# Effect of Smoking on Rat Basilar Artery: Correlation with Inducible Nitric Oxide Synthase and Endothelin Converting Enzyme-1

## Sigara İçilmesinin Rat Basiler Arterine Etkisi: İndüklenebilir Nitrik Oksit Sentez ve Endotelin Konverting Enzim-1 ile Korelasyonu

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**ABSTRACT**

**AIM:** Smoking is an extremely important risk factor for subarachnoid hemorrhage and seems to increase rupture risk of unruptured aneurysms by accelerating their growth rate. The aim of the study was to investigate the effect of smoking on the luminal diameter with wall thicknesses of rat basilar arteries and to detect alterations of inducible nitric oxide synthase and endothelin-converting enzyme -1 in the endothelial cells.

**MATERIAL and METHODS:** Rats were divided into two groups. The level of middle pons slices were embedded in paraffin before they were stained with hematoxylin and eosin. Rabbit anti-human inducible nitric oxide synthase and endothelin converting enzyme-1 antibodies were used.

**RESULTS:** Significant decrease of the vessel luminal diameter and increase of the vessel wall thickness were found in chronic smokers in our study. There was a linear and significant (p= 0,023, r =0,704) correlation between thickness of the wall and endothelin converting enzyme-1 immune reaction. Correlation was not found with inducible nitric oxide synthase (p> 0.05).

**CONCLUSION:** This study on the comparison of vessel luminal diameter and vessel wall thickness with inducible nitric oxide synthase and endothelin converting enzyme-1 immune reactions revealed that the main effect of smoking on the vessel wall is associated with endothelin converting enzyme-1.

**KEYWORDS:** Nitric Oxide Synthase-2, Endothelin Converting Enyzme-1, Smoking, Basilar artery, Subarachnoid haemorrage

**ÖZ**

**AMAÇ:** Sigara içilmesi subaraknoid kanama için önemli bir risk faktörüdür. Kanamamış anevrizmaların büyümesini hızlandırarak kanama risklerini artırmaktadır. Bu çalışmada, sigara içilmesinin rat basiller arter lümen çapı ve duvar kalınlığına etkisi ile endotel hücrelerindeki indüklenebilir nitrik oksid sentaz ve endotelin konverting enzim-1 değişimi araştırıldı.

**YÖNTEM VE GEREÇ:** İki gruba ayrılan ratların pons orta seviye kesitleri hematoksilen eosin ile boyanmak üzere parafine alındı. Rabbit anti-human antikorları ile inducible nitrik oksid sentaz ve endotelin konverting enzim-1 immunohistokimyasal olarak araştırıldı.

**BULGULAR:** Sigara içen grupta lumen çapında azalma ve duvar kalınlığında artış saptandı. Bu grupta damar duvar kalınlığı ve endotelin konverting enzim-1 arasında lineer ve belirgin korelasyon saptandı(p= 0,023, r =0,704). İndüklenebilir nitrik oksid ile korelasyon saptanmadı(p> 0.05).

**SONUÇ:** Bu çalışmada lumen çapı ve damar duvar kalınlığı ile immunohistokimyasal reaksiyon karşılaştırıldığında, sigara içilmesinin esas etkisinin endotelin konverting enzim ile ilişkili olduğu gösterildi.

**ANAHTAR SÖZCÜKLER:** Nitrik oksid sentaz-2, Endotelin konverting enzim-1, Sigara içme, Basiler Arter, Subaraknoid kanama

