



Research Article

Paradoxical Morphometric and Antioxidative Effects of Melatonin on Vasospasm in Experimental Subarachnoid Hemorrhage

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Summary

The purpose of this study is to investigate the histopathologic, biochemical and morphometric effects of melatonin on basilar artery and oxidative balances during subacute vasospasm following a subarachnoid hemorrhage (SAH). Twenty-four male Wistar rats were used in the study. Rats were divided randomly into 4 groups, six rats in each group: no SAH (group I-control), only SAH (group II-control), SAH plus melatonin 5mg/kg (group III), and SAH plus melatonin 10mg/kg (group IV). Melatonin was administered intraperitoneally just after SAH and repeated at 24th hour after as a second dose. Animals were sacrificed 9 days after SAH, and the basilar artery was harvested for morphometric and histopathologic studies. Biochemical assays also were performed in terms of oxidant and antioxidant balance. In group IV area of lumen (AL) was found to be increased suggesting vasodilation ($p<0.05$) compared to the controls. However, there was no significant difference between treated groups with melatonin in terms of AL. No significant changes were observed in levels of total oxidant status (TOS) between the groups, interestingly unexpected increases in TAC levels were observed in “only SAH group” compared to the treatment groups although group II did not receive any treatment ($p<0.05$). The paradox between biochemical and morphometric changes may be explained by insufficient information on density and distribution of melatonin receptors in the brain parenchyma and vascular bed or by asynchronous happening of morphological and biochemical changes.

Key words: Melatonin; Vasospasm; Subarachnoid hemorrhage; Oxidative metabolism; Morphometry; Histopathology

Melatoninin Deneysel Subaraknoid Kanama Sonrası Gelişen Vazospazmda Paradoksal Morfometrik ve Antioksidatif Etkisi

Özet

Bu çalışmanın amacı melatoninin SAK sonrası gelişen subakut vazospazmda oksidatif dengelere ve baziler arterin histopatolojik, biyokimyasal ve morfometrik sonuçlarına etkilerini incelemektir. Bu çalışmada 24 adet erkek Wistar sıçanı kullanılmıştır. Sıçanlar rastgele 4 gruba ayrılmıştır. Her grupta 6 denek kullanılmıştır: SAK olmayan (Grup 1 – kontrol), yalnızca SAK (Grup 2 – kontrol), SAK + melatonin 5 mg/kg(Grup 3) ve SAK + Melatonin 10 mg/kg(Grup 4) Melatonin hemen SAK sonrası ve 2. Doz olarak 24 saat sonra intraperitoneal olarak uygulandı. Denekler SAK sonrası 9. günde sakrifiye edilerek baziller arterden morfometrik ve histopatolojik çalışmalar için örnek alındı. Aynı zamanda oksidan ve

antioksidan denge açısından biyokimyasal analizler yapıldı. Grup 4' de lümen alanının kontrol gruplarına nisbeten vazodilatasyonu düşündürecek tarzda arttığı gözlemlendi.($p<0.005$) Bununla birlikte melatonin ile tedavi edilmiş gruplarda lümen alanında önemli bir değişim gözlemlenmedi. Gruplar arasında total oksidan seviye (TOS) açısından bir farklılık bulunmazken, ilginç olarak yalnızca SAK yapılan grupta total antioksidan seviyelerinde (herhangi bir tedavi almadıkları halde) diğer gruplara göre beklenmedik bir artış izlendi ($p<0.005$). Sonuç olarak biyokimyasal ve morfometrik değişimler arasındaki bu paradoks beyin parankimi ve vasküler yataktaki melatonin reseptörlerinin dağılımı ve yoğunluğundaki yetersiz bilginiz ya da morfolojik ve biyokimyasal değişikliklerin oluşumundaki asenkron bir süreç ile olabileceği muhtemeldir.

