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Mitigating hepatic ischemia and reperfusion injury with polyethylene glycol-enriched Ringer's lactate fluid: insights from an isolated perfused rat model

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Abstract

Background Cold ischemia-reperfusion (IR) injury is a multifactorial process detrimental to liver graft function during liver transplantation (LT). Although flushing hepatic grafts prior to reperfusion have been well explored, the optimal graft rinse solution to prevent cold IR injury remains largely undefined. The aim of this study was to evaluate whether a new rinse solution combining polyethylene glycol PM 35,000 Da (PEG35) with lactated solution (RLS) could mitigate cold IR injury in *Wistar* rats.

Methods Livers were isolated, preserved for 24 h and flushed immediately before ex vivo reperfusion with either RLS or PEG35-enriched RLS. Liver injury, graft function, energy balance, autophagy, oxidative stress as well as inflammatory response were assessed.

Results Flushing hepatic grafts with PEG35-enriched RLS resulted in decreased transaminase levels after cold ischemia. The improved graft function was evidenced by increased bile flow, enhanced BSP clearance, and reduced vascular resistance in these flushed grafts. Phospho-AMPK protein expression, as well as LC3B gene and protein expression were significantly increased compared to those unflushed and flushed only with RLS. PEG35-enriched RLS also maintained the oxidative state, as indicated by reduced activities of antioxidant enzymes and decreased MDA concentration. Additionally, this graft rinse solution down-regulated the inflammatory response by inhibiting the expression of genes involved in the HMGB-1/NF-κB/TNF-α signaling pathway.

Conclusion These data strongly suggest that rinsing liver grafts with PEG35-enriched RLS prior to reperfusion represents a simple and cost-effective strategy to enhance liver functional recovery after cold IR injury. This approach could serve as a viable alternative to RLS in clinical applications, highlighting the need for further research to explore its broader implications.

Keywords Cold ischemia-reperfusion injury, Liver transplantation, Polyethylene glycol PM 35000 Da (PEG35), Graft flushing, Lactated Ringer's solution (RLS), Cold ischemia protection

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Introduction

Liver transplantation (LT) stands as the gold standard treatment for end-stage liver disease [1]. LT process involves subjecting the donated graft to a period of cold ischemia by preserving it in a static cold preservation solution [2]. During this ischemic phase, anaerobic metabolism occurs, leading to energy depletion within the graft. Consequently, dysfunction of ATP-dependent pumps occurs, disrupting ion homeostasis, cellular swelling, acidosis, and cytoskeletal disorganization. Upon reperfusion, reactive oxygen species (ROS) production induces oxidative stress, initiating a cascade of inflammatory responses that ultimately lead to cell death. This irreversible damage is known as cold ischemia-reperfusion (IR) injury, a significant risk factor for poor and non-graft function [3–6].

To mitigate the impact of cold IR injury and improve LT outcomes, hypothermic preservation has been developed [7]. However, this procedure has drawbacks, including toxic metabolic waste accumulation during cold ischemia [8]. In an effort to reduce complications during reperfusion, surgeons have explored graft rinsing before revascularization [9]. Several studies support this novel approach, indicating that pre-reperfusion graft rinsing reduces post-reperfusion damage [10, 11]. Despite its significance, limited clinical research has been conducted on the use of rinsing solutions for graft rescue and conditioning. In clinical practice, Ringer's lactate solution (RLS), a crystalloid solution devoid of additives and buffering capacity, is commonly used for intraoperative volume replacement [12]. Although RLS was not specifically designed to mitigate cold IR injury, it has been shown to improve graft survival and reduce microvascular injury when used for graft flushing prior to reperfusion [13]. However, Adam et al. proposed a more effective alternative to RLS, suggesting that rinsing liver transplants with a macromolecular albumin solution offers better protection against reperfusion damage [9]. Attempts to enhance graft rinse solutions by incorporating specific pharmacological agents led to the emergence of Carolina rinse solution, showing promise in experimental conditions [14]. However, its complex composition and the inclusion of hydroxyethyl starch (HES), causing blood cell aggregation [15], hindered its adoption in routine clinical practice. Other studies explored the effects of rinsing grafts with Normosol-1 and plasmalyte-A solutions showing improvements in liver function and hepatocellular damage, respectively [16, 17]. Yet, substantial improvements in post-transplant outcomes were not consistently observed [18]. A novel rinse solution, called the "Base Solution", containing polyethylene glycol-35 kDa (PEG35) was developed by Zaouali et al., providing strong evidence of PEG35's role in preserving mitochondrial integrity, reducing oxidative stress, and inflammation [19]. However, this graft rinse solution remains in experimental phase and its clinical validation is essential. In a recent study, hydrogen rinse solution was found effective in reducing cold IR injury yet concerns about hydrogen's limited solubility and flammability limited its clinical utility [20]. Numerous solutions have been employed for graft rinsing; however, the optimal composition remains uncertain.

Building upon the established benefits of PEG35 in mitigating IR injury, along with the straightforward composition and the clinical availability of RLS, this study introduces PEG35 into RLS for the first time as a flushing solution for liver grafts before reperfusion. The aim of this research is to evaluate the effectiveness of this novel flushing solution in reducing the susceptibility of rat liver grafts to cold IR injury.

Materials and methods

Animals

Male *Wistar* rats weighing between 200 and 250 g were raised in the animal facility at the Faculty of Pharmacy, Monastir, Tunisia. The rats were provided with free access to food and water and were maintained under standard conditions (temperature, humidity and 12/12 h of light/dark cycle). Ethical approval was obtained from the Research Ethics Committee for Life and Health Sciences at the Higher Institute of Biotechnology of Monastir under the reference CER-SVS/ISBM 05/2020. All experimental procedures were carried out in accordance with experimental ethics.

Liver harvesting and perfusion

Rats were anesthetized by intraperitoneal injection of 24% urethane (0.5 ml/100 g of body weight) before undergoing a median xypho-pubic and transverse bi-subcostal laparotomy. A Venocath 18 G catheter was inserted into the common bile duct. Following the dissection of the aorta, vena cava, and portal vein as outlined in a prior study [21], a Venocath 16 G catheter was placed in the aorta for arterial liver lavage, and a Venocath 14 G catheter was inserted into the portal vein for complete liver wash with cooled Histidine-tryptophan-ketoglutarate (HTK) preservation solution. At the beginning of the liver washing procedure, the inferior and suprahepatic vena cava were promptly incised to allow fluid drainage. Once the washing process was completed, the livers were immediately harvested and stored in HTK preservation solution at 4 °C for 24 h. The rats, still under anesthesia, were euthanized through exsanguination.

