Prevention of Hemolysate-Induced Damage in Cultured Cerebral Endothelial Cells With Mexilitine

Serebral Endotel Hücre Kültüründe Hemolisata Bağlı Oluşan Hasarın Meksilitin ile Önlenmesi

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Abstract: ***Objective:*** Damage to the vascular endothelium is considered to be an important factor in the development of cerebral vasospasm after subarachnoid hemorrhage. Studies have shown that hemolysate causes structural and functional injury in cultured cerebral endothelial cells. Mexilitine is an anti-arrhythmic drug that is widely used in the treatment of ventricular arrythmias, and is known to act as a sodium-channel blocker. However experiments have shown that this compound can also activate ATP-sensitive potassium channels and block calcium channels. Recent in vitro data from studies on liposomes indicate that mexilitine is also a potent antioxidant. The aim of this study was to investigate the potential protective effects of mexilitine on endothelial cells incubated in hemolysate.

Methods: Endothelial cells were isolated from the bovine middle cerebral artery, and primary cell cultures were incubated and passaged weekly. Immunohistochemical staining was used to detect factor VIII-related antigen and acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3'-tetramethylindo-carbocyanine perchlorate (Dil-Ac-LDL), and thus it was confirmed that the cells were endothelial cells. The cells were grown to

Özet: ***Amaç:*** Subaraknoid kanama sonrasında vasküler endotelde oluşan hasar, serebral vasospasm gelişmesinde önemli bir faktördür. Çalışmalar, hemolisatın serebral endotel hücre kültürlerinde yapısal ve fonksiyonel hasara yol açtığını göstermiştir. Meksilitin ventriküler aritmilerin tedavisinde sık kullanılan anti-aritmik bir ilaçtır ve sodyum kanal blokörü olarak rol oynar. Ancak yapılan deneyler bu bileşiğin ATP-duyarlı potasyum kanallarını aktive ettiğini ve kalsiyum kanallarını bloke edebildiğini göstermiştir. Liposomlar üzerinde yapılan son in-vitro çalışmalardan elde edilen bilgiler meksilitinin potent bir antioksidan olduğu lehindedir. Bu çalışmanın amacı hemolisat içinde inkübe edilen endotel hücreleri üzerinde meksilitinin potansiyel koruyucu etkilerinin araştırılmasıdır.

Yöntem: Endotel hücreler sığır orta serebral arterlerinden isole edildi, primer hücre kültürleri haftalık olarak inkübe edildi ve pasajları yapıldı. Faktör VIII-ilişkili antijen ve 1,1'-dioctadecyl-3,3,3'-tetramethylindo-carbocyanine perchlorate (Dil-Ac-LDL) ile işaretlenmiş asetile edilmiş düşük dansiteli lipoproteinleri saptamak için immünhistokimyasal boyama yapıldı. Böylece hücrelerin endotel hücreleri olduğu konfirme edildi.

confluence on coated gelatin plates, and were incubated with Dulbecco's modified Eagle medium. Two plates were kept as controls; two plates were treated with 10^{-4} M hemolysate only; two plates were treated with a combination of 10^{-4} M mexilitine and 10^{-4} M hemolysate; two plates were treated with 10^{-8} M mexilitine and 10^{-4} M hemolysate; and two plates were treated with 10^{-12} M mexilitine and 10^{-4} M hemolysate. The cells were exposed to these specific media for 3 days. Cellular morphology and density were observed throughout this period using reverse-phase microscopy.

Results: Treatment with 10^{-4} M hemolysate alone destroyed almost all cells within 3 days. In the presence of the same concentration of hemolysate (10^{-4} M), 10^{-8} M or 10^{-12} M mexilitine preserved the cells' cobblestone appearance and number. However, addition of 10^{-4} M mexilitine had a deleterious effect. This concentration caused cytoplasmic vacuolization and transformation to spindle-shaped cell morphology.

