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The Effect of Copper on Vasospastic Femoral Artery in Rats

Vazospastik Sıçan Femoral Arterinde Bakırın Etkisi

Eser GUMUS[1], Utku ADILAY[2], Omur GUNALDI[3], Cicek BAYINDIR[4], Burak KAZANCI[2], Bulent GUCLU[2]

[1]Bakirkoy Mazhar Osman Research and Training Hospital for Neurology, Department of Neurosurgery, Istanbul, Turkey
[2]Ministry of Health, Sevket Yilmaz Research and Training Hospital, Department of Neurosurgery, Bursa, Turkey
[3]Cukurova State Hospital, Department of Neurosurgery, Adana, Turkey
[4]Istanbul University, Faculty of Medicine, Division of Neuropathology, Department of Pathology, Istanbul, Turkey

Corresponding Author: Bulent GUCLU / E-mail: guclubulent@hotmail.com

ABSTRACT

AIM: The aim of this study was to investigate the effect of copper, which is the cofactor and regulator of the superoxide dysmutase enzyme, on preventing experimental peripheral vasospasm in rats through antioxidative mechanisms.

MATERIAL and METHODS: Twenty-four female Wistar-Albino rats were divided into 3 groups: group 1 (n=8), control group; group 2 (n=8), vasospasm group; and group 3 (n=8), vasospasm + copper treatment group. Morphometric measurements of wall thickness and lumen diameter of femoral arteries were performed.

RESULTS: Statistical comparison of groups 1 and 2, regarding thickness of vascular walls, revealed a significant increase in group 2 (p=0.037) and regarding lumen diameters, revealed a significant decrease in group 2 (p=0.043). Comparison of diameters of the vascular lumen of groups 1 and 3 showed no significant difference (p=0.19), whereas the comparison of the thicknesses of the vascular walls displayed a significant increase in group 3 (p=0.028). Comparison of groups 2 and 3 regarding diameter of vascular lumens showed a significant decrease in group 2 (p=0.042), whereas group 3 displayed a significant decrease, in terms of thickness of the vascular walls (p=0.029).

CONCLUSION: This study showed quantitatively that copper could prevent the development of experimental peripheral vasospasm in rats.

KEYWORDS: Antioxidant, Cerebral vasospasm, Copper, Rat, Subarachnoid hemorrhage, Superoxide dysmutase

ÖZ

AMAÇ: Bu çalışmanın amacı, süperoksit dismutaz enziminin kofaktörü ve regülatörü olan bakırın sıçanlarda deneysel periferal vazospazmı antioksidatif mekanizmalarla engellemedeki etkisini araştırmakdı.

YÖNTEM ve GEREÇLER: Yirmi dört tane Wistar-Albino sıçanı üç gruba bölündü: birinci grup (n=8), kontrol grubu; ikinci grup (n=8), vazospazm grubu; ve üçüncü grup (n=8), vazospazm + bakır tedavi grubu. Femoral arterlerin duvar kalınlığı ve lümen çapı morfometrik olarak ölçüldü.

BULGULAR: Birinci ve ikinci gruplar damar duvar kalınlığı açısından ölçüldüğünde ikinci grupta istatistiksel olarak bir artış saptandı (p=0,037) ve lümen çapı açısından ikinci grupta bir azalma saptandı (p=0,043). Birinci ve ikinci gruplar damar lümen çapı açısından kıyaslandığında bir fark saptanmadı (p=0,19), ancak üçüncü grupta damar duvar kalınlığında artış saptandı (p=0,028). İkinci ve üçüncü gruplar damar lümen çapı açısında kıyaslandığında ikinci grupta belirgin bir azalma vardı (p=0,042), bununla birlikte damar duvarı kalınlığı açısından üçüncü grupta belirgin bir azalma vardı (p=0,029).

SONUÇ: Bu çalışma, bakırın sıçanlarda deneysel periferal vazospazmı nicel olarak engellediğini göstermiştir.

