Nitric oxide is an essential mediator of the protective effects of remote ischaemic preconditioning in a mouse model of liver ischaemia reperfusion injury

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; C-PTIO, 2-(4-carboxyphenyl) -4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide.potassium salt; IR, ischaemia reperfusion; LDF, laser Doppler flowmetry; MBF, microcirculatory blood flow; NO, nitric oxide; RBC, red blood cell; RIPC, remote ischaemic preconditioning; SEC, sinusoidal endothelial cell; TEM, transmission electron microscopy.

Short title: C-PTIO inhibits remote preconditioning

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ABSTRACT

NO (Nitric oxide) may protect the liver from IR (ischaemia reperfusion) injury. RIPC (remote ischaemic preconditioning) also protects against liver IR injury. The molecular mediator(s) of RIPC are currently unknown. The aim of this study was to assess the role of NO in hindlimb RIPC-induced protection against liver IR injury. Mice were allocated to the following groups: Sham; RIPC: 6 cycles of 4x4 minutes ischaemia/reperfusion of hindlimb; IR: 40 minutes lobar (70%) hepatic ischaemia and 2-hours reperfusion; RIPC+IR: RIPC followed by IR group procedures; C-PTIO+RIPC+IR: C-PTIO (a direct NO scavenger) was administered followed by the RIPC+IR group procedure. Hepatic MBF (microcirculatory blood flow) was measured throughout the experiment. Circulating NOx (nitrite and nitrate) levels, plasma liver transaminases, hepatic histopathological and transmission electron microscopy studies were performed at the end of the experiment. NOx concentrations were significantly elevated ($P < 0.05$) in the RIPC and RIPC+IR groups. Compared to liver IR alone, RIPC+IR preserved hepatic MBF during liver reperfusion ($P < 0.05$). In contrast C-PTIO+RIPC+IR reduced MBF compared to RIPC+IR ($P < 0.05$). RIPC+IR reduced plasma transaminases ($P < 0.05$), histopathological, and ultrastructural features of injury compared to IR alone. The protective effects of RIPC+IR in reducing liver IR injury were abrogated in the group that received antecedent C-PTIO (C-PTIO+RIPC+IR). In conclusion, NO is an essential mediator of the protection afforded by hindlimb RIPC against liver IR injury. The mechanisms underlying this protection involve preservation of the sinusoidal structure and maintenance of blood flow through the hepatic microcirculation.

INTRODUCTION

IR (Ischaemia reperfusion) injury results from a prolonged ischaemic insult followed by restoration of blood perfusion. Hepatic IR injury is a clinical entity with a major contribution to the morbidity and mortality of liver surgery and transplantation. Hepatic IR injury may arise in the setting of systemic hypoxemia (e.g. respiratory failure) or shock states followed by resuscitation (e.g. haemorrhage, sepsis) [1]. The cellular consequences of liver IR injury are hepatocyte and sinusoidal endothelial cell necrosis, apoptosis, or a combination of both [1]. Clinically IR injury leads to liver dysfunction in the mildest cases at one end of the spectrum, and liver failure in severe cases at the other end of the spectrum. One of the therapeutic strategies to lessen liver IR injury is RIPC (remote ischaemic preconditioning) [2]. This concept involves brief ischaemic stimulus of a remote organ or tissue that subsequently affords protection to the liver subjected to a prolonged ischaemic insult [2]. The mechanisms underlying the protective effects of RIPC are currently unknown.

NO (nitric oxide) is an important molecule in IR injury. Previous work has suggested a dichotomous role for NO in IR injury with some studies showing NO worsens IR injury [3,4]; and others pointing to a protective role for NO, reviewed by Phillips *et al* [5]. These opposing effects of the role of NO in IR injury maybe due to the enzymatic source and concentration of the synthesised NO, factors that are in turn influenced by the experimental conditions including, such variables as the length of the ischaemic insult, the organ being studied, the cellular redox status of the organ, time of reperfusion, and in vivo versus in vitro experimental set up. The concentration of released NO is important because it may determine the benefits, or otherwise, of NO in IR injury; with high concentrations of NO promoting the formation of reactive nitrogen species such as peroxynitrite that enhance IR injury; and lower concentrations of NO being protective [6-8].

Direct organ IPC (ischaemic preconditioning) protects against subsequent IR injury at least partially through an NO-dependent mechanism. This has been shown in several organ systems, including the liver [9]. Supporting evidence for the role of NO in mediating the protection offered by direct IPC comes from experiments showing inhibition of NOS (nitric oxide synthase) isoforms by the non-selective NOS inhibitor N-Nitro-L-arginine methyl ester results in abrogation of the protective effects of IPC [10,11]. Additionally, IPC results in increased NOS expression [11,12] with a subsequent increase in the NO oxidation products, nitrite and nitrate, that connotes a rise in NO levels [11,13]. In contrast to the known beneficial effects of NO in direct liver IPC, its role in RIPC of the liver is currently unknown.

We recently showed mice lacking the constitutively expressed endothelial NOS ($eNOS^{-/-}$) enzyme suffer significantly greater levels of liver IR injury compared to wild type animals [14]. However NO production was not measured in that study. NO is a highly reactive free radical molecule, with a short half life that is in the order of 5-10 seconds. Therefore direct measurement of *in vivo* NO production in IR research is impractical and quantification of the reaction products of NO such as nitrite and nitrate is commonly used to reflect the levels of NO synthesis. An additional approach, involves direct elimination of the released NO through the use of the direct NO scavenger C-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.potassium salt) [15]. C-PTIO reacts with NO in a stoichiometric manner to inhibit the actions of NO [15].

The role of synthesised NO in RIPC of the liver is currently unknown. Therefore the aim of the present study was to determine the role of the NO produced during hindlimb RIPC in ameliorating liver IR injury.

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MATERIALS AND METHODS

Animals

The experiments were performed in accordance with the terms of a project license granted by the Home Office (Animals and Scientific Procedures Act 1986). In addition, the experimental protocols were also approved by the institutional animal ethics and welfare committee. Inbred male C57BL/6 mice (Charles River laboratories, UK) were utilised in this study. All mice, aged 8-12 weeks, were kept in a temperature-controlled environment, had a 12 hour light-dark cycle, and were freely allowed standard mouse chow pellets and drinking water.

Hepatic ischaemia reperfusion

An induction chamber was used to anaesthetise the mice using isoflurane. Maintenance of anaesthesia was achieved by the use of 1.5-2.0 % isoflurane via a concentric oronasal mask connected to an anaesthetic circuit. Core body temperature was maintained at 37±0.05 C using a heating pad and monitored by a rectal temperature probe.

Following a midline laparotomy the falciform ligament and the ligament connecting the caudate to the left lobe were divided. 400 µl of 2.5 U/ml heparinised saline was administered into the peritoneal cavity to maintain hydration and prevent thrombus formation in the hepatic circulation during the ensuing ischaemia. The left and median liver lobes, which constitute approximately 70% of the mouse liver, were rendered ischaemic by the application of a microvascular clamp to the pertinent portal triad branch [16]. Successful occlusion of the blood supply in question was confirmed by a change in colour and a reduction in MBF (microcirculatory blood flow) in the affected lobes as measured by LDF (Laser Doppler Flowmetry). At the end of 40 minutes of ischaemia the microvascular clamp was removed and hepatic reperfusion of the ischaemic lobes commenced. A further 400 µl of normal saline was administered into the peritoneal cavity at the start of liver reperfusion to prevent dehydration during the subsequent two hours, during which the abdominal cavity was kept open to permit hepatic MBF measurements using LDF.

