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Effect of Zinc Oxide Nanoparticles on Hepatic Ischemia-Reperfusion Injury: Role of miR-125b Expression in Possible Underlying Mechanisms

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Abstract

Background: This study examined the protective effects of zinc oxide nanoparticles (ZnO-NPs) on hepatic ischemia-reperfusion injury (HIRI) and their possible underlying mechanisms.

Methods: 48 male rats were randomly divided into six groups (n=8): the sham group that received intraperitoneal normal saline solution (Sham), the HIRI group, the control groups pre-treated with 5 and 10 mg/kg ZnO-NPs for 3 consecutive days without surgery (ZnO5) and (ZnO10), the HIRI group pre-treated with 5 mg/kg ZnO-NPs for 3 consecutive days before surgery (HIRI+ZnO5), and the HIRI group pre-treated with 10 mg/kg ZnO-NPs for 3 consecutive days before surgery (HIRI+ZnO10). One hour after reperfusion, serum and tissue samples were collected for biochemical, molecular and histopathological evaluation.

Results: Administration of ZnO-NPs caused significant improvement in the elevated serum concentrations of ALT, AST, TOS and MDA, improved liver histopathology, and increased TNF-α, IL-6, and NF-κB levels in liver tissue compared to HIRI group. In addition, administration of ZnO-NPs increased the expression of miR-125 in liver tissue compared than in the HIRI group.

Conclusion: The administration of ZnO-NPs improved the effect on HIRI by enhancing miR-125b expression and suppressing oxidative stress and inflammatory cytokines.

Keywords: Inflammation, Ischemia-Reperfusion Injury, Mir-125b, Nanoparticles, Zinc Oxide.

Introduction

A frequent consequence of various surgical procedures is hepatic ischemia-reperfusion injury (HIRI), which can lead to morbidity and mortality, especially during liver resection and transplantation surgery (1). Reduced tissue oxygenation during prolonged HIRI results in adenosine triphosphate (ATP) depletion and a subsequent shift to anaerobic metabolism in hepatic resident cells (1). Prolonged ischemic time during transplantation is associated with early rejection, delayed graft function, and an increased risk of complications (2). Therefore, a comprehensive understanding of the molecular mechanisms underlying HIRI could help ensure successful donor liver transplantation during clinical surgery.

A cascade of physiological and biochemical changes occurs during hepatic I/R injury (3). In the ischemia stage, nutrient and oxygen deficiency and metabolic disorders induce mitochondrial dysfunction and trigger energy depletion, leading to injury and death of liver parenchymal cells. In the

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reperfusion stage, blood returns to the liver and exacerbates liver damage by triggering infiltration and activation of various immune cell and by stimulating innate immune and inflammatory molecules, such as natural killer cells, dendritic cells, Kupffer cells, Toll-like receptor 4 (TLR4), reactive oxygen species (ROS) and other cytokines (4).

A set of differentially expressed genes has been identified that mediate biochemical and physiological events triggered by HIRI (5). Recent studies in organ transplant surgery have revealed the regulatory role of microRNAs in ischemia-reperfusion injury (6). MicroRNAs (miRNAs) are a class of short non-coding RNA molecules, 21–30 nucleotides long, that are widely and endogenously expressed in living organisms (7). MiRNAs primarily function post-transcriptionally by mRNA decay and translational repression, mediated through base pairing with the 3' untranslated regions of target mRNAs (8). A novel miRNA with anti-inflammatory potential in HIRI injury diagnosis is miR-125b, which can target multiple inflammatory signals (9). The NF-κB pathway is reported to be downstream of miR-125b, although the specific mechanisms may vary in different situations (10, 11). Previous research has shown that miR-125b has protective effects against HIRI by suppressing the NF-κB signaling pathway (9).

Preventive strategies to mitigate the complications and damages caused by IR during liver surgery or liver transplantation, which can produce irreversible hepatic injury and even trigger a cascade of multiple organ dysfunction, are a concern for researchers in this field (12).

