Research Article

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# Increased endoplasmic reticulum stress might be related to brain damage in hepatic ischemia-reperfusion injury

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**Abstract**

**Objectives:** Our study aimed to investigate the role of endoplasmic reticulum stress (ER) in brain damage following hepatic ischemia-reperfusion (HIR) injury. Specifically, we characterized the expression of markers of ER stress and histopathologic changes in the brain following HIR.
**Methods:** Twelve adults female Wistar rats were divided into two experimental groups equally. Group 1 was designed as the control group, and Group 2 was designed as the HIR group. Blood, liver, and brain tissue samples were collected during the sacrifice. The quantitative ELISA kits were used to detect glucose-regulated protein 78 (GRP-78), activating transcription factor 4 (ATF-4), eukaryotic initiation factor 2 alpha (EIF2-A), caspase-3, caspase-9, and CCAAT/enhancer-binding protein (CEBP) in plasma. Histopathological examination was performed for liver and brain tissues.
**Results:** Higher levels of GRP-78 (p=0.006), ATF4 (p=0.001), and EIF2-A (p=0.007) were detected in group 2. More damage was detected in liver and brain samples in the histopathological examination of group 2 than in group 1.
**Conclusions:** Our results demonstrate that ER stress is involved in developing brain damage following hepatic ischemia-reperfusion injury, as evidenced by increased expression of markers of ER stress and neuronal injury.

**Keywords:** ATF-4; brain damage; ER stress; GRP-78; hepatic ischemia-reperfusion

## Introduction

The liver is central in regulating factors such as fat metabolism, blood glucose levels, and the synthesis of coagulation factors. Therefore, maintaining liver function is crucial for vital functions, including immunity, digestion, and detoxification [1, 2]. Proper vascular part is essential in performing a complex and diverse process in the body. The liver is sensitive to circulatory abnormalities like ischemia due to its complex vascular formation and high metabolic activity. Hepatic ischemia-reperfusion (HIR) injury can be defined as cellular damage and multiple organ dysfunction following the restoration of blood flow [3].

HIR injury can be seen in conditions such as liver transplantation, severe traumatic injuries, and hypovolemic shock. Additionally, liver circulation can be temporarily blocked instantly to reduce postoperative complications during surgery [4]. In an earlier study, HIR injury has been associated with damage to the kidney, lungs, and heart [5]. However, more comprehensive studies about how HIR affects the brain should be understood. Although free oxygen radicals, Kupffer cellular activation, cytokine release, leukocyte migration and activation, adenosine triphosphate (ATP) depletion, sinusoidal endothelial cell damage, irregularities in microcirculation, and activation of the coagulation

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system have been postulated to be associated with the pathogenesis of HIR injury, the exact molecular mechanism is still not widely understood [6–9].

The endoplasmic reticulum (ER) is a vital organelle that performs various functions required for proper cellular activity and survival. Disruptions in the ER's normal processes trigger the unfolded protein response (UPR), an evolutionarily conserved cell stress response [10]. If ER malfunction is severe or persists, these modifications result in cell death [11]. ER, stress has been linked to several parameters, including glucose-regulated protein 78 (GRP-78), activating transcription factor 4 (ATF-4), Eukaryotic initiation factor 2 alpha (EIF2-A), caspase-3, caspase-9, and CCAAT/enhancer-binding protein (CEBP). Changes in biochemical parameters related to ER stress have been consistently reported in different experimental ischemia-reperfusion models, including HIR injury models [12–14]. However, the relationship between ER stress and brain damage in experimental HIR injury models remains unclear.

Biochemical markers are used to identify the presence of endoplasmic reticulum (ER) stress, which occurs when unfolded or misfolded proteins accumulate in the ER. These markers include chaperones such as BiP and Grp94 and enzymes like IRE1, PERK, and ATF6. The activation of these markers triggers unfolded protein response (UPR), which aims to restore protein homeostasis in the ER. However, if the UPR fails to resolve the ER stress, it can lead to cellular dysfunction and apoptosis. Therefore, monitoring biochemical markers of ER stress can provide insight into the underlying mechanisms and potential therapeutic targets for related diseases [15–17].

This study aimed to research ER stress in HIR injuries and how it affects the brain. Therefore, blood levels of GRP-78, ATF-4, EIF2-A, caspase-3, caspase-9, and CEBP have been assessed in adult Wistar rat HIR models. This study may improve our understanding of how HIR injury affects the brain.

