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Authors: Abdulmutalip Karaaslanlı, Mehmet Cudi Tuncer, Fırat Aşır, Tuğcan Korak

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Silymarin protected the cerebral tissue from endoplasmic reticulum stress

Abdulmutalip Karaaslanlı[1], Mehmet Cudi Tuncer[2], Fırat Aşır[3], Tuğcan Korak[4]

[1]*Van Yüzüncü Yıl University, Department of Brain and Neurosurgery, Van, Turkey*

[2]*Dicle University, Faculty of Medicine, Department of Anatomy, Diyarbakir, Turkey*

[3]*Department of Histology and Embryology, Medical Faculty, Dicle University, Diyarbakır, Turkey*

[4]*Department of Medical Biology, Medical Faculty, Kocaeli University, Kocaeli, Turkey*

Address for correspondence: Mehmet Cudi Tuncer, Professor, Ph.D., Chief of Anatomy Department, Dicle University, Medical School, Diyarbakır, Turkey; tel. +90 412 2488001 Ext. 4539 (Faculty room), fax: +90 412 2488440, mobile phone: +90 532 2744926, e-mail: drcudi@hotmail.com

ABSTRACT

Background: Our aim is to explore silymarin's protective effects against endoplasmic reticulum (ER) stress via protein kinase R-like endoplasmic reticulum kinase (PERK) modulation and elucidate potential enriched pathways through in silico analysis of silymarin-associated PERK protein interactors in cerebral ischaemia-reperfusion (IR) injury.

Materials and methods: 30 rats were categorized into three groups: sham, IR and IR + silymarin groups. Cerebral IR damage was not induced. Only the MCA was identified and clamped without further intervention. Sham group received only physiological serum intravenously. IR group, rats were exposed to 2 hours ischaemia and following 3 hours reperfusion. In IR + silymarin group received 1 μg/kg silymarin intravenously (*i.v.*) before inducing cerebral IR. Cerebral tissues were processed for histological tissue preparation. Hematoxylin-Eosin and PERK immunostaining was applied. In Cytoscape software, we imported and integrated the silymarin and PERK protein-protein interaction networks generated

from the STITCH and STRING databases, respectively. Subsequently, Reactome pathway annotation was performed for this intersected network.

Results: In the sham group, neurons were large and round with oval nuclei, and no histopathological changes were observed. In the IR group, neurons and neuroglial cells showed degeneration with pyknotic nuclei, apoptotic bodies, dilated and congested cerebral capillaries, and numerous vacuoles. After silymarin treatment, the IR + silymarin group showed a restoration of normal histology, with more regular neural and neuroglial cells and decreased vessel dilation and congestion. PERK immunoexpression was mainly negative in the sham group, increased in the IR group, and decreased again in the IR + silymarin group. Upon intersecting the interactors of silymarin and PERK, 17 common proteins were identified. Reactome pathway analysis revealed potential impacts of these proteins on key pathways including immune and cytokine signaling, apoptosis, estrogen signaling, and extracellular matrix degradation.

Conclusions: Silymarin's targeting of PERK offers a promising approach to alleviate ER stress and potentially modulate multiple critical pathways in cerebral ischaemia reperfusion, serving as a comprehensive therapeutic strategy for managing cerebral IR injury.

Keywords: cerebral ischaemia reperfusion, ER stress, PERK, reactome pathway, Silymarin

INTRODUCTION

Ischaemia refers to the condition characterized by reduced or absent blood flow to tissues. The lack of oxygen resulting from ischaemia directly causes tissue damage. The failure of oxygen to serve as the final acceptor in the electron transport chain decreases ATP production, which then causes disruptions in membrane transport, acidosis, edema, and degeneration of cell membranes and organelles [6, 16]. Reperfusion after ischaemia, which is the process of restoring blood flow to ischemic tissues, is essential to counteract oxygen deficiency. However, the increased blood flow to tissues during reperfusion results in the accumulation of inflammatory cells, excessive oxygen and calcium overload, and an increase in reactive oxygen species, leading to what is known as ischaemia-reperfusion injury (IRI). Following IRI, a cytokine storm can trigger a systemic inflammatory response, potentially causing multiple organ damage [34, 40]. IRI can occur in various clinical scenarios, including vascular occlusion, myocardial infarction, thrombolytic therapy, orthopedic surgeries, hemorrhagic shock, cardiopulmonary bypass, revascularization, and organ transplantation [4, 22].