Nanotechnology presents new strategies to treat HIRI, with significant improvements in specificity and bioavailability at the site of injury (13). Zinc oxide nanoparticles (ZnO-NPs) are one of the important nanoparticles that have antioxidant and antibacterial effects (14). It is has been suggested that ZnO-NPs exert anti-inflammatory effects by suppressing the expression of pro-inflammatory cytokines (15). In addition, ZnO-NPs can increase the activity of antioxidant enzymes and reduce the concentration of malondialdehyde (MDA) (15). Despite some reports on the beneficial effects of ZnO-NPs, to the best of our knowledge, no study has examined the protective effects of ZnO-NPs against HIRI or their underlying molecular mechanisms in the inflammatory process. Therefore, the current study was designed to examine the protective effects of ZnO-NPs on inflammatory factors through changes in miR-125b expression in HIRI injury in rats.

Materials and Methods

Animals and experimental design

A total of 48 male Wistar albino rats (6 weeks old, 250 ± 30 g) were obtained from the Center for Research, Breeding, and Maintenance of Laboratory Animals, Jundishapur University of Medical Sciences, Ahvaz (Ahvaz, Iran), which adheres to specific pathogen-free (SPF) standards. Animals had free access to food and water under standard conditions, including a 12/12 h light-dark cycle with 55 ± 5% humidity at 22 ± 2 °C. Before the experiments, the rats were fasted for 18 h but had free access to water. All protocols used in this study were approved by the Animal Experimentation Principles of the Animal Welfare Act and the Institutional Animal Ethics Committee of the Shoushtar Faculty of Medical Sciences (IR.SHOUSHTAR.REC.1400.019). The rats were randomly divided into six groups (n=8) including the sham group, which received 1 ml/kg normal saline solution via the intraperitoneal route (i.p.) (Sham); the hepatic ischemia-reperfusion injury group (HIRI); the control group pretreated intraperitoneally with 5 mg/kg ZnO-NPs for 3 consecutive days without surgery (ZnO5); the control group pretreated intraperitoneally with 10 mg/kg ZnO-NPs for 3 consecutive days without surgery (ZnO10); the hepatic ischemia-reperfusion injury group pretreated with 5 mg/kg ZnO-NPs for 3 consecutive days before surgery (HIRI+ZnO5); and the hepatic ischemia-reperfusion injury group pretreated

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intraperitoneally with 10 mg/kg ZnO-NPs for 3 consecutive days before surgery (HIRI+ZnO10) (16, 17). After the experiments, all animals were euthanized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine hydrochloride (15 mg/kg) (Alfasan Co., Woerden-Holland), followed by cardiac exsanguination. Blood from each animal group was collected via cardiac puncture and allowed to coagulate at room temperature. Blood samples were centrifuged at 900 × g for 10 min, and their serum was collected and stored at -20 °C. A part of the liver tissue was immediately removed and after weighing, stored at -80 °C for biochemical analysis. Another part of the liver tissue was fixed in 10% formaldehyde for histopathological evaluation.

Preparation and administration of ZnO-NPs

The suspension of nano-ZnO (10-30 nm) (USnano, Co, USA; CAS number = 1314-13-2) was prepared by sonication in 0.9% saline for 16 minutes and injected intraperitoneally at the doses of 5 and 10 mg/kg of rat body weight (18). Control group just received only 1 ml/kg of 0.9% saline.

To reduce aggregation, the suspension was shacken for 1 minute before each injection. The size of dry powder of nano-ZnO was determined by scanning electron microscopy (SEM) (Hitachi S4160, Co, Japan) (Fig. 1).

