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Does Glp-2 have a protective effect on cerebral ischemia/reperfusion model?

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Background/aim: To investigate the neuroprotective effects of glucagon-like peptide 2 (Glp-2), which increases cerebral blood flow, on the hippocampal complex after cerebral ischemia/reperfusion (I/R) injury in rats.

Materials and methods: Animals were randomized into 4 groups: sham, I/R + 0.9% NaCl, I/R + pre-Glp-2, and I/R + post-Glp-2. Cerebral ischemia was performed via the occlusion of the bilateral internal carotid artery for 40 min and continued with a reperfusion process. At the end of 6 h of reperfusion, animals were decapitated in all groups and brain tissues were removed. Malondialdehyde (MDA) and natural intracellular antioxidant glutathione (GSH) levels and myeloperoxidase (MPO) activities were measured in the left hippocampal tissue. The right hippocampal tissues of all group members were taken for histopathologic study.

Results: MDA levels and MPO activities increased from Group I to Group II and decreased from Group II to Groups III and IV. On the other hand, GSH levels were not significantly different among the groups. The number of apoptotic hippocampal tissue cells increased from Group I to Group II and decreased from Group II to Groups III and IV.

Conclusion: Our preliminary study revealed that Glp-2 treatment may decrease oxidative damage from I/R in cerebral tissue.

Key words: Reperfusion injury, glucagon-like peptide 2, neuroprotective effect, rats

1. Introduction

Ischemia is a pathological process causing a reversible or irreversible cell/tissue injury that develops depending upon the deficiency in circulation of blood to perfuse an organ or tissue (1). Toxic free oxygen radicals are produced in the tissue exposed to ischemia. Although the main target after ischemia is to promote reperfusion and healing of tissue, it is well known that reperfusion triggers more tissue damage. Free oxygen radicals and superoxide radicals cause endothelial damage, increased microvascular permeability, and tissue edema during reperfusion (2–4). As a result, cell membrane permeability decreases and, depending on the ischemia/reperfusion (I/R) time, apoptosis and necrosis develop (5).

Glucagon-like peptide 2 (Glp-2) is a single-chained polypeptide produced from enteroendocrine L cells that are distributed particularly densely in the terminal ileum and colon in the gastrointestinal system. A known major effect of Glp-2 is intestinal growth stimulation. It also increases villus weight and mucosal epithelium thickness. Finally, Glp-2 increases hexose transportation and inhibits apoptosis by decreasing intestinal motility and permeability. Externally administered Glp-2 has many effects on the intestinal system, such as increasing intestinal blood flow (6–9) and intestinal barrier function (10,11). In other studies, Glp-2 has also been found to have an antiinflammatory effect (12,13). Glp-2 presents its effects with Glp-2 receptors. It is shown that these receptors are found mostly in the jejunum, duodenum, ileum, stomach, and colon and also in the hypothalamus (14).

In recent studies, it was proven that Glp-2 causes proliferation of small intestine mucosa and that it has protective and therapeutic effects on intestine and heart I/R. During I/R damage, Glp-2 improves nutrient absorption, increases mucosal DNA content, increases villus height and crypt depth, decreases bacterial

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translocation, and decreases production of reactive oxygen species (15). Experimental studies in rodents have shown that Glp-2 receptors are found in the extrahypothalamic region, which is the center of learning and memory, but their effect on brain I/R is unknown. The purpose of this study was to investigate the neuroprotective effect of Glp-2 on oxidative stress and apoptotic cell death in rat neural tissue subjected to I/R damage.

2. Materials and methods

The study was performed after the consent of the Marmara University Animal Experiments Ethical Committee was obtained. Thirty-two male Sprague Dawley rats weighing 250–280 g each were included in the study. Animals were randomized into 4 groups: Group I (sham, n = 7), Group II (I/R + 0.9% NaCl, n = 9), Group III (I/R + pre-Glp-2, n = 8), and Group IV (I/R + post-Glp-2, n = 8). Rats were fed with a standard diet in a heat- and light-controlled environment.

