RESEARCH ARTICLE

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Overexpression of TRPM2 Channels by Oxidative Stress-Mediated Reperfusion Injury *

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Objective: Since it can be activated by oxidative stress, the TRP melastatin 2 (TRPM2) channel has recently been established as a potential therapeutic target in the fight against oxidative stress-related diseases including diabetes, inflammation, myocardial infarction. The main objective of this study was to investigate the expression of TRPM2 cation channel and histopathological effects in rat cerebral ischemia-reperfusion model.

Materials and Methods: A total of 30 adult male Wistar-Albino rats were used in the study. The animals were divided into 5 groups. In the sham groups bilateral common carotid arteries were uncovered and the rats were decapitated at the 2nd and 6th hours of the experiment. In the ischemia/reperfusion (I/R) groups (I/R-2 and I/R-6), bilateral common carotid arteries were uncovered then were clamped using aneurysm clips for 45 min and reperfusion was induced for 2 and 6 hours, respectively. At the end of the experiment malondialdehyde (MDA) and TRPM2 mRNA levels were measured. The tissues were fixed in formalin solution and then administered routine histological follow-up series and embedded in paraffin blocks. The TUNEL method was used for the determination of apoptotic cells and the avidin-biotin-peroxidase complex was used for the assessment of TRPM2 immunoreactivity.

Results: MDA, apoptosis, and TRPM2 levels were increased in the I/R groups, with a significant increase found in the I/R-6 group compared to the control group. Moreover, experimental ischemia-reperfusion cause an increase in the MDA, apoptosis, and TRPM2 levels in brain tissue.

Conclusion: TRPM2 may play a significant role in the pathophysiology of cerebral I/R injury.

Key Words: *Ischemia, reperfusion, rats, brain, TRPM2, apoptosis*

Oksidatif Stres İlişkili Reperfüzyon Hasarı ile TRPM2 Kanallarının Aşırı Ekspresyonu

Amaç: TRP melastatin 2 (TRPM2) kanalı oksidatif stres tarafından aktive edilebildiğinden diyabet, inflamasyon, miyokard enfarktüsü gibi oksidatif strese bağlı hastalıklarla mücadelede yakın zamanda potansiyel bir terapötik hedef olarak belirlenmiştir. Bu çalışmanın temel amacı bir sıçan serebral iskemi-reperfüzyon modelinde TRPM2 katyon kanalının ekspresyonunu ve histopatolojik etkilerini araştırmaktır.

Gereç ve Yöntem: Çalışmada toplam 30 adet yetişkin erkek Wistar-Albino sıçan kullanıldı. Hayvanlar 5 gruba ayrıldı. Sham gruplarında ise deneyin 2. ve 6. saatlerinde bilateral ana karotid arterler çıkarıldı ve ratlar dekapite edildi. İskemi/reperfüzyon (I/R) gruplarında (I/R-2 ve I/R-6) bilateral ana karotid arterler çıkarıldıktan sonra anevrizma klipleri ile 45 dakika klemplendi ve sırasıyla 2 ve 6 saat reperfüzyon sağlandı. Deney sonunda dokular formalin solüsyonunda sabitlendi, MDA ve TRPM2 mRNA düzeylerinin ölçümü ve ardından rutin histolojik takip serileri uygulandı ve parafin bloklara gömüldü. Apoptotik hücrelerin tespiti için TUNEL yöntemi ve TRPM2 immüno reaktivitesinin değerlendirilmesi için avidin-biyotin-peroksidaz kompleksi kullanıldı.

Bulgular: MDA, apoptoz ve TRPM2 seviyeleri, I/R gruplarında, kontrol grubuna kıyasla I/R-6 grubunda anlamlı bir artış bulundu. Ayrıca deneysel I/R, beyin dokusunda MDA, apoptoz ve TRPM2 seviyelerini arttırdı.

Sonuç: TRPM2, serebral İ/R hasarının patofizyolojisinde önemli bir rol oynayabilir.

Anahtar Kelimeler*: Ischemia, reperfusion, rats, beyin, TRPM2, apoptosis*

Introduction

Brain damage caused by stroke mostly occur by ischemia (1). Ischemia results from the thrombosis formation on ruptured atheromatous plaques that develop mostly in the extracranial and rarely in the intracranial arteries and in their bifurcations (2). The primary goal in the treatment of ischemic stroke is to minimize the brain damage and to prevent secondary brain insults, with the most common treatment methods being antiplatelet, anticoagulant, thrombolytic, anti-edema, and neuroprotective therapies (3).

