


Nitrite enhances liver graft protection against cold ischemia reperfusion injury through a NOS independent pathway

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ABSTRACT

Introduction: Nitrite has been found to protect liver graft from cold preservation injury. However, the cell signaling pathway involved in this protection remains unclear. Here, we attempt to clarify if the NOS pathway by using the NOS inhibitor, L-NAME (L-N^G-Nitroarginine methyl ester).

Animals and methods: Rat livers were conserved for 24 h at 4°C in (IGL-1) solution enriched or not with nitrite at 50 nM. In a third group, rats were pretreated with 50 mg/kg of L-NAME before their liver procurement and preservation in IGL-1 supplemented with nitrite (50 nM) and L-NAME (1 mM). After 24 h of cold storage, rat livers were ex-vivo perfused at 37°C during 2 h. Control livers were perfused without cold storage.

Results: Nitrite effectively protected the rat liver grafts from the onset of cold I/R injury. L-NAME treatment did not abolish the beneficial effects of nitrite. Liver damage, protein oxidation and lipid peroxidation remained at low levels in both nitrite-treated groups when compared to IGL-1 group. Antioxidant enzyme activities and functional parameters were unchanged after NOS inhibition.

Conclusion: Despite NOS inhibition by L-NAME, nitrite can still provide hepatic protection during cold I/R preservation. This suggests that nitrite acts through a NOS-independent pathway.

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KEYWORDS

Sodium nitrite; NOS; IGL-1; ischemia reperfusion injury; antioxidant enzymes

Introduction

It is well established that an ischemia/reperfusion (I/R) injury, inherent to cold preservation, is the main cause of primary dysfunction and primary non-function of liver allograft [1]. In fact, the steps of organ transplantation, from graft harvest to graft revascularization, contribute to enhance organ susceptibility to an I/R injury and further increase tissue vulnerability towards injuries. One of the aforementioned steps is organ preservation in which the goal is to maintain the functional, biochemical and morphological integrity of the graft. Various hypothermic preservation solutions have been formulated to maintain cell viability during ischemia and to allow their early functional resumption on reoxygenation. Although Institut Georges Lopez (IGL-1) liquid extends graft viability, severe organ injury is quite common with prolonged cold storage, and graft dysfunction after transplantation still occurs at significant rates. So, improvement of the composition of preservation solution is fundamental to optimize the outcome of liver transplantation (LT).

Several studies have demonstrated that nitric oxide (NO) effectively protected grafts from the onset of an I/R injury [2–4]. NO is able to induce vasodilatation and so to improve graft revascularization [4]. It could reduce platelet aggregation and leukocytes adhesion which limits inflammatory damage during I/R insult [5,6]. It regulates interleukins and others inflammatory mediators' release [7]. More recently, it has been reported that NO could modulate autophagy and apoptosis [8]. In addition, we have demonstrated that the activation of the endothelial nitric oxide synthase (eNOS) and the production of NO by this enzyme increased liver graft preservation and improve liver function after reperfusion [9–11]. Previous reports have also shown that tissue damage during the early phase of reperfusion appears to be associated with decreased NO availability related to eNOS down-regulation [12]. Taken these facts into account, several teams have sought to optimize the composition of the storage solutions by their supplementation with exogenous source of NO [7,13]. In this sense, the use of nitrite therapy as exogenous source of NO proved to be an useful tool to protect organ

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function and integrity from any I/R damage, such as those encountered in organ transplantation [14].

In our recent report, we confirmed that enrichment of IGL-1 preservation solution with nitrite improved liver graft preservation [15]. However, the pathway by which nitrite mediates its hepatocellular protection remains controversial. Some authors suggested that nitrite activity is dependent on NO production but independent from eNOS activity [14,16,17], whereas others showed that NO production is dependent from eNOS activation [3,9]. Duranski et al. showed that nitrite induced a cardioprotective effect in eNOS-deficient mice submitted to warm I/R demonstrating thus that NO protection is independent from eNOS [14].