Anesthesia of all the rats in the study was performed with urethane (1.25 g/kg, intraperitoneally (IP)). As a surgical procedure, isolation of carotid arteries following tracheostomy was performed in Group I (sham), but clamping was not performed. Carotid arteries in both directions were isolated following a tracheostomy and clamped for 40 min in Groups II, III, and IV. Six hours of reperfusion followed ischemia. In Group II, 0.9% NaCl was administered IP at a rate of 0.3/h for each rat 10 min before ischemia. In Group III, Glp-2 was administered IP at a dose of 5 µg/0.3 mL for each rat 10 min before ischemia. In the treatment following ischemia, a single dose of 5 µg/0.3 mL for each rat was administered IP with the start of reperfusion (Group IV). At the end of 6 h of reperfusion, all animals were decapitated and brain tissues were removed. Tissues were kept at –80 °C until biochemical analysis.

In the second part of the research, the tissues were evaluated histopathologically (apoptosis) for myeloperoxidase (MPO), an indicator of biochemical neutrophil infiltration; for malondialdehyde (MDA), an indicator of lipid damage; and for natural intracellular antioxidant glutathione (GSH). Morphological examinations were performed on the right side and biochemical analyses were performed on the left side of the removed brains.

Tissue samples taken for microscopic analysis were fixed in 10% paraformaldehyde solution and cerebrum and cerebellums were totally blocked. Histopathological changes in the right hippocampal complex were analyzed using hematoxylin and eosin and apoptotic cells were analyzed using terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL).

2.1. MPO activity

Tissue samples were homogenized in 10 mL of frozen potassium phosphate buffer (20 mM K_2HPO_4, pH 6.0). The homogenate was centrifuged at 4 °C for 10 min at 12,000 rpm and solution over the precipitate was discarded. The precipitate was rehomogenized with an equivalent volume of 50 mM K_2HPO_4 containing 0.5% (w/v) hexadecyltrimethylammonium hydroxide (Sigma). MPO activity was evaluated by measuring H_2O_2-dependent oxidation of o-dianisidine 2HCl. One unit of enzyme activity is the amount that causes a change in absorbance of 1.0/min at 37 °C and 460 nm. This is defined as the MPO amount (15).

2.2. MDA and GSH assays

Tissue samples were homogenized in 150 mM KCL at ice-cold temperatures in order to determine MDA and GSH levels. MDA levels were analyzed for lipid peroxidation products and results were expressed as MDA nmol/g tissue (16). GSH was determined spectrophotometrically with Ellman's reactive (17) and results were expressed as GSH µmol/g tissue.

2.3. Histological examination

Comparisons of levels of neuronal cell death were made from the number of morphologically intact cells and the number of black-stained pyknotic cells for the evaluation of apoptosis and necrosis. Sections were stained with Cresyl violet acetate, which shows selective staining of Nissl granules (tigroid bodies and Nissl bodies) in the hippocampal CA_1, CA_2, CA_3, and dentate gyrus (DG) regions. The numbers of cells in each group were counted separately in four hippocampal areas (two areas each per portion) of the hippocampal regions per section. An area was defined as 800 × 600 mm, with the neuronal cell layer of the hippocampal CA_1, CA_2, CA_3, and DG regions set in parallel to the major axis. Mounted slides were examined under a light microscope (Nikon Microscope ECLIPSE E800W, Nikon, Japan) and photographed using a digital camera (Microscope Digital Camera DP20, Olympus, Japan). The photographs were analyzed using an image analysis system. The system used consisted of a PC, hardware, and software. The images were processed by an IBM-compatible personal computer, a high-resolution video monitor, image analysis software (BS200Docu Version 2.0, BAB Imaging Systems, Turkey), camera, and optical microscope. The method requires preliminary software procedures of spatial calibration (micron scale) and setting of color segmentation for quantitative color analysis (18). The number of apoptotic cells was calculated as an average per rat.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software, USA) software. All data were

expressed as mean ± SEM. Data groups were compared with the variance analysis following Tukey's multiple comparison test. The values were accepted as significant at P < 0.05.