Key clinical factors influencing IRI damage include the duration and severity of ischaemia and the rate of reperfusion [27]. Numerous treatment strategies have been investigated in both experimental and clinical studies for IRI, including anti-inflammatory drugs (dexamethasone, prednisone, and tacrolimus), broad-spectrum serine protease inhibitors (aprotinin), Na+/H+ exchange inhibitors (cariporide), anti-apoptotic agents (Bax inhibitor-1), anti-ischemic compounds (trimetazidine), and antioxidants (SOD, CAT, N-acetyl cysteine, vitamins E and D, melatonin) [17, 20]. Despite these various studies, IRI remains an unresolved issue in different clinical settings. Research indicates that the severity of damage correlates with the tissue exposure level to antioxidants. Given that the antioxidant content in tissues decreases due to high levels of ROS during IRI, administering antioxidant agents is viewed as a viable strategy to mitigate IRI [19].

Silymarin is recognized as a potent free radical scavenger with anti-inflammatory and anti-cancer properties [30]. Additionally, various studies have demonstrated that silymarin is effective against a range of biological (bacterial toxins and mycotoxins) and chemical (pesticides, metals, fluoride, cardiotoxic, and hepatotoxic) toxins. In recent years, over 400 articles have been published on the beneficial effects of silymarin and its components [9, 18]. The endoplasmic reticulum (ER) is a crucial cellular organelle responsible for the proper folding and sorting of proteins. Conditions such as glucose deprivation, depletion of calcium stores in the ER, exposure to free radicals, and the accumulation of unfolded/misfolded proteins induce cellular stress and disrupt normal ER functions [5, 28]. In response, the cell initiates the unfolded protein response to manage this stress. However, the molecular mechanisms through which ER stress leads to neuronal survival or death remain unclear. It is especially unclear which effectors predominantly trigger neuronal cell death in conditions like IR injury [5, 14].

Protein kinase R-like endoplasmic reticulum kinase (PERK) is a protein that induce ER stress in the cell [24]. Cerebral ischaemia leads to oxygen and glucose deprivation, disrupting protein folding in the ER, which causes the accumulation of misfolded or unfolded proteins, triggering ER stress. Upon reperfusion, the sudden return of oxygen can exacerbate ER stress, leading to neuronal damage, apoptosis, and further injury. To cope with ER stress, cells activate the unfolded protein response (UPR), a protective mechanism that helps restore ER homeostasis. If the stress is severe or prolonged, the UPR can induce apoptosis. PERK is one of the three main sensors in the UPR. Under ER stress, PERK is activated and phosphorylates eIF2α (eukaryotic

initiation factor 2 alpha), which reduces general protein synthesis to relieve the protein-folding load on the ER. However, prolonged PERK activation can also promote pro-apoptotic pathways, such as the induction of the transcription factor CHOP (C/EBP homologous protein), which leads to cell death [7, 21, 33].

Silymarin has shown the ability to mitigate ER stress in several experimental models. During cerebral ischaemia-reperfusion, silymarin may help reduce the burden on the ER by enhancing protein folding and clearance mechanisms, thus reducing the overall ER stress response. This reduction in ER stress may help suppress excessive activation of the PERK pathway, thereby preventing downstream apoptotic signaling. This modulation of the PERK pathway helps tilt the balance of the UPR towards survival rather than apoptosis, which is essential for protecting neurons during cerebral IRI. In addition, Silymarin's strong antioxidant properties help to reduce the oxidative stress that contributes to ER dysfunction, indirectly affecting the PERK pathway. Its anti-inflammatory action, which inhibits the release of pro-inflammatory cytokines, also reduces the secondary damage caused by ischaemia-reperfusion, limiting the need for a robust ER stress response. For this reason, silymarin exerts a neuroprotective effect in cerebral ischaemia-reperfusion injury by reducing ER stress and modulating the PERK pathway. Its antioxidant and anti-inflammatory properties help decrease the activation of the PERK-eIF2α-CHOP axis, preventing the shift from protective UPR to apoptotic signaling. In this way, silymarin aids in preserving neuronal integrity and function after ischaemic injury, making it a promising compound in the context of brain protection against ischaemia-reperfusion damage [1, 11, 13, 21, 22].

The aim of our research is to role of silymarin treatment on ischaemia-reperfusion injury (IRI) via in silico and immunoexpression analysis.

MATERIALS AND METHODS

Experimental design

The ethical permission was obtained from Local Ethics Committee of Animal Experiment, Dicle University (approval date: 30.01.2024, approval no: 2023/39). In this study, 30 female Wistar albino rats were used. The experimental animals were divided into three groups of 10 rats each. They were fed with unlimited access to food and water, and maintained in a controlled environment with a 12-hour light/dark cycle (from 8:00 AM to 8:00 PM) at a

temperature of 23 ± 2°C. Silymarin (CAS#65666-07-1, Merck, Germany) was obtained commercially for use in the experiments.