The main purpose of the present study was to examine whether the NOS pathway is involved in the protective effect of nitrite against a cold I/R injury in isolated liver by using the NOS inhibitor, L-NAME (L-N^G-Nitroarginine methyl ester).

Animals and methods

Animals

Male Sprague-Dawley rats weighting between 250 and 300 g were used. Animals were housed under periodic cycle of 12 h of light/dark and they had free access to water and rat food. This experimental protocol was performed according to European Union regulations for animal experiments (Directive 86/609 CEE) and after agreement of local ethical committee.

Liver extraction and ex vivo perfusion

Rats were anesthetized with an intraperitoneal injection of urethane (5%). Livers were harvested as described previously [11]. Briefly, after cannulation of the common bile duct, livers were flushed, by cold preservative solution, through a catheter introduced into the aorta. After cooling, a second catheter was inserted into the portal vein to complete organ rinsing and the whole liver was excised and trimmed of surrounding tissues. Then, extracted livers were conserved in 50 mL of preservative solution in a hermetically closed container, for 24 h at 4°C.

After cold preservation, livers were maintained during 20 min at ambient temperature in order to mitigate the rewarming phase in liver graft transplantation. Next, livers were rinsed with 10 mL of Ringer's lactate solution and immediately connected to *ex-vivo* perfusion circuit. Time point 0 min corresponds to the collection of rinse solution effluent which coincides with the connection of the portal catheter to the circuit. Livers were then perfused at 37°C *via* the portal vein in a closed and controlled pressure circuit. The flow was progressively increased over the first 15-min in order to stabilize the portal pressure at 12 mm Hg (Pression

Monitor BP-1; Pression Instruments, Sarasota, FL). The flow was controlled by a peristaltic pump (Minipuls 3; Gilson, France).

After 120 min of normothermic perfusion, vena cava effluent and tissue specimens were collected for biochemical determinations.

Experimental groups

Rats were randomly divided into 4 experimental groups (n = 6 for each):

- Control: After procurement, livers were *ex-vivo* perfused for 120 min as described above without prior cold storage.
- IGL-1: Livers were preserved in IGL-1 storage solution for 24 h and then perfused *ex-vivo* at 37°C during 120 min.
- IGL-1 + Nitrite: Same as IGL-1 group but IGL-1 preservation solution was supplemented with nitrite at 50 nM.
- IGL-1 + Nitrite + NAME: 30 min before liver procurement, L-NAME (50 mg/kg) was injected through the vena cava [4]. Then, livers were flushed and preserved in cold IGL-1 solution supplemented with 50 nM of nitrite and 1 mM of L-NAME [4]. After cold preservation, livers were *ex-vivo* perfused at 37°C during 120 min.

Liver damage evaluation

In order to evaluate liver injury, we measured the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the effluent of rinse solution (time corresponding to 0-min reperfusion) and in the effluent of perfusion solution (time corresponding to 120-min reperfusion). Transaminase activities were determined spectrophotometrically with commercial kit according to provider's indications (Biomerieux, France).

Gamma-glutamyl transferase (GGT) and alkaline phosphatase (PAL) are associated with hepatocellular damage [18,19]. They were measured in liver effluents after 120-min reperfusion, by an UV spectrophotometer at 410 and 405 nm, respectively (Siemens, France).

Bile flow

Liver function was assessed by measuring bile production during 120-min reperfusion. Bile was collected through the cannulated common bile duct, and output was reported as $\mu\text{L}/\text{min}/\text{g}$ of liver [11,20].

Vascular resistance

Vascular resistance is calculated according to the following formula: Vascular resistance = Portal pressure/

portal flow, and expressed as mL/min/g of liver. Portal pressure was fixed at 12 mm Hg during the 120-min perfusion and portal flow was controlled by a peristaltic pump.

Malondialdehyde assay

Malondialdehyde (MDA) is an indicator of membrane's lipid peroxidation induced by oxidative stress which generates oxygen species. MDA is measured in hepatic tissue by means of thiobarbituric acid test [11,20].