ANAHTAR SÖZCÜKLER: Antioksidan, Serebral vazosazm, Bakır, Sıçan, Subaraknoid kanama, Superoksit dismutaz

INTRODUCTION

Cerebral vasospasm (CV) is a major cause of morbidity and mortality occurring after subarachnoid hemorrhage (SAH) (2, 4). Despite great advances in medicine, there is no effective drug treatment for cerebral vasospasm (5). One of the most important theories for pathogenesis of CV is the effect of lipid peroxidation resulting from free radical-mediated injuries (11, 15). Copper (Cu) is a cofactor of superoxide dysmutase (SOD) and regulates the activity of this enzyme, so it can prevent or regulate the vasoconstriction caused by free oxygen radicals. In the literature, various drugs with antioxidant effects have been used to treat cerebral vasospasm either in vivo or in vitro, but no research on copper increasing SOD activity to prevent CV could be found. The aim of this study is to investigate the effect of copper in preventing of experimental vasospasm in the femoral arteries of rats.

MATERIAL and METHODS

This experimental study was performed at the Neurosurgery Animal Laboratory of Bakirkoy Mazhar Osman Research and Training Hospital for Neurology, Neurosurgery and Psychiatry, and Laboratory of Neuropathology Department, Istanbul University.

In this study, 24 female Wistar-Albino rats weighing 180-220 gr. were used. The model of Okada et al. was used as the femoral artery vasospasm model (12). The rats have been divided into 3 groups: group 1 (n=8), control group; group 2 (n=8), vasospasm group; and group 3 (n=8), vasospasm + copper treatment group.

The rats that were selected for the surgical procedure for creating vasospasms in femoral artery were sedated with intraperitoneal 2 mg/kg ketamine HCl (Ketalar vial 50 mg/ml, Pfizer) and placed onto cork blocks in the supine position. The inguinal areas of the rats have been shaved and sterilized with povidine iodine. Two cm longitudinal skin incisions were made and the femoral artery-nerve bundles were exposed. The femoral arteries were dissected from the femoral veins and nerves without being traumatized. A silastic cover was wrapped around a 1-1.5 cm part of the femoral artery and sutured surrounding the artery. Autologous cardiac blood of the subjects was used as whole blood. For the rats in groups 2 and 3, 0.1 cc of percutaneous intracardiac blood was taken with an insulin injector and was placed into the silastic cover, in order to generate the peripheral vasospasm model. The rats in group 3 were subjected to 0.1 mg/kg/day of intraperitoneal copper sulfate for 7 days, and the rats in group 1 were delivered 1 cc 0.9% saline. The dosage of copper sulfate in the current study was similar to the ones applied by Chattopadhyay et al. (3). The rats were fed with rat food for 7 days under normal room temperature (23 ºC) with every rat in a single cage. No subject was excluded from the study due to death or illness.

The subjects were sedated with 2 mg/kg of intraperitoneal ketamine at the end of day 7 and were placed onto cork blocks in a supine position. The old surgical incisions and silastic covers around the femoral arteries were opened and the femoral arteries of the rats were exposed. It has been observed that in all subjects, the femoral artery was in the silastic cover. In the meantime the sternums were shaved, sterilized with povidine iodine, and cut to expose the thorax. The pericardiums were opened, and a puncture was made to the left ventricle of each rat with a green-tipped injection needle. A serum set was fixed to this catheter (the serum set had been placed 10 cm high to establish the physiologic arterial pressure), and a mixture of 100 ml 0.03 M phosphate buffer, 200 ml 4% paraformaldehyde, and 1% glutaraldehyde solution was given to the left ventricles. The given solution had been circulated in the whole circulation until clear fluid was obtained from the right atrium. A small part (1-1.5 cm) of the right femoral artery of all subjects in all groups was cut and taken out for histopathologic, morphometric analysis. After this procedure, the subjects were sacrificed with a megadose of intraperitoneal ketamine.