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Excess reactive oxygen species (ROS) production following ischemia-reperfusion (I/R) injury have a deleterious effect on proteins, lipids, carbohydrates, nucleic acids, and DNA. Therefore, ROS can inactivate intracellular signaling pathways. Moreover, when they reach a certain concentration, they may result in metabolic and cellular disorders by reacting with cellular components (4). On the other hand, oxidative stress, which is defined as the imbalance between the antioxidant defense mechanism and ROS production, has been shown to have a key role in the development of various disorders including cancer, diabetes, ischemia/reperfusion injury, inflammatory diseases, and neurodegenerative diseases and in the etiopathogenesis of ageing-related diseases (5).

Transient receptor potential (TRP) channel constitute a large family of cation-conducting channel proteins. These ion channels are non-selectively permeable to cations including sodium (Na^{+}), calcium (Ca^{+2}), and magnesium (Mg^{+2}) (6). Since it can be activated by oxidative stress, the TRPM2 channel has recently been established as a potential therapeutic target in the fight against oxidative stress-related diseases including diabetes, inflammation, myocardial infarction, and neurodegenerative diseases (7). However, oxidative stress opens the TRPM2 ion channel, thereby increasing intracellular Ca^{+2} concentration (8, 9).

In this study, we aimed to investigate the expression of TRPM2 cation channel in a rat cerebral ischemia-reperfusion model.

Materials and Methods

Research and Publication Ethics: The experimental study was performed at Fırat University Experimental Research Center and Fırat University Medical School Histology and Embryology Laboratory after obtaining an approval from the local ethics committee (Approval No. 63; date, May 9, 2013). The study was completely funded by Fırat University Scientific Research Projects Coordination Unit (Project No. TF.1327).

Animals: The study included 30 adult male Wistar-Albino rats weighing 200-230 g that were aged 8-10 weeks. The animals were obtained from Fırat University Experimental Research Center and were kept at a room temperature of 22-25 °C with a 12/12 h light/dark cycle. The animals were housed individually in special metabolism cages and the floors of the cages were cleaned on a daily basis. The animals were fed standard rat chow with food and tap water provided ad libitum. The chow and the food were given in steel food cups and the water was given using water bottles.

Study Groups: The 30 rats were divided into 5 groups with 6 rats each:

Control group: No intervention was performed.

Sham Group 1 (2 h) (Sham-2): With the rats were placed in the supine position, bilateral common carotid arteries were uncovered via a simple midline cervical incision and then the skin incision was closed using 3-0 silk suture. After 2 h, the rats were decapitated.

Sham Group 2 (6 h) (Sham-6): In the supine position, bilateral common carotid arteries were uncovered and via a simple midline cervical incision and then the skin incision was closed using 3-0 silk suture. After 6 h, the rats were decapitated.

Ischemia/Reperfusion (I/R) Group 1 (2 h) (I/R-2): In the supine position, bilateral common carotid arteries were removed via a simple midline cervical incision and then bilateral arteries were clamped for 45 min using aneurysm clips, followed by 2-h reperfusion. Subsequently, all the rats were decapitated.

Ischemia/Reperfusion (I/R) Group 2 (6 h) (I/R-6): In the supine position, bilateral common carotid arteries were removed via a simple midline cervical incision and then bilateral arteries were clamped for 45 min using aneurysm clips, followed by 6-h reperfusion. Subsequently, all the rats were decapitated (10).

Sampling of brain tissues: At the end of the experiment, all the rats were decapitated under ketamine (75 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia. Shortly afterwards, the brain tissues were removed and were stored at –80 °C until the measurement of Malondialdehyde (MDA), TRPM2, and messenger RNA (mRNA) levels. For histological analysis, the tissues were fixed in formalin solution and then administered routine histological follow-up series and embedded in paraffin blocks.

