Research Article

The Role of Thrombocyte Activation on Early Brain Injury in Experimental Subarachnoid Hemorrhage Model

Ümit KOCAMAN¹, Füsun DEMİRÇİVİ ÖZER², İsmail ORAN³, Mesut METE⁴, İbrahim BURAK ATČI⁵, Tuncer TURHAN⁶, Eren DEMİRTAŞ⁷

¹Van İpekyolu Government Hospital, Neurosurgery, Van, Türkiye ²Tepecik Educational and Research Hospital, Neurosurgery, İzmir, Türkiye ³Ege University Medical School, Radiology, İzmir, Türkiye ⁴Celal Bayar Medical School, Neurosurgery, Manisa, Türkiye ⁵Elazığ Educational and Research Hospital, Neurosurgery, Elazığ, Türkiye ⁶Ege University Medical School, Neurosurgery, İzmir, Türkiye ⁷Micro Pathology Laboratory, Pathology, İzmir, Türkiye

Summary

Aim: Thrombocyte activation is one of the mechanisms blamed for emerging of early brain injury (EBI) soon after subarachnoid hemorrhage (SAH). It is wondered by researching to what extent neuron injury is prevented by making thrombocyte inhibition in SAH model. It shows that the role of trombocyte activation on EBI.

Material and Method: 21 rabbits weighting 3-5 kg are divided in 3 groups 7 each. After ether anesthesia, 0.2 cc arterial blood taking from group 1 and 2 was injected into cisterna magna and created SAH. After SAH, at regular intervals antithrombocyte drug (tirofiban) was given to group 2 intraperitoneally; no treatment was carried on group 1. 0.2 cc SF was injected into cisterna magna in group 3. Decapitation was done in 72nd hour and then ischemic brain map was done at hippocampus level. The amount of ischemic neuron was scored and statistically analyzed.

Results: SAH was detected in all rabbits of group 1 and 2. There are meaningful difference between the group 1 and group 3 of scoring ischemic neuron of hippocampus's CA-3 and CA-4 areas. Whereas in group 2 ,which is a ischemic neuron treatment group, there is a decline in each area, this decline has reached to a statistical means only in CA-4 area.

Conclusion: There can be neuronal loss due to EBI even in the area which is free from blood in experimental SAH. Decreasing the injury of neuron with an antithrombocyte medicine shows that thrombocyte activation plays a great role in pathogenesis of EBI.

Key words: Early brain injury, subarachnoid hemorrhage, thrombocyte

Deneysel Sak Modelinde Trombosit Aktivasyonunun Erken Beyin Hasarı Gelişimindeki Rolü

Özet

Amaç: Subaraknoid kanamadan (SAK) sonra dakikalar içinde başlayan ‘erken beyin hasarını’ ortaya çıkmasında suçlanan mekanizmalarдан birisi artmış trombosit aktivasyonudur. SAK modelinde gerçekleştirilirken bu çalışmada merak edilen, trombosit inhibisyonu yapanlar noktada hasarın ne ölçüde engellenecidir. Bu bize EBI’da trombosit aktivasyonun rolünü gösterir.

Gereç ve Yöntem: 3-5 kg arasında olan 21 tavşan 7’li üç gruba ayrıldı. Ether anestezisi sonrasında, grup 1 ve 2’de tavşanlardan alınan 0.2 cc arteryal kan, sisterna magnaya verilerek SAK gerçekleştirilirdi. SAK’tan sonra belli aralıklarla antitrombosit bir ilaç(tirofiban),
intraperitoneal olarak grup 2'ye verildi; grup 1'e tedavi uygulanmadı. Grup 3'te sisterna magna'ya 0.2 cc SF uygulandı. Tavşanlara 72.saatte dekapitasyon uygulandı; daha sonra hipokampüslär seviyesinde iskemik nöron haritalaması yapıldı. İskemik nöron miktarları puanlandı. Sonuçlar istatistiksel olarak analiz edildi.
Bulgular: İlk iki grupta tüm tavşanlarda SAK saptanmıştır. Hipokampüsün CA-3 ve CA-4 bölgelerinde iskemik nöron puanlarında grup 1 ve grup 3 arasında anlamlı fark vardır. Her iki bölgede de iskemik nöron puanları tedavi grubu olan grup 2'de azalmakla birlikte bu azalma sadece CA-4 bölgesinde istatistiksel anlam ulaşılmıştır.
Sonuç: DeneySEL SAK'da kandan uzak alanlarda bile EBH'na bağlı nöron kaybı olabilmektedir. Nöron hasarının antitrombosit bir ilaçla azaltılabilmesi erken beyin hasarı'nın patogenezinde trombosit aktiveasyonunun önemli rol oynadığını göstermektedir.
Anahtar Kelimeler: Erken beyin hasarı; subaraknoid kanama; trombosit