Conclusion: Mexilitine's unique and diverse activity profile suggests that this agent might help prevent the endothelial cell damage caused by hemolysate after subarachnoid hemorrhage. The ability to stop this damage is a key factor in limiting cerebral vasospasm. The results proved that low-dose mexilitine treatment can prevent the structural and functional endothelial damage caused by hemolysate.

Key words: Cerebral vasospasm, ion channels, lipid peroxidation, mexilitine, subarachnoid hemorrhage

Hücreler jelatin kaplı tabaklarda büyütüldü ve Dulbecco'nun modifiye Eagle ortamında inkübe edildi. İki tabak kontrol olarak tutuldu; iki tabağa 10^{-4} M hemolisat uygulandı; iki tabağa 10^{-4} M hemolisat ve 10^{-4} M meksilitin kombinasyonu uygulandı; iki tabağa 10^{-4} M hemolisat ve 10^{-8} M meksilitin uygulandı; iki tabağa 10^{-4} M hemolisat ve 10^{-12} M meksilitin uygulandı. Hücreler bu özel ortamlarda 3 gün bırakıldılar. Bu süre içerisinde hücre morfolojisi ve dansitesi reverse-phase mikroskopi kullanılarak gözlemlendi.

Bulgular: 10^{-4} M hemolisat tek başına uygulandığında bütün hücreleri 3 gün içinde destrükte etti. Aynı oranda hemolisat (10^{-4} M) ile birlikte 10^{-8} M veya 10^{-12} M meksilitin hücrelerin kaldırım taşı görünümünü ve sayılarını korudu. Ancak 10^{-4} M meksilitin eklenmesinin zararlı etkisi oldu. Bu oran sitoplasmik vakuolizasyon ve iğsi şekilli hücre morfolojisi oluşmasına sebep oldu.

Sonuç: Meksilitinin özgün ve çeşitli etki profili, bu ajanın subaraknoid kanama sonrasında hemolisat nedeniyle oluşan endotel hasarını önlemede etkili olabileceğini düşündürmektedir. Bu hasarı önleyebilmek serebral vasospasmı sınırlamadaki anahtar faktördür. Bu çalışmadan elde edilen bulgular hemolisat nedeniyle oluşan yapısal ve fonksiyonel endotel hasarının mexiletin tedavisiyle önlenebileceğini kanıtlamıştır.

Anahtar kelimeler: İyon kanalları, lipid peroksidasyonu, meksilitin, serebral vasospasm, subaraknoid kanama

INTRODUCTION

The mechanism of the onset of cerebral vasospasm after subarachnoid hemorrhage (SAH) remains controversial. Much research in this area has focused on the prevention and reversal of cerebral vasospasm using therapeutic agents that act on the pathways assumed to be involved in vasoconstriction. Mexilitine is a class 1b antiarrhythmic drug that is widely used to treat ventricular arrhythmias (11,12). The best-known action of this agent is sodium (Na)-channel blockage, which prevents cell depolarization (17). However, experimental studies have shown that mexilitine can also activate ATP-sensitive potassium (K) channels and block calcium (Ca) channels (13,16).

Dohi et al. studied the vasorelaxant effect of mexilitine in the mesenteric resistance arteries of rats, and concluded that the drug induced this effect by inhibiting the transmembrane movement of Ca (6). The range of action that mexilitine exhibits in stabilizing cell membranes, blocking Ca channels, and activating K channels suggests that this agent might promote vasodilation through its impact on vascular smooth muscle cells. Dohi et al. also speculated that mexilitine has a greater inhibitory effect on noradrenaline-activated, verapamil-insensitive Ca channels than on voltage-gated channels (6).

In addition to its effects on various types of ion channels, in a previous study we demonstrated mexilitine's potent antioxidant effect on phospholipid-containing lysosomes (5). The results showed that the alteration of membrane permeability by this agent was related to its low molecular weight and highly lipophilic character. The unique activity profile of mexilitine suggested to us that it might help prevent the endothelial cell damage caused by hemolysate after SAH. Such a

protective effect would be a key factor in limiting cerebral vasospasm. Our aim in the present study was to investigate whether mexilitine protects endothelial cells that have been subjected to hemolysate.