Prior to surgery, general anesthesia was administered to the rats via intramuscular injection of 90 mg/kg ketamine hydrochloride (Ketalar; Pfizer, Istanbul, Turkey) and 10 mg/kg xylazine (Rompun; Bayer, Istanbul, Turkey). The rats were positioned supine on the operating table, and their neck area was disinfected with povidone-iodine. A midline incision was made from the upper edge of the sternum to the hyoid bone using surgical scissors. The incision area was expanded with a tissue retractor, and the trachea was exposed by blunt dissection. The paratracheal muscles were dissected to access the middle cerebral artery (MCA), which was isolated from surrounding tissues. MCA occlusion was achieved by placing a micro bulldog clamp, inducing cerebral ischaemia for 2 hours. After this period, the clamp was removed, tissues were returned to their original anatomical positions, and the skin and subcutaneous fascia were sutured. Reperfusion of cerebral tissues was carried out for 3 hours.

Experimental groups

Sham group: Administered 1 cc of physiological serum intravenously (*i.v.*) before the experiment. Cerebral IR damage was not induced. Only the MCA was identified and clamped without further intervention.

IR group: Administered 1 cc of physiological serum intravenously (*i.v.*) before the experiment. Cerebral IR procedure was conducted (2 hours ischaemia, 3 hours reperfusion).

IR + Silymarin group: Administered 10 μg/kg silymarin intravenously (*i.v.*) before inducing cerebral IR [15]. After administering silymarin, 2 hours of ischaemia and 3 hours of reperfusion were applied.

Histopathological examination

The rats were euthanized by under general anesthesia and sacrificed. Cerebral tissues were fixed in formaldehyde solution, dehydrated in a graded alcohol series (80%, 90%, 96% ethanol), and then embedded in paraffin blocks. To identify histopathological changes in brain tissue, cerebral tissue sections were cut from paraffin blocks, dewaxed and stained with Hematoxylin-Eosin. Sections were cleared in xylene and mounted and examined under a Zeiss Imager Axio A2 photomicroscope.

Immunostaining

Immunohistochemistry with PERK (catalog no: ab79483, Abcam, Cambridge, MA, USA, dilution ratio: 1/100) was performed according to the biotin-streptavidin peroxidase complex method described by Ermiş et al. [8]. Initially, tissue samples were dewaxed in xylene and rehydrated in ethyl alcohol. Endogenous peroxidase activity was blocked by treating the samples with 3% H_2O_2 (catalog no: TA-015-HP, ThermoFischer, Waltham, MA, USA) for 20 minutes. Antigen retrieval was conducted using citrate buffer (pH 6.0) for 10 minutes at 90°C. To block nonspecific proteins, a blocking solution (catalog no: TA-015-UB, ThermoFischer, Waltham, MA, USA) was applied for 8 minutes. Subsequently, the tissue sections were incubated overnight with the diluted primary antibodies, followed by immersion in 1x phosphate-buffered saline (PBS). The sections were then incubated with a biotinylated secondary antibody (catalog no: TP-015-BN, ThermoFischer, Waltham, MA, USA) for 15 minutes, immersed again in 1× PBS, and incubated with streptavidin peroxidase (catalog no: TS-015-HR, ThermoFischer, Waltham, MA, USA) for another 15 minutes. The sections were then treated with a chromogen solution (diaminobenzidine, catalog no: TA-001-HCX, ThermoFischer, Waltham, MA, USA), stained with Gill III hematoxylin for 1 minute. Sections were cleared in xylene and mounted and examined under a Zeiss Imager Axio A2 photomicroscope.

Electron microscopy examination

Lead acetate-uranyl citrate staining was applied to the cerebral sections for electron microscopy. The sections were examined using a Jeol JEM-1010 transmission electron microscope at Dicle University Science and Technology Application and Research Center. Cytopathological changes were photographed using a side-mounted TEM CCD camera (Gatan ES500W Erlangshen).

Silymarin-correlated PERK interactors and reactome pathway analysis

To explore the potential PERK-associated mechanisms through which silymarin might exert its effects in cerebral ischaemia-reperfusion beyond ER stress, a functional annotation analysis was conducted. The silymarin target protein-protein interaction (PPI) network was constructed in Cytoscape (https://cytoscape.org/, version 3.10.2) using the Search Tool for

Interactions of Chemicals (STITCH) database with a medium confidence level (0.400) and the maximum number of interactors. The PERK (UniProtKB/Swiss-Prot ID: Q9NZJ5) PPI network was created using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string-db.org) with medium confidence and up to 200 interactions. The PERK PPI network was then imported into Cytoscape and intersected with the silymarin network. Functional annotations were retrieved for this intersected network, followed by Reactome pathway analysis. A false discovery rate (FDR) < 0.05 was used as the threshold for statistical significance. The top ten Reactome pathways, ranked by increasing FDR values, were visualized.