INTRODUCTION
What are the mysterious effects of aneurysmatic subarachnoid hemorrhage? The most alarming complication of aneurysmatic subarachnoid hemorrhage is rebleeding. This problem can be solved with the help of timely surgery and vasospasm has taken the place of it(1). But, recently, it has been seen that there is no relationship between the patient's clinic and the vasospasm(5). Although the absence of angiographic vasospasm or recovery in angiographic vasospasm with medical treatment, patient's clinic may be worse. Under the circumstances, the main reason which causes deterioration can't be the process of vasospasm(5). The main reason which causes deterioration is the process of early brain injury(5). Early brain injury starts immediately after the aneurysmatic subarachnoid haemorrhage and ends within the first 72 hours; effects can be seen in all periods after aneurysmatic subarachnoid hemorrhage(11). Many pathways are held responsible for early brain injury. Some of them are ischemic pathway, apoptotic pathway and inflammatory pathway(5). A large number of key factors play a role in this process such as increasing intracranial pressure, decreasing blood flow in the brain, lowering of brain oxygenation, losing blood-brain barriers, brain edema and neuronal cell death(5,3). Leukocyte infiltration and thrombocyte aggregation occur in neural tissue in the inflammation pathway(3). Blocking this pathway by using an antithrombothic medicine can reveal the importance of thrombocyte activation on early brain injury.

MATERIAL AND METHODS
This study was done with the Aegean University Animal Experiments' Local Ethical Counsel's agreement dated July 18th, 2009 in the research laboratory of the Aegean University's Medical School. In this study, 21 New Zealand albino rabbits weighing, 3-5 kilograms each, were used. In the study, research was conducted on the rabbits' hippocampi since the hippocampus is highly vascularized and is very responsive to ischemia. Being far from the area of cisternae magna, where aneurysmatic subarachnoid hemorrhage(SAH) was formed, can be valuable for evaluating the vast effects of early brain injury.

The animals were divided into 3 heptad groups. Group 1(experimental SAH group), Group 2(experimental SAH treated with tirofibran group) and Group 3(control group) were put to sleep by ether inhalation anaesthesia. The animals' heart rates and breathing were monitored. When observed stable as hemodynamic, 0.2 cc blood was taken intracardially from the rabbits of Group 1 and 2. Each rabbit was taken into sitting position and the head taken slightly into flexion. The atlantooxipital area was sterilized by rubbing with antiseptic three times. After

715
0.1 cc BOS puncture, 0.2 cc intracardiac blood was injected into the cisternae magna by entering into the atlantooccipital area using an insulin injector. An aneurysmatic subarachnoid hemorrhage model was created on Group 1 and 2. The anti-thrombotic medicine, tirofiban hydrochloride (glycoprotein 2b-3a inhibitor), was given to Group 2 intraperitoneally (shortly thereafter SAH 1.5 mg, 2nd hour 0.25 mg, 1st day and 2nd day 0.25 mg). No treatment was given to Group 1. In Group 3, 0.2 cc serum physiologic was injected into cisterna magna after 0.1 cc BOS puncture. The brains were prepared by decapitation process after general anaesthesia with ether before 72 hours had passed. An ischemic brain map was done after staining with hemotoxylin-eosine by taking coronal incisions at the hippocampus level. If there were no ischemic neurons, ‘0’ point; below 25% , ‘1’ point; between 25-50 %, ‘2’ points; above 50%, ‘3’ points were given. In this evaluation, the Fisher Exact Test was used and grouped as: ‘0 and 1’ points, absence of ischemic neuron;‘2 and 3’ points, existence of ischemic neurons.

RESULTS
We want to give impressive examples from the pathologic preparations before giving the details of our results:

![Picture 1: The macroscopic view from bottom of brain.](image1)

![Picture 2: The view of experimentally created SAH around basilar artery.](image2)

![Picture 3: The view of normal histology of a rabbit hippocampus.](image3)

![Picture 4: Ischemic neurons with dark nuclei.](image4)
The organization of rabbit hippocampi is shown in below:

While following 2 rabbits in group 1, 1 rabbit in group 3 was lost. Limited with basal cisterns, aneurysmatic subarachnoid hemorrhage was detected in the first two Groups.

No ischemic neurons were observed in CA-1 and CA-2 areas of hippocampi in any rabbits.

In Group 1, different degrees of ischemic neurons were detected in all 5 rabbits' CA-3 areas, after the rabbits' hippocampi CA-3 areas were histopathologically examined. This range was 3 points for 4 rabbits and 1 point for 1 rabbit.

