

Ultrastructural Effects of Two Different Preparation Techniques in Vasoconstriction Studies on the Rabbit Basilar Artery

Tavşan Baziler Arter Vazokonstrüksiyon Çalışmasında İki Değişik Tekniğin Ultrastrüktürel Etkileri

İ. SEMİH KESKİL, M. KEMALİ BAYKANER, HURİYE SENCER, NECDET ÇEVİKER,
Z. SEVİM ERCAN

Fatih University Medical School, Department Of Neurosurgery Emek, Ankara (İSK)
Gazi University Medical School, Departments Of Neurosurgery And Pharmacology Beşevler, Ankara (MKB, NÇ, ZSE)
Ankara University Medical Faculty, Electron Microscopy Unit, Sıhhiye, Ankara (HS)

Abstract: When commenting on morphological findings in cerebral vasoconstriction studies, caution is required in two areas, namely the mechanical stresses involved in experimental manipulations, and preparation techniques. Since simultaneous radiological studies are sometimes compared with the results of morphological investigations, clarification of the relationship between morphological findings and the in vivo situation has gained added importance. In the absence of properly planned control groups, many morphological findings that are, in fact, caused by preparation technique may easily be considered "results." In this study, the ultrastructural effects of two different preparation techniques were evaluated on electron microscopic images of the basilar artery. Ultrastructural changes, such as convoluted lamina elastica interna, cytoplasmic vacuole formation in the endothelium, endothelial denudation, and electron-dense smooth muscle cells with swollen mitochondria and abundant collagenous fibers, were also observed, to a lesser degree, in the control groups.

Key Words: Cerebral vasospasm, endothelin 1, smooth muscle, tissue fixation, vascular pathology

Özet: Serebral vazokonstrüksiyon çalışmalarında morfolojik bulguları yorumlarken iki konuya dikkat etmek gerekir; deneysel monüplasyonla oluşabilecek mekanik stresler ve preparasyon teknikleri. Bazı araştırmalarda morfolojik incelemeler eş zamanlı olarak radyolojik bulgularla karşılaştırıldığından bu yasa morfolojik verilerle in-vivo durumun ilişkisinin önemi de giderek artmaktadır. Düzenli planlanmış kontrol gruplar yokluğunda birçok morfolojik bulgu preparasyon teknikleri sonucu kolaylıkla "sonuç" olarak değerlendirilmektedir. Bu çalışmada baziler arterin elektron mikroskopisi görüntüleri iki değişik preparasyon tekniği ile karşılaştırılmıştır. Lamine elastike internede bükülme, endotel sitoplazmasında vaknol formasyonu, endotel soyulması, mitokondiyal şişme ile birlikte elektron-yoğun düz kas hücresi ve artmış kollejen lif ultrestriktürel olarak gözlenmiştir. Bu bulgulara kontrol gruplarda daha az rastlanmıştır.

Anahtar Kelimeler: Doku fiksasyonu, düz kas, endotelin 1, serebral vasospazm, vasküler patoloji

INTRODUCTION

Cerebral vasoconstriction is an important cause of cerebral ischemia. There is little doubt that the pathogenesis of cerebral vasoconstriction includes many vasodilator and vasoconstrictor substances found in both blood and cerebrospinal fluid. Vasospastic arteries exhibit features typical of stiffening of the contractile and noncontractile components of the vessel wall. However, in the acute period light microscope study does not show the degenerative changes in detail, so ultrastructural investigation is necessary. In commenting on the morphological findings of vasoconstriction studies, caution must be taken in two areas, namely experimental manipulations and preparation technique-related mechanical stress (4). Regarding the former area, short-term arterial constriction is known to occur after simple mechanical stimulation of cerebral vessels (1). Concerning the latter; many different harvesting and fixation techniques are available, but the superiority of perfusion-fixation over immersion-fixation for electron microscopic (EM) studies has been well established. Still, various authors, including Eldevik et al. (6) and Gertz et al. (10), have not denied the possibility that significant findings may exist in unexamined portions of the control specimen.