At the end of the reperfusion period (2 hours) the animals were terminated by exsanguination through cardiac puncture and blood collection. The blood was immediately centrifuged at 3500 rpm (1150 g) for 10 minutes. The plasma supernatant was stored at -70 C until assayed for liver transaminases. The liver lobes subjected to IR were harvested at the end of each procedure and stored in 10% formal saline for histopathology processing. Samples for transmission electron microscopy were stored in 1.5% glutaraldehyde and 2% paraformaldehyde in PBS buffer.

Hindlimb preconditioning

An open technique of hindlimb ischaemic preconditioning was used. A longitudinal skin incision was made over the right antero-medial thigh. The femoral vascular bundle (femoral artery and vein) was isolated from the surrounding muscles and was clamped using an operating microscope just proximal to its confluence with the femoral nerve. A protocol of four minutes of limb ischaemia followed by four minutes of reperfusion for a total of six cycles was used [16]. Cessation of blood flow to the hindlimb was confirmed by the change in foot colour and by the reduction in foot MBF measured by LDF.

Experimental groups

Four groups with a minimum of six animals in each were used. All animals underwent laparotomy, mobilization of the liver, and mobilization of the right femoral vascular bundle. In addition some of the groups were each subjected to a specific procedure as follows:

Sham: Only underwent the laparotomy, mobilization of the liver, and mobilization of the right femoral vascular bundle described above.

IR: The median and left hepatic lobes were rendered ischaemic for 40 minutes followed by 2 hours reperfusion, using a microvascular clamp to occlude the portal triad branches to these lobes.

RIPC + IR: The right hindlimb was preconditioned with 6 cycles of 4 minutes ischaemia followed by 4 minutes reperfusion, using a microvascular clamp to occlude the femoral vessels under an operating microscope. This was followed by the IR group procedure described above.

C-PTIO + RIPC + IR: The NO scavenger C-PTIO (Enzo Life Sciences, Exeter, UK) was dissolved in phosphate-buffered saline and was administered intravenously (inferior vena cava) immediately before the first hindlimb ischaemia at a dose of 1 mg/kg in a volume of 50 µl [17] followed by the RIPC + IR group procedure.

The above animal groups were used to assess hepatic MBF, plasma liver enzyme levels, histopathological injury scores, and ultrastructural features of liver IR injury. In addition the sham, IR, RIPC + IR, and an additional animal group subjected to hindlimb RIPC alone, were used to measure plasma levels of nitrite and nitrate (NOx).

Nitrite and nitrate measurements

The plasma NOx (nitrite and nitrate) levels were measured using a commercially available colorimetric assay kit (Cayman Chemical, Michigan, USA). The plasma was first deproteinated using 30 kDa centrifugal filters and diluted 1 in 8 using assay buffer. Total nitrite and nitrate concentrations were determined by the reduction of nitrate to nitrite using nitrate reductase (Cayman Chemical, Michigan, USA) in the presence of enzyme cofactor (Cayman Chemical, Michigan, USA). Greiss reagent was then added to the solution containing nitrite and the resultant colour development was quantified by reading the absorbance at 550 nm. The total NOx (µM) were calculated using the nitrate standard curve.

Assessment of the microcirculation

A dual channel laser Doppler flowmeter (DRT4, Moor Instruments Ltd., Devon, UK) was used to measure MBF (in flux units) in the microvasculature of the liver and hindlimb. For each experiment one probe was placed on a fixed site on the left liver lobe, whilst the second was placed on the sole of the right foot. In both cases the probe was positioned so that it was just in contact with the tissue surface. The probes were mounted in probe holders in order to prevent them from pressing on the microvasculature and therefore occluding the blood flow. Hepatic LDF data were collected at a sampling rate of 1Hz. The wavelength of the laser used was 785nm and measured microcirculatory blood flow in tissue volumes of 0.6-2.3 mm^3. The values at the relevant time points were calculated as the mean of the data recorded over a one minute period. Figure 1 shows the time points of LDF recordings during the study protocol. The hindlimb LDF measurements were made but not recorded, as these were performed only to ensure adequate blood flow interruption to the hindlimb during ischaemia, with recovery of blood flow during hindlimb reperfusion.

Measurement of liver enzymes

Plasma concentrations of ALT (alanine amino transferase) and AST (aspartate aminotransferase) were measured by a standard spectrophotometric method using an automated clinical analyzer (Modular Analytics P, Roche Diagnostic Ltd, West Sussex, UK). These enzymes are sensitive markers of liver injury which are released from injured hepatocytes into the circulation.

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Histopathology

A liver biopsy was taken from the ischaemic left lobe at the end of the experiment for each of the animals and was immediately fixed in 10% formal saline. The fixed tissues were then embedded in paraffin, cut into 3 μm sections and stained with haematoxylin and eosin (H&E). The sections were examined under a light microscope using 50x or 100x magnification for assessment of the degree of liver injury by a liver pathologist blinded to the grouping of the animals. Each H&E sample was scored using two different methods [16]. In the first of these an overall histopathological injury grade was assigned to each sample based on the extent of the injury seen on examining each section:

- grade 0 – minimal or no evidence of injury;
- grade 1 – mild injury characterised by cytoplasmic vacuolization and focal nuclear pyknosis;
- grade 2 – moderate injury exhibiting cytoplasmic vacuolization, confluent areas of hepatocyte ballooning but no frank necrosis, sinusoidal dilatation and congestion, and blurring of intercellular borders;
- grade 3 – moderate to severe injury with areas of coagulative necrosis, cytoplasmic hypereosinophilia, extensive sinusoidal dilatation and congestion;
- grade 4 – severe injury consisting of severe confluent coagulative necrosis, and disintegration of and haemorrhage into hepatic chords leading to loss of tissue architecture.

The second method of analysing the samples consisted of a semi-quantitative evaluation of individual histological features of liver IR injury in each sample [16] (Table 1). The individual features are grouped into three categories based on the cell type affected:

- Vascular injury: sinusoidal dilatation and RBC (red blood cell) extravasation.
- Inflammatory cell infiltrate: neutrophil infiltration.
- Hepatocellular injury: eosinophilia, cytoplasmic vacuolation, liver cell ballooning, blurred intercellular borders, discohesive hepatocytes, nuclear pyknosis, and necrosis.

Each of these features was scored in affected areas identified by the histopathologist in each of the sections.

Transmission electron microscopy

Liver biopsies of approximately 1 mm^3 from the left lobe were fixed in a solution containing 1.5% glutaraldehyde and 2% paraformaldehyde in PBS overnight. An automated tissue processor (Leica EM TP) was utilised to process the samples, culminating in each sample being embedded in Lemix (TAAB Laboratories Ltd) epoxy resin. Semi-thin (1 μm) sections were then cut and stained with 1% Toluidine Blue – 1% Borax and examined under light microscopy to determine the areas to be cut and processed for TEM (transition electron microscopy). Ultrathin (70 nm) sections were stained with 2% aqueous uranyl acetate followed by Reynold's lead citrate, and viewed using a Philips 201 TEM. Representative areas were photographed and the images were interpreted by a TEM scientist who was blinded to the groups. Multiple parameters of ultrastructural IR injury were evaluated [16]. Within hepatocytes these were: mitochondrial damage evident by outer membrane disruption and cristae shortening and disruption; endoplasmic reticulum dilatation and vesiculation; formation of cytosolic vacuoles; formation of phagolysosomes; and abundance of lipid droplets and glycogen granules. In addition, injury of the bile canaliculi was determined by dilatation and loss of the microvilli; and damage to the sinusoids was determined by disruption of the cytoplasm of SECs (sinusoidal endothelial cells), presence of sinusoidal luminal debris, extravasation of RBCs, and obliteration of the space of Disse microvilli.