In Group 2, ischemic neurons were not detected (0 point) in 3 rabbits' CA-3 areas after histopathologic examination of rabbits' hippocampi CA-3 areas. The CA-3 areas of the other 2 rabbits revealed a 1 point effect and a 3 point effect was seen in other 2.

No ischemic neurons were seen (0 point) in CA-3 and CA-4 areas of 6 rabbits from Group 3 as a result of histopathological examination.

Different degrees of ischemic neurons were detected in CA-4 areas of Group 1 rabbits' hippocampi. 4 rabbits had 3 points and 1 rabbit had 0 point.

In Group 2 rabbit hippocampi, a 1 point ischemic neuron was detected in 1 rabbit's CA-4 area. No ischemic neurons were detected (0 point) - as seen in normal brains- in the other 6 rabbits' CA-4 areas. It is thought that these areas were protected.

Data for CA-3 and CA-4 was evaluated statistically. The Fisher Exact Test was used for evaluating the data and for comparing Groups. Ischemic neuron points were classified (0 and 1= non-existent, 2 and 3 points: existent) for evaluating with this test.

The result of the comparison between Group 1 and Group 2's ischemic neurons for CA-3 area was p= 0.11. The result of the comparison between Group 1 and Group 3 was p= 0.015. The result of Group 2 and Group 3 was p= 0.26.

The result of the comparison between Group 1 and Group 2's ischemic neurons for CA-4 area was p= 0.044. The result of the comparison between Group 1 and Group 3 was p=0.01. The result of Group 2 and Group 3 was p= 0.53.
According to the statistical evaluation for CA-3 area, it is observed that Group 1 and Group 3 were significantly and statistically different from each other (p=0.015). But in other comparisons; although ischemic neurons' values were decreasing, the results were not statistically meaningful. In Group 2, which was the ischemic neurons' group treated with medication, the results were thus: Comparing Group 1 and 2, p= 0.11; Group 2 and 3, p= 0.26.

When CA-4 areas was compared in ischemic neuron values, it was thought that Group 1, Group 2 (p= 0.044) and Group 1, Group 3 (p= 0.01) were statistically and meaningfully different from each other. There were no significant findings in comparisons of Group 2 and Group 3 (p= 0.53).

**DISCUSSION**

In spite of all developments in microsurgery, radiology, anaesthesiology and intensive care, aneurysmatic subarachnoid hemorrhage that is related with aneurismal rupture still has high mortality and morbidity rates. Vasospasm is an important complication of aneurysmatic subarachnoid hemorrhage and it plays an important role in the cause of neurologic deficit development after the 3rd- 4th days. Early brain damage is among the most important causes of morbidity and mortality 72 hours after aneurysmatic subarachnoid hemorrhage. Early brain injury should be the first target of future research for struggling with mortality and morbidity after aneurysmatic subarachnoid hemorrhage(11).

Traditional research and treatment are focused on the delaying the incident of cerebral vasospasm after aneurysmatic subarachnoid hemorrhage. But, physiological and cellular incidents of early brain injury make an important contribution on prognosis of patients and may be a more important factor than delayed vasospasm. Early brain injury is the result of physiological instability such as enhanced intracranial pressure and reduced blood flow in brain. Occurrences of global brain ischemia end with dysfunction of blood brain barriers, inflammatory and oxidative cascades and finally neuronal cell death. Consequently, these incidents frequently cause patient's death or serious neurologic deficits. That, despite the serious inhibition of cerebral vasospasm, that complete recovery results cannot be reached causes intense focus on the connection between early brain injury and prognosis. The meaning of research on inhibition of early brain injury cascades should be appraised carefully in respect to treatment of patients who have subarachnoid hemorrhage(2).

Of course, inhibition of cascades is promising in the treatment early brain injury. But the main problem is correctly determining the cascades which are effective early brain injury. Only in this way, can the approach of treatment be determined.

A lot of pathways are related with each other in physiopathology of early brain injury. Here is the physiopathological schema that Cahill and his friends suggest(5,3,8,11):
The article in which Fatima A. Sehba summarizes her detailed research on the physiopathological mechanisms of early brain injury was highly intriguing. In her schema below, it can be seen that early brain injury emerges from multi-reasoned and elaborate mechanisms\(^9\). Of course, most of these mechanisms still need to be proven.

Whereas, in our study of the location of thrombocyte activation in physiopathology, attempts were made to clearly define the relationship between thrombocyte activation and early brain injury which occurred minutes after aneurysmatic subarachnoid hemorrhage. In previous studies, ischemic effects from thrombocyte activation formations throughout the whole brain were not shown. In this research, thrombocyte activation was prevented by a strong anti-thrombotic agent called tirofiban hydrochloride. The effect of this intervention on early brain injury on rabbits that had SAH was scaled based on number of ischemic neurons. From the results, an attempt was made to foresee the importance of thrombocyte activation on physiopathology.