3. Results

3.1. Biochemical analysis

3.1.1. MPO activity

The means and standard deviations of cerebral MPO activity of groups included in the study were determined as 3.33 ± 1.85 nmol/g tissue, 11.80 ± 4.73 nmol/g tissue, 7.64 ± 3.22 nmol/g tissue, and 4.92 ± 0.83 nmol/g tissue, respectively, for the control, I/R, pre-Glp-2, and post-Glp-2 groups. MPO activity value was significantly higher in the I/R group than in the control group (P < 0.001). The MPO activity differences between the I/R group (Group II) and the Glp-2 groups (Groups III and IV) were also significant (P < 0.01) (Figure 1).

3.1.2. MDA levels

Cerebral MDA levels were 15.30 ± 3.12 nmol/g protein, 26.72 ± 14.61 nmol/g protein, 15.48 ± 2.44 nmol/g protein, and 16.57 ± 6.26 nmol/g protein, respectively, for the control, I/R, pre-Glp-2, and post-Glp-2 groups. Statistical significance was determined between Groups I and II (P < 0.05) and between Groups III and IV (P < 0.05) (Figure 2).

3.1.3. GSH levels

Cerebral GSH levels were 1.93 ± 0.70 µmol GSH/g tissue, 1.74 ± 0.45 µmol GSH/g tissue, 1.81 ± 0.41 µmol GSH/g tissue, and 1.68 ± 0.25 µmol GSH/g tissue, respectively, for the control, I/R, pre-Glp-2, and post Glp-2 groups. There was no statistically significant difference between any groups (Figure 3).

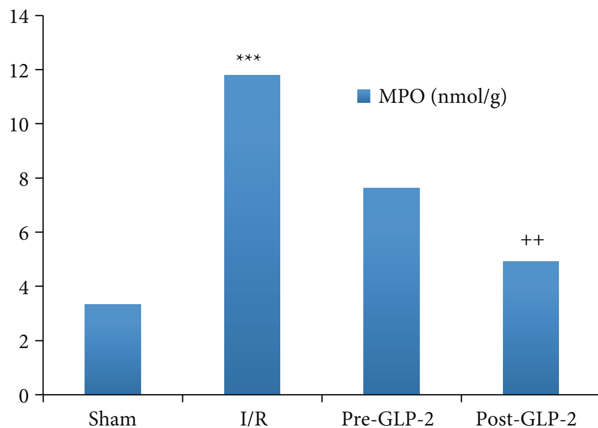

Figure 1. MPO values were significantly higher in the I/R group than in the control group (***: P < 0.001). In treatment groups, only post-Glp-2 treatment significantly decreased the damage (++: P < 0.01).

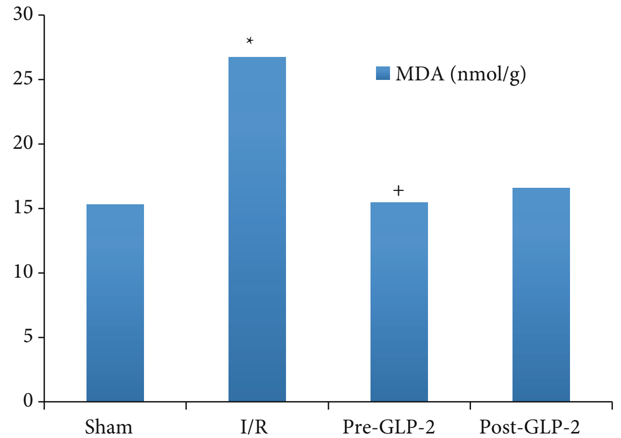

Figure 2. Statistical significance was determined for MDA levels between the control and I/R groups (*: P <0.05) and the I/R and pre-Glp-2 groups (+: P < 0.05).