Statistical analysis

Values are expressed as mean ± SEM (standard error of the mean). ANOVA (one way analysis of variance) with Post Hoc Bonferroni correction for multiple comparisons was used. $P < 0.05$ was considered statistically significant in all analyses.

RESULTS

Hindlimb RIPC significantly increases plasma NOx levels

Both the RIPC and RIPC + IR groups showed a significant increase in plasma NOx levels compared to sham ($P < 0.05$) (figure 2). In addition the RIPC + IR group demonstrated a significant rise in the NOx levels compared to the IR group ($P < 0.05$).

The protective effects of hindlimb RIPC on hepatic microcirculatory blood flow in liver IR are abolished by C-PTIO

Figure 3 shows hepatic MBF for the duration of the experiment. The sham group demonstrated constant hepatic MBF, whilst the IR and RIPC + IR groups displayed a significant reduction of MBF during liver ischaemia. Upon reperfusion the IR group showed no significant improvement in MBF, whilst the RIPC + IR group demonstrated significant recovery of MBF towards baseline values compared to the IR group ($P < 0.05$).

The C-PTIO + RIPC + IR group exhibited reductions in MBF throughout the duration of the experiment. During the first hindlimb ischaemia (HI1) the mean MBF was 93.8% of baseline ($P = 0.038$ vs sham; $P = 0.045$ vs IR; $P > 0.05$ vs RIPC + IR). Similarly, during the first hindlimb reperfusion (HR1) the mean MBF was 93.8% of baseline ($P = 0.025$ vs sham; $P = 0.007$ vs IR; $P > 0.05$ vs RIPC + IR). In comparison there were no significant differences in mean MBF values between the C-PTIO + RIPC + IR and the other groups during the sixth HI and HR. Commencement of liver ischaemia resulted in a significant reduction in MBF in the C-PTIO + RIPC + IR group compared to the sham but not to the IR or RIPC + IR groups. Following reperfusion, MBF in the C-PTIO + RIPC + IR group failed to recover, showing significantly lower MBF values in comparison to the sham and RIPC + IR groups throughout reperfusion ($P < 0.05$). At the end of the 2 hour reperfusion period the mean MBF value was 48.2% of baseline in the C-PTIO + RIPC + IR group compared to 93.5% of baseline in the RIPC + IR group ($P < 0.0001$). There were no significant differences in the mean MBF values between the C-PTIO + RIPC + IR and the IR group throughout the reperfusion period (figure 3).

C-PTIO increases plasma transaminases levels in hindlimb preconditioned mice undergoing liver IR

Liver IR caused a significant rise in the plasma levels of ALT and AST compared to the sham group ($P < 0.05$). The RIPC + IR group showed significant reductions in plasma transaminases levels compared to IR alone ($P < 0.05$). However, intravenous administration of C-PTIO prior to RIPC + IR resulted in a significant increase ($P < 0.05$) in the plasma levels of ALT and AST compared to the RIPC + IR group (figure 4).

C-PTIO non-significantly abrogates the beneficial effects of RIPC on histopathological markers of liver IR injury

The mean overall histopathological grade for each animal group is given in figure 5. The sham group showed minimal signs of liver IR injury with a mean overall grade of 0. Liver IR resulted in a significant increase in the mean overall injury grade (IR group mean overall grade = 1.83) compared to the sham group ($P < 0.05$). In comparison the RIPC + IR group had a mean overall injury score of 1.33, which is not significantly different from the sham or

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IR groups ($P > 0.05$). Administration of C-PTIO prior to RIPC + IR resulted in an increase of the mean overall injury grade to 1.5 ($P < 0.05$ vs sham; $P > 0.05$ vs IR or RIPC + IR).

Assessment of the individual histopathological features of liver IR injury revealed the following in each category (figure 6):

- Vascular injury: mean sinusoidal dilatation scores did not show significant differences between any of the groups. In contrast the mean RBC extravasation score in each of the IR and C-PTIO + RIPC + IR groups, showed a significant increase compared to the sham group ($P < 0.05$) and a non-significant increase compared to the RIPC + IR group.
- Inflammatory cell infiltrate: mean neutrophils infiltration scores did not show significant differences between any of the groups.
- Hepatocellular injury: four of the features assessed under this category (cytoplasmic vacuolation, liver cell ballooning, blurred intercellular borders, discohesive hepatocytes) showed a significant increase in the mean score in each of the IR and C-PTIO + RIPC + IR groups compared to sham ($P < 0.05$). Whilst the mean scores of the IR and C-PTIO + RIPC + IR groups in these 4 features showed a trend to increase compared to the mean scores of the RIPC + IR group, only the difference between the C-PTIO + RIPC + IR and the RIPC + IR groups in cytoplasmic vacuolation reached statistical significance ($P < 0.05$). There were no other statistically significant differences between any of the other groups in any of the remaining features of liver IR injury.

C-PTIO nullifies the protective effects of hindlimb RIPC on ultrastructural damage in liver IR

Figure 7 illustrates representative changes exhibited by each of the animal groups. The sham group showed normal ultrastructural appearances. The IR group exhibited extensive mitochondrial damage, ER dilatation, cytosolic vacuole formation, phagolysosomal formation, lipid droplet formation, glycogen depletion, bile canaliculi dilatation with microvilli damage, and SEC disruption with extravasation of RBCs into the liver parenchyma. In contrast the ultrastructural damage sustained by the RIPC + IR group consisted of ER dilatation, phagolysosomal formation, lipid droplet formation, glycogen depletion, and minimal disruption of SEC and without RBC extravasation.

The administration of C-PTIO prior to RIPC + IR resulted in extensive mitochondrial damage, vacuole formation, lipid droplet formation, bile canaliculi dilatation with microvilli destruction, and homogenous SEC disruption with RBC extravasation. In addition this group exhibited frequent condensed crescent shaped lengths of ER indicative of ER membrane damage secondary to lipid peroxidation.

DISCUSSION

The current study demonstrated for the first time that an NO-dependent pathway mediates the protective effects of hindlimb RIPC in reducing liver damage during the early phase of IR injury. Moreover, the mechanisms underlying this pathway involve preservation of the sinusoidal structure and maintenance of blood flow through the hepatic microcirculation.

The sequence of events in liver IR injury is divided into two phases [18]. The early or acute phase covers the first two hours following reperfusion and is dominated by kupffer cell activation and release of various mediators such as reactive oxygen species and cytokines; and the late phase which commences at about six hours of reperfusion and continues to 48 hours post reperfusion and is characterised by neutrophil accumulation in the liver and

progression of hepatocyte and sinusoidal endothelial cell damage. Similarly, the hepato-protective effects of RIPC are seen over two time windows [2]; the early phase of preconditioning commences immediately following a brief ischaemic stimulus and lasts under four hours. The late phase begins 12-24 hours after preconditioning and lasts 72-96 hours. In this study we assessed the hepato-protective effects of RIPC-induced NO production during the early phases of IR injury and RIPC.

C-PTIO is a nitronyl nitroxide that neutralises the actions of NO in biological systems in a reaction that results in the production of NO_2 and the amino nitroxide C-PTI [15,19]. The substrate C-PTIO directly extinguishes the NO generated by nitric oxide synthase without directly affecting the activity of this enzyme [19]. C-PTIO has been used in vivo to investigate the biological effects of inhibiting NO, including in liver IR research [17,20] where administration of C-PTIO has been shown to abolish the protective effects of nitrite-derived NO [17,20].