In similar researched conducted by Ishikawa, he said, “SAH in basal cisternae increases the interaction of oxygen radicals and P-selectin mediative leucocyte-thrombocyte- endothelial cells in venules of brain surface.” These inflammatory and prothrombogenic responses can be the cause of global brain injury soon after SAH. Furthermore, it was observed microscopically that leukocyte-thrombocyte and endothelial cell adhesion was prevented by ‘p- selectin antibody’, but ischemic effects far away from SAH were not analyzed\(^6\). However, this study contributes to the further understanding of the inflammatory pathway.

A study by Cahil et al, claimed that proteins related with P53 increased and caused apoptosis in the first 24- 72 hours after SAH and this caused neuronal cell death\(^4\). This study is very important for emphasizing the importance apoptotic pathway.

In a study by Kusaka et al, it was observed in rats that permeability of veins, intracranial pressure and brain edema increased within 24 hours after experimentally induced SAH and this caused an increase in mortality. In the study, the phosphorylated levels of VEGF and mitochondrial - activation protein kinase in the cortex reduced after SAH and this caused early brain injury\(^8\).

In a similar study that was done by Sahba et al, it was reported that in the early period after SAH, there was reduction of NO level caused neuronal cell injury that was caused by vascular injury, vasoconstriction and platelet aggregation; this suggestion that there is NO pathway needs to be explained by experimental study\(^10\).

In another study by Karaoğlan et al, protective effect of tirofiban hydrochloride on hippocampal CA-1 neurons after temporary frontal brain ischemia was analyzed. It was concluded by this study that tirofiban has a neuroprotective effect as an antiplatelet and it can be an alternative due to its superiority to other antiplatelet agents that are still being used. Of course, no correlation was seen for SAH\(^7\).
Tirofiban (N-(butylsulfonyl)- o- [4-(4piperidinyl) butyl]- L tyrosine monohydrochloride) is a synthetic, non-peptide inhibitor acting on glycoprotein (GP)IIb/IIIa receptors in human thrombocytes. Therefore, it constitutes as an anticoagulant, specifically an inhibitor of thrombocyte aggregation. Tirofiban inhibits the thrombocyte aggregation depending on the dose and the concentration administered\(^{(8)}\). The formulation is below:

![Tirofiban molecule diagram]

Tirofiban, a strong antiplatelet agent, was used in this study. Although any brain area which was far from the area of SAH could be chosen, the hippocampal area was chosen for evaluation of the ischemic destruction. Because, hippocampus is very sensitive to ischemia and it is a region of highly vascularized. Due to this, in every rabbit's hippocampal area after SAH, a situation of ischemic injury which is a symptom of early brain injury, the effect of the medicine and indirectly the effect of thrombocyte inactivation could be evaluated more objectively.

The data that gathered after evaluating our study, may be a deduction related to this subject.

In hippocampal area's CA-1, CA-2, ischemic injury were not seen. In psychopathology of early brain injury it was not expected that all brain areas affected would diffuse. Therefore, only a part of the hippocampal areas being affected can have a normal result in this respect.

There was a significant difference between Group1 and Group 3 for CA-3 area in statistical analysis (p= 0.015). It can be concluded that a high rate of early brain injury occurred after SAH. There was no meaningful statistical difference between Group 1 and Group 2, although it was on the border of meaningful (p= 0.11). It can be concluded that the effect of tirofiban was limited on CA-3 where early brain injury had occurred. The limited useful effect also can be observed because there was no meaningful difference between Group2 and Group3 (p= 0.26).

There was a meaningful difference between Group 1 and Group 3 in statistical analysis (p= 0.01). We can conclude that there was in high rate of early brain injury after SAH. Again, there was a meaningful difference in Group 1 and Group 2 (p= 0.044). It can be determined that tirofiban was effective in CA-4 area where early brain injury has occurred. That there was no difference between Group 2 and Group 3 was another indicator that tirofiban is effective in CA-4 area (p= 0.53).

**CONCLUSION**

Some conclusions can be made from this research. The most important among them is that the injury of neuron can be reduced with the help of anti-thrombocyte medicine. This shows that thrombocyte activation and aggregation play a great role in early brain injury. Another conclusion is that neuron loss may occur even in a distant area far from where the experimental SAH occurs. This should be enlightening for all physiopathological foundations which define all pathways. It can be said that the main target is to start using preventative medicines which affect these pathways of this syndrome and which are harmonious with basic treatment and care principles of SAH.

**Correspondence to:**
Ümit Kocaman
E-mail: umitkocaman80@gmail.com
REFERENCES

8. Kusaka G. Signaling Pathways for Early Brain Injury After Subarachnoid Hemorrhage.