In this study, our goal was to demonstrate the morphological vascular alterations that stem from preparation technique. Different fixation techniques were applied to both sham operated and Endothelin-1 (ET-1)- treated basilar arteries, since this is an established cerebral vasoconstriction model (3,5,15). ET-1 is a peptide of endothelial cell origin (13) that has potent vasoconstrictor effects. Several reports indicate that it may play an important role in the pathogenesis of cerebral vasoconstriction (2,15,16,18,20), producing sustained angiographic narrowing after intravascular (14) or intracisternal (16) administration by acting directly on smooth muscle cells (16,22). Endothelin levels have been shown to be elevated in the cerebrospinal fluid of patients with subarachnoid hemorrhage (SAH). Furthermore, ET-1 can be measured in the plasma of healthy human subjects, indicating that it is also a circulating compound. Vasospasm can be partially reversed by direct application of monoclonal antibody against ET-1 to the vascular bed. In producing vasoconstriction, ET-1 acts at a site closely coupled to the calcium channel. We chose the basilar artery as the model, since it is one of the semielastic-semimuscular arteries that are most important in the

pathophysiology of cerebral vasoconstriction.

MATERIALS and METHODS

In this study, 18 male New Zealand White rabbits, weighing 2.3-2.8 kg, were randomly divided into 6 groups, and anesthetized with an intravenous injection of Sodium Pentobarbital (30 mg/kg). Animal experimentation was conducted in conformity with the "Guide for the care and use of laboratory animals" (National Institutes of Health publication no: 86-23). Once they were induced, we dissected each animal's inguinal region and catheterized the femoral artery.

Animals in the first group were killed by exsanguination via the jugular veins 30 minutes after saline infusion through the catheter. The entire brain, with basilar artery intact, was quickly removed and placed into a fixative mixture of 2% glutaraldehyde in Sorenson's phosphate buffer (pH 7.4) at 4°C for a minimum of 1 hour. Then the basilar artery was meticulously dissected out under magnification.

The second group underwent craniotomy while under anesthesia, 30 minutes after saline infusion. The basilar arteries were harvested meticulously and immediately placed into the aforementioned fixative solution at 4°C for a minimum of 1 hour. The main difference between the first and second groups was the type of immersion fixation; the basilar artery was removed after euthanasia in the first group, and was removed while the animal was under anesthesia in the second group.

In the third group, 30 minutes after saline infusion we carried out a wide thoracotomy at the level of the fifth intercostal space while the animal was anesthetized. We cannulated the ascending aorta via the left ventricle, opened the right atrium widely, and ligated the descending aorta. Heparin (3,000 IU) was given intravenously for 5 minutes, followed by an infusion of normal saline (150 ml at 22°C) through the ascending aorta for 30 seconds at a pressure of 90 mmHg. Immediately after the blood was washed out, 1 liter of the aforementioned fixative was infused for 10 minutes, also at a constant 90-mmHg pressure. The perfusion-fixated brain was removed and placed into the same fixative solution at 4°C for a minimum of 1 hour. Then, under an operating microscope, the basilar artery was carefully removed.

The 4th, 5th, and 6th groups underwent the same procedures as groups 1, 2, and 3, respectively, but

were infused with ET-1 (0.25 ng total dose, Sigma) for 30 min instead of saline.

Procedure for electron microscopy:

After primary fixation of the specimens, they were washed in phosphate buffer for 5 minutes. Next, they were post-fixed for 1 hour in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), and again briefly rinsed in phosphate buffer for 5 minutes. The specimens were then dehydrated with a graded ethanol series. After this, we stained them en bloc with uranyl acetate-80% ethanol for 30 minutes. The arteries were then embedded in Araldit. Thick sections of approximately 1µm were cut using a Pyramiton (LKB, Stockholm, Sweden) and stained with toluidine blue-borax for light microscopy. Thin sections were cut for electron microscopy (EM) using an ultramicrotome (LKB). These were placed on grids and stained with lead citrate for 3 minutes. Finally, the specimens were examined under an electron microscope (JEOL, 100 B, Tokyo, Japan) without any attempt to assess the resulting images in a quantitative or semiquantitative way. The investigator examining the sections was blind to the preparation protocol used.