Anahtar Kelimeler: Melatonin; Vazospazm; Subaaknoid Kanama; Oksidatif metabolizma; Morfometri; Histopatoloji

INTRODUCTION

Subarachnoid hemorrhage (SAH), one of the types of hemorrhagic stroke in property, constitutes 6-8% of all strokes. Cerebral vasospasm is a pathological contraction in the large arteries located at the base of the brain occurring after SAH and usually associated with the decrease in parenchymal tissue perfusion in the distal part of the affected artery⁽³⁰⁾. Vasospasm reaches the maximum in the 3th to 14th days after SAH and gradually reduces within 2 to 4 weeks. In almost half of cases, vasospasm causes neurological deficits^(8,29). In the in-vivo and in-vitro studies conducted recently; vasospasm development was reported to be multifactorial^(23,24,27). The decreases in the endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) level after SAH, conversion of NO into nitrite and nitrate, increase in free oxygen radicals and hence oxidation of membrane phospholipids, lipid peroxidation, endothelial damage and induction of vascular smooth muscle cell proliferation are causes underlying the vasospasm associated with oxidative metabolism^(3,26,27). Normally, free radicals and their peroxidation products are neutralized by intrinsic antioxidants such as superoxide dismutase (SOD)^(13,34). Probably, this antioxidant system is damaged as a result of increased reactive

oxygen species (ROS) and nitrogen species^(13,31). Melatonin is a hormone that is produced in all mammals, released from the pineal gland and regulates biological rhythms of living organisms^(21,35). Melatonin, whose antioxidant property was proven, may show an impact in wide range of the organisms due to its lipophilic property which enables passing the blood-brain barrier easily and reaching to all cell organelles including the nuclei. This feature provides an advantage to melatonin against the disease that leads degenerative or oxidative changes in the protection of macromolecules and particularly mitochondria and DNA from oxidative damage^(28,35). Our main aim in this experimental study is to examine the effect of melatonin on subarachnoid hemorrhage-induced vasospasm in terms of histopathological, biochemical and morphometric aspects.

MATERIAL AND METHODS

Experimental Procedures:

This study was performed in Experimental Animal Research Laboratory of the Department of Physiology, Faculty of Medicine, Gaziantep University by obtaining permission from the Ethics Committee of Gaziantep University (Vouch number: 06-2008/128, Decision date: 30.06.2008). In the study, male Wistar rats weighing from 250 to 300 gr were used. Rats were fed with the standard

feed and water, and subjected to 12-h light/dark cycle at room temperature ($21 \pm 1^\circ\text{C}$). In this study, a total of 24 Wistar rats were used. The subjects were divided into 4 groups. Table 1 summarizes the treated and control groups.

Surgical procedure:

The subjects were fasted overnight before the procedure and then 50 mg/kg cefazolin sodium (Mustafa Nevzat, Istanbul, Turkey) was administered as a prophylactic. General anesthesia was performed at 37°C by administering 60 mg/kg ketamine hydrochloride intraperitoneally. For the rats to which SAH was planned (groups II, III and IV), occipital muscles were dissected by occipito-cervical midline incision in sterile conditions. One burr hole was opened just above the interparietal-occipital suture and then the cisterna magna was revealed. After about 0.3 ml of CSF was drained, 0.3ml autologous blood taken from the tail artery of rats was injected slowly into the cisterna magna with a 25 gauge needle. The rats were kept with their heads down at 20° for 20 minutes to facilitate the blood settling in the basal cisterns. Once bone defect (mini burr-hole) was plugged with Bone-wax (Ethicon ® Ohio, United States), and then muscle, skin and subcutaneous tissues were sutured in anatomic planes. Afterwards, melatonin (Sigma Chemical Co, St. Louis, MO) was administered intraperitoneally to III. and IV. groups just after skin was sutured and 24 hours after surgery as 2 doses. All subjects were followed in the cages where they were kept in 12-h light/dark cycle and can meet the needs of standard rat chow and water at constant temperature ($21 \pm 1^\circ\text{C}$).

