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The histopathological and stereological assessment of the effect of topically administered leptin on cerebral vasospasm in an experimental subarachnoid hemorrhage model

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1. Introduction

Subarachnoid hemorrhage (SAH) is bleeding into the subarachnoid space, the area between the arachnoid membrane and the pia mater surrounding the brain, for a variety of reasons. It is most commonly secondary to trauma but may also develop spontaneously without any underlying cause. The actual incidence is not known exactly because approximately 10% to 15% of patients with subarachnoid hemorrhage die without any medical intervention (1). SAH occurs most commonly in people aged 60 and over. In some studies, it was reported that it is more frequent in males in autumn and in women in spring (2,3). Although the incidence of SAH in women is more frequent, it was reported that the male/female ratio is 4/1 in the first decade of life, equal in the fifth decade, and ten times higher in women in the sixth decade (4).

Despite the improvements in diagnosis and treatment strategies, SAH is still an important cause of morbidity and mortality. In patients with SAH, familial predisposition is an important risk factor. Studies have revealed that the incidence of SAH in first- and second-degree relatives of SAH patients is higher than that in normal populations (5–7).

Cerebral vasospasm is a pathological chain of events resulting from slowly developing narrowing in the large cerebral arteries and late neurological loss due to brain ischemia or infarction or both (8). Although many methods of treatment of cerebral vasospasm have been reported, treatment methods are still controversial.

Leptin is a polypeptide that can be carried in the blood freely and bound to protein; it involves 167 amino acids secreted from various tissues and fat cells, weighs 16 kDa, and constitutes a specific blood level in the plasma (9,10).

Background/aim: Cerebral vasospasm is a term that refers to prolonged, slowly progressing but reversible pathological narrowing of cerebral arteries occurring several days after subarachnoid hemorrhage (SAH), usually accompanied by a decrease in perfusion distal to the affected artery. Leptin is an endogenous polypeptide hormone that can be carried freely and bound to protein in the blood.

Materials and methods: We investigated the superiority of topical application of leptin that may make a contribution to the development of new treatment modalities for unconscious patients in brain injury intensive care units and its preventive effect, which is considered to have multifactorial pathogenesis on cerebral vasospasm occurring after SAH via stereological studies of the basilar artery.

Results: When mean serum leptin levels of the groups were compared, statistically significant differences were observed between the control and topical leptin-treated groups in favor of the treated groups with respect to serum leptin levels (P < 0.05). In the topical leptin-applied group, a significant difference in favor of vasodilatation was observed in the measurements of the basilar artery luminal area (P < 0.05).

Conclusion: In accordance with the results, the topical administration of leptin can be used in the prevention of vasospasm, especially in unconscious patients with subarachnoid hemorrhage.

Key words: Subarachnoid hemorrhage, cerebral vasospasm, leptin, basilar artery
Neuroprotection and neurogenesis effects of leptin were reported in recent studies. Exogenous leptin administration after ischemic neuronal damage affects the development of neuronal stem cells and leads to an increase in the number of neurons, glia cells, and blood vessels in the perilesional cortex (14,15). Vascular endothelium is considered to be the target organ for leptin and various scientific studies have been conducted to determine its functions (16). It has been demonstrated that intravenous administration of leptin increases serum nitrite/nitrate concentration and that exogenous leptin induces nitric oxide (NO) and may lead to vasodilatation (17). Empirical studies have shown that synchronous exogenous leptin administration with N-monomethyl-L-arginine (L-NMMA), a NO synthase inhibitor, may lead to vasodilatation (18,19). It has been reported in empirical studies that leptin reaches therapeutic levels at 30 min in cerebrospinal fluid (CSF) and at 60 min in serum after topical administration of 10 µL of a 1.2 mg/mL solution (20). In this study, it was aimed to investigate the possible topical treatment effects of leptin on cerebral vasospasm after SAH.

2. Materials and methods
Forty female Sprague-Dawley rats weighing between 280 and 300 g were used in the study. All subjects were kept in standard laboratory conditions (12/12-h light/dark cycle) and fed with a standard diet. This study was approved by the experimental animal ethics committee of Ondokuz Mayıs University (dated 02.06.2012 with number 02-2012/01) and the study was conducted at the Ondokuz Mayıs University Experimental Animals Research Center.

2.1. Experimental design
- **Group 1**: Control group (n = 8)
- **Group 2**: SAH (+), leptin (-), sacrificed on day 3 (n = 8)
- **Group 3**: SAH (+), leptin (+), sacrificed on day 3 (n = 8)
- **Group 4**: SAH (+), leptin (-), sacrificed on day 7 (n = 8)
- **Group 5**: SAH (+), leptin (+), sacrificed on day 7 (n = 8)