# Materials and methods

### Animal exposure

Before the experiments, 12 female Wistar albino rats (11–12 weeks old, weighing 200–224 g) were acclimatized for one week. Individual cages were used for the animals, kept at a constant room temperature (24 °C) with 12/12 h light/dark cycles, and fed standard rat chow. During the 12 h preceding the experiments, only water was provided. The Sivas Cumhuriyet University Local Ethics Committee for Animal Experiments consented (Decision No. 65202830-050.04.04-642/26.04.2022). The institution's ethical guidelines carried out all procedures.

### Hepatic ischemia-reperfusion model

Twelve adults female Wistar rats were divided equally into two experimental groups to compare the effects of HIR on blood levels of ER stress parameters and histopathologic changes in liver and brain tissues. Group 1 was the control group (only laparotomy), while Group 2 was the HIR group (performed 30 min of ischemia and 120 min of reperfusion procedure). The experimental ischemia-reperfusion model in rats developed by Koc et al. was used by us [18]. 60 mg/kg of sodium pentobarbital (Penbital, Bioveta, IvanovicenaHané, Czechia) was administered intraperitoneally to rats to cause anesthesia. Throughout the operations, the animals breathed on their own. Povidone iodine was used to shave and prepare the midabdominal region. Only a laparotomy was conducted and closed in Group 1 (n=6) afterward. In Group 2 (n=6), a laparotomy was done, and a vascular clamp occluded the hepatic pedicle for 30 min. Reperfusion was then induced for 120 min after the clamp was released. With a median abdominal incision, a laparotomy was carried out. The intestinal loops were lateralized and protected with a wet gauze sponge to make it simple to reach the hepatoduodenal ligament. After that, the liver hilum was easily accessible by lifting the right-left and middle lobes upward. Bulldog micro clamps were applied for 30 min, obliterating the hepatic artery, portal vein, and biliary tract on the hepatoduodenal ligament. After 30 min, the clamps were removed to allow for hepatic reperfusion.

### End of experiment

All rats were given intramuscular injections of 15 mg/kg xylazine (Rompun®, Bayer, Türkiye) and 90 mg/kg ketamine hydrochloride (Ketalar®, Pfizer Türkiye) to induce anesthesia. For euthanasia, sodium pentobarbital (Penbital, Bioveta, IvanovicenaHané, Çekya) was subsequently given intraperitoneally at 10 mg/100 g body weight. Liver tissue was taken out right away after euthanasia. Rats' skulls were also gently opened, and the cerebral cortex was removed. Before the rats were euthanized, blood samples were taken via heart puncture from the sedated animals. Blood samples were collected in lavender top tubes (Greiner). Centrifugation separated the plasma fractions (2,349 g, 10 min, 4 °C). They were quickly aliquoted and kept at −80 °C after that (Wise Cryo, Seoul, South Korea).

### Determination of GRP-78, ATF-4, EIF2-A, caspase-3, caspase-9 and CEBP concentrations

To measure biomarker concentrations in plasma, quantitative ELISA kits (BT LAB, Shanghai Korain Biotech, Shanghai, China) were applied. Thermo Scientific MULTISKAN FC microplate reader was used to measure the absorbance at 450 nm, which was used to determine the concentrations. The tests were conducted by the manufacturer's instructions in the user manual. The detection ranges of the GRP-78, ATF-4, EIF2-A, caspase-3, caspase-9, and CEBP were 0.45 ng/mL–30 ng/

mL, 0.16–10 ng/mL, 7–1,500 ng/L, 0.05–20 ng/mL, 0.05–20 ng/mL, 156–10.000 ng/L, respectively. The intra-assay and inter-assay coefficient of variation values of the GRP-78, ATF-4, EIF2-A, caspase-3, caspase-9, and CEBP were <8 % and <10 %, respectively.