Sulfhydryl and carbonyl proteins assays

Sulfhydryl compounds (PSH) are scavengers of reactive oxygen species [21,22] whereas protein carbonyls are produced consecutively to lipid peroxidation but essentially to glyco and protein-oxidation after oxidative stress [23]. PSH were assayed by Ellman's test using DTNB (5,5'-Dithionitrobenzoic acid) and measured spectrophotometrically at 412 nm [24,25]. Carbonyl proteins were measured spectrophotometrically at 350 and 375 nm after derivatization in presence of 2,4-dinitrophenylhydrazine (DNPH) [23,26].

Antioxidant enzymes activities in the hepatic tissue

The total superoxide dismutase (Cu-Zn SOD and Mn SOD) activity was assessed by following the inhibition of pyrogallol oxidation according to the method of Marklund and Marklund [27]. It was measured at 420 nm and expressed as U/mg protein (one unit of SOD was defined as the enzyme amount causing 50% inhibition of pyrogallol oxidation).

The catalase (CAT) activity was assessed by the method of Claiborne [28], which consists of following the decomposition rate of H₂O₂ spectrophotometrically

at 240 nm [28]. It was expressed as μmol of decomposed H₂O₂/min/mg of protein.

We also appraised the activity of glutathione peroxidase (GPx) and the rate of glutathione (GSH) following Flohe and Gunzler reaction by measuring the reduction of the absorbance of GSH at 412 nm in presence of H₂O₂ [29,30]. GSH was expressed as μg/mg of protein and GPx activity was expressed as μmol GSH/min/mg of protein.

Statistical analysis

Results were expressed as mean values ± standard errors (SEM). Differences between groups were examined for statistical significance using Kruskal – Wallis non parametric test followed by Dunn's multiple comparison test (Graph Pad Prism software, version 6 for Windows). Data was considered statistically significant as p-value <0.05.

Results

We conducted these experiments to explore whether NOS inhibition could influence the anti ischemic effect of nitrite. As shown in Figures 1 and 2, significant reduction of ALT and AST activities were observed in IGL-1 + Nitrite group both before and after normothermic *ex vivo* reperfusion as compared to IGL-1 group (p < 0.05, respectively). Regarding ALT, we observed 27.8 ± 2.5 vs 89.7 ± 2.7 U/L (p < 0.05) before reperfusion (Figure 1 A). For AST, we found 44 ± 8 vs 221 ± 28 U/L (p < 0.05) at 0 min (Figure 1(b)). After 120 min of reperfusion, we found 90.6 ± 5.5 vs 388.1 ± 51.6 U/L (p < 0.05) for ALT (Figure 2(a)) and 157.6 ± 31.1 vs 323.8 ± 47.8 U/L (p < 0.05) for AST (Figure 2(b)). Concerning GGT activity, we found 8.30 ± 0.11 vs 7.07 ± 0.14 U/L for IGL-1 and IGL-1 + Nitrite groups, respectively (p < 0.05, Figure 2 C). For