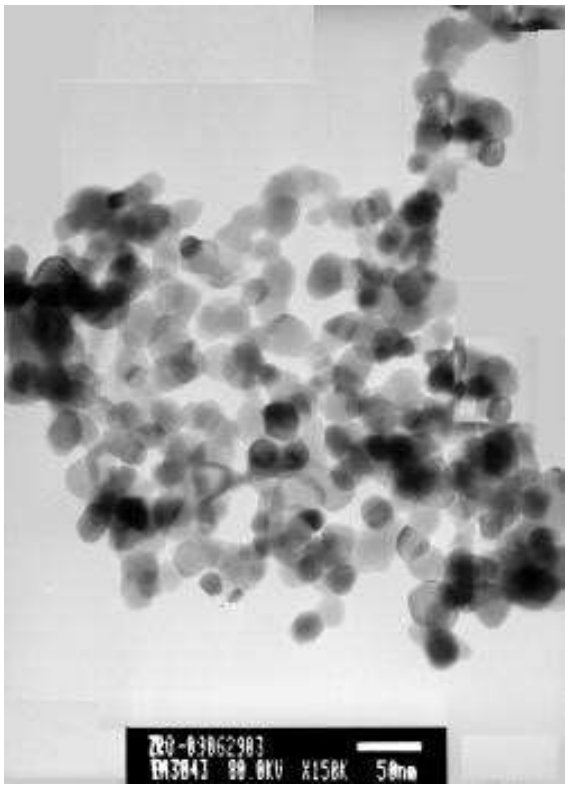

Fig. 1. Nano-ZnO image provided by the SEM (. ZnO: zinc oxide; SEM: scanning electron microscopy.

Animal HIRI model

After anesthetizing rats with ketamine (60 mg/kg) and xylazine hydrochloride (15 mg/kg) (Alfasan Co., Woerden, Holland), a 70% warm hepatic ischemia/reperfusion (1 h/6 h) model was established according to the previous studies (19, 20). After 45 min of ischemia, the clamp was removed and the abdominal muscles followed by the abdominal skin was sutured. Then the animals were reperfused for 60 min.

Biochemical detection of aminotransferase and oxidative stress markers

The serum concentrations of hepatic transaminases (ALT and AST) were measured using commercial kits (Pars Azmoon, IR, Iran) according to the

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manufacturer's instructions, with a serum autoanalyzer (BT-1500-A-A, Rome Italy) (19). Malondialdehyde (MDA) concentrations were determined using the thiobarbituric acid-reactive substances assay, as previously described (21).

Inflammatory cytokines and NF-κB levels

The liver tissue was manually homogenized in cold PBS (pH 7.5). After that, the debris was removed by centrifugation at 4000 g for 10 min at 4 °C, and supernatants were recovered. The levels of IL-6, TNF-α, and NF-κB in the supernatant samples were measured using commercial ELISA kits (Neobioscience Bioengineering Co., Shenzhen, China) following the manufacturer's protocols. The absorbance of the samples was read at 450 nm with a microplate reader (Biotek, USA) (22).

Total RNA isolation and real-time PCR

Total RNA from liver tissue was extracted using RNeasy plus Mini kit (Qiagen, Netherlands) according to the supplier's protocol. Determination of RNA concentration and quality was done using K5500 MicroSpectrophotometer. Synthesis of complementary DNA (cDNA) was performed using approximately 1 µg of total RNA with miScript II RT kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions. mRNA expression levels were evaluated by quantitative RT-PCR (qRT-PCR) analysis applying the SYBR Green QuantiTect RT-PCR kit (23). Relative mRNA/microRNA expression levels were normalized to the housekeeping small RNA, RNU6. Real-time PCR was performed under the following conditions: initial denaturation at 95 °C for 3 min, 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min). The relative fold change in expression was calculated using the ΔΔCt method.

Histopathological analysis

Fixed liver tissue was embedded in paraffin wax, serially sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E). Images were captured and analyzed using a light microscope.

Statistical analysis

Data were expressed as the mean ± SD of at least eight independent experiments. Statistical analysis was performed using SPSS version 24.0 software (SPSS Inc., Chicago, IL). Statistical significance was considered at $p<0.05$ and was calculated using a one-way ANOVA test.

Results

Hepatic transaminases and oxidative stress markers

The pattern of ALT and AST changes was similar in the experimental groups. The highest concentration of aminotransferase enzymes was observed in the HIRI group compared to other groups ($P< 0.05$). The concentration of these enzymes in the HIRI+ZnO5 and the HIRI+ZnO10 groups showed a significant decrease compared to the HIRI group ($P< 0.05$) (Figs. 2a & 2b).

