Bosphorus Medical Journal
Boğaziçi Tıp Dergisi

Original Article

DOI: 10.14744/bmj.2022.87049

Bosphorus Med J 2023;10(1):19–25

The Role of Progesterone in the Regulation of miR-30a and miR-143 in Rat Models of Subarachnoid Hemorrhage: A Study of Dose-Related Effects

Subaraknoid Kanama Modellerinde miR-30a ve miR-143'ün Düzenlenmesinde Progesteronun Rolü: Doza Bağlı Etki Üzerine Bir Çalışma

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Cite this article as: Yavuz AY, Ari BÇ, Çelik SE. The Role of Progesterone in the Regulation of miR-30a and miR-143 in Rat Models of Subarachnoid Hemorrhage: A Study of Dose-Related Effects. Bosphorus Med J 2023;10(1):19–25.

Received: 31.08.2022
Revision: 31.08.2022
Accepted: 21.09.2022

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ABSTRACT

Objectives: Brain damage in the early-period after subarachnoid hemorrhage (SAH) is a significant cause of mortality; however, cell proliferation, apoptosis, and inflammatory cascades play roles in preventing. This study aimed to determine the effect of progesterone on miR-30a and miR-143 expressions after SAH.

Methods: The research was conducted on 40 Sprague-Dawley rats. They were assigned to four groups: Group 1 with SAH (n=10); Group 2, SAH+8mg progesterone (n=10); Group 3, SAH+10mg progesterone (n=10); and Group 4, the controls (n=10). Experimental SAH was created by cisternal injection model. RNAs were isolated from brain tissues and miRNA expression levels were determined by Quantitative Real-Time PCR.

Results: There was a 176-fold and 126-fold decrease in miR-30a and miR-143 expressions of male rats with SAH ($p<0.05$). In the male rats administered with 8 mg and 16 mg progesterone, 39-fold and 2.4-fold decreases were found in miR-30a expressions ($p<0.05$). In the expression levels, there were a 2.3-fold decrease and a 15-fold increase in miR-30a and miR-143 in females ($p<0.05$). In the female rats administered with 8 mg and 16 mg progesterone, a 1.25-fold increase and 4.5-fold decrease were found in miR-30a expressions ($p<0.05$); however, 1400-fold and 400-fold increases were found in miR-143 expressions ($p<0.05$) on the females with 8 mg and 16 mg progesterone.

Conclusion: The decrease in miR-30a and miR-143 expressions in the males with SAH approached the normal limits by increasing doses of progesterone. We think that the molecular basis of this dose-dependent effect of progesterone in miR-30a and miR-143 expressions should be investigated in further studies.

Keywords: Acute brain injury; miR-143; miR-30a; neuroprotection; progesterone; subarachnoid hemorrhage.

ÖZET

Amaç: Subaraknoid kanama (SAK) sonrası erken dönemde beyin hasarı önemli bir mortalite nedenidir ancak hücre proliferasyonu, apopitoz, inflamatuvar kaskadların önlenmesinde rol oynar. Bu çalışmada, SAK sonrası miR-30a ve miR-143 ekspresyonu üzerine progesteronun etkisinin belirlenmesi amaçlandı.

Yöntem: Araştırma, 40 Sprague-Dawley sıçanı üzerinde gerçekleştirildi. Dört gruba ayrıldılar: SAK'lı Grup 1 (n=10); Grup 2, SAK+8 mg progesteron (n=10); Grup 3, SAK+10 mg progesteron (n=10); ve Grup 4, kontroller (n=10). Deneysel SAK, sarnıç enjeksiyon modeli ile oluşturuldu. RNA'lar beyin dokularından izole edildi ve miRNA ekspresyon seviyeleri Quantitative real-time polimeraz zincir reaksiyonu ile belirlendi.

Bulgular: SAK'lı erkek sıçanların miR-30a ve miR-143 ekspresyonlarında 176 kat ve 126 kat azalma oldu ($p<0,05$). 8 mg ve 16 mg progesteron uygulanan erkek sıçanlarda miR-30a ekspresyonlarında 39 kat ve 2,4 kat azalma tespit

edildi (p<0,05). Ekspresyon düzeylerinde kadınlarda miR-30a ve miR-143'te 2,3 kat azalma ve 15 kat artış vardı (p<0,05). 8 mg ve 16 mg progesteron verilen dişi sıçanlarda miR-30a ekspresyonlarında 1,25 kat artış ve 4,5 kat azalma bulundu (p<0,05); ancak 8 mg ve 16 mg progesteron ile kadınlarda miR-143 ekspresyonlarında 1.400 kat ve 400 kat artışlar (p<0,05) bulundu.

