TESIS DOCTORAL

EL PRECONDICIONAMIENTO ISQUÉMICO COMO ESTRATEGIA QUIRÚRGICA ÚTIL EN EL TRASPLANTE HEPÁTICO CON INJERTO DE TAMAÑO REDUCIDO

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4. RESULTADOS

4.1. PRIMER ESTUDIO

PROTECTION OF REDUCED-SIZE LIVER FOR TRANSPLANTATION

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El PC resultó en unos niveles de transaminasas y de grado 3 de necrosis menores, respecto el grupo ROLT; el estudio histológico mostró menos áreas de necrosis y más pequeñas en el grupo precondicionado. Por otro lado, el PC aumentó el índice de marcaje de PCNA y los niveles de HGF en plasma y en hígado.

El PC no fue capaz de modificar el "pool" de nucleótidos de adenina al final del periodo de isquemia fría y después de la reperfusión, por lo que la relación ATP/ADP y la carga energética fueron del mismo orden que en grupo trasplante.

En cuanto a los parámetros de estrés oxidativo, el PC redujo todos ellos (MDA, LPO y H_2O_2) después del ROLT, pero no fue a través de cambios en el sistema xantina/XOD, ya que el PC no modificó la actividad XDH/XOD, ni la acumulación de hipoxantina y xantina. En cambio, la inhibición de la células de kupffer en el grupo trasplante redujo los parámetros de estrés oxidativo, lesión hepática y mejoró la regeneración hepática (PCNA y HGF). Los niveles de IL-6 y TNF-α, que aumentaron después del trasplante no fueron modificados con la aplicación del PC.

La modulación en la síntesis de NO en animales trasplantados con o sin PC indicó la implicación de este mediador en la protección del PC frente a la lesión por I/R hepática y regeneración en ROLT.

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Protection of Reduced-Size Liver for Transplantation

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The shortage of available organs for liver transplantation has motivated the development of new surgical techniques such as reduced-size liver transplantation. Ischemia-reperfusion (I/R) associated with liver transplantation impairs liver regeneration. Ischemic preconditioning is effective against I/R injury in clinical practice of liver tumour resections. The present study evaluated the effect of ischemic preconditioning on reduced-size liver for transplantation and attempted to identify the underlying protective mechanisms. Hepatic injury and regeneration (transaminases, proliferating cell nuclear antigen [PCNA] labeling index, and hepatocyte growth factor [HGF]) were assessed after reduced-size orthotopic liver transplantation (ROLT). Energy metabolism, oxidative stress, tumor necrosis factor-α (TNF) and interleukin-6 (IL-6) were examined as possible mechanisms involved in liver regeneration. Ischemic preconditioning reduced transaminase levels and increased HGF levels and the percentage of PCNA-positive hepatocytes after ROLT. This was associated with a decrease in oxidative stress following ROLT, whereas energy metabolism and hepatic IL-6 and TNF release were unchanged. The benefits of ischemic preconditioning on hepatic injury and liver regeneration could be mediated, at least partially by nitric oxide. These results suggest a new potential application of ischemic preconditioning in reduced-size liver transplantation.

Key words: Hepatic growth factor, interleukin-6, resection, regeneration, tumor necrosis factor

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Introduction

Shortage of donor organs remains a major obstacle to the widespread application of liver transplantation in patients with end-stage liver disease (1–3). Living-related liver transplantation (LRLT) has been developed to increase the number of donor livers (1,4,5). The benefits of LRLT are the access to transplantation without registration on a waiting list and transplantation of a graft of quality with a short ischemic time (6–8). On the other hand, the major concern over application of LRLT for adults is graft-size disparity (9–11). Liver hepatectomy needs posterior regeneration to restore the liver/body ratio (9,12,13). The ability of the liver to restore major tissue loss involves numerous interacting cells and a complex network of mediators (2,13,14). Hepatocyte growth factor (HGF) has been considered as a potent mitogen and plays a crucial role in liver regeneration (13,15,16). Indeed, HGF concentrations increase in several kinds of liver disease in which liver regeneration occurs (16–18).

Ischemia-reperfusion (I/R), which is inevitable in liver transplantation, significantly reduces the DNA synthesis rate and liver regeneration after hepatectomy (12,19,20). During I/R the store of ATP, which is necessary for DNA synthesis, is depleted (12,21). Reactive oxygen species (ROS) originating after reperfusion induce DNA damage and inhibit cell division (12,20,21). Experimental evidence indicates that interleukin-6 (IL-6) exerts potent anti-inflammatory actions in hepatic I/R processes and may induce hepatocyte proliferation *in vivo* (2,19,22). Moreover, poor regeneration in reduced-size liver grafts has been related to decreased production of IL-6 (2). On the other hand, the mechanisms of action of IL-6 in promoting liver regeneration appear to be different from those involved in modulation of reperfusion injury (19,22). The modulation of hepatic I/R injury by IL-6 was thought to be related to down-regulation of tumor necrosis factor-α (TNF). The regenerating capacity of IL-6 may be related with its effect on hepatocytes (19,22).

Brief episodes of ischemia and reperfusion elicit organ tolerance to longer subsequent periods of ischemia. This phenomenon, known as ischemic preconditioning, first described in the heart (23), has been documented in the liver in warm and cold ischemia (24,25). The use of ischemic preconditioning in reduced-size liver transplantation should be considered, as Clavien et al. reported the first clinical application of ischemic preconditioning during normothermic ischemia associated with hepatic resections (26). In addition, results of experimental hepatic ischemia without liver resection suggest that ischemic preconditioning could modulate the negative effects of I/R on hepatic regeneration (24,27–30). Thus, ischemic preconditioning protects

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Table 1: Flow chart of the interventions and measurements corresponding to protocols 1, 2, 3 and 4. Interventions: reduced-size orthotopic liver transplantation (ROLT), PC + ROLT: ROLT with previous ischemic preconditioning, I: cold ischemia, PC + I: I with previous ischemic preconditioning, ROLT + $GdCl_3$: ROLT treated with $GdCl_3$, ROLT + NO: ROLT treated with NO donor, PC + ROLT + NAME: PC + ROLT treated with L-NAME, I + NO: I treated with NO donor, PC + I + NAME: PC + I treated with L-NAME. HGF, hepatocyte growth factor; MDA, malondialdehyde; LPO, lipid hydroperoxides; H_2O_2, hydrogen peroxide; PCNA, proliferating cell nuclear antigen; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; XDH/XOD, xanthine dehydrogenase/xanthine oxidase

INTERVENTIONS	MEASUREMENTS
PROTOCOL 1. Effect of ischemic preconditioning on hepatic I/R injury and liver regeneration.	
Group 1. Sham Group 2. ROLT Group 3. PC+ ROLT	Plasma: Transaminases, HGF Liver: MDA, LPO, H_2O_2,PCNA, HGF, IL-6, TNF-α, Histology
PROTOCOL 2. Effect of ischemic preconditionig on ROS-generating systems	
2.1. Xanthine/XOD Group 4. Control Group 5. I Group 6. PC+ I Groups 1- 3 (PROTOCOL 1)	Liver: XDH/XOD, Xanthine,Hypoxanthine
2.2. Kupffer cells Group 7. ROLT + $GdCl_3$	Plasma: Transaminases, HGF Liver: MDA, LPO, H_2O_2,PCNA, HGF, IL-6, TNF-α, Histology
PROTOCOL 3. Effect of ischemic preconditioning on energy metabolism	
Groups 1-6 (PROTOCOL 1 and 2.1)	Liver: ATP, Adenine nucleotides, Energy balance, Energy charge
PROTOCOL 4. Role of NO in ischemic preconditioning	
4.1. Hepatic injury and liver regeneration Group 8. ROLT + NO Group 9. PC+ ROLT + NAME	Plasma: Transaminases, HGF Liver: MDA, LPO, H_2O_2, PCNA, HGF, IL-6, TNF-α, Histology, XDH/XOD, Xanthine Hypoxanthine
4.2. Xanthine/ XOD Group 10. I + NO Group 11. PC + I + NAME	Liver: XDH/XOD, Xanthine. Hypoxanthine

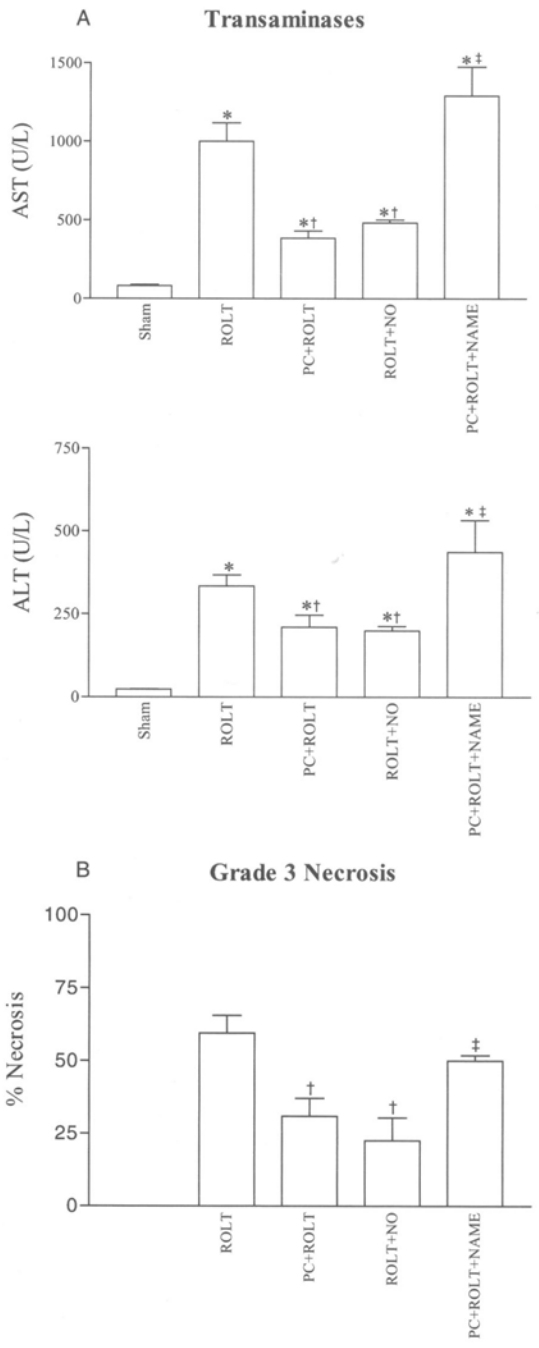

Figure 1: Transaminase (AST and ALT) (A) and percentage of grade 3 necrosis (B) reduced-size orthotopic liver transplantation (ROLT), PC + ROLT: ROLT with previous ischemic preconditioning; ROLT + NO: ROLT treated with NO donor; PC + ROLT + NAME: PC + ROLT treated with L-NAME. $*p < 0.05$ vs. sham, $\dagger p < 0.05$ vs. ROLT; $\ddagger p < 0.05$ vs. PC + ROLT.

against the deleterious effect of ischemia on ATP degradation in experimental models of warm ischemia (24,27). In addition, this surgical strategy, through nitric oxide (NO), protects against the deleterious effects of both ROS and TNF on hepatic I/R injury (28–30).

We report the results of an experimental study aimed to evaluate the effectiveness of ischemic preconditioning on hepatic I/R injury and hepatic regeneration in reduced-size liver transplantation.

Materials and Methods

Experimental animals

Male Sprague-Dawley rats weighing 200–250 g were used as donors and recipients. All animals were anesthetized with isoflurane. This study respected the European Union regulations for animal experiments (EC guideline 86/609/CEE).

Experimental design

Protocol 1. Effect of ischemic preconditioning on hepatic I/R injury and liver regeneration

To evaluate the effect of preconditioning on hepatic I/R injury and liver regeneration, animals were distributed into the following experimental groups:

1. Sham (n = 6): Animals were subjected to anesthesia, transversal laparotomy, and silk ligatures in the right suprarenal vein, and hepatic artery.

2. Reduced-size orthotopic liver transplantation (ROLT) (n = 12, six transplantations): Liver reduction was achieved by removing the left lateral lobe and the two caudate lobes just before harvesting the liver, which resulted in a 40% reduction of the liver mass (1). The pedicle of the left lateral lobe was ligated with 5–0 silk ligature, and the lobe was removed. Two caudate lobes were separately removed with the ligation (31,32). The donors livers were flushed and preserved with cold (4°C) University of Wisconsin (UW) for 1 h (1). Reduced-size orthotopic liver transplantation was performed according to the Kamada's cuff technique, without hepatic artery reconstruction (33). The time of the anhepatic phase was 17–20 min (34).

3. Ischemic preconditioning + reduced-size orthotopic liver transplantation (PC + ROLT) (n = 12, six transplantations): To induce ischemic preconditioning, the blood flow of the donor liver was interrupted by placing a bulldog clamp at the portal vein and hepatic artery for 10 min, followed by reflow for 10 min (35). Following the same surgical procedure as for group 2, liver lobes were resected and flushed and preserved with cold (4°C) UW solution for 1 h.

Twenty-four hours after transplantation, plasma and liver samples were collected. Hepatic injury was determined by analysis of transaminases (aspartate aminotransferase, AST, and alanine aminotransferase, ALT) in plasma. Lipid peroxidation (malondialdehyde, MDA, and lipid hydroperoxides, LPO) and H_2O_2 levels were measured in the liver. To evaluate hepatic regeneration, the proliferating cell nuclear antigen (PCNA)-labeling index was determined in liver and HGF levels were measured in plasma and liver samples. IL-6 and TNF were evaluated in liver samples. Histological analyses in liver were also performed.

Protocol 2. Effect of ischemic preconditioning on ROS-generating systems

Xanthine/xanthine oxidase (XOD). To evaluate the effect of ischemic preconditioning on the accumulation of both hypoxanthine and xanthine, and the conversion of xanthine dehydrogenase (XDH) to XOD in reduced-size

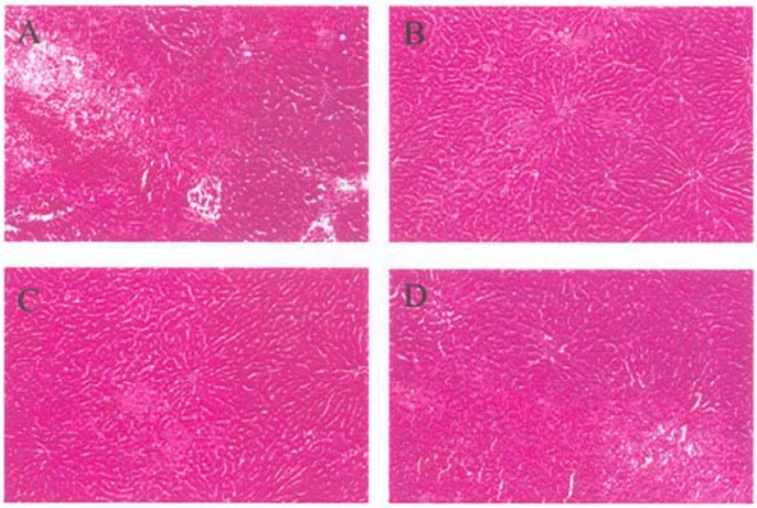

Figure 2: Histological lesions in (A–D) liver. (A) Reduced-size orthotopic liver transplantation (ROLT), extensive and multifocal areas of coagulative necrosis of hepatocytes with disruption of hepatic cords, infiltration of neutrophils and hemorrhage. (B) PC + ROLT (ROLT with previous ischemic preconditioning), focal and small areas of hepatocyte necrosis. (C) ROLT + NO (ROLT treated with NO donor) and (D) PC + ROLT + NAME (PC + ROLT treated with L-NAME): Histological lesions similar to PC + ROLT and ROLT, respectively. (H&E, Original magnification ×240).

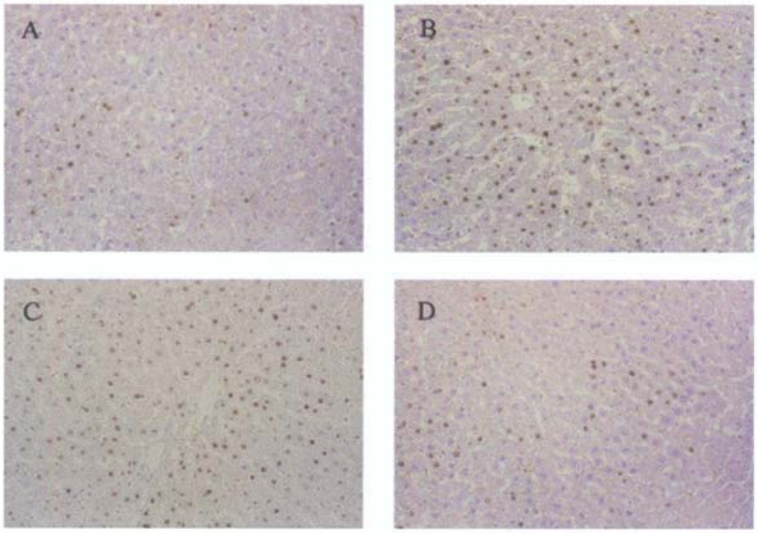

Figure 3: Immunocytochemical staining of PCNA-positive hepatocytes. Reduced-size orthotopic liver transplantation (ROLT) (A) showed lower positive cells number than PC + ROLT (B). ROLT + NO (C) and PC + ROLT + NAME (D) showed an amount of PCNA-positive staining hepatocytes similar to PC + ROLT and ROLT, respectively. PC + ROLT: ROLT with previous ischemic preconditioning; ROLT + NO: ROLT treated with NO donor; PC + ROLT + NAME: PC + ROLT treated with L-NAME. (Original magnification ×500).

liver grafts during cold ischemia, animals were distributed into the following experimental groups:

4. Control (n = 6): Reduced-size livers were flushed with cold UW solution.

5. Ischemia (I) (n = 6): Reduced-size livers were flushed with cold UW solution and then stored in UW solution for 1 h at 4°C.

6. Ischemic preconditioning + ischemia (PC + I) (n = 6): Same as group 5 but with previous preconditioning induced by 10 min of ischemia followed by 10 min of reperfusion.

After cold ischemia, liver samples were processed to measure XDH/XOD, hypoxanthine, and xanthine. These biochemical parameters were also measured in liver samples corresponding to groups 1–3 from protocol 1 to evaluate the effect of ischemic preconditioning on xanthine/XOD after reperfusion.