The femoral artery specimens were placed in 10% buffered formaldehyde and then put into a tissue processor and treated with formalin for fixation purposes. After the specimens were dehydrated with graded alcohol, they went through a xylene stage, and paraffin was applied. All procedures up to this stage took 24 hours. Then the specimens placed into paraffin were taken and frozen as paraffin blocks. Each tissue block was serially sectioned perpendicular to the femoral artery lumen. The thickness of the sections was 6 micrometers, and they were deparaffinized in an incubator at 60 ºC for 1 hour. The deparaffinization process was continued with xylene (which was applied 3 times). The sections were treated with graded alcohol, rehydrated, washed with water and stained with toluidine blue. The preparations were examined under x100, x200, and x400 magnification with an Olympus (Olympus BX7, Japan) microscope and photographed for morphometric analysis. Vascular wall thicknesses and lumen areas were measured as unit values by the Image J 1.34 program. Measurements were carried out on the photographs taken at x40 magnification. To determine wall and artery thicknesses, 10 different areas of each artery wall was measured; the mean value of 10 different measurements was taken as the thickness of an artery wall. To determine the lumen diameter of each artery, diameters of 5 different lumen areas of each artery were measured, and the mean value of 5 different measurements was taken as the lumen diameter of an artery.

Statistical Evaluation

Morphometric measurements of vascular wall thicknesses and vascular lumen diameters were done for all groups, and statistical comparisons were done using SPSS (Version 11.5) software with independent Student's t test. The measurements were expressed as the mean values ± standard deviations. The groups were compared through the calculation of p values; values with $p < 0.05$ were accepted as statistically significant.

RESULTS

When resected femoral arteries were analyzed under a surgical microscope, development of different levels of vasospasm was observed in the rats of groups 2 and 3, whereas no such changes were observed in group 1.

Histopathologic Changes

The femoral artery sections of all groups were examined under a light microscope. In Group 1, it was observed that the arteries had a thin and smooth endothelium, thin and unfolded internal elastic lamina, and concentric smooth muscle cells. In Group 2, significant narrowing in the diameter of the lumen, significant thickening in the vascular walls, disruption of endothelial integrities, folding in the internal elastic lamina, and vacuolization in muscle layers were observed (Figure 1). In Group 3, it was confirmed that the structures of the femoral arteries were similar to the control groups, they had thin and smooth endotheliums, thin and mildly folded internal elastic lamina, and concentric smooth muscle cells (Figure 2).

Morphometric Analysis

After the calculation of mean values for the wall thicknesses and lumen diameters of the femoral arteries of all groups, the groups were compared with each other (Table I). The statistical comparison of groups 1 and 2 showed a significant increase in wall thickness in group 2 ($p=0.037$). This indicated

Table I: Table Showing Comparisons of Femoral Artery Wall Thickness and Lumen Diameter Mean Values of All Groups

	Groups	n	Min.	Max.	Mean value(units)	Std.
Lumen diameter	**Group 1**	8	524.00	942.00	658.00	142.94
	Group 2	8	191.00	479.00	259.87	96.10
	Group 3	8	556.00	920.00	638.13	117.41
Wall thickness	**Group 1**	8	39.00	133.00	78.50	32.94
	Group 2	8	164.00	210.00	195.00	15.48
	Group 3	8	84.00	144.00	101.75	19.53

Min: *minimum,* ***Max:*** *maximum,* ***Std:*** *standard deviation.*

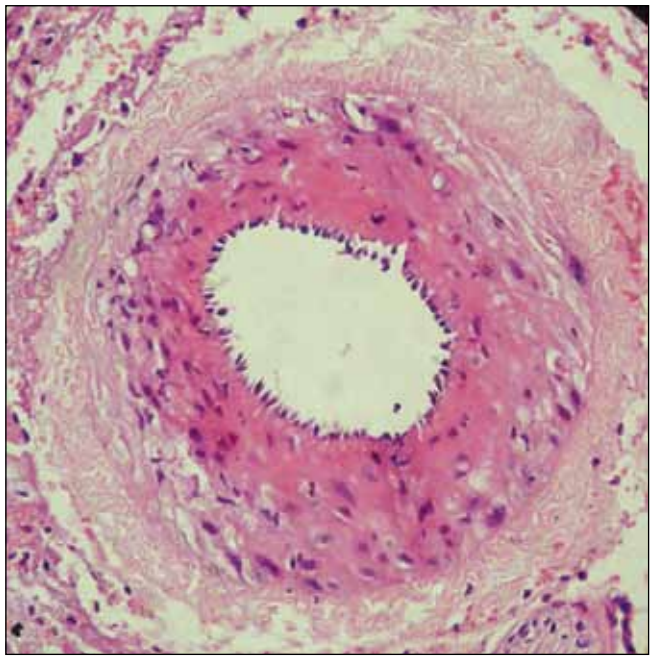

Figure 1: The lumen of the femoral artery of a rat in group 2 (vasospasm group): Significant narrowing in the diameter of lumen and significant thickening in the vascular wall, disruption of endothelial integrity, folding in the internal elastic lamina and vacuolizations in muscle layer were observed (H&E, x40).

