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Original Article

Results of a hemostatic agent to potentiate ischemia-induced skin flap necrosis in an experimental rat model

Deneysel sıçan modelinde iskemiye bağlı deri flep nekrozunda hemostatik ajan kullanımı

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Abstract

Aim: The uses of plant based hemostatic agents are increasing for obtaining operative field hemostasis. However, their effects on vascularly challenged tissue is not known. The aim of this study was to investigate the effect, if any, of plant-based hemostatic agent, Ankaferd (ABS) on flap viability in a rat model.

Material and Methods: **Twenty rats underwent McFarlane flaps under general anesthesia. Ten rats in Group I received no** other treatment. Ten rats in Group II received ABS to the underside of flaps. Viable flap area was determined in scintigraphic images and percentage of viable flap area pixel size to the total flap area pixel size was calculated. Additionally all flap areas were digitally photographed and underwent histopathologic evaluation.

Results: Scintigraphic analysis has shown limited viability at proximal pedicle zone of flap in Group II compared to Group I. The mean area of flap survival percentage was calculated in Group I and in Group II was as follows respectively 56.33 ±9.94%, 26.27±7.05%. Differences between groups were statistically significant ($p<0.001$). Similarly, Group II has shown significantly smaller area of viable tissue percentage compared to Group I (26.81±5.55%, versus 59.66±12.04%, $p<0.001$) by digital photographic analysis. Histopathologic results were significantly high in Group II compared to Group I ($p<0.001$) whereas neovascularization was significantly low in Group II compared to Group I ($p<0.001$).

Conclusion: Despite its efficacy in surgical hemostasis, ABS use is associated with an increased incidence of distal tip necrosis in dorsal rat skin flaps. Therefore ABS use in marginally perfused tissues particularly in random skin flaps should be cautious.

Keywords: flap viability; Ankaferd; ABS (Ankaferd Blood Stopper); hemostatic agent

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Öz

Amaç: Operasyon alanlarında hemostazı elde etmek için bitki bazlı hemostatik ajanların kullanımı artmaktadır. Ancak bu ajanların vasküler kanlanmanın önemli olduğu durumlarda kullanımı ile ilgili herhangi çalışma bulunmamaktadır. Kullanımı son yıllarda yaygınlaşan, bitki bazlı bir topikal hemostatik ajan olan Ankaferd'in (ABS) McFarlane flep viyabilitesi üzerindeki etkisini sıçan modelinde inceledik.

Gereç ve Yöntemler: Yirmi sıçanda McFarlane tarzı flepler genel anestezi altında eleve edildi. Sıçanların 10'u Grup I olarak atandı ve başka ek tedavi uygulanmadı. Grup II'deki 10 sıçanın fleplerinin alt yüzlerine ABS uygulandı. Flepler orijinal yerlerine adapte edildikten sonraki 7. günde flap viabilitesi için sintigrafik çekim ve dijital olarak çekilen fotoğraflarla topografik analiz yapıldı. Canlı flep alanları sintigrafik görüntülerde belirlendi ve bu alanların pikselleri total flep alanına oranlandı. Sonrasında flep dokuları histopatolojik olarak değerlendirildi.

Bulgular: Sintigrafik analizde, Grup I ile karşılaştırıldığında, Grup II'de flepin proksimal sap kesiminde sınırlı alanda viyabilite gözlendi ve flep viyabilitesi yüzdesi Grup I ve Grup II için sırasıyla, %56.33±9.94, %26.27±7.05 olarak bulundu. Grup II'de gözlenen nekrotik doku yüzdesi Grup I'e oranla anlamlı olarak yüksekti ($p < 0.001$). Aynı şekilde makroskopik gözlemle uyumlu olarak ABS ile tedavi edilen sıçanlar kontrol grubu ile karşılaştırıldığında, topografik analizinde bulunan canlı doku alanı anlamlı olarak daha küçüktü (%26.81±5.55, karşın %59.66±12.04, $p<0.001$). Benzer şekilde histopatolojik sonuçlarda, ABS grubunda kontrol grubuyla karşılaştırıldığında histopatolojik değişikliklerde anlamlı artış ($p < 0.001$), neovaskülarizasyonda anlamlı azalma saptandı ($p < 0.001$).