The role of NO in mediating the protective effects of RIPC on liver IR injury is currently unknown. In the present study we showed that hindlimb RIPC significantly elevates circulating levels of NOx (nitrite and nitrate) and we showed hindlimb RIPC significantly attenuates liver IR injury. Taken together this indicates hindlimb RIPC-induced NO generation is responsible for the protective effects of RIPC against liver IR injury. Our group previously measured NOx levels following RIPC + IR in a rabbit model [21]. We showed the plasma NOx levels were non-significantly lowered following hepatic IR alone compared to the sham group. Hindlimb RIPC prior to hepatic IR resulted in an increase in NOx levels compared to the IR alone group [21]. These results indicated RIPC prior to liver IR prevented a reduction in NOx plasma levels compared to IR alone. In the current study, we are the first group to demonstrate that RIPC-induced NO production mediates the protective effects of RIPC on liver IR injury.

With regards to the role of NO in mediating the protective effects of RIPC in other organs, various experimental designs have been utilised to elicit this in different combinations of RIPC sites and organs subjected to index ischaemic insults. Shahid et al [22] showed femoral artery preconditioning prior to myocardial ischaemia significantly reduces myocardial infarct size and improves cardiac function compared to non-preconditioned animals. Administration of the NOS inhibitor L-NAME abrogated the protection furnished by preconditioning [22]. Similarly, infusion of L-NAME prior to hindlimb RIPC abolished its protective effects against subsequent abdominal adipocutaneous flap ischaemia [23]. Other NOS inhibitors such as N omega-nitro-L-arginine (L-NNA) and S-methylthiosulfourea have also been shown to block the protective effects of femoral artery RIPC, but in these studies the index ischaemic insult was global brain ischaemia [24,25]. The use of NOS knockout animals has been limited to a study in which $iNOS^{-/-}$ mice lost the protection afforded by hindlimb RIPC on myocardial IR compared to wild type animals [26].

The present study showed significant reductions in hepatic MBF during liver reperfusion in the C-PTIO + RIPC + IR compared to the RIPC + IR group, indicating that NO produced by hindlimb RIPC preserves the hepatic microcirculation. In further support of a role for RIPC-induced NO in preserving hepatic MBF our group previously demonstrated failure of restoration of hepatic MBF, during 2 hours of liver reperfusion, in $eNOS^{-/-}$ mice subjected to RIPC + IR compared to wild type mice subjected to the same treatment [14]. The mechanisms underlying these protective effects of NO on the microcirculation are likely to be multifactorial. In this study we demonstrated, through an increase in RBC extravasation and sinusoidal ultrastructural damage, SEC damage was worse in the C-PTIO + RIPC + IR compared to the RIPC + IR group, which would have contributed to the reduction in blood flow within the sinusoids. In addition, we previously showed NO significantly increases hepatic MBF in healthy and steatotic livers during direct liver IPC [27,28] and our results here

indicate RIPC has a similar effect. NO is a vasodilator [29] and is known to induce an increase in MBF in chronically ischaemic limbs which is significantly reduced by administration of C-PTIO [30]. Similarly, in our experiments the inhibition of NO through the use of C-PTIO was the cause of the reduction in hepatic MBF seen during reperfusion in the C-PTIO + RIPC + IR group. C-PTIO was administered prior to the first cycle of hindlimb RIPC and interestingly resulted in a small but significant hepatic MBF reduction prior to any liver ischaemia induction, only during the first hindlimb RIPC cycle though, compared to the sham and IR groups. This is likely to be a transient effect of C-PTIO administration as hepatic MBF values were not significantly different from other groups during the second to the sixth hindlimb RIPC cycles.

The results of the current study show that NO is a pre-requisite for the full manifestations of the benefits of RIPC on liver IR injury, as neutralisation of NO with C-PTIO abolished the protection of RIPC. The plasma liver enzymes showed a significant elevation in the C-PTIO + RIPC + IR compared to the RIPC + IR group. There was a trend showing a reduction in the overall histopathological grade and in the score of 8 individual features of liver IR injury, in the RIPC + IR compared to the IR group; however the difference was not statistically significant. Similarly, although the overall histopathological grade and 7 individual histopathological features of liver IR injury, under the categories of vascular and hepatocellular injury, showed increased mean scores in the C-PTIO + RIPC + IR compared to the RIPC + IR group; in only one of these features, cytoplasmic vacuolation, was the difference statistically significant. The reason for this lack of statistical significance in the histopathological scores is due to the fact that the liver biopsies for histopathological assessments were performed at 2 hours of reperfusion, when the IR-induced histopathological changes are only beginning to occur, but before exhibiting the maximal damage normally observed at a later time point of reperfusion [31-33]. Furthermore, at this point in time of reperfusion, and despite showing a trend to this effect, RIPC does not significantly alter histopathological features of IR injury [16]. Previous work indicates histopathological manifestations of liver IR injury exhibit significant features of damage at reperfusion periods of between 6 to 24 hours [31-33].

The ultrastructural changes in the C-PTIO + RIPC + IR group showed increased mitochondrial damage, ER membrane lipid peroxidation, and increased SEC damage compared to the RIPC + IR group. NO has many protective functions within cells. In liver IR injury NO has been shown to decrease hepatic injury by preserving the mitochondria and antioxidant enzyme activity [34-36], hence providing an explanation for the increase in mitochondrial damage and ER membrane lipid peroxidation observed in our study. Moreover, NO is one of the main endothelial cell homeostatic factors, fulfilling a range of functions to maintain the endothelium physiological status quo, including anti-inflammatory actions [29]. Therefore inhibition of NO in the C-PTIO + RIPC + IR group in our study led to SEC damage to an extent not seen in the RIPC + IR group.

Further support for a protective role for RIPC-induced NO production comes from our previous work using $eNOS^{-/-}$ and wild type mice [14]. In these experiments we demonstrated that eNOS is an essential prerequisite for the protective effects of hindlimb RIPC on liver IR injury, as $eNOS^{-/-}$ animals fail to exhibit any protection against liver IR injury when subjected to hindlimb RIPC prior to liver IR injury [14]. Additionally the protection of RIPC in wild type mice at 2 hours of reperfusion is likely due to an increase in eNOS activity rather than protein upregulation, as liver and hindlimb eNOS expression was equal among all the wild type animal groups [14]. Moreover, numerous published studies have shown a significant reduction in liver IR injury with the administration of various NO donors [37-43]. Whilst these results collectively suggest a protective role for NO in ameliorating liver IR injury; the

results of the current study show for the first time that RIPC-induced protection against liver IR injury is mediated by NO generation.

In conclusion and for the first time, we have shown an NO-dependent pathway mediates the protective effects of hindlimb RIPC on liver IR injury. The mechanisms underlying this protection involve preservation of the sinusoidal structure and maintenance of blood flow through the hepatic microcirculation.

AUTHOR CONTRIBUTION: Mahmoud Abu-Amara was responsible for study conception/design, data acquisition/analysis, and manuscript generation. Shi Yu Yang contributed to study conception/design, and the analysis and interpretation of data. Alberto Quaglia performed the histopathology assessment of all the liver biopsies whilst blinded to the animal groups. Peter Rowley processed and photographed the liver biopsies using TEM, and performed the assessement of all the TEM images whilst blinded to the animal groups. Achala de Mel performed all the NOx measurements. Niteen Tapuria assisted in data acquisition and analysis. Alexander Seifalian, Brian Davidson, and Barry Fuller were responsible for conception/design, and interpretation of the study's data. They revised the manuscript for publication. All authors approved the manuscript for publication.

