ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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THE EFFECTS OF COMBINATION OF RADIOFREQUENCY AND PULSED MAGNETIC FIELD ON CAROTID ARTERIA ISCHEMIA AND REPERFUSION INDUCED BRAIN INJURY: A PRELIMINARY REPORT

RADYOFREKANS VE DARBELİ MANYETİK ALAN KOMBİNASYONUNUN KAROTİS ARTER İSKEMİ REPERFÜZYON KAYNAKLI BEYİN HASARI ÜZERİNDEKİ ETKİLERİ: BİR ÖN RAPOR

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Öz

Amaç

Beyindeki aterosklerotik plağın aracılık ettiği iskemi (IS) nedeniyle serebrovasküler olay serebral korteks, hipokampus ve serebellum dokularında inflamasyonu tetikleyebilir. Radyofrekans elektromanyetik alan (RF-EMF) ve darbeli manyetik alan (PMF) uygulamaları vasküler endotel tabakasından nitrik oksit oluşumunu artırabilir. Bu ön çalışmanın amacı, iskemi nedeniyle beynin farklı dokularında meydana gelen hasarı azaltmaktır.

Gereç ve Yöntem

Her bir grupta bir tane rat olacak şekilde toplamda 9 adet rat; sham, profilaktik RF, PMF, RF+PMF ve terapötik RF-EMF, PMF, RF-EMF+PMF, profilaktik ve terapötik RF-EMF+PMF ve yalnızca IS uygulanan gruplara ayırt edilmiştir. Profilaktik/terapötik RF-EMF ve PMF gruplarının tekli veya kombine uygulamalarında ratlar IS amaçlı 30 dakikalık karotis arter oklüzyonu öncesi ve sonrasında 30 dakikalık manyetik alan maruziyetleri için deney ünitesine alındı. Sakrifikasyondan sonra alınan beyin dokusu (serebral korteks ve hipokampüs) ile beyincik dokularında, histopatolojik olarak hematoksilin-eozin boyama; immünohistokimyasal analizler ile de brain derivated neurotrophic factor (BDNF), tumor necrosis factor-alpha (TNF-α), mammalian target of rapamycin (mTOR) ve inducible nitric oxide synthase (iNOS) ekspresyonlarına bakıldı. Bulgular: IS grubunda histopatolojik olarak belirgin hiperemi, ödem, kanama ve nöronal dejenerasyon

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saptandı. Ayrıca immünohistokimyasal olarak TNF-α, mTOR, iNOS artışı ve BDNF boyamasında azalma gözlendi. Profilaktik ve/veya terapötik RF-EMF ve/veya PMF uygulamaları tüm bu parametreleri tersine çevirmiştir. En fazla düzelme Profilaktik+Terapötik RF-EMF+PMF grubunda gözlendi.

Sonuç
Sonuç olarak RF-EMF ve PMF ile her iki beyin dokusu kısımları ve beyincik dokularında IS'ye bağlı inflamasyon tablosunun gerilemesi nörolojik defisitlerin oluşması, öğrenme ve hafıza mekanizmalarının devamlılığı ve denge fonksiyonlarının korunması açısından önemlidir.

Anahtar Kelimeler: Serebral iskemi, eNOS, iNOS, mTOR, Darbeli manyetik alan, Radyofrekans elektromanyetik alan

Abstract

Objective
Cerebrovascular accident due to ischemia (IS) mediated by atherosclerotic plaque in the brain can trigger inflammation in the cerebral cortex, hippocampus and cerebellum tissues. Radiofrequency electromagnetic field (RF-EMF) and pulsed magnetic field (PMF) applications can increase nitric oxide formation from the vascular endothelial layer. The aim of this preliminary study is to reduce the damage caused by IS in different tissues of the brain by magnetic field applications.

Material and Method
A total of 9 rats, one rat in each group; sham, prophylactic RF, PMF, RF+PMF and therapeutic RF-EMF, PMF, RF-EMF+PMF, prophylactic and therapeutic RF-EMF+PMF and IS-only groups were distinguished. In single or combined applications of prophylactic/therapeutic RF-EMF and PMF groups, rats were taken to the experimental unit for 30 minutes of magnetic field exposure before and after 30 minutes of carotid artery occlusion for IS purposes. Histopathological hematoxylin-eosin staining in brain tissue (cerebral cortex and hippocampus) and cerebellum tissues taken after sacrification; With immunohistochemical analysis, brain derived neurotrophic factor (BDNF), tumor necrosis factor-alpha (TNF-α), mammalian target of rapamycin (mTOR) and inducible nitric oxide synthase (iNOS) expressions were examined.

Results
Histopathologically significant hyperemia, edema, bleeding and neuronal degeneration were detected in the IS group. Additionally, immunohistochemically, an increase in TNF-α, mTOR, iNOS and a decrease in BDNF staining were observed. Prophylactic and/or therapeutic RF-EMF and/or PMF applications reversed all these parameters. The greatest improvement was observed in the Prophylactic+Therapeutic RF-EMF+PMF group.

Conclusion
As a result, the regression of IS-related inflammation in both brain tissue parts and cerebellar tissues with RF-EMF and PMF is important in terms of the formation of neurological deficits, the continuity of learning and memory mechanisms, and the preservation of balance functions.

Keywords: Cerebral ischemia, eNOS, iNOS, mTOR, Pulsed magnetic field, Radiofrequency electromagnetic field

Introduction

The term "cerebrovascular accident" is a term used for cerebral vascular occlusion, also called "stroke" (1). The World Health Organization defines cerebrovascular disease, called "stroke", as clinical manifestations lasting 24 hours or longer or the development of death due to a focal or globally rapidly developing cerebral disorder (2, 3). In the classification made according to the "Trial of Organization in Acute Stroke Treatment" study published in 1993, the causes were specified as large artery atherosclerosis (thrombosis and embolism), cardio-embolism, small vessel occlusion, and other etiologies, in order of frequency (4). In large artery atherosclerosis (thrombosis and embolism), ischemia (IS) develops due to thrombosis that occurs with the destabilization of atheroma plaques in the intracranial vessels and their bifurcation areas, especially extracranial. This atherothrombotic state leads to stenosis or occlusion of the vessel. Various neurological deficits can be observed in patients, depending on the neuronal damage and location distal to the occluded area as neurological deficits and learning and memory disorders (4-6). IS is a restriction in blood flow to any tissue by ischemic conditions and turbulent flow-induced damage in vascular structure by reperfusion (7). It is a pathology that causes a lack of oxygen required for cellular metabolism. IS usually results from pathologies related to blood vessels, resulting in tissue damage or dysfunction,

namely hypoxia and microvascular dysfunction (8). The persistence of hypoxic conditions in distal tissue due to IS triggers intracellular pathways, including hypoxia-inducible factor-1 alpha (Hif-1α), which can lead to the expression of markers that trigger oxidative stress, inflammation, and apoptosis. While promptly resolving the pathological process causing IS allows reperfusion, the turbulent and strong current formed in the distal part of the narrowed vessel can also damage the endothelium and initiate the aforementioned process (7). Nitric oxide (NO), also known as endothelial-derived relaxation factor, is synthesized from arginine molecules via the endothelial nitric oxide synthase (eNOS) enzyme, by increasing cyclic guanylate monophosphate levels through the enzyme guanylate cyclase, resulting in vasodilation, neurotransmitter effect, antimicrobial effector molecule and immunomodulatory effect (9,10). Moreover, it has effects such as regulation of vasomotor tone, platelet activation, inhibition of adhesion and aggregation (11, 12). There are also some studies in the literature showing that treatments to increase NO levels are effective in preventing IS-induced brain tissue damage (13).

