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The Haemostatic Effects of Ankaferd Blood Stopper on Mammalian Brain Parenchyma: An Experimental Study

Ankaferd Kanama Durdurucunun Beyin Parankiminde Hemostatik Etkisi: Deneysel Çalışma

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ABSTRACT

Aim: Haemostasis is a vital stage for the success of the surgery. Although Ankaferd Blood Stopper (ABS), a low-cost and reliable agent, is used in many surgeries, it is not yet available for use in the intracranial area. This study aims to reveal ABS's cytotoxic effects and safety profile in mammalian brain parenchyma.

Material and Methods: 30 Wistar Albino rats were divided into three groups consisting of 10 rats. Haemostasis was achieved with saline in group 1, 50% diluted ABS in group 2, and 100% ABS in group 3 in bleeding caused by damage to the brain parenchyma. Urotensin, Antithrombin III (AT3) and fibrinogen were studied in blood samples taken before surgery and during sacrification. In addition, the histologic examination was performed after the sacrification of rats and injury scores were assessed.

Results: Fibrinogen levels in groups 2 and 3 were significantly higher than group 1 in blood samples taken before surgery. There was a significant increase in urotensin during sacrification compared to the pre-surgical period in all three groups. (p=0.005) Slight injury in group 2, mild injury in group 3, and severe injury in group 1 were statistically significantly higher. (p=0.005) These results indicate that the use of 50% diluted ABS is safe.

Conclusion: ABS, used for the first time in the mammalian brain parenchyma, was evaluated as safe in rats. Compared to haemostatic matrix agents, in addition to safety and efficacy, its low cost might increase its clinical use in the future.

Keywords: Ankaferd Blood Stopper, Cranial surgery, Haemostasis

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ÖZ

Amaç: Hemostaz, ameliyatın başarısı için hayati bir aşamadır. Düşük maliyetli ve güvenilir bir ajan olan Ankaferd kanama durdurucu (AKD) pek çok ameliyatta kullanılmasına rağmen henüz kafa içi bölgede kullanıma sunulmamıştır. Bu çalışma, AKD'nun sitotoksik etkilerini ve memeli beyin parankimasında güvenlik profilini ortaya çıkarmayı amaçlamaktadır.

Gereç ve Yöntemler: 30 Wistar Albino sıçan, 10 sıçandan oluşan üç gruba ayrıldı. Beyin parankim hasarına bağlı kanamalarda grup 1'de salin, grup 2'de %50 seyreltilmiş AKD ve grup 3'te %100 AKD ile hemostaz sağlandı. Ameliyat öncesi ve sakrifiye edilirken alınan kan örneklerinde ürotensin, Antitrombin III (AT3) ve fibrinojen çalışıldı. Ayrıca sıçanların sakrifiye edilmesinden sonra histolojik inceleme yapıldı ve yaralanma skorları değerlendirildi.

Bulgular: Ameliyat öncesi alınan kan örneklerinde grup 2 ve 3'teki fibrinojen düzeyleri grup 1'e göre anlamlı derecede yüksekti. Her üç grupta da ameliyat öncesi döneme kıyasla sakrifikasyon sırasında ürotensin'de önemli bir artış vardı. (p=0,005) Grup 2'de hafif yaralanma, grup 3'te hafif yaralanma ve grup 1'de ciddi yaralanma istatistiksel olarak anlamlı derecede yüksekti. (p=0.005) Bu sonuçlar %50 seyreltilmiş AKD kullanımının güvenli olduğunu göstermektedir.

Sonuç: Memeli beyin parankiminde ilk kez kullanılan AKD, sıçanlarda güvenli olarak değerlendirildi. Hemostatik matriks ajanlarla karşılaştırıldığında, güvenlik ve etkinliğe ek olarak, düşük maliyeti gelecekte klinik kullanımını artırabilir.

