ACETAZOLAMIDE PROTECTS STEATOTIC LIVER GRAFTS AGAINST COLD ISCHEMIA REPERFUSION INJURY

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Running Title: Acetazolamide protects fatty liver against ischemic damage

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Abbreviations: AZ: acetazolamide; CA, carbonic anhydrase; eNOS: endothelial nitric oxide synthase; ERK, extracellular signal regulated kinases; IGL-1 solution, Institute of Georges Lopez-1 solution; IRI: ischemia reperfusion injury; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinases

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Abstract

Ischemia reperfusion injury (IRI) is a primary concern in liver transplantation especially when steatosis is present. Acetazolamide (AZ), a specific carbonic anhydrases (CAs) inhibitor, has been suggested to protect against hypoxia. Here, we hypothesized that AZ administration could be efficient to protect fatty livers against cold IRI. Obese Zucker rats livers were preserved in Institut Georges Lopez (IGL-1) storage solution for 24 hours at 4°C and “ex vivo” perfused for 2 hours at 37°C. Alternatively, rats were also treated with intravenous injection of AZ (30 mg/kg) before liver recovery. Liver injury, hepatic function and vascular resistance were determined. CA II protein levels and CA hydratase activity were assessed, as well as other parameters involved in IRI (endothelial nitric oxide synthase (eNOS), mitogen activated protein kinases (MAPKs) family, hypoxic inductible factor 1 alpha (HIF-1α) and erythropoietin (Epo)). We demonstrated that AZ administration protects efficiently steatotic liver against cold IRI. AZ protection was associated with better function, decreased vascular resistance and activation of eNOS. This was consistent with an effective MAPKs inactivation. Finally, no effect on HIF-1α/Epo pathway was observed. The present study demonstrated that AZ administration is a suitable pharmacological strategy for preserving fatty liver grafts against cold IRI.
Introduction

Limited pool of donor organs for liver transplantation has led to the acceptance of marginal livers such as steatotic ones, despite their higher risk of early allograft dysfunction (EAD) and non-function. In fact, steatotic liver grafts are associated with an EAD rate of 60% compared with less than 5% for nonsteatotic grafts (Selzner et al., 2000; Selzner and Clavien, 2001; Farrell et al., 2008). This is due to their poor tolerance to ischemia reperfusion injury (IRI) (Deschenes, 2013), evidenced by the exacerbated oxidative stress, mitochondrial damage, and microcirculatory alterations (Selzner and Clavien, 2001; Casillas-Ramirez et al., 2006).

Multiple mechanisms are involved in the decreased tolerance of steatotic livers to ischemic injury, and consequently, various pharmacological strategies need to be combined to effectively protect fatty livers. Particular experimental pharmacological strategies to rescue steatotic livers consisted in the improvement of hepatic microcirculation (Hakamada et al., 1997; Caraceni et al., 1999), the inhibition of oxygen-free radical-mediated injury (Nakano et al., 1997; Pesonen et al., 1998; Luo et al., 2012), and the inhibition of proteasome system with bortezomib (Ramachandran et al., 2012; Zaouali et al., 2013). In clinical practice, only a few pharmacological protective strategies, consisting of bezafibrate, an activator of PPAR-\(\alpha\) and \(\beta/\delta\), were used to treat human living donors for liver transplantation (Nakamuta et al., 2005). There are other drugs that could potentially be taken by living donors, but their significant side effects are limiting their use (Chalasani, 2005; Liu et al., 2013). These observations point to the need for more effective and safe drugs for preventing the steatotic liver from hepatic IRI.