Sacrification of Rats and Preparation of Samples:

The subjects were followed for nine days and then sacrificed by giving the higher dose of ketamine hydrochloride (50mg/kg) intramuscularly. After removing the brain tissues, the portions of the pons containing the basilar artery were extracted by cutting

from upper and lower anatomical limits of the pons. The brain stem with basilar artery was kept in 10% formalin solution for three days without damaging the basilar artery for histopathological examination with the purpose of fixation. The samples with the length of 4 mm and the width of 3 mm prepared under the light microscope from these tissues were delivered to the Department of Pathology as embedded in paraffin by remaining the basilar artery in its bed.

The remaining brain tissue was homogenized at 16.000 rpm for 2 minutes at $+4^\circ\text{C}$ with a homogenizer (SOW's ® Shanghai, China) in an ice cold 0.1 M Tris HCl buffer (pH 7.5) in order to examine total antioxidant capacity (TAC), total oxidant status (TOS) and oxidative stress index (OSI).

Morphometric measurements of Basilar Artery:

After paraffin blocks were prepared, four of 0.5 micron thick sections were taken from the basilar artery of each animal. They were coded according to their order. These sections were stained with hematoxylin eosin following deparaffinization and photographed and examined under the light microscope (Olympus BX50, Japan). The prepares were examined at 200X and 400X magnification in the Department of Pathology, Faculty of Medicine, Gaziantep University and photographs of all sections were taken. In these photos obtained, basilar artery lumen area was measured in square millimeter using a program, MIPAV (Medical Image Processing, Analysis and Visualization Center for Information Technology (CIT), National Institutes of Health (NIH), Version: 3.1.3 2007-03-30).

Brain TAC and TOS Assay:

Total antioxidant and oxidant capacity were measured by Erel method notified in the literature. Oxidative Stress Index (OSI)

was calculated by dividing as (TOS) / (TAC)⁽¹¹⁾.

Statistical Methods:

Data analysis was made with the statistical program, SPSS for Windows Release 22.0. The non-parametric test, Kruskal-

Wallis variance analysis test, was used for all intergroup analysis. Pair wise comparisons between groups were analyzed by Mann-Withey-U test which is also a non-parametric test. In these comparisons, p values less than 0.05 were considered as statistically significant.

Table 1: Groups and treatment protocols in rat models of subarachnoid hemorrhage

Groups*	Treatment protocols
I	No SAH and SS (i.p.)
II	Only SAH and no treatment
III	SAH and Melatonin 5 mg/kg/i.p. just after surgery and 24 hours after as 2 doses
IV	SAH and Melatonin 10 mg/kg/i.p. just after surgery and 24 hours after as 2 doses

*Each group had 6 rats.

Abbreviations: SAH: Subarachnoid hemorrhage, SS: Saline solution, i.p.: Intraperitoneally

RESULTS

In our study, a total of 24 subjects were used, and were followed for 9 days. Appropriate model of subarachnoid hemorrhage was observed to be formed in the macroscopic and microscopic examinations of brain tissue of subjects.

Effects of SAH and Melatonin on Biochemical Parameters:

When total antioxidant and oxidant measurements were analyzed using Kruskal-Wallis analysis of variance (Table 2), it was observed that there were significant differences between groups in terms of TAC and OSI values ($p < 0.05$). Thus, pair wise comparisons between groups were considered necessary and

there was a significant difference in group II according to comparisons (Table 3). In these comparisons, we noticed that TAC values of only SAH (II) group ($2.78 \pm 0.03 \mu\text{mol/l}$) were higher than the MLT treated groups (III and IV), $1.95 \pm 0.06 \mu\text{mol/l}$ and $2.01 \pm 0.18 \mu\text{mol/l}$, respectively.