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Figure legends

Figure 1. Laser Doppler Flowmetry (LDF) measurements. ↑ indicates time points of LDF measurements. The mean of four LDF readings over a one minute period was recorded at each time point indicated. Note during cycles 1 to 6 of HI and HR the hindlimb LDF measurements were made but not recorded, as these were performed only to ensure adequate blood flow interruption to the hindlimb during HI, with recovery of blood flow during HR. *BL*, baseline; *HI*, hindlimb ischaemia; *HR*, hindlimb reperfusion; *BLI*, beginning of liver ischaemia; *ELI*, end of liver ischaemia; *BR*, beginning of liver reperfusion; *R30* to *R120* - liver reperfusion at 30, 60, 90, and 120 min post ischaemia.

Figure 2. Plasma nitrite and nitrate (NOx) levels in wild type mice ($n \geq 6$ per group). *$P <$ 0.05 vs sham. #$P < 0.05$ vs IR.

Figure 3. Hepatic microcirculatory blood flow, measured by LDF, is illustrated as mean percentage compared to pre-ischaemic baseline. Significant ($P < 0.05$) inter-group differences are shown vertically below each time point on the x-axis. *BL*, baseline; *HI*, hindlimb ischaemia; *HR*, hindlimb reperfusion; *BLI*, beginning of liver ischaemia; *ELI*, end of liver ischaemia; *BR*, beginning of liver reperfusion; *R30 to R120*, liver reperfusion at 30, 60, 90, and 120 min post ischaemia.

Figure 4. Plasma liver enzyme levels. (**A**) Alanine aminotransferase (ALT). *$P < 0.05$ vs IR, RIPC+IR, or C-PTIO (C-PTIO+RIPC+IR group). (**B**) Aspartate aminotransferase (AST). *P < 0.05 vs IR, RIPC+IR, or C-PTIO (C-PTIO+RIPC+IR group).

Figure 5. Group means ± SEM of overall histopathological grades (n = 6 per group). C-PTIO = C-PTIO+RIPC+IR group. *NS*; not significant.

Reference: CS2010/0598 (2)

Figure 6. Histopathological scores of individual features of liver IR injury. Each bar chart is the mean histopathological score of six animals in that group. The error bars are the standard error of the means. Only significant differences are labelled: $*P < 0.05$ vs sham; $\#P < 0.05$ vs C-PTIO + RIPC + IR. All remaining comparisons did not reach statistical significance.

Figure 7. Hepatic transmission electron micrographs. The liver samples were taken at the end of the procedure in each group. **(A) & (B)** Sham group showing normal endoplasmic reticulum (er), bile canaliculi with microvilli (bc), mitochondria (m), glycogen (gl), and sinusoidal endothelial cells (sec) with microvilli in space of Disse (sdm). Note cytosolic clarification in the lower hepatocyte in **(A)**. **(C) & (D)** IR group demonstrating extensively damaged mitochondria (dm) with some merging into vacuoles (vc), dilatation and vesiculation of endoplasmic reticulum (der), lysosomes (ly) and phagolysosomes (pl), lipid droplets (ld), and severely damaged sinusoidal endothelial cells (dsec) associated with extravasated red blood cells (erbc) and plasma (ep). **(E) & (F)** RIPC + IR group demonstrating undamaged pleomorhic mitochondria (m), lipid droplets (ld), lysosomes (ly) and phagolysosomes (pl), normal (er) and dilated (der) endoplasmic reticulum, bile canaliculi with microvilli (bc), glycogen (gl), and damaged sinusoidal endothelial cells (sec) but without RBC extravasation. **(G) & (H)** C-PTIO + RIPC + IR group showing damaged mitochondria (dm), vacuolation (vc), lipid droplets (ld), severely damaged sinusoidal endothelial cells (dsec) associated with red blood cell extravasation (erbc), and condensed crescent shaped lengths of endoplasmic reticulum indicative of lipid peroxidation of the endoplasmic reticulum membrane (ler).

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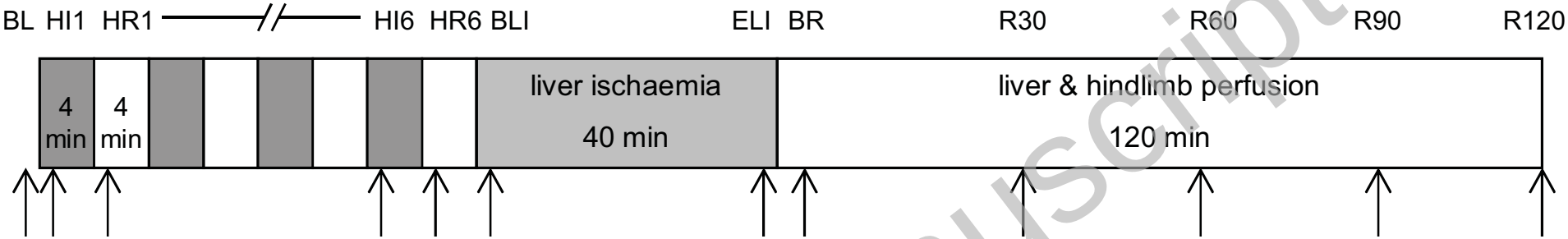

Figure 1.

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Table 1. Scoring criteria for individual histopathological features of liver injury.

Histopathological features of liver injury	Score (S)	Interpretation of scores
Vascular injury		
Sinusoidal dilatation	0	None
	1	Rarely seen
	2	Frequent perivenular
	3	Frequent perivenular midzonal
	4	Frequent panlobular
Red blood cell extravasation / haemorrhage	0	None
	1	Rarely seen
	2	Frequent perivenular
	3	Frequent perivenular midzonal
	4	Frequent panlobular
Inflammatory cell infiltrate		
Neutrophil infiltration	0	None
	1	Rarely seen
	2	Scattered in some lobules
	3	Scattered in most lobules
	4	On the verge of confluence or with some confluence
	5	Dense clusters
Hepatocellular injury		
Eosinophilic changes of hepatocytes	0	None
	1	Rarely seen
	2	Scattered in some lobules
	3	Scattered in most lobules
	4	Widespread
Cytoplasmic vacuolation	0	None
	1	Rarely seen
	2	Scattered in some lobules
	3	Scattered in most lobules
	4	Widespread
Liver cell ballooning	0	None
	1	Focal, spotted after extensive search
	2	Some lobules affected
	3	Most lobules affected
	4	Widespread
Blurring or loss of intercellular borders	0	None
	1	Rarely seen
	2	Scattered in some lobules
	3	Scattered in most lobules
	4	Widespread
Discohesive hepatocytes	1	Mild
	2	Moderate
	3	Severe
Nuclear pyknosis	0	None
	1	Rarely seen
	2	Scattered in some lobules
	3	Scattered in most lobules
	4	Widespread

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Cell necrosis	0	None
	1	1-2 apoptotic bodies
	2	> 3 apoptotic bodies
	3	Focal necrosis with 1-2 foci seen
	4	Focal necrosis with 3 or more foci seen
	5	One confluent area of necrosis
	6	Multiple bridging confluent areas of cellular death
	7	Areas of necrosis exceed areas of viable parenchyma
	8	No or very focal viable parenchyma identified