Carbonic anhydrases (CAs) and their inhibitors are relevant in many physiological processes and diseases. In mammals, there are 16 isoforms with different catalytic
activity, tissue concentration and subcellular localization and three of them are expressed in the liver (CA II, CA VA and CA XVI) (Pastorekova et al., 2004; Swenson, 2014). In 1956, AZ was the first non-mercurial diuretic to be used clinically. AZ inhibits CAs in the proximal tubule of the nephron, which leads to the inhibition of protons (H⁺) secretion and bicarbonate (HCO₃⁻) excretion triggering the movement of the isotonically water and augmented diuresis (Supuran, 2011). AZ has been used in many hypertensive-related diseases (Supuran, 2011) and recently, it has been reported that AZ protect kidney against IRI through NO activation (An et al., 2013).

Here, it has been evaluated if the treatment with AZ before liver recovery (AZ preconditioning) could be a useful tool to better protect fatty liver grafts against cold IRI. Moreover, the potential mechanisms were investigated.
Materials and methods

Animals

Male homozygous obese Zucker rats, aged 9 to 10 weeks were purchased from Charles River (France) and housed at 22°C with free access to water and standard chow. All experiments were approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 697/14), University of Barcelona, and were conducted according to European Union regulations for animal experiments (Directive 86/609 CEE). All procedures were performed under isoflurane anesthesia inhalation.

Liver procurement

The surgical technique was performed as previously described (Ben Abdennebi et al., 1998). Briefly, after cannulation of the common bile duct, livers were flushed with chilled preservation solution (4°C) by the mean of catheter insertion into the aorta. After cooling, a second catheter was inserted into the portal vein to complete liver rinsing and the whole liver was excised and trimmed of surrounding tissues. Forty milliliters of preservation solution were infused through the aorta and the portal vein. Then, the livers were preserved with further 130 ml of the same solution for 24h at 4°C.

Liver perfusion

Fatty livers were perfused at 37°C via the portal vein in a closed and controlled pressure circuit. Time point 0 was established when the portal catheter was satisfactorily connected to the circuit. During the first 15 minutes of perfusion (initial equilibration period), the flow was progressively increased in order to stabilize the portal pressure at 12mm Hg (Pression Monitor BP-1; Pression Instruments, Sarasota, FL). The flow was controlled by a peristaltic pump (Minipuls 3; Gilson, France). The reperfusion liquid
(150 ml for each perfusion) consisted of a cell culture medium (William’s medium E; BioWhittaker, Barcelona, Spain) with a Krebs-Heinseleit–like electrolyte composition enriched with 5% albumin as oncotic supply. The medium was continuously gassed with 95% O₂ and 5% CO₂ gas mixture and subsequently passed through a heat exchanger (37ºC) and a bubble trap prior to entering the liver. After 120 minutes of normothermic reperfusion, the effluent perfusion fluid was collected for biochemical determination and fatty livers were sampled.

**Drug treatment:**

AZ (Sigma Aldrich (A6011) Spain) was dissolved in NaOH 1M, then pH was adjusted to 9.6 and distilled water was added to reach the final concentration of 60 mg/ml. AZ was injected at 30 mg/kg 10 min before liver procurement, according data reported by Ichikawa et al. (Ichikawa et al., 1998).

**Experimental groups:**

All animals were randomly distributed into different experimental groups, as indicated below:

*Protocol 1: Effect of AZ in liver injury after 24h of cold storage in IGL-1 preservation solution*

1) Group 1: Control 1 (Ctr 1) (n=4): Control livers were flushed via the portal vein with Ringer’s lactate solution immediately after laparotomy without cold storage;
2) Group 2 (n=5): IGL-1: Livers were preserved for 24 hr in IGL-1 solution;
3) Group 3 (n=5): AZ: Livers were pretreated with AZ intravenously at 30 mg/kg, 10 min before liver procurement and then preserved for 24 h in IGL-1.
After 24h cold storage in IGL-1, steatotic livers were removed from preserved solution and flushed at room temperature with 20 ml of Ringer Lactate solution. This flushed liquid is aliquoted and stored for biochemical determination (AST and ALT).