Radiofrequency electromagnetic field (RF-EMF) and pulsed magnetic field (PMF) cause vasodilation by increasing the eNOS enzyme expressions in the endothelial layer, which is the innermost layer of the vascular structures in the area where they are applied (it also has the feature of being able to pass behind the bone tissue), resulting in an increase in NO synthesis, and hypoxia inducible factor-1 alpha (Hif-1α) mediated vascular endothelial growth factor (VEGF) levels contribute to angiogenesis (14-16). The possible mechanism of these fields on carotid artery IS-induced brain injury was shown in Figure 1. The aim of this study is to conduct a preliminary investigation to establish the effectiveness of.1 Hz, 50% duty cycle, 0.5 mT PMF and 27.12 MHz frequency RF-EMF on carotid artery IS induced brain and cerebellar injury.

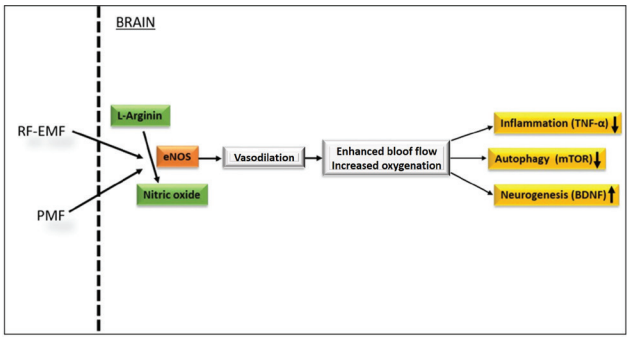

Figure 1
The possible mechanism of RF-EMF and PMF on carotid artery ischemia induced brain injury.
RF-EMF: Radiofrequency electromagnetic field, PMF: Pulsed magnetic field, TNF-α: Tumor necrosis factor alpha, mTOR: Mammalian target of rapamycin, BDNF: Brain derivated neurotrophic factor

Material and Method

Ethical Approval

All experiments conducted in this study were carried out in accordance with the ARRIVE (Animal Research: Reporting in Live Experiments) 2.0 guidelines for animal research and were approved by the Suleyman Demirel University, Isparta Committee on Animal Research (Approval No. 2022-08/108). This research was supported by the Scientific Research Fund of Suleyman Demirel University, under Project Number TSG-2022-8783.

RF-EMF and PMF Setup

The 0.5 mT magnetic field value aimed for PMF is generated using a set of two coils under each Eurotype-2 cage. In order to generate RF-EMF, circuit was fed with 12 Vdc to obtain radiations with two 27.12 MHz RF antenna with an aimed electric field value of 10 V/m. 0.8 W RF output was obtained from the 27.12 MHz PCB antenna. Experiments were contucted by feeding and tuning the circuits to give an aimed electric field intensity of 10 V/m in each cage.

In the scifience literature, it is emphasized that such exposure mechanisms should be isolated from the external environment. Pre-study measurements were made in the electromagnetic isolated room (Faraday cage), where the experiments were carried out. Shielding effectiveness is measured as (80 dB) for the operating frequency. PMF measurement setup is given in Figure 2.

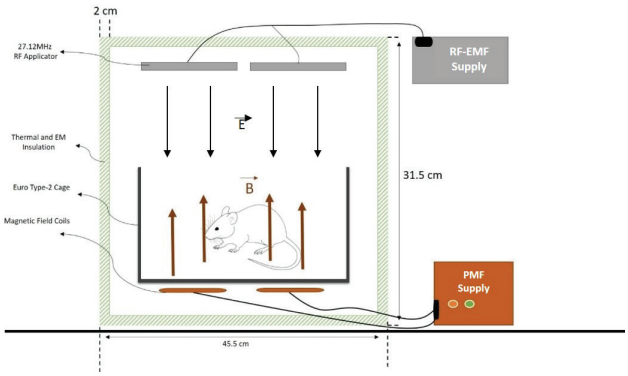

Figure 2
RF-EMF+PMF application setup.
RF-EMF: Radiofrequency electromagnetic field, PMF: Pulsed magnetic field

Study animals and design of experiment

In this preliminary study, 9 rats obtained from Suleyman Demirel University Experimental Animals Production and Experimental Research Center were divided into the following groups. IS were applied to each group except sham group, and the groups were as follows:

1-Sham, 2-Prophylactic RF-EMF, 3-Prophylactic PMF, 4-Prophylactic RF-EMF+PMF, 5- Therapeutic RF-EMF, 6-Therapeutic PMF, 7-Therapeutic RF-EMF+PMF, 8-Prophylactic and Therapeutic RF-EMF+PMF, 9-IS applied group.

Before all surgical procedures and sacrifices, all rats were administered 80-100 mg/kg ketamine (Ketalar, Pfizer, Turkey) and 10 mg/kg xylazine (Xylazinbio %2, Bioveta, Czech Republic) to induce anesthesia.

In the sham group, the rats were placed in the RF-EMF and PMF unit for 0-30 minutes without operating the device. After 30 minutes, the rats underwent neck dissection under anesthesia without carotid artery dissection. Following dissection, the animals were kept in the RF-EMF and PMF unit for an additional 30 minutes without the device operating, and then the rats were sacrificed at the end (Table 1).