### Histopathologic examination

After ischemia-reperfusion, brain and liver tissues were removed and preserved in 10 % neutral buffered formalin (Merck KGaA, Darmstadt, Germany). Following fixation, the tissues were maintained in hot paraffin by being passed through increasing concentrations of ethyl alcohol (Merck KGaA, Darmstadt, Germany) (70 %, 80 %, 96 %, 100 %), followed by xylol (Merck KGaA, Darmstadt, Germany), as is done for standard tissue follow-up analysis under a light microscope. The liver and brain tissues, whose tissue follow-up procedure was finished, were embedded in microscope slides and cut into 4–5 µm sections using a microtome (Leica RM2255; Leica Microsystems GmbH, Wetzlar, Germany) before being stained with the common hematoxylin-eosin (H&E) method and examined under a light microscope (Olympus BX51, Japan). The variations in the liver tissue between the groups were seen and recorded during the light microscopic analysis of the liver tissues. All samples' histological alterations in the frontal brain area, brain hippocampus region, and plexus choroideus tissues were scored semi-quantitatively using a scale from 0 to 3. Hemorrhage, congestion, necrosis, edema, neuronal loss, and inflammation were six different histopathological factors assessed and graded: Negligible is 0; mild is 1; moderate is 2; and common is 3. Each brain sample's histopathology score was calculated by averaging the results of the six factors. Scoring was performed according to Gülmez et al. [18].

### Statistical analyses

Data gathered for the study was examined using GraphPad Prism 7.01 for Windows (GraphPad Software, La Jolla, CA, USA). The Shapiro-Wilks normality test was used to assess the data's normality. The mean and standard deviation (SD) represent numerical variables. The levels of GRP-78, ATF-4, EIF2-A, caspase-3, caspase-9, and CEBP were compared between groups using the t-test. The Mann-Whitney U test was used to compare the histopathologic score differences between the groups. Statistical significance was defined as $p<0.05$.

**Table 1:** Comparison of laboratory parameters related with endoplasmic reticulum stress between groups.

| Variables | Control (n=6) | HIR group (n=6) | p-Value |
|---|---|---|---|
| GRP-78, ng/mL | 8.76 ± 1.37 | 12.2 ± 2.07 | 0.006 |
| ATF4, ng/mL | 5.60 ± 0.64 | 7.64 ± 0.9 | 0.001 |
| EIF2 alpha, ng/L | 76.7 ± 31.4 | 142 ± 35.8 | 0.007 |
| Caspase-3, ng/mL | 2.46 ± 0.50 | 2.96 ± 1.03 | 0.306 |
| Caspase-9, ng/mL | 2.24 ± 0.45 | 2.61 ± 0.42 | 0.173 |
| CEBP, ng/L | 368 ± 93 | 476 ± 76 | 0.052 |

ATF4, activating transcription factor 4; GRP-78, glucose regulated protein 78; EIF2 alpha, eukaryotic initiation factor 2; CEBP, CCAAT/enhancer-binding proteins. Differences between groups were compared by using the Student's t-test. $p<0.05$ was statistically significant.

## Results

In the biochemical evaluation, the HIR group had significantly higher GRP-78, ATF4, and EIF2-A than the control group. Caspase-3 and caspase-9 levels did not differ statistically significantly between the groups Table 1 and Figure 1.

In the histopathologic examination, significant ischemic damage with hepatocyte loss (short arrow) and sinusoid enlargements (arrow) in the pericentral and midzonal areas (B, B1, B2) was determined in the experimental group liver tissue compared to the control group. In the liver tissue of the experimental group, neutrophils were observed in the middle zonal region. In the control group, non-ischemic liver slices show normal histology. Hepatic lateral sections show normal histology (Figure 2).

Hemorrhage, congestion, necrosis, edema, neuronal death, and inflammation significantly varied over the experimental and control groups' brain tissues. In the experimental groups after ischemia, shrunken neurons (arrowhead), spaces with neuronal loss (dashed arrowhead), increased perivascular areas (star) with neutrophil infiltration (arrow), and damaged ependymal cells (red arrowhead) due to ischemia are observed Figure 3 and Table 2.

## Discussions

In the present study, a rat model of HIR injury was performed to examine brain and liver histopathological findings and the changes in some biomarkers related to ER stress. This study showed higher levels of GRP-78, ATF4, and EIF2-A in the HIR group compared to the control group. We also found ischemic damage and a reduction in hepatocyte density in the pericentral and mid-zonal regions of the liver in the HIR group. In addition, we detected shrinking neurons, cavities after neuron loss, and perivascular and ependymal cell damage in brain tissues in the HIR group.