After the cold ischemia preservation period, the isolated liver grafts underwent ex vivo reperfusion at 37 °C in a closed circuit through the portal vein for 120 min. The livers were perfused with a solution containing

Krebs-Heinseleit bicarbonate buffer (pH 7.4), 5% albumin, and a mixture of 5% CO_2 and 95% O_2.

Following ex vivo normothermic reperfusion, the liver tissue was divided into three segments. One segment was fixed in 10% formalin for subsequent histological and immunofluorescence analysis. Another segment was stored in RNA Later for transcriptome analysis, while the remaining segment was frozen at -80 °C for biochemical assessments.

Experimental groups

The experimental groups were as follows ($n = 7$):

Group 1 (sham): The livers were promptly washed out and harvested, then immediately, subjected to ex vivo perfusion without undergoing cold preservation.

Group 2 (cold IR): Following a 24-h period of cold ischemia in HTK preservation solution, the liver underwent ex vivo reperfusion without any rinsing procedure.

Group 3 (RLS): Similar to the cold IR group, but in this case, the livers were rinsed with 50 ml of RLS before undergoing a 120-min ex vivo reperfusion at 37 °C. The RLS solution was administered through the portal vein at room temperature.

Group 4 (RLS + PEG35): Similar to the RLS group, but the livers were rinsed with 50 ml of RLS enriched with 5 g/L of PEG35 [19] at room temperature.

Liver injury and function

Liver transaminase assay

Liver cytolysis was evaluated by assessing transaminase activity [22], following 120 min of ex vivo normothermic perfusion. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in portal vein effluents were determined using Biomaghreb kits (Tunisia), with results quantified in U/L.

Bile output

Liver function was assessed by measuring cumulative bile production during a 120-min normothermic perfusion [23]. Bile was collected through the cannulated bile duct, and its volume was determined by weighing. Bile flow was quantified as µL/g liver/120 min.

Bromosulfophthalein clearance

Hepatic clearance involved the addition of 1 mg of bromosulfophthalein (BSP, Sigma Aldrich) to the Krebs-Heinseleit medium 30 min after initiating perfusion. The percentage of BSP excreted in bile was then calculated based on the administered dose of BSP. BSP concentration in both the perfusate and bile was measured at 580 nm. BSP clearance was determined as a percentage of perfusate content (t_{120}bile / t_{30}perfusate * 100) [24].

Vascular resistance

The portal pressure was maintained constant at 12 mmHg throughout the ex vivo perfusion time. Vascular resistance was calculated as the ratio of portal pressure to perfusion flow. Vascular resistance was expressed in mmHg.min.g.ml^{-1}, as previously reported [25].

Antioxidant enzymes activities measurement

The total activity of superoxide dismutase (Cu-Zn SOD and Mn SOD) was evaluated using the method outlined by Marklund et al. [26]. This assay assesses the capacity of SOD to inhibit the autoxidation of pyrogallol, with SOD activity measured at 420 nm and expressed as U/mg protein. Catalase activity (CAT) was determined following the procedure described by Claiborne et al. [27]. This method involves monitoring the breakdown of hydrogen peroxide (H_2O_2), with CAT activity measured at 240 nm and expressed as µmol of H_2O_2 decomposed/min/mg protein. Glutathione peroxidase (GPx) activity was assessed by according to the method introduced by Flohe et al. [28]. GPx activity was determined by measuring the oxidation of glutathione (GSH) into glutathione disulfide (GSSG) at 412 nm and expressed as µmoL of oxidized GSH/min/mg protein.

Malondialdehyde assay

Lipid peroxidation was assessed in the hepatic tissues by the determination of malondialdehyde (MDA) formation with the reaction at 530 nm [24]. It was represented as nmol/mg of protein.

Histological analyses

Liver specimens were fixed in 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) (RAL-Diagnostics). Images were captured using a Leica DFC320 R2 digital camera. Damage assessment involved counting morphological changes in randomly selected microscopic fields from each group's samples, following the classification by t'Hart et al. [29]: (1) Normal rectangular form, (2) rounder hepatocytes with larger sinusoidal spaces, (3) vacuolization, (4) nuclear pyknosis, and (5) necrosis.

Immunofluorescence analysis of AMPK and LC3 protein expression

Paraffin-embedded liver sections were permeabilized with 1% Triton X-100 for 30 min, and blocked with normal goat serum for 1 h at 37 °C. Subsequently, tissue samples were incubated overnight at 4 °C with rabbit anti Adenosine monophosphate-activated protein kinase (AMPK) (1:100, cell signalling, #2535), rabbit

Table 1 Primer sequences

Gene name	Forward primer sequence	Reverse primer sequence
SOD	5′-GCC TCC CTG ACC TGC CTT AC-3′	5′-GCA TGA TCT GCG CGT TAA TG-3′
Catalase	5′-CCC AGA AGC CTA AGA ATG CAA-3′	5′-GCT TTT CCC TTG GCA GCT ATG-3′
GPx	5′-GTG TTC CAG TGC GCA GAT ACA-3′	5′-GGG CTT CTA TAT CGG GTT CGA-3
NF-kB	5′-GGCAGCACTCCTTATCAAC-3′	5′-GGTGTC-GTCCCATCGTAG-3′
HMGB-1	5′-TATGGCAAAAGCGGACAAGG-3′	5′-CTTCGCAACAT-CACCAATGGA-3′
TNF-α	5′-CCCTCACACTCAGAT-CATCTTCT-3′	5′-GCTACGAC-GTGGGCTACAG-3′
LC3	5′-GAGTGGAAGATGTCCGGCTC-3′	5′-CCAGGAG-GAAGAAGGCTTGG-3′

anti-microtubule-associated proteins1A/1B light chain 3B (LC3B) (1:100, Sigma-Aldrich, #L7543). Following this, a secondary antibody, Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:500, Thermo Fisher, Invitrogen, #A32731), were applied for 1 h at 37 °C in the absence of light. Microscopic analysis and image capture were conducted using a Carl Zeiss system.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from rat livers with TRItidy-G reagent (AppliChem, TRItidy G™, Italy), according to the manufacturer's instructions. cDNA was synthesized using the M-MLV RT enzyme kit according to the manufacturer's protocol ((MMLV, Thermoscientific Invitrogen™, France). Quantitative real-time reverse transcription PCR (qRT–PCR) was performed using StepOne Software from Thermo Fisher Scientific. The $2^{-(\Delta\Delta CT)}$ method, as described by Livak et al. in 2013, was employed to determine relative gene expression, with β-actin serving as the reference gene. This analysis was conducted using SYBR® Green PCR Master Mix (Applied BiosystemsTM) and the primers outlined in Table 1.