Effect of SAH and Melatonin on Histopathologic findings

Histopathologic appearances of basilar artery of groups I, II, III, and IV were shown in Figure 1. Similar images were present in the microscopic examination of subjects in the groups treated with 5 mg and 10 mg melatonin by performing SAH (groups III and IV) compared to the control group (group I). It was observed that vessel lumens were coated with a single row of

endothelial layer and did not cause irregularity in the internal elastic lamina which is the bottom layer of endothelium layer. Also, significant morphological changes were not observed histopathologically in the smooth muscle cells of medial layer and adventitia layer. In the untreated group after SAH (group II), significant histopathological differences were not observed macroscopically compared to the control group although the slight narrowing of the lumen due to vasospasm was observed compared to the treatment groups. Therefore, significant histopathological difference was not observed between the treatment and untreated control groups I and II except mild vasospasm (Fig. 1).

Morphometric Effects of Melatonin on Basilar Artery

Areas of lumen (AL) were measured with MIPAV program (Table 4). When pair wise comparisons between groups were performed it was observed that 10 mg dose of melatonin after SAH significantly increased the lumen areas of basilar arteries ($p < 0.05$) in group IV ($32259.33 \pm 3801.06 \text{ mm}^2$) compared to the control groups that SAH was not observed ($19201.17 \pm 3037.32 \text{ mm}^2$) and SAH+SS-treated group ($17993.50 \pm 3213.58 \text{ mm}^2$). However, there was no difference between the groups treated with melatonin. In the comparison between SAH-free and SAH+SS treated groups, there was no statistically significant difference in terms of the parameter related to the measurement of lumen area ($p > 0.05$) although a 6.3% narrowing in the internal area were found in the group with SAH compared to the control without SAH (Table 5).

Table 2. Results of the comparisons of TAC, TOS and OSI values among the groups

Groups	TAC($\mu\text{mol/l}$) mean $\pm$ SEM	TOS($\mu\text{mol/l}$) mean $\pm$ SEM	OSI ratio mean $\pm$ SEM
Group I	2,40 $\pm$ 0,23	30,46 $\pm$ 2,18	13,19 $\pm$ 1,28
Group II	2,78 $\pm$ 0,03	26,14 $\pm$ 1,23	9,38 $\pm$ 0,38
Group III	1,95 $\pm$ 0,06	25,72 $\pm$ 1,22	13,29 $\pm$ 0,83
Group IV	2,01 $\pm$ 0,18	27,89 $\pm$ 1,71	14,53 $\pm$ 1,59
p value	0.003*	0,192	0.022*

Abbreviations: MLT: Melatonin, SEM: Standart Error of the Mean, SAH: Subarachnoid Hemorrhage, SS: Saline Solution
 ** Kruskal-Wallis Variance Analysis Test, * p values below 0.05 were considered to be statistically significant.

Table 3. Obtained p values and comparison results of TAC and OSI **

Compaired Groups	TAC p value and result	OSI p value and result
I-II	0,154	0,030 (I>II)*
I-III	0,106	0,947
I-IV	0,212	0,524
II-III	0,001 (II>III)*	0,004 (II<III)*
II-IV	0,008 (II>IV)*	0,022 (II<IV)*
III-IV	0,760	0,509

** Mann-Whitney U test , *p values below 0.05 were considered to be statistically significant

Abbreviations: “<” less than or “>” greater than in terms of mean±SEM

Table 4. Arterial lumen area values of the basilar arteries (mm²)*

Groups	AL (mean±SEM)
I (No SAH)	19201,17± 3037,32
II (SAH+SS)	17993,50 ± 3213,58
III (SAH+5mg MLT)	30636,67± 4879,79
IV (SAH+10mg MLT)	32259,33 ± 3801,06

Abbreviations: AL: area of lumen , SEM: Standart Error of the Mean

*MIPAV image analysis software

Table 5. Obtained p values of AL in paired comparisons **

Compared Groups	P values	Results
I-II	0,790	
I-III	0,080	
I-IV	0,024*	I<IV
II-III	0,060	
II-IV	0,017*	II<IV
III-IV	0,729	