In IS-applied groups, the trachea and the adjacent vascular sheath were reached with a longitudinal incision just above the trachea of the rats. The carotid artery and vagus nerve were carefully separated from the jugular vein. Then, the carotid artery and vagus nerve were separated and the carotid artery was occluded using 4/0 silk. The 4/0 silk was rotated on itself to provide occlusion and fixed in this way. After the 30 minutes occlusion time expired, it was opened by turning in the opposite direction (Figure 3). In single or combined applications of prophylactic/therapeutic RF-EMF and PMF groups; rats were placed in Eurotype-2 and put into the experimental unit for 30 minutes magnetic field exposure before and after 30 minute IS period. After the abdominal incision, rats were euthanized with surgical exsanguination. Brain tissues were taken after decapitation and collected for hematoxylin-eosin (HE) staining and immunohistochemical analyses as brain derived neurotrophic factor (BDNF), tumor necrosis factor-alpha (TNF-α), the mammalian target of rapamycin (mTOR) and inducible nitric oxide synthase (iNOS) expressions.

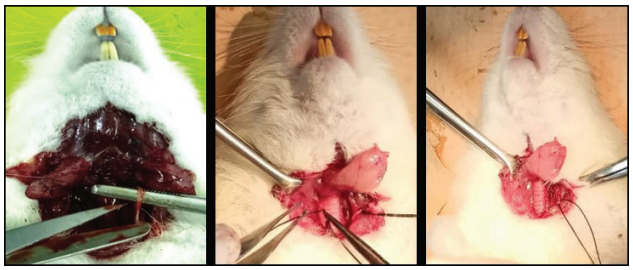

Figure 3
The surgical procedure of the experiment

Histopathological Analysis

The brain and cerebellum tissue samples were

Table 1 Schematic of the RF-EMF and PMF applied experimental groups

	0.min	30.min	
Sham	RF-EMF (-) PMF (-)	ND	RF-EMF (-) PMF (-)
Prophylactic RF-EMF	RF-EMF (+) PMF (-)	IS	RF-EMF (-) PMF (-)
Prophylactic PMF	RF-EMF (-) PMF (+)	IS	RF-EMF (-) PMF (-)
Prophylactic RF-EMF+PMF	RF-EMF (+) PMF (+)	IS	RF-EMF (-) PMF (-)
Therapeutic RF-EMF	RF-EMF (-) PMF (-)	IS	RF-EMF (+) PMF (-)
Therapeutic PMF	RF-EMF (-) PMF (-)	IS	RF-EMF (-) PMF (+)
Therapeutic RF-EMF + PMF	RF-EMF (-) PMF (-)	IS	RF-EMF (+) PMF (+)
Prophylactic RF-EMF+PMF + Therapeutic RF-EMF + PMF	RF-EMF (+) PMF (+)	IS	RF-EMF (+) PMF (+)
IS	RF-EMF (-) PMF (-)	IS	RF-EMF (-) PMF (-)

RF-EMF: Radiofrequency electromagnetic field, PMF: Pulsed magnetic field, IS: Ischemia, , ND: Neck dissection

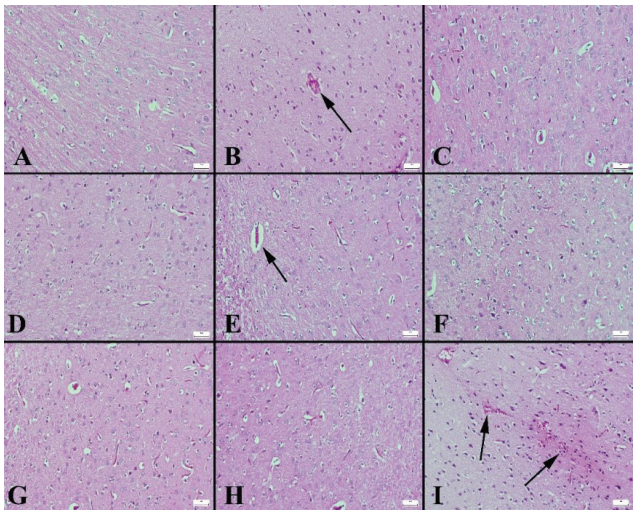

Figure 4
Histopathological appearance of the cerebral cortices between the groups.
(A) Normal brain histology in the sham group. (B) Marked hyperemia and microhemorrhage around the blood vessel (arrow) in prophylactic RF-EMF. (C) Decreased pathological findings in prophylactic PMF (D) Almost normal brain histoarchitecture in prophylactic RF-EMF+PMF. (E) Marked hyperemia and edema (arrow) in therapeutic RF-EMF (F) Decreased hyperemia in the therapeutic PMF group. (G) Slight hyperemia in therapeutic RF-EMF+PMF (H) Almost normal brain histology in prophylactic + therapeutic RF-EMF+PMF (I) Marked hyperemia and hemorrhage (arrows) in the IS group. HE, Scale bars=50µm.

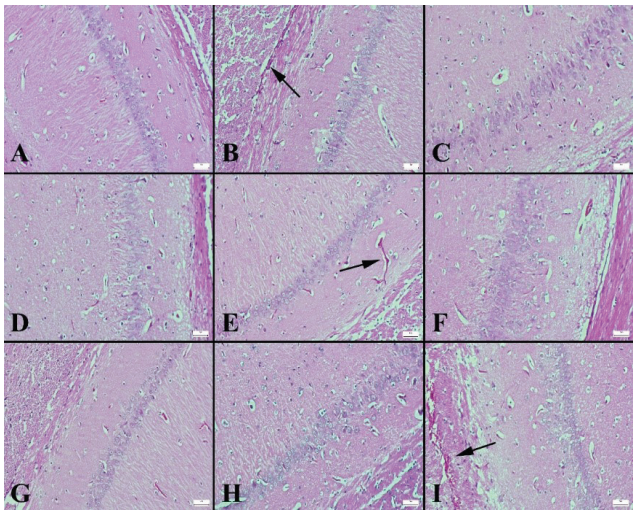

Figure 5
Representative microscopical figures of the hippocampus between the groups.
(A) Normal hippocampal histology in the sham group. (B) Marked hyperemia and hemorrhage near the hippocampus (arrow) in the prophylactic RF-EMF group. (C) Moderate hyperemia but no hemorrhage in the prophylactic PMF group. (D) Decreased pathological findings in the prophylactic RF-EMF+PMF group. (E) Marked hyperemia and edema (arrow) in the therapeutic RF-EMF group. (F) Slight hyperemia in the therapeutic PMF group. (G) Decreased pathological findings in the therapeutic RF-EMF+PMF group. (H) Almost normal brain histology in prophylactic + therapeutic RF-EMF+PMF group. (I) Marked hemorrhage (arrow) hyperemia and edema in the IS group. HE, Scale bars=50µm.

collected and fixed in 10% formalin solution. Then routinely processed with a fully automatic tissue processor, and rotary microtome were used to cut 5 µm thick sections from the paraffin blocks (Leica RM2155, Leica Microsystems, Wetzlar, Germany). The sections were then deparaffinized, rehydrated with ethanol graded in decreasing concentrations, stained with HE, cleaned in xylene, and covered.