RESULTS

Histochemical findings

Sections of cerebral cortex were stained with Hematoxylin and Eosin staining and shown in Figure 1. In sham group, neurons were large, round with oval nuclei. Neuroglial cells were smaller. Capillaries were regular. Pyramidal cells were prominent in sections. No histopathology was observed in sham group (Fig. 1A). In IR group, neurons and neuroglial cells were degenerated with pyknotic nuclei. Apoptotic bodies were observed in neurons. Cerebral capillaries were dilated and congested. Numerous vacuoles were observed in cerebral cortex (Fig. 1B). after silymarin treatment, adverse effects of IR were histologically restored in IR + silymarin group. neural and neuroglial cells were more regular and histologically normal. Dilation and congestion of vessels were decreased compared to TB group (Fig. 1C).

Immunohistochemical findings

Sections of cerebral cortex were stained to illustrate PERK immunoexpression to show ER stress in groups (Fig. 2). In sham group, PERK immunoreactivity was mainly negative in neurons, neuroglial cells and in vascular endothelial cells (Fig. 2A). Compared to sham group, PERK expression was increased in IR group. neurons and neuroglial cells in cerebral cortex showed positive PERK expression. The expression was evident in endothelial cells of vessels (Fig. 2B). In IR + silymarin group, PERK expression was decreased after silymarin treatment compared to IR group. PERK immunoexpression was mainly negative in neurons and neuroglial cells of cerebral cortex (Fig. 2C).

Ultrastructural findings

Electron micrographs of cerebral cortex were illustrated in Figure (Fig. 3). In sham group, neurons were regular with membrane integrity, homogenous nucleus content (Fig. 3A). Compared to sham group, IR group showed shrunken, dark and fragmented nuclei, loss of membrane integrity, cytosolic disruptions, smaller cell bodies and apoptotic bodies (Fig. 3B). In IR + silymarin group, the nucleus and membrane of neuron appeared to be normal. no fragmented nuclei and disrupted cytosolic structures were observed. Apoptotic bodies were decreased (Fig. 3C).

The reactome pathway annotation of Silymarin-associated PERK interactors

Upon intersecting the interactors of silymarin and PERK, 17 common proteins were identified. These proteins include Catenin Beta 1 (CTNNB1), Cyclin D1 (CCND1), Fibronectin 1 (FN1), SHC Adaptor Protein 1 (SHC1), Tumor Protein P53 (TP53), Poly(ADP-Ribose) Polymerase 1 (PARP1), Caspase 8 (CASP8), Cadherin 1 (CDH1), Matrix Metallopeptidase 2 (MMP2), Prostaglandin-Endoperoxide Synthase 2 (PTGS2), Cyclin Dependent Kinase Inhibitor 1B (CDKN1B), CDKN1A, CASP9, Hypoxia Inducible Factor 1 Subunit Alpha (HIF1A), CCNB1, MMP9, and CASP3. Reactome pathway analysis performed on these proteins yielded the following top ten pathway annotations and their respective FDR values: signaling by interleukins (FDR: 2.71E-11), cytokine signaling in immune system (FDR: 2.78E-11), interleukin-4 (IL4) and interleukin-13 (IL13) signaling (FDR: 2.78E-11), immune system (FDR: 7.2E-11), signal transduction (FDR: 2.26E-9), apoptosis (FDR: 2.2E-6), extra-nuclear estrogen signaling (FDR: 2.2E-6), disease (FDR: 4.46E-6), apoptotic cleavage of cellular proteins (FDR: 9.47E-6), and degradation of the extracellular matrix (ECM) (FDR: 2.24E-5) (Fig 4).

DISCUSSION

The histopathological findings of this study showed that after treatment with silymarin, the IR + silymarin group exhibited a marked histological improvement. The neurons and neuroglial cells appeared more regular and histologically normal, with reduced dilation and

congestion of the blood vessels compared to the IR group. This suggests that silymarin treatment mitigated the adverse histological effects of IR.

Cerebral IR injury disrupts the neuronal health due to generation of reactive oxygen species, mitochondrial dysfunction, inflammation and tissue damage [2]. Guo et al. [12] found that cerebral IR increased expression of ER stress markers such as CHOP and GRP78 proteins. Additionally, they stated that neuronal regeneration and death was increased in cell culture. In an experimental cerebral IR rat model, neuronal apoptosis, hypoxic changes, area of cerebral infarction, cell death were increased in brain tissues following cerebral IR injury [25]. Gelen et al. [10] conducted acute cerebral IR of rats and investigated ER stress. The authors pointed out that oxidative stress, serum inflammation, intracellular edema, and neuronal degeneration was prominent in cerebral tissues after IR. Moreover, CHOP expression (an ER stress marker) decreased in IR group, indicating high ER stress level. In a study of cerebral IR on mice, cerebral infarction size, cerebral water content, neuronal apoptosis rate, neuronal loss, neuronal nucleus shrinkage, cell swelling, and edema were increased in cerebral tissues of mice following IR injury. The authors also indicated that