Protocol 2: Effect of AZ in fatty livers injury after cold IRI

To examine the effect of AZ in liver injury and the underlying mechanisms, fatty livers were subjected to 2-hr normoxic reperfusion, in the following groups:

1) Control group (Ctr 2) (n=4): After procurement, steatotic livers were ex-vivo perfused for 2h as described above without prior cold storage.

2) IGL-1 group (n=6): Fatty livers were preserved in IGL-1 preservation solution for 24 hours at 4ºC and then subjected to 2 h of normothermic reperfusion at 37ºC.

3) AZ (n=6): Zucker Ob rats were pretreated by intravenous administration of acetazolamide (AZ) at 30 mg/kg, 10 min before liver procurement. Then, livers were preserved 24h in IGL-1 solution and finally ex-vivo perfused for 2 hours at 37ºC.

Liver injury: transaminase assay.

Hepatic injury was assessed in terms of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in washout liquid or perfusate effluent with commercial kits from RAL (Barcelona, Spain). Briefly, 100 μL was added to 1 ml of the substrate provided by the commercial kit and then transaminases activity was measured at 340 nm with an UV spectrometer and calculated following the supplier’s instructions. Results were normalized using a commercial calibrator (Biocal, RAL, Barcelona, Spain).

Liver function: Bile production and hepatic clearance
Liver function was assessed by measuring bile production and hepatic clearance of bromosulfophthalein (BSP). Bile was collected through the cannulated commun bile duct, and output was reported as μl/g of liver after 120 min of reperfusion.

BSP clearance was assessed as previously reported (Zaouali et al., 2011). Briefly, thirty minutes after the onset of perfusion, 1 ml of BSP (Sigma Aldrich, Barcelona, Spain) at 10 mg/ml was added to the 150 ml of the perfusate. After 120 min of reperfusion, the concentration of BSP in bile was measured at 580 nm with a UV-visible spectrometer and divided by the concentration of BSP in the perfusate at t=30. The result was expressed by percentage ((t120 bile/t30 perfusate)*100).

**Vascular resistance**

Vascular resistance was defined as the ratio of portal venous pressure, which was maintained at 12 mmHg during the reperfusion to flow rate, and expressed in mmHg/min per gram of liver/mL. Perfusion flow rate was assessed continuously throughout the reperfusion period and expressed as mL/min per gram of liver.

**Western blotting technique**

Liver tissue was homogenized in HEPES buffer and proteins were separated by SDS-PAGE and transferred to PVDF membranes as previously described (Bejaoui et al., 2014). Membranes were immunoblotted over night at 4 °C using the following antibodies: anti-carbonic anhydrase II (ab115306, abcam, UK), anti-p-SARK/JNK (Thr183/Tyr185), anti-p-p38 MAP kinase (Thr180/Tyr182, #9211), anti-p-p44/42 MAPK (Erk1/2, Thr202/Tyr204, #9101); the above antibodies were all purchased from Cell Signaling (Danvers, MA), anti-eNOS (610296, Transduction Laboratories, Lexington KY), and anti-b-actin (A5316, Sigma Chemical, St. Louis, MO, USA). After
washing, bound antibody was detected after incubation for 1 h at room temperature with the corresponding secondary antibody linked to horseradish peroxidase. Bound complexes were detected and quantified by scanning densitometry.

**Real-time qRT-PCR**

Total liver RNA was isolated using the TRIzol reagent (Invitrogen). Reverse transcription was realized on a 1 µg RNA sample using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The reaction included incubation at 25 °C (5 min), at 42 °C (30 min) and 85 °C (5 min) and then cDNA was stored at -80 °C. Subsequent PCR amplification was conducted in the iCycler iQ Multi-Color Real-Time PCR (Bio-Rad Laboratories) using SsoAdvanced Universal SYBR Green Supermix and the following rat primers for HIF-1α: forward, 5’- TCAAGTCAGCAACGTGGAAG -3’ and reverse, 3’- GTCAGCTGTGTCGGAGCTAT-5´ and its target erythropoietin (Epo): forward, 5'-CCAGCCACCAGAGGTCTTC -3’ and reverse, 3’-GTGTCGCTATGAAAGACGT-5’. Reactions were carried out in duplicate and threshold cycle values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The ratio of HIF-1α and Epo relative expression to GAPDH was calculated by the ΔCt formula.