RESULTS

Basic ultrastructural findings were evaluated in order, from the luminal to the abluminal side. Findings for the groups assessed with similar fixation techniques were reported together, and, to avoid an

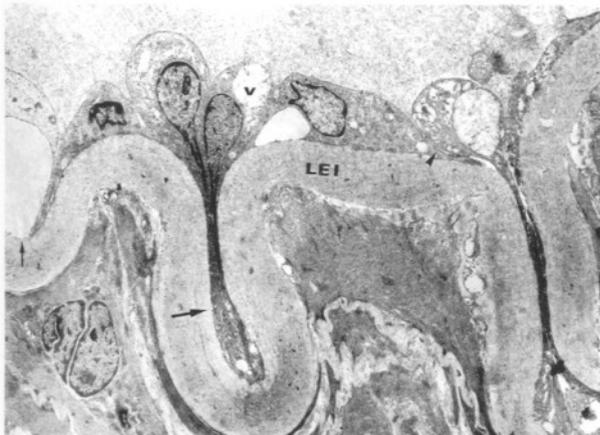


Figure 1: A basilar artery from the first group. The lamina elastica interna (LEI) is corrugated; endothelial cells are round, separated (small arrow), contain vacuoles (V), and tight junctions are separated (x 6100 arrow head). Part of an endothelial cell squeezed between LEI folds appears electron-dense (large arrow).

unnecessary abundance of data, only images from control vessels were shown and discussed.

Groups 1 and 4:

We observed no differences between the groups on light microscopy. On EM examination, endothelial cells appeared rounded, and frequently contained cytoplasmic vacuoles. There was occasional evidence of subendothelial "bleb" formation due to separation and invagination of the cell membrane (Figure 1). Focally, some cells were even detached (Figure 2) and had fallen off into the lumen (Figure 2, inset). The lamina elastica interna (LEI) was corrugated. Occasional endothelial cells and smooth muscle cells (SMC) were squeezed into an invaginated portion of LEI, and these appeared more electron-dense, even necrotic (Figure 1). Increased amounts of collagen were observed in the widened intercellular space (Figure 2). The adventitia was composed of densely packed collagen fibers and fibril-synthesizing stromal cells (Figure 3), some of which were also necrotic. These findings were considered to represent early degenerative changes.

Groups 2 and 5:

Again, no difference was observed between the groups on light microscopy. On EM study, the endothelial cells were generally flat, and were rounded only at the nuclear regions. They frequently contained cytoplasmic vacuoles and condensed organelles. At some places, folding of the

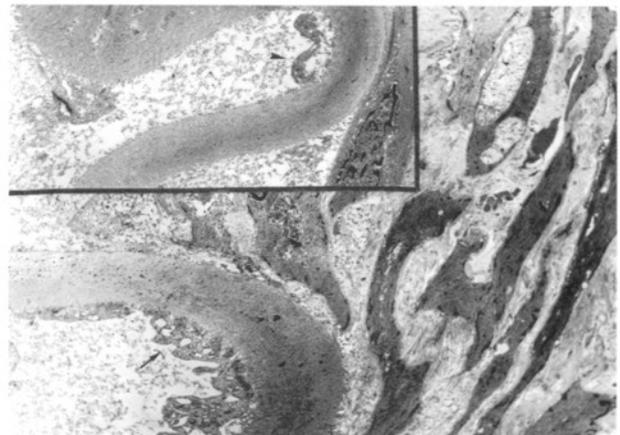


Figure 2: A basilar artery from the first group. Vacuolated endothelial cells (arrow) and both thin and separated smooth muscle cells are observed in the media, together with increased deposition of collagen in the expanded intercellular space. Inset shows an endothelial cell (E) detaching from the vessel wall (arrow head). x 4700 (inset x 5500)

intracytoplasmic membrane was abundant (Figures 4,5) and the tight junctions were disrupted. The LEI was thicker than that observed in groups 3 and 6. Its luminal margin had a granular and moth-eaten appearance, while the abluminal side was relatively homogeneous (Figure 4). Corrugated LEI squeezed the cells mildly but did not give rise to electron-dense or necrotic areas (Figure 4). Occasional regions lacking endothelium and LEI were observed, together with attached platelets (Figure 7, inset) and "irregular" thin LEI (Figure 7). Collagenous fibers were scanty in the narrow interstitial spaces among SMCs (Figure 6), which were joined by their external

laminae. The adventitia was more crowded than that of groups 3 and 6, in terms of cells and collagen fibers per unit area (Figure 6, inset).