MDA Assay: A buffer solution containing 0.42 g Tris-Base + 1.43 g Tris-HCl + 3 gr KCl, and 0.5 mL Tween 20 was dissolved in 250 mL of distilled water and was used for the homogenization of the tissue samples. The tissue was ground by adding 5 mL of homogenization buffer per X g of tissue samples. The homogenate was centrifuged at 5.000 rpm for 5 min. Subsequently, 1 mL of the supernatant was transferred into a fresh tube and then was added with 1 mL of 10% TCA (10 g TCA was dissolved in 100 mL of distilled water), followed by the addition of 1 mL of 0.6% TBA (0.6 g TBA was dissolved in 100 mL of distilled water,1 mL of distilled water, and 0.5 mL of 4% HCl, (4 mL of HCl was dissolved in 100 mL of distilled water), respectively. The mixture was incubated at 90-95 °C for 120 min. Following the incubation process, the tubes were cooled off to room temperature and were vortexed with 3 mL butanol. After cooling, the tubes were centrifuged at 5,000 rpm for 5 min and then the top (red-pink) layer was removed and pipetted into a quartz cuvette and measured at 532 nm against butanol as blank. The absorbance value was calculated based on the X: ($measured_{ABS}$ +0.0344)/0.0492 formula. The obtained value was multiplied by 5 (because the tissue homogenate was prepared with 5 mL of buffer) and the resultant value was accepted as the tissue weight in the homogenate.

TUNEL Staining: Paraffin sections at 4-6 µm thickness were mounted on polylysine slides. Apoptotic

cells were determined using ApopTag plus Peroxidase in Situ Apoptosis Detection Kit (Chemicon, cat no: S7101, USA) in accordance with the guidelines provided by the manufacturer.

The sections were deparaffinized with xylene, passed through graded alcohol series, and then rinsed in phosphate buffered saline (PBS). They were then incubated in 0.05% proteinase K for 10 min and were reincubated in 3% hydrogen peroxidase in water for 5 min to block endogenous peroxidase activity. Between each incubation step, the sections were rinsed in PBS. A 6-min incubation with Equilibration Buffer was followed by a 60-min incubation at 37 °C in a humidity chamber (70% µL Reaction Buffer + 30% TdT Enzyme). The sections were incubated in stop/wash buffer for 10 min to terminate the reaction and then were incubated with anti-digoxigenin-peroxidase for 30 min. Apoptotic cells were visualized by incubating the cells in the 3,3-diaminobenzidine. (DAB) substrate. The sections were counterstained with Harris' hematoxylin and then were occluded with appropriate occluding solutions. For negative controls, distilled reaction buffer instead of TdT was used.

In the evaluation of TUNEL staining, the cells stained with blue in Harris' hematoxylin were accepted as normal cells and the cells stained with brown were accepted as apoptotic cells. At 10x magnification, a minimum of 500 normal and apoptotic cells were counted in a randomly selected field. The apoptotic index was calculated as the percentage of apoptotic cells to total (normal + apoptotic) cells in a given field and was used for statistical analyses.

Immunohistochemical Staining: The immunoreactivity of TRPM2 in brain tissue was determined using avidin-biotin-peroxidase complex (ABC). Paraffin sections at 5-6 µm thickness were mounted on polylysine slides and then were incubated in H_2O_2 to block endogenous peroxidase activity. The sections were deparaffinized with xylene, passed through graded alcohol series, and then boiled in a microwave (750 W) in citrate buffer (pH 6.0) for 12 min for antigen retrieval. A 5-min incubation with Ultra V Block (TA-125-UB, Lab Vision Corporation, USA) was performed to block background staining, followed by a 60-min incubation with the primary antibody (Rabbit Anti-TRPM2 antibody, ab101738, Abcam, Cambridge, UK). Subsequently, the sections were incubated with the secondary antibody (biotinylated Goat Anti-Poliyvalent (anti-mouse / rabbit IgG), TP–125-BN, Lab Vision Corporation, USA) in humid chamber at room temperature for 30 min. After incubation with Streptavidin Alkaline Phosphatase (TS-060-AP, Lab Vision Corporation, USA) in humid chamber at room temperature for 30 min, the sections were immersed in distilled water. Fast-Red Substrate System (Lab Vision Corporation, USA) solution was instilled on the tissues and following the detection of an image signal on the light microscope, the sections were simultaneously rinsed with distilled water. The sections were then counterstained with Mayer's hematoxylin, rinsed with distilled water, and occluded with the appropriate occluding solution (Large Volume Vision Mount, Lab Vision Corporation, USA). For positive controls, the tissues were compared with breast tissues. For negative control, PBS instead of primary antibody was used and the other steps of the procedure were applied in the same manner. In the evaluation of immunohistochemical staining, a histoscore was calculated using the formula (histoscore = prevalence x severity) based on the prevalence (0.1: <%25, 0.4:%26-50, 0.6:%51-75, 0.9:%76-100) and the severity (0: absent, +0.5: very low, +1: low, +2: moderate, +3: extensive) of the immunoreactivity.