The evaluation of histopathological alterations was done under a light microscope. The brain, hippocampus and cerebellum's histopathological lesions received semiquantitative scoring. Hyperemia, hemorrhage, gliosis, and neuronal damage were assessed for this purpose. According to the severity, the descriptions were assigned scores ranging from 0 to 3. The scoring system for the histology results is 0: Normal, 1: slight, 2: moderate, 3: severe lesions (17).

Immunohistochemical Examination

Sections taken onto polylysine slides were immunostained with Recombinant Anti-BDNF antibody [EPR1292] (ab108319); Recombinant Anti-iNOS antibody [EPR16635] (ab178945) and Recombinant Anti-TNF alpha antibody [EPR19147] (ab183218) Abcam, Cambridge, UK), mTOR (mTOR antibody (ab32028 Abcam (Cambridge, UK)) using the streptavidin-biotin technique. We utilized 1/100 dilutions of all primary antibodies. Sections were immunohistochemically stained with biotinylated secondary antibodies and streptavidin-alkaline phosphatase conjugate after incubation with 60 minutes primary. The secondary antibody and chromogen were both taken from a ready-to-use commercial kit called EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436) (Abcam, Cambridge, UK). For negative controls the primary antiserum step was replaced with the dilution solution. All examinations were conducted on samples that were blinded by a specialist pathologist from another university. The following grading scale was applied for the semiquantitative analysis of the

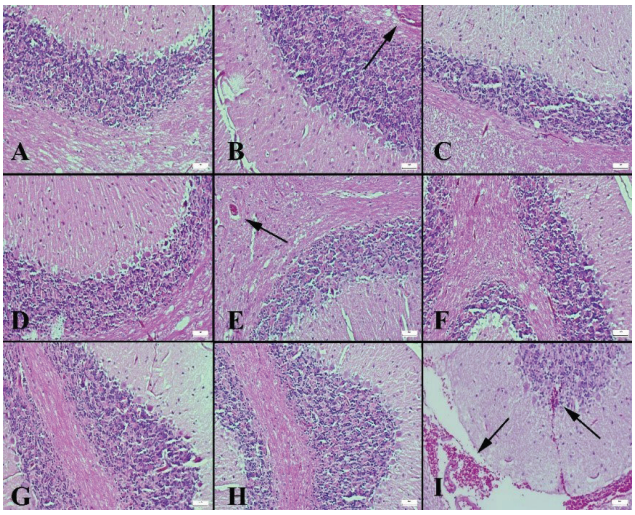

Figure 6
Microscopical appearance of the cerebellums between the groups.
(A) Normal cerebellar histoarchitecture in the sham group. (B) Marked hyperemia (arrow) in the prophylactic RF-EMF group. (C) Decreased pathological findings in the prophylactic PMF group. (D) Almost normal cerebellar histology in prophylactic RF-EMF+PMF group. (E) Marked hyperemia and edema (arrow) in the therapeutic RF-EMF group. (F) Slight hyperemia in the therapeutic PMF group. (G) Markedly decreased pathological findings in the therapeutic RF-EMF+PMF group. (H) Almost normal brain histology in prophylactic + therapeutic RF-EMF+PMF group. (I) Marked meningeal and parenchymal hemorrhage (arrows) in IS group. HE, Scale bars=50μm.

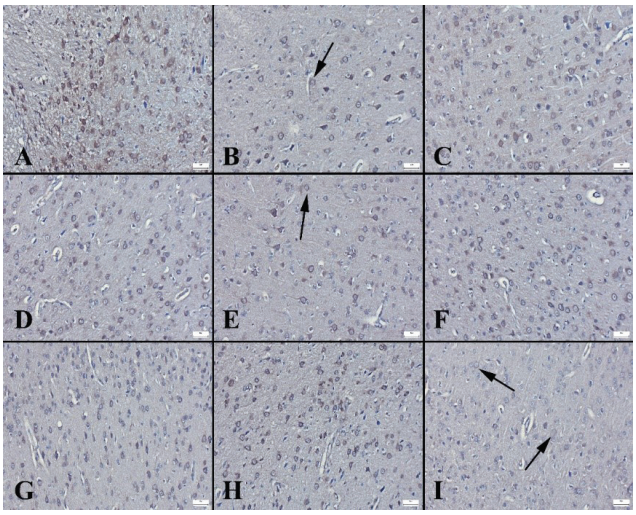

Figure 7
BDNF expressions in brains among the groups.
(A) Marked expressions in neurons in the sham group. (B) Markedly decreased expression in neurons (arrow) in the prophylactic RF-EMF group. (C) Slight increase expressions in the prophylactic PMF group. (D) Moderate increase in neurons in the prophylactic RF-EMF+PMF group. (E) Slight increase in the therapeutic RF-EMF group. (F) Increase in expression in the therapeutic PMF group. (G) Increase expressions in the therapeutic RF-EMF+PMF group. (H) Marked increase in expressions in prophylactic + therapeutic RF-EMF+PMF group. (I) Totally decreased expressions in the IS group. Streptavidin biotin peroxidase method, Scale bars=50μm.

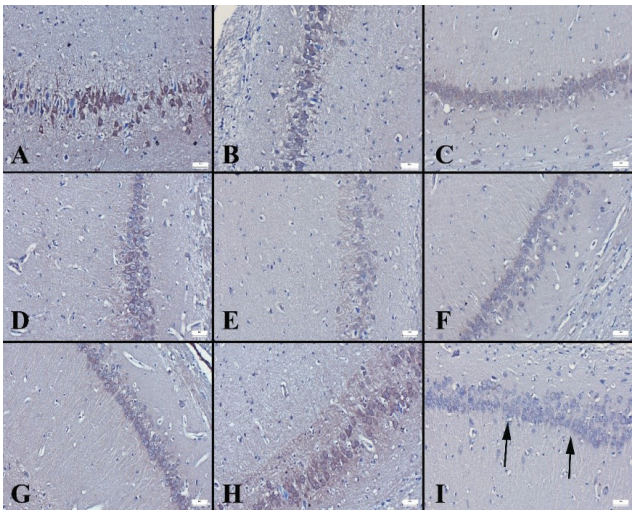

Figure 8
BDNF expressions in the hippocampus between the groups.
(A) Marked expressions in neurons in the sham group. (B) Markedly decreased expression in neurons in the prophylactic RF-EMF group. (C) Slight increase in expressions in the prophylactic PMF group. (D) Moderate increased expressions in neurons in the prophylactic RF-EMF+PMF group. (E) Slight increase in the therapeutic RF-EMF group. (F) Increase in expression in the therapeutic PMF group. (G) Increase expressions in the therapeutic RF-EMF+PMF group. (H) Marked increase in expressions in prophylactic + therapeutic RF-EMF+PMF group. (I) Totally decreased expressions in neurons (arrows) in the IS group. Streptavidin biotin peroxidase method, Scale bars=50μm.