Sonuç: SAK'lı erkeklerde miR-30a ve miR-143 ekspresyonlarındaki azalma, progesteron dozlarının artmasıyla normal sınırlara yaklaşmıştır. Progesteronun miR-30a ve miR-143 ekspresyonlarındaki bu doza bağımlı etkisinin moleküler temelinin ileri çalışmalarda araştırılması gerektiğini düşünmekteyiz.

Anahtar sözcükler: Akut beyin hasarı; miR-30a; miR-143; nöroproteksiyon; progesterone; subaraknoid hemoraji.

Subarachnoid hemorrhage (SAH) occurs in the subarachnoid space due to arterial problems. Early brain damage, which may arise in the first 3–4 days after SAH, is one of the most important causes of morbidity and mortality, and weakens the prognosis as well.[1] The pathophysiological mechanisms for this process include widespread ischemia, dysfunction in the blood-brain barrier (BBB), and neural cell death caused by the activation of inflammatory and oxidative cascades that follow increased intracranial pressure or decreased cerebral blood flow.[2] Progesterone has been shown to be a neuroprotective steroid that reduces brain edema in the first 24 h after SAH, helps repair the BBB, reduces the risk of early brain damage by reducing the expression of matrix metalloproteinase-9 (MMP-9) and caspase-3.[3-5]

MicroRNAs (miRNA) are small (20–24 nucleotides) but functional RNAs that regulate protein synthesis.[6] Although they are known to have metabolic activities, there is limited knowledge about their interactions with progesterone receptors (PR) on neurons and glial cells in the central nervous system (CNS).[7] It has been found that miRNAs are involved in several genetic regulatory mechanisms for cell proliferation, differentiation, necrosis, and apoptosis in the CNS; animal studies have shown that their expression levels vary in cerebral ischemia models. It is known that the increase or decrease in miRNA levels is effective in the prevention and treatment of hemorrhagic stroke.[8] Based on these observations, miRNAs were also examined in the pathophysiology of early brain damage after SAH and were shown to have potential effects.[3,9] Studies have demonstrated that progesterone regulates the expression of various miRNA in different cell types.[10-12] Although the decreases in progesterone receptor expression were associated with a reduction of miR-30a expression, there are no studies on the effects of progesterone on miR-30a expression.[13] It has been shown that progesterone regulates the expression of miR-143 and cell proliferation in granulosa and endometrial epithelial cells.[10-12] It is critical to determine the effects of progesterone on miR-30a and miR-143 expression in different disease groups to reveal the relationships between progesterone and these miRNAs. Therefore, this study investigated the effects of progesterone administration on miR-30a and miR-143 expression after SAH.

Methods

Subjects

This study involved a total of 40 Sprague-Dawley rats (20 male and 20 female), which weighed 270–300 g, were aged 6–8 weeks, and were housed in Aziz Sancar Institute of Experimental Medicine Laboratory at Istanbul University. The rats had free access to pellet food and water and were kept in a room with controlled temperature (22–25°C) and light/dark cycle (12:12 h). The animals were divided into four groups: Three experimental groups and one control group, each consisting of 10 animals in Figure 1. In Group 1, rats were subjected to SAH. In Group 2, rats were subjected to SAH and were given 8 mg progesterone subsequently. In Group 3, rats were subjected to SAH and were given 16 mg progesterone subsequently. Group 4 was the control group with no SAH or progesterone administered. For general anesthesia, 50 mg/kg ketamine hydrochloride and 10mg/kg xylazine hydrochloride were administered to all animals before the surgical procedures. Then, the rats were subjected to SAH through the intracisternal injection model. Standardization was achieved in the control group by injecting 0.15cc of 0.9% saline solution into the cisterna magna of the animals in 30 s. Progesterone was administered to the rats at the 0th h through the intraperitoneal route. Vital signs, respiration, and circulation were monitored for 6 h in all groups. After the follow-up period, euthanasia and decapitation were performed to conduct molecular analyses on brain tissues. The surgeries were conducted by experts in Aziz Sancar Institute of Experimental Medicine Laboratory at Istanbul University and the Department of Neurosurgery in Okmeydani Research and Training Hospital at the University of Health Sciences, Istanbul, Türkiye. The isolation and assessment of miRNAs were performed by Anatolia Ge-