Statistical analysis

Data were presented as means ± SD. Statistical analyses were conducted using one-way ANOVA, followed by Tukey post hoc test GraphPad software (version Prism 8.4.2). $p < 0.05$ was considered statistically significant. Basic Intensity Quantification was performed using Image J [30].

Results

Rinsing hepatic grafts with PEG35-enriched RLS preserves cell integrity

The assessment of hepatocellular damage, utilizing liver enzymes ALT and AST, revealed a significant increase in perfusate ALT and AST levels in rats subjected to Cold IR as compared to the sham group (Fig. 1A and B). Flushing the grafts with RLS did not significantly affect ALT and AST levels compared to the Cold IR group. However, rinsing livers with PEG35-enriched RLS before reperfusion resulted in a significant reduction in ALT levels compared to both the Cold IR and RLS groups, and a decrease in AST levels compared to the Cold IR group.

Rinsing hepatic grafts with PEG35-enriched RLS ameliorates hepatic function

Liver function assessment included measurement of bile production, vascular resistance and BSP clearance. As depicted in Fig. 2A, liver grafts exposed to Cold IR exhibited a significant reduction in bile production compared to the sham group. No significant difference was observed between livers subjected to Cold IR and those rinsed with RLS alone. However, rinsing grafts with RLS-enriched PEG35 resulted in a significant enhancement in bile production compared to both Cold IR and RLS groups.

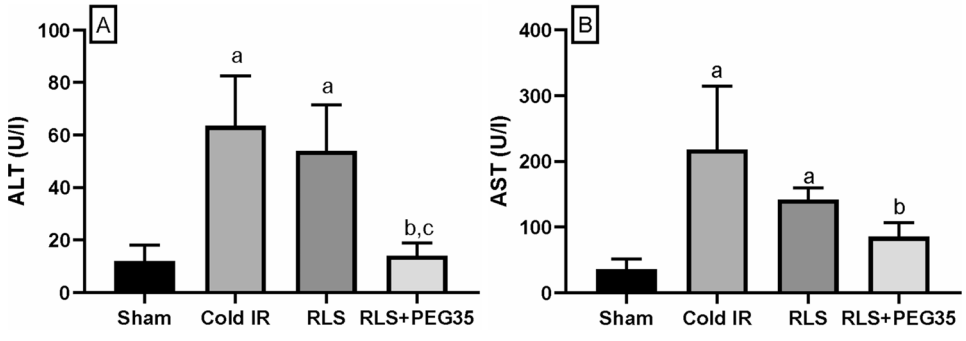

Fig. 1 PEG35-enriched RLS limits the enzyme leakage. Alanine aminotransferase (**A**, ALT) and aspartate aminotransferase (**B**, AST) activities in the liver effluent obtained after 120 min of ex vivo reperfusion. Sham: livers harvested from untreated rats and immediately submitted to 120 min of ex vivo reperfusion, Cold IR: livers subjected to cold ischemia for 24 h at 4 °C and submitted to 120 min of ex vivo reperfusion, RLS: livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution before undergoing a 120-min of ex vivo reperfusion, RLS + PEG35: livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution enriched with PEG35 at 5 g/L before undergoing a 120-min of ex vivo reperfusion. Data were expressed as means ± SD ($n = 8$ for each group). $^{a}P < 0.05$ vs. Sham; $^{b}P < 0.05$ vs. Cold IR; $^{c}P < 0.05$ vs. RLS

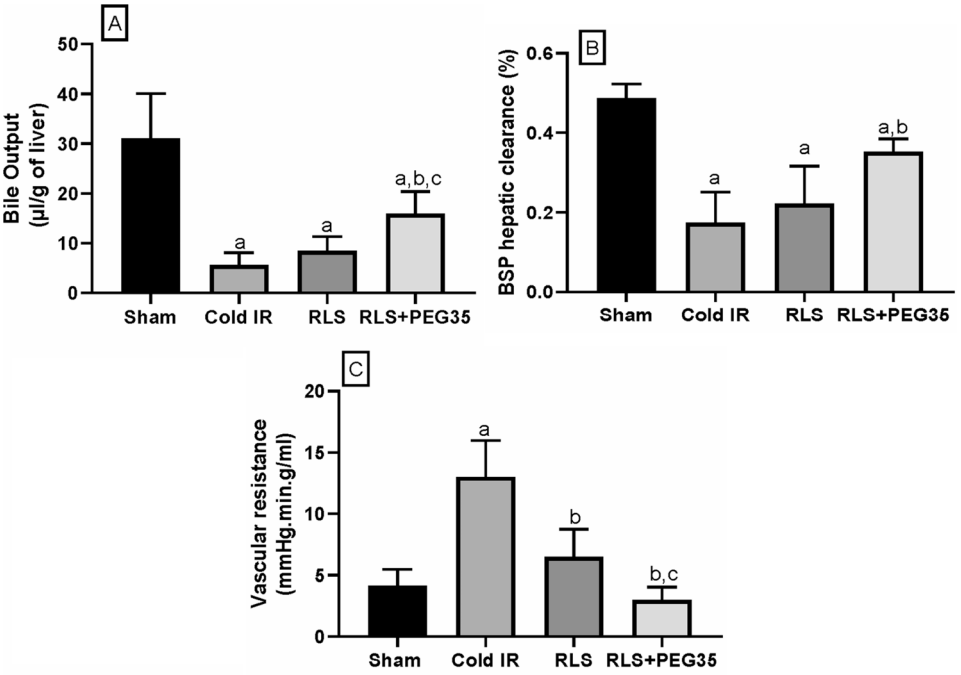

Fig. 2 PEG35-enriched RLS improves liver function. Liver function expressed as bile flow (**A**), bromosulfophthalein clearance (**B**) and vascular resistance (**C**) in livers harvested from untreated rats and immediately submitted to 120 min of ex vivo reperfusion (Sham), in livers subjected to cold ischemia for 24 h at 4 °C and submitted to 120 min of ex vivo reperfusion (Cold IR), in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution before undergoing a 120-min of ex vivo reperfusion (RLS) and in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution enriched with PEG35 at 5 g/L before undergoing a 120-min of ex vivo reperfusion (RLS + PEG35). Data were expressed as means ± SD ($n = 8$ for each group). $^{a}P < 0.05$vs.Sham; $^{b}P < 0.05$vs. Cold IR; $^{c}P < 0.05$ vs. RLS

In Fig. 2B, liver grafts exposed to Cold IR significantly reduced hepatic BSP clearance compared to sham group. Rinsing the livers with RLS alone had no significant effect on BSP clearance compared with the Cold IR group. In contrast, rinsing grafts with PEG35 enriched with RLS increased biliary clearance of BSP compared with the Cold IR group.