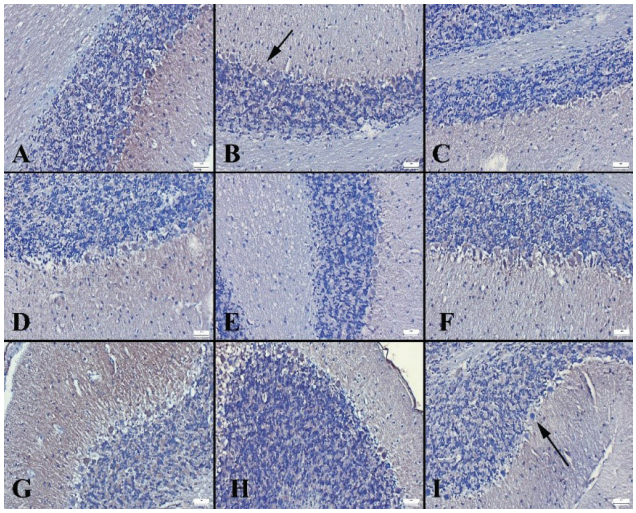

Figure 9
BDNF expressions in cerebellums between the groups.
(A) Marked expressions especially Purkinje cells in the sham group. (B) Markedly decreased expression in Purkinje cells (arrow) in neurons in the prophylactic RF-EMF group. (C) Slight increase expressions in the prophylactic PMF group. (D) Moderate increase in neurons in the prophylactic RF-EMF+PMF group. (E) Slight increase in the therapeutic RF-EMF group. (F) Increase in expression in the therapeutic PMF group. (G) Increase expressions in the therapeutic RF-EMF+PMF group. (H) Marked increase in expressions in prophylactic + therapeutic RF-EMF+PMF group. (I) Totally decreased expressions in neurons (arrows) in the IS group. Streptavidin biotin peroxidase method, Scale bars=50µm.

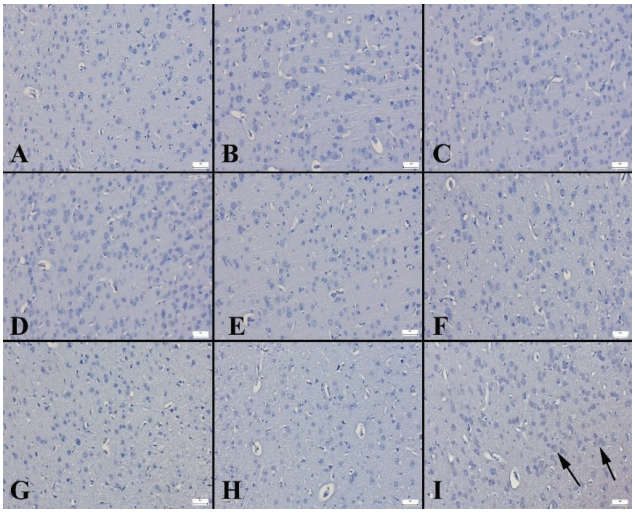

Figure 10
iNOS expressions in brains among the groups.
(A) Negative expressions in neurons in the sham group. (B) No expression in neurons in the prophylactic RF-EMF group. (C) Negative expressions in the prophylactic PMF group. (D) No expression in neurons in the prophylactic RF-EMF+PMF group. (E) Negative expression in the therapeutic RF-EMF group. (F) No expression in the therapeutic PMF group. (G) No expressions in the therapeutic RF-EMF+PMF group. (H) Negative expressions in the prophylactic + therapeutic RF-EMF+PMF group. (I) Slight increase in expressions in the IS group. Streptavidin biotin peroxidase method, Scale bars=50µm.

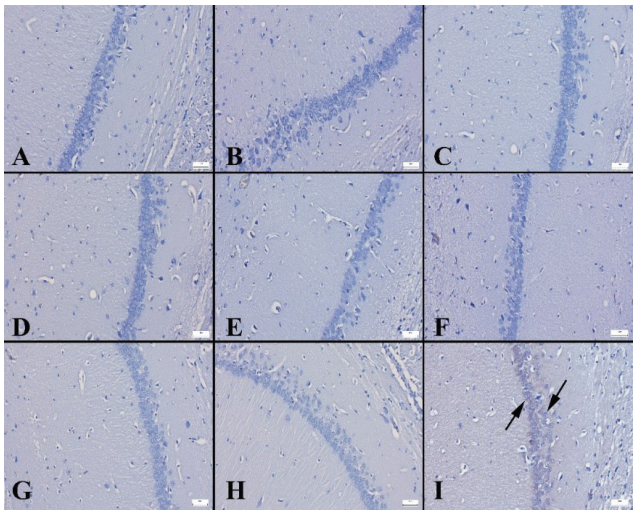

Figure 11
iNOS expressions in hippocampus among the groups.
(A) Negative expressions in neurons in the sham group. (B) No expression in neurons in the prophylactic RF-EMF group. (C) Negative expressions in the prophylactic PMF group. (D) No expression in neurons in the prophylactic RF-EMF+PMF group. (E) Negative expression in the therapeutic RF-EMF group. (F) No expression in the therapeutic PMF group. (G) No expressions in the therapeutic RF-EMF+PMF group. (H) Negative expressions in the prophylactic+therapeutic RF-EMF+PMF group. (I) Slight increase in expressions in the IS group. Streptavidin biotin peroxidase method, Scale bars=50µm.

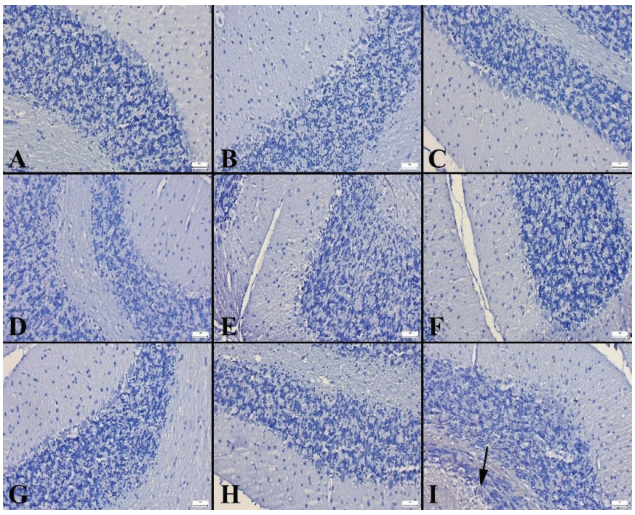

Figure 12
iNOS expressions in cerebellum among the groups.
(A) Negative expressions in neurons in the sham group. (B) No expression in neurons in the prophylactic RF-EMF group. (C) Negative expressions in the prophylactic PMF group. (D) No expression in neurons in the prophylactic RF-EMF+PMF group. (E) Negative expression in the therapeutic RF-EMF group. (F) No expression in the therapeutic PMF group. (G) No expressions in the therapeutic RF-EMF+PMF group. (H) Negative expressions in the prophylactic+therapeutic RF-EMF+PMF group. (I) Slight increase in expressions in the IS group. Streptavidin biotin peroxidase method, Scale bars=50µm.