MATERIALS AND METHODS

Brains of freshly slaughtered cows were acquired and endothelial cells were isolated from the bovine middle cerebral artery (BMCA-EC). The vessels were placed in a standard sterile medium composed of Dulbecco's modified eagle medium and 1% bovine serum albumin fraction V (DMEM-BSA). The blood was immediately flushed from the arterial lumen using DMEM-BSA, and the middle cerebral arteries were cut into 1- to 2-cm lengths. The vessel segments were turned inside out under a dissecting microscope, and were incubated in 0.1% collagenase solution in phosphate-buffered saline (PBS) for 30 min at 37 °C. Next, the solution containing suspended cells was separated from the tissue residue and collected, and then centrifuged for 10 min at 10,000 rpm. The suspended cells were then washed twice with DMEM-BSA. Gelatin-coated plates were inoculated with the cells, and these were incubated at room temperature for 2-3 hours. After this, the primary cultures of endothelial cells were incubated for 2 days at 37 °C in 5% CO_2. Cell cloning was carried out and 20 colonies were trypsinized, incubated, and passaged weekly. At the third passage, cells were transferred to two well-chamber slides (Lab Miles Laboratories; Naperville, IL, USA) and were immunohistochemically stained for factor VIII-related antigen and acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3'-tetramethylindo-carbocyanine perchlorate (Dil-Ac-LDL) in order to confirm their identity as endothelial cells. Cell lines that stained positive for factor VIII-related antigen and had typical cobblestone morphology selected for further study (Figure 1). The staining was primarily localized to the perinuclear region. The confirmed cells were then grown in DMEM for 4 days to confluency. After this, the medium was changed two or three times weekly.

For hemolysate preparation, rabbit arterial blood was centrifuged at 4°C and 800 G ($1.118x10^{-5}xrxrpm^2$) for 7 min. The supernatant was discarded, and the erythrocyte fraction was washed three times with cold normal saline (4 °C) at a volume ratio of approximately 1:5. The erythrocytes were then lysed by four freeze/thaw cycles. After diluting them 2:1 with distilled water, the erythrocytes were centrifuged at 6000 G for 30 min and the supernatant (hemolysate) was collected. The purity and concentration of oxyhemoglobin were determined spectrophotometrically.

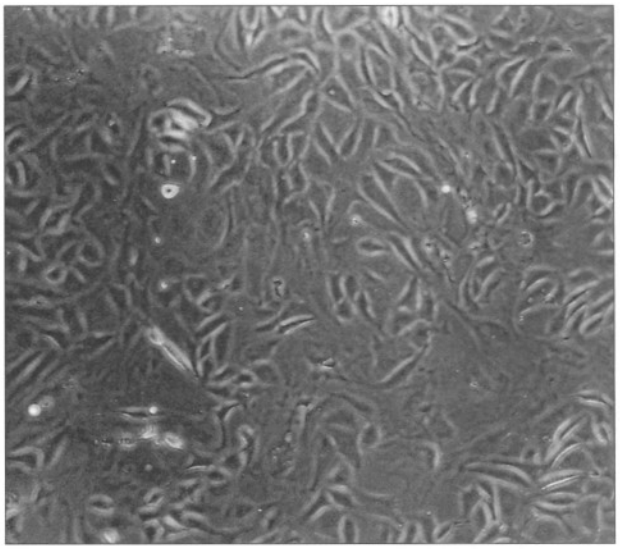

Figure 1: A control plate of endothelial cells shows healthy cobblestone morphology.

