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Original Article *Özgün Araştırma*

PROGESTERONE INCREASES THE ISCHEMIC DAMAGE IN MALE RATS WITH CEREBRAL ISCHEMIA REPERFUSION INJURY

Progesteron Serebral İskemi Yapılan Erkek Ratlarda İskemik Hasarı Artırır

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ABSTRACT

Objective: In the current literature, there are few accepted pharmacological treatment methods for acute ischemic stroke. This study was conducted to investigate the effects of progesterone on transient ischemia / reperfusion injury in male rats.

Material and Methods: A total of 25 Wistar albino male and young rats were divided into 5 groups called Control group, acute stage groups (Sham-A and PRG-A), and chronic stage groups (Sham-C and PRG-C), randomly and their internal carotid arteries were compressed using temporary aneurysm clips for 30 minutes. At 4 hours after removal of the clips, progesterone was injected to the animals of the PRG-A and PRG-C group via intraperitoneal route. After sacrifice of all animals, pyknotic and necrotic neuronal cells were counted in hippocampal cornu amnonis (CA)1, CA2, CA3 and parietal cortical regions, histopathologically. Tissue interleukin (IL)-6, IL-10, caspase-3, and hypoxia-inducible factor-1 (HIF1) gene expression levels were evaluated using real time polymerase chain reaction assay.

Results: Histopathological and biochemical findings revealed that progesterone has no healing effects on ischaemic neuronal tissue damage in either acute or chronic period. Moreover, progesterone was found to significantly increase symptoms of ischaemia in both acute and chronic periods compared to healthy control group and even compared to Sham groups where I/R injury was applied and no experimental agent was administered.

Conclusion: At the end of this study, it was thought that progesterone had no therapeutic effect on cerebral ischemia / reperfusion injury in male sex rats and it could lead to increase it further, unfortunately.

***Keywords**: Progesterone, stroke, interleukin, HIF1, apoptosis*

ÖZ

Amaç: Günümüzde akut iskemik inme tedavisine yönelik literatürde çok az sayıda kabul edilmiş etkili farmakolojik tedavi yöntemi bulunmaktadır. Bu deneysel çalışma progesteronun erkek ratlarda oluşturulan geçici iskemi/ reperfüzyon hasarı üzerine olan etkilerini araştırmak amacıyla yapıldı.

Gereç ve Yöntemler: Kontrol grubu (n=5) dışında, 20 adet Wistar albino erkek sıçanı (Sham-A, Sham-C, PRG-A, PRG-C) dört gruba dağıtıldı ve geçici anevrizma klipleri 30 dakika boyunca internal karotid artere uygulandı. Klipler alındıktan dört saat sonra, PRG-A ve PRG-C gruplarına intraperitoneal olarak progesteron uygulandı. Tüm hayvanlar sakrifiye edildikten sonra, hipokampal cornu ammnonis CA 1, CA2, CA3 ve parietal korteksteki piknotik ve nekrotik nöronal hücreler histopatolojik olarak sayıldı. Ek olarak, doku interlökin (IL)-6, IL-10, kaspaz-3, HIF1 gen ekspresyon seviyeleri ve real time polimeraz zincir reaksiyonu değerlendirildi.

Bulgular: Progesteronun iskeminin hem akut hemde kronik döneminde iskemik nöronal doku hasarı üzerinde histopatolojik ve biyokimyasal veriler bakımından iyileştirici etkilerinin olmadığı görüldü. Hatta progesteronun iskemi bulgularını hem akut dönemde ve hem de kronik dönemde sağlıklı dokulara göre ve hatta sadece cerrahi girişim yapılıp hiçbir deneysel ajan verilmeyen deney gruplarına göre belirgin şekilde arttırdığı gözlendi.

Sonuç: Bu çalışma sonunda progesteronun erkek cinsiyetteki ratlarda oluşturulan serebral iskemi/reperfüzyon hasarında tedavi edici etkinliğinin olmadığı ve bu hasarı daha da artırdığı düşünüldü.

***Anahtar Kelimeler**: Progesteron, inme, interlökin, HIF1, apopitozis*

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INTRODUCTION

In recent literature, although the most of studies have aimed to develop the new neuroprotective treatment modalities to reduce the progression of neuronal damage in acute ischaemic stroke, there are found very few accepted effective pharmacological treatment methods (such as tissue plasminogen activator). Unfortunately, several promising drugs have been evaluated as unsuccessful in clinical trials because of side-effects and/or therapeutic limitations (1,2). In various experimental ischaemic injury and cerebral trauma models, it has been shown that progesterone, which is an endogenous steroid hormone, could have pharmacotherapeutic effects. These studies have demonstrated that progesterone reduced the cerebral adenosin and excitator amino acid response and had an antioxidant and "scavenger" effect (2-5). In addition, progesteron could reduce the brain infarct by possibly modulating expressions of some proteins (such as vascular endothelial growth factor, glial fibrillar acidic protein, and matrix metalloproteinase-9). It also may repaire the blood-brain barrier by decreasing MMP expressions and may reduce the apoptosis by modulation of the PI3K/Akt pathway (3-5).