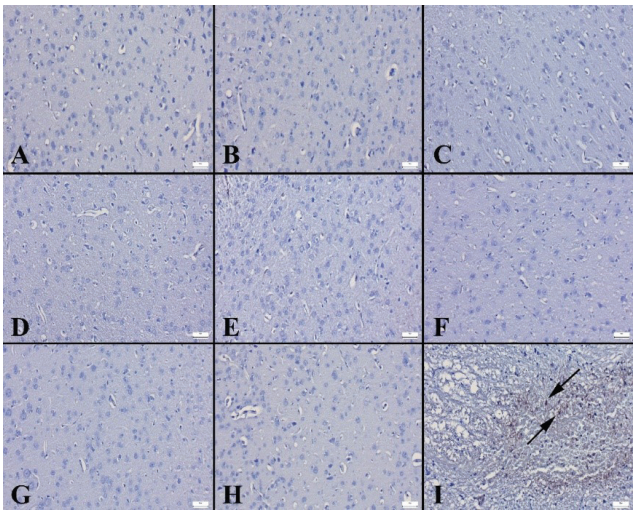

Figure 13
mTOR expressions in brains among the groups.
(A) Negative expressions in neurons in the sham group. (B) No expression in neurons in the prophylactic RF-EMF group. (C) Negative expressions in the prophylactic PMF group. (D) No expression in neurons in the prophylactic RF-EMF+PMF group. (E) Negative expression in the therapeutic RF-EMF group. (F) No expression in the therapeutic PMF group. (G) No expressions in the therapeutic RF-EMF+PMF group. (H) Negative expressions in the prophylactic+therapeutic RF-EMF+PMF group. (I) Moderately increased expressions in neurons (arrows) in the IS group. Streptavidin biotin peroxidase method, Scale bars=50µm.

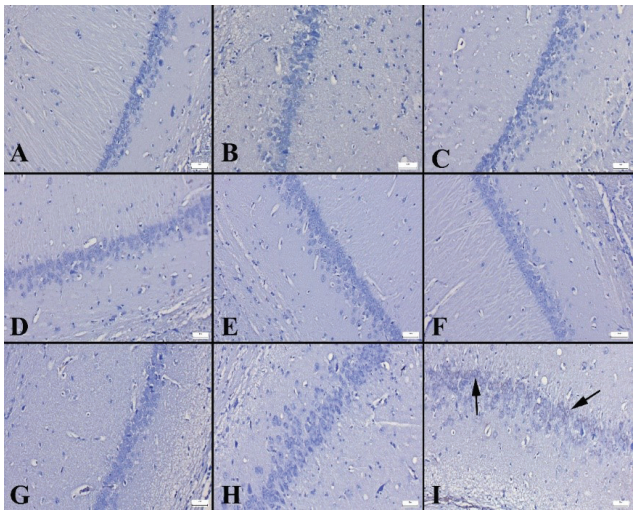

Figure 14
mTOR expressions in the hippocampus among the groups.
(A) Negative expressions in neurons in the sham group. (B) No expression in neurons in the prophylactic RF-EMF group. (C) Negative expressions in the prophylactic PMF group. (D) No expression in neurons in prophylactic RF-EMF+PMF group. (E) Negative expression in the therapeutic RF-EMF group. (F) No expression in the therapeutic PMF+IR group. (G) No expressions in the therapeutic RF-EMF+PMF group. (H) Negative expressions in the prophylactic+therapeutic RF-EMF+PMF group 8. (I) Moderately increased expressions in neurons (arrows) in the IS group. Streptavidin biotin peroxidase method, Scale bars=50µm.

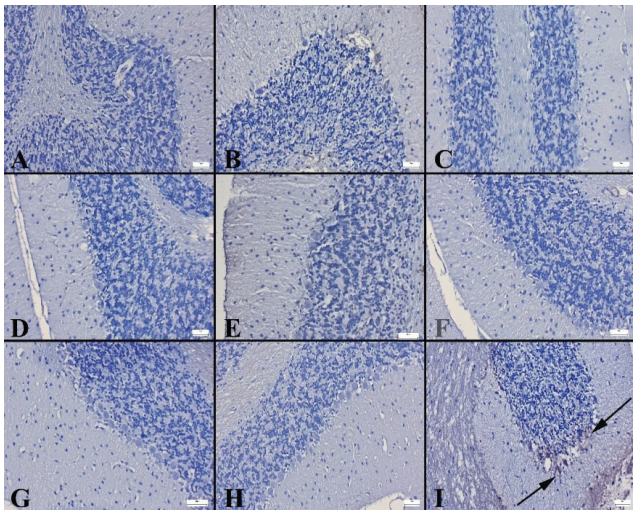

Figure 15
mTOR expressions in the cerebellum among the groups.
(A) Negative expressions in neurons in the sham group. (B) No expression in neurons in the prophylactic RF-EMF group. (C) Negative expressions in the prophylactic PMF group. (D) No expression in neurons in the prophylactic RF-EMF+PMF group. (E) Negative expression in the therapeutic RF-EMF group. (F) No expression in the therapeutic PMF group. (G) No expressions in the therapeutic RF-EMF+PMF group. (H) Negative expressions in the prophylactic+therapeutic RF-EMF+PMF group. (I) Moderately increased expressions in neurons (arrows) in the IS group. Streptavidin biotin peroxidase method, Scale bars=50µm.