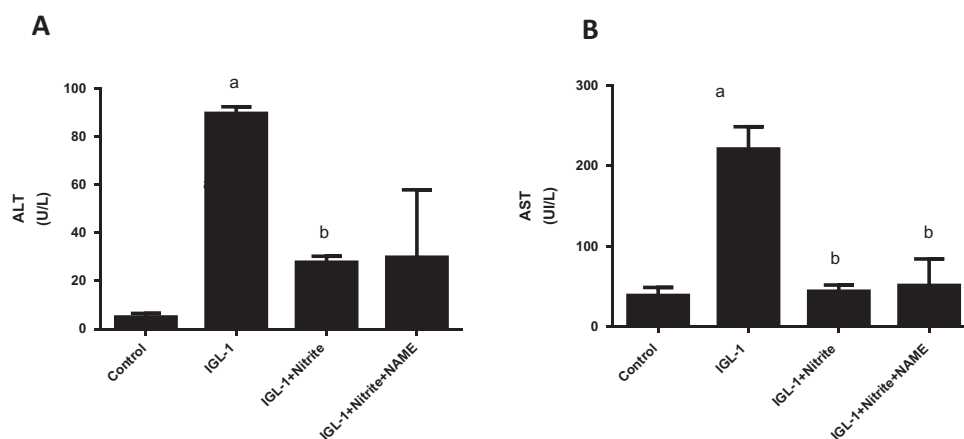


Figure 1. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in liver effluent obtained after 24 h of cold preservation (a, b). Livers' rat (n = 6) were flushed and preserved in IGL-1 solution (4°C for 24 h) supplemented with 50 nM of nitrite or with 50 nM of nitrite + 1 mM of L-NAME (L-N^G-Nitroarginine methyl ester). Sham: livers were flushed and perfused *ex vivo* without cold storage. Data are expressed as means ± SE (n = 6 for each group). a: p < 0.05 vs Sham; b: p < 0.05 vs IGL-1.

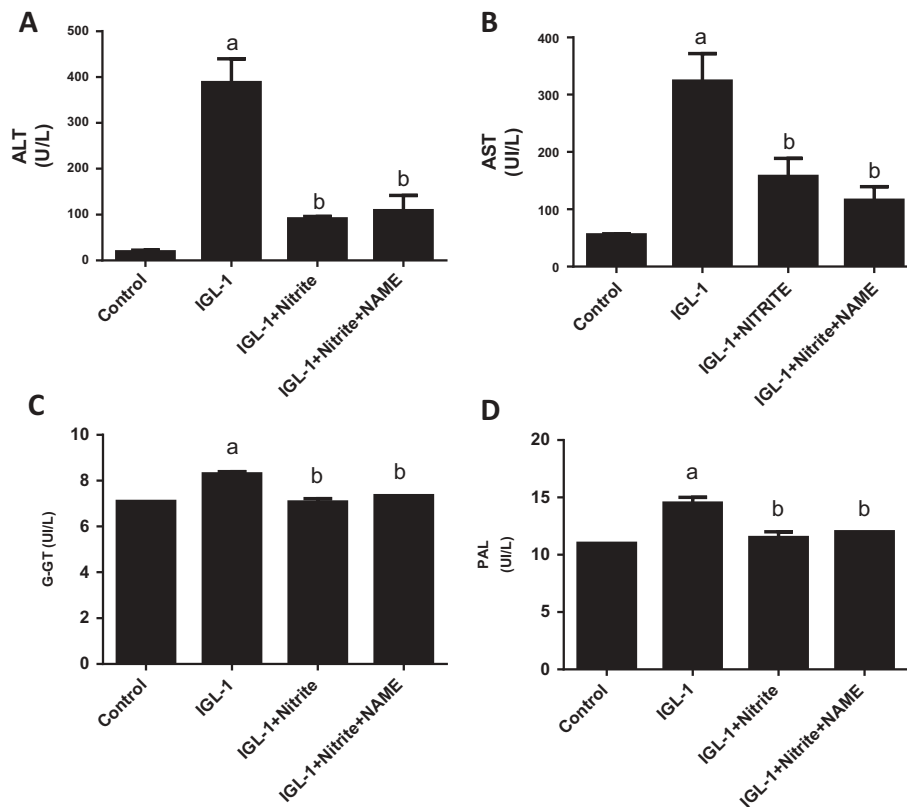


Figure 2. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in liver effluent obtained after 24 h of cold preservation and 120 min of normothermic reperfusion (a, b), Gamma-glutamyl transferase (G-GT) (c), and alkaline phosphatase (PAL) (d) after of normothermic reperfusion. Livers' rat (n = 6) were flushed and preserved in IGL-1 solution (4° C for 24 h) supplemented with 50 nM of nitrite or with 50 nM of nitrite + 1 mM of L-NAME (L-N^G-Nitroarginine methyl ester). Sham: livers were flushed and perfused *ex vivo* without cold storage. Data are expressed as means \pm SE (n = 6 for each group). a: $p < 0.05$ vs Sham; b: $p < 0.05$ vs IGL-1.