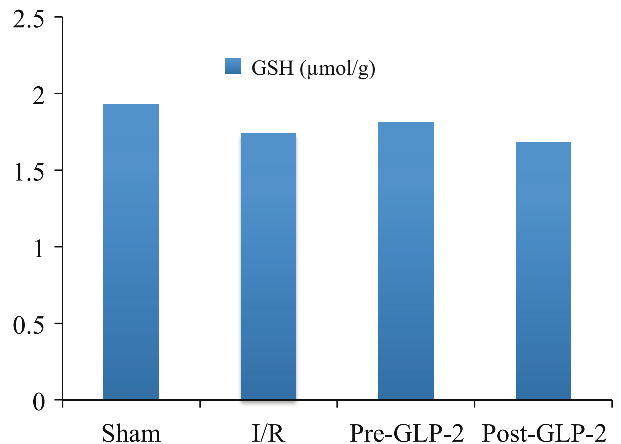

Figure 3. There was no statistically significant difference among groups in GSH levels.

3.2. Histopathologic results

Severe degenerative changes and shrinkage in cytoplasm and extensively dark pyknotic nuclei were seen in the neuronal cells of Group II (I/R). Groups III and IV had fewer degenerative changes and only slight shrinkage in cytoplasm and nuclei when compared with Group II (P < 0.05) (Figure 4).

The cell counts (dark pyknotic cell changes) of histopathological changes in the groups are given in the Table.

Group II (I/R) sections were stained with Cresyl violet acetate, which showed very dark pyknotic nuclei and selective staining of Nissl granules (tigroid bodies and Nissl bodies) in the hippocampal tissue. The number of Nissl granules showed significant decrease in Groups III and IV as compared with Group II (Figure 5).