During the HIR process, various functional changes increase hepatic cellular destruction [19]. Suakıtıcı et al. showed that HIR caused some harmful histopathological changes in the livers of rats. The liver tissue necrosis, vacuolization, and congestion scores were significantly higher in the experimental group than in the control group [20]. Crockett et al. detected that the HIR group had sinusoidal congestion, cytoplasmic necrosis, neutrophil infiltration, and increased alanine transaminase (ALT) levels in rats [21]. In their rat experimental research, Serracino-Inglott et al. discovered polymorphonuclear leucocyte (PMNL) infiltration, hepatocyte necrosis, sinusoidal enlargement, and an elevation in alanine transaminase (ALT) and

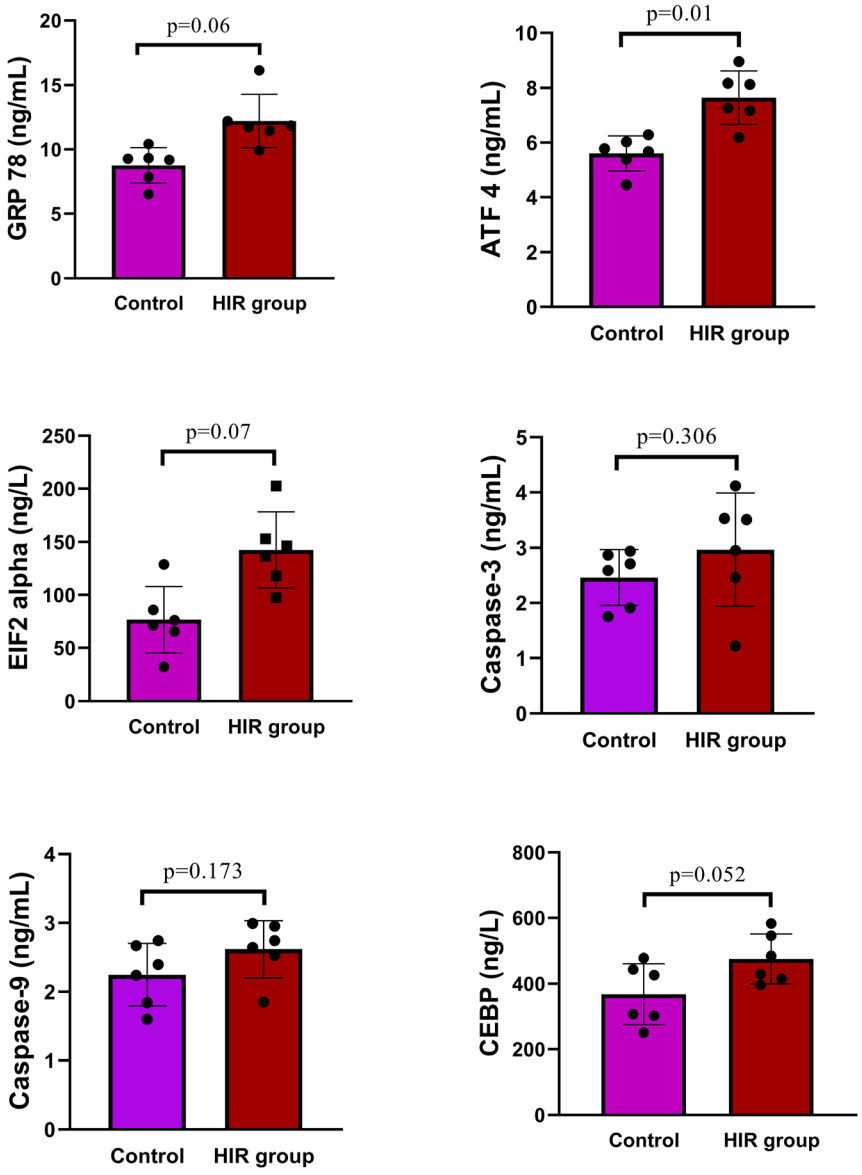


**Figure 1:** Box plot of glucose-regulated protein 78 (GRP-78), activating transcription factor 4 (ATF-4), eukaryotic initiation factor 2 alpha (EIF2-A), caspase-3, caspase-9, and CCAAT/enhancer-binding protein (CEBP) concentrations.

aspartate transaminase (AST) levels [22]. Our study found that the liver's histopathological examination in the HIR group demonstrated ischemic damage in the pericentral and mid-zonal regions. In the histopathological examination of brain tissues after the HIR process, we found shrinking neurons, cavities after neuron loss, and perivascular and ependymal cell damage. These histopathological changes show that our experimental HIR model was performed successfully.