Groups 3 and 6:

Light microscopy (Figure 8) clearly showed that the basilar artery had a wider lumen in this group (Figure 8, right and inset) compared to groups 2 and 5, which were normal in appearance (Figure 8, left). The endothelial cells appeared to be thin, and were rich in cytoplasmic intermediate microfilament

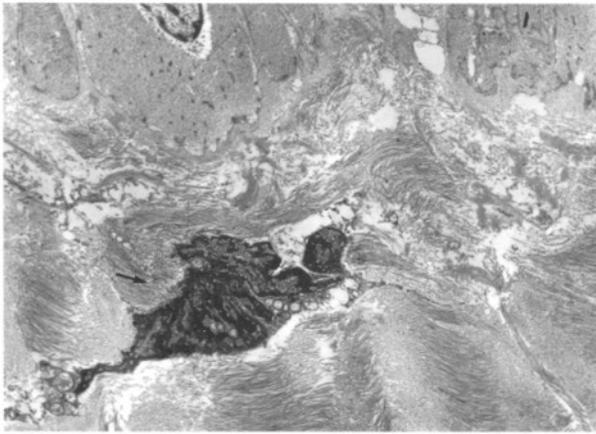


Figure 3: A basilar artery from the first group. The adventitia, visible underneath smooth muscle cells, is composed of densely packed, abundant collagenous fibers and pyknotic fibril-synthesizing stromal cells (arrow). x 7300

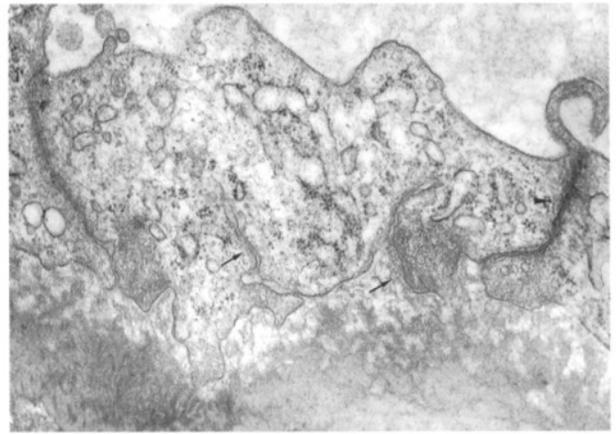


Figure 5: A basilar artery from the second group. At higher magnification, the endothelium displays intracytoplasmic membrane folds (arrows), condensed organelles, and undisrupted, electron-dense, tight junctions (open arrow). x 48000

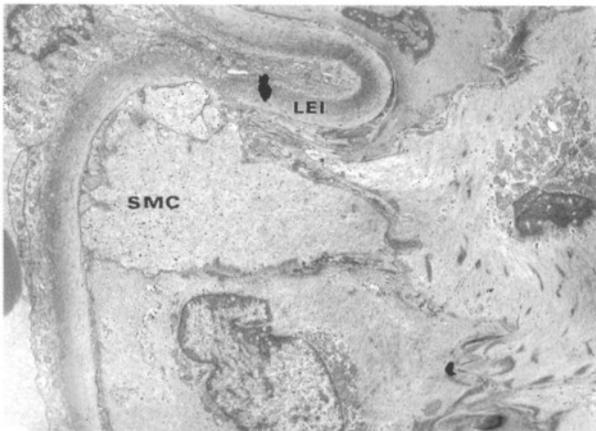


Figure 4: A basilar artery from the second group. Flat endothelial cells are seen on the luminal side of lamina elastica interna (LEI), which appeared somewhat corrugated and squeezed. The LEI is also granular at the luminal margin. Collagenous fibers were scanty in the narrow interstitial spaces among smooth muscle cells (SMC). x 8200