Kupffer cells

To evaluate the role of Kupffer cells in ROS generation the following group was examined:

7. Reduced-size orthotopic liver transplantation + gadolinium chloride (ROLT + GdCl3) (n = 12, six transplantations): Same as group 2, but with previous administration of GdCl3 (10 mg/kg, i.v.) in donor rats to inactivate Kupffer cells, 48 h and 24 h before hepatectomy (36).

24 h after transplantation, plasma and liver samples were collected. Biochemical determinations and histological analyses were the same as those described in protocol 1.

Protocol 3. Effect of ischemic preconditioning on energy metabolism

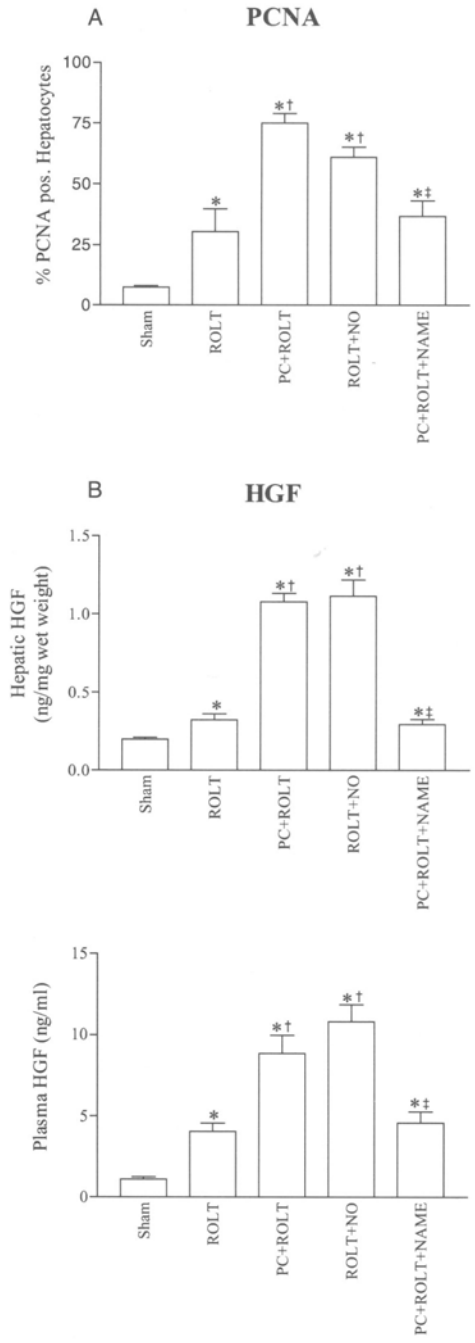

Figure 4: Percentage of proliferating cell nuclear antigen (PCNA)-positive hepatocytes (A) and hepatocyte growth factor (HGF) levels (B): Reduced-size orthotopic liver transplantation (ROLT), PC + ROLT: ROLT with previous ischemic preconditioning; ROLT + NO: ROLT treated with NO donor; PC + ROLT + NAME: PC + ROLT treated with L-NAME. $*p < 0.05$ vs. sham; $\dagger p < 0.05$ vs. ROLT; $\ddagger p < 0.05$ vs. PC + ROLT.

To evaluate whether the benefits of ischemic preconditioning could be explained by changes in nucleotide metabolism, ATP, adenine nucleotides (ATP + ADP + AMP), energy balance (ATP/ADP ratio) and energy charge (ATP + [1/2]ADP/ATP + ADP + AMP) were evaluated in liver samples corresponding to groups 1–6 mentioned earlier.

Protocol 4. Role of NO in ischemic preconditioning

Hepatic injury and liver regeneration. To study whether the benefits of ischemic preconditioning on hepatic I/R injury and liver regeneration could be mediated by NO, the following experimental groups were established:

8. Reduced-size orthotopic liver transplantation + NO donor (ROLT + NO) (n = 12, six transplantations): Same as group 2, but with previous administration of the NO donor, spermine NONOate (10 mg/kg i.v.) (27).

9. Ischemic preconditioning + reduced-size orthotopic liver transplantation + NAME (PC + ROLT + NAME) (n = 12, six transplantations): Same as group 3, but with previous administration of a NO synthesis inhibitor, Nw-nitro-L-arginine methyl ester (NAME). As previously reported, 10 mM of L-NAME was administered through the portal vein when the liver grafts were harvested, and 20 mg/kg was injected i.v. into the recipients just after reperfusion (25).

Twenty-four hours after transplantation, plasma and liver samples were collected. Biochemical determinations and histological analyses were the same as those described in protocol 1.

Xanthine/XOD. To evaluate the effect of NO on xanthine/XOD, the following groups were examined:

10. Ischemia + NO donor (I + NO) (n = 6): Same as group 5, but with previous administration of the NO donor, spermine NONOate (10 mg/kg i.v) (27).

11. Ischemic preconditioning + ischemia + NAME (PC + I + NAME) (n = 6): Same as group 6 but with previous administration of 10 mM of L-NAME through the portal vein when the liver grafts were harvested (25).

After cold ischemia, hypoxanthine, xanthine and XDH/XOD were measured in liver samples. These biochemical parameters were also measured in liver samples corresponding to groups 8 and 9 mentioned earlier.

The interventions and measurements corresponding to protocols 1, 2, 3 and 4 are summarized in Table 1.

Biochemical determinations

Transaminase assay. Hepatic injury was evaluated by measurements of transaminases in plasma using a commercial kit from Boehringer Mannheim (Munich, Germany).

Lipid peroxidation assay. Lipid peroxidation was used as an indirect measurement of the oxidative injury induced by ROS (28,37–39). Lipid peroxidation was followed by the determination of malondialdehyde (MDA) and lipid hydroperoxides (LPO). After protein precipitation, the formation of MDA was measured by using the thiobarbituric reaction (28). To analyze LPO, liver homogenates were deproteinated and extracted in acidic conditions (37,38). Lipid hydroperoxide levels were determined using a commercial kit (Oxis Research, Portland, OR).

H_2O_2 measurement. Liver tissues were homogenized in 0.033 M Na_2HPO_4 and 0.9% KCl. After centrifugation, the supernatants were used for H_2O_2

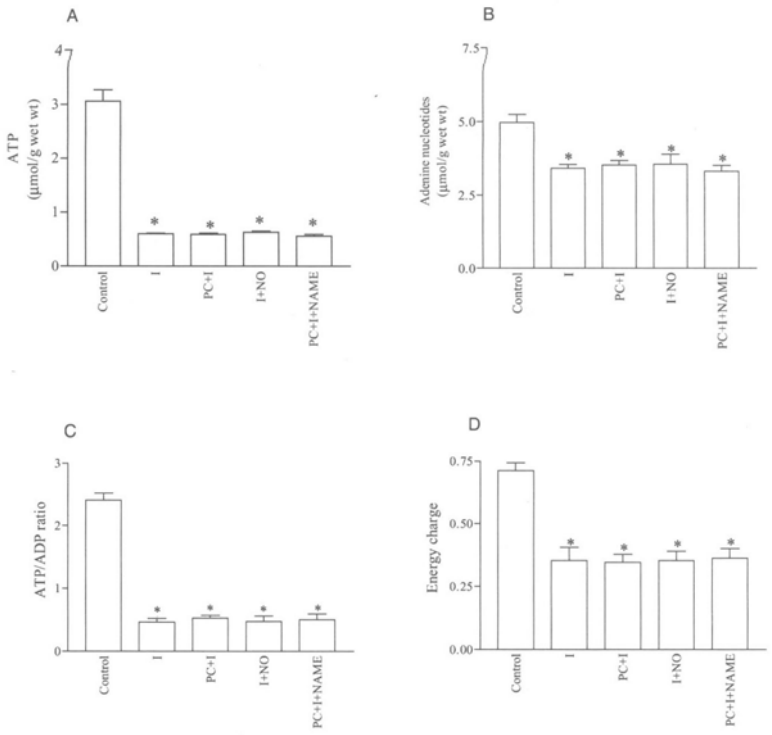

Figure 5: ATP (A), adenine nucleotides (ATP + ADP + AMP) (B), ATP/ADP ratio (C) and energy charge (D) in liver. I: cold ischemia, PC + I: I with previous ischemic preconditioning; I + NO: I treated with NO donor, PC + I + NAME: PC + I treated with L-NAME: $*p < 0.05$ vs. control.

analysis (40,41). H_2O_2 levels were measured using a commercial kit (Oxis Research).

XDH and XOD. Liver tissues were homogenized in 0.1 M Tris, containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol, and 0.5 mg/L leupeptin. After centrifugation the supernatant was chromatographed on a Sephadex G-25 80 column in the same buffer. Liver XDH/XOD activity was measured spectrophotometrically at 292 nm on the basis of uric acid formation in the presence and absence of NAD^+, respectively. Xanthine was used as substrate (28).

Nucleotide analysis

Livers were freeze-clamped and immediately homogenized in 3.6% perchloric acid solution, and after centrifugation, 50 μL of the supernatant was injected into Waters 717 Plus Autosampler liquid chromatographic equipment. Nucleotide profiles were obtained using a reverse-phase Spherisob ODS column (C_{18}, 5-μm particle size, 15 × 0.4 cm; Teknokroma, Sant Cugat, Spain) coupled to a 600 HPLC system (Waters, Milford, MA) equipped with a Waters 996 Photodiode Array Detector (Waters, Milford, MA, USA). The absorbance was monitored at 254 nm. Nucleotide separation was allowed to proceed in an isocratic fashion with 100 mmol/L ammonium phosphate, until ATP, ADP, hypoxanthine/xanthine, and AMP were separated (27).

Interleukin assay

Liver samples were homogenized in 50 mM Tris, containing 150 mM NaCl, triton X-100, and a protease inhibitor cocktail (Roche, Basel, Switzerland) (42). Hepatic IL-6 levels were detected using a commercial enzyme-linked immunosorbent assay kit (rat IL-6 ELISA kit, Biosource, Camarillo, CA) (42).

TNF assay

Liver tissues were homogenized in 50 mM phosphate buffer. Hepatic TNF levels were measured using a commercial immunoassay kit of rat TNF-α from Biosource (43).

PCNA labeling index

Proliferating cell nuclear antigen is a stable cell-cycle nuclear protein which is expressed in the late G1 and throughout the S-phase of the mitotic cycle. Briefly, after fixation with formalin and paraffin embedding of the liver tissue, the 3-μm sections were incubated with the anti-PCNA antibody (clone PC10; dilution 1 : 20; Dako GMbh, Hamburg, Germany). The immunohistochemistry was performed using a commercial kit (DAKO Envision + System, peroxidase (DAB); Dako GMbh, Hamburg, Germany). The proliferation index of PCNA-stained biopsy specimens was determined in 30 high-power fields. Data were expressed as the percentage of PCNA-stained hepatocytes per total number of hepatocytes (2,19).

Assay for HGF

Liver tissues were homogenized in 20 mM Tris[hydroxymethyl]aminomethane-HCl solution, containing 2 M NaCl, 1 mM phenylmethylsulphonyl fluoride, 1 mM ethylenediaminetetraacetic acid, and 0.1% polyoxyethylenesorbitan mono-oleate (17,44). Plasma and hepatic HGF were measured by the ELISA technique, as provided by the Institute of Immunology (Tokyo, Japan) (17).

Histology

Liver samples were processed and stained with hematoxylin-eosin according to standard procedures. For the severity of hepatic injury, hematoxylin and eosin-stained sections were evaluated by a point-counting method using an ordinal scale as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting in cytoplasmatic vacuolation and focal

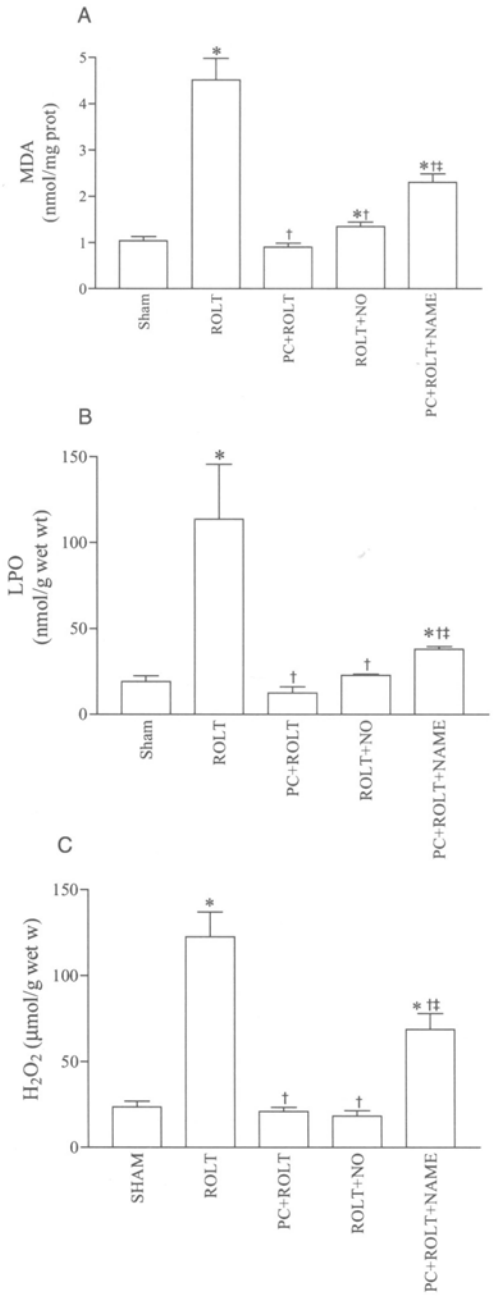

Figure 6: Malondialdehyde (MDA) (A), lipid hydroperoxides (LPO) (B) and H_2O_2 (C) in liver. Reduced-size orthotopic liver transplantation (ROLT), PC + ROLT: ROLT with previous ischemic preconditioning; ROLT + NO: ROLT treated with NO donor; PC + ROLT + NAME: PC + ROLT treated with L-NAME. $*p < 0.05$ vs. sham; $\dagger p < 0.05$ vs. ROLT; $\ddagger p < 0.05$ vs. PC + ROLT.

nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmatic hypereosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration. Forty high-power fields were investigated per slide to determine the percentage of necrotic cells (22,45).

Statistics

Data are expressed as means ± standard error. Statistical comparison was performed with analysis of variance, followed by the Student Newman-Keuls test. $p < 0.05$ was considered significant.

Results

Effect of ischemic preconditioning on hepatic I/R injury and liver regeneration

Ischemic preconditioning conferred protection against hepatic injury. Transaminase levels and the percentage of grade 3 necrosis were significantly lower in the preconditioned group than in the un-preconditioned group (see Figure 1). The histological study of the liver after ROLT revealed multifocal and extensive areas of coagulative necrosis randomly distributed throughout the hepatic parenchyma (Figure 2A). In contrast, fewer and smaller areas of hepatocyte necrosis were observed when ischemic preconditioning was carried out (PC + ROLT, 2B). In regards to the parameters of liver regeneration, ischemic preconditioning (PC + ROLT, Figure 3B) increased the number of PCNA-positive hepatocytes compared with the results obtained in the ROLT group (Figure 3A). The proliferation index after ROLT was 30.3% ± 10.2% vs. 75.1% ± 4.4% when ischemic preconditioning was carried out (Figure 4). In addition, this surgical strategy increased hepatic HGF levels and similar results were observed for plasma HGF release (see Figure 4).

Effect of ischemic preconditioning on energy metabolism

As shown in Figure 5, ATP and adenine nucleotides decreased in reduced-size liver grafts as a consequence of cold ischemia. The effect of cold ischemia on adenine nucleotide pool was not modified by previous ischemic preconditioning. ATP/ADP ratio and energy charge were similar in the preconditioned and un-preconditioned livers. The pattern of nucleotide metabolism found in all groups after cold ischemia was similar to that observed after reperfusion (data non shown).

Effect of ischemic preconditioning on ROS, IL-6 and TNF

Ischemic preconditioning reduced the increases in hepatic MDA, LPO and H_2O_2 levels after ROLT (Figure 6). The effect of ischemic preconditioning on ROS-generating systems including xanthine/XOD and Kupffer cells was evaluated. As shown in Figure 7 (A), in reduced-size liver grafts XDH was converted to the oxygen radical-producing form XOD, as a consequence of cold ischemia (I). XOD was activated after reperfusion (ROLT) (Figure 7B). Hypoxanthine was highly accumulated during ischemia, but decreased after reperfusion. Xanthine levels increased after cold

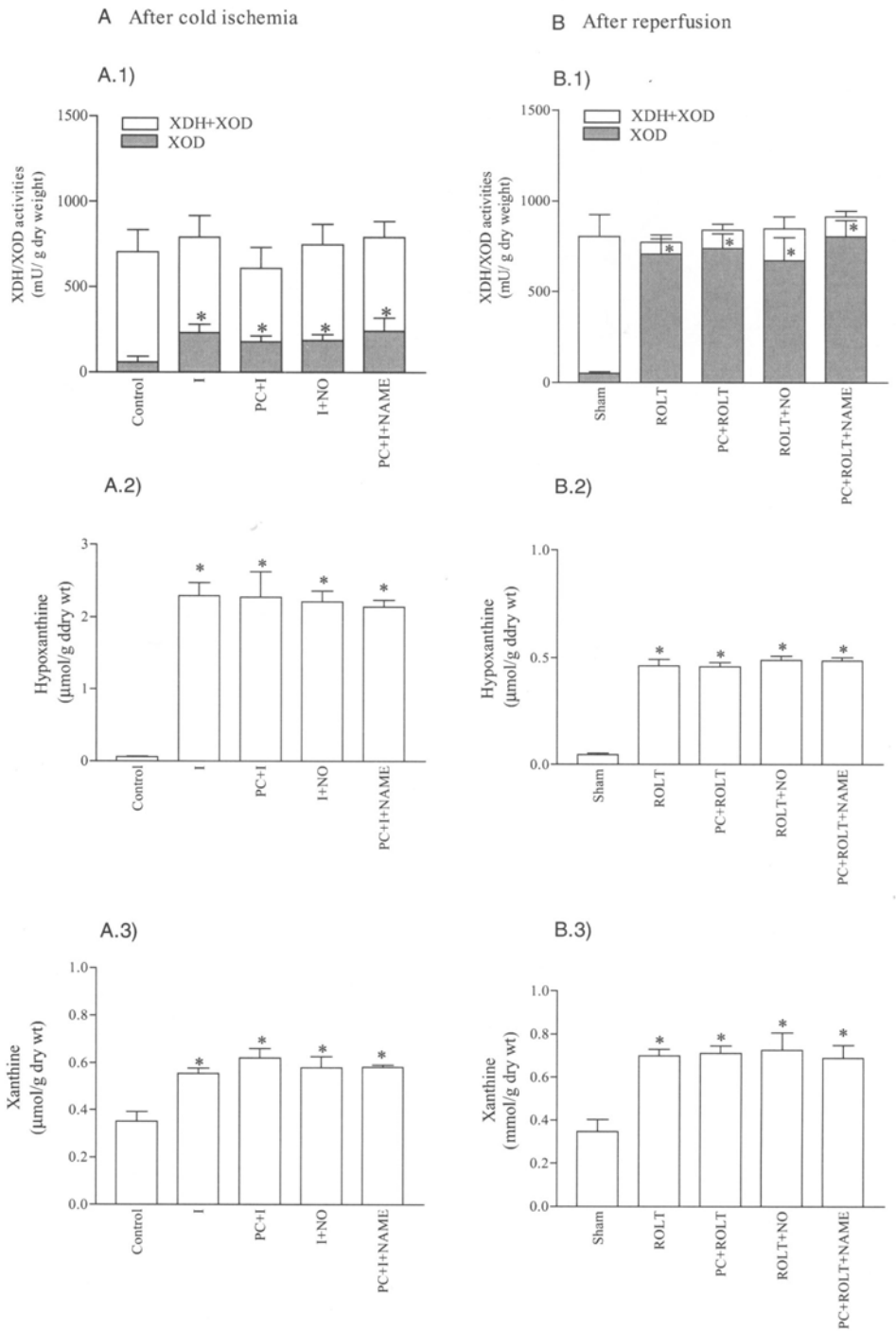

Figure 7: XDH/XOD, hypoxanthine and xanthine in liver after cold ischemia (A) and after reperfusion (B). I: cold ischemia, PC + I: I with previous ischemic preconditioning; I + NO: I treated with NO donor; PC + I + NAME: PC + I treated with L-NAME; reduced-size orthotopic liver transplantation (ROLT), PC + ROLT: ROLT with previous ischemic preconditioning; ROLT + NO: ROLT treated with NO donor; PC + ROLT + NAME: PC + ROLT treated with L-NAME. $*p < 0.05$ vs. control/sham.