ER, stress is a cellular response to various conditions, including ischemia-reperfusion injury in the liver and brain. During ischemia, cells are deprived of oxygen and nutrients, accumulating unfolded or misfolded proteins in the ER. Upon reperfusion, a sudden influx of oxygen and nutrients can further exacerbate ER stress, triggering a cascade of events that can ultimately lead to cell death [23]. A study by Zhang et al. showed that HIR caused ER stress, leading to oxidative stress-related cell death [24].

We also detected a higher expression of biomarkers related to ER stress in our study. Previously seen that ER stress associated with HIR injury has a detrimental effect on the rat's brain in an experimental model [23]. It's well known that ER stress can lead to cognitive impairment and neuronal cell death in animal models [24]. Yu et al. found that HIR led to ER stress in the hippocampus, a brain region important for learning and memory that inhibiting ER stress with a pharmacological chaperone improved cognitive function [25]. In their experimental rat HIR model, Zhu et al. showed that HIR impairs the blood-brain barrier by inhibiting brain microvascular endothelial cell proliferation [4]. Additionally, Tan

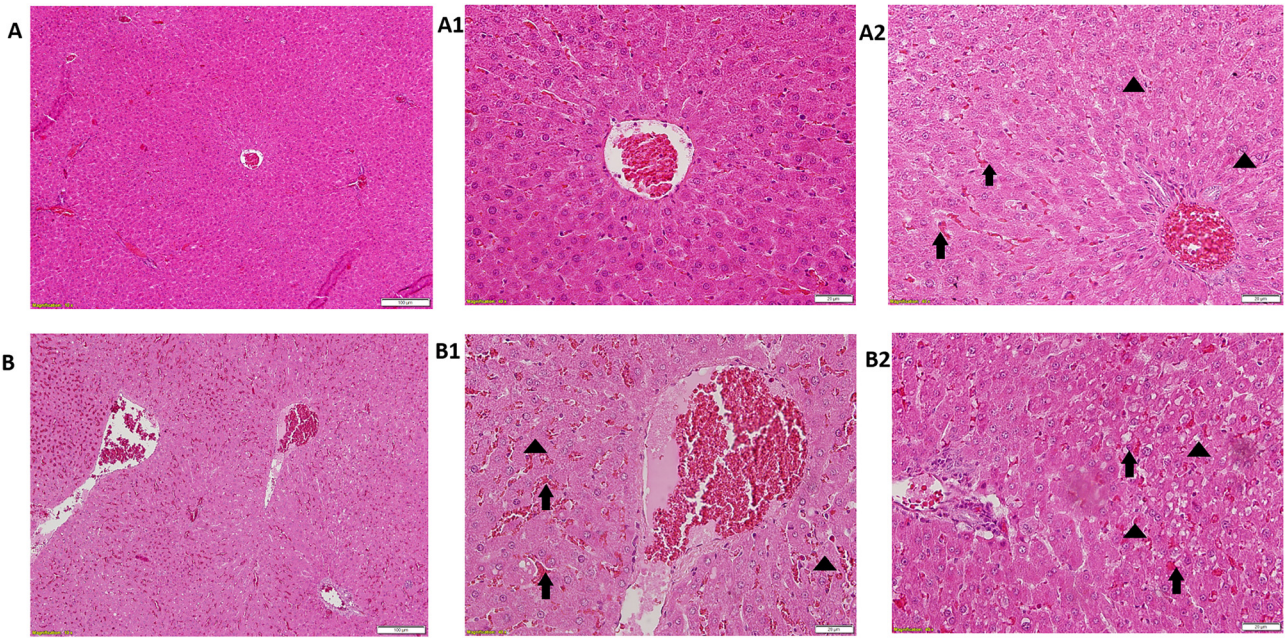


**Figure 2:** Liver histopathology in established experimental (B, B1, B2) and control groups (A, A1, A2). Liver tissues were stained with H&E. A, A1, and A2 correspond to liver tissue sections of the control group, and B, B1, and B2 to ischemic liver tissue sections. Enlarged sinusoidal areas (arrow) and hepatocytes with vacuolized cytoplasm (arrowhead) in the ischemic liver compared to control groups are striking (A and B images were acquired with 10× and A1, A2, B1, and B2 images with 40× objective lenses).