PAL activity, we detected 14.5 ± 0.5 vs 11.5 ± 0.5 for IGL-1 and IGL-1 + Nitrite groups, respectively ($p < 0.05$, (Figure 2(d)). About IGL-1 + Nitrite + L-NAME group, we noted for ALT: 30 ± 28 U/L and 109 ± 31 U/L at respectively 0 min and 120 min; for AST: 51 ± 33 U/L and 116 ± 23 U/L at respectively 0 min and 120 min; for GGT and PAL at 120 min: 7.35 ± 0.01 U/L and 12.01 ± 0.01 U/L, respectively. No statistical differences were found between IGL-1 + Nitrite and IGL-1 + Nitrite + L-Name groups for ALT and AST activities at both 0 min and 120 min of reperfusion.

Nitrite was also found to protect hepatic function. This is evidenced by the improvement of bile flow (6.41 ± 0.07 vs 36.01 ± 7.22 $\mu\text{L}/\text{min}/\text{g}$ for IGL-1 and IGL-1 + Nitrite groups respectively, $p < 0.05$) (Figure 3 (a)), as well as the attenuation of portal resistance (3.54 ± 0.18 vs 0.85 ± 0.11 mm Hg.min/mL/g for IGL-1 and IGL-1 + Nitrite groups respectively, $p < 0.05$) (Figure 3(b)). The use of L-NAME didn't induce significant changes in any of these parameters as compared to IGL-1 + nitrite group. Actually, we noted 46.04 ± 2.43 $\mu\text{L}/\text{min}/\text{g}$ for bile flow and 1.08 ± 0.10 mm Hg.min/mL/g for portal resistance.

The next step of the study explored the relationship between oxidative stress, nitrite and NOS. We

found that, comparing with IGL-1 group, nitrite supplementation markedly reduced lipid and protein oxidation as attested by lower levels of MDA (3.28 ± 0.32 vs 1.35 ± 0.28 nmol/mg protein, respectively, $p < 0.05$) (Figure 4(a)) and of carbonyl proteins (0.36 ± 0.06 vs 0.17 ± 0.01 nmol/mg protein, respectively, $p < 0.05$) (Figure 4(b)) on one hand, and a higher level of PSH (3.64 ± 0.42 vs 7.19 ± 0.17 $\mu\text{g}/\text{mg}$ protein, respectively, $p < 0.05$) (Figure 4(c)), on the other hand. In line with this, livers stored in IGL-1 + nitrite solution exhibited a marked rise of the activity of antioxidant enzymes catalase (221.05 ± 3.34 $\mu\text{mol H}_2\text{O}_2/\text{min}/\text{mg}$ protein) (Figure 5(a)), GPx (24.16 ± 1.10 $\mu\text{mol GSH}/\text{min}/\text{mg}$ protein) (Figure 5(b)), SOD (4.73 ± 0.42 U/mg protein) (Figure 5(c)) and of GSH level as well (0.65 ± 0.07 $\mu\text{g}/\text{mg}$ protein) (Figure 5(d)) when referred to those conserved in IGL-1 (177 ± 3.4 $\mu\text{mol H}_2\text{O}_2/\text{min}/\text{mg}$ protein, 5.44 ± 0.36 $\mu\text{mol GSH}/\text{min}/\text{mg}$ protein, 3.53 ± 0.22 U/mg protein, 0.37 ± 0.07 $\mu\text{g}/\text{mg}$ protein, respectively). Upon L-NAME association to nitrite, no statistical changes were found in the hepatic oxidative status as compared to nitrite alone. We noted 1.09 ± 0.05 nmol/mg protein for MDA, 0.06 ± 0.01 nmol/mg protein for carbonyl proteins, 8.24 ± 1.17 $\mu\text{g}/\text{mg}$ protein for PSH, 236.09 ± 7.38 $\mu\text{mol H}_2\text{O}_2/\text{min}/\text{mg}$ protein for CAT, 30.90 ± 3.67 $\mu\text{mol GSH}/$