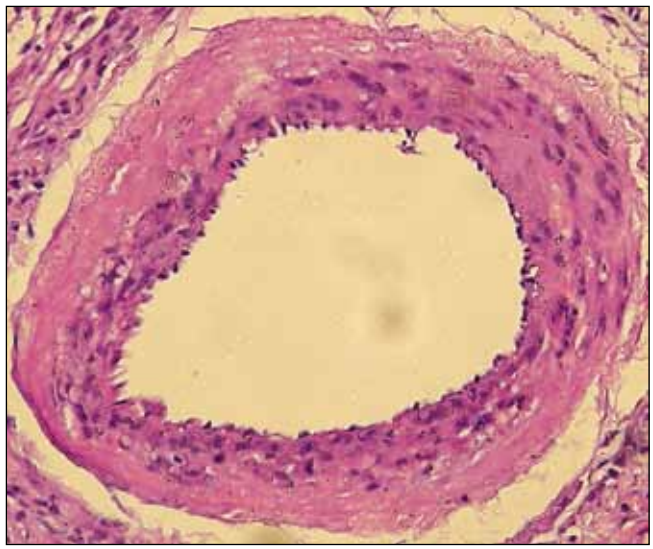

Figure 2: The lumen of the femoral artery of a rat in group 3 (vasospasm + copper treatment group):
Structure of the femoral artery was similar to the control group; they had a thin and smooth endothelium, thin and mildly folded internal elastic lamina and concentric smooth muscle cells (H&E, x40).

the development of a remarkable vasospasm upon delivery of blood to the periarterial areas compared with the control group. Statistical comparison of groups 1 and 2 regarding lumen diameters revealed a significant decrease in group 2 (p=0.043) (Table II). While no significant difference could be observed between the mean vascular lumen diameters of groups 1 and 3 (p=0.19), a significant increase in group 3 was observed in terms of mean vascular wall thickness (p=0.028) (Table III). This finding indicated that the group receiving copper treatment demonstrated a remarkable muscular thickening, but there was no accompanying narrowing in lumen diameter. A significant lumen-narrowing in favor of group 2 was found during the statistical comparison of groups 2 and 3 for mean artery lumen diameter (p=0.042), while a significant increase of wall thickness in favor of group 2 was also found (p=0.029) (Table IV). This indicated the effectiveness of copper treatment in the prevention of vasospasm femoral arteries of rats.

DISCUSSION

CV is the clinical and radiological entity occurring in the distal areas of the affected artery with decreasing perfusion by pathological vasospasm caused by blood and blood products collected in the cerebral cistern after SAH (1, 2, 11**).** The symptoms of clinical CV usually start on the day 4 after SAH and peak at days 7-8; many of them are resolved around the end of week 4. In experimental studies, it has been shown that blood injected into subarachnoid distance causes vasospasm (7, 12, 13). The pathophysiology of a cerebral vasospasm is extremely complicated and multifactorial (8, 10). In vitro and in vivo studies showed that in the development of vasospasm, the main responsible factor is oxyhemoglobin. Oxyhemoglobin causes the artery smooth muscles to contract through several mechanisms (9, 12, 17).

Table II: Table Showing Mean Values of Femoral Artery Wall Thicknesses and Lumen Diameters in Groups 1 and 2

	Groups	Mean values (units)	P value
Lumen diameter	**Group 1**	658.00	-
	Group 2	259.87	0.037
Wall thickness	**Group 1**	78.50	-
	Group 2	195.00	0.043

p<0.05 was found betweeen groups 1 and 2 for the mean values of femoral artery wall thickness and lumen diameter.