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## INTRODUCTION

Smoking is one of the most important risk factors for subarachnoid hemorrhage (SAH) and seems to increase rupture risk of unruptured aneurysms by accelerating their growth rate (21,22,39). The prevalence of smoking in patients who have suffered from SAH is higher than that in general adult population (20,21,39,54). The effect of smoking on the incidence and severity of delayed symptomatic cerebral ischemia following SAH has been investigated by many investigators, according to whom smoking does not significantly affect outcome after SAH (19,21,28,33). On the contrary, Pobereskin et al. have shown that increased vasospasm in smokers may reduce the severity of the initial hemorrhage and that the risk of death from SAH in smokers is almost half that of nonsmokers (37). More recent experimental and clinical data strongly demonstrate evidence for increased oxidative stress being responsible for endothelial dysfunction in chronic smokers (34,35). Endothelial cells regulate vascular tone by secreting paracrine mediators including nitric oxide (NO) and endothelins (11,14). There are a number of possible factors involved in the pathophysiology of cerebral vasospasm, including endothelial injury or dysfunction (16,29). NO released from the endothelium or adventitia of cerebral vessels plays a key role as a mediator of vascular tone. Alterations in vascular tone and dilatation are predominantly affected by alterations in baseline NO. NO decrease in SAH may contribute to certain processes, including vasoconstriction, and decreased cerebral blood flow (CBF), cerebral ischemia (2,3,42). Inducible nitric oxide synthase (iNOS) is expressed in arteries during inflammation and may contribute to vascular dysfunction (41,55). Endothelin-1 (ET-1) seems to represent the contractile component in a network to modulate arterial tone, with NO representing the relaxant component (8). ET-1 is synthesized by vascular endothelial or smooth muscle cells. Endothelin Converting Enzyme-1 (ECE-1) is localized predominantly to endothelial cells, the apparent primary source of ET-1 (56). The synthesis of ET-1 seems to be enhanced in the central nervous system in pathological conditions. Increased levels of ET-1 have been found in cerebrospinal fluid (CSF)and brain tissue after trauma, ischemia, and SAH (25,27).

The aim of this animal-model study was to investigate the effect of smoking on the luminal diameter and wall thicknesses of basilar arteries and to detect alterations in expression of iNOS and ECE-1 in the endothelial cells.

## MATERIALS and METHODS

We studied 24 male Sprague-Dawley rats weighing 380 to 440 gr. Experimental protocols were approved by our institution's animal care committee. Rats were divided into two groups: group 1, exposed to cigarette smoke for eight weeks (12 rats) and group 2, control (12 rats)

### Smoking Protocols

We used the Modified Walton machine for experimental smoking to provide a continuous exposure to cigarette smoke for eight weeks (4). Cigarettes (containing 0.9 mg nicotine and 12 mg tar each) were used to generate smoke. Two cigarettes were used subsequently in each exposure to achieve a continuous animal exposure of 12 min. The first cigarette was kept lit for 6 min and removed and then second cigarette was inserted and kept lit for another 6 min. Non-smoking rats in group 1 were kept in the same environment (in the smoking machine without smoking) and duration (for eight weeks).

### Tissue preparation and examination:

The rats were anesthetized with pentobarbital sodium (Nembutal, Abbot: 50 mg/kg) before they were decapitated and their brains were removed and fixed in paraformaldehyde for at least 2 days. Blocks of tissues were taken by cutting the brain stem above and below the pons. The samples were subdivided at the level of middle pons (Figure1). These slices about 5mm thick were embedded in paraffin before they were stained with hematoxylin and eosin. Sectioned slices were obtained at 6 micron thickness. The vascular luminal diameter and thickness of wall were examined by light microscopy and photographed, and planimetric measurements were performed. Non-smoking rats were included in control group.

### Immunohistochemistry

Rabbit anti-human iNOS (sc 651, Santa Cruz Biotechnology, Santa Cruz, Ca) and ECE-1 (sc 27557, Santa Cruz Biotechnology, Santa Cruz, Ca) antibodies were used to detect iNOS and ECE-1. Antibody binding was visualized by use of biotinylated secondary antibody, avidin-horseradish peroxidase, and diaminobenzidine (Santa Cruz Biotechnology). Immunoreactivity for iNOS and ECE-1was mainly determined in the endothelial cells of the vessel wall. A semiquantitative scale was used for

*Figure 1:* The samples were subdivided into two segments at the level of middle pons.

**Table II:** The distribution of the frequencies of the ECE-1 and iNOS semiquantitative values in group 1 and group 2.

| Staining intensity | Group 1 (n= 10) (Smoker rat) | Group 2 ( n=11) (Control rat) |
|---|---|---|
| **ECE-1** | | |
| 1 | 1 (10%) | 5 (45,5%) |
| 2 | 1 (10%) | 5 (45,5%) |
| 3 | 8 (80%) | 1 (9%) |
| **iNOS** | | |
| 1 | 0 (0%) | 0 (0%) |
| 2 | 0 (0%) | 3 (27,3%) |
| 3 | 10 (100%) | 8 ( 72,7%) |

immunohistochemical evaluation of iNOS and ECE-1 by the pathologist in a blind way. The staining of the endothelial cells were graded as 0, absent staining; +1, weak staining; +2, moderate staining; +3, strong staining (Table II).

### Statistical analysis

Statistical analysis was performed via the SPSS 11.0 software for Windows. A value of p of less than 0.05 was considered statistically significant. Simple descriptives and frequency analysis were used to define the minimum, maximum, mean, values, and standard deviation. Student's t-test was used for the comparison of vessel diameter, vessel wall thickness, ECE and NOS between the smokers group and those of the control group. Correlation studies were performed by the Pearson correlation test.