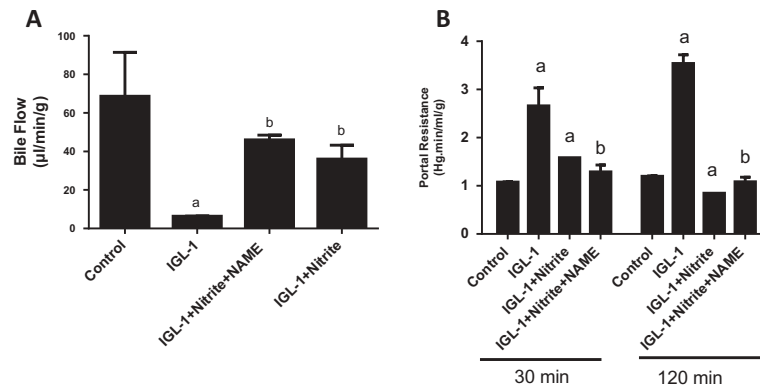


Figure 3. Bile output (a) and portal resistance (b), after 120 min of normothermic reperfusion. Livers' rat (n = 6) were flushed and preserved in IGL-1 solution (4°C for 24 h) supplemented with 50 nM of nitrite or with 50 nM of nitrite + 1 mM of L-NAME (L-N^G-Nitroarginine methyl ester). Sham: livers were flushed and perfused *ex vivo* without cold storage. Data are expressed as means ± SE (n = 6 for each group). a: p < 0.05 vs Sham; b: p < 0.05 vs IGL-1.