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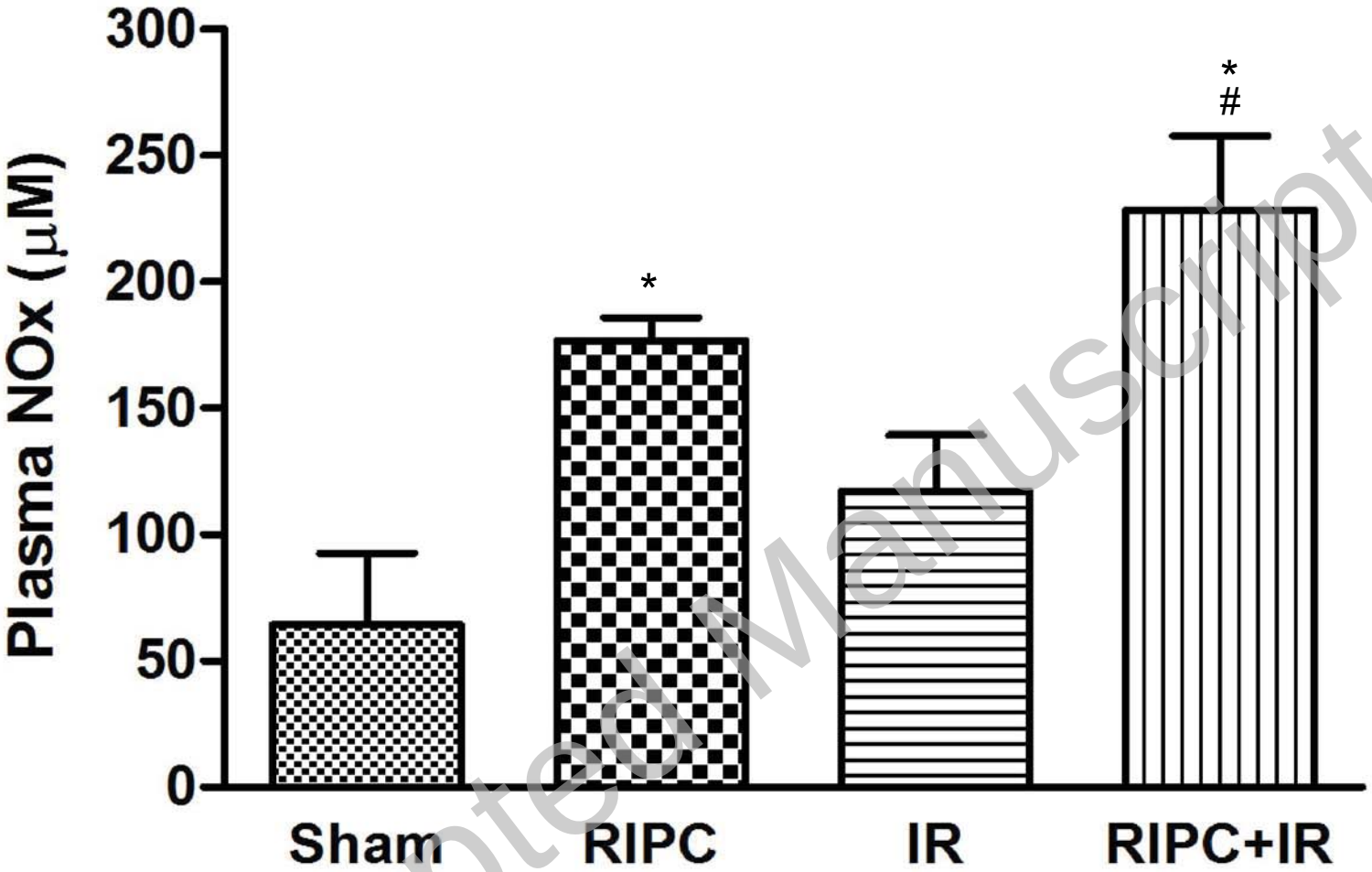

Figure 2.

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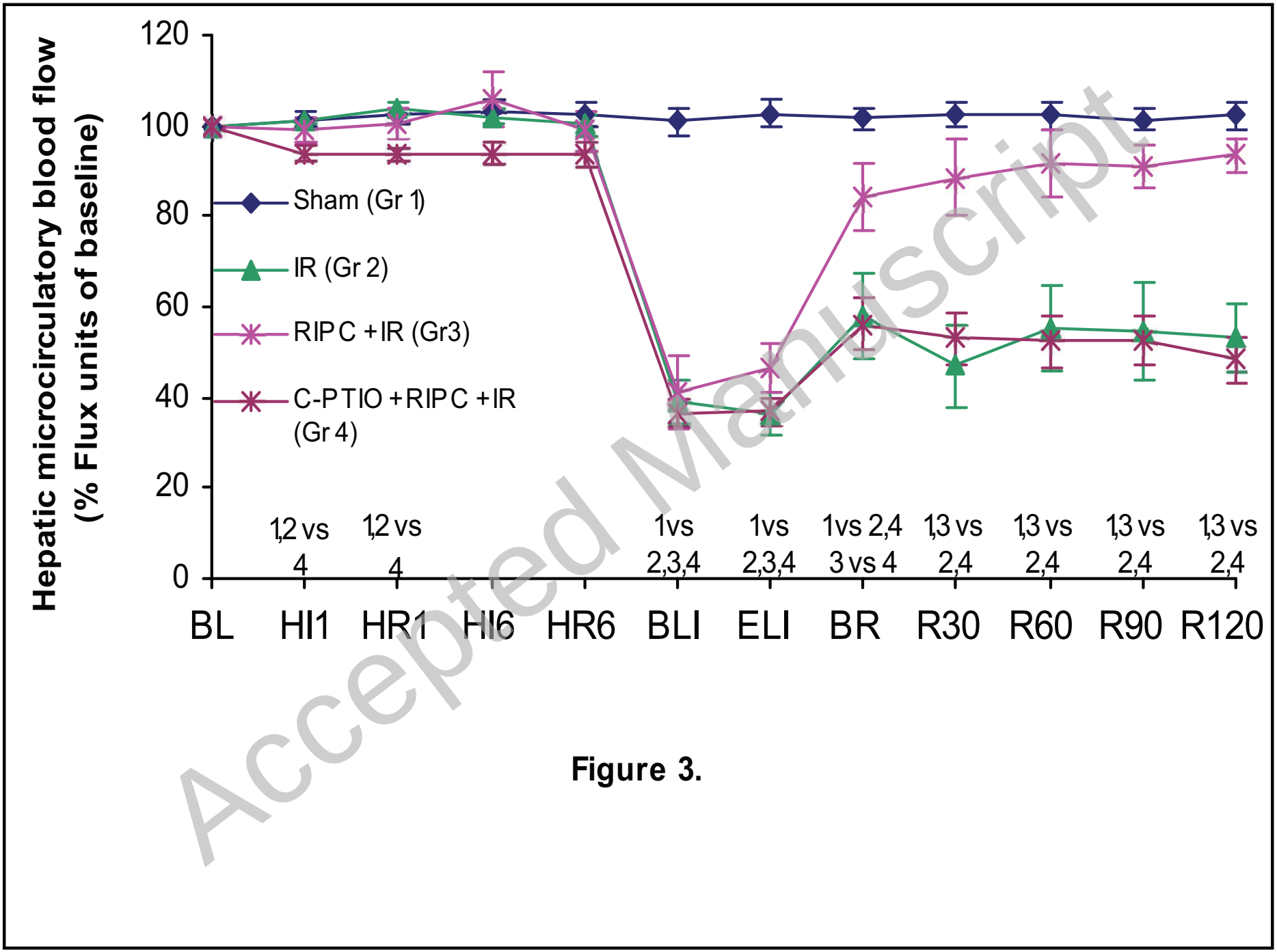