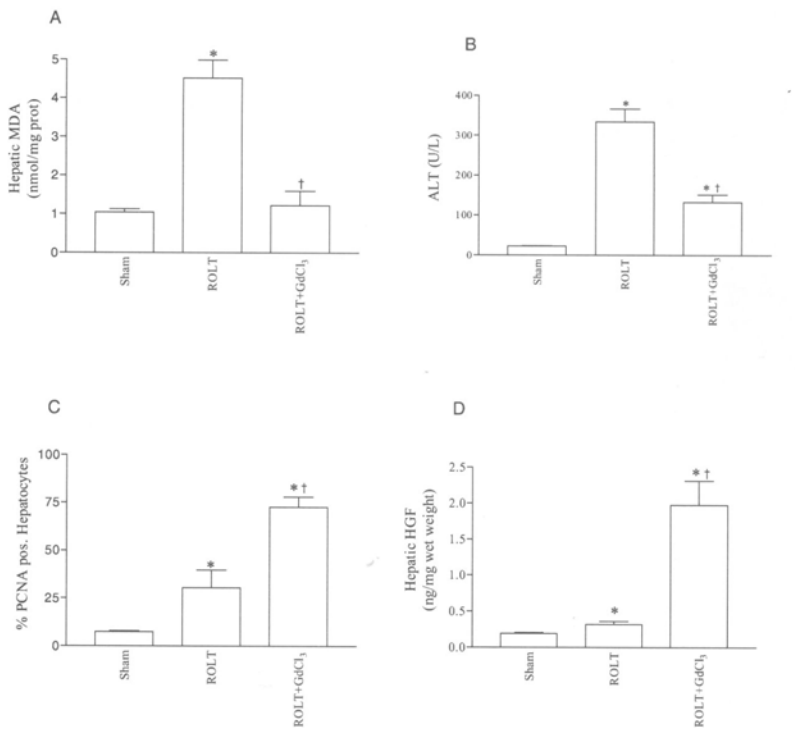

Figure 8: Malondialdehyde (MDA) (A), alanine aminotransferase (ALT) (B), percentage of proliferating cell nuclear antigen (PCNA) -positive hepatocytes (C) and hepatocyte growth factor (HGF) (D) after Kupffer cells inhibition. Reduced-size orthotopic liver transplantation (ROLT), ROLT + $GdCl_3$: ROLT treated with $GdCl_3$. $*p < 0.05$ vs. sham, $\dagger p < 0.05$ vs. ROLT.

ischemia and remained high after reperfusion. Ischemic preconditioning did not modify XDH/XOD activity nor the accumulation of hypoxanthine and xanthine. The inhibition of Kupffer cells reduced the increases in all the biochemical parameters of oxidative stress after ROLT. This was associated with a reduction in hepatic injury and improvement in liver regeneration (Figure 8). Figure 8 reports MDA levels as a parameter of oxidative stress, ALT levels as a parameter of hepatic injury and a percentage of PCNA-positive hepatocytes and hepatic HGF levels as parameters of liver regeneration. The other parameters of oxidative stress (LPO and H_2O_2), showed the same pattern as MDA. Similarly, the parameters of hepatic injury (ALT, and histology) and regeneration (plasma HGF) followed the same pattern as AST and hepatic HGF, respectively.

Both IL-6 and TNF increased after ROLT (Figure 9). Ischemic preconditioning did not modify the IL-6 and TNF levels.

Role of NO in the benefits of ischemic preconditioning on hepatic I/R and liver regeneration

The parameters of hepatic injury (Figures 1 and 2) and liver regeneration (Figures 3 and 4) in ROLT + NO and PC + ROLT + NAME groups were comparable to those observed in PC + ROLT and ROLT, respectively. The effect of NO on ROS and the ROS-generating system, xanthine/XOD, was investigated. Nitric oxide reduced the MDA, LPO, and H_2O_2 levels following ROLT (Figure 6). However, NO synthesis inhibition did not completely revert the benefits of ischemic preconditioning on oxidative stress. Nitric oxide donor pretreatment and L-NAME did not modify xanthine/XOD (Figure 7). Similar results were observed for nucleotide metabolism and cytokine levels (see Figures 5 and 9).

Discussion

Numerous investigations have demonstrated that HGF promotes hepatic growth and regeneration (1,13,15,16,46–48). In the context of liver transplantation, exogenous HGF administration to recipients of reduced-size liver graft enhances early restoration of liver volume, stimulates hepatic regeneration, and provides protection from rejection injury (1,46–48). The results of the present study indicate that both ischemic preconditioning and NO donor treatments protect against hepatic I/R injury and improve hepatic regeneration in ROLT. These surgical and pharmacological treatments promote the release of the growth factors responsible for liver regeneration such as HGF. However, there is no significant overlap between ischemic preconditioning and NO. The results of the present study indicate that NO contributes to some but not all the benefits of ischemic preconditioning. In fact, the inhibition of NO did not completely revert the effect of ischemic preconditioning on oxidative stress.

Results obtained from experimental models of normothermic ischemia combined with partial hepatectomy

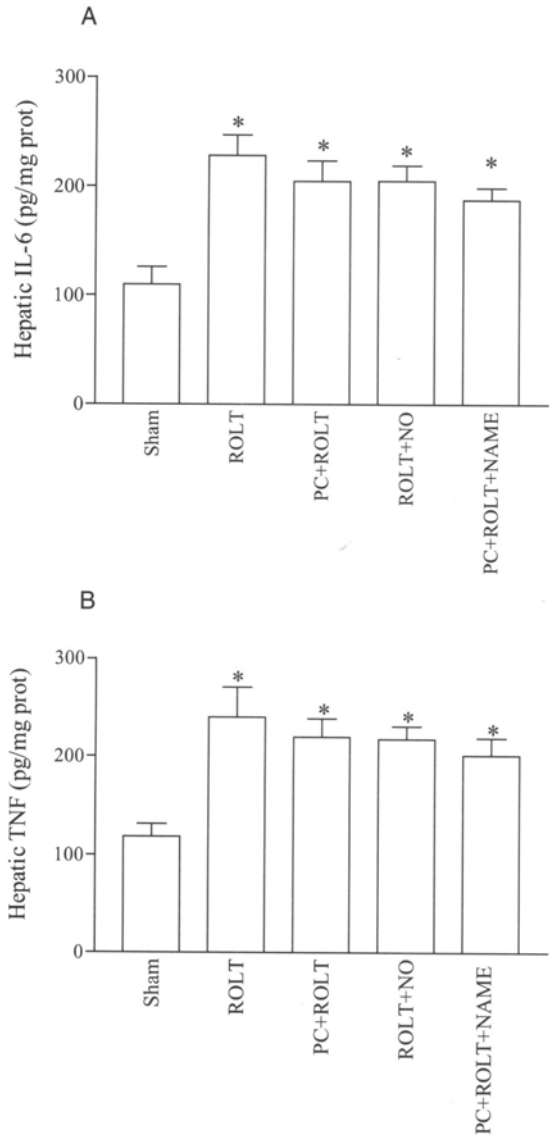

Figure 9: IL-6 (A) and TNF (B) in liver. Reduced-size orthotopic liver transplantation (ROLT), PC + ROLT: ROLT with previous ischemic preconditioning; ROLT + NO: ROLT treated with NO donor; PC + ROLT + NAME: PC + ROLT treated with L-NAME. $*p < 0.05$ vs. sham.

indicated that ROS damage lipoproteins and peptides, cause lipid peroxidation, and inhibit DNA synthesis (12,20). It has been suggested that ROS may damage membrane receptors for growth factors and inhibit metabolic pathways of liver regeneration (20). Thus, it could be expected that ischemic preconditioning, by reducing ROS generation in ROLT, could improve the regenerative response of livers. The involvement of XOD in stress oxidative and hepatic injury associated with liver transplantation is well known (35,49,50). High levels of either hypoxanthine or xanthine have been considered as markers of low survival rates in liver transplantation (51–53). The benefits of ischemic preconditioning on xanthine/XOD have been previously reported (35,54). In this line, ischemic preconditioning reduced the conversion of XDH to XOD and limited the accumulation of xanthine in nonreduced liver grafts during 6 h of cold ischemia, and the authors suggested that the inhibition of XOD activity caused by ischemic preconditioning could be mediated by NO (54). In fact, NO has been reported to inhibit XOD activity *in vivo* in endothelial cells (55,56) and activated macrophages (57). Results obtained by Ichimori et al. (58) indicates that NO reacts with XOD/XDH and converts the enzyme to the inactive desulfo-form in anaerobic conditions. It has also been reported by other authors that XOD converts NO to nitroxyl in the presence of hypoxanthine in anaerobic conditions. In these conditions, XOD lost its activity (59). However, in our hands, the reduction in ROS caused by ischemic preconditioning in reduced-size liver grafts could not be explained either by differences in XDH/XOD activity or by differences in hypoxanthine or xanthine accumulation. The different results concerning the effect of ischemic preconditioning and NO on xanthine/XOD could be explained by differences in the experimental models evaluated, including the duration of cold ischemia. There is evidence that ischemic preconditioning reduces oxidative stress via Kupffer cells (60,61). The results of the present study indicate that the inhibition of Kupffer cells reduced the oxidative stress following ROLT. This resulted in beneficial effects on liver injury and regeneration, as previously reported in experimental models of partial hepatectomy (62,63). However, the possibility that ischemic preconditioning could modulate other ROS-generating systems including mitochondria (64) and antioxidant systems such as glutathione (28) should not be discounted.

Results obtained from an experimental model of 70% of hepatectomy indicated that hepatic regeneration was closely correlated to the ATP levels of the remaining liver (65). In contrast to normothermic conditions (24,27), our results suggest that ischemic preconditioning neither affects the adenine nucleotide pool of liver cells during cold ischemia nor modifies the ability of hepatocytes to regenerate ATP following reperfusion. Thus, the protective effect of ischemic preconditioning on liver regeneration could not be explained on the basis of the preservation of adenine nucleotides.

Next, we investigated whether ischemic preconditioning could protect against hepatic I/R injury and improve hepatic regeneration by stimulating IL-6 release. This could prevent the deleterious effects of TNF on the liver and promote liver regeneration following ROLT. Indeed, the reduction in TNF release following hepatic I/R induced by

ischemic preconditioning has been previously reported in normothermic and cold ischemia conditions without hepatectomy (28–30). However, in our hands, ischemic preconditioning did not modify the levels of either IL-6 or TNF in reduced-size liver grafts after transplantation. In addition to the injurious effects of TNF in hepatic I/R, results obtained in partial hepatectomy models suggest that this cytokine may play a critical role in liver regeneration (66–68). The results of the present study suggest that neither IL-6 nor TNF is crucial in hepatic I/R injury and liver regeneration in ROLT, as the differences in hepatic injury and liver regeneration observed in all groups of the study were not reflected in changes in these two cytokines. This is consistent with the observations reported by other authors, indicating that hepatocyte proliferation can be induced by at least two different pathways (69,70): compensatory regeneration which is TNF and IL-6-dependent, and mitogen-induced direct proliferation which does not require TNF or IL-6. When knockout TNF and IL-6 mice were treated with primary mitogens, hepatocyte proliferation was the same as in wild-type mice (69).

Ischemic preconditioning has been applied successfully in humans in normothermic conditions associated with hepatic resections. The results of the present study suggest a new potential application of ischemic preconditioning in living-related liver transplantation.

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4.2. SEGUNDO ESTUDIO

HOW ISCHEMIC PRECONDITIONING PROTECTS SMALL LIVER GRAFTS

Franco-Gou R, Roselló-Catafau J, Casillas-Ramirez A, Massip-Salcedo M, Rimola A, Calvo N, Bartrons R, Peralta C

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Los resultados obtenidos con antagonistas de los receptores de IL-1 y la determinación de los niveles de mRNA de IL-1 mostraron la participación de la IL-1-α en el síndrome de I/R asociado al ROLT, además establecieron una relación entre la IL-1α y los factores de crecimiento. Así pues, la IL-1α redujo los niveles de HGF y aumentó los niveles de TGF-β, influyendo negativamente en el proceso de la regeneración hepática. El precondicionamiento isquémcio (PC) (a través del NO) y el tratamiento con donadores de NO inhibieron la producción hepática de IL-1, protegiendo así frente a los efectos perjudiciales de esta interleuquina sobre la lesión y regeneración hepática. Además, por otra vía independiente del NO, el PC dio lugar a una inducción de la expresión de HSP70 y de HO-1. La HO-1 protegió frente a la lesión por I/R hepática y por regeneración y en cambio el papel protector de la HSP70 fue exclusivamente relacionado con la proliferación hepatocitaria. La inhibición de la síntesis de NO en el grupo PC no modificó los efectos del PC sobre ambas HSPs. Estos resultados explican los mecanismos protectores de PC y indican que, además del PC, estrategias encaminadas a modular la acción de la IL-1 y/o las HSPs podrían ser consideradas en situaciones clínicas que requieran regeneración hepática, como es el caso del trasplante hepático con injerto de tamaño reducido.

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Original Paper

How ischaemic preconditioning protects small liver grafts

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Abstract

Interleukin-1 (IL-1) and transforming growth factor-β (TGFβ) are key inhibitors of hepatocyte proliferation after hepatectomy. IL-1 inhibition by heat shock proteins (HSPs) has been reported in inflammatory processes. A recent study indicated the benefits of ischaemic preconditioning in reduced-size orthotopic liver transplantation (ROLT). The present study examined: (a) the effect of ischaemic preconditioning on IL-1 and TGFβ in ROLT; (b) whether preconditioning protects small liver grafts through HSP induction; and (c) whether the potential benefits of preconditioning on HSP is related to IL-1 inhibition. Our results, obtained with an IL-1 receptor antagonist, indicated the injurious effects of IL-1 in ischaemia-reperfusion (I/R) injury and established a relationship between IL-1 and growth factors. Thus, IL-1 reduced hepatocyte growth factor (HGF) and promoted TGFβ release, thus contributing to the impaired liver regeneration associated with ROLT. Preconditioning inhibited IL-1 through nitric oxide (NO), thereby protecting against the injurious effects of IL-1. In addition, by another pathway independent of NO, preconditioning induced HSP70 and haem-oxygenase-1 (HO-1). HO-1 protected against I/R injury and liver regeneration, whereas the benefits resulting from HSP70 were mainly related to hepatocyte proliferation. These results suggest a mechanism that explains the effectiveness of preconditioning in ROLT. They suggest, too, that other strategies, in addition to preconditioning, that modulate IL-1 and/or HSPs could be considered in clinical situations requiring liver regeneration such as small liver grafts.

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Keywords: reduced-size liver transplantation; ischaemia-reperfusion; liver regeneration; IL-1; HSP; oxidative stress

Introduction

Living-related liver transplantation was developed to alleviate the mortality resulting from the scarcity of suitable cadaveric grafts [1,2]. The main problem in using living-related liver transplantation for adults is graft size disparity [3,4]. In addition, ischaemia-reperfusion (I/R), which is inevitable in liver transplantation, reduces liver regeneration after hepatectomy [5,6].

Ischaemic preconditioning, i.e. a short period of ischaemia followed by a brief period of reperfusion before a sustained ischaemic insult, improved hepatic regeneration in an experimental model of reduced-size liver transplantation [7]. This surgical strategy promoted the release of hepatocyte growth factor (HGF). However, hepatocyte growth is controlled by both growth-promoting and growth-inhibiting factors [8,9].

Transforming growth factor β (TGFβ), a potent inhibitor of hepatocyte DNA synthesis [10–12] counterbalances the stimulatory effects of mitogens such as HGF during liver regeneration [10,13]. However, TGFβ does not seem to be the sole or the most significant negative regulator of hepatocyte replication. In fact, interleukin-1 (IL-1) is the main inhibitor of hepatocyte proliferation after partial hepatectomy without ischaemia [14].

IL-1 biosynthesis is down-regulated through a mechanism related to the induction of heat shock proteins (HSPs) [15–19]. The toxicity of IL-1 for pancreatic cells can be prevented by haem-oxygenase-1 (HO-1) activators [16,17]. Over-expression of HSP70 limits lipopolysaccharide (LPS)-induced production of IL-1 [18]. *In vitro* and *in vivo* studies indicate the ability of HSPs to inhibit IL-1 release in lung cells [15,19].