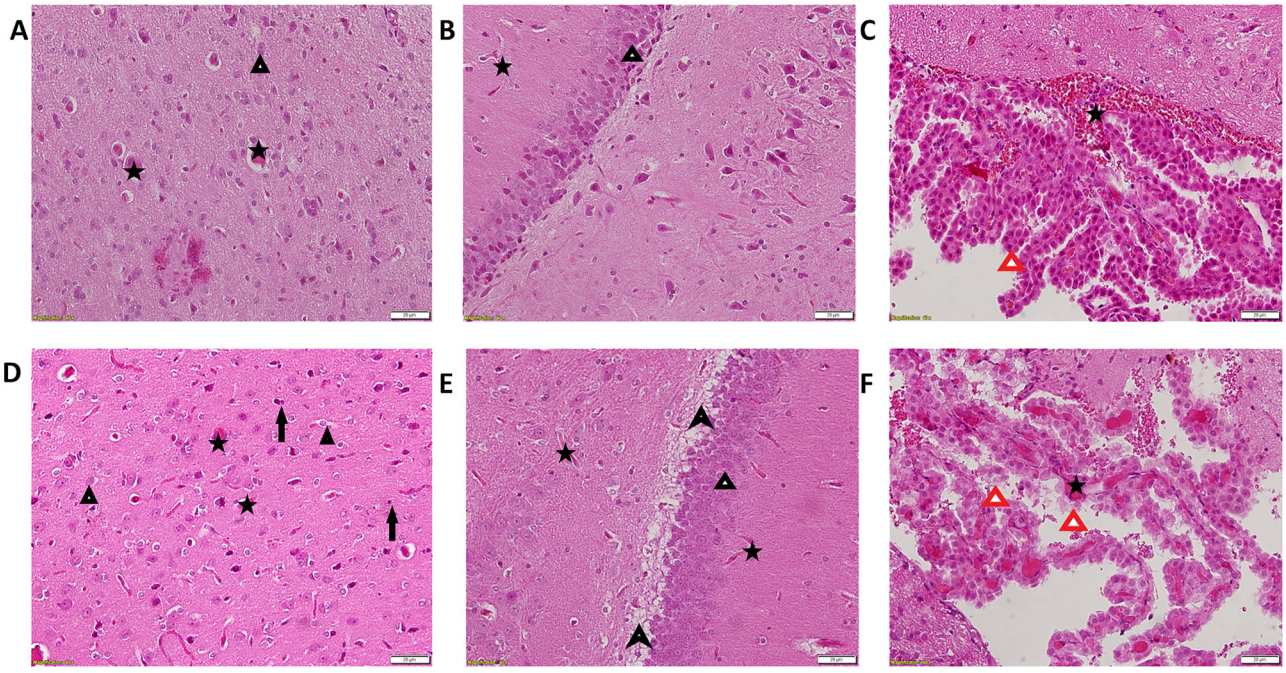


**Figure 3:** Light microscopic evaluation of the frontal brain (A, D), hippocampus (B, E), and plexus choroideus (C, F) regions after ischemia with H&E staining. Control group normal-appearing neurons with brain tissue (A), hippocampus (B), plexus choroideus (C) with ischemia-damaged brain tissue (D), hippocampus (E), and plexus choroideus (F) with shrunken neurons with nuclear shrinkage (arrowhead), extinction of neurons spaces (dashed arrowhead), increased perivascular distances (star) with neutrophil infiltration (arrow) and standard and damaged ependymal cells (red arrowhead) and enlarged blood vessels (star) due to ischemia (magnification ×40).

**Table 2:** Comparisons between groups in terms of histopathologic scores of brain damage.

| Parameters | Control | HIR | p-Value |
|---|---|---|---|
| Hemorrhage | 0,1,0,0,0,1 | 1,2,2,3,2,2 | 0.004 |
| Congestion | 0,0,0,0,0,0 | 3,2,2,2,3,3 | 0.002 |
| Necrosis | 0,0,0,0,1,0 | 1,2,2,1,2,2 | 0.004 |
| Edema | 0,1,1,0,0,0 | 2,1,1,1,2,2 | 0.015 |
| Neuronal loss | 0,0,0,0,1,0 | 2,2,3,3,2,2 | 0.002 |
| Inflammation | 0,0,1,0,0,0 | 2,2,3,2,2,2 | 0.002 |

Negligible: 0; mild: 1; moderate: 2; common: 3.

et al. reported that ER stress significantly contributes to post-traumatic brain injury [26]. These studies suggest ER stress is vital to brain damage following HIR. Accordingly, with the literature, we think that the pathogenesis of brain damage after HIR may be related to increased ER stress markers.