Polymerase Chain Reaction (PCR) Assay

Total RNA Isolation and Complementary DNA (cDNA) Synthesis: The GeneJet RNA Purification kit (cat. No: K0731, Thermo Scientific, Lithuania) was used for isolating RNA from brain tissue, in accordance with the guidelines provided by the manufacturer. The RNA pellet was rinsed once in 75% ethanol, air dried, and resuspended in 10-30 µL of RNase-free water. Complementary DNA (cDNA) synthesis was conducted using a High-Capacity cDNA Reverse Transcription Kit (cat no: 4368814, Applied Biosystems, Fostercity, USA), with a 10 µL reaction volume which contained 5 µL RNA sample, 1 µL 10XRT buffer, 1 µL 10XRT random primers, 2.1 µL nuclease-free water, 0.4 µL 25X dNTP mix, and 0.5 µL MultiScribe™ Reverse Transcriptase enzyme. The PCR was performed for cDNA synthesis at 25 °C for 10 min, at 37 °C for 120 min, and at 85 °C for 5 min using a Veriti 96 well thermal cycler (Applied Biosystems, Fostercity, USA).

Gene Expression Analysis: Gene expression analysis was performed using SYBR-green based primers (Qiagen, USA). Table 1 presents the primers studied and their properties. The mixture needed for gene expression analysis was made with iTaq universal SYBR green supermix (cat. No. 172-5121, Bio-Rad, USA). Measurement of gene expression levels was performed using Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Fostercity, USA). For the gene expression analysis, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat. No. QT00079247, Qiagen, USA) was utilized as control gene (housekeeping). Following the quantitative RT-PCR analyses, the differences in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1. Catalog numbers of the genes analyzed with quantitative RT-PCR.

Gene	Rattus norvegicus
TRPM2	PPR55663A
GAPDH	PPR06557B

Statistical Analysis: Data were analyzed using IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY: IBM Corp.). All data were expressed as mean ± standard deviation (SD). Groups were compared using One-way ANOVA followed by post-hoc Tukey's test. A *p* value of <0.05 was considered significant.

Results

MDA Assay: The MDA levels were similar in the control, Sham-2, and Sham-6 groups. However, although no significant difference was found between the I/R-2 and I/R-6 groups, the MDA levels were significantly increased in the I/R-2 and I/R-6 groups compared to the control group ($p<0.05$) (Figure 1).

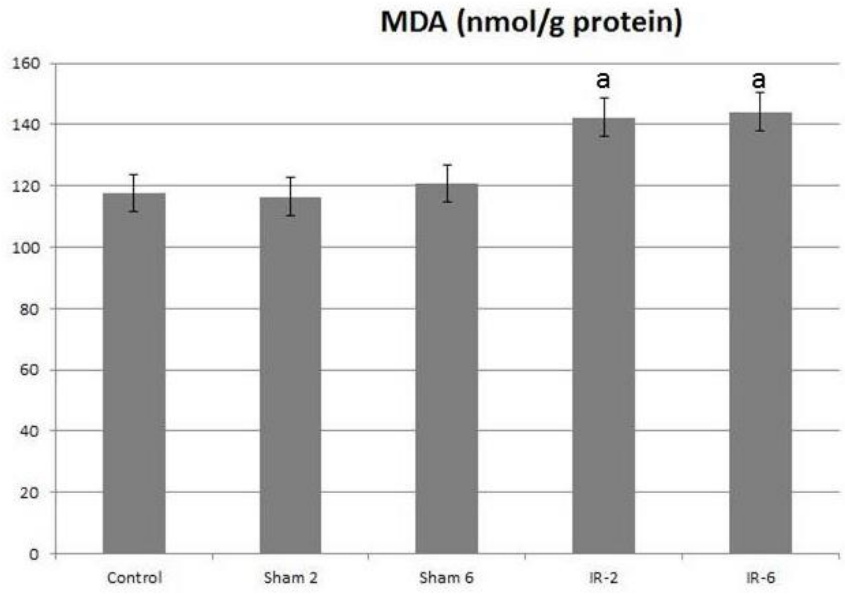

Figure 1. MDA levels in all groups Data were expressed as mean ± SD **a:** Compared to the control group ($p<0.05$).