Ischaemia-reperfusion group showed high expression of endoplasmic reticulum stress markers GRP78/Bip and Caspase 12 compared to sham group [32]. Zille et al. [39] showed that cerebral stroke caused neuronal injuries with dark shrunken, half-moon nuclei, many vacuoles and cytosolic small bodies at ultrastructural level. A similar study showed that cerebral ischaemia caused neuronal shrinkage, atypical neurons, cytoplasmic edema and paucity of organelles [31]. Consistent with previous study, we observed adverse pathological changes in cerebral sections of IR group compared to sham group including pyknotic neurons and neuroglial cells, apoptotic bodies in neurons, at microscopic and ultrastructural analysis of cerebral images. However, silymarin improved the histopathology caused by cerebral IR after silymarin treatment, indicating neuroprotective effects of silymarin against cerebral IR injury.

To assess ER stress in cerebral IR, we analyzed the immunoexpression of PERK. Our findings of PERK immunoexpression in this study showed that in sham group, PERK immunoreactivity was predominantly negative cerebral cortex cells, indicating minimal ER stress while in the IR group, there was a notable increase in PERK expression, signifying elevated ER stress. After silymarin treatment in the IR + silymarin group, the PERK immunoexpression was mainly negative in the neurons and neuroglial cells of the cerebral

cortex. This protective effect of silymarin is consistent with its known antioxidative and anti-inflammatory properties, which may help in stabilizing cellular functions and promoting recovery following cerebral IR injury.

Following the immunohistochemical validation that silymarin targets PERK to reduce ER stress and improve outcomes in cerebral ischaemia reperfusion, we conducted an in-silico analysis to identify potential pathways affected by this targeting. The results suggest that silymarin's influence on PERK may potentially impact immune and cytokine signaling, apoptosis, extra-nuclear estrogen signaling, and extracellular matrix degradation pathways. The significance of immune and interleukin (IL) signaling in cerebral ischaemia/stroke has been emphasized in numerous studies [35, 38]. For instance, IL-4 has been shown to play a role in M2 polarization and facilitate the long-term recovery of microglia following ischemic stroke. Mice deficient in IL-4 show increased M1 polarization of microglia and macrophages, experience larger infarct sizes, and suffer from more pronounced functional impairments following cerebral ischaemia. Conversely, administration of recombinant IL-4 can alleviate these adverse outcomes [23]. After a stroke, IL-13 influences microglia and infiltrating macrophages in the brain, managing the shift in their polarization from an anti-inflammatory to a pro-inflammatory state [3]. Other critical ILs, such as IL-10, are crucial in reducing ischemic damage by promoting anti-inflammatory responses, angiogenesis, neurogenesis, and the activation, proliferation, and regulation of immune cells [38]. During cerebral IR injury, neuronal cell shrinkage can result in membrane blebbing and chromatin condensation, leading to apoptosis, one of the key cell death mechanisms in IR. Along with apoptosis, it has been emphasized that the simultaneous regulation of other cell death mechanisms, such as autophagy, necrosis, and ferroptosis, is suggested to be an effective therapeutic strategy for preventing cerebral IR [37]. Moreover, the importance of ECM degradation and estrogen in cerebral IR have been highlighted in several studies [26, 36]. These findings suggest that, in addition to mitigating ER stress, the influence of silymarin on PERK could modulate these critical pathways and potentially provide a multifaceted therapeutic approach for cerebral IR injury.

While our study demonstrates the neuroprotective effects of silymarin in mitigating ER stress, several limitations should be acknowledged. First, the study was conducted in an experimental model, which may not fully capture the complexities of ER stress in human cerebral tissue. Further research using human clinical data is necessary to validate the

translational relevance of these findings. Second, the exact molecular mechanisms through which silymarin exerts its protective effects on ER stress were not fully elucidated, leaving room for future studies to explore these pathways in greater detail. Finally, our study focused on acute ER stress, and the long-term effects of silymarin on chronic ER stress and neurodegenerative processes remain to be investigated.

CONCLUSIONS

Silymarin's targeting of PERK not only mitigates ER stress but also potentially influences critical pathways including immune signaling, apoptosis, and extracellular matrix degradation in cerebral ischaemia reperfusion. Therefore, silymarin may present a comprehensive therapeutic strategy for managing cerebral IR injury.

ARTICLE INFORMATION AND DECLARATIONS

Data availability statement

All data and analyses related to the study can be obtained from the corresponding author.