In the current study, GRP-78 levels were statistically significantly higher in the HIR group compared with the control group. The effect of increasing levels of GRP-78 was investigated previously. This chaperone protein plays a vital role in the UPR on the brain, kidney, and retinal damage following ischemia-reperfusion procedures [27]. It also indicated that one of the most crucial cellular defense mechanisms to preserve homeostasis is the increase of GRP-78 in ER stress [28]. Some inactive proteins are bound to the GRP-78 complex in the normal physiological state. When ER homeostasis is impaired, GRP-78 dissociates from the complex and releases other proteins such as PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE-1) [29, 30]. According to Ouyang et al., overexpressing GRP-78 protects primary astrocytes against ischemia injury in-vitro and maintains mitochondrial function [31]. Zhang et al. discovered in their investigations that GRP-78 overexpression after ER stress triggered autophagy and shielded the neural cells from ischemia-reperfusion injury [32]. We believe the increase of GRP-78 is a defense mechanism that attempts to prevent cellular damage related to ER stress following HIR. However, further research is needed to determine the optimal method for increasing GRP-78 levels and to evaluate the safety and efficacy of this approach; these findings suggest that increasing GRP-78 levels may be a potential therapeutic strategy for improving brain damage following HIR.

In this study, we detected a significant increase in EIF2-A levels in the HIR group compared to the control group. PERK is a type I transmembrane protein member of the EIF2-A kinase family [33]. To promote cell survival, the PERK signaling pathway is essential for preventing the uncontrolled accumulation of unfolded proteins in the ER [34]. Tian et al. reported that, in their mice and cell culture models, phosphorylation of EIF2-A mitigates ER stress and hepatocyte necroptosis in acute liver injury [35]. Wang et al. detected that the lack of PERK-induced phosphorylation of EIF2-A and subsequent inhibition of translation is an essential factor leading to neurological damage in cerebral ischemia [36]. Accordingly, elevated EIF2-A levels may protect the neuronal cells against the detrimental effects of ER stress-related to HIR.

Our study detected significantly increased ATF-4 levels in the HIR group. ATF-4 regulates the expression of many genes that allow cells to survive against stressors such as hypoxia or amino acid deficiency. Under chronic stress, ATF-4 promotes the induction of apoptosis. ATF4 also activates CCAAT-enhancer-binding protein (CEBP), which is required for ER-stress-mediated apoptosis [37, 38]. Rao et al. reported that inhibition of the ATF4-CEBP pathway in mouse hepatocytes is critical in low-dose lipopolysaccharide preconditioning to protect against HIR injury, preventing apoptosis cascades [39]. In this study, we think cell deaths in the brain and the liver may develop with the increase in ATF-4 and CEBP due to the development of ER stress after HIR.

This study detected no differences between groups in caspase-3 and caspase-9 levels. Discordant results have been reported regarding the caspase-3 and caspase-9 levels in experimental HIR models of rats [39–42]. HIR is a complex process involving multiple pathways and mechanisms, including oxidative stress and inflammation, resulting in cell death. Different cell death pathways, such as necrosis, apoptosis, and necroptosis, have been associated with HIR. It is well-known that caspases are an essential modulator of the apoptosis process. Accordingly, other cell death pathways are more prominent than apoptosis in HIR injury [42].

Several limitations of this study should be noted. First, the findings from this study may not generalize to other animal species or human populations. Second, the sample size was relatively small, which may limit the statistical power and generalizability of the results. Third, the ER biomarkers did not evaluate in the tissue samples. Fourth, the study was conducted in a laboratory setting, and the results may need to reflect the complexity and variability of real-world conditions.

## Conclusions

Higher levels of ER stress-related biomarkers were determined after HIR. Additionally, this procedure caused histopathological changes in the different parts of the liver and brain. We hypothesized that reducing ER stress may protect the brain after an HIR injury. This study could provide theoretical and experimental guidance for investigating novel HIR-induced brain damage treatment. Future research with

larger sample sizes, different doses, treatment durations, and more ecologically valid study designs must confirm and extend these findings.

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