Previous results indicate that ischaemic preconditioning induces HSP70 over-expression in isolated hepatocytes [20] and reduces hepatic IL-1 production under normothermic conditions [21]. However, to our knowledge, the possibility that preconditioning protects in reduced-size liver transplantation by inducing changes in HSP and/or IL-1 release has not been tested previously.

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We report the results of an experimental study designed to evaluate: (a) the effect of ischaemic preconditioning on TGFβ and IL-1; (b) whether preconditioning protects in reduced-size liver transplantation through HSP induction; and (c) whether the potential benefit of preconditioning for HSPs is related to IL-1 inhibition. Ischaemic preconditioning has been successfully used clinically under normothermic conditions for hepatic tumour resections [22–24]. In our knowledge, there is only one study indicating the benefits of ischaemic preconditioning in reduced-size liver transplantation, but the underlying protective mechanisms were not investigated [7]. If preconditioning could be understood at the molecular level, it might be possible to develop new surgical and/or pharmacological treatments in reduced-size liver transplantation.

Materials and methods

Experimental animals

Male Sprague–Dawley rats (200–250 g) were anaesthetized with isoflurane. Research procedures complied with European Union regulations for animal experiments (EU Guideline 86/609/EEC).

Experimental design

Protocol 1. IL-1 in the benefits of preconditioning for hepatic I/R injury and liver regeneration in ROLT 24 h after transplantation

Effect of preconditioning on the injurious effects of IL-1 in ROLT

1. Sham ($n = 6$): silk ligatures in the right suprarenal vein, and hepatic artery.
2. Reduced-size orthotopic liver transplantation (ROLT) ($n = 12$, six transplantations): liver was reduced by removing the left lateral lobe and the two caudate lobes and preserved with cold University of Wisconsin (UW) solution for 1 h [7]. ROLT was performed according to the Kamada'cuff technique [25].
3. ROLT + IL-1ra ($n = 12$): like group 2, but with interleukin-1 receptor antagonist (Amgen Biologicals, Thousand Oaks, CA), 40 mg/kg i.v. just after reperfusion [21].
4. PC + ROLT ($n = 12$): like group 2 but with ischaemic preconditioning (PC) induced by 10 min of ischaemia followed by 10 min of reperfusion before the livers were flushed with UW solution [7].
5. PC + ROLT + IL-1α ($n = 12$): like group 4, but with recombinant IL-1α (Prepotech EC, Rocky Hill, NJ), 5 µg/kg i.p. just after reperfusion [26,27].

Role of nitric oxide (NO) in the benefits of preconditioning on IL-1 release

6. ROLT + NO ($n = 12$): like group 2, but with spermine NONOate (Cayman Chemical, Ann Arbor, MI), 10 mg/kg i.v. 5 min before the livers were flushed with UW solution [7].
7. PC + ROLT + NAME ($n = 12$): like group 4, but with a NO synthesis inhibitor, Nω-nitro-L-arginine methyl ester (NAME) (Sigma Chemical, St. Louis, MO), 10 mM when the liver grafts were harvested, and 20 mg/kg i.v. just after reperfusion [7].
8. PC + ROLT + NAME + IL-1ra ($n = 12$): like group 7, but with IL-1ra, 40 mg/kg i.v. just after reperfusion [7,21].

Twenty-four hours after transplantation, plasma and liver samples were collected. Hepatic injury was evaluated by determination of transaminases in the plasma. IL-1, malondialdehyde (MDA), proliferating cell nuclear antigen (PCNA) labelling index, HGF and TGFβ levels were evaluated in liver. Tissue accumulation of nitrite and nitrate was determined. Histological analysis in the liver was also performed.

Protocol 2. HSPs in the benefits of preconditioning on hepatic I/R injury and liver regeneration in ROLT 24 h after transplantation

Effect of preconditioning on HSPs HSP90, HSP70 and HO-1 levels were analysed by western blot in liver from groups 1, 2, 4 and 7 of Protocol 1.

Role of HSPs To study whether the changes in HSPs induced by preconditioning are reflected in amelioration of I/R injury and liver regeneration, the following experimental groups were studied:

9. PC + ROLT + HSP70inh ($n = 12$): like group 4, but with a HSP inhibitor, quercetin (Sigma Chemical), 100 mg/kg i.p. 2 h before preconditioning [28,29]. In addition to HSP70, quercetin inhibits the expression of HSP90 and HSP25 [30,31].
10. PC + ROLT + HO-1inh ($n = 12$): like group 4, but with a HO inhibitor, Zinc(II) Protoporphyrin IX (Oxis Research, Portland, OR), 20 mg/kg i.p. 24 h before preconditioning [32]. In addition to HO-1, Zinc(II) Protoporphyrin IX inhibits all HO activity mediated by HO-2 and HO-3 [33,34].

The doses of quercetin and Zinc(II) Protoporphyrin IX used in the present study were effective in evaluating the role of HSP70 and HO-1 in different experimental models of I/R [28,29,32]. Control experiments were performed at a higher dose of these inhibitors, and the results obtained were similar. Quercetin and the other drugs were prepared in dimethylsulphoxide and saline, respectively, as in previous reports [32,35–37] and following the manufacturer's instructions. Control experiments were performed with the vehicle used for the different drugs. Under our conditions, the vehicle used did not modify the post-transplantation outcomes.

Twenty-four hours after transplantation, plasma and liver samples were collected. Biochemical determinations and histological analyses were the same as those

described in Protocol 1. The effect of quercetin on HSP90, HSP70, HO-1 and HSP25 was also evaluated.

Protocol 3. HSPs and IL-1 in ROLT 6 h after transplantation

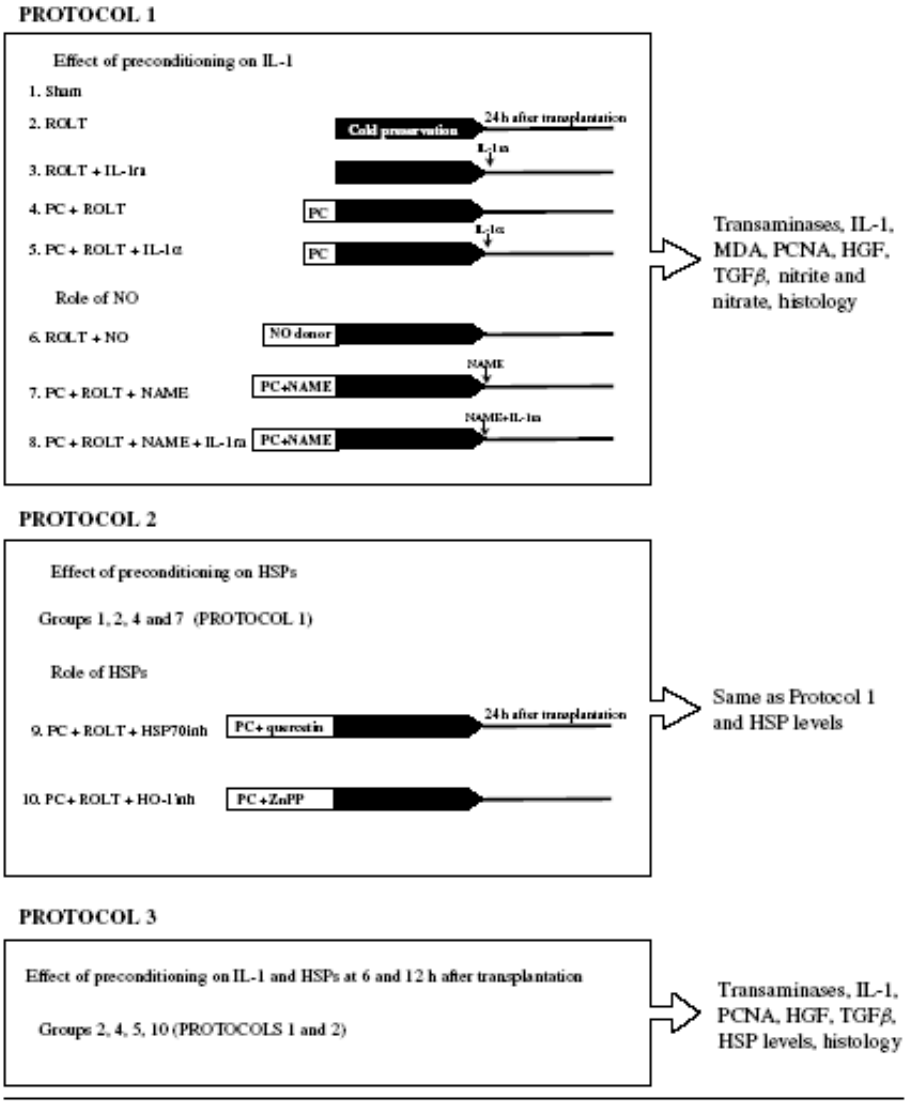
To evaluate whether the changes in IL-1 and HSPs caused by preconditioning 24 h after transplantation (Protocols 1 and 2) occurred at earlier time points, a surgical procedure similar to that used for groups 2, 4, 5 and 10 was carried out, but the samples were obtained at 6 and 12 h after transplantation and processed to determine transaminases, IL-1, PCNA labelling index, HGF and TGFβ levels, HSP levels and grade 3 necrosis.

The interventions and measurements for Protocols 1, 2 and 3 are summarized in Table 1.

Biochemical determinations

- *Transaminase assay*. Transaminases were measured using a commercial kit from Boehringer-Mannheim (Munich, Germany).
- *Lipid peroxidation assay*. Lipid peroxidation was determined by measuring the formation of MDA with the thiobarbiturate reaction [7].
- *Interleukin assay*. IL-1 levels were measured as previously reported [21]. Commercial kits from Amersham Life Sciences (Amersham, UK) were used.
- *Determination of nitrite and nitrate*. NO production was determined by tissue accumulation of nitrite and nitrate [37,38].
- *PCNA labelling index*. Immunohistochemistry was performed using a commercial kit [DAKO Envision +System, peroxidase (DAB); Dako GMbh, Germany] [7].

Table 1. Flow chart of the interventions and measurements corresponding to Protocols 1, 2 and 3



- *Assay for HGF*. HGF levels were measured by the enzyme-linked immunosorbent technique (Institute of Immunology, Tokyo, Japan) [7].
- *Assay for TGFβ*. Total and active TGFβ levels were measured as previously reported [39,40]. Commercial kits from R&D Systems (Minneapolis, MN) were used.

Western blot analysis for HSP

Equal amounts of protein were separated by SDS–PAGE and transferred to PVDF membranes. Western blotting was performed with primary antibodies against HSP90 and HSP70 (BD Transduction Laboratories, San José, CA), HO-1 and β-actin (Sigma Chemical, St. Louis, MO) and HSP25 (StressGen, Vancouver, BC). Signals were detected by enhanced chemiluminescence and quantified by scanning densitometry. All signals were standardized to the corresponding Ponceau S [41].

Quantitative real-time PCR for *IL-1α* messenger RNA

Total RNA from liver samples was obtained using the isolation kit Ultraspec (Biotecx Laboratories, Houston, TX) and was reverse-transcribed using a Ready-To-Go You-Prime First-Strand Beads Kit and Random Hexamer primers (Amersham, Uppsala, Sweden). *IL-1α* was specifically amplified by real-time PCR, using the probe/primer set (Rn00556700_ml) for rat *IL-1α* (NM_017019) (Applied Biosystems, Foster City, CA). Gene expression of *IL-1α* relative to *β-Actin* (probe/primer: Rn00667869_ml, Applied Biosystems) was calculated using the $\Delta\Delta C_T$ method [42].

Histology

To determine the severity of hepatic injury, haematoxylin and eosin (H&E)-stained sections were evaluated by a point-counting method on an ordinal scale [7].

Statistics

Data are expressed as means ± standard error. Statistical comparison was performed with analysis of variance, followed by the Student–Newman–Keuls test. $p < 0.05$ was considered significant.

Results

IL-1 in the benefits of preconditioning on hepatic I/R injury and liver regeneration in ROLT 24 h after transplantation

No differences in hepatic IL-1β levels were observed in any of the groups evaluated. Unlike IL-1β, hepatic IL-1α levels did increase after ROLT over the results obtained in the Sham group. (Figure 1A). IL-1 receptor antagonist attenuated the increases in transaminases (Figure 2A) and grade 3 necrosis (Figure 2B). Preconditioning reduced IL-1α (Figure 1A) and protected against hepatic injury (Figure 2A, B). These benefits were reverted when IL-1α was administered (PC + ROLT + IL-1α).

Like preconditioning, NO pre-treatment reduced IL-1α release and *IL-1α* mRNA expression (Figure 1A, B, respectively) and protected against hepatic injury (Figure 2). NO synthesis inhibition (PC + ROLT + NAME) abolished the benefits of preconditioning on IL-1α release and on the parameters of hepatic injury; and IL-1 receptor antagonist supplementation (PC + NAME + IL-1ra) prevented the injurious effects of NO synthesis inhibition. The amount of NO in the liver was also checked. Thus, significantly more NO (reflected in the values of tissue nitrites and nitrates) was found after preconditioning and after NO pre-treatment than in the Sham group (Figure 3A). L-NAME reduced the increased nitrite and nitrate levels observed in the preconditioned group.

Histological study of the liver after ROLT revealed multifocal and extensive areas of coagulative necrosis randomly distributed throughout the hepatic paren-

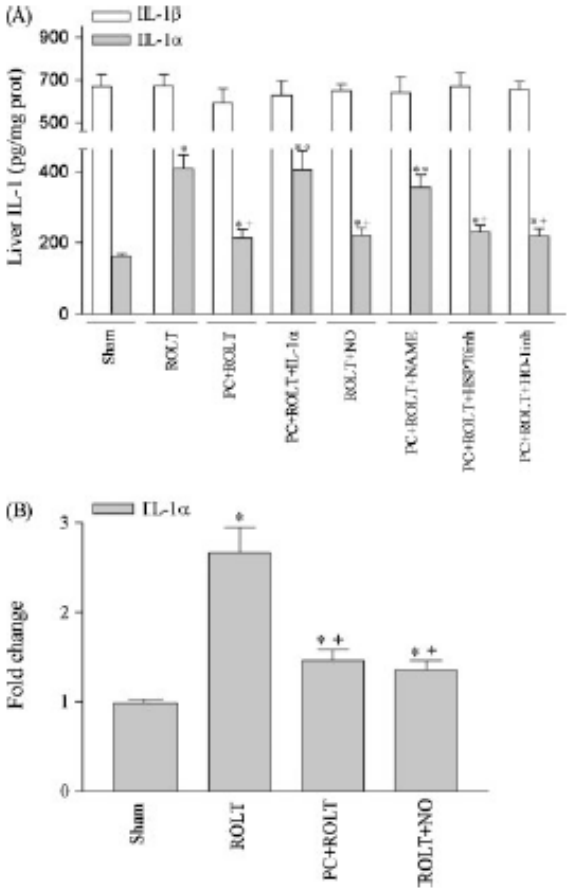


Figure 1. (A) IL-1 (IL-1α and IL-1β) and (B) *IL-1α* mRNA expression levels (the data represent the fold change vs. Sham group) in liver 24 h after transplantation. *$p < 0.05$ vs. Sham; +$p < 0.05$ vs. ROLT; ◦$p < 0.05$ vs. PC + ROLT

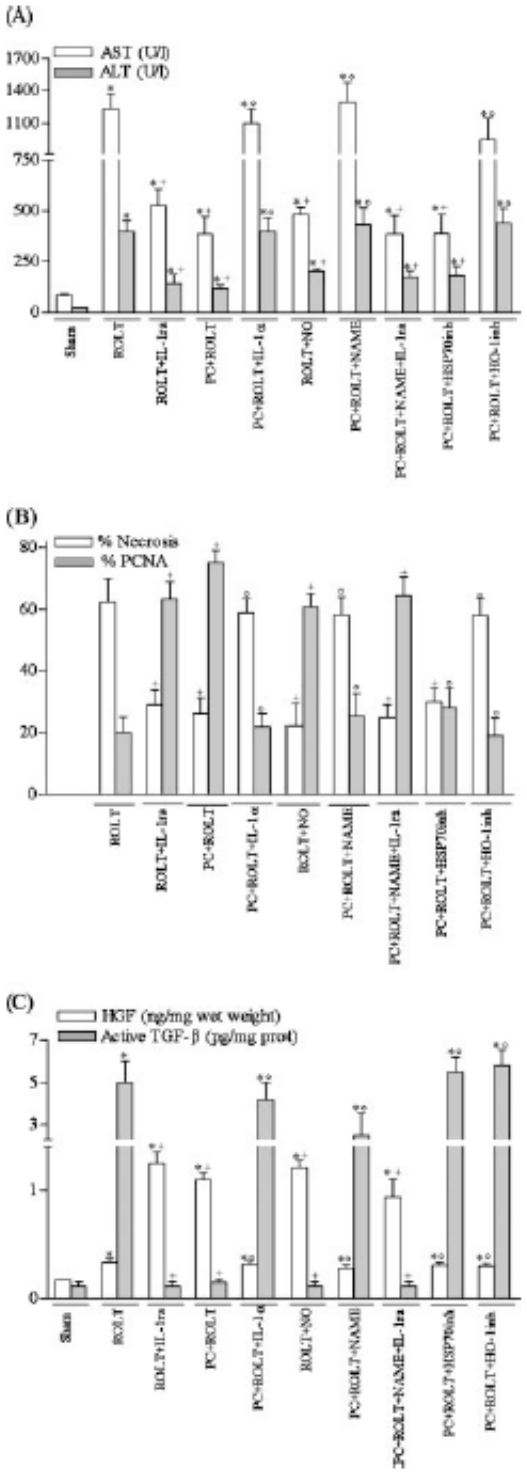


Figure 2. (A) Transaminase, (B) grade 3 necrosis and percentage of PCNA-positive hepatocytes, and (C) hepatic HGF and TGFβ levels 24 h after transplantation. $^{*}p < 0.05$ vs. Sham; $^{+}p < 0.05$ vs. ROLT; $^{\circ}p < 0.05$ vs. PC + ROLT

chyma (Figure 4A). However, fewer and smaller areas of coagulative hepatocyte necrosis were observed in ROLT + IL-1ra, PC + ROLT and PC + ROLT + NAME + IL-1ra groups (Figure 4B, C and D, respectively).