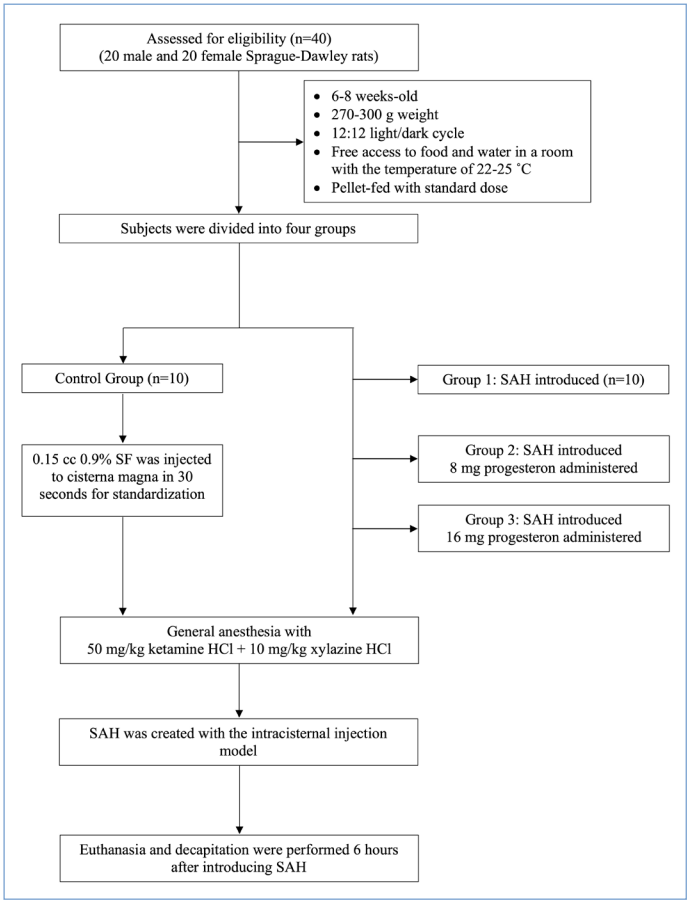

Figure 1. STROBE diagram for the evaluation of the animals.

neworks Laboratory Anatolia Tanı ve Biyoteknoloji, Istanbul, Türkiye). The approval for the study was granted by the Local Ethics Committee for Animal Experiments at Istanbul University (approval number: 2015/69) and was conducted in accordance with the Declaration of Helsinki. The study was not supported financially by an outside organization.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) for miR-30a and miR-143

A 50 mg sample of cerebral tissue was isolated by microdissection and placed into a microcentrifuge tube (0.5–1.5 mL). The samples were supplemented with 400 µL of GT Buffer to break up tissues and 20 µL of proteinase K to degrade proteins: The tubes were sealed with parafilm, vortexed, and incubated at 56°C for 45 min. The samples were cooled to 4°C and centrifuged at 12500 rpm for 5 min. After mixing the upper phase with 520 µL Bead Buffer on Magrev stand (Anatolia Biotechnology, Istanbul, Türkiye), the sample was incubated at room temperature for 15 min. Then, a 750µL of Inhibitor Removal (IR) Buffer was added to the sample and homogenized. After the IR process, 800µL W Buffer and 100µL DNAse I (5U) were added to remove the DNA in the sample while the beads were magnetized. The samples were shaken and incubated for 10 min at 37°C. A 200 µL of LB Buffer was added to each tube, incubated at room temperature for 5 min, and magnetized. Then, the beads were magnetized and homogenized with 750 µL IR Buffer. After the IR process, the beads were washed with 750 µL W2 Buffer twice. Elution was performed by incubating the beads with an 80 µL Elution Buffer at 95°C for 10 min.