Sonuç: Çalışmamızın sonucunda cerrahi hemostazdaki etkinliğine rağmen, ABS kullanımının özellikle cilt flepleri gibi marjinal olarak perfüze dokularda flep viyabilitesine olumsuz etkisi bulunmuştur. Dolayısıyla, vasküler damarlanmanın önemli olduğu dokularda ABS kullanımında dikkatli olunmalıdır.

Anahtar Kelimeler: flep canlılığı; Ankaferd; ABS (Ankaferd Blood Stopper); hemostatik ajan

Introduction

Studies on skin flap survival mechanisms have been conducted abundantly over the last 50 years, however; still little progress has been made. One of the most important complications of flap surgery is ischemia, which often results in necrosis. To reduce the possibility of necrosis, to prevent necrosis and to reduce ischemic conditions, many drugs, methods or situations have been assessed.

Topical hemostatic agents have now become an important assistant in many surgical procedures for reducing intraoperative and postoperative bleeding and also in nonsurgical bleedings. The hemostasis definitely has an outstanding role attaining in favorable postoperative outcomes. Ankaferd blood stopper (ABS) (Ankaferd Drug Inc., Istanbul, Turkey) is a plant-based topical hemostatic agent, which is increasingly used as an adjunctive for obtaining operative field hemostasis. And also it is the first topical hemostatic agent regarding the red blood cell– fibrinogen interactions tested in the clinical trials . It has been shown that ABS-induced pharmacological modulation of fundamental erythroid proteins (ankyrin, spectrin, actin) can cause vital erythroid accumulation by affecting fibrinogen gamma .

The effectiveness of ABS has been demonstrated in a wide range of studies including different types of injuries, in clinical applications such as in oral and maxillofacial surgery, and in orthopedics and plastic surgery . Its efficiency has been shown in to control bleeding and healing in colorectal anastomosis and in dermal wounds . However, the ideal hemostatic agent is the one that reliably controls bleeding in the operative field and does not incite deleterious effects directly attributable to its application. Considering this wide-ranging utility of ABS in hemostasis in a variety of surgical procedures, one may wonder whether tissue survival in vascularly challenged areas, could be adversely affected. Therefore, the aim of this study was to investigate the effect of ABS, if any, on tissue survival using a rat model.

Material and Methods

Experimental Design

All the experimental protocols used in this study were conducted according to international regulations and

declarations concerning animal care guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the study was approved by the ethics committee institutional review board–approved. Twenty male, Wistar albino rats weighing between 250-300g were used. They were distributed randomly into two equal groups of 10. The rats were kept individually in separate cages in a room with controlled light and temperature and they received standard chow and water ad libitum. The rats were anesthetized by a combination of intramuscularly injected ketamine hydrochloride (87.5mg/kg) and xylazine hydrochloride (12.5 mg/kg) and the surgical procedures were performed under sterile conditions. The dorsum of the animals was depilated and caudal based random pattern McFarlane-style skin flaps, each with a length- to-width ratio of 3:1 and measuring 9×3cm were raised from the dorsum of both groups. The flap was then repositioned on its original position and the closure was performed by simple stitches using 4-0 polypropylene sutures (Propilen®, Dogsan, Turkey). Group I was the control group, in which was only exposed to McFarlane flaps were elevated and repositioned. Group II was the study group and after the McFarlane flap elevation, a thin layer of ABS was applied to the underside of flaps and then the flaps were adapted to its original place and similarly sutured in place using 4-0 polypropylene sutures.

Calculating Mean Areas of Flap Survival

The outer boundaries of each flap were outlined, identified according to appearance, pliability, and texture indicative of non-viability. At post-operative 7th day all rats were digitally photographed and a scintigraphy of the flap survival was obtained.

Topographic Analysis

The viability of the flaps was daily observed. The digital photographs of the flap were taken from a standard distance. The percentage of skin flap necrosis area was calculated on the seventh postoperative day by means of Digimizer image analysis software (Med- Calc Software, Ostend, Belgium). The demarcation between viable and necrotic tissues was identified and the percentage of the viable flap area was calculated (Figure 1).

