/PAP in hepatic transplant patients. Plasma samples were taken before surgery, during reperfusion and on the1st and 2nd days after surgery. SLPI and PAP index were calculated for each patient relative to plasma sample values obtained before the surgery. Data represent the mean±SEM of the control untreated and ALA-treated groups. *p<0.05, Wilcoxon matched-pairs signed rank test.

Figure 4. Post-reperfusion syndrome in liver transplant patients treated or without ALA. Post-reperfusion syndrome was defined as persistent hypotension (decrease in more than 30% of the anhepatic phase), asystole or arrhythmias, patients who required a continuous vasopressor infusion during the intra-operative period. Other presentations of PRS included prolonged or recurrent fibrinolysis that required treatment with antifibrinolytic agents.. *p<0.05, Chi-square test, two-sided.

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Table 1. Demographic clinical data of transplanted patients[1]

	Untreated n=18	*ALA-treated n=22*	*P =*
Recipient Age (years)	61 ± 11.6	58 ± 10.3	0.226[a]
Recipient gender (% Male)	61	64	0.870[b]
Pre-transplant serum creatinine (mg/dl)	1.1 ± 0.72	1.3 ± 1.03	0.495[c]
Pre-transplant serum Total Bilirubin (mg/dl)	3.8 ± 4.42	5.9 ± 6.04	0.229[c]
Pre-transplant serum ALT (UI/L)	226 ±229	140 ± 198.3	0.210[c]
MELD	24 (22-25)	25 (23-29)	0.114[a]
Child Pugh	10 (5.0-12.0)	10 (6.8-12.0)	0.749[a]
CIT (h)	7 ± 1.5	7 ± 1.7	0.678[c]
WIT (min)	56 ± 7.0	67 ± 28.9	0.117[c]
Donor Age (years)	40 ± 16.7	41 ± 15.2	0.804[a]
Donor gender (% Male)	56	64	0.604[b]

[1]Data represent the mean ± SD for recipient and donor age, cold ischemia time (CIT), warm ischemia time (WIT), pre-transplant serum creatinine, total bilirubin and alanine transaminase (ALT); Median (interquartile range) for model for end-stage liver disease (MELD) and Child Pugh score; or percentage for recipient and donor gender. There were no statistically significant differences among the groups; [a]Mann Whitney test; [b]Chi-square; [c]Unpaired t test; two-tailed.

Table 2. Demographic and clinical data of ECD transplanted patients[1]

	Untreated n=9	ALA-treated n=10	*P* =
Recipient Age (years)	65 ± 4.2	60 ± 9.4	0.458[a]
Recipient gender (% Male)	67	70	0.876[b]
Pre-transplant serum creatinine (mg/dl)	0.9 ± 0.34	1.6 ± 1.40	0.182 [c]
Pre-transplant serum Total Bilirubin (mg/dl)	1.9 ± 1.1	6.8 ± 6.3[2]	0.034 [c]
Pre-transplant serum ALT (UI/L)	176 ± 243	173 ± 214	0.975 [c]
MELD	24 (22-25)	26 (24-29)	0.035[a]
Child Pugh	7 (5.0-12.0)	9.5 (6.0-12.0)	0.589[a]
CIT (h)	7 ± 1.7	7 ± 1.4	0.691[c]
WIT (min)	55 ± 6.8	58 ± 7.9	0.357[c]
Donor Age (years)	54 ± 8.4	54 ± 7.5	0.795[a]
Donor gender (% Male)	67	50	0.462[b]

[1]Data represent the mean ± SD for recipient and donor age, cold ischemia time (CIT), warm ischemia time (WIT), pre-transplant serum creatinine, total bilirubin and alanine transaminase (ALT); Median (interquartile range) for model for end-stage liver disease (MELD) and Child Pugh score; or percentage for recipient and donor gender. [a]Mann Whitney test; [b]Chi-square; [c]Unpaired t test; two-tailed.