Anahtar Sözcükler: Ankaferd kanama durdurucu, Kraniyal Cerrahi, Hemostaz

INTRODUCTION

Ankaferd Blood Stopper® (ABS) is a mixture of five plant roots: Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum, Urtica dioica, and Thymus vulgaris (1). ABS does not affect standard physiological individual coagulation systems and shows its haemostatic effect in less than a second by forming an encapsulated protein network for erythrocyte aggregation. Therefore, topical administration this agent, which does not affect coagulation systems such as prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, thrombin time (TT), reptilase time, anti-Xa essay, antithrombin III activity, and can also be used in patients within average coagulation values or with primary or secondary haemostatic disturbances. On the other hand, by upregulating the GATA/FOG (Friend of GATA) transcription system, Ankaferd impacts erythroid functions and urotensin II. As a significant element of Ankaferd, Urotensin II expresses the connection between active erythroid cells and injured vascular endothelium adhesive proteins (2).

Massive bleeding while performing surgical operations can be related to a higher risk of morbidity and mortality. In terms of avoiding blood loss, various haemostatic agents such as tranexamic acid (TXA), human gelatine - thrombin matrix (Floseal®), polyethene glycol hydrogel (Coseal®) have been widely used in clinical practice (3-5). However, even though the beneficial effect of topical haemostatic agents in reducing bleeding have been well reported, the topics associated with low systemic absorption, side effects and cost-effectiveness are still the interest areas of the researchers (3).

While ABS has been used in traditional Turkish medicine for centuries, it has recently been approved by the Turkish Ministry of Health for medical use (6, 7). It is used for lung, cardiac surgery, gastrointestinal system, urological surgeries, ENT surgery, and dentistry (8-18). It also has anti-inflammatory and antioxidant properties (19). The agent is available in ampoule, spray and tampon forms. However, it is not yet used for cranial surgery. This study aims to reveal the cytotoxicity and feasibility of the agent in the mammalian brain, which can be used in many delicate human body tissues.

MATERIAL and METHODS

Rats (Animals)

Approval for the study was obtained from the Ethics Committee for Experimental Animals at Pamukkale University School of Medicine by the European Community Council Directive 86/609/ECC for the care and use of laboratory animals. Experiments were performed on 30 female 250-300 gr Wistar Albino adult rats purchased from the Pamukkale University Experimental Animal Production Centre. The animals were housed in an air-conditioned room (temperature: 21 ± 2 C°), under a 12-h light/ dark cycle, with free access to food and water. Every effort was made to minimise animals' suffering and reduce the number of animals used.

Surgical Procedure

Rats were divided into 3 groups: saline (group 1), 50% diluted ABS (group 2) and 100% ABS (group 3). Under intraperitoneal anaesthesia, a 1 ml blood sample was taken from each rat to study urotensin, AT3 and fibrinogen and 3 mm diameter craniotomies were performed in the left frontal region as standard in all rats. A 2 mm deep injury was created with an insulin needle following the dura opening. In the first group, bleeding was stopped with saline, with 2 ml of 50% diluted ABS in the second group and 100% ABS in the third group. The closure was performed following standard surgical rules. Three rats (Rat 7 in group 1 and rats 4 and 6 in group 3) died after surgical interventions. During the study period, there was no wound dehiscence.

Histopathological Analysis

Sacrification was performed on the 15th postoperative day. Before anaesthesia, a 1 ml blood sample was taken from

each rat to study urotensin, AT3 and fibrinogen. With 30 mg/kg ketamine HCl and 6 mg/kg xylazine HCl, Rats were anaesthetised by intraperitoneal injection. Brain tissues were fixed in 10% neutral formalin for 72 hours. Tissues were washed under running water for 1 hour. Dehydration with alcohol and transparency of the tissue with xylene were performed. Following that, the tissues were embedded in paraffin. Transverse and coronal 5 μm thick sections passing through the damaged area were taken with a microtome (Leica RM-2125). Haematoxylin-Eosin (H&E) (Merck, Germany) staining was performed. All sections taken were evaluated under a light microscope. Histological images of the tissues were recorded by scanning five random areas in the damaged area with a 10X objective. In these 5 points, the most common injury type was accepted for scoring. Histological scoring for tissues was performed according to the following criteria for groups. Frontal cortical damage was assessed by five different morphological parameters: neuronal morphological changes (shrinkage of the cell body, pyknoses of the nucleus, disappearance of the nucleolus and loss of Nissl substance in the cytoplasm with extensive eosinophilia), neuronal loss, cytotoxic oedema, vasogenic oedema, and inflammatory cell infiltration into the cerebral cortex. By degree of changes (0, 1, 2, 3, and 4 points for each score 0% <25%, 25–50%, 50–75%, and 75–100%, respectively) and severity of injury (score 0 = normal histology)., score 1 = slight, 2 = mild, 3 = moderate, and 4 = severe changes) (20).