2.2. Surgical procedure
General anesthesia was achieved with an intraperitoneal injection of a ketamine (50 mg/kg) (Ketalar, Eczacibaşı) and xylazine (10 mg/kg) (Rompun 2%, Bayer) mixture in overnight fasted rats. Anesthesia was adjusted to make rats unresponsive to pain and support spontaneous ventilation. Additional doses were given if needed. Body temperatures of rats during the experiment were controlled by a rectal temperature probe and kept constant at 37 °C. After maintaining general anesthesia, rats were shaved and a stereotactic frame was placed with the head flexed at 45°. The surgical site was cleaned with polyvinylpyrrolidone iodine. A 2-cm midline occipitocervical flat incision was made, the occipital muscles and posterior cervical muscles were dissected, and the atlantooccipital membrane was exposed (Figure 1). Rats were placed in the supine position and left inguinal regions were cleaned with polyvinylpyrrolidone iodine. After skin incision, the left femoral artery was exposed. After femoral artery catheterization, 0.1 mL of nonheparinized autologous arterial blood was taken and rats were placed in the prone position again. The cisterna magna was entered through the atlantooccipital membrane with a 25-gauge needle. After extracting 0.1 mL of CSF, arterial blood in the same amount was injected slowly for 30 s (Figure 2). Muscle tissue and skin were sutured and the surgical site was closed (Figure 3). Rats were placed in a 45° Trendelenburg position to achieve intracisternal clot formation for 15 min. Rats were kept in cages after fully waking up. Before the procedure, all subjects were given prophylactic 50 mg/kg i.p. cephalosporin. Polyvinylpyrrolidone iodine dressing was performed for the surgical wound sites daily until the rats were sacrificed.

2.3. Leptin administration
The day on which SAH occurred was considered day 0. The first dose of 10 µL of 1.2 mg/mL leptin in phosphate-buffered saline solution (Sigma-P4417) was administered...
to the same eye of the subjects in group 3 three times and those in group 5 seven times every 24 h.

2.4. Measurement of serum leptin levels
Rat leptin standard solutions were prepared in accordance with the test protocol. Rat serum samples were diluted 20-fold in Eppendorf tubes and made ready for the analysis. The Rat Leptin ELISA (BioVender, Cat. No:RD2910001200R) kit procedure was followed for the serum samples obtained from all subjects (Figure 4).

2.5. Histological and stereological procedures
The chest cavity was opened under general anesthesia using ketamine and xylazine. For the measurement of serum leptin levels, intracardiac blood (3 mL) was taken from the heart and samples were centrifuged, and then serum was separated and stored at −20 °C. After taking the blood, the descending aortas of rats were closed with a clamp. An intracatheter was placed in the left ventricle. Initially, 200 mL of heparin-containing saline solution was given. The fluid returning to the heart following brain perfusion was drained by opening the right atrium. Vascular structures were fixed by giving 10% formalin solution (200 mL). The brain and brain stem were removed by performing a large craniectomy. For stereological analysis, prelabeled tubes containing 10% formalin were monitored via routine light microscopy. Samples were prepared from the monitored tissues and embedded in paraffin. After preparing the paraffin blocks, sections of 15 µm were taken from each block using microtome in accordance with the principle of systematic random sampling, one of the basic principles of stereology. After a routine histological follow-up, sections were stained with hematoxylin and eosin. Field measurements were done on the stained sections by using the Cavalier method (Stereoinvestigator 9.0, MicroBrightField, Colchester, VT, USA) in the Stereology Workstation. For these measurements, a point-counting grid was used. The distance between the points on the grid was determined as 20 µm × 20 µm according to preliminary examination. While making calculations on the sections, the point-counting grid was placed randomly at the relevant sections on serial cross images and all points intersecting the related structure were counted. The areas belonging to the basilar artery lumen and the tunica media layer were restricted. Two different markers were used for two different fields, and the field measurements were performed (Figure 5). Point density of the point-counting grid was determined by considering the number of serial light microscopic images specified by an appropriate coefficient of error (21,22). Counting was performed in all groups according to the point density determined by the coefficient of error calculated in the pilot study. Obtained results were put in the relevant place in the following formula and field measurements of above-mentioned parameters were calculated (23):

Figure 3. Surgical site was sutured and closed after the surgical procedure.

Figure 4. Performing ELISA procedure for the serum samples.
Area = a/p (µm × µm) × (SP) µm²

a/p: the space between two points

SP: total number of points intersecting the structure

2.6. Statistical analysis

Statistical analysis was performed using SPSS 20.0 for Windows. A normality test was done prior to analysis. The normality test revealed that data were normally distributed. Data were analyzed using one-way analysis of variance (ANOVA). The Tukey test was used for multiple comparisons. For comparisons of groups, a 0.05 significance level was used.

3. Results

Serum leptin levels (Table 1; Figure 6), basilar artery lumen area (Table 2; Figure 7A) and t. media thickness of the groups (Table 3; Figure 7B) are shown. Histopathological appearances of basilar arteries are shown in Figure 8. In accordance with the data obtained from this study, a statistically significant difference was observed between group 1 and topical leptin-treated groups 2 and 5 in favor of the treated groups (P < 0.05). These results show that topical administration of leptin finds its way into the systemic circulation.

When group 5, which was treated with topical leptin for 7 days after SAH, was compared with group 1 and group 4, a statistically significant difference was observed in favor of vasodilatation in the basilar artery lumen area measurements (P < 0.05), whereas no statistically significant difference was observed between media layers (P > 0.05). The highest media/lumen ratio was in group 4 (0.46), whereas the lowest ratio was in group 5 (0.18).