The isolated RNAs were reverse-transcribed to cDNA via the qScript miRNA cDNA Synthesis Kit (Quanta Bio). To detect nonspecific mRNA binding, cDNAs were synthesized with and without the Poly-A tail. For cDNA synthesis, 10 µL Poly-A Tailing Reaction (from above), 9 µL microRNA cDNA Reaction Mix, and 1 µL qScript Reverse Transcriptase were incubated for 20 min at 42°C and 5 min at 85°C. For Poly-A tail, 2 µL Poly (A) Tailing Buffer (5X), 7µL RNA (up to 1 µg total RNA or miRNA-enriched RNA), 1 µL Nuclease-Free Water, and 1 µL Poly (A) Polymerase were mixed and incubated at 37°C for 60 min and 70°C for 5 min. The resulting cDNAs were used for qRT-PCR with Perfecta SYBR Green Super Mix using LightCycler 480 real-time PCR System (Roche Diagnostics, Mannheim, Germany). The primers were prepared using the primer design tool with the following sequences: 5'-GCAACTGTAAACATCCTCGACT-3' (F), 5'-CAGCTGCAAACATCCGACTG-3' (R) for miR-30a and 5'-TGCATCTCTGGTCAGTTGGG-3' (F), 5'-GCTGCAGAACATCTTCTCCCTT-3' (R) for miR-143. For qRT-PCR, the components were kept at 95°C for 2 min, and then amplification was performed with the PCR protocol with 40 cycles. Each cycle included denaturation at 95°C for 5 s, annealing at 60°C for 15 s, and synthesis at 70°C for 30 s; and melting was performed from 60°C to 90°C. After the reaction, the presence of specific gene products was confirmed by the basic relative quantification analysis. The Ct values for miR-30a and miR-143 were normalized based on the expression level of miR-24 housekeeping miRNA. The expression of miR-24 has been reported to be stable in many tissues.[14] Using the Ct values obtained from the system, the variations were analyzed with the 2-ΔΔCt method.

Statistical Analysis

The data were analyzed using the Statistical Packages for the Social Sciences (SPSS, version 22, IBM Corp., Armonk, NY, USA). $p<0.05$ was considered significant. The Mann–Whitney U test was used in all paired comparisons between

the female and male rats in the control and experimental groups. The Kruskal–Wallis H test was used to compare the expression levels of the female and male rats in all groups.

Results

The results of qRT-PCR for miRNAs were evaluated separately according to the sex of the animals. For each sex, the statistical comparisons were performed between the ΔΔCt values of experimental groups and the controls using the binding values of miR-30a and miR-143. The expression of miR-30a was reduced approximately 176-fold in the male rats with SAH (Group 1) compared to that in the controls; the difference was statistically significant ($p<0.05$). The expression of miR-143 was reduced approximately 126-fold in male rats with SAH (Group 1) compared to that in the controls; however, the difference was not statistically significant ($p>0.05$). The expression of miR-30a was reduced approximately 39-fold in the male rats with SAH+8mg progesterone (Group 2) compared to that in the controls; the difference was statistically significant ($p<0.05$). The expression of miR-143 was reduced approximately 3-fold in the male rats with SAH+8mg progesterone (Group 2) compared to that in the controls; the difference was not statistically significant ($p>0.05$). The expression of miR-30a was reduced approximately 2.4-fold in the male rats with SAH+16mg progesterone (Group 3) compared to that in the controls with no significant difference ($p>0.05$). The expression of miR-143 was reduced approximately 4.5-fold in the male rats with SAH+16mg progesterone (Group 3) compared to that in the controls; this decrease was not statistically significant ($p>0.05$) in Figure 2.

The expression of miR-30a was reduced approximately 2.3-fold in the female rats with SAH (Group 1) compared to that in the controls; the difference was statistically significant ($p<0.05$). The expression of miR-143 was increased approximately 15-fold in the female rats with SAH (Group 1) compared to that in the controls; the difference was statistically significant ($p<0.05$). The expression of miR-30a was increased approximately 1.25-fold in the female rats with SAH+8mg progesterone (Group 2) compared to that in the controls; the difference was statistically significant ($p<0.05$). The expression of miR-143 was increased approximately 1400-fold in the female rats with SAH+8 mg progesterone (Group 2) compared to that in the controls; the difference was statistically significant ($p<0.05$).

The expression of miR-30a was reduced approximately 4.5-fold in the female rats with SAH+16mg progesterone (Group 3) compared to that in the controls, and the difference was statistically significant ($p<0.05$). The expression of miR-143 was increased approximately 400-fold in the female rats with SAH+16mg progesterone (Group 3) compared to that in the controls; this increase was statistically significant ($p<0.05$) in Figure 3.