As described above, the BMCA-ECs were grown to confluence in separate sets of eight-well gelatin-coated plates and then incubated in DMEM. Two plates were kept as controls; two plates were treated with 10^{-4} M hemolysate only; two plates were treated with a combination of 10^{-4} M mexilitine and 10^{-4} M hemolysate; two plates were treated with 10^{-8} M mexilitine and 10^{-4} M hemolysate; and two plates were treated with 10^{-12} M mexilitine and 10^{-4} M hemolysate. To assess the cytotoxic effects of hemolysate and the impact of mexilitine, cells were exposed to these specific media for 3 days. During this period, cellular morphology and density were observed using reverse-phase microscopy.

RESULTS

Treatment of cultures with 10^{-4} M hemolysate damaged the endothelial cells within 24 hours, and

destroyed almost all cells in 3 days. During the destruction process, the cells gradually changed from normal cobblestone appearance to spindle-shaped morphology (Figure 2). In the presence of this same concentration of hemolysate (10^{-4} M), treatment with 10^{-8} M and with 10^{-12} M mexilitine preserved the cells' cobblestone appearance and numbers (Figure 3). However, the10^{-4} M mexilitine concentration we tested had a deleterious effect on the cells. It caused vacuolization and transformation to spindle-shaped cell morphology (Figure 4).

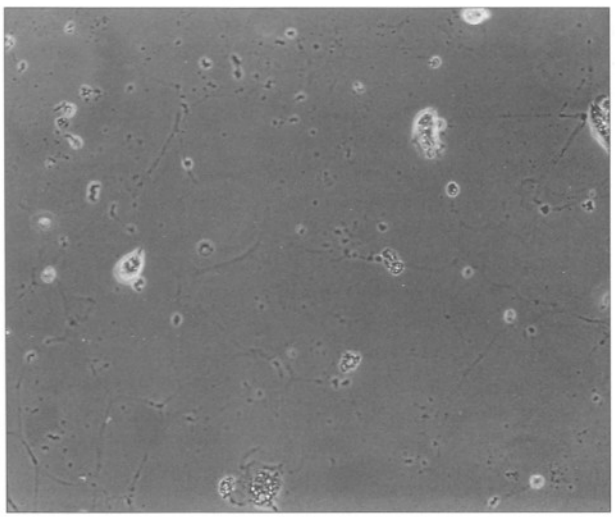

Figure 2: Treatment of cultures with 10^{-4} M hemolysate damaged the endothelial cells within 24 hours, and virtually eliminated almost all the destroyed cells by 3 days. Over the course of their destruction, the cells gradually changed from their normal cobblestone appearance to spindle-shaped morphology.

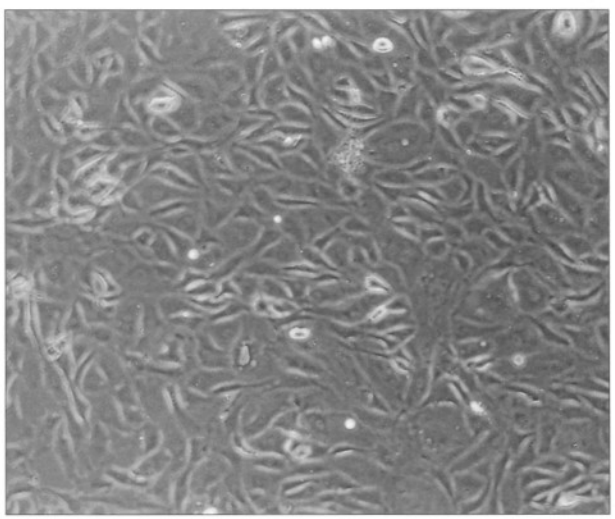

Figure 3: Treatment of cultures subjected to 10^{-4} M hemolysate with 10^{-8} M or 10^{-12} M mexilitine preserved the cells' cobblestone appearance and numbers.