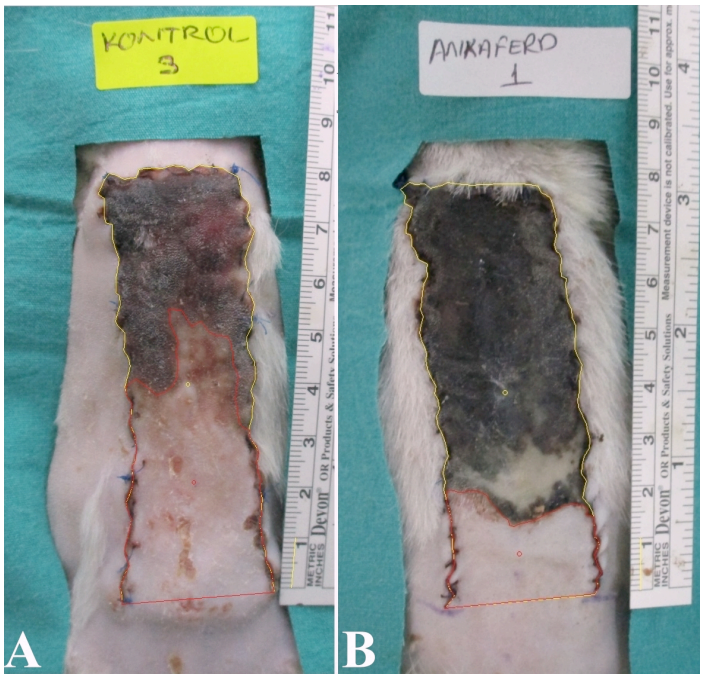

Figure 1. Topographic analysis of rats from a) Group I with 54.2 % of flap viability and b) Group II with 22.5 % of flap viability by topographic analysis.

Radionuclide Scintigraphic Analysis

On the seventh postoperative day after intramuscular (IM) ketamine and xylazine 2% injections, the scintigraphic images were obtained. The dynamic images were showing the tissue perfusion, were acquired simultaneously under the gamma camera (Siemens e.cam, USA) equipped with pinhole collimator after the administration of 1 mCi of Tc-99m pertechnetate through the tail vein. After the perfusion images, the blood pool and late blood pool phases were acquired. The blood pool images were acquired just after the perfusion images and the late blood pool images were acquired after the clearance of heart and large vascular tissue. Because of the systemically given radioactivity, to prevent the background radioactivity scattering to the flap area and also the superficial tissue perfusion increase due to superficial bleeding the lead layers were placed under the flap, contacting to the flap pedicle and the whole body of the rat was closed with lead layers. In order to prevent contamination with the radioactive material in the lead layer, the layer was wrapped with a sheet of water-proof nylon outer face paper. This cover was replaced with a new one after each acquisition and the contamination was prevented by radioactive material. A separate image was acquired with a radioactive marker to determine the distal tip of the flap due to the expected perfusion defects at the distal part of the flap (Figure 2).

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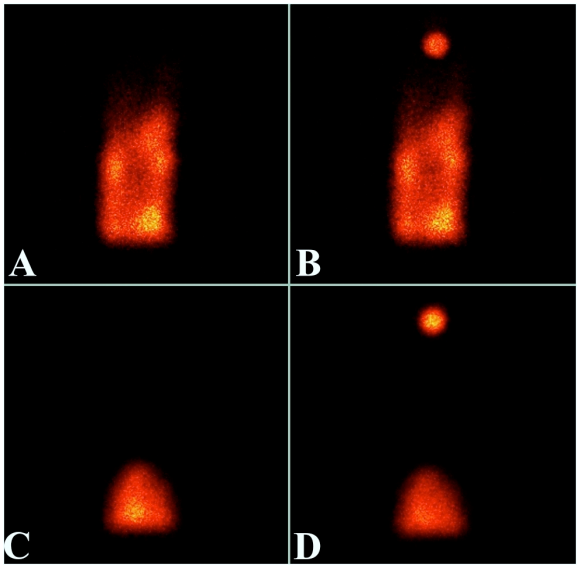

Figure 2. Flap viability on scintigraphic image of a rat from a) Group I b) same rat image from Group I with radioactive marker shown on the top of the image c) Group II d) same rat image from Group II with radioactive marker shown on the top of the image.

The images were first assessed visually for the hyperemic areas. After that by semi-automatically drawn region of interests (ROI) on the late blood pool phase images were the pixel size of the viable flap area were acquired. The manual ROIs were drawn over the total flap area through images with radioactive marker, and the pixel size of the total flap area were obtained and the ratio of the viable flap area was calculated (Figure 3). The percentages of necrosis were calculated by subtracting the viable area from the total flap area.