The pattern of MDA and TOS changes in the experimental groups was similar. There was no significant change in MDA and TOS levels between the control and ZnO5 and ZnO10 groups ($P> 0.05$). The highest levels of MDA and TOS was found in the HIRI group, and the lowest levels of MDA and TOS was found in the HIRI+ZnO10 group ($P< 0.05$) (Fig. 2c &2d).

Cytokines and NF-κB levels

The highest levels of cytokines IL-6, TNF-α, and NF-κB were observed in the HIRI group compared to the other experimental groups ($P< 0.05$). The levels of cytokines and NF-κB showed a significant increase after treatment with ZnO5 and ZnO10 compared to the sham group ($P< 0.05$). In addition, a significant decrease in the level of IL-6, TNF-α, and NF-κB was observed in HIRI + ZnO5 and HIRI +ZnO10 compared to the HIRI group ($P< 0.05$) (Fig. 3).

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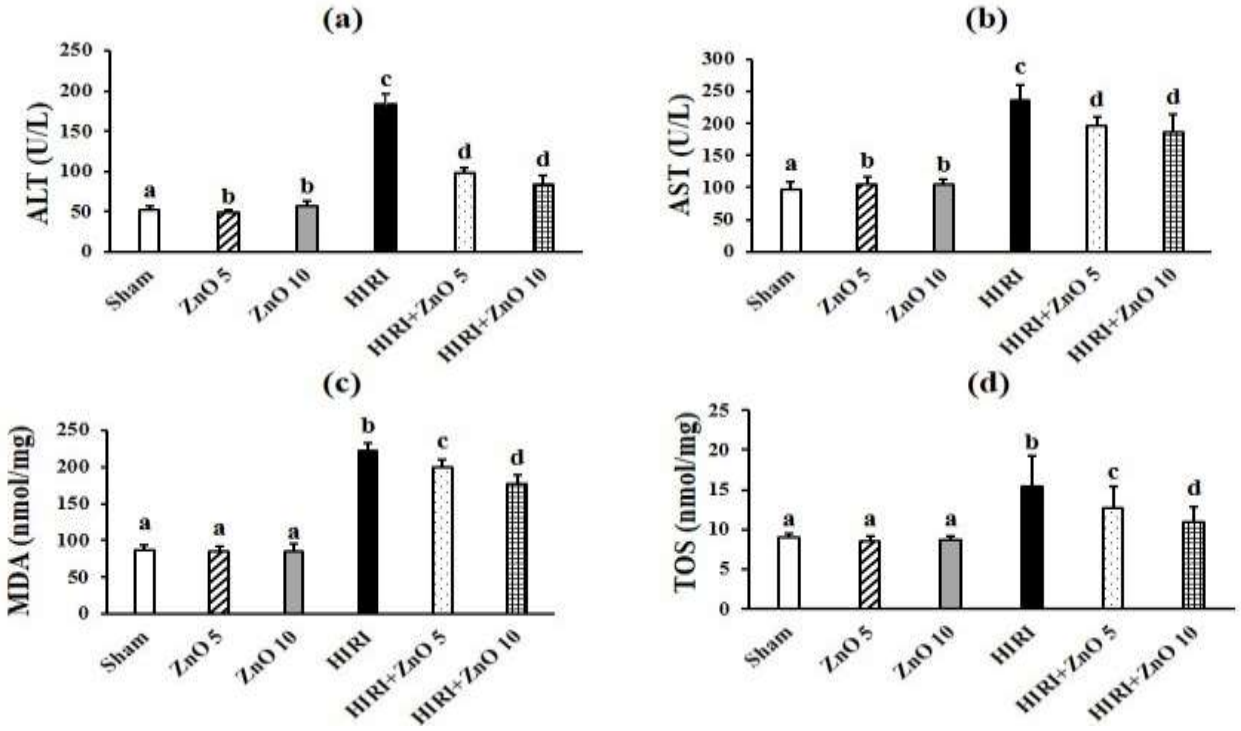

Fig. 2. Changes in the levels of alanine transaminase (ALT) (a), aspartate transaminase (AST) (b), malondialdehyde level (MDA) (c), and total oxidative stress (TOS) (d) levels in the experimental groups. Different letters in each group indicate significant differences, mean±SD, (n=8). HIRI: hepatic ischemia reperfusion injury.