Statistical Analysis

Data analysis was done in IBM SPSS 11.5 program. As descriptive statistics, mean ± standard deviation was used for quantitative variables and number (percentage) was used for qualitative variables. The Chi-square test was used when it was desired to see whether there was a statistically significant relationship between two qualitative variables. For the quantitative variable, whether there is a difference between the categories of the qualitative variable with more than two categories, if the normal distribution assumptions are met, the One-Way ANOVA test is used; if not, the Kruskal Wallis H test is used. The Wilcoxon Signed-Rank test was used to determine whether there was a difference between two quantitative dependent variables (such as while alive-ex) since the assumptions of normal distribution were not met. The statistical significance level was taken as 0.05.

RESULTS

All of the descriptive values in the study are summarised in Table 1.

Table 1: Descriptive data of the study.

Variables		
Group, n (%)	Group 1	10 (33.3 %)
	Group 2	10 (33.3 %)
	Group 3	10 (33.3 %)
Brain Injury Score, n (%)	Normal Histology	0 (0.0 %)
	Slight injury	9 (33.3 %)
	Mild injury	15 (55.6 %)
	Moderate injury	3 (11.1 %)
	Severe injury	0 (0.0 %)
Urotensin, Alive	Mean±SD	142.59±12.36
	Median (Min.-Max.)	143.97 (116.16-172.13)
Urotensin, Post sacrification	Mean±SD	191.40±131.03
	Median (Min.-Max.)	159.91 (141.36-840.00)
AT3, Alive	Mean±SD	2.29±0.31
	Median (Min.-Max.)	2.30 (1.39-2.96)
AT3, Post sacrification	Mean±SD	2.30±0.30
	Median (Min.-Max.)	2.28 (1.75-3.15)
Fibrinogen, Alive	Mean±SD	3.44±1.95
	Median (Min.-Max.)	4.25 (0.38-6.00)
Fibrinogen, Post sacrification	Mean±SD	4.13±1.73
	Median (Min.-Max.)	4.69 (0.38-5.88)

SD: Standard deviation, **Min:** Minimum, **Max:** Maximum.

Table 2: The comparisons between groups

Variables		Groups			p value
		Group 1	Group 2	Group 3	
Brain Injury Score, n (%)	Slight	1 (11.1)	7 (70.0)	1 (12.5)	**0.005[a]**
	Mild	5 (55.6)	3 (30.0)	7 (87.5)	
	Moderate	3 (33.3)	0 (0.0)	0 (0.0)	
Urotensin, Alive	Mean±SD	140.10±9.73	147.93±17.04	140.36±9.28	0.354[b]
	Median (Min.-Max.)	143.41 (125.06-156.71)	148.68 (116.16-172.13)	142.19 (122.59-151.70)	
Urotensin, Post sacrification	Mean±SD	232.74±228.11	168.80±15.99	173.13±25.85	0.464[c]
	Median (Min.-Max.)	154.96 (141.36-840.00)	164.48 (147.13-195.34)	162.27 (152.63-214.61)	
AT3, Alive	Mean±SD	2.25±0.36	2.34±0.32	2.28±0.28	0.866[b]
	Median (Min.-Max.)	2.26 (1.39-2.73)	2.28 (1.87-2.96)	2.36 (1.85-2.61)	
AT3, Post sacrification	Mean±SD	2.30±0.17	2.35±0.41	2.25±0.27	0.781[b]
	Median (Min.-Max.)	2.26 (2.08-2.63)	2.37 (1.75-3.15)	2.23 (1.78-2.57)	
Fibrinogen, Alive	Mean±SD	1.73±1.86	4.39±1.02	4.63±1.11	**0.005[c]**
	Median (Min.-Max.)	0.38 (0.38-4.62)	4.25 (3.07-6.00)	4.71 (2.27-5.81)	
Fibrinogen, Post sacrification	Mean±SD	3.50±1.86	4.73±1.60	4.08±1.70	0.098[c]
	Median (Min.-Max.)	4.09 (0.38-5.30)	5.04 (0.38-5.88)	4.46 (0.38-5.67)	