Table III: Table Showing Mean Values of Femoral Artery Wall Thicknesses and Lumen Diameters in Groups 1 and 3

	Groups	Mean values (units)	P
Lumen diameter	**Group 1**	658.00	-
	Group 3	638.13	0.19
Wall thickness	**Group 1**	78.50	-
	Group 3	101.75	0.028

p>0.05 was found between groups 1 and 3 for the mean vascular lumen diameters. p<0.05 was found between groups 1 and 3 for mean vascular wall thickness.

Table IV: A Significant Lumen Narrowing for Group 2 was Found During the Statistical Comparison of Group 2 and Group 3 for Median Artery Lümen Diameter (p<0.05), While no Difference was Observed Between These Two Groups in the Comparison of Artery Wall Thickness (p>0.05)

	Groups	Mean values (units)	P
Lumen diameter	**Group 2**	259.87	-
	Group 3	638.13	0.042
Wall thickness	**Group 2**	195.00	-
	Group 3	101.75	0.029

Okada et al. developed the chronic peripheral femoral artery vasospasm method on rats. It has been reported that this method shows similarity with cerebral vasospasm. In the present study, as Okada et al. described in the peripheric vasospasm model, autologous arterial full blood was used to create vasospasm (12).

After SAH, free radicals cause the peroxidation of polyunsaturated fat acids and stimulate the chain reaction of lipid chain peroxydation (9). In the very early stages, the lipid peroxidation products affect the cerebral artery wall and form inflammatory changes (myonecrosis and subintimal proliferation). The lipid peroxidation also forms various eicosanoids and free radicals by causing the decrease of membrane phospholipids (phosphatidylcholine and phosphatidylethanolamine). These events add to the inflammatory changes in the artery wall. It has been shown that the lipidperoxide levels determined in the analysis of cerebrospinal fluid (CSF) of SAH patients match the degree of the vasospasm (6). Sano et al. showed that intracisternal injection of lipid hydroxyperoxide causes vasospasm (16).

Copper is present in the organism bound to antioxidants, cytochrome-C oxidase, catalase, and SOD and participates in the structure of monoaminooxidase, ascorbic acid oxidase and urokinase enzymes. In recent years some studies have been published about the effect of copper on superoxide dismutase. Sansinanea et al. stated that the SOD activity increased in rats that were given 0.2% copper sulfate (CuSO4) solution (14).

Superoxide Dismutase (SOD) is an enzyme that repairs cells and reduces the damage done by superoxide, the most common free radical in the body. Studies have shown that SOD acts as both an antioxidant and anti-inflammatory in the body, neutralizing free radicals. Superoxide dismutase helps the body to use zinc, copper, and manganese. There are two types of SOD: copper/zinc (Cu/Zn) SOD and manganese (Mn) SOD. Each type of SOD plays a different role in keeping cells healthy. Cu/Zn SOD protects the cells' cytoplasm, and Mn SOD protects their mitochondria from free radical damage. The idea of using CuSO4 in this study was created on the hypothesis that it could prevent vasospasm by improving antioxidant defense mechanism and decreasing the biological effects of oxidant materials by increasing the activity of SOD enzyme.

This study showed quantitatively that copper could prevent the development of experimental peripheral vasospasm in rats and the vasospasm group compared to control group showed significant thickening in artery walls and narrowing of artery lumens. When comparing the control group and copper treatment group, despite having no difference between artery lumen diameters, the artery wall thickness in copper treatment group had significantly increased when compared to control group. When vasospasm and copper treatment groups were compared, the artery lumen diameters of the copper treatment group were significantly larger than those of the vasospasm group, but no significant difference could be determined in artery wall thicknesses. However, the results had no necessary direct implications to intracranial arteries or the occurrence of vasospasm in humans in the context of aneurysmal SAH. Many further studies in humans using randomized series are needed to better understand the effect of copper in preventing development of cerebral vasospasm in humans after SAH and to determine copper's effective dose to prevent vasospasm. Yet we had limited conclusions because the results had no necessary direct implication for intracranial arteries and the occurrence of vasospasm in humans in the context of aneurysmal SAH.

ETHICS in PUBLISHING

This experimental study was performed with the approval obtained from the Ethical Committee of Istanbul University,

Istanbul School of Medicine, Experimental Medicine Research Institution.

ABBREVIATIONS

Subarachnoid hemorrhage (SAH), Cerebral vasospasm (CV), Superoxide dismutase (SOD).

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