### RESULTS

Two rats in the smoker group (group 1) and 1 rat in control group (group 2) died during the experiment. Evaluation was made for group 1 with 10 rats and group 2 with 11 rats.

### Vasospastic change in basilar artery

There was marked narrowing in the lumens of basilar arteries in group 1 compared to group 2. The diameter of the artery lumen in group 1 ranged between 96 and 131 micrometers ($\mu$m). The mean vessel luminal diameter was 117.42±11.57 (mean±SD) $\mu$m. The diameter of the artery lumen in group 2 ranged between 123 and 168 $\mu$m and the mean luminal diameter was 147.82±13.02 $\mu$m. The mean vessel luminal diameter of the smokers group was significantly lower than the control group (p<0.0001).

The mean of the thickness of the vessel was 29.52±4.79 $\mu$m (range, 21 to 37 $\mu$m)in Group 1 and 28.09±5.84 $\mu$m (range, 21 to 34 $\mu$m) in Group 2 (Table I) Although the mean vessel thickness increased in the smokers group, it was not statistically significant (p>0.05). Thickening of the arterial wall involved the tunica media and adventitia. No additional histological change was noted in the arterial walls in these groups.

### Immunohistochemistry

*ECE-1:* Staining was weak in 5 of 11, moderate in 5 and strong in 1 in the control group. In the smoking group, staining was strong in 8 of 10 rats, moderate in 1 and weak in 1. The mean ECE-1 staining was 2.70±0.67 in the smokers group and 1.64±0.67 in the control group. The staining intensity of ECE-1 was significantly higher in the smokers group (p=0.002). There was a significant (p= 0.023, r =0.704) linear

**Table I:** The comparison of vessel lumen diameters, wall thicknesses of the basilar arteries and ECE-1, iNOS immunohistchemical staining in group 1 and group 2.

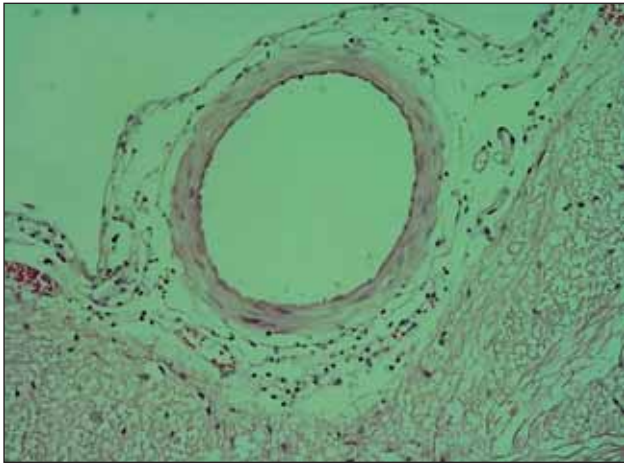| | Vessel diameter | Vessel thickness | ECE | NOS |
|---|---|---|---|---|
| Group 1 (smokers) | 117.42±11.57 | 29.52±4.79 | 2.70±0.67 | 3.00±0.0 |
| Group 2 (control) | 147.82±13.02 | 28.09±5.84 | 1.64±0.67 | 2.73±0.46 |
| p value | p<0.0001 | p>0.05 | p= 0.002 | p=0.082 |

**Figure 2:** *The distribution of the frequencies of the ECE-1 and iNOS semiquantitative values in group 1 and group 2.*

correlation between the thickness of the vessel wall and immunostaining for ECE-1 (Figure 3A,4A)

*iNOS:* All the rats were strongly stained in group 1. 8 of 11 rats were strongly and 3 of 11 rats moderately stained in group 2. Mean iNOS staining was 3.00 in the smokers group and 2.73±0.46 in the control group. Although iNOS staining was increased in the smokers group, there was no significant difference between the two groups (p=0.082), (Figure 3B,4B).

## DISCUSSION

CBF is a very important parameter for stroke and SAH. Smoking appears to have a significant biphasic affect on the tone in the cerebral vasculature. This is due to pial vasodilatation in the acute phase and decreased CBF in chronic phase (26,38). Various studies assert that NO at cerebral artery is responsible for pial dilatation during smoking (3,16).