Table 3. Variables after surgery

Variable	*Total = 40*	*Untreated n=18*	*ALA-treated n=22*	*[1]P =*
Severe PRS	9	7 (38.9 %)	2 (9.0 %)	0.025
1st week dialysis	6	3 (16.7 %)	3 (13.6 %)	0.790
Rejections (at 1st month post-transplant)	4	3 (16.7 %)	1 (4.5 %)	0.204
Deaths (at 6th month post-transplant)	3	2 (11.1 %)	1 (4.5 %)	0.433

[1]Chi-square test, two-sided.

Table 4. Biochemical parameters after surgery[1]

	Day after surgery	*Untreated n=18*	*ALA-treated n=22*	*p*
Creatinine (mg/dl)	***1***	1.7 ± 1.30	1.5 ± 0.64	0.23
	2	1.5 ± 1.02	1.4 ± 0.60	0.30
	3	1.3 ± 0.97	1.3 ± 0.75	0.48
	7	1.0 ± 0.96	1.1 ± 0.87	0.35
	30	1.4 ± 1.21	1.3 ± 0.46	0.28
Total Bilirubin (mg/dl)	1	5.8 ± 5.75	6.2 ± 4.96	0.77
	2	4.2 ± 5.40	4.7 ± 5.03	0.75
	3	3.6 ± 3.64	4.8 ± 6.01	0.46
	7	2.4 ± 1.87	3.3 ± 5.24	0.49
	30	1.15 ± 0.63	1.2 ± 0.77	0.99
ALT (UI/L)	1	740 ± 988	1229 ± 1459	0.23
	2	510 ± 648	800 ± 974	0.29
	3	290 ± 220	515 ± 533	0.10
	7	85 ± 87	161 ± 178	0.11
	30	57 ± 61	47 ± 56	0.61

[1]Data represent the mean ± SD for creatinine, total bilirubin and ALT at different time points after surgery. There were no statistically significant differences among the groups. Two-tailed, unpaired t test, for each time point.

Table 5. Clinical endpoints after surgery for transplanted with Expanded Criteria Donors[1,2]

	Day	*Untreated n=9*	*ALA-treated n=10*	*p*
PRS		5 (55%)	1 (10%)	0.033
Creatinine (mg/dl)	***1***	1.60 ± 1.44	1.70 ± 0.75	0.846
	2	1.54 ± 1.14	1.35 ± 0.58	0.647
	3	1.32 ± 0.96	1.36 ± 0.95	0.928
	7	1.02 ± 1.09	1.42 ± 1.17	0.448
	30	1.77 ± 1.60	1.32 ± 0.53	0.467
Total Bilirubin (mg/dl)	***1***	5.78 ± 7.27	6.43 ± 5.68	0.829
	2	4.81 ± 7.39	3.70 ± 2.73	0.661
	3	3.96 ± 4.88	3.28 ± 2.72	0.710
	7	2.67 ± 1.99	3.03 ± 3.10	0.768
	30	1.51 ± 0.66	0.94 ± 0.47	0.047
ALT (UI/L)	***1***	523 ± 274	731 ± 695	0.415
	2	396 ± 283	463 ± 383	0.673
	3	257 ± 196	333 ± 365	0.587
	7	98 ± 109	163 ± 211	0.416
	30	84 ± 76	50 ± 74	0.353
Dialysis	***7***	3 (33.3%)	1 (10%)	0.213
Rejections	***30***	2 (22.2%)	0 (0%)	0.115
Deaths	***180***	2 (22.2%)	1 (10 %)	0.466

[1]Data represent the mean ± SD for creatinine, total bilirubin and ALT at different time points after surgery. Unpaired *t* test, two-tailed, was used.

[2]Data represent number of patients that suffered PRS, rejection at 30 days after transplantation, need of dialysis during the first week, number of death patients at 6th month after transplantation. Chi-square test, two-tailed was used.