Discussion

The most prominent cause of mortality after SAH is rebleeding that may occur within the first 3 days. Bleeding causes the activation of pathophysiological cascades, leading to widespread neuronal cell death, diffuse stroke, and early brain damage.[15] Considering that the early-period brain damage that develops after SAH may be due to genomic

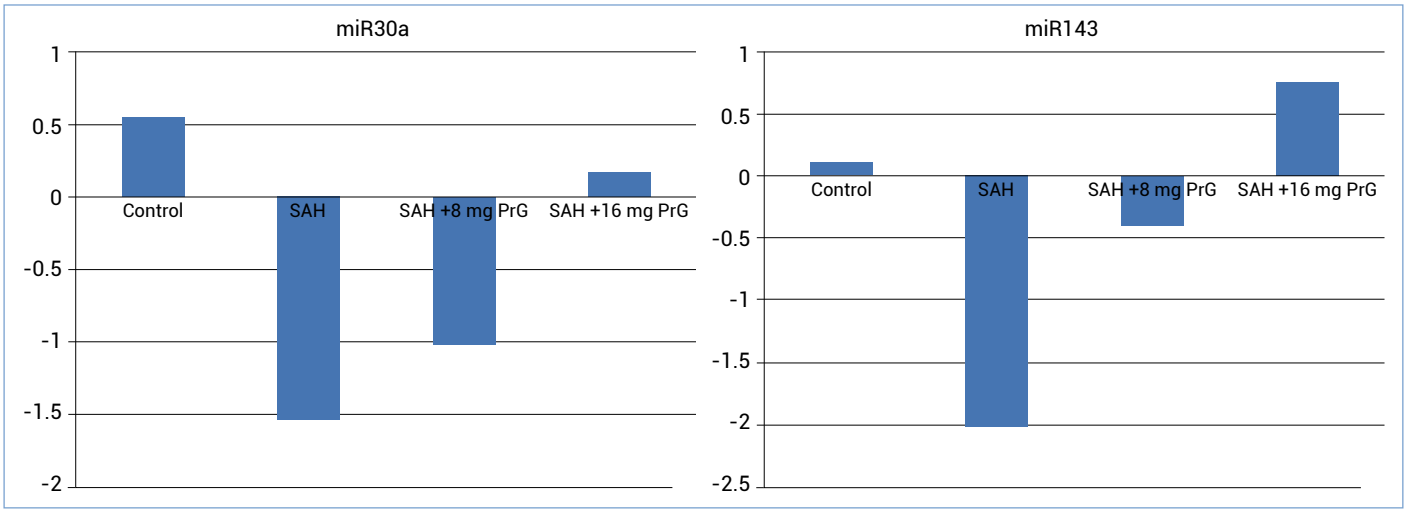

Figure 2. The expression levels of miR-30a and miR-143 in male rats by study groups. In the graphical representation of relative change in expression levels, values are given in $\log_{10}$.