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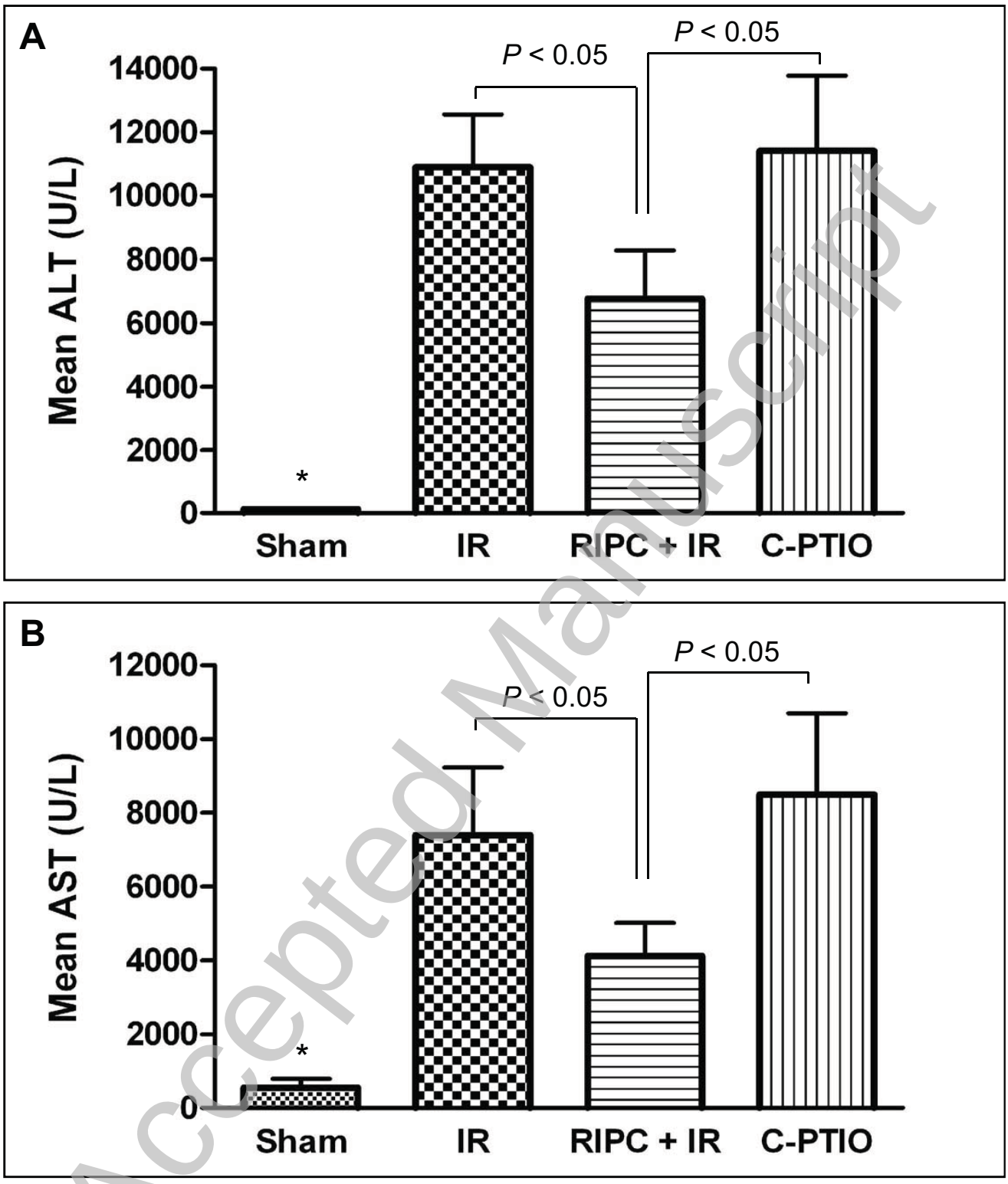

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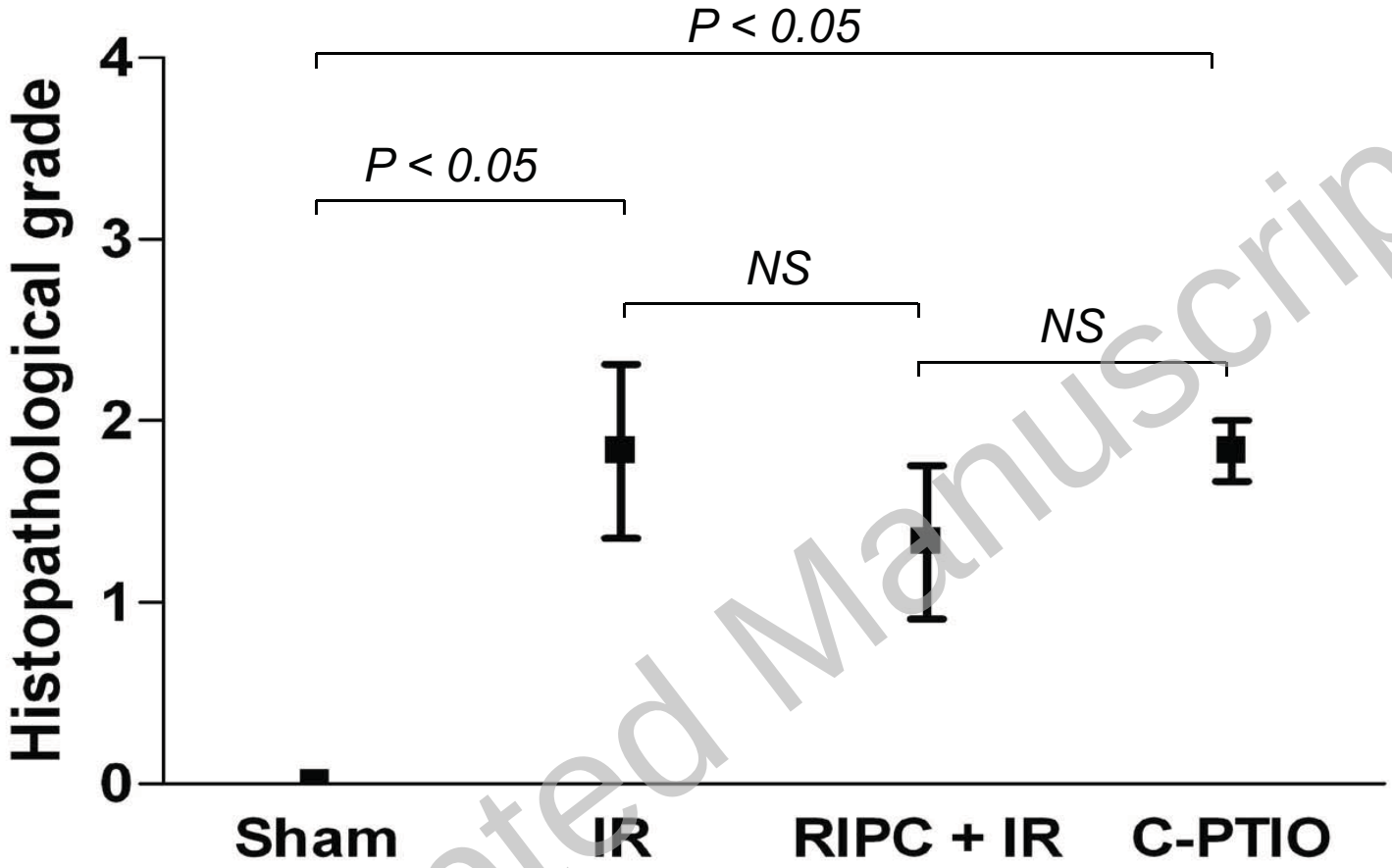