According to Fig. 2C, vascular resistance was significantly increased in the Cold IR compared to the sham group. Compared to the Cold IR group, rinsing grafts with RLS alone significantly decreased vascular resistance. However, the most important reduction in vascular resistance was shown when the grafts were flushed with RLS-enriched PEG35 compared to the Cold IR and RLS groups.

Rinsing hepatic grafts with PEG35-enriched RLS maintains hepatic architecture

As shown in Fig. 3A and B, histological examination revealed significant damage in the Cold IR group, characterized by vacuolization, necrosis, and disintegration of liver architecture. The RLS group displayed sinusoidal dilatation, while this effect was significantly mitigated in the RLS group enriched with PEG35.

Rinsing hepatic grafts with PEG35-enriched RLS improves hepatic energy balance

Fluorescence intensity associated with phospho-AMPK protein expression showed a significantly reduced level in the Cold IR group compared to the sham group. Rinsing with RLS alone had no significant effect, while PEG35-enriched RLS improved energy balance, evidenced by a significant increase in phosphor-AMPK fluorescence intensity compared to both groups (Fig. 4).

Rinsing hepatic grafts with PEG35-enriched RLS enhances autophagy

Autophagy marker (LC-3) gene and protein expression decreased in livers subjected to Cold IR. Rinsing with RLS alone showed no significant difference, but PEG35-enriched RLS induced substantial LC3 gene and protein expression compared to other groups (Fig. 5A, B and C).

Rinsing hepatic grafts with PEG35-enriched RLS protects against oxidative damage

Antioxidant enzyme activities (SOD, GPx, CAT) were significantly higher in livers exposed to Cold IR, reduced by RLS flushing, and significantly decreased with PEG35-enriched RLS compared to Cold IR and RLS groups (Fig. 6A, B, C). Enzymatic antioxidant gene expressions (SOD, GPx, CAT) followed a similar profile (Fig. 6D, E,

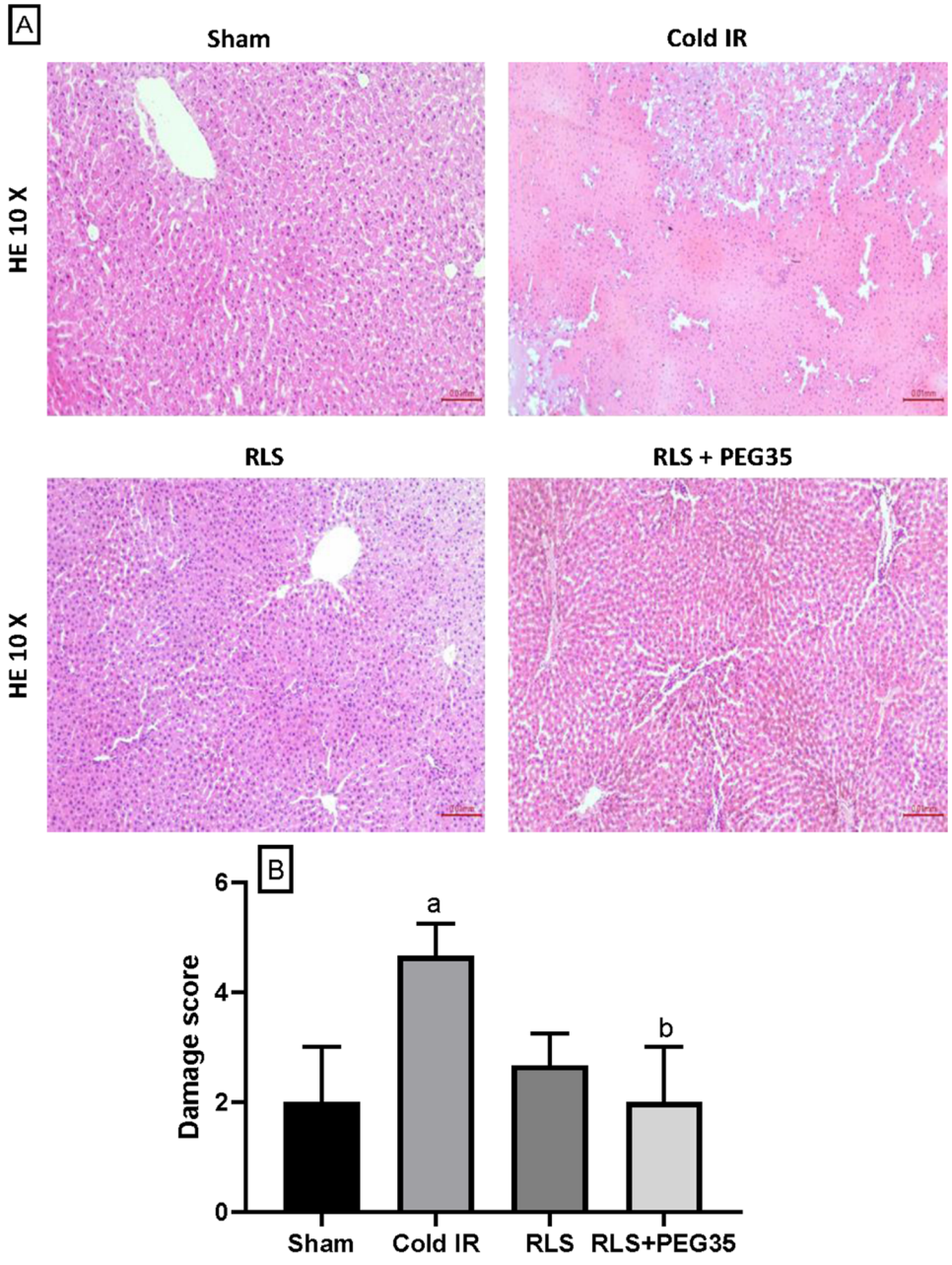

Fig. 3 PEG35-enriched RLS prevents histological lesions. Hematoxylin eosin stained sections (**A**) were used for the evaluation of histological lesions (**B**) (magnification × 10) (scale bars = 100 µm) in livers harvested from untreated rats and immediately submitted to 120 min of ex vivo reperfusion (Sham), in livers subjected to cold ischemia for 24 h at 4 °C and submitted to 120 min of ex vivo normothermic reperfusion (Cold IR), in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution before undergoing a 120-min of ex vivo reperfusion (RLS) and in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution enriched with PEG35 at 5 g/L before undergoing a 120-min of ex vivo reperfusion (RLS + PEG35). Data were expressed as means ± SD ($n = 8$ for each group). $^{a}P < 0.05$ vs. Sham; $^{b}P < 0.05$ vs. Cold IR; $^{c}P < 0.05$ vs. RLS