Despite all these beneficial effects, several experimental studies conducted with male rats and ovariectomised female rats demonstrated that progesterone could have negative effects on acute ischaemic injury and would lead to a worse outcome (6-9).

In this experimental study, it was aimed to explore the effects of progesterone on transient ischaemia/reperfusion (I / R) injury created in male rats.

MATERIALS AND METHODS

For use of animal subjects, approval for the study was granted by Local Ethics Committee (Date: 15.04.2015; Decision No: 15/42) and all study procedures were constructed with this approval. The minimum number of animals necessary for statistical analysis to be applied in the testing of the drug was defined as 25.

In this study, progesterone (Progestan 50 mg/ mL, Koçak Farma, Turkey) was administered intraperitoneally at the dose of 8 mg/kg.

The animals were randomly separated into groups as described below:

Control group (n=5): This group was consisted of healthy subjects on which I/R injury was not performed and no pharmacological agent was administered. (This group was constructed to show that how close the therapeutic effect of the progesterone compares to the normal, healthy brain and to determine the efficacy of the ischemic injury created in the stroke model of this study)

Changes in stroke in the acute period (i.e. 3 days after the surgical procedure) were examined in 2 sub-groups:

SHAM-A group (n=5): I/R injury was applied but no experimental agent was administered to the subjects of this group.

PRG-A group (n=5): I/R injury was applied and intraperitoneal 8 mg/kg/day progesterone was injected for three days to the subjects of this group.

Changes in stroke in the chronic period (i.e. 10 days after the surgical procedure) were examined in 2 sub-groups:

SHAM-C group (n=5): I/R injury was applied but no experimental agent was administered to the subjects of this group.

PRG-C group (n=5): I/R injury was applied and intraperitoneal 8 mg/kg/day progesterone was injected for ten days to the subjects of this group.

For sedational anaesthesia, 40 mg/kg ketamine (Ketalar®; Pfizer) and 5 mg/kg xylazine (Rompun® %2; Bayer) were administered via intraperitoneal route.

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Surgical Procedure

At the experimental stage, sedational anaesthesia with anaesthetic drugs was administered to the animals via the intramuscular route. Then, a vertical incision was made on the neck of each animal, both internal carotid arteries were exposed, and these vessels were compressed for 30 minutes using temporary aneurysm clips (Figure 1).

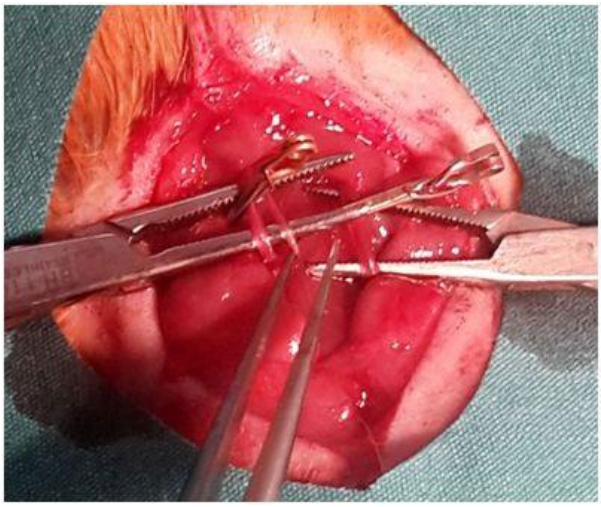

Figure 1: Both internal carotid arteries were exposed, and these vessels were closed for 30 minutes using aneurysm clips

At the end of 30 minutes, the clips were removed and blood flow was again permitted in these vessels. The rats were then aroused from the sedation anaesthesia and kept at normal room temperature for the duration of the study. When 4 hours had elapsed after the taken of the clips, 8 mg / kg progesterone was given to PRG-A and PRG-C subjects intraperitoneally as a single dose daily. (In clinical experience, many of the patients have admitted to the hospital 4 hours after the occurence of the stroke. For this reason, it was considered injecting the progesterone to the animals 4 hours after the clippage to mimic clinical practice.)