TUNEL Staining: TUNEL positivity was observed in the nuclei of neuroglial cells, whereas no TUNEL positivity was observed in the negative controls (Figure 2a). TUNEL positivity was similar in the control (Figure 2b), Sham-2 (Figure 2c), and Sham-6 (Figure 2d) groups. However, although no significant increase was found in the I/R-2 group (Figure 2e) with regards to TUNEL positivity a significant increase was found in the I/R-6 group (Figure 2e) compared to the control group ($p<0.05$) (Figure 2, 3).

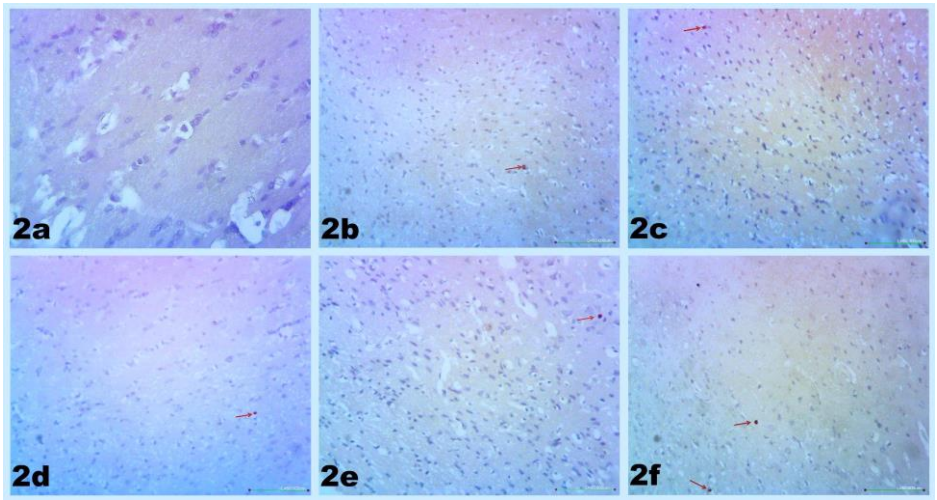

Figure 2. a. TUNEL-negative control, **b:** TUNEL-positive cells in the control group, **c:** TUNEL-positive cells in the Sham-2 group, **d:** TUNEL-positive cells in the Sham-6 group, **e:** TUNEL-positive cells in the I/R-2 group, **f:** TUNEL-positive cells in the I/R-6 group.

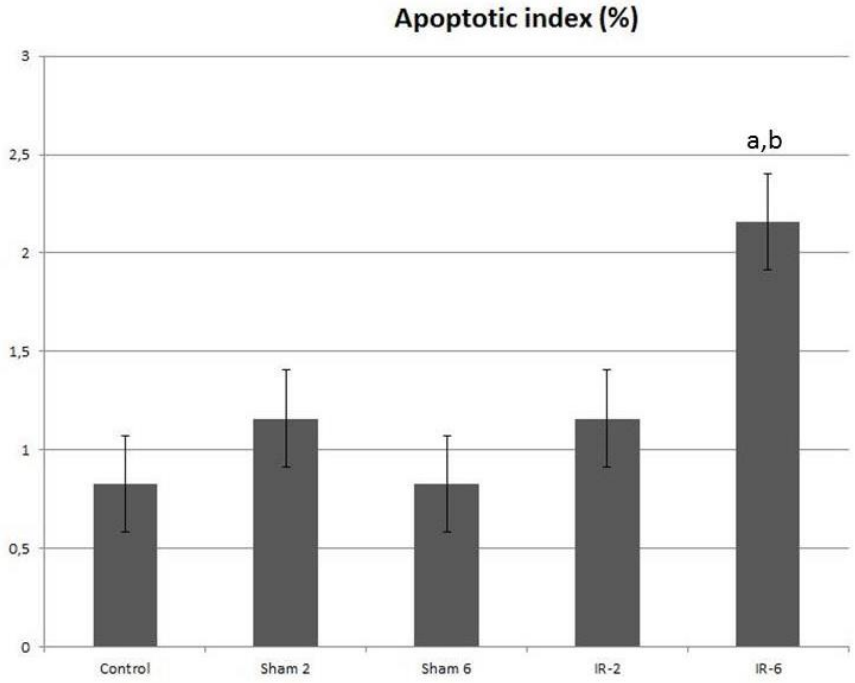

Figure 3. Apoptotic Index (%) Data were expressed as mean ± SD **a:** Compared to the control group, **b:** Compared to the I/R-6 group ($p<0.05$)

PCR Assay: The TRPM2 mRNA levels were similar in the control, Sham-2, and Sham-6 groups. However, a significant increase was observed in the I/R-2 group and a significant decrease was found in the I/R-6 group compared to the control group ($p<0.05$ for both) (Figure 4).