Abbreviations: “<” less than or “>” greater than in terms of mean±SEM

** Mann-Whitney U test , *p values below 0.05 were considered to be statistically significant

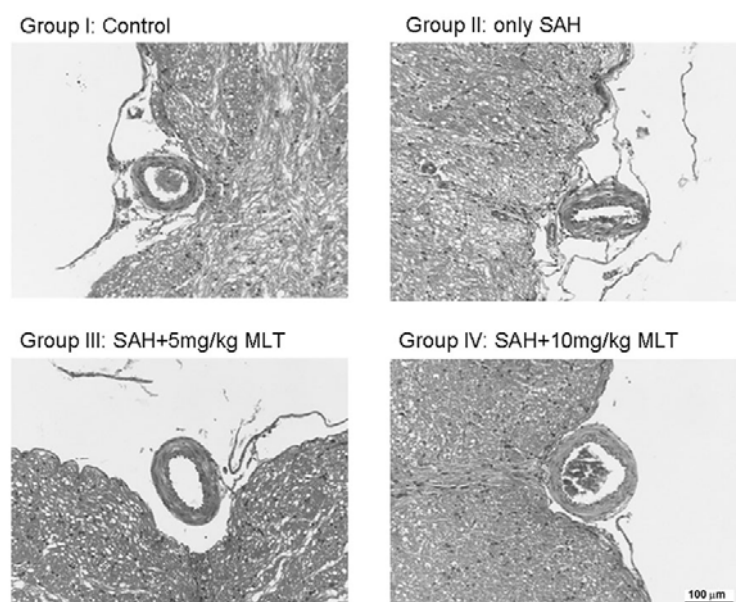


Figure 1: Representingn photograph of light microscopic appearance of a cross-sectional area of the basilar arteries in (H&E, X200). While it was seen significant reduction in luminal diameter and marked thickening of the vessel wall and endothelial cells and substantial corrugation of the internal elastic lamina of the basilar artery in SAH, SAH plus placebo groups, the microscopic appearance of group SAH plus melatonin was similar in appearance of normal vessels in the control group.

DISCUSSION

Today, in patients with subarachnoid hemorrhage, cerebral vasospasm in the subacute period (3-14 days), as a multifactorial phenomenon, is one of the main problems in the practice of neurological surgery, especially cerebrovascular surgery. Several vasoactive substances released after vasospasm into the medium determines the course of the disease with proinflammatory, proapoptotic, vasoconstrictor and many other unknown effects. Mechanisms of early brain injury after subarachnoid hemorrhage^(5,14). All of the agents forming vasospasm following SAH show their effects by increasing intracellular Ca^{++} concentrations and/or increasing the sensitization of smooth muscle contractile proteins to calcium^(18,36). Melatonin is one of the essential elements of antioxidant defense system of the organism. It protects cellular compartments against oxidative damage as an effective antioxidant and free radical scavenger^(2,15,32,33). It can pass easily through all the barriers in the body due to the fact that it is both fat and water soluble and access easily to all intracellular components. Protection of DNA against oxidative damage thanks to this property gives advantage⁽³⁷⁾. In addition, melatonin is a calmodulin antagonist preventing myosin phosphorylation and consequently vascular smooth muscle contractions by binding intracellular Ca^{++} calmodulin complex⁽¹⁶⁾. The purpose of the current treatment for cerebral vasospasm is to increase cerebral blood flow by dilating affected vessels directly or indirectly⁽¹⁰⁾. Among drug treatments developed in recent years for the prevention of the unintended consequences of delayed ischemic deficits, magnesium sulfate, statin agents (simvastatin, pravastatin), L-arginine, mexiletine, the adenosine A1 receptor agonists, clazosent and free radical scavengers (edaravone, the