Throughout the study, the rats were kept in separate group cages at normal room temperature with a 12-hour light and dark cycle and free access to food and drinking water.

At the end of the sheduled time, under sedation anaesthesia all subjects were sacrificed by exsanguination from the aorta. The subjects were then decapitatied and their brain tissues were removed.

Right hippocampus taken from each animal for histopathological examination was fixed in 10% neutral buffered formalin at room temperature and left hippocampus taken for real-time polymerase chain reaction (PCR) gene expression analysis was immediately frozen at -30˚C and stored until assay.

Histopathological Examination

The tissue samples were dehydrated after 48 hours and embedded in paraffin blocks, from which slices 4-5µm in thickness were then cut. These slices were stained using the routine hematoxyline and eosin (H&E) staining method and slides obtained were evaluated with a binocular microscope (Olympus BX51; Olympus, Tokyo, Japan) by a pathologist who blinded to the experimental groups and the agent substances under investigation.

Pyknotic and necrotic neurons were counted and photographed in the hippocampus cornu ammonis (CA) 1, CA2, CA3 sections and the adjacent parietal cortex areas of these preparates.

PCR Gene Expression Analysis

For PCR gene expression analysis, the tissue samples kept at -30˚C were homogenised in a homogenisator. The hydrolysis probes master PCR mix (Hydrolysis Probes Master and SYBR Green Master, Roche® Molecular Biochemicals) prepared from the tissue homogenates were placed into the PCR array (LightCycler 480, Roche® Molecular Biochemicals) and gene-expression levels of the caspase-3, hypoxia-inducible factor-1 (HIF1), interleukin (IL)-6 and IL-10 levels in the injured brain tissue were obtained according to the manufacturer's data sheet.

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Statistical Analysis

The optimal number of animals was determined by the minimum number of animals allowed as stipulated by the ethical rules. Additionally, this number was determined to be the lowest number that can be analyzed statistically.

From the data of the acute period, parietal cortex and CA1, CA2, CA3 necrotic neuron count results and the IL-6, IL-10 and HIF1levels and from the chronic period data, IL-6, IL-10 and HIF1 levels were seen not to have normal distribution between the groups and were not homogenous. In the statistical analyses of the findings, the difference among the groups was determined using the Kruskall-Wallis test, and in the comparison of differences between paired groups, the Mann Whitney U test and Bonferroni Correction test were used ($p < 0.05$ and $p < 0.017$, respectively). The caspase-3 levels in the acute period and the parietal cortex, CA1, CA2, CA3 necrotic neuron count and caspase-3 levels in chronic period were determined to be normally distributed between the groups and were homogenous. To determine the difference among groups, One-Way Analysis of Variance (ANOVA) test was applied ($p < 0.05$). To compare the differences between the paired groups, Tukey Multiple Comparisons test and Bonferroni Correction test was used ($p < 0.017$). The acute and chronic period findings of each group were compared using Paired Samples t test and Wilcoxon Signed Ranks test ($p<0.05$).

RESULTS

Light Microscope

On histopathological examination of control group, no oedema or inflammatory cell infiltration was seen at the neuropil or perivascular region of the hippocampus and parietal cortex of the brain. However, severe oedema around vascular structures and deterioration of typical cellular arrangement with an elevated number of pyknotic neurons were seen on histopathological slices of the Sham-A and Sham-C groups. Neuronal necrosis and perivascular oedema in the PRG-A and PRG-C groups were observed much more than in Sham-A and Sham-C groups. (Figure 2, Figure 3).