a: Chi-square test, **b:** One Way ANOVA test, **c:** Kruskal Wallis H test.

Table 3: P-values for live-post sacrification comparisons for each group separately.

Variables		Groups		
		Group 1	Group 2	Group 3
Urotensin	**p value**	**0.011**	**0.036**	**0.028**
AT3	**p value**	0.594	0.575	0.753
Fibrinogen	**p value**	0.093	0.263	0.463

Biochemical Examination

There was no significant difference in urotensin (alive, post sacrification), AT3 (alive, post sacrification) and fibrinogen (post sacrification) values between the three groups. However, fibrinogen levels in the samples taken before surgery were significantly higher in groups 2 and 3 compared to group 1 (p=0.005) (Table 2).

When the samples were taken while alive and during sacrification were compared within the groups, no significant difference was observed in AT3 and fibrinogen levels, while a statistically significant increase was observed in urotensin levels in each group (p=0.011, p=0.036 and p=0.028, respectively) (Table 3).

Histopathological Examination

In the light microscopic examination of H&E-stained sections, congestion areas were evident in the frontal cortex in Group 1. The congestion in the damaged area was quite intense. Neurodegeneration and loss of neuropil were detected in the damaged area. Haemorrhage and oedema in the blood vessels were quite prominent. Neuroglial cell increase in this region and eosinophilia in the cytoplasm in some pyramidal cells were detected (Figure 1A,B). In Group 2, it was determined that pyramidal cell degeneration was less in the damaged area. Neuropil damage, vascular congestion, and oedema were seen less frequently than in the other groups (Figure 1C,D). Although pycnotic changes were detected in the pyramidal cells in the damaged area in group 3, the general morphological changes were better than group 1. Although neuropil loss, intercellular and vascular oedema was less than Group 1, it was more than Group 2 (Figure 1E,F).

The slight injury in group 2 was significantly higher than the other groups (p=0.005). Mild injury in group 3 was significantly higher than in the other groups. (p=0.005). Moderate injury in group 1 was significantly higher than in the other groups (p=0.005). These results reveal that the slightest cell injury was in group 2 (Table 2).

DISCUSSION

Haemostasis is a vital stage in neurosurgery, as it is in all surgeries that should be done carefully. Although it is a process that can be managed with simple and inexpensive materials (oxidised regenerated cellulose) most of the time, additional haemostatic agents may be required in some cases. Haemostatic matrix agents in current use are costly products in the current state of health economics. For this reason, ABS was used on spine dura by some authors (21-