**Histology**

Liver samples were fixed in 10% neutral buffered formalin and embedded in Paraplast, and 5-µm sections were stained with hematoxylin and eosin according to standard procedures. Histological evaluation was graded semiquantitatively from 0 (no damage) to 4 (severe cellular damage, such as vacuolization, cell dissociation, cell swelling and disintegration of hepatic architecture)
**Carbonic anhydrase activity**

**Sample preparation**

Each liver sample (approximately, 300 mg) was homogenized in 0.05 M Tris-HCl buffer, pH 7.5. The homogenate was centrifuged twice for 30 min at 12000×g. The resulting supernatant was subject to protein determination and assayed for CO$_2$ hydratase activity.

**Protein determination**

Protein concentration was determined using the Bio-Rad Protein Assay, based on the method of Bradford (Bradford, 1976)

**Carbonic Anhydrases Assay**

CAs activity assay was a modification of the procedure described by Chirica et al (Chirica et al., 1997). The assay was based on the monitoring of pH variation due to the catalyzed conversion of CO$_2$ to bicarbonate. Bromothymol blue was used as the indicator of pH variation. The assay was performed at 0°C adding 1.0 mL ice-cold CO$_2$-saturated water to 1.0 mL mixtures of 25 mM Tris-SO$_4$ buffer. The CO$_2$-saturated solution was prepared by bubbling CO$_2$ into 100 mL distilled water for approximately 3 h. The CO$_2$ solution was chilled in an ice water bath. 50 μL of the liver extract were added to one tube, and an equivalent amount of buffer was added to the second tube as control. One milliliter of CO$_2$ solution was added very quickly and simultaneously a stopwatch was started. The time required for the solution to change from blue to yellow was recorded (transition point of bromothymol blue is pH 6–7). The production of hydrogen ions during the CO$_2$ hydration reaction lowers the pH of the solution until the color transition point of the dye is reached. The time required for the color change is inversely related to the quantity of carbonic anhydrase present in the sample. Detecting the color change is somewhat subjective but the error for triple measurements was in the
range of 0–1 s difference for the catalyzed reaction. Wilbur-Anderson units were calculated according to the following definition: One Wilbur-Anderson unit (WAU) of activity is defined as \((T_0 - T)/T\), where \(T_0\) (uncatalyzed reaction) and \(T\) (catalyzed reaction) are recorded as the time (in seconds) required for the pH to drop from 8.3 to the transition point of the dye in a control buffer and in the presence of enzyme, respectively.

**Fat content:**

Liver tissues were homogenized in 1/10 homobuffer (220 mM mannitol, 70 mM sucrose, 0,1 mM EDTA, 3 mM TRIS and 0,1% de bovine serum albumin (Fatty Acid Free), pH 7.4)) and then diluted 4 times in PBS. Triglycerides, free cholesterol and fatty free acids were determined colimetrically at the Hospital Clinic, Barcelona, Spain.