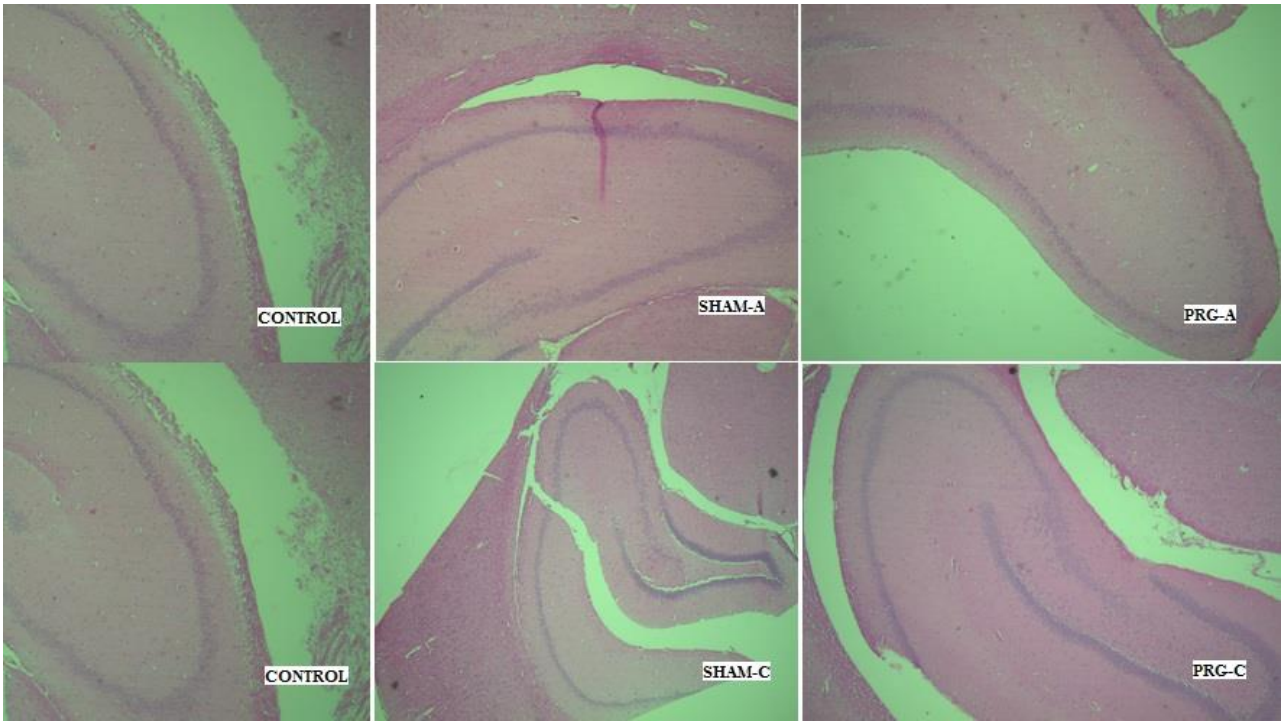

Figure 2: Figure demonstrates the histopathological microphotographs of hippocampal layers for each group

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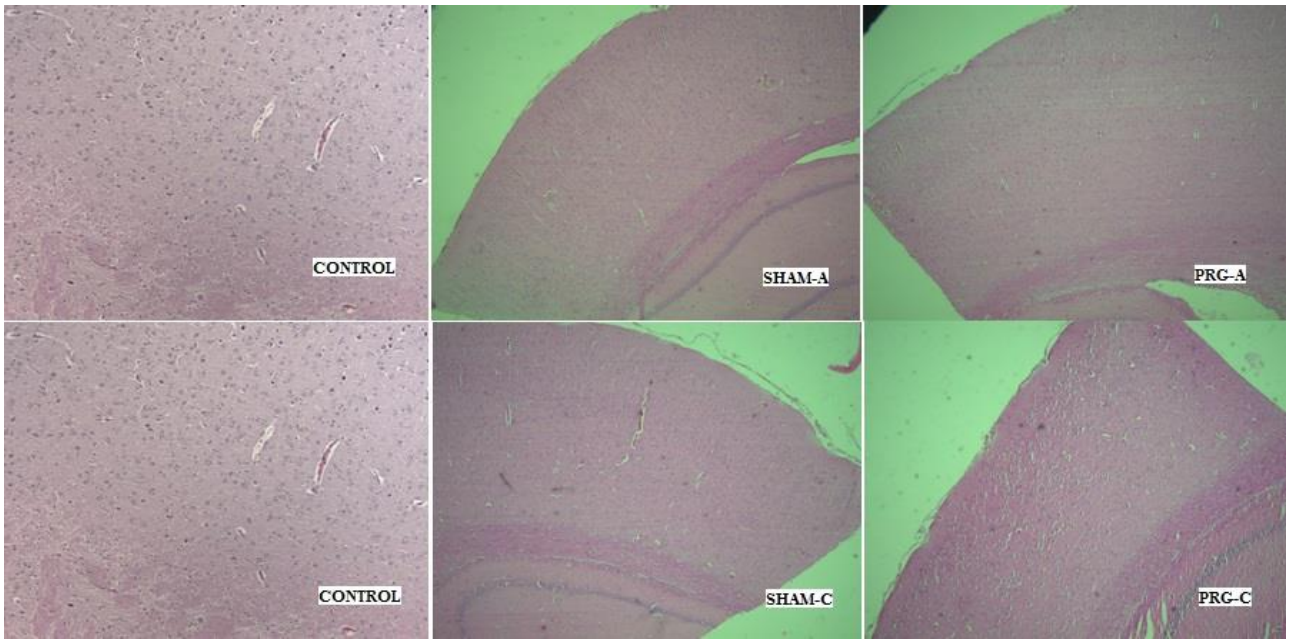

Figure 3: Figure shows the histopathological microphotographs of parietal cortex of each group.