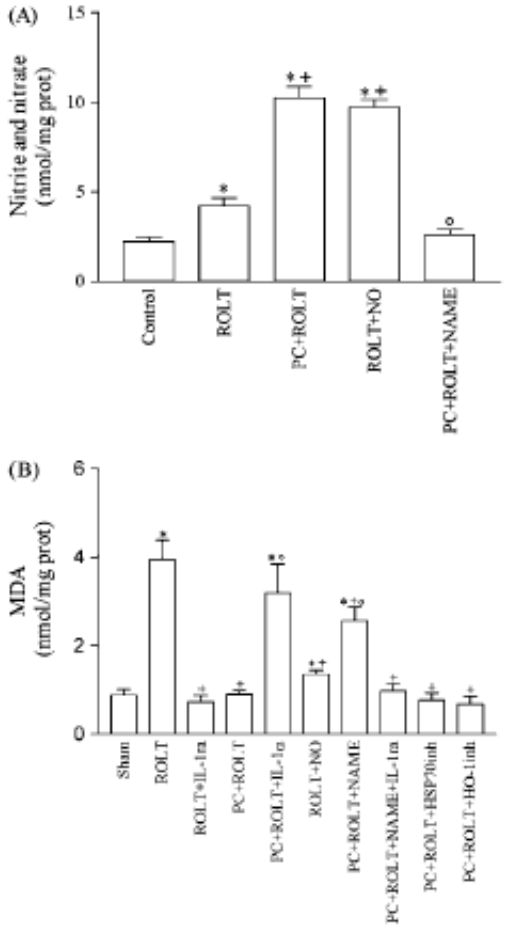


Figure 3. (A) Nitrite and nitrate and (B) MDA levels in liver 24 h after transplantation. $^{*}p < 0.05$ vs. Sham; $^{+}p < 0.05$ vs. ROLT; $^{\circ}p < 0.05$ vs. PC + ROLT

For the parameters of liver regeneration, IL-1 receptor antagonist, preconditioning and NO pre-treatment increased both PCNA-positive hepatocytes and HGF, and reduced active TGFβ levels (Figures 2, 5). Total hepatic TGFβ levels were similar in all groups (data not shown). NO inhibition in the preconditioned group (PC + ROLT + NAME) and IL-1α administration (PC + ROLT + IL-1α) abolished the benefits of preconditioning on liver regeneration, resulting in proliferation index, HGF and TGFβ levels similar to those for the ROLT group. However, IL-1 receptor antagonist supplementation in the PC + ROLT + NAME group (PC + ROLT + NAME + IL-1ra) returned the parameters of liver regeneration to those for the PC + ROLT group (Figures 2, 5).

HSPs in the benefits of preconditioning on hepatic I/R injury and liver regeneration in ROLT 24 h after transplantation

As shown in Figure 6A, no differences in HSP90 levels as a consequence of ROLT were found.

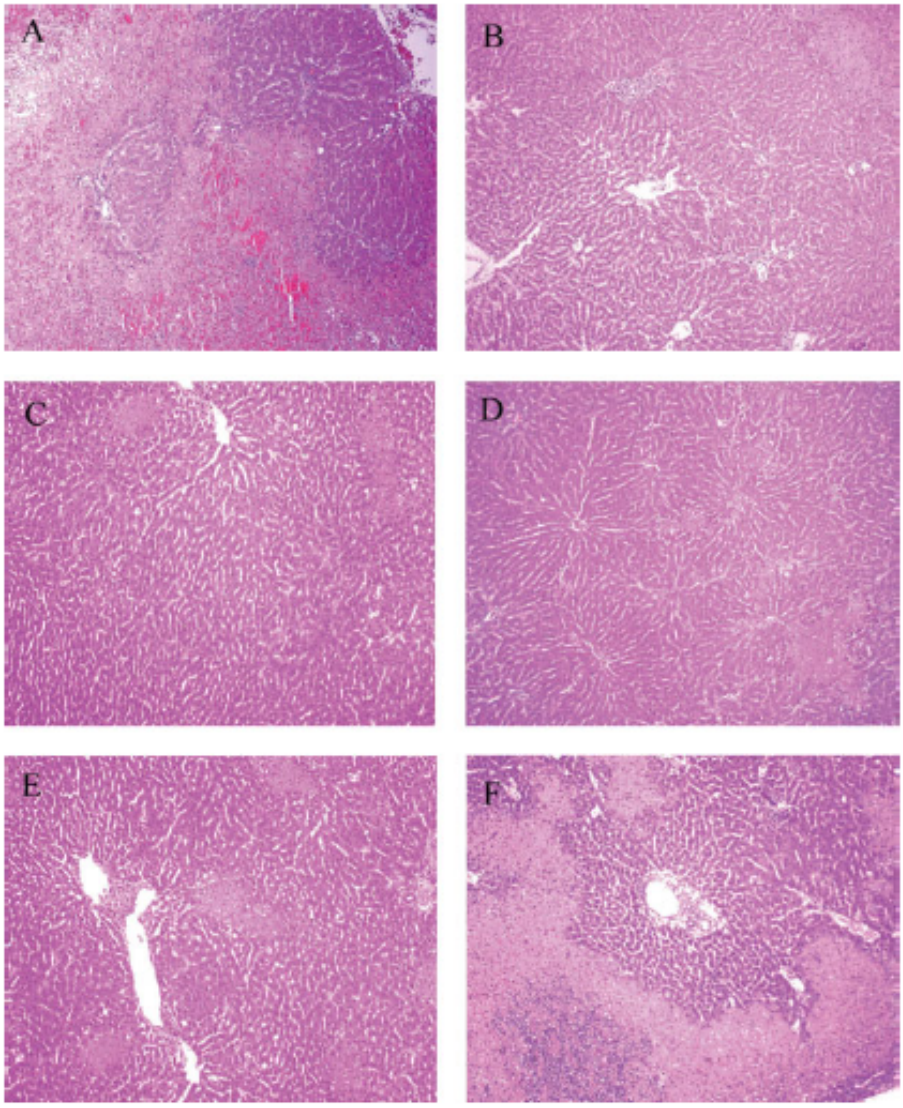


Figure 4. Histological lesions in liver 24 h after transplantation. (A) ROLT, extensive and multifocal areas of coagulative necrosis of hepatocytes with disruption of hepatic cords, infiltration of neutrophils and haemorrhage. (B) ROLT + IL-1ra, focal and small areas of hepatocyte necrosis. (C) PC + ROLT and (D) PC + ROLT + L-NAME + IL-1ra: similar to ROLT + IL-1ra. (E) PC + ROLT + HSP70inh and (F) PC + ROLT + HO-1inh: similar to ROLT + IL-1ra and ROLT, respectively (H&E, ×240)

Preconditioning increased both HSP70 and HO-1 levels more than the ROLT group did. The effect of preconditioning on HSP was not modified when NO synthesis was inhibited (see Figure 6A). Under our conditions, quercetin inhibited HSP70 (Figure 6B). However, similar HSP90, HO-1 and HSP25 levels were observed in PC + ROLT and PC + ROLT + HSP70inh groups.

In contrast to the results obtained with HSP70 inhibition, HO inhibition reverted the benefits of preconditioning on hepatic injury (Figures 2, 4). HSP inhibition in the preconditioned group (PC + ROLT + HSP70, PC + ROLT + HO-1) reduced HGF and increased TGFβ, resulting in HGF, TGFβ and proliferation values similar to those for the unpreconditioned group (ROLT) (Figures 2, 5).

The effect of HSP induction caused by preconditioning on IL-1α levels was tested. HSP inhibition (PC + ROLT + HSP70inh, PC + ROLT + HO-1inh) did not modify the reduced IL-1α levels induced by preconditioning (PC + ROLT) (Figure 1A). The role of IL-1 and HSPs in the benefits of preconditioning on oxidative stress was also evaluated. IL-1 receptor antagonist, preconditioning and NO protected against oxidative stress (Figure 3B). NO synthesis inhibition (PC + ROLT + NAME) and IL-1α administration (PC + ROLT + IL-1α) abolished the benefits of preconditioning on MDA levels; IL-1 receptor antagonist supplementation in the PC + ROLT + NAME group (PC + ROLT + NAME + IL-1ra) resulted in MDA levels comparable to those found in the PC + ROLT group. HSP inhibition (PC + ROLT +

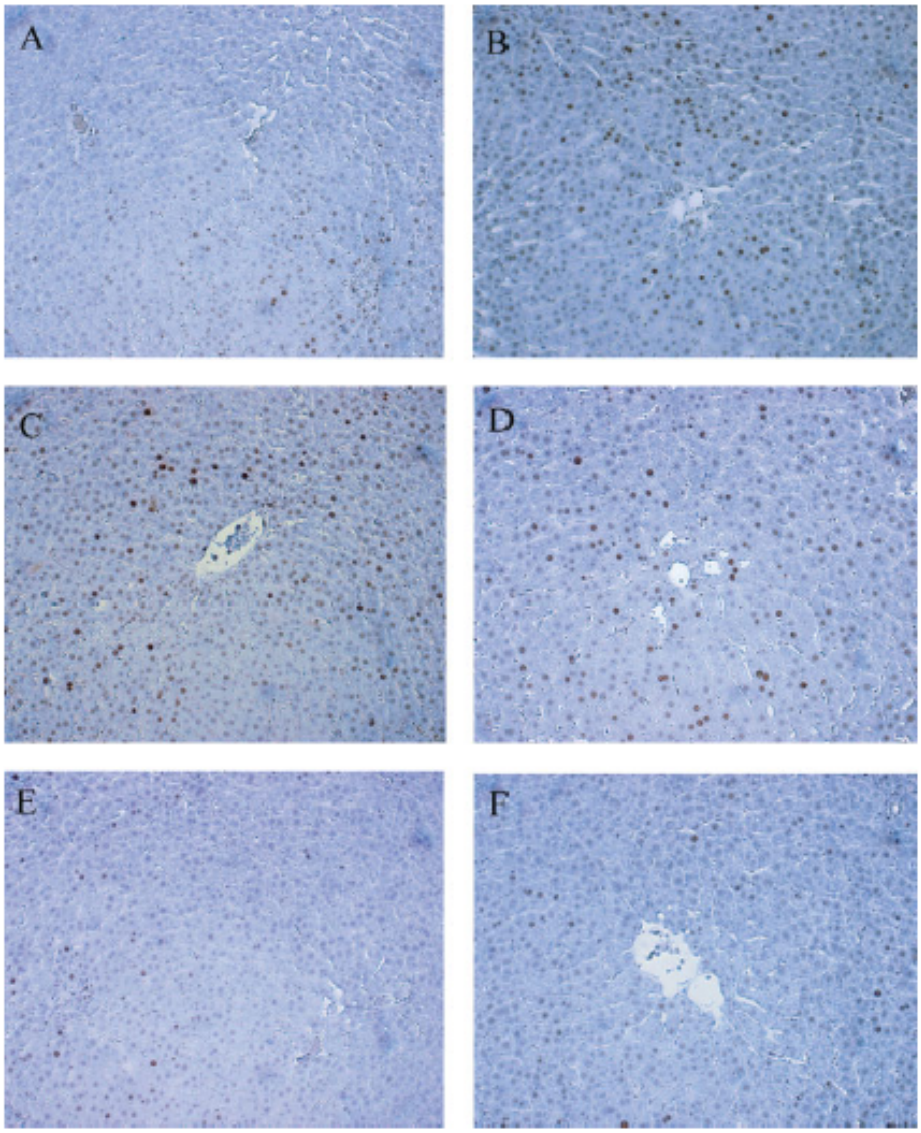


Figure 5. Immunocytochemical staining of PCNA-positive hepatocytes 24 h after transplantation. ROLT (A): lower positive cell number than ROLT + IL-1ra (B). PC + ROLT (C) and PC + ROLT + L-NAME + IL1-ra (D): amount of PCNA-positive staining hepatocytes similar to ROLT + IL-1ra. PC + ROLT + HSP72inh (E) and PC + ROLT + HO-1inh (F): PCNA-positive staining hepatocytes similar to ROLT (×500)

HSP70inh, PC + ROLT + HO-1inh) did not modify the benefits of preconditioning (PC + ROLT) on oxidative stress (Figure 3B). Other parameters of oxidative stress (lipid hydroperoxides and H_2O_2) showed the same pattern as MDA (data not shown).

HSPs and IL-1 in ROLT 6 h after transplantation

The benefits of preconditioning on hepatic I/R injury and liver regeneration observed 24 h after transplantation were also found at 6 h. Preconditioning reduced transaminase levels and grade 3 necrosis. This surgical strategy reduced TGFβ and increased HGF and the percentage of PCNA-positive hepatocytes (Figure 7). At 6 h after transplantation, no differences in HSP70 levels were observed in any of the groups evaluated (Figure 8A). As at 24 h, preconditioning reduced IL-1α and increased HO-1 levels 6 h after transplantation (Figure 8). IL-1α administration and HO inhibition in the preconditioned group abolished the benefits of preconditioning on the parameters of hepatic injury and liver regeneration (Figure 7). HO inhibition did not affect the benefits of preconditioning on IL-1α release 6 h after transplantation (Figure 8B). Similar results were observed 12 h after transplantation.

Discussion

Higher TGFβ and lower HGF after portal branch ligation may suppress hepatocyte proliferation, thus

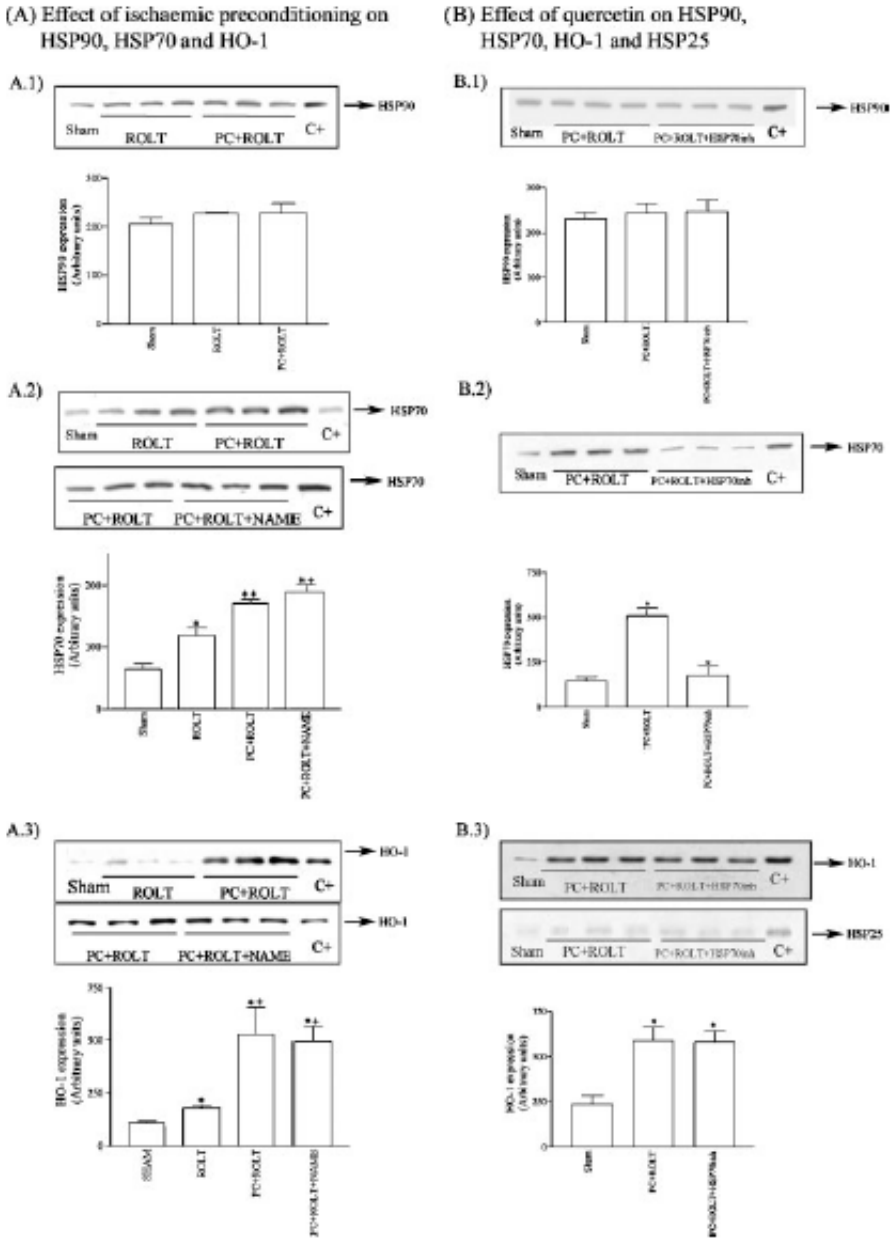


Figure 6. (A) Effect of preconditioning on HSP90 (A.1), HSP70 (A.2) and HO-1 (A.3) and (B) effect of quercetin on HSP90 (B.1), HSP70 (B.2), HO-1 and HSP25 (B.3) in liver 24 h after transplantation. The upper panels show one representative blot of three independent experiments and the lower panels show densitometric evaluation of the independent western blot. $^{*}p < 0.05$ vs. Sham; $^{+}p < 0.05$ vs. ROLT, $^{\circ}p < 0.05$ vs. PC + ROLT

delaying liver regeneration [43]. Our results suggest that the benefits of preconditioning and NO donors on liver regeneration in ROLT were due to a balance between TGFβ and HGF. Both strategies reduced TGFβ levels after ROLT, which was associated with increased HGF levels.

In addition to growth factors, partial hepatectomy induces activation of cytokines [44–46]. IL-1β has been put forward as a possible down-regulator of hepatocyte proliferation in *in vitro* and experimental models of hepatectomy without ischaemia, as has TGFβ [11,14,47]. Our results indicate that IL-1β does not seem to play a role in mediating I/R injury and liver regeneration associated with ROLT. However, this was not the case for IL-1α. Interestingly, strategies such as the use of IL-1 receptor antagonist, aimed at modulating the action of IL-1α, may be important for attenuation of hepatic I/R injury and amelioration of liver regeneration associated with ROLT. In addition to ascertaining the function of IL-1 in ROLT, the results gave information on the availability of IL-1 in modulating the growth factors HGF and TGFβ.