In SAH patients, there is a strong correlation between local narrowing of arteries and decreased CBF that worsens the clinical grading (15,23). In addition to changes in CBF that correlated with the degree of cerebral vasospasm, the canine model of subarachnoid hemorrhage showed significantly decreased basilar artery diameter and CBF (1). Although CBF was not measured in this study, a significant decrease was found in basilar artery lumen diameter in smokers. The mean wall thickness was found to have increased in the smoking group due to vascular constriction.

NO is synthesized from L-arginine by NO synthase (NOS). There are three types of NOS: neuronal NOS (NOS-1 or nNOS), inducible NOS (NOS-2 or iNOS) and endothelial NOS (NOS-3 or eNOS) (5,17).
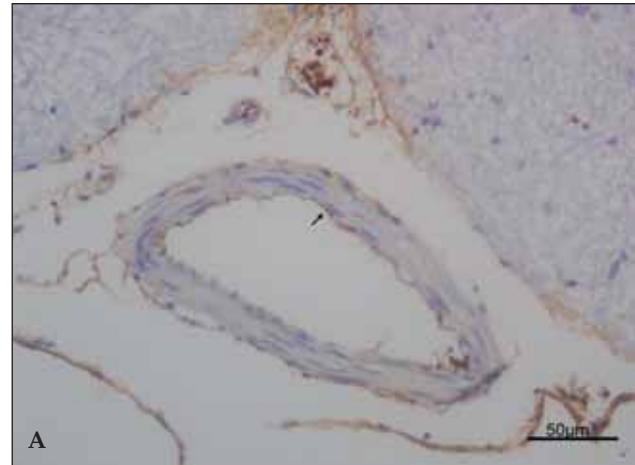


**Figure 3:** *The weak immunohistochemical expression(+1) of ECE-1 (**A**) and moderate (+2) iNOS (**B**) in the endothelial cells of the vessels in control group (arrow) (x100).*

Administration of NO donor after experimental SAH model restores CBF and decreases ischemic glutamate release (43). Administration of L-arginine has been proposed as a potential treatment for delayed cerebral vasospasm (24,48). Infusion of L-arginine did not influence the incidence and degree of vasospasm but this infusion markedly increased CBF in an animal model of SAH (36). Continuous release of NO from endothelial cells is necessary to maintain resting cerebrovascular tone and basal CBF (49,53). Binding of NO by hemoglobin results in decrease of NO at arterial wall, and the decrease in NO at the arterial wall is a putative cause of vasospasm (6,18,48).

INOS is an important modulator of NO activity, and is expressed by smooth muscle cells during the critical early phase of vasospasm (46,47). Sayama et al. showed that the smooth muscle layer is not the primary site of iNOS expression after SAH and that the main sources are vascular endothelial cells and
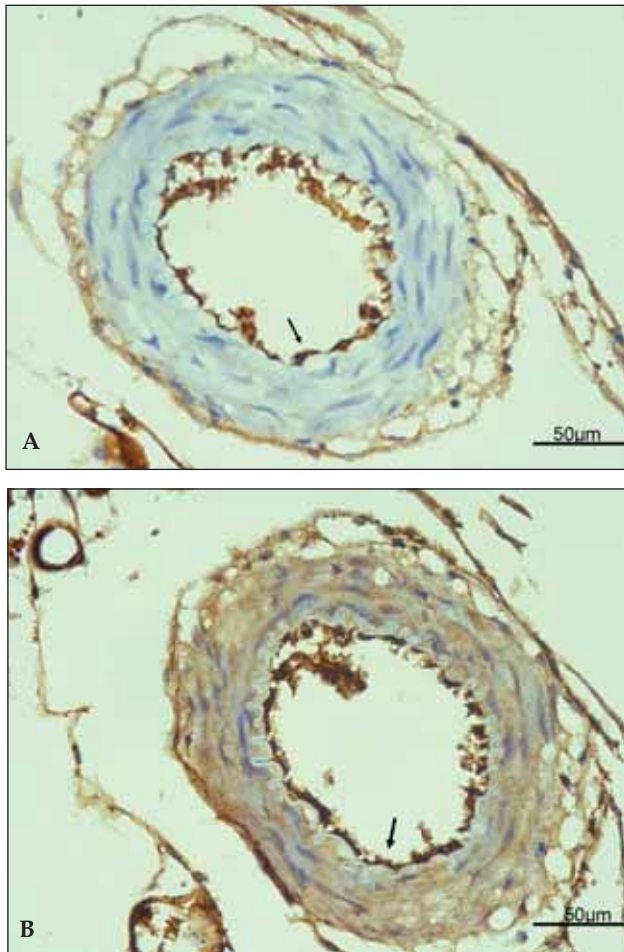
*Figure 4: The strong immunohistochemical expression (+3) of ECE-1 (A) and iNOS (B) in the endothelial cells of the vessels in smoker group (arrow) (x100).*