Histopathological Examination

Acute period findings: The necrotic neuron count results in the CA1, CA2, CA3 areas of the hippocampus, and the parietal cortex were found to be different among the groups (X^2=11.818, p=0.003; X^2=11.681, p=0.003; X^2=1.791, p=0.003 and X^2=12.727, p=0.002, respectively) (Table 1, Table 2) (Figure 4).

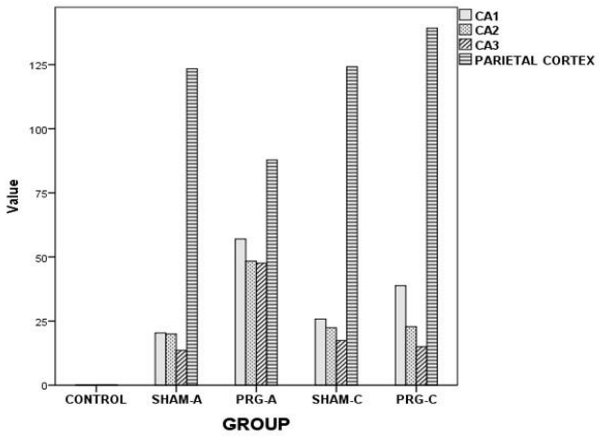

Figure 4: Each bar presents count values of necrotic and pyknotic neurons at parietal cortex and hippocampus. (CA: cornu ammonis)

Table 1: Table demonstrates the necrotic neuron count values and PCR gene expression analysis results of the groups (CA: Cornu ammonis, HIF: hypoxia-inducible factor, IL: interleukin)

GROUP	Variable	Minimum	Maximum	Mean (†) / Median	Standard deviation
CONTROL	CA1	0	0	0	0.00
	CA2	0	0	0	0.00
	CA3	0	1	0	0.45
	PARIETAL CORTEX	0	1	0	0.45
	CASPASE3	0.00	0.12	0.08†	0.04
	HIF1	0.98	2.27	1.74	0.52
	IL6	0.00	0.85	0.11	0.38
	IL10	0.00	0.00	0.00	0.00
SHAM-A	CA1	15	36	17	8.79
	CA2	16	24	19	3.81
	CA3	6	24	10	8.32
	PARIETAL CORTEX	110	137	125	10.36
	CASPASE3	0.01	0.24	0.14†	0.10
	HIF1	0.22	11.18	5.29	4.06
	IL6	0.00	0.05	0.03	0.02
	IL10	0.00	0.00	0.00	0.00
PRG-A	CA1	33	120	36	37.21
	CA2	23	112	30	37.57
	CA3	17	110	36	36.57
	PARIETAL CORTEX	75	95	88	7.98
	CASPASE3	0.00	0.12	0.05†	0.05
	HIF1	0.00	4.22	2.73	1.63
	IL6	0.00	0.03	0.02	0.01
	IL10	0.00	0.00	0.00	0.00
SHAM-C	CA1	17	45	25.80†	11.08
	CA2	12	34	22.40†	8.65
	CA3	10	26	17.40†	5.98
	PARIETAL CORTEX	106	162	124.20†	22.54
	CASPASE3	0.05	0.18	0.13†	0.05
	HIF1	0.04	11.65	4.29	4.74
	IL6	0.00	0.00	0.00	0.00
	IL10	0.00	0.00	0.00	0.00
PRG-C	CA1	22	65	38.80†	19.41
	CA2	13	39	22.80†	11.05
	CA3	8	26	15.00†	7.68
	PARIETAL CORTEX	128	151	139.20†	9.49
	CASPASE3	0.00	0.08	0.03†	0.03
	HIF1	0.00	1.99	0.66	0.81
	IL6	0.00	0.01	0.00	0.00
	IL10	0.00	0.00	0.00	0.00

(†) Mean Value

Table 2: Table shows the comparison results of the histopathological and biochemical findings of the groups. *One Way-Analysis of Variance test and Kruskall-Wallis test, p<0.05* (CA: cornu ammonis, HIF: hypoxia-inducible factor, IL: interleukin, F=F score, X^2=Chi-Square score)