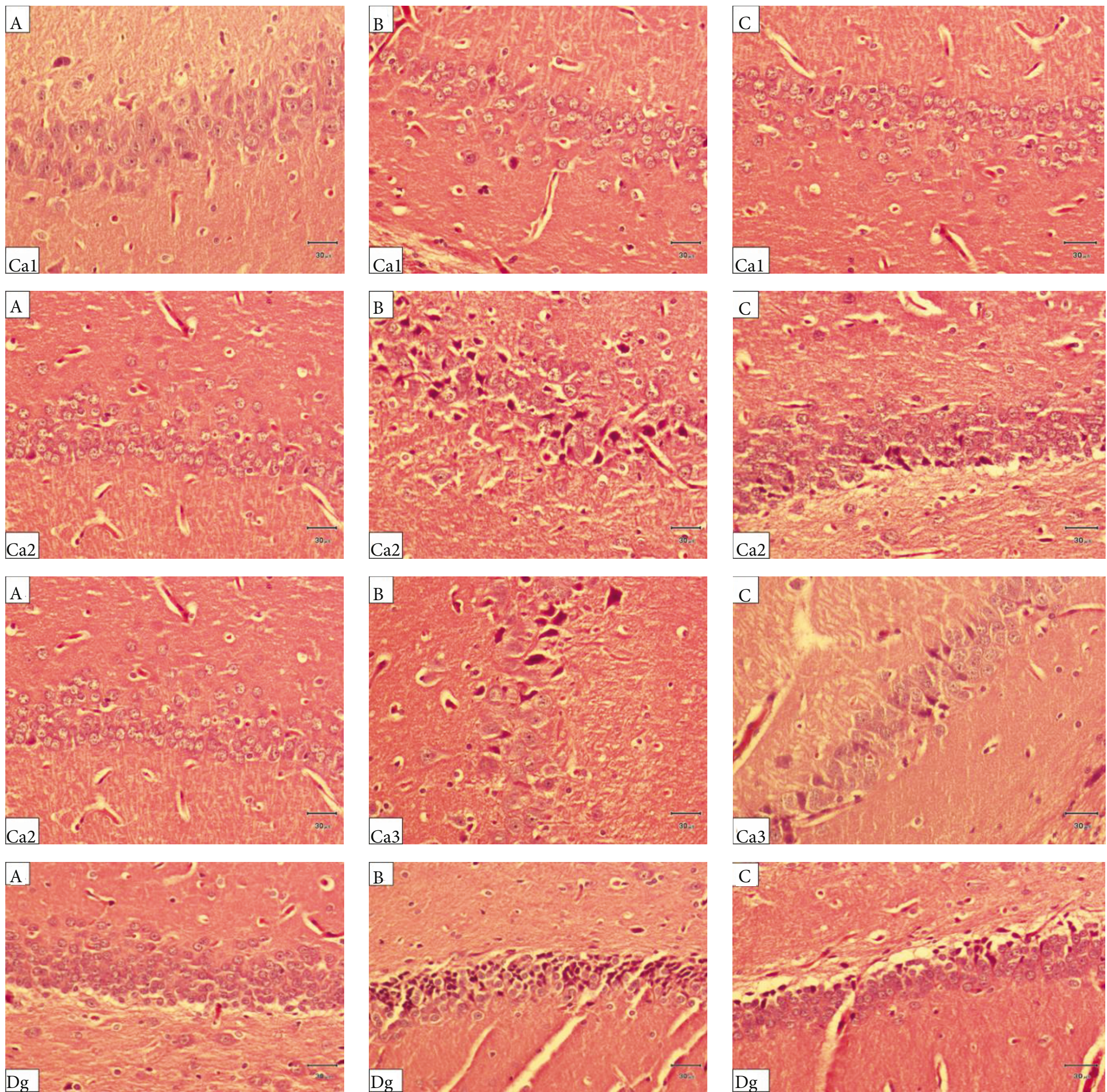

Figure 4. H&E staining. **A-** Hippocampal tissue histology in control rats. **B-** Hippocampal tissue histology in Group II rats (|I/R). Severe degenerative changes, shrunken cytoplasm, and extensively dark pyknotic nuclei are seen in neuronal cells. **C-** Hippocampal tissue histology in Group III (I/R + pre-Glp-2) and Group IV (I/R + post-Glp-2) rats. Fewer degenerative changes and slight shrinkage in cytoplasm and nuclei are seen in neuronal cells. Scale bar = 30 µm.

4. Discussion

In the present study, we showed the neuroprotective effect of Glp-2 on I/R injury of brain tissue in a rat model. The enteroendocrine hormone became popular after its discovery in the 1970s (3). Most studies focused on the intestinal tissue where it is secreted (2,3). In the studies performed by Deniz et al., Glp-2 increased blood flow of the small intestine as well as the carotid arteries and regulated intestinal blood flow during both pre- and post-Glp-2 treatments in experimental intestinal I/R damage (7). The increase of carotid blood flow after 30 min of ischemia via Glp-2 administration attracted the researchers' interest, although it has a short half-life. Glp-2 also increased cerebral and intestinal blood flow in experimental studies (6–8).

Reperfusion damage after thrombolytic treatment of cerebral ischemia is a current issue in paralyzed patients (19). There are publications showing that ischemic damage

Table. Histopathological changes (dark pyknotic cell changes) of all groups.

Region	Groups			
	I (Sham)	II (I/R + 0.9% NaCl)	III (I/R + pre-GLP-2)	IV (I/R + post-GLP-2)
CA_1	8	29	17	14
CA_2	1	171	103	72
CA_3	6	55	22	19
DG	2	201	100	102
Total	17	456	242*	207*

*: P < 0.05 compared to Group II.