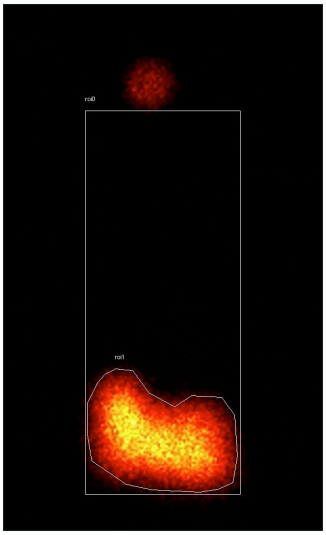

Figure 3. The manual rectangular ROI (roi0) drawn over the total flap area via images with radioactive marker, and the pixel size of the total flap area were obtained. The pixel size of the viable flap area is determined by the semi-automatically drawn region of interest (roi1) and the ratio of the viable flap to the total area was calculated.

Histopathologic Examination

The animals were adequately anesthetized using high dose of intraperitoneal ketamine and xylazine and the flap tissue was raised before sacrification and immediately fixed in 10% buffered formalin. Following routine tissue procedures, a 5-µm section was taken with a microtome device and paraffin embedded and stained with hematoxylin-eosin and Masson's trichrome. For each flap tissue, the degree of neovascularization, inflammation, oedema, and necrosis were scored from 0 (none) to 4 (severe) (score 0: none, score 1: mild, score 2: positive, score 3: strong positive, score 4: severe positive) for the non-viable flap area, the demarcation zone and the viable flap area. Photomicrographs were taken using light microscopy AxioScope.A1 (Carl Zeiss, Oberkochen, Germany) at x 5-fold magnification.

Data Analysis

Data analysis was performed using Statistical Package for Social Sciences for Windows software (SPSS version 23.0, SPSS Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used to examine whether the variables are normally distributed or not. The descriptive statistics are given as mean ± standard deviation for the numeric variables with normal distribution and for the numeric variables without normal distribution as median (minimum-maximum) values. The differences between the groups were analyzed by the Mann-Whitney U test. A value of $p < 0.05$ was accepted as statistically significant.

Results

Operative Results

During the course of the experiment, all rats were noted to be healthy. On post-operative 7th day, there was a clear demarcation zone between the viable and necrotic tissue. The necrotic skin was noted to be black, firm with no bleeding when cut, whereas the surviving skin was soft, yellow, and hairy and bled when cut. The majority of skin flaps in Group II survived, with small areas of viability in the pedicular zone and large areas of distal tip necrosis.

Histologic Results

The sections were obtained from the necrotic area, the demarcation zone and the pedicle zone in both groups. The flap zones of the groups were compared between each other according to the number of neovascularization in the tissues, and oedema, inflammation and necrosis scores (Table 1). All the parameters were significantly different between 2 groups (Table 1). The tissue oedema, inflammation and necrosis scores were significantly higher in Group II compared to Group I, whereas the neovascularization was significantly lower in Group II (Figure 4).

Table 1. The histopathologic comparison of the flap zones are presented in both groups.

	Neovascularization†	p value	Oedema	p value	Inflammation	p value	Necrosis	p value
Group I Necrotic zone	6.50±8.8	p<0.01	2 (0-2)	p<0.001	1.50 (0-4)	p<0.001	3 (0-4)	p<0.001
Group II Necrotic zone	0.00±0		4 (4-4)		4 (4-4)		4 (4-4)	
Group I Demarcation zone	14.30±6.1	p<0.001	2 (0-4)	p<0.001	1 (0-4)	p<0.01	1 (0-2)	p<0.001
Group II Demarcation zone	0.00±0		4 (4-4)		3 (3-4)		3 (3-4)	
Group I Pedicle zone	23.50±5.5	p<0.001	0 (0-2)	p<0.001	0 (0-1)	p<0.001	0 (0-1)	p<.001
Group II Pedicle zone	6.80±2.9		3 (2-3)		3 (1-3)		3 (1-3)	

According to the Mann-Whitney U test, p value of <0.05 was considered statistically significant. For neovascularization, †, the values are expressed in mean±standard deviation; for oedema, inflammation, necrosis the values are expressed in median (minimum-maximum).