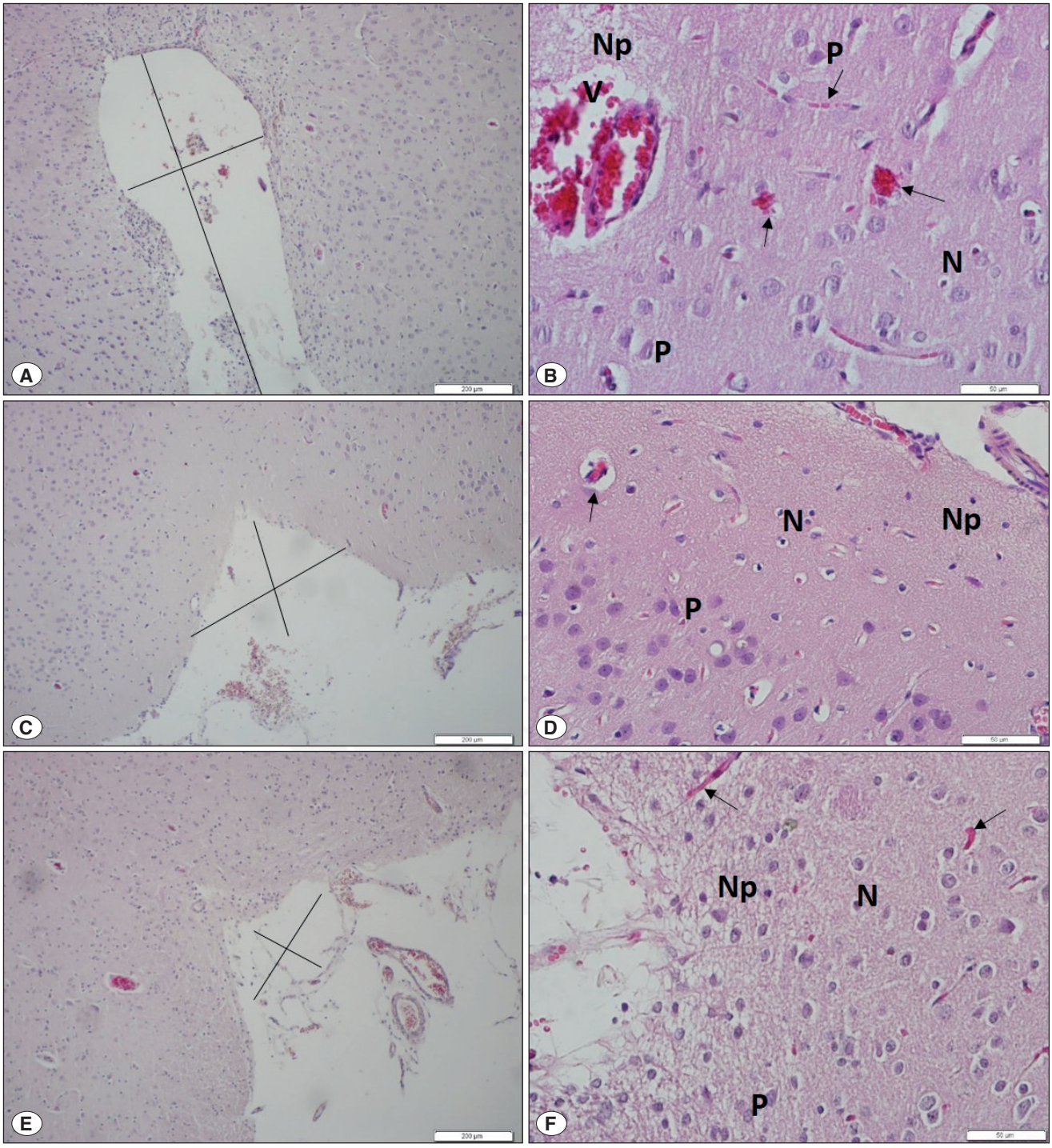

Figure 1: Representative histological changes in brain tissue. H&E image of group 1: **A**-10X, **B**-40X magnification; H&E image belonging to group 2: **C**-10X, **D**-40X magnification; H&E image belonging to group 3: **E**-10X, **F**-40X magnification; V: Blood Vessel, N: Neuroglia, P: Pyramidal cell, Np: Neuropil, arrow: Congestion areas.

23) but tested for the first time in the mammalian brain in our study and was shown to have no cell toxicity. Therefore, ABS is a candidate agent for use in the human brain due to its low cost and high efficiency.

Okumus et al. punctured the femoral vein, treated it with ABS tampon, and declared no histopathological changes in neurovascular structures. This study encouraged us to use ABS in the mammalian brain parenchyma (24). In our study,

the slight injury was most frequently observed in group 2, the mild injury was most commonly observed in group 3, and moderate injury was most commonly observed in group 1. A statistically significant difference was also revealed (p=0.005). These results prove that cytotoxicity is the least in rats in which 50% diluted form of ABS is used. Thus, it has been shown that the agent with known hemostatic activity is safe for use in mammalian brain tissue.