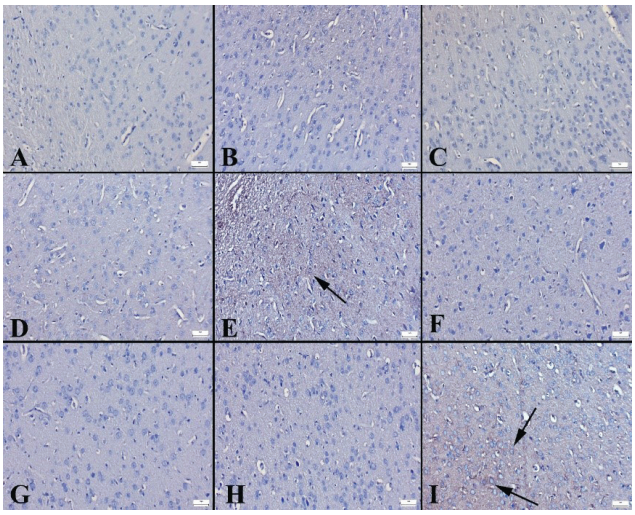

Figure 16
TNF-α expressions in brains between the groups.
(A) Negative expressions in neurons in the sham group. (B) No expression in neurons in the prophylactic RF-EMF group. (C) Negative expressions in the prophylactic PMF group. (D) No expression in neurons in the prophylactic RF-EMF+PMF group. (E) Slight expression in neurons (arrow) in the therapeutic RF-EMF group. (F) No expression in group therapeutic PMF. (G) No expressions in the therapeutic RF-EMF+PMF group. (H) Negative expressions in the prophylactic+therapeutic RF-EMF+PMF group. (I) Moderately increased expressions in neurons (arrows) in the IS group. Streptavidin biotin peroxidase method, Scale bars=50µm.

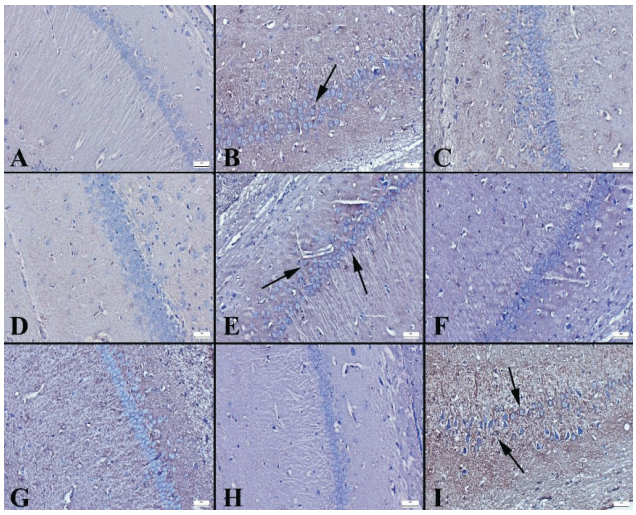

Figure 17
TNF-α expressions in the hippocampus between the groups.
(A) Negative expressions in neurons in the sham group. (B) Slight expression in neurons (arrow) in the prophylactic RF-EMF group. (C) Decreased expressions in the prophylactic PMF group. (D) No expression in neurons in the prophylactic RF-EMF+PMF group. (E) Moderate expression in neurons (arrows) in the therapeutic RF-EMF group. (F) Decreased expression in the therapeutic PMF group. (G) Slight expressions in the therapeutic RF-EMF+PMF group. (H) Negative expressions in the prophylactic+therapeutic RF-EMF+PMF group. (I) Marked increased expressions in neurons (arrows) in the IS group. Streptavidin biotin peroxidase method, Scale bars=50µm

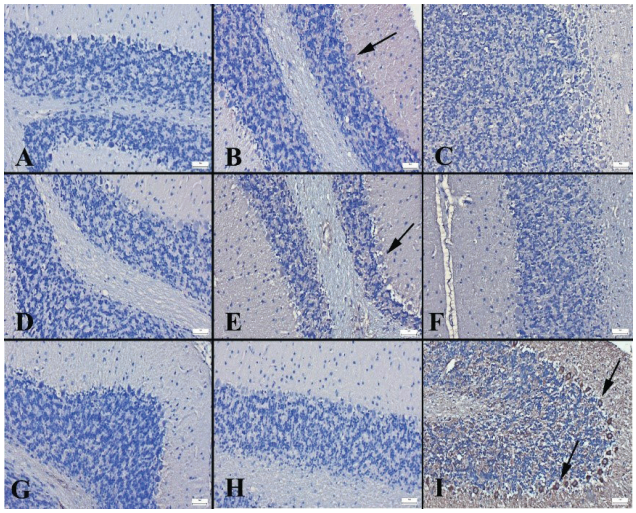

Figure 18
TNF-α expressions in cerebellums among the groups.
(A) Negative expressions in neurons in the sham group. (B) Slight expression in Purkinje cells (arrow) in the prophylactic RF-EMF group. (C) Decreased expressions in the prophylactic PMF group. (D) No expression in neurons in the prophylactic RF-EMF+PMF group. (E) Moderate expression in neurons (arrow) in the therapeutic RF-EMF group. (F) Decreased expression in the therapeutic PMF group. (G) Negative expressions in the therapeutic RF-EMF+PMF group. (H) No expressions in the prophylactic+therapeutic RF-EMF+PMF group. (I) Marked increased expressions in Purkinje cells (arrows) and neurons in the IS group. Streptavidin biotin peroxidase method, Scale bars=50µm.

immunohistochemical scores: [0] negative staining, [1] focal and weak staining, [2] diffuse and weak staining, and [3] diffuse and marked staining (18). In each section, 10 separate areas were examined under 40X objective magnification. The Database Manual Cell Sens Life Science Imaging Software System (Olympus Co., Tokyo, Japan) was used for microphotography and morphometric analysis.

Results

Histopathological and Immunohistochemical Findings of in Vivo Study

Histopathological Findings

At the histopathological examination of the normal cerebral cortex, hippocampus, and cerebellum tissue architecture was observed in the sham group. Marked pathological findings such as hyperemia, edema, hemorrhage, and neuronal degeneration were diagnosed in the IS group. Decreased severity of the lesions was observed in prophylactic and/or therapeutic RF-EMF and/or PMF applied groups. The most marked amelioration was noticed in the Prophylactic+Therapeutic RF-EMF+PMF group (Figure 4-6).

Immunohistochemical Findings

Examination of BDNF-stained cerebral cortex, hippocampus, and cerebellum sections revealed that while marked expressions were observed in the sham group, decreased expressions were noticed in the IS group. Increased expressions were observed between the prophylactic and/or therapeutic RF-EMF and/or PMF applied groups. The most marked increase was noticed in the Prophylactic+Therapeutic RF-EMF+PMF group (Figs. 7-9).

At the examination of iNOS immunostained slides, no or very slight expressions were observed in all groups except the IS group. In the ISgroup, slight expressions were noticed in cerebral cortex, hippocampus, and cerebellum neurons (Figs. 10-12).

During the examination of mTOR immunostained slides expressions were only observed in the hemorrhage areas in the IS group. Neurons expressed mTOR near the lesion, and expression decreased with the treatments (Fig. 13-15).

At the TNF-α immunostained slides, although negative expressions were observed in the sham group, increased expressions were noticed in the IS group. Negative expressions were seen in all the other groups except the Therapeutic RF-EMF group. Only slight expressions were noticed in the Therapeutic RF-EMF group (Fig. 16-18).