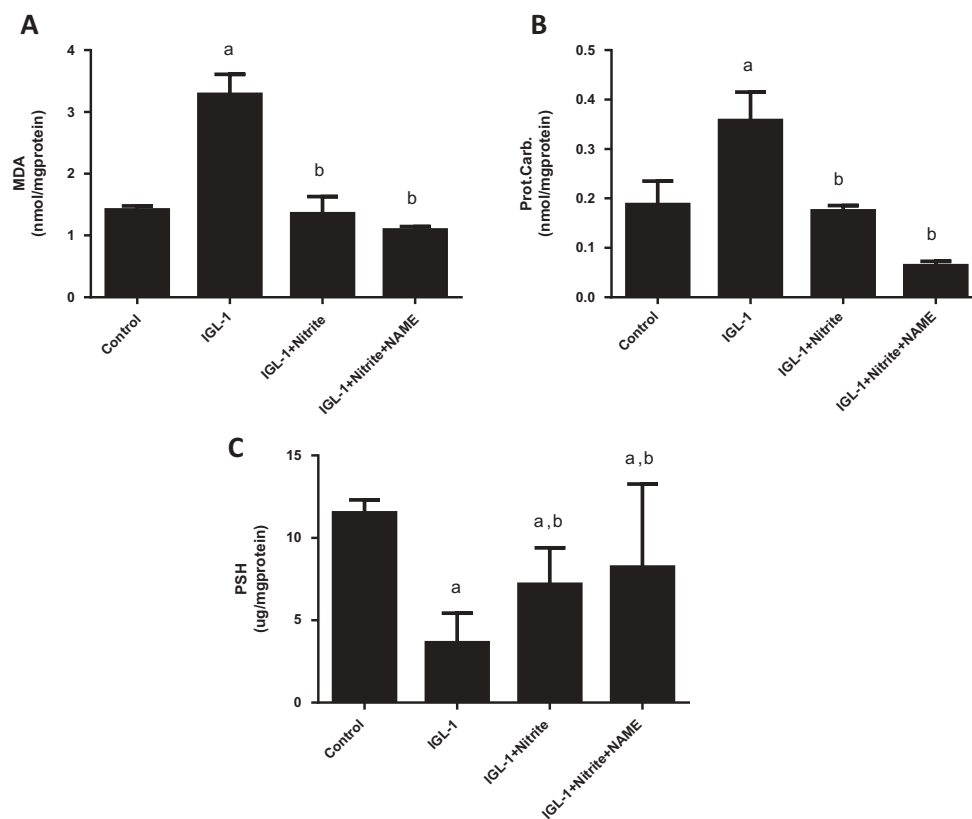


Figure 4. Lipid peroxidation expressed as malondialdehyde (MDA) activity (a), carbonyl proteins (Prot Carb) (b), and sulfhydryl proteins (PSH) levels (c) in liver tissues after normothermic reperfusion. Livers' rat (n = 6) were flushed and preserved in IGL-1 solution (4°C for 24 h) supplemented with 50 nM of nitrite or with 50 nM of nitrite + 1 mM of L-NAME (L-N^G-Nitroarginine methyl ester). Sham: livers were flushed and perfused *ex vivo* without cold storage. Data are expressed as means ± SE (n = 6 for each group). a: p < 0.05 vs Sham; b: p < 0.05 vs IGL-1.

min/mg protein for GPx, 4.81 ± 0.47 U/mg protein for SOD and 0.57 ± 0.01 µg/mg protein for GSH.

Discussion

An Ischemia-reperfusion injury associated to liver surgery remains until now an unresolved problem. Damages are caused by warm ischemia during graft procurement and implementation, cold ischemia during

preservation and reperfusion [4]. All of these steps are unavoidable during LT. Many groups are focusing of strategies reducing I/R injury in order to limit primary graft dysfunction or non-function [31]. Otherwise, it is widely valued that I/R associated with LT induces endothelial insult characterized by marked trouble in NO homeostasis [9,16,32]. The decrease in NO bioavailability appears early after reperfusion, and seems to be the consequence of the disruption of NO synthesis and