adventitial layer (41). They reported improvement in the process of vasoconstrictive changes on experimental SAH model in rats using aminoguanidin which is a selective inhibitor of iNOS and mentioned that iNOS is important in vasospasm. Widenka et al. showed that iNOS was present in endothelial cells, smooth-muscle cells and adventitial cells (55). At seven days after experimental SAH, their results revealed a positive correlation between the induction of iNOS and the degree of vasospasm. They stated that iNOS is the possible key factor for chronic vasospasm in the experimental SAH model. There was no significant difference in iNOS immunoexpression in our study, despite the significant changes observed on vessel wall diameters between smoker and nonsmoker rats. The arterial diameter of the chronic smoker group was significantly constricted compared to that of the control group. However iNOS immune reaction at vessel wall was about the same as the control group. Mean vessel lumen diameter and iNOS immune staining was $117.42 \pm 11.57$ and $3.00 \pm 0.0$ in the smokers group and $147.82 \pm 13.02$ and $2.73 \pm 0.46$ in the control group. No correlation was found between decrease of vessel diameter and iNOS immunostaining.

In this study, we considered that demonstrating the change of iNOS, which is thought to be important for vascular tonus, in the rat basilar artery, could be useful to understand vasospasm in smoking SAH patients. Although there is a significant difference of luminal diameter between the smoker and control groups, iNOS does not seem contribute to this process. We believe that evaluation of other NOS (neuronal and endothelial) for complex vascular tonus could provide additional information as well.

The system between endothelium-derived constricting and relaxing factors is important for regulating and preserving the vascular tonus of the normal and pathologic cerebrovascular system. ET-1 was first identified in the culture of supernatant of porcine aortic endothelial cells (57). There is an increasing evidence for a central role of the polypeptide ET-1 in the pathophysiological cascade leading to this vasospasm. This is supported by a correlation of cerebral vasospasm with increasing levels of ET-1, ET-3, and big ET-1 in the CSF or plasma of SAH patients (56). Increased levels of ET-1 have been found in CSF and brain tissue after SAH (7,44). The protease that catalyzes the conversion of big ET-1 to ET-1 was termed "endothelin-converting enzyme" (ECE). There are two specific ECE subtypes, ECE-1 and ECE-2 (9,56). Recently, two ECE-1 isoforms have been identified and termed ECE-1 alpha and beta, which are alternatively spliced products of the same gene and share the same C-terminal domain (45,51). In most tissues, expression of the ECE-1 subtype seems to exceed that of ECE-2. ECE-2 is localized in intracellular compartments while ECE-1 is a membrane-bound ectoenzyme. Accordingly, a large amount of ET-1 released from endothelial and parenchymal cells seems to be processed predominantly by ECE-1 activity (56). Thus, ECE-1 has been regarded as an integral component of ET-1 processing in endothelial cells. ET-1 is an extremely potent agent that plays a key role on long-lasting contractile effect on cerebral vessels (12,13,40,52). Knowing that ECE-1 activity is important in vasospasm, we examined basilar artery diameter and found a correlation between vascular diameter and ECE-1 activity.

Nicotine causes endothelial dysfunction, resulting in the attenuation of endothelium- dependent vasodilatation. This is consistent with endothelium-dependent vasodilatation and with previous papers (32,50). The precise mechanism of smoking-related endothelial dysfunction is not well understood but is probably multifactorial. Although vasospastic vessels showed a relatively normal number of endothelial cells, these cells exhibit features of structural damage such as vacuolation, loss of tight junction, and wall necrosis (10,30,31). These pathological processes support the concept of a toxic factor.

A significant decrease of the vessel diameter and increase of the vessel wall thickness was found in chronic smokers in our study. There was a significant (p= 0,023, r =0,704) linear correlation between thickness of the wall and ECE-1 immunoreaction. Correlation was not found with iNOS (p> 0.05). The main effect of smoker processes on the vessel wall is associated with ECE-1 but not with iNOS. This preliminary study shows that the relatively decreased degree of initial hemorrhage and decreased death risk in smoker SAH patients may be due to increase in ECE-1 increase and vasospasm. NO and endothelin subtypes should be studied in the new SAH model for smoker rats in further studies.

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