**Statistical analysis**

The results were analyzed using a one-way ANOVA (Analysis of Variance), with three levels (Control, IGL-1 solution and AZ preconditioning). This approach allowed us to assess whether there was any significant difference in the effects among the three levels and then to explore where such differences existed, based on pairwise comparisons. Adjustments for multiple comparisons were based on the Tukey test. The assumption of normality of residuals and homogeneity of variances was checked using Shapiro-Wilk and Bartlett tests, respectively. For all test, p-value <0,05 were considered statistically significant.
Results

We determined transaminases as a tool for predicting organ damage after cold preservation (Teramoto et al., 1993; Pantazi et al., 2014). Fig. 1 reveals ALT and AST releases in liver perfusate after 24 hr of liver graft storage. The higher transaminases levels released by steatotic livers after 24 hr of cold preservation in IGL-1 solutions group confirm the high vulnerability of fatty livers to cold ischemia (14,3 ± 1,5 versus 74,5 ± 7 U/L for ALT and 18,2 ± 5,5 versus 90,8 ± 23,1 U/L for AST). A significant reduction in transaminases levels in the flushing effluent of fatty livers was observed when AZ was administered previously to graft procurement (41,8 ± 15 versus 75,5 ± 7 U/L for ALT and 45 ± 16,8 versus 90,8 ± 23,1 U/L for AST).

To investigate the possible effect of AZ preconditioning against reperfusion injury in steatotic livers we measured AST/ALT levels in livers preserved for 24 hr in IGL-1 solution followed by 2 hr normoxic reperfusion (37ºC). We also assessed histological analysis. Fig. 2A shows the perfusate AST/ALT levels after 2 hr of normothermic reperfusion in steatotic livers preserved in IGL-1 with or without AZ pretreatment. AST/ALT levels increased in IGL-1 group compared to control group (158 ± 61,7 versus 29 ± 10,9 U/L for ALT and 338,2 ± 156,5 U/L for AST). On the other hand, AZ pretreatment significantly reduced transaminases levels (62 ± 33,5 versus 158 ± 61,7 for ALT and 76 ± 39,7 versus 338 ± 156,5 for AST). This decrease in liver injury was furthermore confirmed by the histological findings (Fig. 2B). The histological study of control steatotic livers (Ctr 2) showed grade II to III of fatty infiltration, with hepatocyte integrity maintenance. In IGL-1 group, fatty infiltration was conserved and severe cellular damage was seen, whereas in AZ group decreased cellular damage was observed when compared to IGL-1 group.
Hepatic function was assessed by bile production and BSP clearance in bile. The prevention of liver reperfusion injury exerted by AZ was consistent with a significant improvement of liver function. As shown in Fig. 3 bile production and BSP clearance decreased in preserved steatotic livers when compared to control ones. In all cases, AZ favored a significant recovery of bile output and % BSP at 2-hr reperfusion when compared to IGL-1 group (26 ± 3.7 versus 8.7 ± 3.9 µL/g.liver/120 min for bile output and 15.2 ± 6.8 versus 38 ± 2.1 of % of BSP).

To investigate the relationship between AZ administration and CAs inhibition, we assessed CAs activity (Fig. 4A). Our results confirm that AZ pretreatment at the dose of 30 mg/kg was efficient to inhibit CAs hydratase activity (0.103 ± 0.034 in IGL-1 group versus 0.020 ± 0.007 WAU/mg protein in AZ group). Moreover, we examined the potential effect of AZ in the regulation of CA II expression, as we have previously shown that its addition to IGL-1 solution protects steatotic livers against IRI (Bejaoui et al., 2015). Surprisingly, we observed that AZ induced a significant induction of CAII protein levels (Fig. 4B).

Recently, several studies have provided evidence that CAs inhibitors of the sulfonamide/ sulfamate type have been considered to be potential antiobesity drugs (Supuran et al., 2008). Thus, we have quantified the fat content by measuring triglycerides, free fatty acids and free cholesterol in liver tissue and we have found that AZ reduced significantly free cholesterol and moderately triglycerides when compared to IGL-1 group after two hours of reperfusion (Fig. 4C).

AZ is indicated in many hypertensive related diseases and it is also well known that enhanced liver resistance during reperfusion is associated with the poor tolerance of steatotic grafts to IRI. Our results show that AZ preconditioning induced a significant reduction in vascular resistance (Fig. 5A). We speculated that this significant reduction
could be correlated with the generation of NO, a well-known vasodilator mediator. For this reason, we determined eNOS activity in steatotic livers subjected to cold ischemia and reperfusion. As indicated in Fig. 5B, we observed a significant eNOS activation when AZ was administered prior to cold ischemia.