Ethics statement

The ethical permission was obtained from Local Ethics Committee of Animal Experiment, Dicle University (approval date: 30.01.2024, approval no: 2023/39).

Author contributions

AK, MCT and FA participated in all stages of the experimental procedure of the article. Histopathological evaluations of the study were performed by MCT and FA. Genetic analysis of the study was performed by TK. All authors participated in writing the article and shaping its final version.

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Conflict of interest

None declared.

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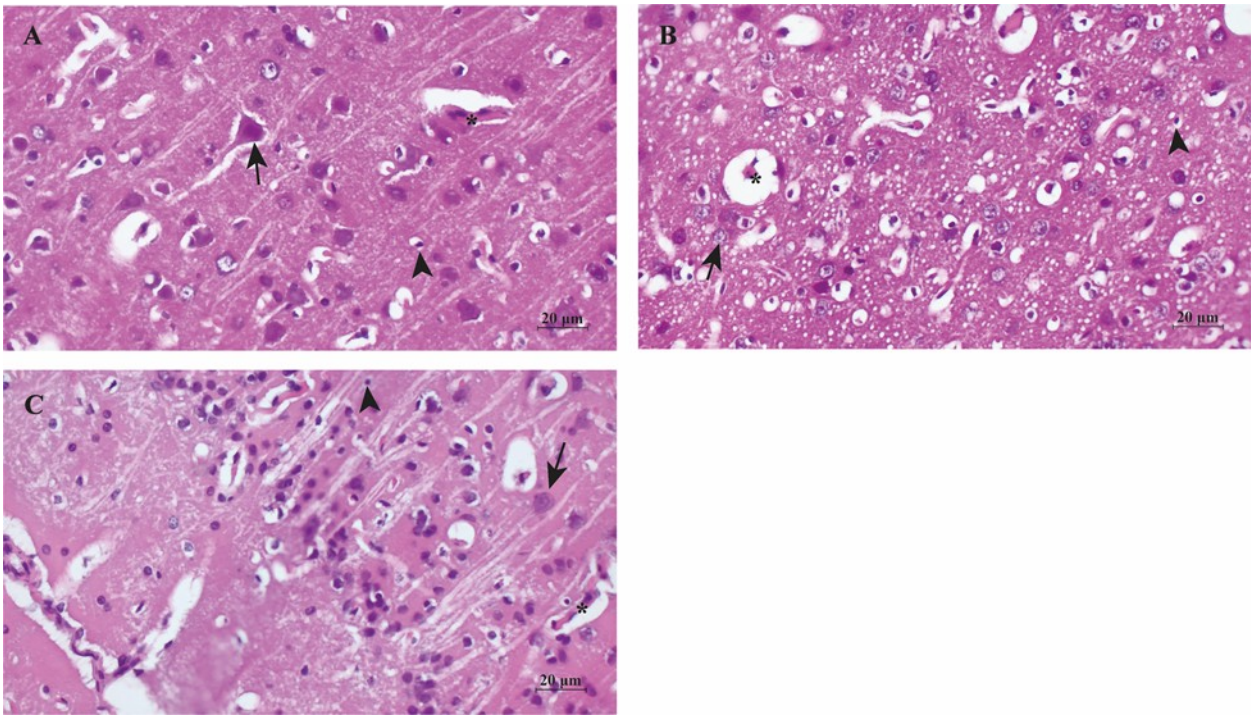

Figure 1. Cross sections of cerebral cortex in groups; **A.** Sham group; **B.** IR group; **C.** IR + Silymarin group; arrow: neurons, arrowhead: neuroglia, asterisk: capillary, haematoxylin and eosin staining, scale bar: 20 μm, magnification: 40×. IR — ischaemia-reperfusion injury.