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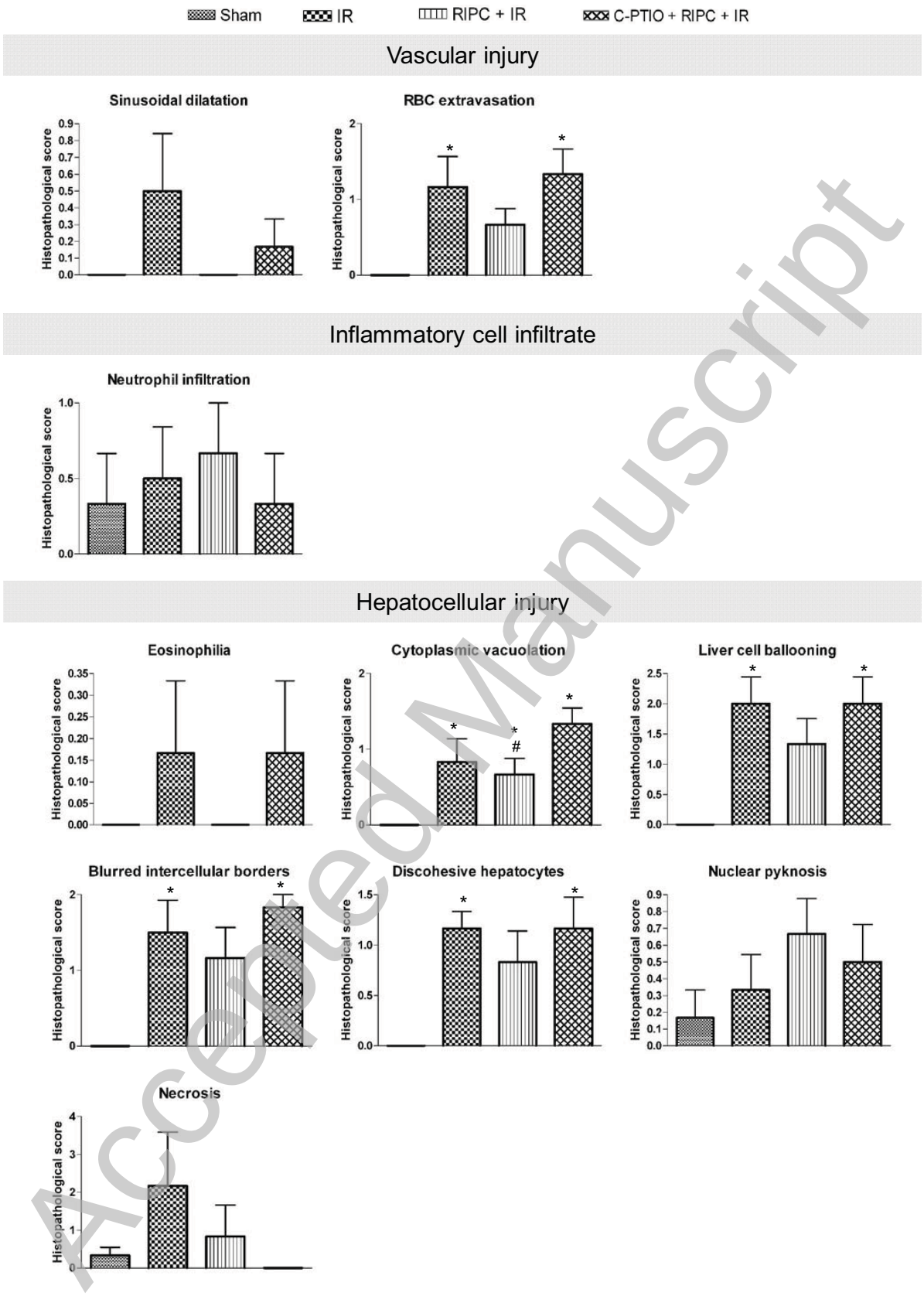

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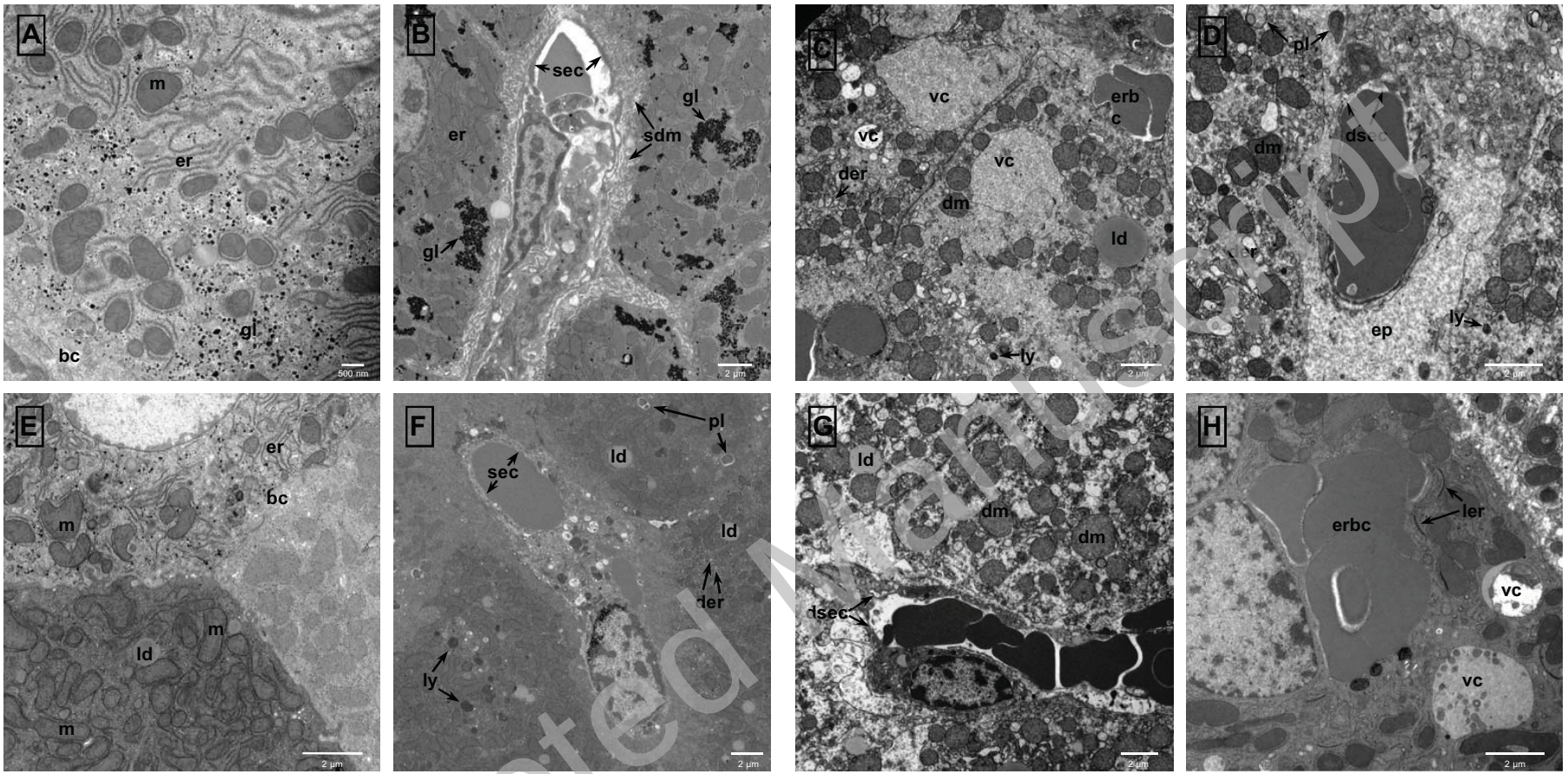

Figure 7.