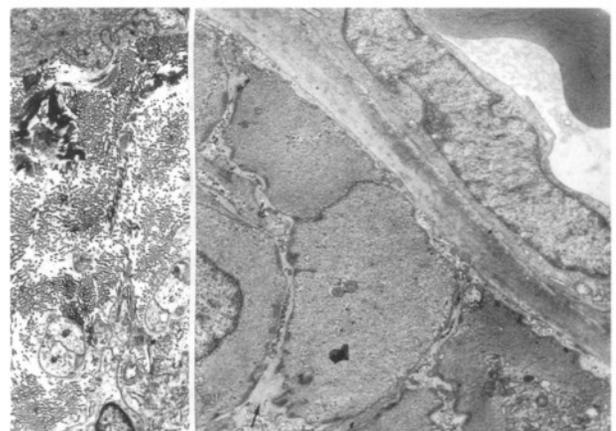


Figure 6: A basilar artery from the second group. Note the narrow interstitial spaces among the smooth muscle cells and scanty collagenous fibers. Smooth muscle cells appear to be joined by the external laminae (arrow). Inset shows the adventitia crowded with collagenous fibers. x 11600 (inset x 7800)

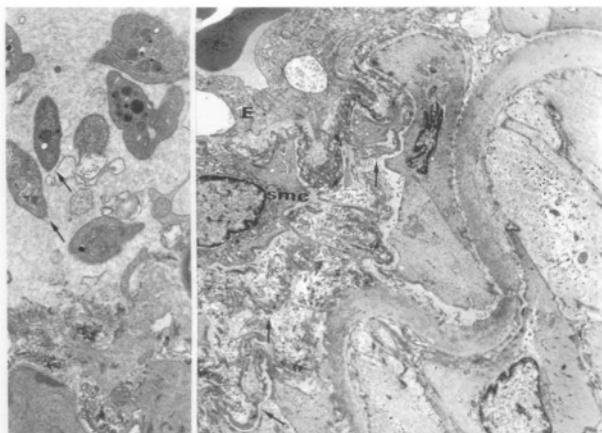


Figure 7: A basilar artery from the second group. This likely traumatized region shows a thin and irregular elastic lamina (arrows) associated with smooth muscle cells (SMC) and endothelial cells (E). Inset shows platelets (arrows) attacking the region of SMCs that is lacking the protection offered by endothelium and LEI. x 6700 (inset x 10600).

bundles. On the basal side, short and sparse but "regular" endothelium extensions were observed stretching into the LEI (Figure 10). The most marked finding was the abundance of swollen mitochondria. The LEI was flat, again with a granular and moth-eaten appearance, and had a homogeneous abluminal margin (Figures 9,10). The SMCs were also thin, and the external lamina was thickened, thus connecting the separate cells (Figure 9). The interstitial space was devoid of collagen fibers (Figure

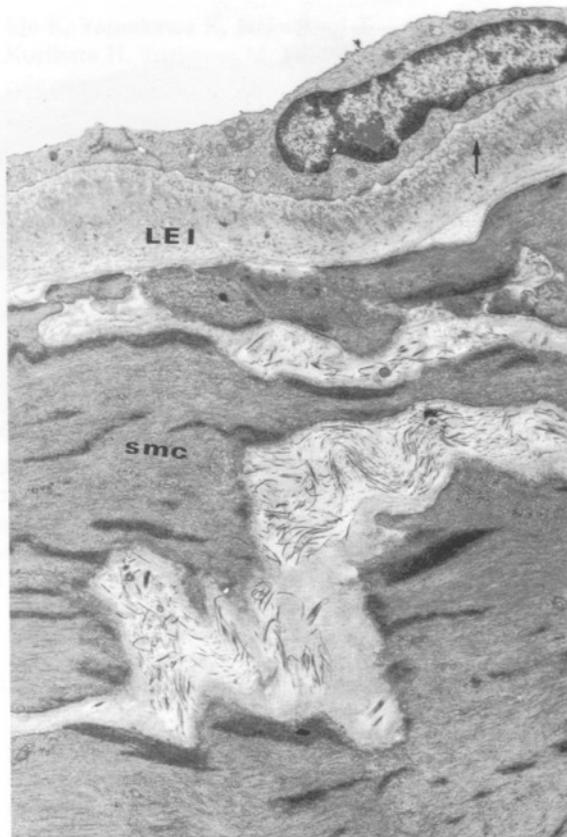


Figure 9: A basilar artery from the third group. Flat endothelial cells are rich in cytoplasmic intermediate microfilaments. The lamina elastica interna (LEI) has a porous appearance at the luminal margin (arrow), while it is relatively homogeneous on the abluminal side. Smooth muscle cells (SMCs) are also thin and separated from each other. x 11800