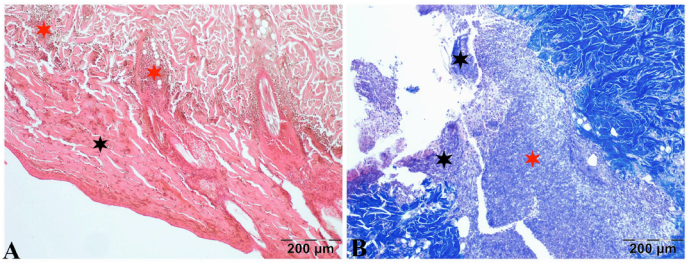

Figure 4. The representative photomicrographs of the flaps in Group II showing the necrosis (black stars) and the inflammation (red star) a) in Haematoxylin-Eosin x400 and b) in Masson's trichrome stain. Scale Bar: 200 µm.

Scintigraphic Examination

The pixel size of the total flap area calculated on scintigraphic images was in 8227.5 ± 3765.64 pixels in Group I versus 7740.6 ±1531.38 pixels in Group II (Table 2). However, there was no statistically significant difference between the study groups in terms of pixel size of the total flap area (p=0.257).

The percentages of viable flap area evaluated in sintigraphic study were as follows; 56.33 ± 9.94 % in Group I and 27.24 ± 7.05 % in Group II (Figure 5). The differences between 2 groups were statistically significant (p < 0.001).

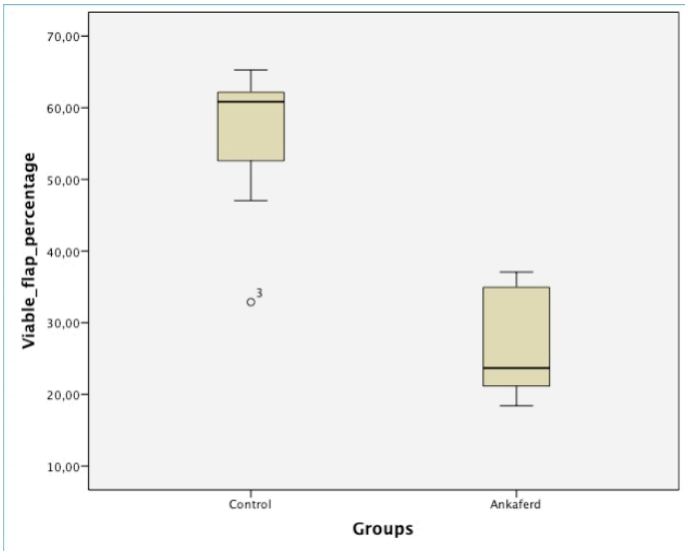

Figure 5. The viable flap percentages for 2 groups are presented.

Topographic Analysis

On postoperative day 7, the total area of the flap was 21.14 ± 2.21 cm2 in Group I and 19.14± 1.80 cm2 in Group II. The differences between 2 groups were not significant in terms of total area (p > 0.05) (Table 2). The viable flap area in Group I was 12.23 ± 1.2 cm2 in Group I and 5.11 ± 1.03 cm2 in Group II. The viable flap area differences between groups were statistically significant (p<0.001). The percentages (mean±SD) of the viable in flap area, evaluated on digital images by the Digimizer analysis program, in each group were as follows for Group I 59.66±12.04 % and for Group II 26.81±5.55 %. There was a statistical significant difference in flap survival between groups (p < 0.001)(Table 2).

Table 2. The percentages of viable flap areas and the total flap areas are presented by scintigraphic and topographic analysis.

	Group I	Group II	p value
Percentage of Viable Flap Area by Scintigraphy	56.33±9.94 %	26.27±7.05 %	p<0.001
Percentage of Viable Flap by Topographic Analysis	59.66±12.04 %	26.81±5.55 %	p<0.001
Total Flap Area in Pixels by scintigraphy	8287.50±3765.64	7740.60±1531.38	p>0.05
Total Flap area by Topographic Analysis	21.14±2.2 cm2	19.14±1.8 cm2	p>0.05

Discussion

Random pattern skin flaps are frequently used in reconstructive surgery for the open wounds. However, the most common complication of flap surgery, the skin flap necrosis remains as an important drawback. Although the exact mechanism of necrosis occurring in the distal part of the skin flap is unknown; vasospasm, thrombosis and insufficient blood flow

are thought to be the main reasons. The partial flap necrosis is caused by blood vessel injury, which leads to the blood flow abnormality, proceeding with hemodynamic instability. The distal part is commonly affected due to the diminished vascular perfusion at the distal portion. To improve the flap viability, numerous experimental studies with many treatment methods and various agents have been carried out to increase the flap survival and it is still a challenging issue .