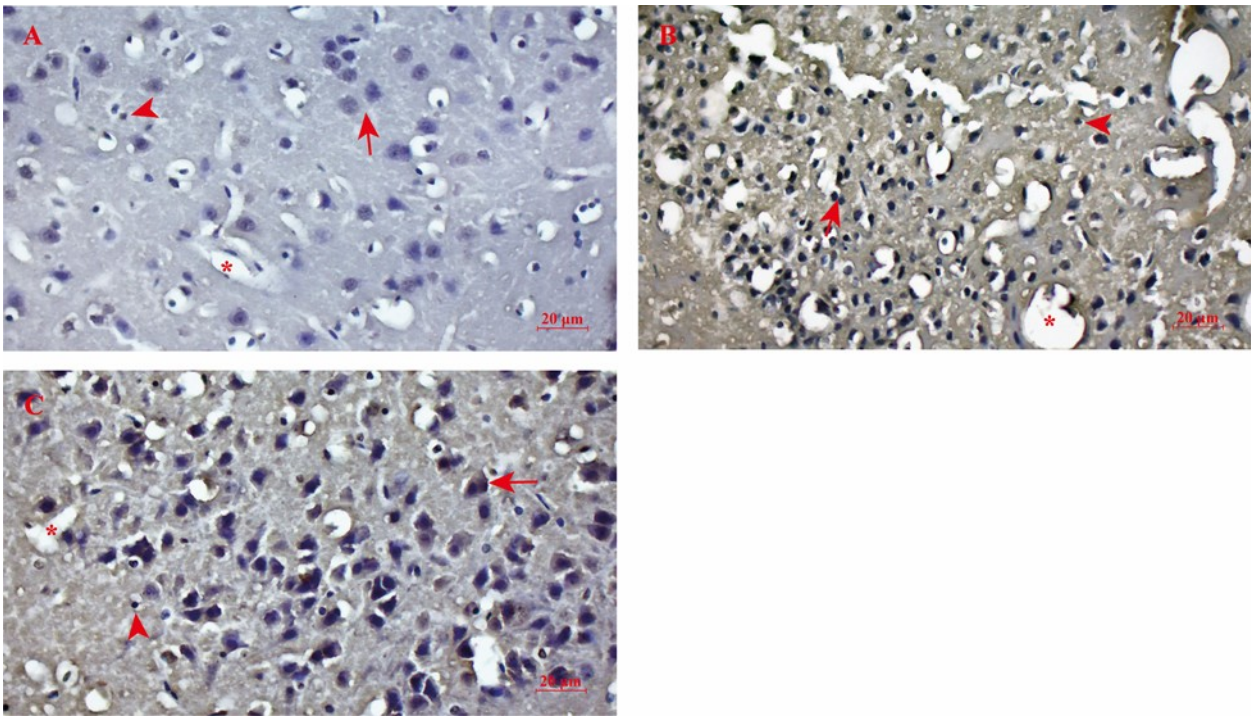

Figure 2. PERK immunoactivity in cross sections of cerebral cortex in groups; **A.** Sham group; **B.** IR group; **C.** IR + silymarin group; arrow: neurons, arrowhead: neuroglia, asterisk: capillary, PERK immune staining, scale bar: 20 μm, magnification: 40×. IR — ischaemia-reperfusion injury; PERK — protein kinase R-like endoplasmic reticulum kinase.