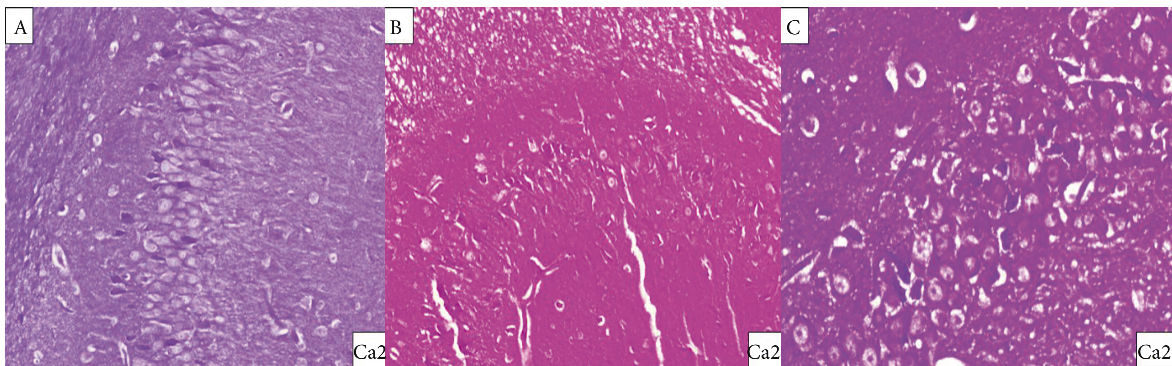

Figure 5. Cresyl violet acetate stained sections, CA2 region. **A-** Hippocampal tissue histology in control rats. **B-** Hippocampal tissue histology in Group II rats (I/R). Sections were stained with Cresyl violet acetate, which shows very dark pyknotic nuclei and selective staining of Nissl granules (tigroid bodies and Nissl bodies) in the hippocampal tissue. **C-** Hippocampal tissue histology in Group III (I/R + pre-Glp-2) and Group IV (I/R + post-Glp-2) rats. Decreased Nissl granules. Cresyl violet acetate 200×.

in the brain occurs in a short period (20,21). Cerebral ischemia may cause swelling of the cells and disrupts the blood–brain barrier. Increasing reactive oxygen species may contribute to endothelial and cell dysfunction. As a result, permeability of the blood–brain barrier increases and life-threatening cerebral edema develops (22,23). In many studies, it has been shown that I/R in the brain is related to lipid peroxidation that causes oxidative destruction of the cell membrane and provides autocatalytic mechanisms (24,25). GSH is an intracellular tripeptide composed of glutamic acid, cysteine, and glycine. GSH is an important intracellular antioxidant and is found in low concentrations at extracellular distances (26). It reduces the harmful effects of endogenous and exogenous oxidants by maintaining oxidoreduction balance of the cell (27,28). In our study, there was no significant change in hippocampal GSH values following Glp-2 treatment. It is possible that GSH did not have a role in decreasing damage and another mechanism decreased the increased damage.

When free oxygen radicals formed during ischemia exceed the defense capacity of the organism, they react with polyunsaturated fat acids and lipid peroxidation occurs (29). MDA is the last product of lipid peroxidation and it is accepted as one of the most sensitive indicators for lipid peroxidation in the case of I/R (30–32). In a study by Cirak et al., MDA levels increased in brain tissue in rats in the first 2 h following head trauma and then decreased with time; melatonin use decreased serum MDA levels (33). In our study, cerebral MDA values significantly decreased in Glp-2 treatment applied to the pre-Glp-2 group.

The important role of leukocytes in the I/R model has been shown in many studies. MPO is a lysosomal enzyme released from leukocytes as a response to oxidative stress. Zhang et al. reported that MPO level was an independent indicator for coronary arterial disease and that it could be used for screening as a noninvasive method (34). MPO uses nitric oxide as a catalytic substrate and contributes to endothelial dysfunction by causing protein nitration

(35). In our study, hippocampal MPO activities following Glp-2 treatment were significantly different between the control and I/R groups (P < 0.001) and between the I/R and post-Glp-2 groups (P < 0.01). In our study, pre- and post-GLP-2 treatment reduced the increasing MPO values linked to ischemia. This reduction was only significant for the post-Glp-2 treatment. The greater efficacy of post-Glp-2 treatment compared to pre-Glp-2 treatment may be due to the half-life of Glp-2. As the damage is more intense during the reperfusion period of I/R, the post-Glp-2 treatment in this period may have had a continued active effect compared to the pre-Glp-2 treatment. These results prove that Glp-2 treatment has a regulatory effect on neutrophil functions during the I/R process.

The pathological specimens of hippocampal tissues in ischemic rats (shrinkage in cytoplasm, extensively dark pyknotic nuclei, and Nissl bodies) (Group II) showed more apoptotic changes compared to Glp-2 treated rats (Groups III and IV) (P < 0.05).

We think that Glp-2 treatment decreases oxidative damage in cerebral I/R and that it might be used in the treatment of I/R injuries.

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