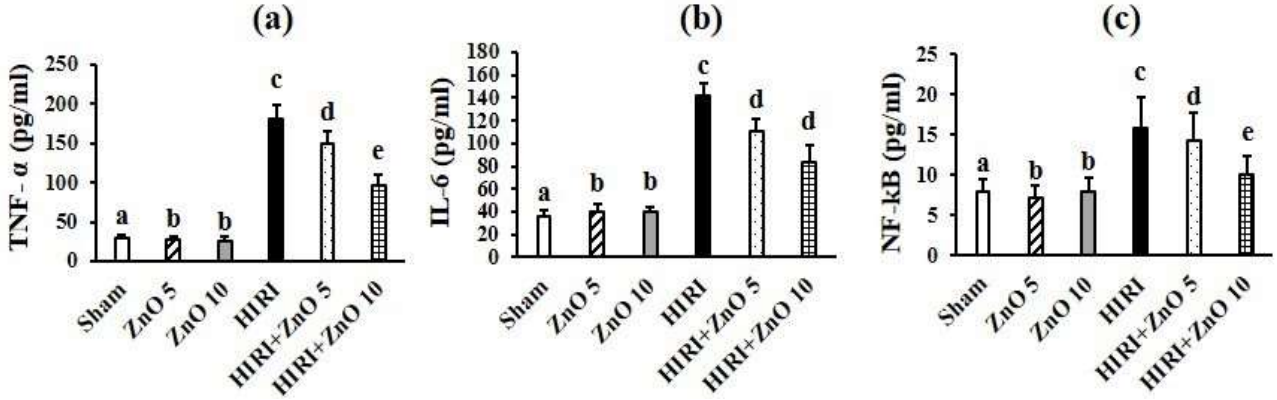

Fig. 3. Changes in the levels of IL-6 (a), TNF-α (b), and NF- κB (c), in the experimental groups. Different letters in each group indicate significant differences, mean±SD, (n=8). TNF-α: Tumor necrosis factor-α; IL-6: interleukin-6; HIRI: hepatic ischemia reperfusion injury; NF-κB: nuclear factor-kappa B.

Expression of miR-125b

Expression of miR-125b in the ZnO5 and ZnO10 groups showed a significant increase compared to the sham group. The miR-125b expression in the HIRI and HIRI + ZnO5 groups was significantly decreased compared to the control group ($P< 0.05$) (Fig. 4).

Liver Histopathology

Microscopic examination of liver tissue sections obtained from rats subjected to HIRI revealed coagulative necrosis of hepatocytes in the centrilobular and midzonal areas. There was a large area of hemorrhage, and sinusoids were focally congested with red blood cells. Hepatocytes showed loss of intercellular borders. Inflammatory infiltration and ballooning degeneration were also prominent.

In HIRI + ZnO5 group, whilst a decrease in necrosis and hemorrhage areas was observed, hepatocyte ballooning and inflammatory cell

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infiltration were still visible. The histologic injury features in this group were lower than those in HIRI group; however, these changes were still significantly more severe than in the HIRI + ZnO10 group.

As compared to HIRI group, changes observed in the HIRI+ZnO10 group were mild and limited to some reversible damage foci including cell swelling and fat accumulation. Moreover, no significant necrosis areas were detected. Pretreatment with ZnO10 considerably improved these parameters and showed better results than ZnO5. The sham group had normal histologic appearance.

Histopathological examination of the liver sections obtained from ZnO5 and ZnO10 groups also showed classical hepatic lobules (Fig. 5).