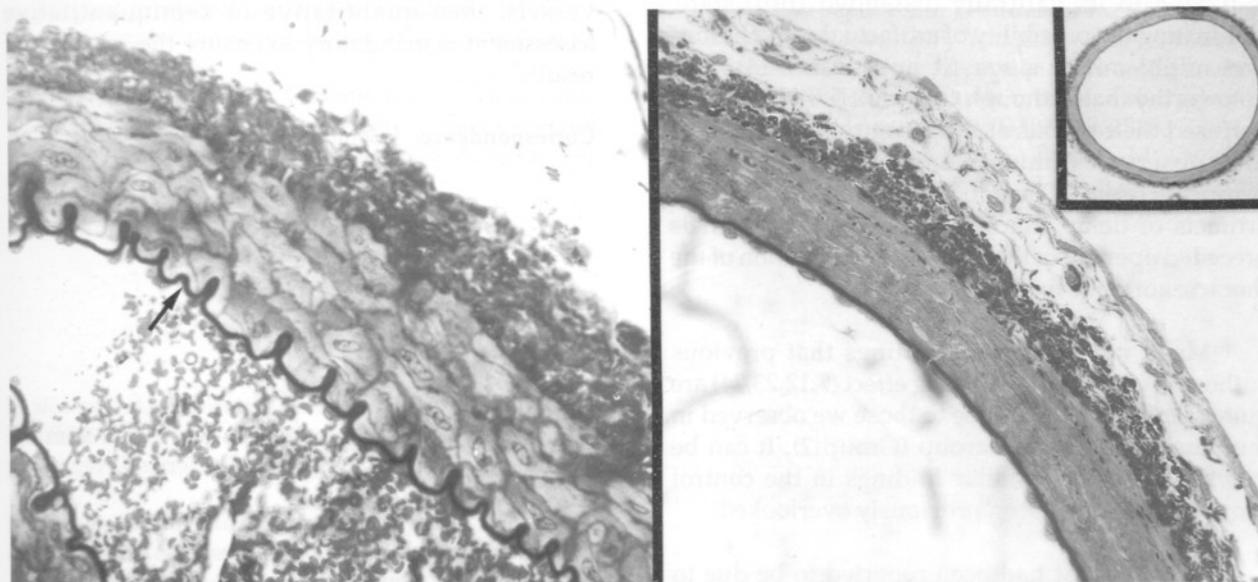


Figure 8: Basilar arteries from the second (left) and third (right) groups. On the left, the artery is narrow and the lamina elastica interna (arrow) is corrugated, while on the right the artery is flat, and has a wide lumen and flat LEI. x 400

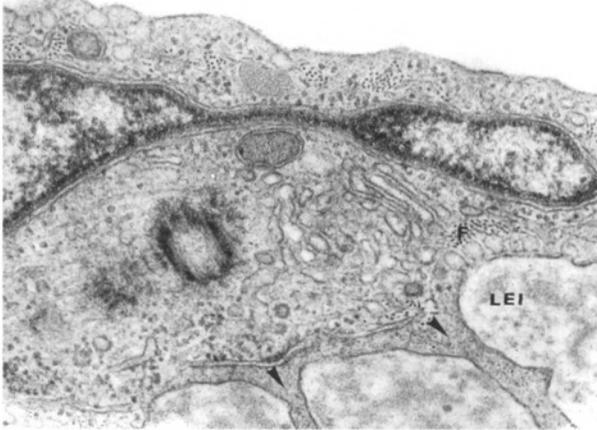


Figure 10: A basilar artery from the third group. At higher magnification, endothelial cells are shown to have both cytoplasmic intermediate filaments (F) and sparse, short, but regular extensions entering the lamina elastica interna (LEI) (arrow heads). x 73300