	Acute stage		Chronic stage	
Variable	**F (†) / X^2**	**p**	**F (†) / X^2**	**p**
CA1	11.818	0.003	11.714†	0.002
CA2	11.681	0.003	12.966†	0.001
CA3	11.791	0.003	13.701†	0.001
PARIETAL CORTEX	12.727	0.002	145.849†	<0.001
CASPASE-3	2.315†	0.141	6.997†	0.010
HIF1	4.820	0.090	5.049	0.080
IL6	1.460	0.482	7.596	0.022
IL10	0.156	0.925	0.426	0.808

(†) F value

Table 3: Paired group comparison results of the histopathological and biochemical findings of the groups. *Tukey Multiple Comparisons test, Mann-Whitney U test and Bonferroni Correction test, p <0.017* (CA: cornu ammonis, HIF: hypoxia-inducible factor, IL: interleukin, MD: mean difference, Z: Z score)

		Acute stage		Chronic stage	
Group (I/ J)	**Variable**	**Z**	**p**	**MD (†) / Z**	**p**
CONTROL /SHAM	CA1	-2.78	0.005	-25.80†	0.021
	CA2	-2.79	0.005	-22.40†	0.002
	CA3	-2.69	0.007	-17.20†	0.001
	PARIETAL CORTEX	-2.69	0.007	-124.00†	<0.001
	CASPASE-3	-	-	-0.05†	0.199
	IL6	-	-	-2.62	0.009
CONTROL/ PRG	CA1	-2.78	0.005	-38.80†	0.001
	CA2	-2.78	0.005	-22.80†	0.002
	CA3	-2.69	0.007	-14.80†	0.004
	PARIETAL CORTEX	-2.69	0.007	-139.00†	<0.001
	CASPASE-3	-	-	0.05†	0.181
	IL6	-	-	-1.78	0.075
SHAM/ PRG	CA1	-2.09	0.036	-13.00†	0.286
	CA2	-2.00	0.045	-0.40†	0.997
	CA3	-2.19	0.028	2.40†	0.782
	PARIETAL CORTEX	-2.61	0.009	-15.00†	0.253
	CASPASE-3	-	-	0.10†	0.007
	IL6	-	-	-0.97	0.332

(†) Mean difference value

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In the comparison of the paired groups, the necrotic neuron count values of the hippocampal layers and the parietal cortex were statistically different between the Control and Sham-A groups and between the Control and PRG-A groups ($p < 0.017$) (Table 3). The necrotic neuron count results of the parietal cortex were statistically different only between the Sham-A and PRG-A groups ($Z = -2.61$, $p = 0.009$).

Chronic Period Findings: The results of the necrotic neuron count in the CA1, CA2, CA3areas of the hippocampus and in the parietal cortex were different among the groups ($F = 11.714$, $p = 0.002$; $F = 12.966$, $p = 0.001$; $F = 13.701$, $p = 0.001$ and $F = 145.849$, $p < 0.001$, respectively) (Table 1, Table 2) (Figure 4).

Binary comparison results of the groups showed that necrotic neuron count values of the hippocampus and the parietal cortex were different between the Control and Sham-C groups and between the Control and PRG-C groups. Between the Sham-C and PRG-C groups, no statistically significant difference was determined (Table 3).

PCR Gene Expression Analysis

Acute period findings: Tissue caspase-3, HIF1, IL-6 and IL-10 gene expression level values were not different among the groups (Table 1, Table 2, Table 3) (Figure 5).