During cold ischemia, MAPKs are activated and are responsible for the induction of cellular damage (King et al., 2009). We found that fatty liver preservation in IGL-1 solution resulted in marked induction of MAPKs, when compared with controls, as evidenced by up-regulation of pERK, p-p38 and pJNK. AZ administration prior to cold ischemia reperfusion induced an important reduction of MAPKs (Fig 6).

Finally, we investigated whether AZ preconditioning protective effect could be related to HIF-1α up-regulation. Recently, AZ has been shown to protect kidney against IRI through HIF-1α induction (An et al., 2013). Moreover, HIF-1α upregulation was associated with better preservation of steatotic and non steatotic liver grafts (Zaouali et al., 2010a). Our results show that HIF-1α and its target erythropoietin (Epo) mRNA expression were downregulated after cold ischemia reperfusion. However, AZ pretreatment did not enhance HIF-1α or Epo mRNA expression.
Discussion

Here, we demonstrated that AZ is a promising drug to prevent the deleterious effects of IRI in steatotic livers. Its beneficial effects are reflected by a significant prevention of liver injury and improvement of hepatic function, decreasing the steatotic liver grafts vulnerability to IRI. In our work, steatotic livers were cold preserved in IGL-1 solution pretreated or not with AZ. The rational of the proposed protocol was to induce a pharmacological PC against the subsequent cold storage and reperfusion injury. This could be relevant in clinical situation of brain-dead donors and steatotic livers, both being risk factor in liver transplantation.

The diminished hepatic injury in rats pretreated with AZ was concomitant with a significant decrease in CA hydratase activity and an enhanced expression level of CAII. This is in line with our previous observation showing that CAII addition to IGL-1 solution prevented IRI in steatotic livers which was associated with enhanced CAII expression (Bejaoui et al., 2015). Thus, AZ protective mechanism may be related to enhanced CAII protein expression rather than CAs hydratase activity inhibition.

An important factor for the susceptibility of steatotic livers to IRI is the distortion and narrowing of hepatic sinusoids due to the reduced luminal diameter (up to 50%). This fact leads to alterations of blood flow and microcirculation, hindering the suitable revascularization of the graft (Fukumori et al., 1999; Liu et al., 2013).

Clearly, our results showed that: 1) AZ decreased vascular resistance through eNOS activation; 2) AZ vasodilator effect is correlated to CA inhibition. In literature, the mechanism of AZ induced vasodilatation is controversial because in some cases it was showed to be independent of eNOS activation (Kiss et al., 1999); in others AZ increased NO production through eNOS activation (Tuettenberg et al., 2001; An et al., 2013), and others showed that AZ induced vasodilation is independant of CA inhibition (Hohne et
Recently, Rasmus Aamand et al. have shown that CAII is able to generate vasoactive NO from nitrite at high rates and with no strict requirement for low O$_2$ (Aamand et al., 2009). Interestingly, the addition of AZ increased significantly the CA-catalyzed NO production. The CA-catalyzed NO production is significantly higher at acidic conditions similar to those found during ischemia (pH=5.9) and the reaction also occurs under anaerobic conditions. The authors hypothesize that CAII has two active site, one for CO$_2$ and the other for nitrite and that AZ may increase the affinity for the substrate nitrite by occupying non-productive binding sites on the enzyme. Unfortunately, our experimental model does not permit to corroborate this hypothesis due to the difficulty of determining NO levels and the nitrite-reducing CAs activity in liver tissue during cold ischemia and reperfusion. In our experimental model, we determined CAs activity that corresponded to the CO$_2$ hydratation activity. Further determinations such as eNOS inhibition when AZ was used could provide more information. Also, the isolated rat aortic rings model might be more suitable to examine this hypothesis.