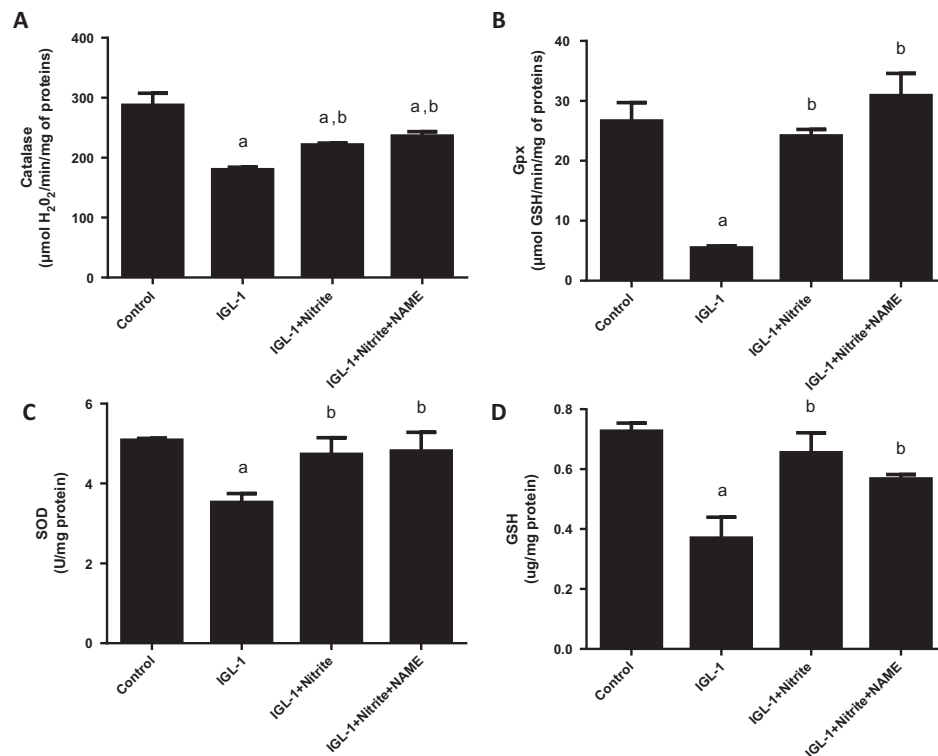


Figure 5. Evaluation of antioxidant enzymes catalase (CAT) (a), glutathione peroxidase (GPX) (b), superoxide dismutase (SOD) (c), reduced glutathione (GSH) (d) activities. Livers' rat ($n = 6$) were flushed and preserved in IGL-1 solution (4°C for 24 h) supplemented with 50 nM of nitrite or with 50 nM of nitrite + 1 mM of L-NAME (L-N^G-Nitroarginine methyl ester). Sham: livers were flushed and perfused *ex vivo* without cold storage. Data are expressed as means \pm SE ($n = 6$ for each group). a: $p < 0.05$ vs Sham; b: $p < 0.05$ vs IGL-1.

the inactivation of NO by the overproduction of superoxide anion, or both. It is largely appreciated that eNOS activation induces liver protection [33].

In the present study, we aimed to determine if the NOS pathway is involved in the protective effect of nitrite against cold I/R injury in isolated liver by using the NOS inhibitor, L-NAME. We found that nitrite supplementation to IGL-1 solution at 50 nM improves all biochemical, oxidative stress and functional parameters of the liver graft, and that L-NAME addition to nitrite enriched IGL-1 solution didn't abolish the protective effect of nitrite whereas Name addition to IGL-1 solution without nitrite didn't shown any significant results from IGL-1 group (results are not shown). These results suggest that nitrite could act through an independent NOS pathway. Our results are in accordance with those of Wei Li et al. demonstrating that the use of nitrite induced protection against cold I/R injury in liver graft [34]. Also, they support Duranski et al. hypothesis [14] stating that nitrite induces its protecting issues on liver and heart through a pathway independent from eNOS. This team has reported that nitrite remains cytoprotective even in eNOS-deficient mouse and the effect is dependent on NO production. Nevertheless, other authors found that nitrite did not protect against renal I/R injury [35].

The strongest data supporting the thesis that nitrite could serve as an important source of endocrine NO

appear from studies of I/R although the precise mechanism of how it induces tissue protection is still misleading. Endogenous NO is synthesized from L-arginine in an oxidation reaction, catalyzed by the NOS enzymes [3]. This complex reaction requires the presence of co-substrates O₂ and the reduced form of nicotinamide adenine dinucleotide phosphate, as well as many cofactors including flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin. *In vivo*, nitrite is considered as a catabolism product of NO. Indeed, NO is inactivated primarily by superoxide anion, but other pathways could be implicated. The first one is related to the autoxidation of NO to nitrite and then to nitrate. Other pathways may be mediated by metal-catalyzed oxidation reactions. The copper-containing protein ceruloplasmin (P-Cu²⁺) has been found to quickly oxidize NO to nitrite in physiological conditions. Besides to the P-Cu²⁺-mediated reaction, ferrous deoxygenated hemoglobin rapidly converts NO to nitrate [36,37]. However during ischemic condition, some investigations have emphasized the ability for nitrite to serve as a biological precursor of NO in the vascular compartment. This allowed some studies to demonstrate that nitrite can protect or reverse tissue damage during ischemia, especially in settings characterized by loss of NO bioavailability such those encountered during cold preservation of organs. Nitrite is reduced to NO during hypoxia and acidosis by the enzymatic action of xanthine

oxidoreductase (XOR) and by heme proteins such as deoxyhemoglobin, myoglobin, and tissue heme [38–40]. The L-NAME has been shown, *in vitro* and *in vivo*, to be potent inhibitor of NOS and the production of NO [41]. Therefore, our results suggest that nitrite action would be mediated through these ischemia-activated pathways and not through NOS activation.

Taken together, our results show that nitrite may be an efficient additive to cold preservation solution to fill up the losses of NO and to correct NO disorders associated with organ storage. The mechanism of action of nitrite seems to be independent from NOS pathway.

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Disclosure statement

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