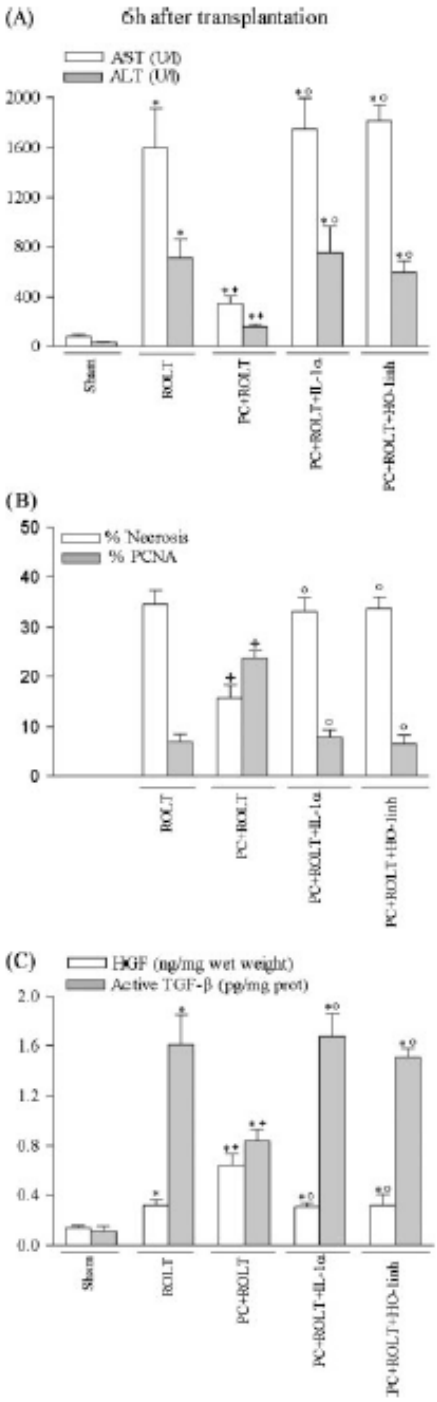


Figure 7. (A) Transaminase, (B) grade 3 necrosis and percentage of PCNA-positive hepatocytes, and (C) hepatic HGF and TGFβ levels 6 h after transplantation. $^{*}p < 0.05$ vs. Sham; $^{+}p < 0.05$ vs. ROLT; $^{\circ}p < 0.05$ vs. PC + ROLT

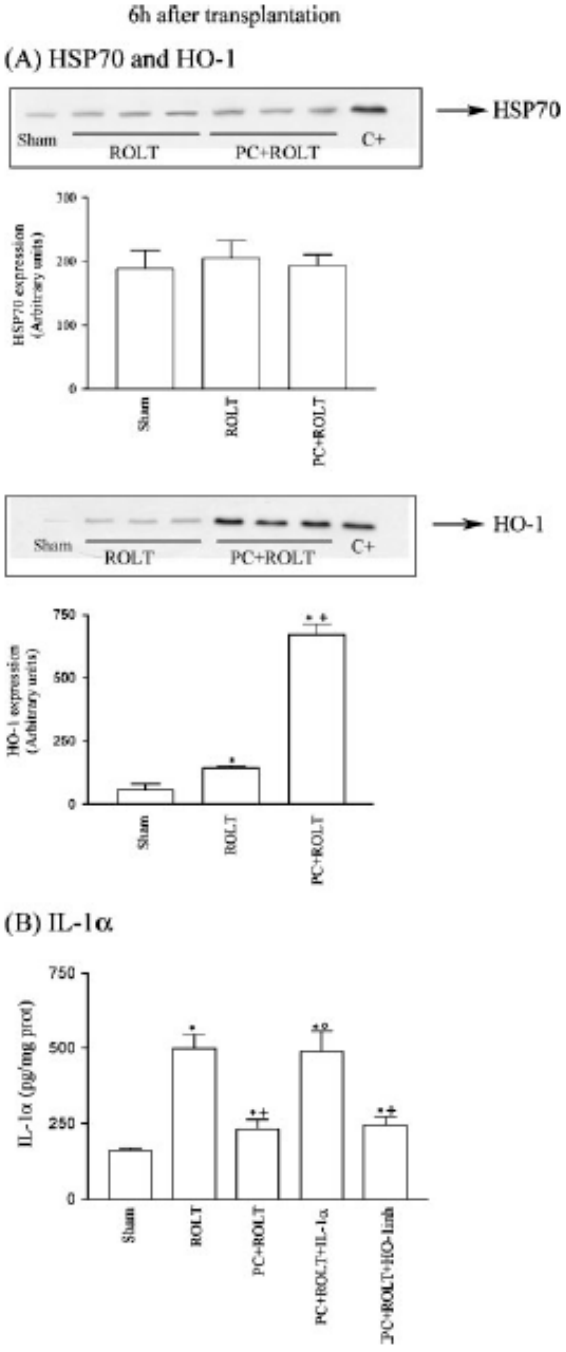


Figure 8. (A) HSP70 and HO-1 and (B) IL-1 levels in liver 6 h after transplantation. The upper panels show one representative blot of three independent experiments and the lower panels show densitometric evaluation of the independent western blot. $^{*}p < 0.05$ vs. Sham; $^{+}p < 0.05$ vs. ROLT; $^{\circ}p < 0.05$ vs. PC + ROLT

Like IL-1 receptor antagonist pre-treatment, preconditioning can be considered a strategy for modulating the injurious effects of IL-1 on hepatic I/R injury and liver regeneration associated with ROLT. Given the role of NO in preconditioning [7,48] and its possible role on IL-1 release [49–51], we studied whether the benefits of preconditioning on the injurious role of IL-1 in ROLT are mediated by NO. Experimental results seem to confirm this hypothesis. NO synthesis inhibition in the preconditioned group increased IL-1α

release after ROLT, abolishing the benefits of preconditioning on hepatic injury, oxidative stress and liver regeneration. However, if we inhibited IL-1 action with IL-1 receptor antagonist, the injurious effect of NO inhibition disappeared. In addition, NO donor administration to ROLT simulated the benefits of preconditioning on hepatic injury and liver regeneration.

Altogether, our results indicate that preconditioning, through NO, inhibits IL-1 release, thereby protecting against hepatic I/R and ameliorating liver regeneration associated with ROLT. This results in the regulation of both growth factors and oxidative stress. Although further studies will be required to establish a relationship between growth factors and oxidative stress in ROLT, this possibility should not be ruled out. In fact, in experimental models of hypoxia/reoxygenation in hepatocytes [52] and hepatic I/R [53], HGF pre-treatment inhibited reactive oxygen species production. Conversely, TGFβ induced an oxidative stress process in hepatocytes [54,55]. The injurious effects of oxidative stress on liver regeneration are well known [6,56].

Data obtained in pancreatic [16,17] and lung cells [19], and results obtained in experimental models of toxicity induced by LPS [15,18], indicate that the induction of HSP is responsible for IL-1 biosynthesis inhibition. In addition, the role of NO in regulating HSP expression in different experimental models of I/R has been reported previously [57–59]. Thus, taking all of these findings into account, we hypothesize that preconditioning through NO induces HSP over-expression. This would reduce IL-1 release, thus improving hepatic I/R injury and liver regeneration following ROLT. Our results in ROLT seem not to confirm this hypothesis. In our hands, the benefits of preconditioning on IL-1α seem not to be dependent on HSPs. Thus, we suggest a mechanism to explain the protection offered by preconditioning, as summarized in Figure 9. Preconditioning through NO inhibits IL-1α release. The benefits of IL-1α inhibition on I/R injury and liver regeneration can be explained, at least partially, by the regulation of growth factors and/or oxidative stress. In addition, preconditioning by another pathway independent of NO protects against hepatic I/R injury and ameliorates liver regeneration associated with ROLT. This second pathway seems likely to be HO-1 and HSP70 induction. HO-1 protects against hepatic I/R and ameliorates liver regeneration associated with ROLT. Given the results obtained 6, 12 and 24 h after transplantation, HO-1 induction seems key to maintaining the protection of liver grafts throughout reperfusion. The protection conferred by HSP70 in preconditioning is mainly related to cellular proliferation processes and was only seen at prolonged reperfusion periods. It should be noted that, in addition to HSP70, other protective mechanisms of preconditioning should not be discarded. The HSP70 inhibitor used in the present study, quercetin, also inhibits protein kinase C [60,61]. It is known that protein kinase C modulates intracellular signals in preconditioned livers and liver after transplantation [62,63]. Similarly, due to the properties of Zinc(II) Protoporphyrin IX, the involvement of HO-2 and HO-3 in the benefits of preconditioning should not be ruled out. The results of the present study may open the way to new surgical and/or pharmacological strategies to protect small liver grafts effectively from the deleterious effects of I/R on hepatic injury and liver regeneration.

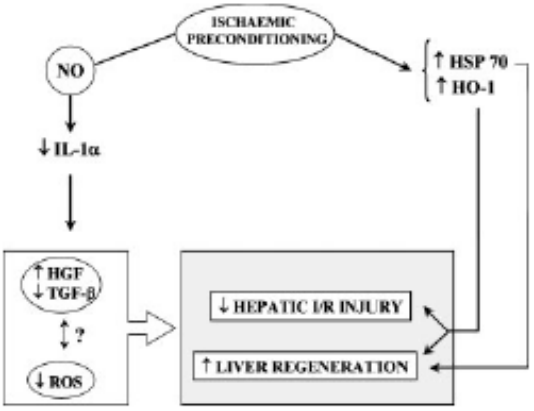


Figure 9. Diagram of suggested mechanisms by which preconditioning modulates hepatic injury and liver regeneration in ROLT 24 h after transplantation

Acknowledgements

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4.3. TERCER ESTUDIO

PROTECTION AGAINST LUNG DAMAGE IN REDUCED-SIZE LIVER TRANSPLANTATION

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Critical Care Medicine, En prensa

En el hígado, la inhibición de las células de kupffer, el PC y el NO disminuyeron los niveles de IL-1α tras un trasplante hepático con injerto de tamaño reducido (ROLT). El PC, a través del NO, redujo la liberación de IL-1 y ejerció un papel protector frente a la lesión pulmonar asociada al ROLT. La inhibición de la síntesis de NO en el grupo con PC llevó a un aumento en los niveles de IL-1 y aumentó el daño pulmonar después del ROLT, mientras que el tratamiento con el antagonista del receptor de IL-1 (IL-1ra) protegió frente a los efectos adversos resultantes de la inhibición de la síntesis de NO. Además, la administración de un donador de NO resultó en unos parámetros similares a aquellos encontrados en el grupo PC por lo que respecta a los niveles de IL-1 y lesión pulmonar. Los beneficios observados en pulmón como consecuencia de la inhibición de la IL-1 parecen estar ligados al efecto que presenta esta citoquina sobre un mecanismo endógeno que neutraliza la acción del TNF-α, como son los receptores solubles del TNF-α. Así pues, estrategias que inhiben la acción de la IL-1, como son el tratamiento con IL-1ra, el PC y el tratamiento con el donador de NO, provocan un aumento en los niveles plasmáticos de sTNFR2 y disminuyen los niveles sistémicos de TNF-α libre, después del ROLT. De igual manera, la inhibición de la síntesis de NO en el grupo con PC, que provocó un aumento en los niveles de IL-1 y en el daño pulmonar, redujo los niveles de sTNFR2 en plasma y aumentó los niveles de TNF-α libre. Estos efectos adversos desparecieron cuando se inhibió la acción de la IL-1.

PROTECTION AGAINST LUNG DAMAGE IN REDUCED-SIZE LIVER TRANSPLANTATION

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ABSTRACT

Objective: This study examined the effect of ischemic preconditioning on pulmonary damage associated with reduced-size orthotopic liver transplantation (ROLT) and attempted to identify the underlying protective mechanisms. *Design*: Randomized and controlled animals study. *Setting*: Experimental laboratory. *Subjects*: Male Sprague-Dawley rats. *Interventions*: Lung damage was evaluated in ROLT with or without preconditioning. Nitric oxide (NO) and interleukin-1 (IL-1) actions were altered pharmacologically. *Measurements and Main Results*. IL-1, tumor necrosis factor-α (TNF), soluble TNF receptors (sTNFR) and inflammatory response in lung were measured after ROLT. Our results indicate the involvement of IL-1 in the lung damage following ROLT. Ischemic preconditioning, mediated by NO, reduced IL-1 release and protected against lung damage. NO synthesis inhibition in the preconditioned group led to increased IL-1 levels and increased lung damage following ROLT, while the addition of IL-1 receptor antagonist protected against the injurious effects of NO inhibition. In addition, NO pre-treatment gave similar results in terms of IL-1-α, and lung protection to those found in preconditioning. The benefits to the lung attributable to IL-1 inhibition might be linked to the effect of this cytokine on sTNFR, an endogenous mechanism that modulates systemic TNF actions. In fact, strategies aimed at inhibiting IL-1 action, including IL-1 receptor antagonist, ischemic preconditioning, and NO donor, increased systemic sTNFR2 and decreased free TNF, following ROLT. Similarly, NO synthesis inhibition in the preconditioned group, which increased IL-1α and lung damage, reduced systemic sTNFR2, and increased free TNF levels. These injurious effects were avoided when IL-1 action was inhibited. *Conclusion*: Ischemic preconditioning and pharmacological strategies that simulate its benefits protected against lung damage in an experimental model of ROLT. Our results also suggest a potential relationship between NO, IL-1 and TNF/sTNF in the benefits of preconditioning on the lung damage associated with ROLT.

INTRODUCTION

Ischemic preconditioning, induced by brief ischemia and reperfusion periods, protects the liver and the lung against a subsequent sustained hepatic I/R in warm ischemia associated with tumor hepatic resections and in liver transplantation from non-reduced size liver graft (1-5). Preconditioning by inhibition of endothelin (ET) production reduced the systemic release of Kupffer cell-associated tumor necrosis factor-α (TNF), thus preventing lung P-selectin up-regulation and subsequent pulmonary damage (3-5). Moreover, preconditioning modulates other systems involved in both local and systemic disorders including xanthine-xanthine oxidase (XOD) (1,2). Preconditioning was more effective than the addition of XOD inhibitors to the preservation solutions or anti-selectin, anti-ET or anti-TNF therapies in preventing hepatic I/R injury (1,2,5). There is evidence that the benefits of preconditioning on these mechanisms responsible for local and systemic disorders associated with hepatic I/R depend on the release of nitric oxide (NO) (1-5). A recent experimental study from our group showed the benefits of ischemic preconditioning on hepatic injury associated with reduced-size liver transplantation (6). However, it is not known whether ischemic preconditioning is effective only locally (liver) or if it would also modulate the potential lung damage associated with reduced-size liver transplantation.

In addition to TNF, ET, and xanthine/XOD, the injurious role of interleukin-1 (IL-1) in the lung damage associated with hepatic I/R is well established (7-10). The data obtained in experimental models of hepatic I/R that mimic the warm ischemia associated with tumor hepatic resections indicate that IL-1 action inhibition ameliorated pulmonary injury (8), and IL-1 produced in the liver appears to stimulate the alveolar macrophages of the lung and induce pulmonary injury (10). On the basis of these reports, there would seem to be a real possibility that IL-1 is responsible for the lung damage associated with reduced-size liver transplantation.

The relationship between NO generation and hepatic IL-1 production has been demonstrated in various inflammatory processes (11-14). Thus, the inhibition of NO synthesis increased IL-1 generation in experimental models of hepatic I/R that mimic the warm ischemia associated with tumor hepatic resections (11-13), and inducible NO synthase-deficient animals showed high IL-1 levels in response to lipopolysaccharide (14). However, the hypothesis that the NO released during preconditioning might modulate the lung damage associated with reduced-size liver transplantation through its action on hepatic IL-1 release remains unconfirmed.

Several analyses of systemic disorders associated with inflammatory processes indicate that increases in IL-1 occur in parallel with high TNF levels (10,15,16). It has been widely demonstrated that TNF mediates inflammation by two distinct cell-surface receptors (TNFR1 and TNFR2), as observed during rejection episodes and impaired graft function after liver transplantation (17-20). These two receptors can also be present in soluble form (sTNFR: sTNFR1 and sTNFR2), since the extracellular part of both receptors is shed by proteolytic cleavage and circulates as sTNFR (natural inhibitors of TNF). This then binds the TNF in plasma, thus preventing its deleterious effects (21-24). In fact, treatment with sTNFR stems plasma TNF increase and prevents acute lung injury following cardiopulmonary bypass (25) and after intestinal ischemia (26). The injurious effects of IL-1 on systemic disorders associated with hepatic I/R might be explained by the effect of this cytokine on systemic TNF/sTNFR levels following I/R. Thus, IL-1 receptor antagonist treatment reduced plasma TNF release in experimental model of hepatic I/R that mimic the warm ischemia associated with tumor hepatic resections (15). In addition, IL-1 down-regulated the expression of receptors for TNF in cell lines from human fibroblastoid and cervical carcinoma, which may reduce sTNFR release (27-29). Thus, it would appear that strategies aimed at modulating IL-1 action might be useful to regulate TNF/sTNFR levels and to reduce the systemic diseases associated with hepatic I/R processes.

Accordingly, we report the results of an experimental study aimed at evaluating 1) the role of IL-1 in the lung damage associated with reduced-size liver transplantation; 2) the potential benefits of ischemic preconditioning for the lung; 3) whether such protection is related to the IL-1 inhibition by NO; and 4) whether the modulation of IL-1 action by NO results in changes in systemic TNF/sTNR levels.

MATERIAL AND METHODS

Experimental animals

Male Sprague-Dawley rats weighing 200 to 250 g were used as donors and recipients. All animals were anesthetized with isoflurane. This study respected the European Union regulations for animal experiments (EC guideline 86/609/CEE).

Experimental design

Role of IL-1 in lung damage associated with ROLT

1) Sham (n=6): Animals were subjected to anesthesia, transversal laparotomy, and silk ligatures in the right suprarenal vein, and hepatic artery.

2) Reduced-size orthotopic liver transplantation (ROLT) (n=12, 6 transplantations): Liver reduction was achieved by removing the left lateral lobe and the two caudate lobes just before harvesting the liver, which resulted in a 40% reduction in liver mass. The pedicle of the left lateral lobe was ligated with 5-0 silk ligature, and the lobe was removed. Two caudate lobes were separately removed with the ligation. The donors' livers were flushed and preserved with cold (4ºC) University of Wisconsin (UW) solution for 1 h (6). ROLT was performed according to the Kamada's cuff technique (30). The time of the anhepatic phase was 17-20 min (6), which practically coincided with the times of warm ischemia suffered by the graft between extraction from the preservation solution and the restoration of portal flow.