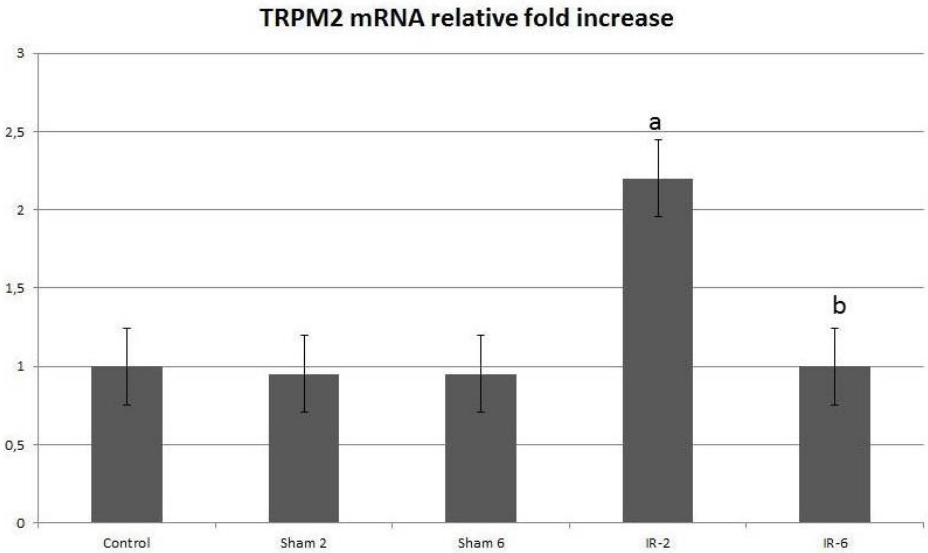

Figure 4. TRPM2 mRNA levels in all groups Data were expressed as mean ± SD **a:** Compared to the control group, **b:** Compared to the I/R-6 group ($p<0.05$)

TRPM2 Immunoreactivity: TRPM2 immunoreactivity was detected in the neuronal (red arrow) and neuroglial (black arrow) cells. However, no TRPM2 immunoreactivity was detected in the negative controls (Figure 5a). TRPM2 immunoreactivity was similar in the control (Figure 5b), Sham-2 (Figure 5c), and Sham-6 (Figure 5d) groups and was significantly increased in the I/R-2 (Figure 5d) and I/R-6 (Figure 5e) groups compared to the control group ($p<0.05$). Moreover, it was also significantly increased in the I/R-2 group compared to the I/R-6 group ($p<0.05$) (Figure 5, 6).