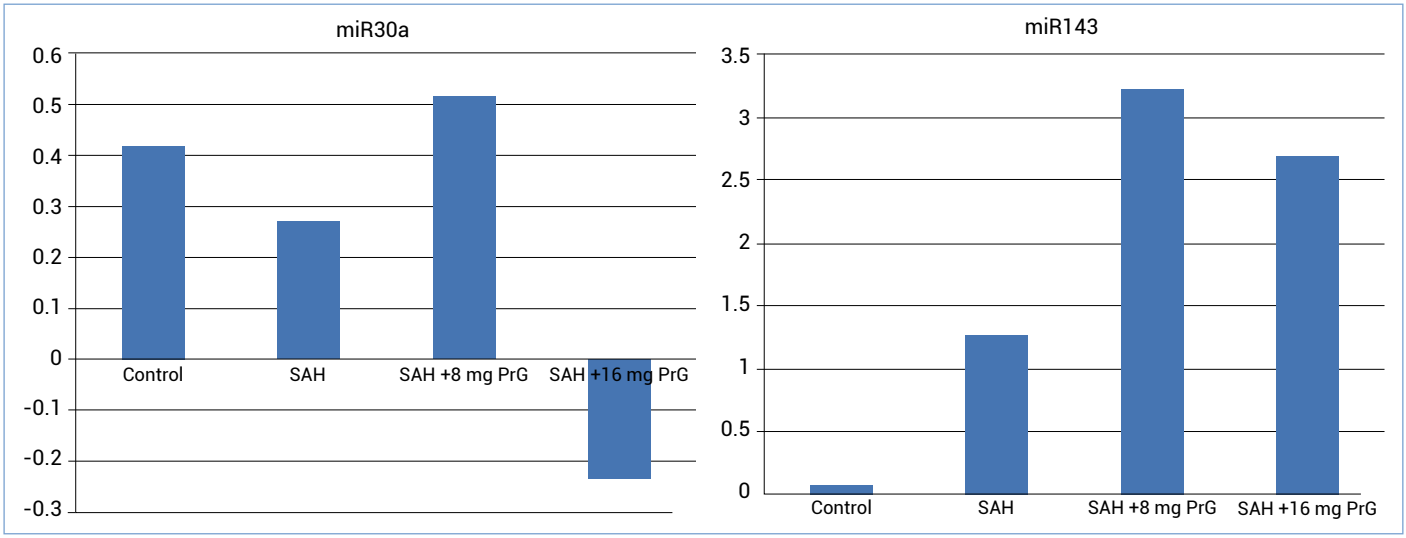

Figure 3. The expression levels of miR-30a and miR-143 in female rats by study groups. In the graphical representation of relative change in expression levels, values are given in $\log_{10}$.

regulation involving miRNAs, our study aimed to provide a potential therapeutic perspective for preventing injury, morbidity, and mortality by inhibiting this cascade with a neuroprotective agent. An intracisternal injection model was chosen to introduce SAH due to the ease of application, low cost, and low mortality rates. A decrease in miR-30a expression was found after SAH in the male rats. This decrease was partly stopped in the groups administered with 8 mg progesterone and the difference was statistically significant ($p<0.05$). Although administering 16 mg progesterone increased miR-30a expression, the difference was not statistically significant. These results indicate that a significant decrease in expression of miR-30a was observed in the male rats at the 6th h after SAH; this decrease can be inhibited with 8 mg progesterone but not with 16 mg progesterone. In the female rats, a reduction in miR-30a expression at the 6th h after SAH was found in those that did not receive progesterone and those that received 8 mg progesterone. However, there was an increase in those that received 16 mg progesterone. Our findings supported that progesterone had an inhibitory effect in the females at 8 mg dose but not at 16 mg. Regarding the miR-143 expression, we found no increase after SAH in the male rat groups. In the female rats, an increase in miR-143 expression was detected in the 6th h after SAH, but a progesterone effect could not be demonstrated.

miRNAs constitute a recent focus of genetics studies to clarify the pathophysiology of diseases and to develop new treatments. miRNAs are small, non-coding RNAs made up of 22 nucleotides that bind to and regulate the function of mRNAs. Some miRNAs have been associated with the pathophysiology of aneurysmal SAH through their role in processes such as leukocyte activation, extracellular matrix organization, TGF-β signaling, smooth muscle cell proliferation, angiogenesis, apoptosis, and oxidative stress response. Studies have demonstrated that the changes in blood levels of miRNAs in the patients with SAH enable the predictions about the pathophysiology, diagnosis, prognosis, and the alternative treatment protocols of early brain injury. The effects of up or down-regulation of miRNAs are determined by whether the target protein's expression is induced by binding to the 3' untranslated region of the target mRNA in the post-translation phase or binding to specific promoter regions in the cell nucleus.[16,17] The miR-30 family of miRNAs is one of the miRNAs that are significantly expressed after and lead to the progression of SAH. This family has a well-defined role in angiogenesis and was found to increase in cerebral vessels after SAH. Still, its effects on the pathophysiology of SAH have not been adequately elucidated yet.[16-18] miR-30a belongs to the miR-30 family, is localized on chromosome 6q13, and is produced from the intron transcription unit. miR-30a is widely known to prevent extracellular matrix degradation, apoptosis, and increasing arterial branching. When it is up-regulated in neurons, it has been observed that it prevents the development and maturation in the prefrontal cortex by decreasing brain-derived neurotrophic factor. Its expression levels in cortical neurons were found to decrease significantly after ischemic stroke and they were found to prevent the ischemic neuronal damage by reducing the expression of the heat-shock protein A5.[17-19] The only study that examined the time-dependent change in miRNA levels

after SAH reported a brain-tissue-specific up-regulation of miR-30a.[17] Our study showed that miR-30a was downregulated after SAH, and this downregulation can be inhibited by progesterone in a dose-dependent manner. We think that the downregulation of miR-30a might have occurred due to its interaction with progesterone and the classical PR.