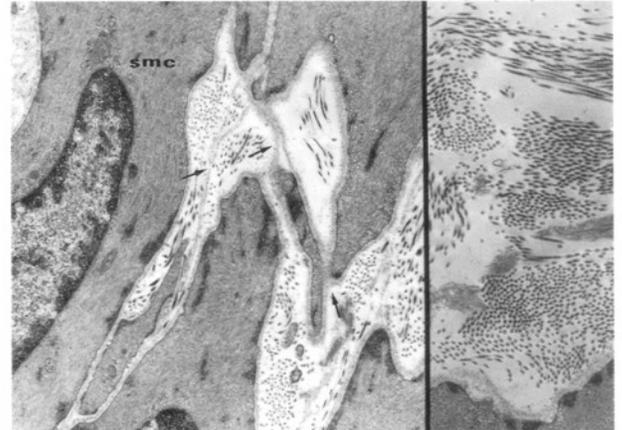


Figure 11: A basilar artery from the third group. Although the smooth muscle cells (SMC) are thin and widely separated, the external lamina forms a connection between them (arrows). Inset shows the scarcity of collagenous fibers compared to Figure 6. x 15800 (inset x 16800).

11), and the adventitia was also loose, containing sparse cells and collagen fibers.

DISCUSSION

The outstanding benefit of perfusion-fixation is that cells are fixed at a definite stage of biological activity. Since many cells are fixated at various stages, the observer is able to grasp a form of *in vivo* image. Authors have stated that intimal folding can be avoided by keeping the intraarterial perfusion pressure above 80 mmHg (4,9). This way the artery is fixated in its naturally distended (full) state, eliminating the possibility of artifactual convolutions that might mimic a spastic appearance (4). It is noteworthy that, although Greenhill & Stehbens (11) perfused their rabbits at these controlled pressures, they observed luminal convolutions in normal carotid arteries. These corrugations were likely artifacts of delay, since sacrifice of their animals preceded opening of the chest and cannulation of the thoracic aorta for fixative infusion.

Many morphological findings that previous authors have attributed to drug effect (7,12,23,24) are quite similar in appearance to those we observed in the immersion-fixation group (Group 2). It can be inferred that some similar findings in the control groups might have been previously overlooked.

Findings that had been reported to be due to the applied agents (3,7,8,9,12,17,19,21) were not entirely absent in the control specimens of our study,

although they were less marked. Even in our perfusion-fixed control group (Group 3), slight damage was evident in the form of swollen mitochondria. Mechanical trauma or delay in the start of the perfusion fixation process might have induced these changes, as could the pH levels of the solutions used, since ultrastructure is so sensitive to pH.

CONCLUSION

If ultrastructural comparison must be made between various pharmacological agent-treated vessels, then quantitative or semiquantitative assessment is mandatory to ensure the accuracy of results.

Correspondence: İ. Semih Keskil
Fethiye Sokak No: 4/6
Gaziosmanpaşa
06700 Ankara, Turkey
Phone : 312-446 45 94
Fax : 312-221 32 76

REFERENCES

1. Arutiunov AI, Baron MA, Majorova NA: The role of mechanic factors in the pathogenesis of short-term and prolonged spasm of the cerebral arteries. *J Neurosurg* 40 , 459-472, 1974
2. Asano T, Ikegami Y, Suzuki Y, Satoh S: Endothelin and the production of cerebral vasospasm in dogs. *Biochem Biophys Res Commun* 159 , 1345-1351, 1989
3. Baykaner MK: Evidence for the participation of vascular endothelium originated humoral factors to