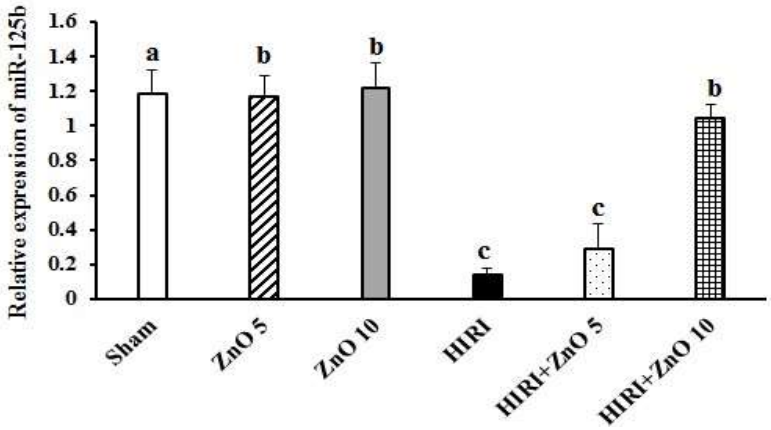

Fig. 4. Expression levels of miR-125b in different experimental groups. Different letters in each group indicate significant differences, mean±SD, (n=8). HIRI: hepatic ischemia reperfusion injury.

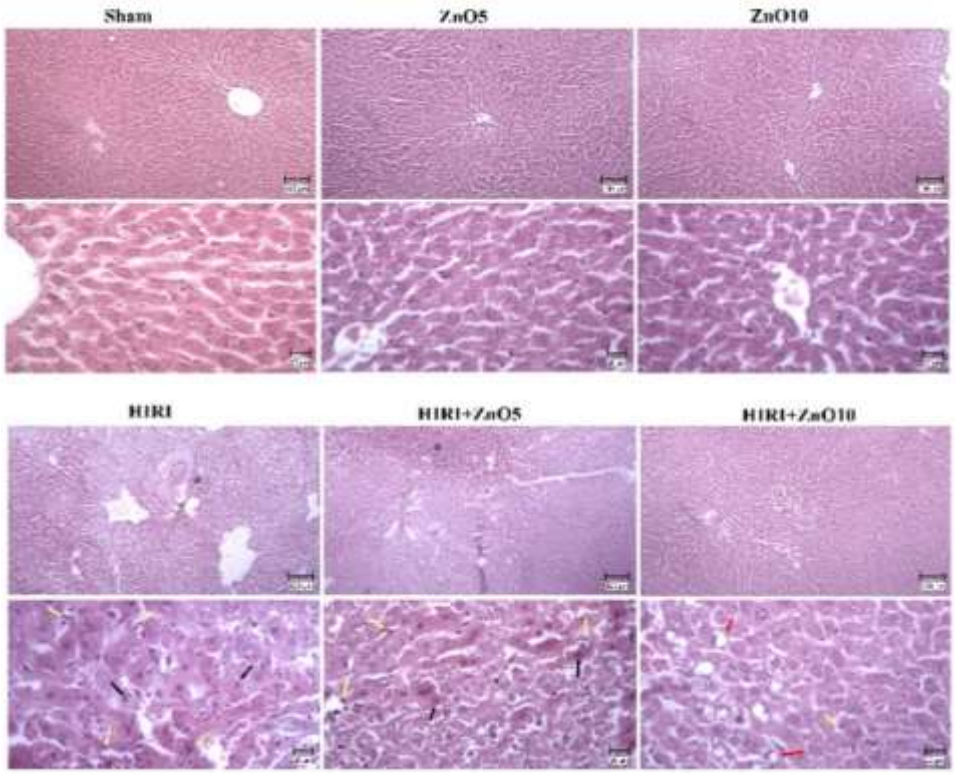

Fig. 5. Histopathology of hepatic lobules (Hematoxylin and Eosin, Bars: 100 & 20 µm). Sham group: normal histological structure. Notice hepatocytes are arranged radially *around a small central vein*. HIRI group: notice large area of coagulative necrosis and hemorrhage (black asterisk), inflammatory cell infiltration (yellow arrow) and ballooning degeneration (black arrow). HIRI+ZnO5: notice necrotic area inflammatory infiltration and hepatocytes ballooning. HIRI+ZnO10: notice the least histopathological features of injury.