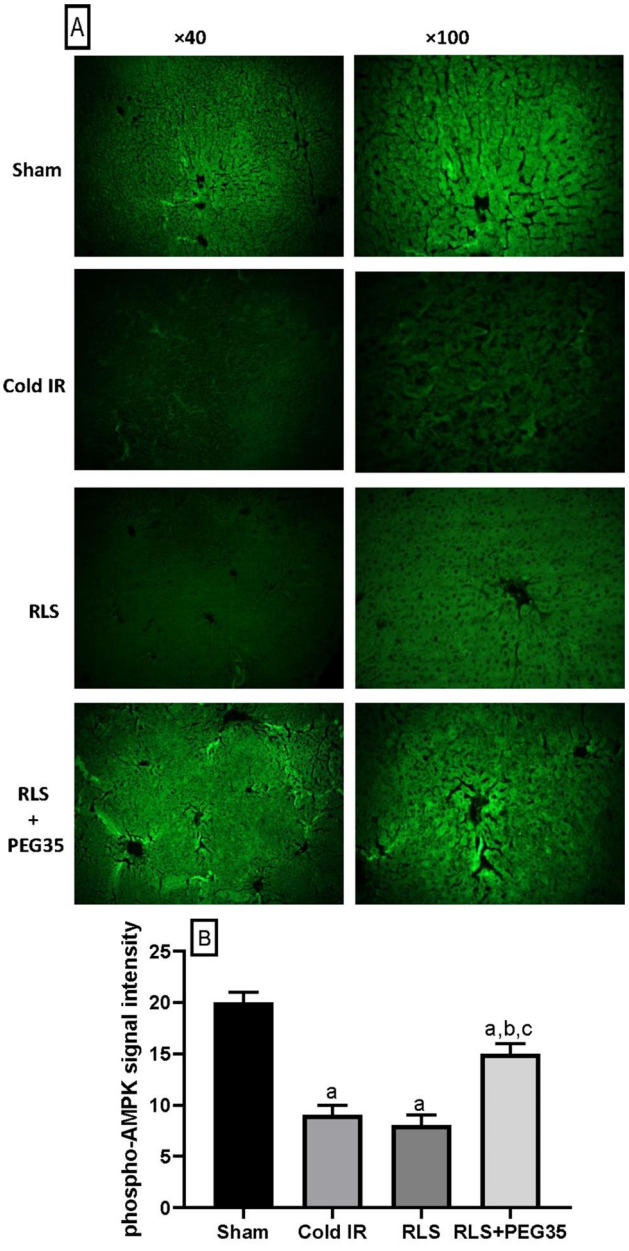

Fig. 4 PEG35-enriched RLS prevents liver energy metabolism. Phosphorylated adenosine monophosphate-activated protein kinase protein (AMPK) expression by immunofluorescence (original magnification ×40, ×100) (**A**) and AMPK signal intensity (**B**) was evaluated in livers harvested from untreated rats and immediately submitted to 120 min of ex vivo reperfusion (Sham), in livers subjected to cold ischemia for 24 h at 4 °C and submitted to 120 min of ex vivo reperfusion (Cold IR), in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution before undergoing a 120-min of ex vivo reperfusion (RLS) and in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution enriched with PEG35 at 5 g/L before undergoing a 120-min of ex vivo reperfusion (RLS + PEG35). Data were expressed as means ± SD ($n = 8$ for each group). [a] $P < 0.05$ vs. Sham; [b] $P < 0.05$ vs. Cold IR; [c] $P < 0.05$ vs. RLS

F), and MDA concentrations were significantly reduced with PEG35-enriched RLS compared to the Cold IR group (Fig. 6G).

Rinsing hepatic grafts with PEG35-enriched RLS attenuates cold ischemia-induced inflammation

Inflammation-related gene expressions, including High Mobility Group Box 1 (HMGB-1), Nuclear factor kappa B (NF-kB) and Tumour Necrosis Factor alpha (TNF-α), were highest in livers subjected to Cold IR in comparison to Sham group. HMGB-1 and NF-kB gene expressions were significantly reduced by RLS flushing compared to the cold IR group, whereas TNF-α expression showed no significant reduction. Rinsing grafts with PEG35-enriched RLS further decreased these gene expressions compared to both the Cold IR and RLS groups (Fig. 7).

Discussion

Although LT techniques are becoming more standardized, there are still ongoing controversies surrounding various aspects of the procedure [31]. The rinsing of grafts after cold preservation has emerged as a potential factor influencing liver function outcomes after reperfusion. In this study, we explored the effects of a novel graft rinse solution combining RLS with PEG35. Using an ex vivo perfusion model, we found that PEG35 supplementation to RLS for normothermic liver rinse after hypothermic preservation effectively maintained membrane integrity, preserved energy metabolism, promoted autophagy, alleviated oxidative stress, and reduced inflammation.

The intricate pathophysiology of cold IR injury involves significant metabolic and structural changes leading to substantial tissue damage [32, 33]. In this regard, our findings revealed that exposure to cold ischemia in HTK solution increased the release of ALT/AST in the perfusate compared to the sham group. Notably, rinsing liver grafts with PEG35-enriched RLS resulted in a greater reduction in transaminase levels compared to RLS alone, suggesting a lower degree of hepatocyte cytolysis. The histological study corroborates these findings, revealing less damages in hepatic grafts rinsed with this rinse solution compared to other groups. Additionally, they underscore the critical role of incorporating PEG in effectively protecting hepatocytes, maintaining their membrane integrity, and restoring their inherent viability and functionality [34, 35].

Monitoring bile production is widely accepted as a reliable parameter for assessing the integrity and energy status of ex vivo perfused livers [36]. Preservation of liver grafts in HTK solution resulted in decreased bile flow compared to the sham group. Conversely, flushing grafts with normothermic RLS prior to ex vivo reperfusion improved biliary output, consistent with previous