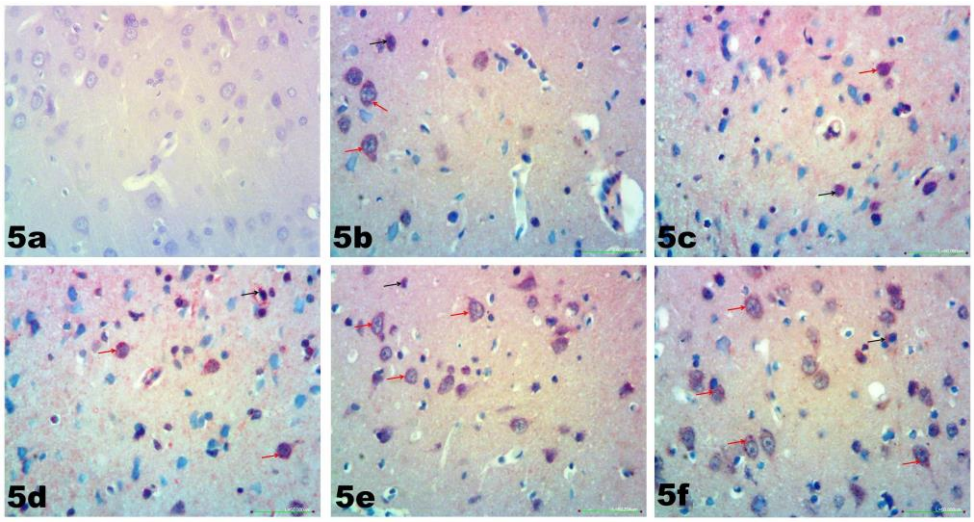

Figure 5. a: TRPM2-negative control, **b:** TRPM2 immunoreactivity in the control group, **c:** TRPM2 immunoreactivity in the Sham-2 group, **d:** TRPM2 immunoreactivity in the Sham-6 group, **e:** TRPM2 immunoreactivity in the I/R-2 group, **f:** TRPM2 immunoreactivity in the I/R-6 group.

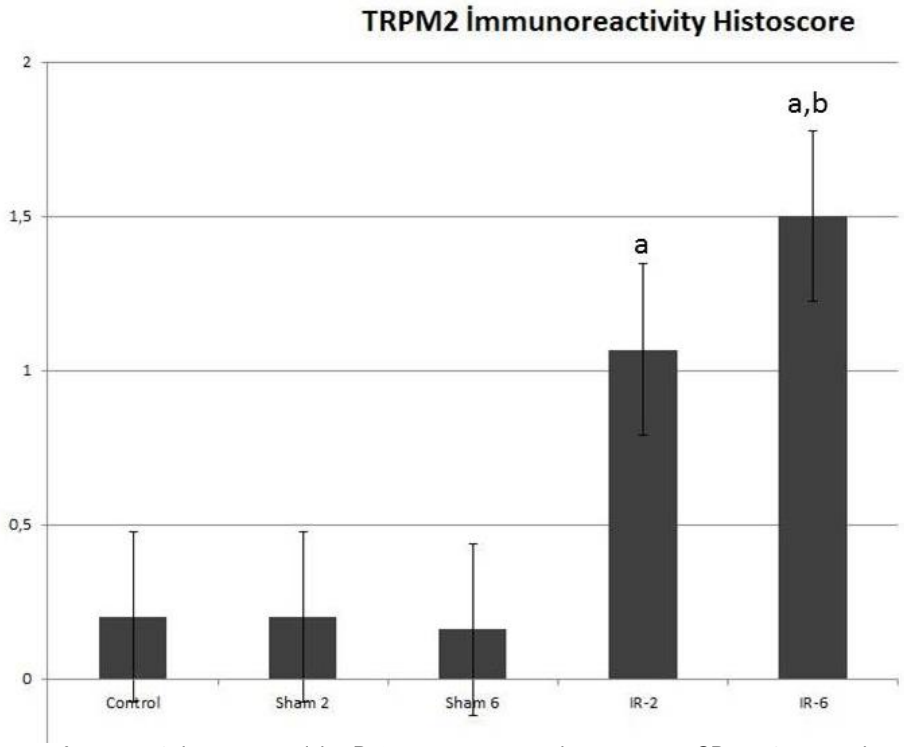

Figure 6. Histoscore for TRPM2 immunoreactivity Data were expressed as mean ± SD **a:** Compared to the control group, **b:** Compared to the I/R-6 group ($p<0.05$)

Discussion

Ischemic stroke is a clinical condition that rapidly develops clinical signs of focal or global cerebral dysfunction, manifests with symptoms lasting 24 h or longer, and may result in death with no apparent cause other than of vascular origin (10). Brain damage caused by stroke may occur by two mechanisms including ischemia and hemorrhage. Ischemic stroke, which is characterized by oxygen and glucose deprivation resulting from reduced blood supply, accounts for approximately 80% of all strokes. Moreover, the effect of ischemia on brain is highly rapid since the brain depends on glucose as its main source of energy and cannot store it and thus the brain shifts from aerobic to anaerobic metabolism in the absence of glucose (1). On the other hand, embolism and thrombosis lead to cerebral artery occlusion, thereby resulting in ischemia in the tissue supplied by the artery. In addition, hypoxia

and anoxia play a key role in the development of neuronal cell damage (11-14).

The primary goal in the treatment of ischemic stroke is to minimize the brain damage and to prevent secondary brain insults. Moreover, a second objective is to prevent acute complications and the most common treatment methods include antiplatelet, anticoagulant, thrombolytic, anti-edema, and neuroprotective therapies. Among the antiplatelet therapies, aspirin is the most commonly studied agent in patients with acute ischemic stroke. However, there is insufficient documentation regarding the effects of ticlopidine, clopidogrel, dipyridamole, and glycoprotein IIb/IIIa antagonists and the methods used in the treatment of the acute phase of the disease (3).