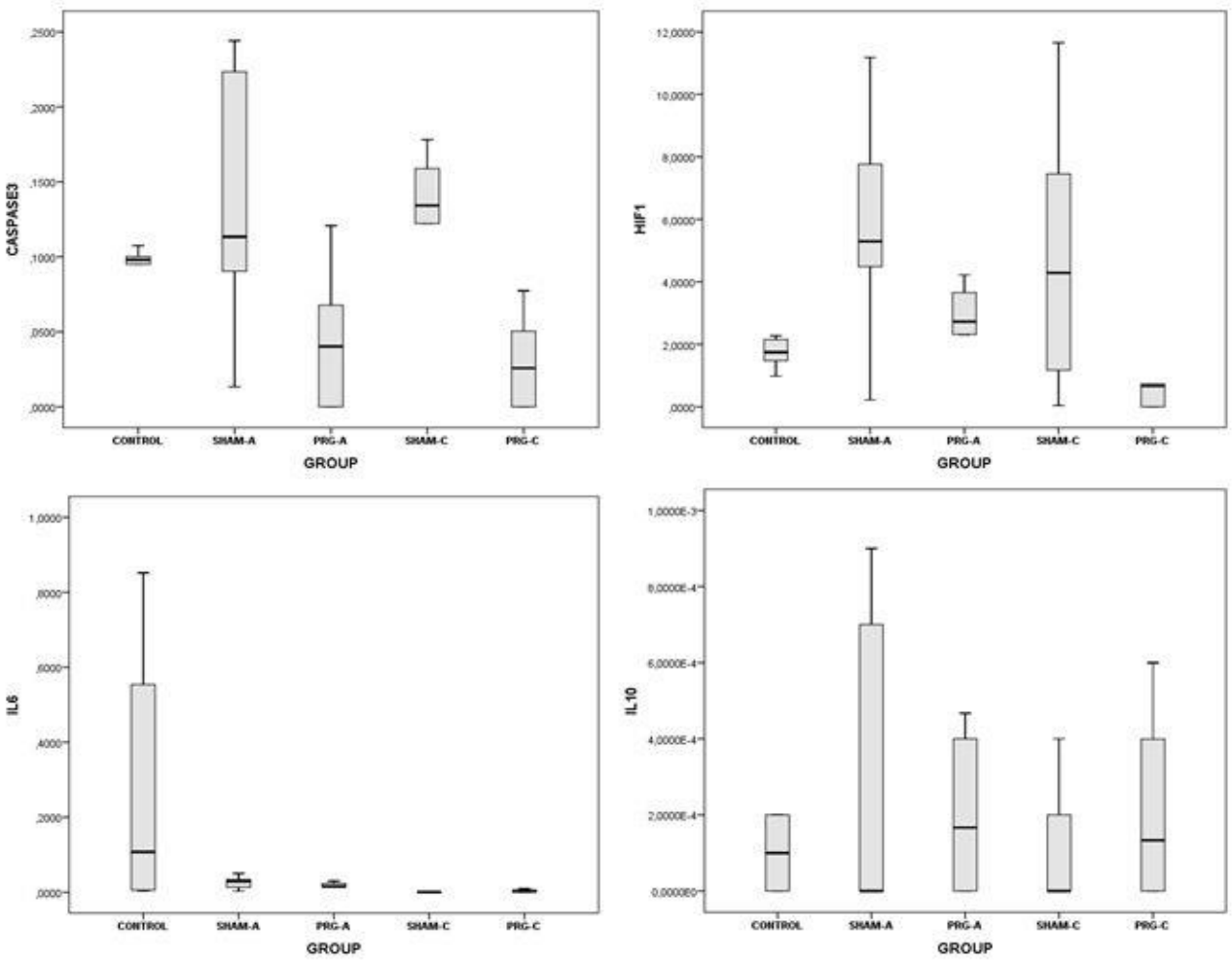

Figure 5: Bars represent the tissue caspase-3, HIF1, IL-6 and IL-10 gene expression level values i of both periods groups. (IL: interleukin, HIF: hypoxia-inducible factor)

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Chronic period findings: The caspase-3 and IL-6 gene expression levels examined in the cerebral tissues were found statistically different among the groups (F = 6.997, p = 0.010; X^2 = 7.596, p = 0.022) (Table 1 and 2) (Figure 5). In binary comparison of groups, a significant difference was found in respect of caspase-3 level values between the Sham-C and PRG-C groups (F = 0.010, p = 0.007) and in respect of IL-6 level values between the Control and Sham-C groups (Z = -2.62, p= 0.009) statistically (Table 3).

The Comparison Results of the Acute and Chronic Period Findings for Each Group

In the comparison of the results of the administered progesterone groups, a difference was determined in the necrotic neuron count in the parietal cortex (Z=-2.619, p=0.009). These findings suggested that progesterone increased the number of necrotic neurons in the parietal cortex in the chronic period of ischaemic injury. As a result of the comparison of the findings of Sham groups, the results of IL-6 gene expression level were observed to be different (Z = -2.619, p = 0.009) (Table 4).

Table 4: Table demonstrates the comparison results of the acute period and chronic period findings for each group. *Paired Samples t test and Wilcoxon Signed Ranks test, p <0.05* (CA: cornu ammonis, HIF: hypoxia-inducible factor, IL: interleukin, t: t score, Z: Z score)

Variable	PRG-A/ PRG-C		SHAM-A/ SHAM-C	
	t (†) / Z	p	t (†) / Z	p
CA1	-1.149	0.251	-1.467	0.142
CA2	-1.567	0.117	-0.424	0.671
CA3	-2.193	0.028	-0.838	0.402
PARIETAL CORTEX	-2.619	0.009	-0.522	0.602
CASPASE-3	-0.323†	0.746	0.154†	0.881
HIF1	-1.798	0.072	0.522	0.602
IL6	-1.798	0.072	-2.619	0.009
IL10	-0.108	0.914	-0.471	0.638