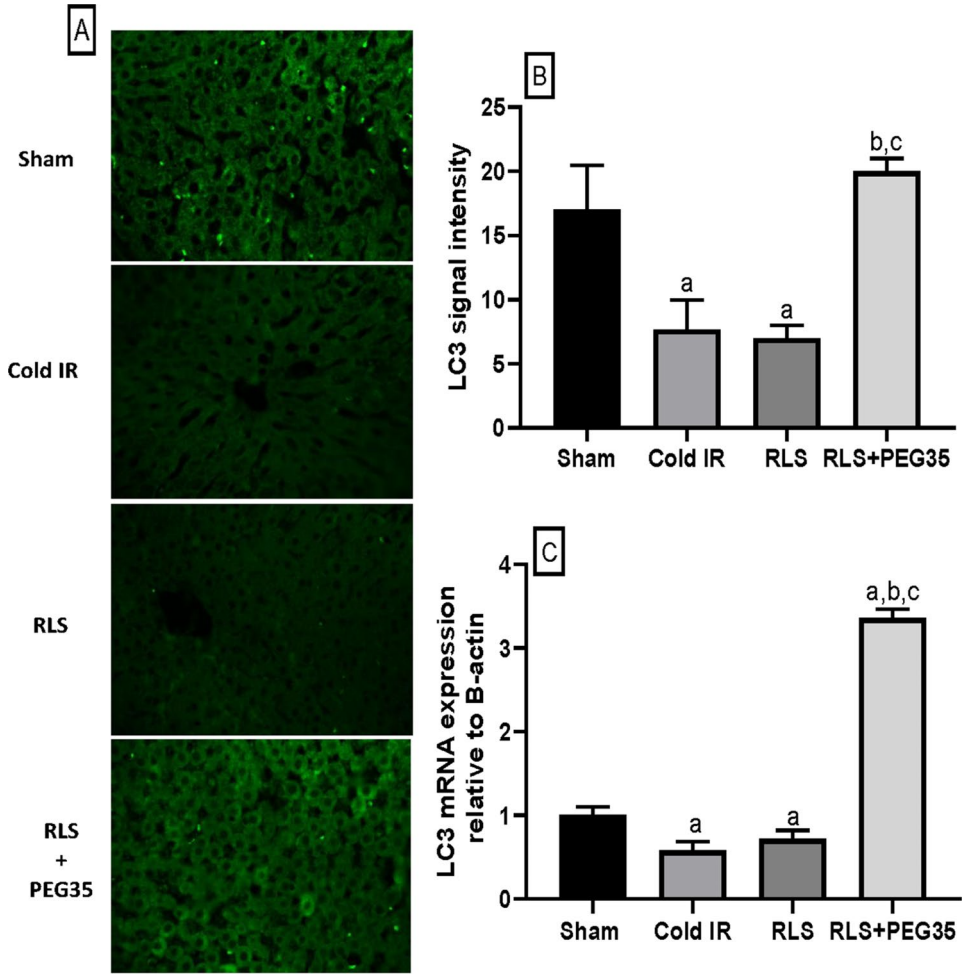

Fig. 5 PEG35-enriched RLS promotes autophagy process: Microtubule-associated protein 1 A/1B-light chain 3 (LC3, **A**) protein expression by immunofluorescence (original magnification ×400), LC3 signal intensity (**B**) and LC3 mRNA expression (**C**) were evaluated in livers harvested from untreated rats and immediately submitted to 120 min of ex vivo reperfusion (Sham), in livers subjected to cold ischemia for 24 h at 4 °C and submitted to 120 min of ex vivo reperfusion (Cold IR), in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution before undergoing a 120-min of ex vivo reperfusion (RLS) and in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution enriched with PEG35 at 5 g/L before undergoing a 120-min of ex vivo reperfusion (RLS + PEG35). Data were expressed as means ± SD ($n = 8$ for each group). $^{a}P < 0.05$ vs. Sham; $^{b}P < 0.05$ vs. Cold IR; $^{c}P < 0.05$ vs. RLS

research suggesting that warm RLS may mitigate the effect of cold IR injury [37]. Notably, flushing grafts with PEG35-enriched RLS demonstrated a significant improvement in bile production after 24 h of cold ischemia compared to RLS group. This improvement, which goes hand in hand with the enhancement in BSP hepatic clearance noted after rinsing grafts with this PEG35-enriched RLS, is associated with the preservation of liver function. These findings provide further validation of the beneficial effects of the flushing solution on liver function [19].

Elevation in post-transplant vascular resistance is associated with an increased incidence of primary graft dysfunction and non-function in LT [38]. Liver grafts stored in HTK solution exhibited higher vascular resistance than those of the sham group in our study. Flushing the liver grafts with RLS decreased vascular resistance during reperfusion compared to the Cold IR group. Importantly, flushing hepatic grafts with PEG35-enriched RLS improved the reduction in vascular resistance compared to RLS alone. This improvement in microvascular function may be attributed to the beneficial effects of the washing step as well as the local effects of PEG35 on endothelial cells, including enhanced nitric oxide production and maintenance of colloidal oncotic pressure [39–41].

To investigate the mechanism by which pre-reperfusion rinsing of grafts with PEG35-enriched RLS mitigates cold IR injury, our study focused on examining the role of AMP-activated protein kinase (AMPK), a key enzyme involved in the regulation of autophagy processes, oxidative stress, and inflammation. It plays a significant role