3) Reduced-size orthotopic liver transplantation+gadolinium chloride (ROLT+$GdCl_3$) (n=12, 6 transplantations): As in group 2, but with previous administration of $GdCl_3$ (Sigma Chemical, St. Louis, MO) (10 mg/kg, i.v.) in donor rats to inactivate Kupffer cells, 48 hours and 24 hours before hepatectomy (6).

4) Reduced-size orthotopic liver transplantation+Interleukin-1 receptor antagonist (ROLT+IL-1ra) (n= 12, 6 transplantations): As in group 2, but treated with IL-1ra (Amgen Biologicals, Thousand Oaks, CA) (40 mg/kg, i.v.) just after reperfusion (13).

Role of NO involved in ischemic preconditioning on IL-1 release following ROLT

5) Ischemic Preconditioning+Reduced-size orthotopic liver transplantation (PC**+**ROLT) (n=12, 6 transplantations): To induce ischemic preconditioning, the blood flow of the donor liver was interrupted by placing a bulldog clamp at the portal vein and hepatic artery for 10 min, followed by reflow for 10 min (13). Following the same surgical procedure as for group 2, liver lobes were resected and flushed and preserved with cold (4ºC) UW solution for 1 h.

6) Reduced-size orthotopic liver transplantation + NO donor (ROLT+NO) (n=12, 6 transplantations): As in group 2, but with previous administration of the nitric oxide (NO) donor, spermine NONOate (Cayman Chemical, Ann Arbor, MI) (10mg/kg i.v.) (10). In contrast with other NO donors, spermine NONOate is a spontaneous NO donor that releases NO independently of enzymatic catalysis, and it does not require activation in the tissue (31,32).

7) Ischemic Preconditioning+ Reduced-size orthotopic liver transplantation + NAME (PC+ROLT+NAME) (n=12, 6 transplantations): As in group 5, but with previous administration of an NO synthesis inhibitor, Nω-nitro-L-arginine methyl ester (NAME) (Sigma Chemical, St. Louis, MO). As previously reported, 10mM of L-NAME was administered through the portal vein when the liver grafts were harvested, and 20mg/kg was injected i.v. into the recipients just after reperfusion (6).

8) Preconditioning+Reduced-size orthotopic liver transplantation+NAME+IL-1ra (PC+ROLT+NAME+IL-1ra) (n=12, 6 transplantations): As in group 7, but treated with IL-1ra (40 mg/kg, i.v.) just after reperfusion (6,13).

Twenty-four hours after transplantation, plasma and lung samples were collected. Malondialdehyde (MDA) levels and myelopreroxidase (MPO) activity were measured in the lung. IL-1α and IL-1β levels were measured in liver samples. Total (bound and free) TNF-α, free TNF-α, and sTNFR (sTNFR1 and sTNFR2) were determined in plasma. Histological analyses in lung were also performed.

Biochemical determinations

Lipid peroxidation assay. Lipid peroxidation in the lung was used as an indirect measurement of the oxidative injury induced by reactive oxygen species (33). After protein precipitation, the formation of malondialdehyde (MDA) was measured by using the thiobarbiturate reaction (13,34).

Myeloperoxidase assay. Lung MPO has been used as a marker of pulmonary neutrophil infiltration and activation (35,36). MPO levels were determined photometrically using 3,3',5,5'-tetramethylbenzidine as substrate (37).

Interleukin assay. Liver samples were processed as previously reported (13) and IL-1α and IL-1β levels were measured using Enzyme-linked immunosorbent assay (ELISA) kits from Amersham Life Sciences (Amersham, UK).

TNF assay. Free TNF-α levels in plasma were measured using a commercial ELISA kit from Biosource (Camarillo, CA, USA). To measure the total (bound and free) TNF-α levels in plasma, a commercial competitive enzyme immunoassay kit from Chemicon International (Temecula, CA) was used.

sTNFR1 and sTNFR2 assay. The soluble TNF-α receptors (sTNFR1 and sTNFR2) levels in plasma were measured using commercial ELISA kits from R&D systems (Minneapolis, MN,USA) .

Histology

For the histological studies the lung was first perfused with a fixative solution (10% neutral-buffered formalin) at a pressure of 25 cmH_2O. Lung samples were fixed in 10% formalin for at least 18 h before being processed and stained with hematoxylin-eosin according to standard procedures (38,39).

Statistics

Data are expressed as means $\pm$ standard deviations, and compared statistically by analysis of variance, followed by Student-Newman-Keuls. $p<0.05$ was considered significant.

RESULTS

No differences in hepatic IL-1β levels were observed in any of the groups evaluated (Fig. 1A). Unlike IL-1β, hepatic IL-1α levels after ROLT were found to increase to a higher level than those recorded in the Sham group (Fig. 1B). Given the evidence indicating that these cells are one of the main sources of IL-1 (40), this study evaluated whether IL-1α levels observed after ROLT may be dependent on Kupffer cells. As shown in Fig. 1B, $GdCl_3$ pre-treatment reduced this significant increase in IL-1α levels observed after ROLT.

Pulmonary neutrophil accumulation and oxidative stress, as estimated by pulmonary MPO and MDA levels increased significantly after ROLT compared with the results obtained in the Sham group (Fig. 2). Lung damage after ROLT is multifactorial. In addition to the consequences on lung damage derived from hepatic I/R, atelectasis, diaphragm injury, surgical procedures and other factors could injure the lungs. In order to minimize the potential effects of the surgical manipulation and the lung damage caused by the sample collection we compared the data obtained in the ROLT group with those obtained in the Sham group (these animals were subjected to a fictitious operation. The anesthesia and surgical times were not statistically different between the Sham and the other groups). In addition, the lung (corresponding to all groups, including Sham) was first perfused with a fixative solution before being processed for histological examinations.

The involvement of IL-1α in the inflammatory response and in the lung damage associated with ROLT was evaluated. IL-1 receptor antagonist treatment (ROLT+IL-1ra) attenuated the increases in lung MPO and MDA after ROLT. Ischemic preconditioning (PC+ROLT) reduced IL-1α (Fig. 1B) and protected against lung injury associated with ROLT (Fig. 2). NO donor treatment (ROLT+NO) kept IL-1α, MPO and MDA levels at the same levels as in ischemic preconditioning. NO synthesis inhibition (PC+ROLT+NAME) abolished the benefits of ischemic preconditioning on IL-1α

release and on the parameters of lung damage. This is shown by the increases in IL-1α, MPO and MDA levels, which are of the same order as those observed after ROLT; and IL-1 receptor antagonist supplementation (PC+NAME+IL1ra) prevented the injurious effects of NO synthesis inhibition, giving biochemical parameters of lung damage similar to those observed in PC+ROLT group.

Histological changes in the lung following ROLT were in keeping with the biochemical study. The lung integrity in the Sham group was preserved (Fig. 3A). The ROLT and PC+ROLT+NAME groups showed margination and adhesion of neutrophils to the endothelium, a diffuse augmentation of the cellularity in the alveolar walls, and a marked or moderate thickening of the alveolar walls (Fig. 3B and 3C). By contrast, no apparent vascular margination of neutrophils and only a slight and non-diffuse thickening of alveolar walls was observed in the ROLT+IL-1ra, PC+ROLT, ROLT+NO and PC+NAME+IL-1ra groups (Figs. 3D and 3E).

The effect of ischemic preconditioning on TNF and sTNFR following ROLT was investigated. Total TNF (free and bound) was similar in all groups (Fig. 4). However, a significant increase in plasma free TNF levels was found after ROLT. This was reduced when ischemic preconditioning (PC+ROLT) or NO donor pre-treatment (ROLT+NO) were carried out (see Fig. 4). NO synthesis inhibition (PC+ROLT+NAME) eliminated the benefits of ischemic preconditioning on plasma TNF release, leading to free TNF values comparable to those observed in the ROLT group. Next, we considered the possibility that the beneficial effects of preconditioning on systemic free TNF might be related to changes in sTNFR. The increases in sTNFR1 levels after ROLT were not modified by ischemic preconditioning, NO donor (NO+ROLT) o L-NAME treatment (PC+ROLT+NAME) (Fig. 5A). However, this was not the case of sTNFR2 (Fig. 5B). Ischemic preconditioning and NO donor treatment increased the sTNFR2 levels more than the ROLT group did. NO synthesis inhibition (PC+ROLT+NAME) abolished the benefits of ischemic preconditioning on sTNFR2, leading to sTNFR2 values similar to those observed in the ROLT group. Interestingly, an association between free TNF and

sTNFR2 levels was observed. Thus, reduced systemic free TNF levels (Fig. 4B) were associated with high sTNFR2 levels (Fig. 5B), and inversely, increased systemic free TNF levels were associated with low sTNF2 levels. As shown in Fig. 6, the ratio of TNF to sTNFR2 (TNF/sTNFR2) fell in the groups in which lung protection was observed (PC+ROLT, ROLT+NO, ROLT+IL-1ra and PC+NAME+IL-1ra), whereas TNF/sTNFR2 ratio increased in the groups in which pulmonary damage was observed (ROLT and PC+ROLT+NAME).

These results indicate that ischemic preconditioning, mediated by NO 1) reduced IL-1 release and thus protected against the lung damage associated with ROLT and 2) regulated systemic TNF/sTNFR2 levels. Given these results and the data in the literature indicating that IL-1 might modulate TNF/sTNFR (15,27-29), we assessed whether the inhibition of IL-1 release by ischemic preconditioning protected against the lung damage associated with ROLT through the regulation of TNF/sTNFR2. Our results indicate that IL-1 action inhibition by IL-1 receptor antagonist (ROLT+IL-1ra), which protected against lung damage (Fig. 2), reduced systemic free TNF and increased sTNFR2 (Figs. 4 and 5), suggesting a relationship between IL-1, TNF/sTNFR2 and pulmonary damage. Because NO generated by preconditioning reduced IL-1, TNF/sTNFR2 and pulmonary damage, NO synthesis inhibition (PC+NAME+ROLT) increased IL-1, TNF/sTNFR2 and lung damage, as the same manner as in ROLT group. However, IL-1 action inhibition in PC+NAME group (PC+NAME+ROLT+IL1ra) prevented the injurious effects of L-NAME on TNF/sTNFR2 and pulmonary damage, resulting in similar values to those found in the PC+ROLT group.

All the results described above were recorded 24h after transplantation. Control experiments to assess whether the consequences of ROLT on lung damage are transitory, indicate that the parameters of lung damage seen 24h after transplantation (MPO, oxidative stress, and the alterations in the pulmonary integrity seen by histological study) were exacerbated 48h later. In addition, the benefits of ischemic

preconditioning on lung damage observed 24h after transplantation were maintained days later.

DISCUSSION

Experimental data indicate that pulmonary damage associated with hepatic I/R is mediated by neutrophil infiltration and the release of inflammatory mediators including reactive oxygen species (41,42). The fall in the oxidative stress and neutrophil infiltration observed in lung tissue after IL-1 receptor antagonist treatment points to the involvement of IL-1α- which seems to be dependent on Kupffer cells- in the lung damage associated with ROLT. Ischemic preconditioning reduced hepatic IL-1α release and protected against lung damage associated with ROLT. NO synthesis inhibition in the preconditioned group, which increased IL-1α, abolished the benefits of ischemic preconditioning on the lung damage associated with ROLT. However, when we inhibited IL-1 action with IL-1 receptor antagonist, the injurious effects of NO inhibition on lung damage disappeared. In addition, NO donor administration to ROLT resulted in reduced hepatic IL-1α, and biochemical and histological parameters of lung damage similar to those in the preconditioned group. The results of the present study indicate that ischemic preconditioning, through NO, inhibited hepatic IL-1α release, thus protecting against the lung damage associated with ROLT. In an experimental model of I/R that mimics the warm ischemia associated with tumour hepatic resections, IL-1 produced in liver following reperfusion was implicated in systemic TNF release (15). Furthermore, lung damage associated with liver transplantation from non-reduced size liver graft appears to be linked to systemic TNF release (43). We therefore examined whether the benefits as regards lung damage resulting from IL-1 action inhibition could be explained by changes in systemic TNF/sTNF levels. To this end, we assessed the effect of ischemic preconditioning on systemic TNF/sTNF levels following ROLT.

In most studies based on inflammatory processes (17,44,45), increases in TNF are paralleled by similar increases in sTNFR. Ischemic preconditioning is associated with a down-regulation of systemic free TNF levels; therefore, a similar down-regulation

of sTNFR release may be expected. However, an equally plausible hypothesis is that the processes which led to the enhanced release of sTNFR are stimulated by ischemic preconditioning. In fact sTNFR supplementation, in situations where there is insufficient production of endogenous sTNFR, would reduce the free TNF levels, thus inhibiting the deleterious effects of TNF (21-24). Our results are in line with this second hypothesis since following preconditioning, sTNFR2 release was up-regulated and free TNF release was down-regulated, thereby reducing the ratio of TNF/sTNFR2 and suggesting a decrease in the bioavailability of TNF. This could contribute to the ischemic preconditioning tolerance against lung damage associated with ROLT. Furthermore, we established a relation between IL-1, lung damage and sTNFR in ROLT. In fact, IL-1-ra pre-treatment, ischemic preconditioning and NO donor treatment (which inhibited IL-1 action and protected against lung damage) increased systemic sTNFR2 and decreased TNF, resulting in low systemic TNF/sTNFR2 levels following ROLT. Similarly, any treatment that increases IL-1, should increase TNF/sTNFR2 and lung damage. In fact, NO synthesis inhibition in the preconditioned group, which resulted in high IL-1α levels, increased both TNF/sTNFR2 and the biochemical parameters of lung damage at levels of the same order as those observed in the ROLT group. However, when we inhibited IL-1 action with IL-1 receptor antagonist, the injurious effects on lung damage disappeared and this was associated with systemic sTNFR, TNF and TNF/sTNFR2 levels as in preconditioned group.

Based on the findings reported here, we suggest a mechanism that might explain the benefits of ischemic preconditioning in preventing the lung damage associated with ROLT. This surgical strategy, through NO, can inhibit IL-1 release, which in turn would regulate the systemic TNF/sTNFR2 release and protect against lung damage associated with ROLT. Further studies based on the use of TNFR2-deficient rat, antibodies to neutralize sTNFR2, and the blockade of shedding of TNFR2, are required to determine whether the up-regulation of sTNFR2 is needed for the tolerance of ischemic preconditioning against the lung damage associated with ROLT. However,

such studies would be difficult to interpret. Thus, if we used TNFR-deficient rat, it would not be possible to determine the relative contribution of sTNF release blockade versus the absent membrane-bound TNFR. A further possibility would involve the design of antibodies that neutralize sTNFR, though not the membrane-bound TNFR, as this would present numerous difficulties. Alternatively, TNFR shedding might be prevented, although this might result in an exaggerated response to TNF, not only from the decrease in sTNFR2, but also because of the enhanced cellular responsiveness to TNF (secondary to the increase in membrane-bound TNFR).

The results of the present study indicate: 1) the involvement of IL-1 in the lung damage associated with ROLT; 2) the underlying protective mechanisms of ischemic preconditioning on lung damage associated with ROLT, based on the inhibition of IL-1 action by NO; 3) The capacity of ischemic preconditioning to regulate systemic TNF/sTNF following ROLT; 4) a potential relationship between NO, IL-1 and TNF/sTNF in the benefits of ischemic preconditioning on the lung damage associated with ROLT. These results suggest that surgical strategies, including ischemic preconditioning aimed at regulating IL-1 action, could modulate systemic TNF/sTNFR levels and protect against the pulmonary damage in an experimental model of reduced-size liver transplantation. The benefits of ischemic preconditioning on lung damage could also be simulated by pharmacological strategies including NO donor and IL-1 receptor antagonist treatment.

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Figure legends

Figure 1. IL-1β (A) and IL-1α (B) levels in liver. *P<0.05 vs. Sham; +P<0.05 vs. ROLT; °P<0.05 vs. PC+ROLT.

Figure 2. MPO and MDA levels in lung. *P<0.05 vs. Sham; +P<0.05 vs. ROLT; °P<0.05 vs. PC+ROLT.

Figure 3. Histological lesions in lung. A) Sham: No pulmonary lesions. B) ROLT: Marked thickening of alveolar walls and vascular margination of neutrophils. C) PC+ROLT+NAME: Moderate thickening of alveolar walls with margination of neutrophils to the endothelium. D) PC+ROLT: Slight thickening of alveolar walls. E) PC+NAME+ROLT+IL-1ra: Histological lesions comparable to PC+ROLT. (H&E, Original magnification).

Figure 4. Total (bound and free) TNF-α and free TNF-α levels in plasma. *P<0.05 vs. Sham; +P<0.05 vs. ROLT; °P<0.05 vs. PC+ROLT.

Figure 5. Soluble TNF-α receptors (sTNFR1 and sTNFR2) in plasma. *P<0.05 vs. Sham; +P<0.05 vs. ROLT; °P<0.05 vs. PC+ROLT.

Figure 6. TNF/sTNFR2 ratio. This parameter is expressed as the relation between plasma TNF-α levels and plasma sTNR2 ×1000. *P<0.05 vs. Sham; +P<0.05 vs. ROLT; °P<0.05 vs. PC+ROLT.