(†) t value

DISCUSSION

Alkayed et al reported that ovariectomized female rats had infarct of the same dimensions as that of male rats. With this result, it was thought that the protection of nerve tissue in females was most probably due to estrogen, originating from an activity of female sex steroids. At the end of the study, it was thought that female animals with normal hormone levels experienced higher tissue perfusion during ischaemia. It was suggested that after ischaemic brain damage in female rats, more positive results were obtained because of the probable neuroprotective and blood flow protective effects of estrogen (6). Moreover, Murphy et al reported that in ovariectomized female rats, progesterone treatment exogenously administered in the range of physiological and supraphysiological peak plasma levels could not improve brain damage following vascular stroke. It was stated in this study

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that chronic hormone exposure at high doses could even worsen the infarct volume. Thus, with these findings, they suggested that it had no neuroprotective effect to the ischaemic brain of the female rat when a single dose of progesterone was applied and there was a harmful effect of exogenous steroid in experimental stroke associated with dose or length of exposure. In addition, they reported that only chronic dose progesterone application caused striatal damage and therefore the duration of exposure could be important, and peak steroid levels were also stated to be important (7). Additionally, in a study by Coomber et al, there was neither a beneficial nor a harmful effect on the damage formed after focal cerebral ischaemia with the administration of progesterone alone at physiological doses. It was concluded that progesterone at physiological doses administered continuously before cerebral ischaemia in previously ovariectomized female rats was neither benficial nor harmful (8). In support of those findings, in a study by Wong et al it was stated that although progesterone could reduce the volume of the ischaemic lesion in adult ovariectomized females, the incidence of stroke-related death was increased. In the emergence of negative effects in these subjects, it was recommended that the levels of endogenous hormone reserves should be taken into consideration (9).

From examination of the histopathological findings of the present study, the progesterone was seen to have significantly increased the symptoms of ischaemia in both the acute and chronic periods compared to healthy tissue and compared to the sham group where only an ischemia reperfusion injury was created and no pharmacological agent was administered. Progesterone administered in the chronic period in particular was determined to have increased neuronal necrosis in ischaemic tissue and especially in the parietal cortex more than in the acute period. In fact, it was considered that the progesterone provided no protection to the neurons in both the hippocampus and the adjacent parietal cortex in the acute and chronic periods of cerebral ischaemia/reperfusion injury, and even increased neuronal death in these brain areas.

It has been generally considered in literature that HIF1 is the important factor of cellular response against to the hypoxia and it could enhance and promote many target genes and it can also promote apoptotic cell death (10). On the other hand, recent studies have pointed out that neuronal apoptosis can be promoted by IL-6. Moreover, IL-6 may aggravate the injury of the brain tissue in the stroke patient, whereas IL-10 can prevent neuronal apoptosis and promote the repair of the cells (11-13). It has been demonstrated that suppression of HIF1 and IL-6 by progesterone early after hypoxia represents neuroprotective effect (2,14).

When the PCR gene expression findings of this study were reviewed, it was seen that in the acute and chronic period of cerebral ischaemia/reperfusion injury, progesterone seemed to decrease the caspase-3 and HIF1 levels in the cerebral tissues compared to the sham group but this finding was not statistically significant. Furthermore, progesteron did not affect the IL-10 level values in both periods. Moreover, the histopathological examination results supported these findings, and in the light of all these findings, it can be considered that progesterone has no therapeutic effect on the cerebral ischaemia reperfusion injury model created in male rats and it could possibly have exacerbated the ischaemic injury.

There were some limitations in this study. First, the further biochemical analyses methods (such as. Western Blot) and histopathological analysis methods (such as electron microscopy, immunofluorescent microscopy immunohistochemistry) that could shed light on the mechanisms of the effect of progesterone were not used because of financial restrictions. Secondly, because the progesterone given at 8 mg/kg at 4 h after ischemia failed to improve the cells and the cytokines, it is strongly suggested that progesterone at various doses, administered in various ways (e.g.,

intrathecal) and at various times should be tested in future studies. A third limitation was that female and/or ovariectomized rats where ischaemia/ reperfusion injury was created were not included in this study, and therefore the the effects of progesterone obtained from male rats could not be compared with these groups. Fourth limitation, the serum oestrogen and progesterone levels of the animals were not tested before and after the injury model. Final limitation was that because of the short study duration, no functional test was performed to the rats to determine their neurological deficit level (15). Therefore, it could be strongly suggested that these functional tests should be assessed in ongoing experiments.

In conclusion, the results of this study showed that progesterone did not reduce the effects of ischemia / reperfusion injury in the male rat brain and could even increase it.

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