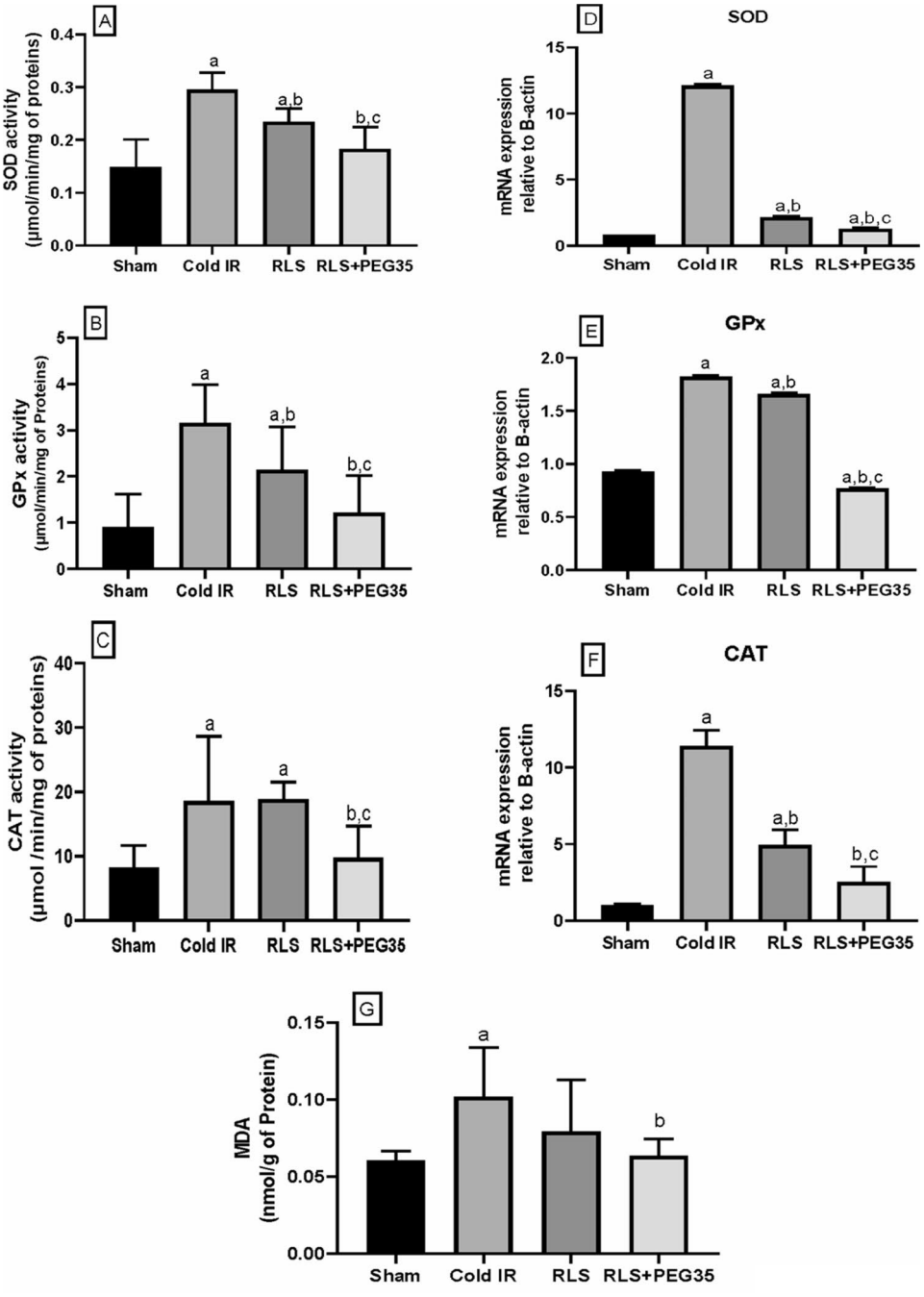

Fig. 6 PEG35-enriched RLS prevents oxidative stress and lipid peroxidation. The antioxidant enzymes: superoxide dismutase (**A**, SOD), glutathione peroxidase (**B**, GPX), catalase (**C**, CAT) activities, their mRNA expression (**D**, SOD; **E**, GPX and **F**, CAT respectively) and Hepatic malondialdehyde (MDA) levels (**G**) were measured in livers harvested from untreated rats and immediately submitted to 120 min of ex vivo reperfusion (Sham), in livers subjected to cold ischemia for 24 h at 4 °C and submitted to 120 min of ex vivo reperfusion (Cold IR), in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution before undergoing a 120-min of ex vivo reperfusion (RLS) and in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution enriched with PEG35 at 5 g/L before undergoing a 120-min of ex vivo reperfusion (RLS + PEG35). Data were expressed as means ± SD ($n = 8$ for each group). $^{a}P < 0.05$ vs. Sham; $^{b}P < 0.05$ vs. Cold IR; $^{c}P < 0.05$ vs. RLS

in maintaining metabolic balance and counteracting the depletion of ATP that occurs during cold IR injury [42, 43]. Our findings revealed a decrease in AMPK protein level after livers preservation in HTK solution compared to the sham group. However, the addition of PEG35 to RLS led to an improvement in AMPK phosphorylation. Activation of AMPK is known to stimulate catabolic pathways that generate ATP and inhibit anabolic pathways that could deplete ATP [44]. Rinsing liver grafts with PEG35-enriched RLS resulted in increased AMPK

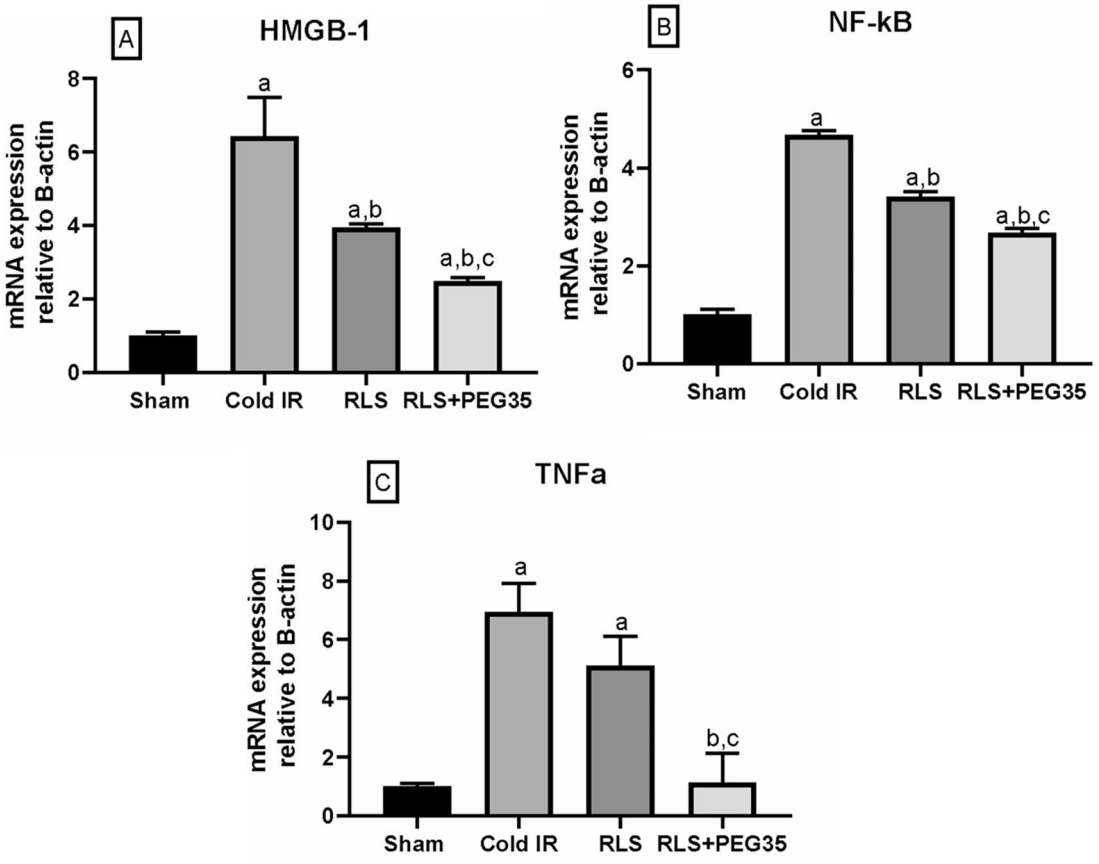

Fig. 7 PEG35-enriched RLS attenuates inflammatory response. Gene expression of high mobility group box – 1 (HMGB-1, **A**), Nuclear factor kappa B (NF-κB, **B**) and Tumor necrosis factor alpha (TNF-α) in liver tissue by RT-PCR were assessed in livers harvested from untreated rats and immediately submitted to 120 min of ex vivo reperfusion (Sham), in livers subjected to cold ischemia for 24 h at 4 °C and submitted to 120 min of ex vivo reperfusion (Cold IR), in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution before undergoing a 120-min of ex vivo reperfusion (RLS) and in livers subjected to cold ischemia for 24 h at 4 °C and rinsed with Ringer's lactate solution enriched with PEG35 at 5 g/L before undergoing a 120-min of ex vivo reperfusion (RLS + PEG35). Data were expressed as means ± SD ($n = 8$ for each group). [a] $P < 0.05$ vs. Sham; [b] $P < 0.05$ vs. Cold IR; [c] $P < 0.05$ vs. RLS

phosphorylation, as well as enhanced gene and protein expression of LC3B, highlighting PEG35's potential to activate AMPK and trigger cytoprotective autophagy, which aids in the elimination of defective mitochondria [45]. Therefore, our results suggest that the beneficial effects of pre-reperfusion rinsing with PEG35-enriched RLS in attenuating cold IR injury may be mediated, at least in part, through the activation of AMPK and the induction of cytoprotective autophagy mechanisms aimed at preserving liver function [46].