ROS lead to cell death in addition to direct neuronal damage by activating the pathways that trigger the excitotoxic damage and apoptotic process. Apoptotic neuronal cell death has been studied in both in vivo and in vitro global/focal I/R models. Apoptosis results from the activation of cell survival/death signaling pathways (15).

Oxidative stress, which is defined as the imbalance between the antioxidant defense mechanism and ROS production, has been shown to have a major role in the development of various disorders including cancer, diabetes, I/R injury, inflammatory diseases, and neurodegenerative diseases and in the etiopathogenesis of ageing-related diseases (5). Moreover, recent reports have indicated that oxidative and nitrative stress play a key role in the development of brain injury induced by I/R (16-19). Nevertheless, despite the extensive documentation of I/R injury, the factors contributing to high prevalence of cerebral I/R injury and its etiopathology remain unclear. Therefore, the present study aimed to contribute to the literature on the etiopathology of cerebral I/R injury.

In our study, the MDA level in the brain tissue was measured spectrophotometrically and the measurements indicated that the MDA levels were similar in the control, Sham-2, and Sham-6 groups, whereas they were significantly increased in the I/R-2 and I/R-6 groups compared to the control group.

MDA is the end product of lipid peroxidation and also a key marker of oxidative injury (10). Moreover, oxidative stress is regarded as a primary factor that further aggravates cerebral ischemic injury (20-23).

Brain tissue is relatively more susceptible to the detrimental effects of ROS due to its low regeneration capacity and high metabolic rate when compared to other tissues (24).

Abd-Elsameea et al. (25) conducted a similar study in 2014 to evaluate the effects of I/R in an experimental rat model and reported that the MDA levels were significantly increased and also the metformin treatment decreased the MDA levels. Similarly, numerous studies also indicated that the MDA levels were increased after ischemia (26, 27).

Experimental studies have shown that oxidative stress plays a key role in the cerebral injury caused by ischemia (28, 29). In addition, recent reports indicated that mitochondrial dysfunction and oxidative stress play the major role in the pathogenesis of acute ischemic stroke (30, 31).

In our study, the increased TUNEL positivity in the I/R-6 group can be accepted as an indicator of the DNA damage caused by oxidative stress, considering that ischemia can result in metabolic and structural changes within the cell. On the other hand, reduction or loss of blood supply to tissue leads to reduced oxidative phosphorylation, thereby leading to depletion of energy stores. Subsequently, the Na- K ATPase pump in the plasma membrane is inhibited and oxidative stress alters calcium homeostasis and the mitochondrial membrane potential. These alterations, in turn, result in mitochondrial and DNA damage, ultimately leading to programmed cell death or apoptosis (32-34).

In our study, the TRPM2 immunoreactivity was significantly increased in the I/R-2 and IR-6 groups compared to the control group.

Literature indicates that there are several extracellular factors that activate the TRPM2 cation channels, such as oxidative stress, ADPR/NAD+ metabolism, and tumor necrosis factor alpha (TNF-α) (8). Of these, oxidative stress opens the TRPM2 ion channels, thereby increasing intracellular Ca^{+2} concentration (8,9). Moreover, since the TRPM2 channel is activated by oxidative stress, TRPM2 has recently emerged as a potential therapeutic target in the fight against oxidative stress-related diseases including diabetes, inflammation, myocardial infarction, and neurodegenerative diseases (9, 35).

The oxidative stress induced by neurological disorders is known to originate from the oxidized phospholipids in the brain. In our study, the increased MDA levels implicate that these phospholipids are likely to be oxidized in this way. (9, 36-41).

The major limitations of the present study are that it is a single-center experience and represented by a small sample number, but our results are relevant to inspire for further studies with larger samples. The lack of use of TRPM2 channel antagonists is one of the shortcomings of our study. Larger studies using TRPM2 channel antagonists are needed

In conclusion, the results indicated that the experimental I/R model increased the MDA, apoptosis, and TRPM2 expression in the brain tissue and that TRPM2 may have a key role in the pathophysiology of cerebral I/R injury. Further large-scale studies investigating TRPM2 blockers and/or inhibitors are needed to evaluate the therapeutic options based on TRPM2 in the treatment of ischemic stroke.

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