Table 1. Serum leptin levels of the groups.

<table>
<thead>
<tr>
<th>Serum leptin levels (pg/mL)</th>
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</thead>
<tbody>
<tr>
<td>Groups</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
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</tbody>
</table>

Figure 6. Serum leptin levels of the groups are seen.
4. Discussion
The pathogenesis of cerebral vasospasm cannot be explained clearly but it is considered to be multifactorial. Cerebral vasospasm is one of the most important causes of morbidity and mortality in today's brain surgery. This can be attributed to ineffective treatments and their undesirable detrimental effects (24).

The main problem in patients with SAH is symptomatic brain ischemia developing in the subacute period (between 3 and 14 days) as a result of persistent vasospasm in the intracranial artery. Despite many experimental and clinical studies, effective treatment and prevention method could not be found (25). The presence of cerebral vasospasm in the first 2 weeks after SAH increases mortality up to 1.5–3 times (26).

Unlike the peripheral system arteries, adventitia layers are not well developed in intracranial arteries in the subarachnoid space. Erythrocyte cell infiltration is seen in pores of these arteries, which are fed through CSF from adventitia. Thus, a situation similar to SAH and vasospasm in humans can be provided. In addition, since collateral circulation is well developed in animals, neurological deficit resulting from vasospasm occurs rarely. Another advantage of using the basilar artery is that the posterior circulation of animals is well developed. In our study, a SAH model, the most preferred and widely used method, was used via autologous blood injection (27).

Angiography, transcranial Doppler, and histological measurements are commonly used methods in the evaluation of experimental vasospasm. On the other hand, other methods such as measurements of regional cerebral blood flow and transcranial magnetic stimulations have also been reported (28–32). Since they are reliable and give quantitative results, we carried out evaluations using stereological measurements in our study.

Table 2. The area of the basilar artery lumen.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Basilar artery lumen area (µm²)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,192,640 ± 162,068</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>935,600 ± 159,247</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1,060,866 ± 115,310</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>622,666 ± 106,287</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>974,333 ± 145,644</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Tunica media thickness of the basilar artery.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Thickness of tunica media (µm)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>278,070 ± 46,222</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>277,150 ± 52,939</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>226,950 ± 39,604</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>217,600 ± 40,638</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>196,800 ± 25,243</td>
<td></td>
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</tbody>
</table>

Figure 7. Lumen area (A) and tunica media thickness (B) of basilar artery of the groups.
Leptin is a hormone that is produced by the ob gene and released into the plasma (12,33). After finding its way into the bloodstream, it passes the blood–brain barrier and reaches the central nervous system through its specific receptors and reduces food intake and increases energy expenditure (34).

Nakagawa et al. and Matsuda et al. showed that synchronously administered exogenous leptin and N-monomethyl-L-arginine (L-NMMA), a NO synthase inhibitor, can lead to vasodilatation (16,17).

In his study, Raymond showed peak CSF and serum leptin levels at 30 and 60 min respectively after administering 10 µL of a 1.2 mg/mL solution of rat leptin eye drops (19).

In our study, we investigated the superiority of a topical application (eye drops) of leptin that may make

**Figure 8.** Histopathological view of the basilar arteries of the groups is seen. *; lumen of the artery. Hematoxylin and eosin staining.
a contribution to the development of new treatment modalities for unconscious patients in brain injury intensive care units and its preventive effect, which is considered to have multifactorial pathogenesis on cerebral vasospasm occurring after SAH.

When tunica media area/luminal areas ratio were evaluated, having the highest and lowest rates in group 4 (0.46) and group 5 (0.18) respectively suggested that morphological changes occurred on the 7th day. Having the highest and lowest rates in group 4 (0.46) and group 5 (0.18) respectively also suggested that possible luminal narrowing and expansion are controlled by thickness in the tunica media as well as other intracellular means.

Watson et al. stated that leptin exhibits antioxidant activity indirectly by increasing the levels of catalase, glutathione peroxidase, and glutathione reductase, which are antioxidant enzymes (33). We are of the opinion that the intracellular change caused by leptin is responsible for this result. This approach is consistent with those reported by Lembo et al. (34).

Avraham et al. reported that leptin has significant neuroprotective, neurogenetic, and angiogenetic effects after ischemia (13). The results obtained from that study may have resulted from the possible angiogenetic effect of leptin. We are of the opinion that the current angiogenetic effect of leptin occurs as an increase in lumen diameter and subsequently vascular endothelial growth factor.

As well as its vasodilator effect, antioxidant, angiogenetic, and neuroprotective effects of leptin increase the success rate of the treatment. The topical applicability of leptin in unconscious patients with SAH for the prevention of cerebral vasospasm should be considered and much research on this issue must be planned.

Therapeutic doses recommended in the literature were administered in this study. It was shown in the measurements of serum leptin levels that leptin finds its way into the systemic circulation effectively after topically administration (eye drops). It was also shown that topical administration of leptin leads to vasodilatation in basilar artery vasospasm resulting from experimental SAH.

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