- cerebral vasospasm. *Acta Neurochirurg* 124, 135-138, 1993
4. Clark JM, Glasgow S: Luminal surface of distended arteries by scanning electron microscopy; eliminating configuration and technical artifacts. *Br J Exp Pathol* 57, 129-135, 1976
 5. Çeviker N, Keskil S, Ercan ZS, Uluoğlu Ö, Baykaner MK, Alp H: Pharmacological and pathophysiological effects of periadventitial stripping on immediate postoperative vasoconstriction in guinea pigs. *Neurosurg Rev* 20: 45-50, 1997
 6. Eldevik OP, Kristiensen K, Torvik A: Subarachnoid hemorrhage and cerebrovascular spasm. Morphological study of intracranial arteries based on animal experiments and human autopsies. *J Neurosurg* 55, 869-876, 1981
 7. Espinosa F, Weir B, Shnitka T, Overton T, Boisvert D: A randomized placebo-controlled double-blind trial of nimodipine after SAH in monkeys: Part 2. Pathological findings. *J Neurosurg* 60, 1176-1185, 1984.
 8. Fein JM, Flor WJ, Cohan SL, Parkhurst J: Sequential changes of vascular ultrastructure in experimental cerebral vasospasm. Myonecrosis of subarachnoid arteries. *J Neurosurg* 41, 49-58, 1974
 9. Findlay JM, Weir BKA, Kanamaru K, Espinosa F: Arterial wall changes in cerebral vasospasm. *Neurosurgery* 25, 736-746, 1989
 10. Gertz SD, Rennels ML, Forbes MS: Preparation of vascular endothelium for scanning electron microscopy: A comparison of the effect of perfusion and immersion fixation. *Microsc (Oxf)* 105, 309-313, 1975
 11. Greenhill NS, Stehbens WE: Scanning electron-microscopic study of the inner surface of experimental aneurysm in rabbits. *Atherosclerosis* 45, 319-330, 1982
 12. Hacıyakupoğlu S, Kaya M, Çetinalp E, Yücesoy A: Effect of prostacyclin and adenosine triphosphate on vasospasm of the canine basilar artery. *Surg Neurol* 24, 126-140, 1985
 13. Hickey KA, Rubanyi G, Paul RJ, Highsmith RF: Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. *Am J Physiol* 248, 550-556, 1985
 14. Ide K, Yamakawa K, Nakagomi T, Sasaki T, Saito I, Kurihara H, Yozisumi M, Yazaki Y, Takakura K: The role of endothelin in the pathogenesis of vasospasm following subarachnoid haemorrhage. *Neurol Res* 11: 101-104, 1989
 15. Keskil IS, Baykaner MK, Sencer H, Ataoğlu Ö, Ilgıt E, Erdoğan B, Çeviker N, Ercan ZS: Alteration by Iloprost of the vasospastic effects of Endothelin 1 in rabbit cerebral vessels. *Neuroreport* 5, 1089-1092, 1994
 16. Kobayashi H, Hayashi M, Kobayashi S, Kabuto M, Handa Y, Kawano H: Effect of endothelin on the canine basilar artery. *Neurosurgery* 27: 357-361, 1990
 17. Mayberg MR, Okada T, Bark DH: The significance of morphological changes in cerebral arteries after subarachnoid hemorrhage. *J Neurosurg* 72, 626-633, 1990
 18. Mima T, Yanagisawa M, Shigeno T, Saito A, Goto K, Takakura K, Masaki T: Endothelin acts in feline and canine cerebral arteries from the adventitial side. *Stroke* 20, 1553-1556, 1989
 19. Nakagomi T, Kassell NF, Sasaki T, Lehman RM, Hongo K, Ogawa H, Vollmer DG: Time course of blood-arterial wall barrier disruption following experimental subarachnoid hemorrhage. *Acta Neurochir (Wien)* 98, 176-183, 1989
 20. Papadopoulos SM, Gilbert LL, Webb RC, D'Amato C: Characterization of contractile responses to endothelin in human cerebral arteries: Implications for cerebral vasospasm. *Neurosurgery* 26, 810-815, 1990
 21. Roberts JC, McCrossan MV, Jones HB: The case for perfusion fixation of large tissue samples for ultrastructural pathology. *Ultrastruc Pathol* 14, 177-191, 1990
 22. Satio A, Shiba R, Kimura S, Yanagisawa M, Goto K, Masaki T: Vasoconstrictor response of large cerebral arteries of cats to endothelin, and endothelin-derived vasoactive peptide. *Eur J Pharmacol* 162, 353-358, 1989
 23. Sasaki T, Kassell NF, Yamashita M, Fujiwara S, Zuccarello M: Barrier disruption in the major cerebral arteries following experimental subarachnoid hemorrhage. *J Neurosurg* 63, 433-440, 1985
 24. Tanabe Y, Sahato K, Ito T, Takada M: Cerebral vasospasm and ultrastructural changes in cerebral arterial wall. *J Neurosurg* 49, 229-238, 1978