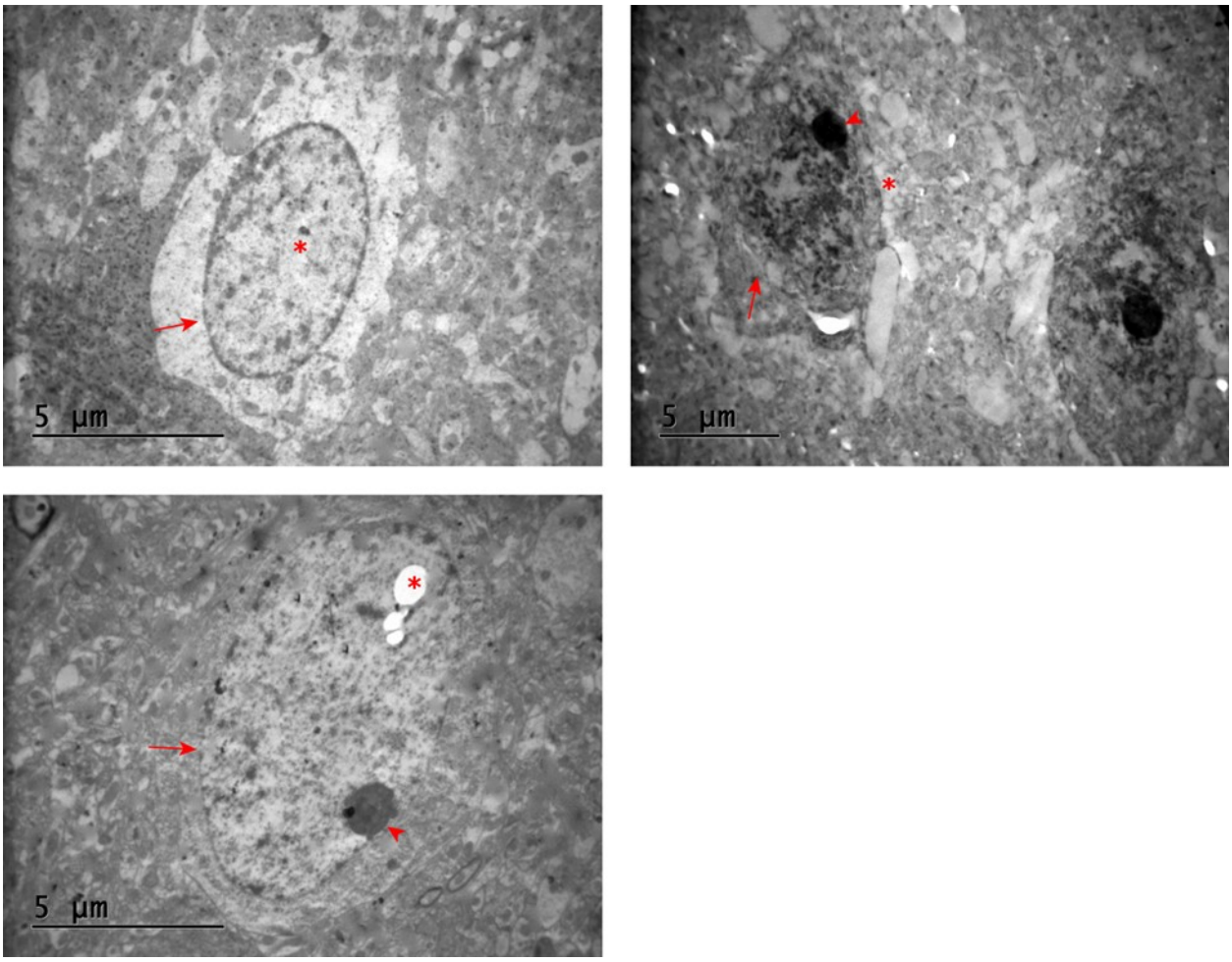

Figure 3. Transmission electron microscopy of neurons in **A.** Sham group; **B.** IR group; **C.** IR + silymarin group; arrow: membrane, arrowhead: apoptotic bodies, asterisk: small bodies, scale bar: 5 μm. IR — ischaemia-reperfusion injury.

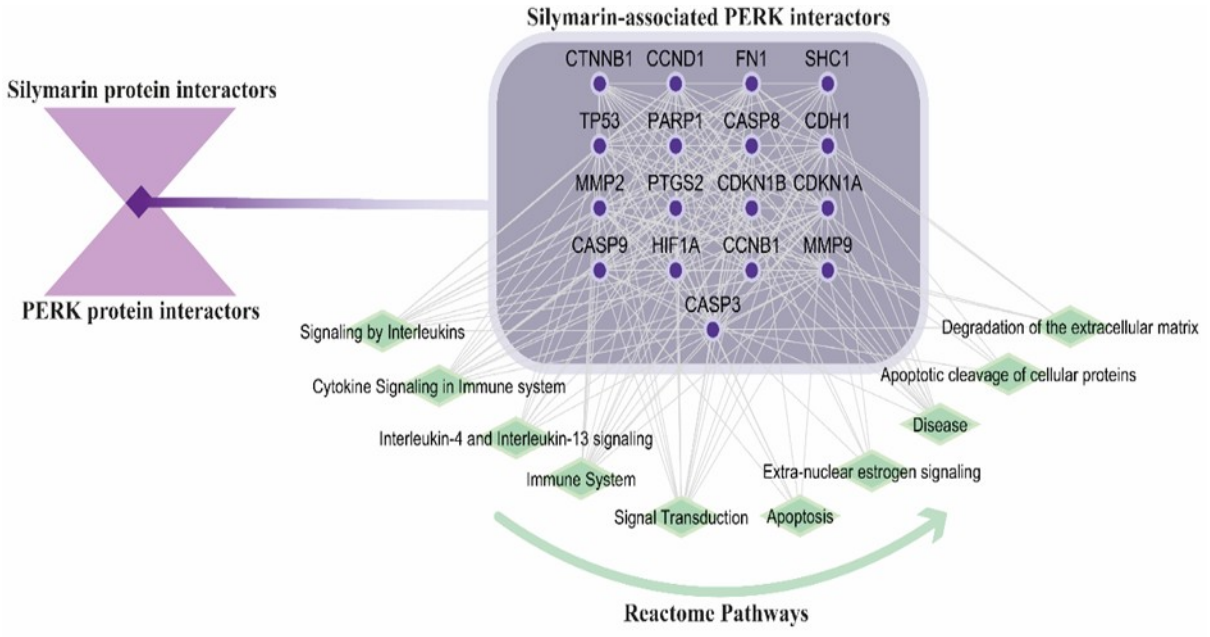

Figure 4. Top reactome pathways enriched in silymarin and perk intersecting proteins. The 17 purple nodes represent the common protein interactors of silymarin and PERK, while the green nodes represent the associated Reactome pathways. The Reactome pathways are ordered from left to right according to increasing FDR values. FDR — false discovery rate; PERK — protein kinase R-like endoplasmic reticulum kinase.