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Discussion

As the main cause of liver damage after transplantation, HIRI initiates an NF- κB-mediated inflammatory cascade via phosphorylation and nuclear translocation (24).

In this study, the protective effects of ZnO-NPs in HIRI were evaluated via their effect on miR-125b expression and the suppression of inflammatory response and oxidative stress. The main findings of this study were that hepatic ischemia-reperfusion injury significantly enhanced aminotransferase enzymes, altered the redox state, and increased inflammatory response in liver tissue, significantly deteriorated liver histopathology, and downregulated the expression of miR-125b gene in the liver tissue. Moreover, administration of ZnO5 and ZnO10 in the HIRI model caused significant attenuation of aminotransferase enzymes, restored redox state, and reduced the inflammatory response, improved tissue injury, and upregulated the miR-125b gene in the liver tissue.

Previous studies reported that overactivation of immune cells such as neutrophils, during HIRI is a critical event in initiating liver damage. During reperfusion, TNF-α and IL-6 act as continuous stimulators of hepatic neutrophil infiltration and the inflammatory response (25, 26). This enhanced the inflammatory response further exacerbates oxidative stress and initiates a vicious cycle that eventually culminates in hepatocyte death and liver dysfunction (27).

Activation of the NF-κB signaling pathway is the main pathway that leads to the activation of the inflammatory response and oxidative stress in HIRI (28). Therefore, suppression of this signaling pathway has become one of the main targets for protection against HIRI (29, 30). Huang et al. reported that miR-125b could suppress the NF-κB pathway by increasing p-IκBα protein levels and reducing the proinflammatory response and aminotransferase enzymes in HIRI (9).

Some studies have confirmed that miR-125b upregulation inhibits NF-κB signaling, which prevents the release of pro-inflammatory cytokines and may protect against HIRI (31, 32). Considering that ZnO-NPs can have a stimulating effect on miR-125b expression (33), it is possible that the improvement in oxidative stress conditions, immune reactions, liver injury, and aminotransferase enzymes after ZnO-NP treatment in the HIRI model was due to increased miR-125b expression followed by the suppression of the NF-κB signaling pathway. The decrease in NF-κB protein levels and cytokine levels after ZnO-NP treatment confirmed this hypothesis.

The ZnO-NP-enhanced mechanism was remarkable, and this tremendous effect was achieved by directly entering the intracellular region, leading to the modulation of many metabolic and antioxidant enzymes and transcription factors. In contrast, ZnO-NPs have a direct anti-inflammatory effect that inhibits the expression of inflammatory cytokines, including TNF-α and IL-6, which are known to induce ROS (34). Awadalla et al. reported that ZnO-NPs exert cytoprotective effects against ischemia-induced kidney injury by upregulating antioxidant genes and downregulating inflammatory cytokines (35).

The results of this study revealed that administration of ZnO-NPs significantly attenuated hepatic ischemia-reperfusion injury in a rat model. This positive effect could be attributed to the enhancement of miR-125b expression and suppression of oxidative stress and inflammatory cytokines. Moreover, high-dose ZnO-NPs (10 mg/kg) offered a more powerful hepatoprotective effect than low-dose ZnO-NPs (5 mg/kg). Therefore, ZnO-NPs, as therapeutic agents can be considered a promising strategy in HIRI conditions. However, the use of nanoparticles in ischemia-reperfusion (IR) injury is still in its initial stages and requires further research.

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Authors' contributions
All authors read and approved the final manuscript and have participated in collection of data. FS was a major contributor in writing and editing the manuscript and has made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. SAM has made substantial contributions to conception, design and or interpretation of data, and was a major contributor in editing of the manuscript. He has studied protocol planning and has supervised the study. AR has performed the histopathological analysis of the liver, and has made contribution to interpretation of its data. MB has studied protocol planning and made contributions to analyze and interpretation of data.

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Conflicts of Interest
The authors declare no conflict of interest.

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