Discussion

Cerebrovascular diseases are a group of conditions associated with a high mortality rate and significant morbidity. These diseases can trigger cellular damage mechanisms such as inflammation, apoptosis, and necrosis due to hypoxia, which often results from the narrowing of blood vessels, including atherosclerosis, commonly affecting vascular structures. A decrease in oxygen supply to the distant part of a specific region may occur (19-21). Prolonged oxygen deficiency due to these conditions can progressively worsen, leading to clinical symptoms such as neurological deficits or cognitive impairments in patients (22, 23). Scientists are investigating various compounds with antioxidant, anti-inflammatory, anti-apoptotic, and anti-necrotic properties to protect brain tissues downstream of the occluded area (23, 24). In cases of vascular damage,

particularly in the endothelial layer, eNOS-mediated NO synthesis cannot occur, which impairs vascular tone and may lead to vasoconstriction (25, 26). Promoting endothelial proliferation, repairing vascular structures, and ensuring the continuity of vasodilation are crucial (27). Cerebral hypoperfusion can worsen clinical presentations, causing falls, strokes, cognitive impairment, and increased mortality (28). Nitric oxide (NO), synthesized from the amino acid arginine, plays vital roles as a vasodilator, neurotransmitter, immunomodulator, regulator of vasomotor tone, controller of cell proliferation, and inhibitor of platelet activation, adhesion, and aggregation (9, 11, 29, 30). Increasing the synthesis and secretion of NO from the intact endothelium, both proximal and distal to the occluded area, is crucial in reducing damage caused by ischemic lesions (31). Advanced molecular studies were planned as a result of the histopathological and immunohistochemical results obtained in this preliminary study, which aimed to validate the efficacy of the methods used individually or in combination, prior to conducting more detailed research.

In this preliminary study, the histopathological results of the single or combined prophylactic/therapeutic application of RF-EMF and PMF methods in the carotid artery occlusion model; the observation of hyperemic, hemorrhagic foci and inflammatory events with neuronal degeneration in the cerebral cortex, hippocampus, and cerebellum tissues. All of these findings show that the experimental model has been implemented. Observation of reductions in these markers has also shown that applications can protect tissues against damage with anti-inflammatory activity. As shown in the literature, PMF treatment had anti-inflammatory action against rotator cuff injury and osteoarthritis by decreasing inflammatory responses and enhancing cellular inflammatory mechanisms (33, 34).

Many biomarkers can be used to show progressive inflammatory reactions in tissues. It is known that the increase in prooxidant or proinflammatory substance synthesis is triggered especially in the distal of the occlusion. These substances increase the expression of some important cytokines through their receptors in the membrane of the cells (38). For example, the expression of TNF-α, an acute phase reactant, is generally increased in acute events or inflammatory lesions in the chronic active background (39). In this study, the TNF-α levels that increase with IS were reset in all groups, but some expression was observed only in the group that used RF-EMF for therapeutic purposes, indicating that RF-EMF alone could not provide a reducing but sufficient amount of reduction for acute ischemic conditions. In the prophylactic applications made in this study, it has also been proven that NO can be protected by increasing the readiness of the tissues against damage due to the stated effects of increased secretion before the lesion. In another study by Bragin et al., RF-EMF therapy attenuates high intracranial pressure-induced pathological microcirculatory changes, tissue hypoxia, blood-brain barrier degradation, and neuronal necrosis (40). In addition, the fact that this situation can be prevented at the maximum level in the combined application may be an indicator of why the methods used in this study were preferred together.

The increase in iNOS expressions, especially in the area of inflammation, in the ischemic group and the reduction of these expressions in all treatment groups may be another indication that the methods contribute to the anti-inflammatory effectiveness (41). In this situation, the vasodilator activity and the distribution of inflammatory cytokines that occur in the area of inflammation from the event site to the systemic circulation may play a role.

As it is known, when TNF-α binds to its own receptor on the cell surface, it can initiate apoptosis directly via enhancement of caspase-8 expressions, while it can also trigger autophagy through mTOR gene expressions (42, 43). The reduction of these increases in IS-related expressions detected within the scope of the preliminary study with RF-EMF and PMF applications may be secondary to a direct decrease in TNF-α levels, as well as inhibiting specific gene expression such as mTOR. This situation needs to be proved by more detailed molecular methods to be used in future studies.

It is known that BDNF levels, an important protective neurotrophic factor, may decrease in injury groups (44). The preservation of the expression levels of this important marker, whose expression is decreased in tissues due to neuroinflammation, in RF-EMF and PMF applied groups shows that the methods can provide neuroprotection with their anti-inflammatory activities. The preservation of BDNF expressions, especially in the hippocampal tissue, will be of great importance for the continuity of conditions that require intellectual capacity, such as learning and memory (45).

As a result, regression of the inflammatory scene due to IS in all three tissues with RF-EMF and PMF is important in terms of the occurrence of neurological deficits, the continuity of learning and memory mechanisms, and the preservation of balance functions. Considering the

results that have been discovered in this study, it is a fact that RF-EMF and PMF applications are applied in combination and more detailed studies with Western Blot or PCR methods are needed with performing water maze studies investigating learning and memory mechanisms. In our next study, a study in which the methods were applied together, in which the distal of the artery with carotid artery occlusion and the changes in the cerebral cortex on that side were examined with detailed analysis as mentioned above was planned and presented to the relevant units as a project.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
All experiments conducted in this study were carried out in accordance with the ARRIVE (Animal Research: Reporting in Live Experiments) 2.0 guidelines for animal research and were approved by the Suleyman Demirel University, Isparta Committee on Animal Research (Approval No. 2022-08/108).

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Availability of Data and Materials
Data can be requested from the authors.

Authors Contributions
RT: Planning of the study; Processing of Data; Experiment Planning; Experiment Implementation; Research; Methodology; validation; visualization; Writing the Article.

HA: Planning of the study; Experiment Planning; Experiment Implementation; Formal Analysis; Obtaining Financing; Research; Methodology; Project management; Provision of Resources; Audit; validation; Editing the article.

DU: Experiment Planning; Experiment Implementation; Methodology.

SA: Experiment Planning; Formal Analysis; Research; visualization; Writing the Article.

MDU: Experiment Planning; Experiment Implementation; Formal Analysis; Research; visualization; Writing the Article.

ASO: Planning of the study; Research; Methodology; Formal Analysis; visualization; Writing the Article.

SC: Study planning; Research; Methodology; Experiment Implementation; Formal Analysis; visualization; Writing the Article.

OO: Formal Analysis; Research; visualization; Writing the Article.

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