Recently, it has been proposed that the use of selective CAs inhibitors is useful for the development of new antiobesity drugs (Supuran et al., 2008). Quantification of fat content in liver after reperfusion showed that AZ reduce moderately steatosis. This is an interesting result because it gives new insights into the use of CAs inhibitors in defatting cocktails in machine perfusion (Nagrath et al., 2009).

MAPKs consist of (1) Extracellular signal-regulated kinases, ERK 1/2, (2) c-Jun N-terminal kinases 1/2 (JNK 1/2) and (3) p38 MAPK and are signal transducers that transmit messages from the cell surface to the nucleus in response to oxidative and other environmental stress. In case of IRI, MAPKs are well known mediators of stress responses, as they have been associated with upregulation of proinflammatory and cell
death pathways. MAPKs are activated during hypothermia and several minutes after reperfusion and inhibition of their activation has been associated with ameliorated hepatic injury (Yoshinari et al., 2001; Kobayashi et al., 2002; Xu et al., 2005; Zaouali et al., 2010b). In line with this, we report that AZ protective effect has been mediated through an overwhelming decrease in p-ERK, p-p38 and p-JNK.

Hepatic steatosis was shown to contribute to augmented oxidative stress, leading to NF-κB inactivation and impaired HIF1-α induction and thereby increased susceptibility to hypoxic injury (Anavi et al., 2012). HIF-1α is a key transcriptional factor mediating cellular adaptation under stress conditions including IRI. Indeed, it plays an important role in fatty liver protection against cold IRI and its protective action is related to its downstream proteins including Epo and eNOS (Zaouali et al., 2010a; Eipel et al., 2012). Moreover, HIF-1α has been implicated in the AZ beneficial effects against renal IRI (An et al., 2013) and in case of acute mountain sickness (Xu et al., 2009). However, in our experimental conditions results show that AZ protective effects are not related to HIF up-regulation but future investigations for clarifying the effects on HIF are needed.

In conclusion, our results show that AZ preconditioning is a promising strategy for improving the fatty liver graft viability. The beneficial effect was associated with decreased vascular resistance, activation of eNOS and the prevention of MAPKs family activation.
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Authorship Contributions:

Participated in research design: Bejaoui and Catafau

Conducted experiments: Bejaoui, Pantazi, De Luca, Panisello and Serafin

Performed data analysis: Catafau, Folch-Puy, Capasso and Supuran

Wrote or contributed to the writing of the manuscript: Bejaoui, Catafau, Capasso and Supuran
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LEGENDS

Figure 1: Hepatic injury measured as AST and ALT levels after 24h cold storage in IGL-1 solution. AZ administration significantly decreased AST/ALT levels. Ctr1: liver flushed without cold preservation; IGL-1: liver preserved in IGL-1 solution; AZ: liver pretreated with AZ at 30 mg/kg and preserved in IGL-1 solution for 24 hours at 4 ºC. * p < 0.05 vs Ctr1; # p < 0.05 vs IGL-1

Figure 2: Hepatic injury after 2h-normotermic “ex-vivo” perfusion measured by AST and ALT levels (A) and by hematoxylin and eosin staining (B). AZ preconditioning attenuated AST/ALT levels in contrast to IGL-1 group. Histological analysis show that cold ischemia in IGL-1 solution and reperfusion increased hepatic damage, whereas AZ pretreatment diminished hepatic damage; Ctr2: Liver flushed and perfused “ex-vivo” without cold preservation; IGL-1: liver preserved in IGL-1 solution (4 ºC, 24 h) and subjected to 2h- normothermic “ex vivo” perfusion; AZ: animals pre-treated with AZ (30 mg/kg) before fatty liver cold storage in IGL-1 solution (4 ºC, 24 h) and following to 2h- normothermic “ex vivo” perfusion. * p < 0.05 vs Ctr 2; # p < 0.05 vs IGL-1