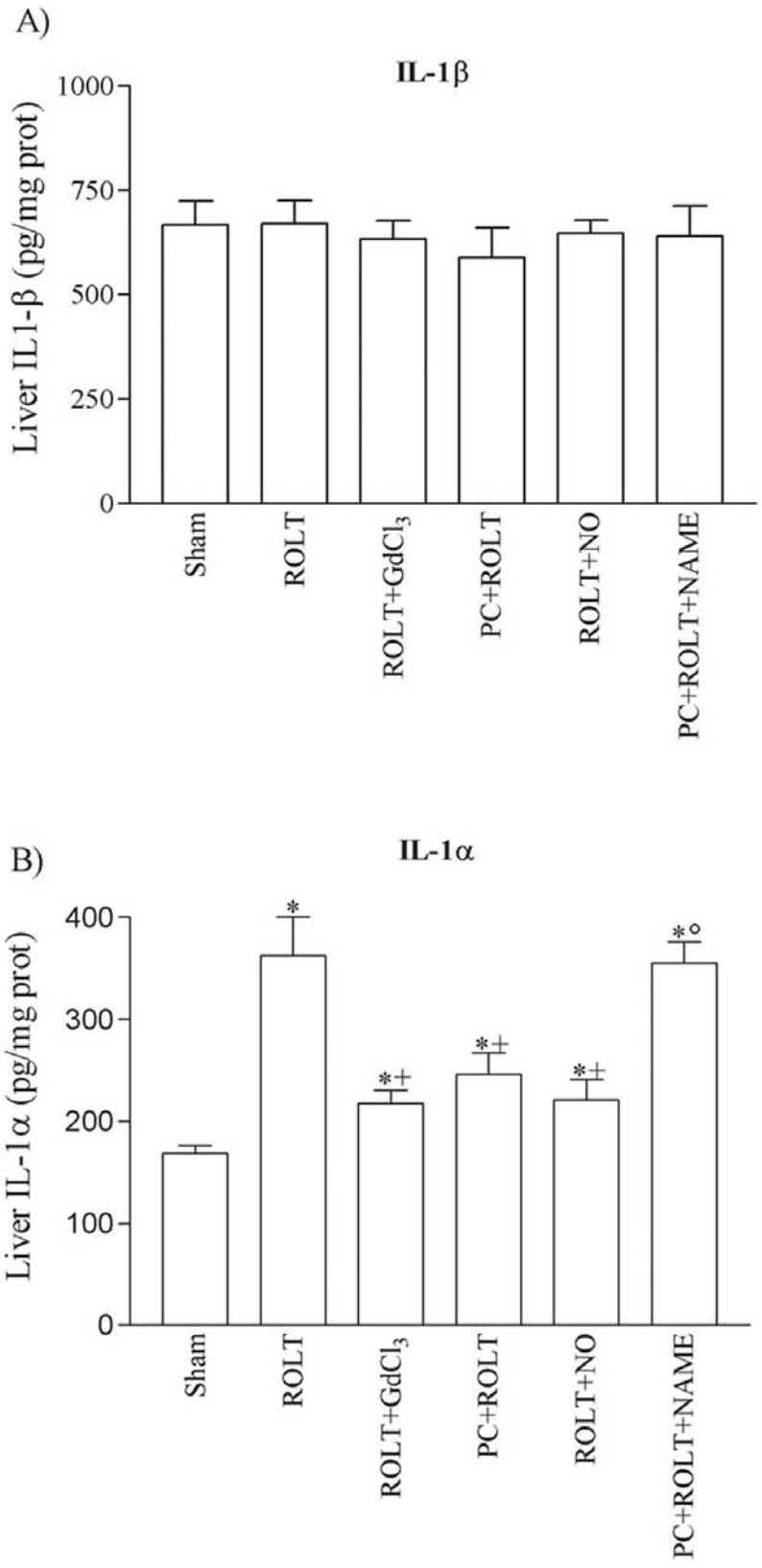

Fig. 1
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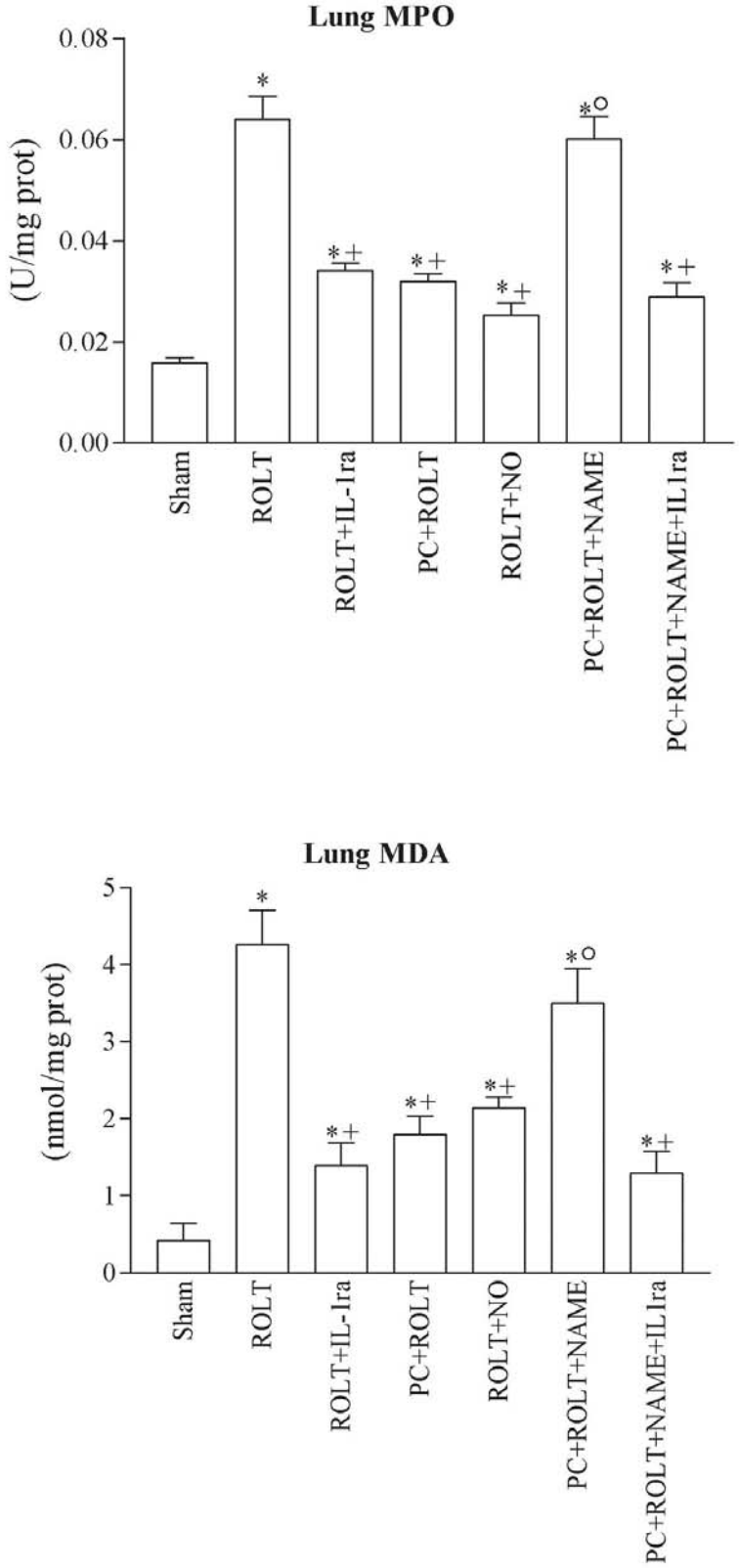

Fig 2
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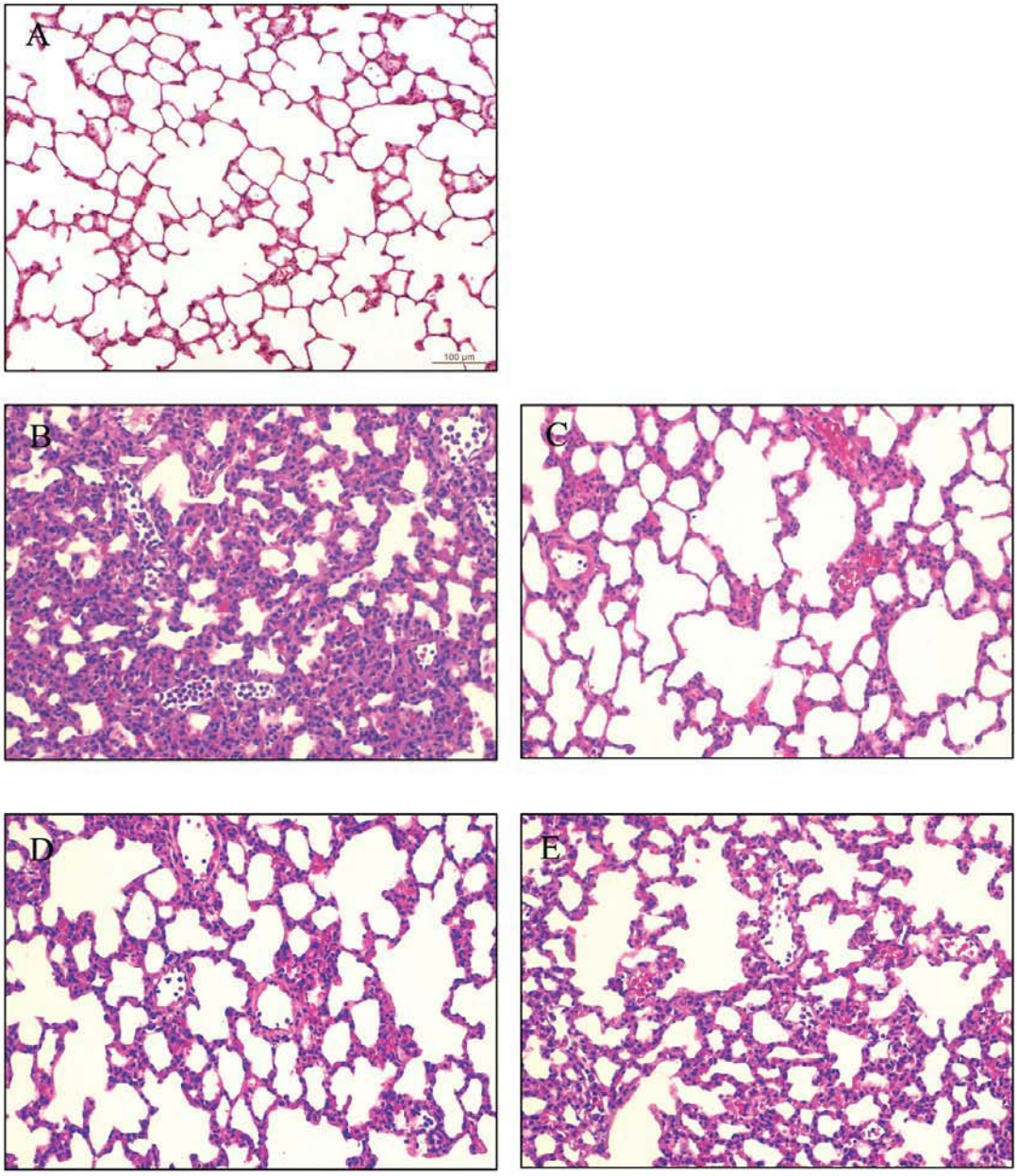

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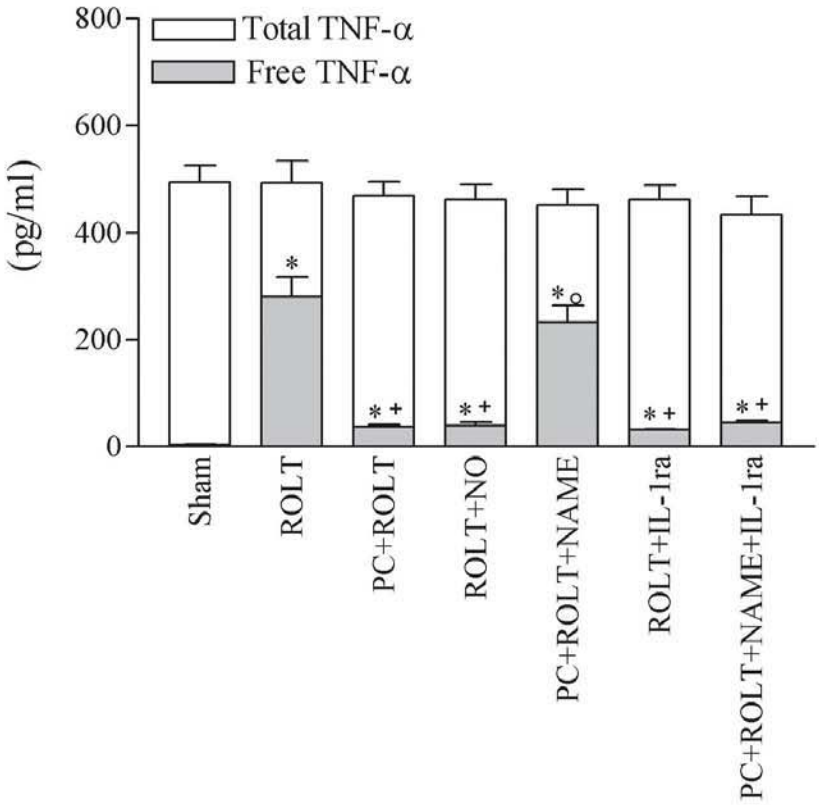

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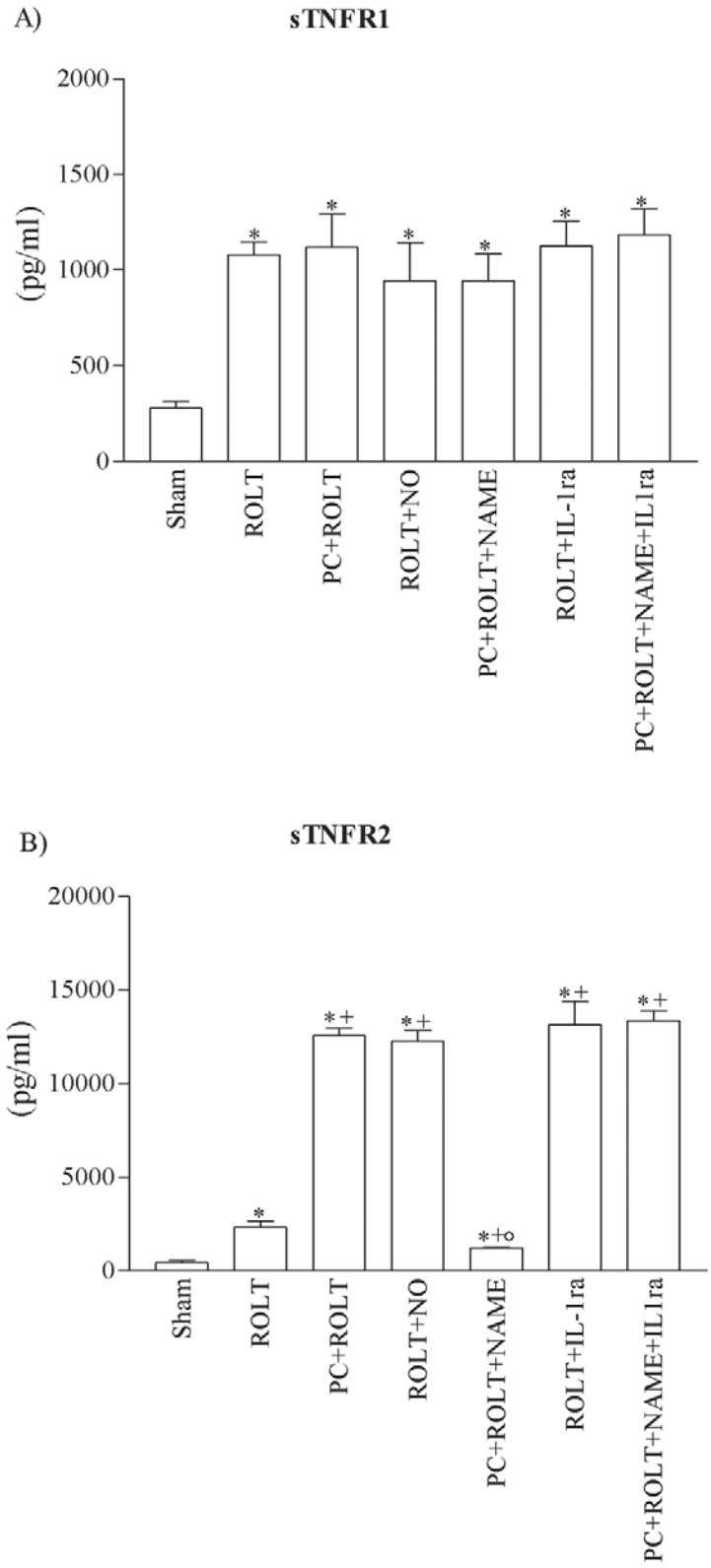

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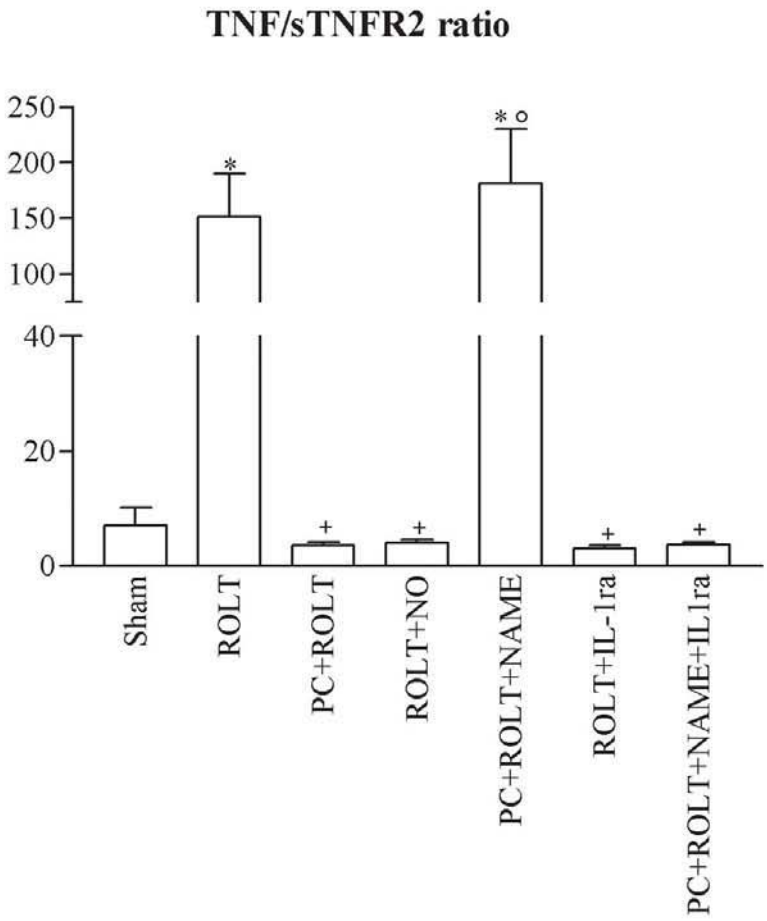

Fig 6
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