erythropoietin and melatonin) which are popular in recent years come forward^(17,20,22,25). In most of these studies, angiographic vasospasm reduced significantly, but no significant change was observed in the clinical outcomes of patients. This condition revealed that there might be other ignored factors in the pathogenesis of vasospasm. In this study, we investigated the effects of melatonin⁽⁶⁾, which is said to decrease the vasospasm by inhibiting lipid peroxidation with its strong antioxidant property, in biochemical and morphometric aspects on the vessel lumen area and the total antioxidant levels at lower (5 mg) and higher (10 mg) doses in the basilar artery of rats that experimental SAH was formed. To better understand the effects of melatonin in the CNS, it is necessary to know primarily the regions to which it binds and shows affinity. In a study conducted by Dubocovich et al, the presence of the receptors to which melatonin binded both in the brain parenchyma and vascular bed were emphasized⁽⁹⁾. It has been reported in this and similar studies, melatonin had two receptor subtypes in mammals named MT1 (1a) and MT2 (1b) which could be defined⁽⁷⁾. Meanwhile, in a study on the brain parenchyma and vascular beds, all of these areas were examined in terms of receptor distribution of melatonin or melatonin binding sites by using radioligand named 2-¹²⁵I iodomelatonin⁽³⁸⁾. In conclusion, MT1 was found especially in SCN, PT and paraventricular thalamic tissues and MT2 has been observed to be higher in tissues such as SCN, hippocampus, retina⁽⁸⁾. When the effects of melatonin on cerebral vascular bed were investigated, biphasic concentration-response curve was obtained; this shows that there are more than one receptor in this environment⁽¹⁹⁾. In this study, we have applied 5 and 10 mg/kg doses of melatonin in the treatment groups which were reported to be useful in the literature in order to reveal the dose-

dependent effects of melatonin. In our study, TAC and TOS levels were measured in the brain tissue homogenates. But we observed unexpectedly an important increase in TAC values in the untreated SAH group. The reason for that, these levels have not emerged significantly; can be explained by receptor density localized in regions which very small areas or volumes such as PT and SCN in the brain parenchyma and these areas had a small volume in whole brain homogenate. Thus, activation of receptors only in these small areas may have failed to reduce the severity of oxidative stress. To overcome this, forcing the therapeutic dose range of melatonin by applying higher doses such as 200 mg/kg may be helpful in terms of observing the effects of melatonin.

When this study was concerned in terms of morphometry of the vascular lumen, significant increases in vessel area were observed in the SAH group to which 10 mg/kg melatonin was administered compared to control groups ($p < 0.05$). In comparison between the control (I and II) groups, statistical difference was not found in terms of morphometric measurements ($p > 0.05$). However, there are some publications in literature defending that melatonin did not have any positive effect on vascular spasm as well as the publications expressing the positive effect of melatonin^(4,12).

In a study conducted by Aladag et al, increase in the levels of nitric oxide and decrease in the level of arginase and oxidative stress were found in rats underwent SAH and treated with 20 mg / kg melatonin and a significant increase in basilar artery diameter was observed compared with the control group⁽¹⁾. It has been shown in the study of Aydın et al. on rabbits underwent SAH and treated with 5 mg/kg melatonin that melatonin prevented the vasospasm and vascular endothelial apoptosis formed by SAH⁽⁴⁾.

As a result; melatonin is a molecule often highlighted in the literature due to its well-

known antioxidant effects. However, our study does not support the present findings in the literature in all aspects. In order to solve this paradox, the diffuse effects of melatonin are still questionable despite the affinity to CNS is known regionally as of receptor distributions. There has been no adequate number of scientific studies on receptor distribution in CNS and vascular bed yet. So, the effects on vasospasm may be questioned because of the contradictions in the literature. Performing dose-dependent studies on melatonin with more than two doses and wide dosage range would be beneficial in terms of determination of the therapeutic dose range. TAC is a general indicator of the level of oxidative stability, thus it is unlikely that local changes can be ignored.

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