miR-143 originates from the 18th chromosome; it has been shown to be expressed in vascular smooth muscle, cardiac muscle, and endothelial cells and involved in the proliferation and differentiation of vascular smooth muscle cells.[17,18] It was observed that miR-143 knock-out mice had thinner arterial walls, lower blood pressure, and neo-intima formation was negatively affected in response to vascular damage. This observation suggests that miR-143 has a role in shaping the vascular smooth muscle phenotype. In addition, a decreased expression of miR-143 was found in carotid artery damage.[20] The only study examining the role of miR-143 in the pathophysiology of SAH reported an increase in its expression after SAH. Based on this study and considering the lack of comprehensive studies on SAH, we examined miR-143 as well; however, we found no post-SAH effect in rats of both sexes.

It has also been found that progesterone increases neuronal plasticity, reduces damage caused by lipid peroxidation with its membrane-stabilizing effect, exerts a neuroprotective effect after brain damage and ischemic events by suppressing neuron hyperexcitability, and contributes to myelinization.[7,21-23] Progesterone was shown to reduce brain edema within the first 24 h after SAH, play a role in repairing the BBB, and decrease MMP-9 and caspase-3 expression.[4] In addition, differences in serum progesterone concentrations have been shown to have differential effects on neuroprotection, independent of the type, and the severity of neurodegenerative damage.[24] It is known that progesterone exhibits its effect through classical PR A and B, which are few on neurons. Some miRNAs also contribute to the neuronal plasticity produced by progesterone by interacting with these receptors. In the CNS, regions have been found where some miRNAs can bind to mRNAs of classical PRs and provide PR modulation, but our knowledge on this subject is still limited.[7,25] Especially in a study conducted by Pieczora et al.,[26] miR-30a expression was observed in neural tissue. Our study demonstrated that progesterone induced the expression of miR-30a after SAH, and 8 mg progesterone had a therapeutic effect in both sexes.

One of the limitations of our study was that progesterone levels specific to menstrual cycles were not determined in females. In the future studies, different results can be obtained by determining the menstrual cycles in females. On the other hand, our study will guide future studies as there are not many studies on miR-30a in intracerebral physiology or pathologies. The literature includes studies on miRNA expression in cerebral physiology or pathologies such as CNS development, brain damage, and cerebral ischemia, indicating that miRNAs can show gender-dependent dimorphism in males and females.[27-30] However, there are no gender-variable studies on miRNA expression levels after SAH yet. Hence, we think that our study may constitute a primer for more comprehensive future studies. Our results indicate that understanding the regulation of miRNAs is essential in guiding future diagnosis and treatments.

Although there are studies showing that miRNAs play a role in the pathophysiology of early brain damage, these mechanisms have been fully understood yet. Our study showed that miR-30a expression decreases after SAH, and this decrease can be inhibited by progesterone. However, an effect of miR-143 on the SAH mechanism could not be demonstrated. The results of this study warrant future studies with a broader perspective and larger sample size that account for sex differences, aimed at the pathophysiological mechanisms of early brain damage and the therapeutic benefits of progesterone through their genetic regulation and inhibition.

Disclosures

Ethics Committee Approval: The study was approved by the Local Ethics Committee for Animal Experiments at Istanbul University (approval number: 2015/69).

Acknowledgments: Our special thanks are extended to the staff of Aziz Sancar Institute of Experimental Medicine at Istanbul University and Anotolia Geneworks Laboratory.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The study was performed in the Department of Neurosurgery at University of Health Sciences, Okmeydani Research and Training Hospital and in the Department of Neurology at Bahcesehir University Medical Faculty.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – A.Y.Y., S.E.Ç.; Design – A.Y.Y., S.E.Ç.; Supervision – S.E.Ç.; Materials – A.Y.Y., S.E.Ç.; Data collection &/or processing – A.Y.Y.; Analysis and/or interpretation – A.Y.Y., B.Ç.A., S.E.Ç.; Literature search – B.Ç.A.; Writing – A.Y.Y., B.Ç.A.; Critical review – A.Y.Y., B.Ç.A.

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