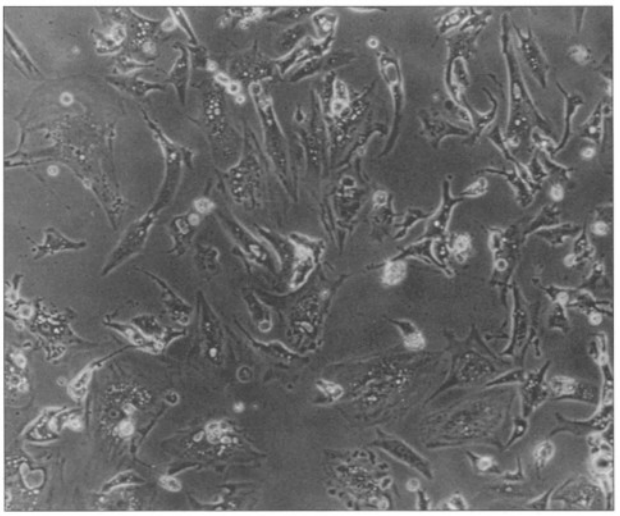

Figure 4: The higher concentration of mexilitine (10^{-4} M) had a deleterious effect on the cells. It caused vacuolization and transformation to spindle-shaped cell morphology.

DISCUSSION

The prolonged vasoconstriction that occurs after SAH presents a number of very unusual pharmacological problems. This response develops very slowly with a delay of several days after the initial stimulus, and it is generally agreed that the etiology is multifactorial. The source of the agent or agents responsible for cerebral vasospasm is most likely blood, and hemoglobin (Hb) is one of the prime suspects (4). Research has shown that, after SAH, Hb can penetrate the arterial wall and alter endothelial function (7). Endothelial cells contain both vasodilatory (nitrous oxide [NO]) and vasoconstrictive factors (prostacyclin, endothelin), and a balance of these is necessary to maintain a vascular tone. Injury to these cells is an important step in vasospasm, since this balance is upset and the resultant changes lead to vasoconstriction. Phospholipase A2, Ca, free radicals, and the arachidonic acid pathway are also known to be important elements in the endothelial cell damage (2,3,8,9). Oxyhemoglobin acts as the trigger for many of the processes that are involved (15). It initiates Ca entry into the cell and activates

phospholipase A2, which results in the production of free radicals and lipid peroxidation (1). Endothelin-1 (ET-1) is the main vasoconstrictive factor produced by endothelial cells (7,10). Experiments have shown that ET-1 secretion increases in a dose-dependent manner relative to Hb levels (14). Hemoglobin binds extracellular NO, and this action diminishes inhibitory NO feedback on endothelial cells and augments ET-1 secretion. As noted above, oxyhemoglobin causes morphological changes in endothelial cells by acting as a catalyst in the formation of oxygen free radicals. These reactive oxygen species react with lipid membranes, proteins, nucleic acids and extracellular matrix material to destroy cellular integrity. These cytotoxic effects result in apoptosis, which begins with compression of chromatin and aggregation at the nuclear margin, cytoplasmic condensation, and convolution of the nuclear and cellular membranes. In the second stage of apoptosis, the cytoplasm and nucleus fragment into membrane-bound vesicles that contain tightly packed intact organelles and portions of condensed nucleoplasm. Finally, apoptotic bodies are engulfed by neighboring cells and eventually rupture.

Protection of the endothelium from these cytotoxic agents effectively attenuates SAH-induced vasospasm. Our experiment showed that 10^{-8} M and 10^{-12} M concentrations of mexilitine preserve the morphology and density of endothelium cells incubated with 10^{-4} M hemolysate. This protection may be due to the unique activity profile of this agent, which includes Ca-channel blockage, activation of ATP-sensitive K channels, and antioxidant effects. In contrast, the higher concentration of mexilitine that we tested did not protect the endothelial cells. It is possible that the higher degree of Na-channel blockage under these conditions disrupts the balance between extracellular and intracellular osmolarity.

CONCLUSION

Our findings show that mexilitine treatment can prevent hemolysate-induced endothelial damage. The drug achieves this by blocking Ca channels, by activating ATP-sensitive K channels, and by antioxidant action. This important effect suggests that mexilitine may be valuable in the treatment of cerebral vasospasm.

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