Yilmaz et al. showed that the epidural fibrosis of ABS increased in the rat laminectomy model (22). Erdoğan et al. compared the efficacy of Microporous Polysaccharide Hemospheres (MPH) and ABS on epidural fibrosis in a laminectomy model in rats. It has been determined that MPH reduces epidural scar formation and adhesion, while ABS increases it (21). Kuruoglu et al. also compared Momordica Charantia (MC) and ABS in the laminectomy model. They found that both agents were ineffective in preventing peridural fibrosis, but they mentioned that MC could promote new bone formation and angiogenesis in laminectomy rats (23). It is a fact that ABS, which was found to not prevent or increase epidural fibrosis in three different studies, is not suitable for use in the spinal area. Although this effect does not cause neurological problems in the cranial region, it may force clinicians to distinguish between tumour and normal tissue in radiological imaging for follow-up purposes. This possible situation can only be evaluated if ABS enters clinical use in the cranial area.

During ABS applications, prothrombin time (PT), activated partial thromboplastin time (aPTT), and coagulation factors are within average values. However, Beyazıt et al. showed in their study that the thrombin time (TT) was prolonged due to the increase in fibrinogen gamma (25). In our study, no significant change was found in fibrinogen levels. Okay et al. reported an increase in PT, aPTT, and TT attributed the antithrombin activity of ABS and attributed its Antithrombin effects to its high iron content (26). However, our study found no significant difference in AT3 levels after topical ABS application.

The Urotensin II receptor is one of the proteins identified in ABS. It links the trauma-damaged vessel wall, adhesive proteins, and activated red blood cells. The theoretical mechanism underlying the pleiotropic effects of Ankaferd in the hemostasis process were obtained by advanced scientific methods (in vitro and in vivo studies with MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time-of-Flight) proteomic molecular analyzes, cytometric methods, transcription analyses, and ultrastructural studies (27). The significant increase in urotensin levels in all three groups in our study proves that ABS does not provide hemostatic efficiency in this way.

Although hemostatic matrix agents are reported as reliable and safe in many publications, complications such as susceptibility to thromboembolic events, formation of the intracranial cystic cavity, and acute cerebral oedema have also been reported (5, 27-33). Apart from these, the cost of hemostatic matrix agents is also a fundamental problem in developing countries. Therefore, the cost of one ampoule of ABS being less than ten US dollars will also be a reasonable preference if it enters clinical use.

There are some limitations to our study. First, this study mainly focused on cytotoxicity. However, no data could be obtained on whether the agent used would cause abscess formation and what kind of artefact might cause in the follow-up imaging of the patient who underwent surgery.

The agents for hemostasis, which is the vital step of surgical interventions, should be low cost and effective and should not be cytotoxic. In this study, in which ABS was tested for the first time in the mammalian brain parenchyma, it was demonstrated that it was not cytotoxic. It is also an essential advantage for a hemostatic agent that it does not affect the coagulation steps. However, additional supportive studies are needed for its use in human brains.

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Author Contributions

Concept: **Ahmet Koluman,** Design: **Emrah Egemen, Başak Ünver Koluman,** Data Collection or processing: **Ümit Akın Dere, Nazlı Çil, Yücel Doğruel, Esin Avcı, Başak Ünver Koluman, Emine Tural,** Analysis or Interpretation: **Batuhan Bakırarar,** Literature Search: **Fatih Yakar,** Writing: **Emrah Egemen, Fatih Yakar, Başak Ünver Koluman,** Approval: **Emrah Egemen, Ahmet Koluman.**

Conflicts of Interest

All authors declare no conflict of interests.

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Ethical Approval

Approval for the study was obtained from the Ethics Committee for Experimental Animals at Pamukkale University School of Medicine (PAUHADYEK-2019/27)

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