Oxidative stress significantly impacts the integrity and function of liver grafts after cold IR injury [25]. In response, the organ activates intracellular biological defense mechanisms, involving both enzymatic and non-enzymatic antioxidants [47]. Our results revealed remarkable antioxidants enzymatic activity in liver grafts subjected to cold IR injury without rinsing. Rinsing liver grafts with RLS significantly decreased antioxidant enzyme activity and reduced lipid peroxidation compared to reperfused grafts without rinsing. The most relevant reduction in these enzymatic activities was observed following rinsing grafts with PEG35-enriched RLS compared to RLS. This result may be related to PEG35's ability to directly eliminate ROS and activate AMPK, inhibiting ROS production [48, 49]. Additionally, PEG35's protective effects may be attributed to its ability to maintain mitochondrial integrity and enhance key mitochondrial biomarkers [50].

HMGB-1 modulation by AMPK is known to play a crucial role in inflammation processes [51]. Our results showed overexpression of HMGB-1 genes in livers exposed to liver preservation in HTK without rinsing compared to the sham group. Rinsing liver grafts with RLS decreased the expression levels of inflammation-related genes, including TLR-4, NF-kB, and TNF-α, compared to Cold IR group. This reduction in inflammation was further pronounced when grafts were rinsed with PEG35-enriched RLS compared to the RLS group. PEG35 has been shown to reduce leukocyte infiltration and pro-inflammatory cytokine levels by activating AMPK,

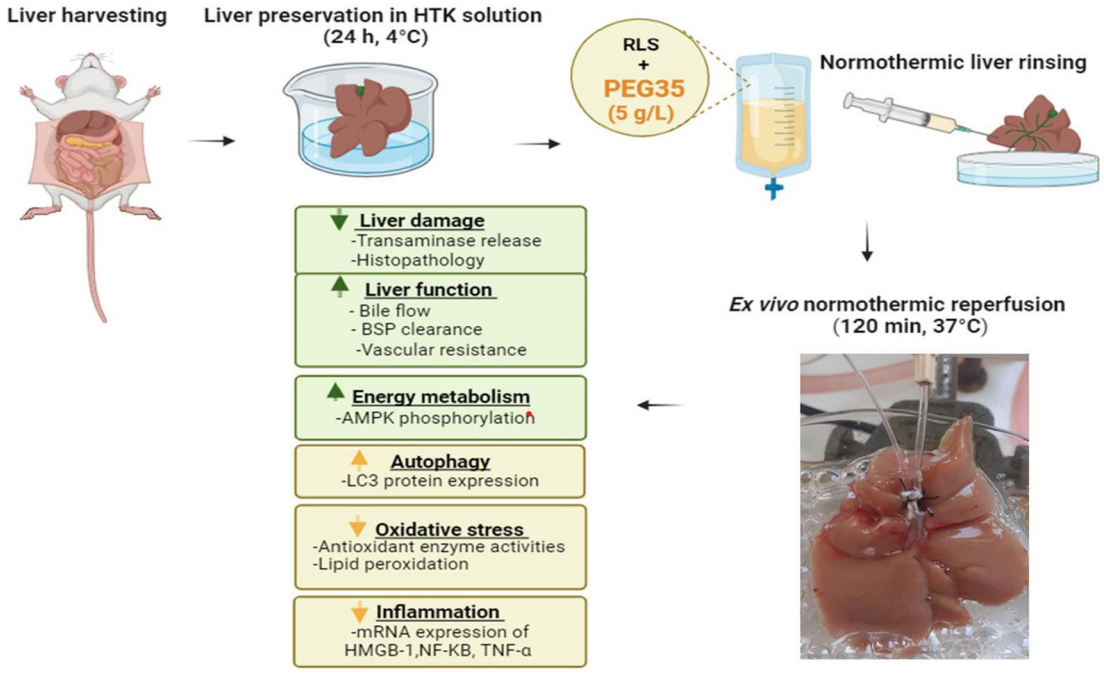

Fig. 8 PEG35-Enriched RLS ensures functional liver grafts recovery. Flushing liver grafts with PEG35-enriched RLS boosts cell membrane integrity, enhances liver function, and triggers AMPK activation. This, in turn, promotes autophagy, mitigates oxidative stress, and alleviates inflammation caused by cold IR injury

contributing to its anti-inflammatory effects [52, 53]. These findings came in agreement with Ferrero-Andres A who reported that PEG35 could reduce leukocyte infiltration and pro-inflammatory cytokine levels by activating AMPK, contributing to its anti-inflammatory effects [53]. Taken together, our research shows that AMPK activation could be the primary mechanism by which the use of PEG35-enriched RLS solution in liver graft rinsing produces its effects. These findings provide evidence supporting the benefits of using a PEG35-enriched rinsing solution in LT to reduce cold IR injury (Fig. 8).

Conclusion

In conclusion, we have demonstrated that adding PEG35 to RLS protects cellular integrity, reduces oxidative stress, and modulates key processes such as inflammation and autophagy. These findings highlight the potential of PEG35-enriched RLS as a novel and effective strategy to counteract cold IR injury, a critical factor in LT, and pave the way for future applications in clinical practice.

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Author contributions
IBJ carried out the experiment, performed the measurements and wrote the manuscript. MAZ designed the study, analysed of the results and the wrote of the manuscript. RC contributed to the qRT PCR measurements. SB contributed to the immunofluorescence measurements. AC contributed to the biochemical measurements. HBA designed the study, analysed the data and wrote the manuscript. All authors contributed to the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All animal experiments in this study were carried out in accordance with the guidelines of the Animal Ethics Committee for Life and Health Sciences at the Higher Institute of Biotechnology of Monastir under the reference CER-SVS/ISBM 05/2020.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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