Figure 3: Effect of AZ preconditioning in liver function expressed as bile output (A) and % of BSP excretion in bile (B) in steatotic livers after 120 min of normothermic “ex vivo” perfusion. AZ administration ameliorated liver function when compared to liver grafts preserved in IGL-1 solution. Ctr 2: Liver flushed and perfused “ex-vivo” without cold preservation; IGL-1: liver preserved in IGL-1 solution (4 ºC, 24 h) and subjected to 2h- normothermic “ex vivo” perfusion. AZ: animals pretreated with AZ before liver cold storage in IGL-1 solution (4 ºC, 24 h) and following to 2h-normothermic “ex vivo” perfusion. * p < 0.05 vs Ctr 2; # p < 0.05 vs IGL-1
Figure 4: Effect of AZ preconditioning in CA II protein expression (A) CAs activity (B) and fat content (C). AZ administration decreased CA activity and augmented the expression of CA II. Interestingly, AZ reduced moderately triglycerides and free fatty acids and diminished significantly free cholesterol levels when compared to IGL-1 group. Ctr 2: Liver flushed and perfused “ex-vivo” without cold preservation; IGL-1: liver preserved in IGL-1 solution (4 ºC, 24 h) and subjected to 2h- normothermic “ex vivo” perfusion. AZ: animals pretreated with AZ before liver cold storage in IGL-1 solution (4 ºC, 24 h) and following to 2h- normothermic “ex vivo” perfusion. * p < 0.05 vs Ctr 2; # p < 0.05 vs IGL-1

Figure 5: Effect of AZ pretreatment in liver vascular resistance (A) and eNOS activity (B) in steatotic liver after 120 min of normothermic “ex vivo” perfusion. AZ pretreatment significantly reduced vascular resistance and promoted eNOS in contrast to IGL-1 group. Ctr2: Liver flushed and perfused “ex-vivo” without cold preservation; IGL-1: liver preserved in IGL-1 solution (4 ºC, 24 h) and subjected to 2h- normothermic “ex vivo” perfusion; AZ: animals pre-treated with AZ before liver cold storage in IGL-1 solution (4 ºC, 24 h) and following to 2h- normothermic “ex vivo” perfusion. * p < 0.05 vs Ctr 2; # p < 0.05 vs IGL-1

Figure 6: The role of AZ pretreatment in MAPKs activation: Western blotting and densitometric analysis of p-38 (A); p-ERK (B) and p-JNK (C) in steatotic liver after 120 min of normothermic “ex vivo” perfusion. AZ prevented MAPKs activation. Ctr2: Liver flushed and perfused “ex-vivo” without cold preservation; IGL-1: liver preserved
in IGL-1 solution (4 ºC, 24 h) and subjected to 2h- normothermic “ex vivo” perfusion; AZ: animals pre-treated with AZ before liver cold storage in IGL-1 solution (4 ºC, 24 h) and following to 2h- normothermic “ex vivo” perfusion. * p < 0.05 vs Ctr1; # p < 0.05 vs IGL-1

**Figure 7**: mRNA expression of HIF-1α (A) and its target Epo (B) in steatotic liver after 120 min of normothermic “ex vivo” perfusion. Cold ischemia reperfusion significantly decreased HIF-1α and Epo mRNA expression both in IGL-1 and AZ groups. Ctr2: Liver flushed and perfused “ex-vivo” without cold preservation; IGL-1: liver preserved in IGL-1 solution (4 ºC, 24 h) and subjected to 2h- normothermic “ex vivo” perfusion; AZ: animals pre-treated with AZ before liver cold storage in IGL-1 solution (4 ºC, 24 h) and following to 2h- normothermic “ex vivo” perfusion.* p < 0.05 vs Ctr 2.
Figure 2

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B
Figure 3
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Figure 5
Figure 6