ABS is a standardized mixture of the plants Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum, and Urtica dioica which is a widely used hemostatic agent. Through its ingredients it has been shown that Thymus vulgaris is an antioxidative via its effects on lipid peroxidation ; Glycyrrhiza glabra decreases vascular endothelial growth factor production and cytokine-induced neovascularization ; Vitis vinifera has antiatherosclerotic effects ; Alpinia officinarum prevents nitric oxide production and Urtica dioica induces nitric oxide in the endothelium and causes vasodilation . Other than these the anti-oxidant and anti-mutagenic activities and anti-inflammatory effects of ABS have been recently demonstrated. Its efficiency in wound healing and in tendon healing have been discussed in randomized clinical studies and also its use in septorhinoplasty, in split thickness skin graft, in cleft-palate patients have been reported in a report with individual cases . Furthermore, a study demonstrated that on an aortotomy wound, ABS treatment provided hemostasis whilst preserving vascular patency . These encouraging results led the authors of this study to explore the effect of this agent on flap survival in a rat model. Former studies had not verified the qualitative ischemic effects of ABS on vascular-damaged tissues.

To obtain a consistent and uniform technique, we have used McFarlane-style skin flaps planned to fixed anatomical points with a flap size of 3x9 cm keeping the aspect ratio of 1: 3. McFarlane et al. found that, the ratio of necrosis area to total flap area ranged from 22% to 50% . Similar rates of necrosis was reported in a study of Myers and Cherry . Likely, in our study, we used the flap size of 3x9 cm and we found that the ratio of necrosis area in the control group to the total flap area was similar to the rate of necrosis in the literature . Similar to the literature, scintigraphic examination was found to be compatible with the topographic analysis . Furthermore, there was no statistically significant difference in terms of the total flap areas in both groups in scintigraphy ($p>0.05$) and also in topographic analysis ($p>0.05$) (Table 2).

ABS have demonstrated safety and efficacy in a variety of surgical procedures with the profound hemostatic effect and also its topical application has been reported to shorten the duration of bleeding . Similarly, in our study its application has shortened the duration of bleeding. However, in our study, other than subjective effectiveness of ABS on duration of bleeding that we have observed, we didn't evaluate this subject deeply, as it has been deeply discussed elsewhere .

However, our results of the macroscopic analysis, the topographic analysis and the scintigraphic evaluation have shown that the percentages of necrotic areas were significantly higher in Group II compared to Group I (Table 2).

In the pathophysiology of flap necrosis, the triggering event is the separation of the feeding blood supply and the sympathetic nerves. Following elevation of the flap, there are two main factors responsible for the pathogenesis of necrosis in the distal part of the flap; the first one is the decreased nutrition in the distal flap due to sympathetic activation process with diminished blood flow and the second one is the reperfusion injury occurring after 6-12 hours of ischemia when circulation is restored. Moreover, in the ischemic tissues, with anaerobic glycolization, reactive oxygen radicals (ROS) occur. Increased radicals directly lead to lipid peroxidation in the cell membrane and show toxic effects such as acute inflammation, leukocyte accumulation and adhesion, resulting in endothelial damage, which result in microvascular collapse and usually necrosis occurs .

The basic mechanism of action for ABS is the formation of an encapsulated protein web, which represents the focal point for vital erythrocyte masses . During the blood coagulation, the red blood cells make an almost impermeable seal in a clot and bind to the fibrinogen via a beta3-containing integrin, with almost similar affinity as platelets . However, despite the several tissue protective effects that the agent has , possibly the clot formation by the protein network resulting the impermeable seal may feasibly increased the microvascular collapse which may explain the increased incidence of outsized necrosis in dorsal rat skin flaps. Though this was a preliminary study we believe that the flap survival was decreased either by its cytotoxic effects on normal cells

with DNA damage, apoptosis and cytotoxicity by generating ROS activity or increased microvascular collapse via red blood cell– fibrinogen interactions.

Conclusion

Our study results demonstrate the use of a rat model that reliably mimics a common clinical scenario for random skin flaps, and the provision of topographic, scintigraphic and histopathologic data. Our data unequivocally demonstrated that the application of ABS was associated with an increased incidence of distal necrosis in dorsal rat skin flaps. This is particularly important because of its increasingly used modality for maintaining hemostasis. However, as the experience with ABS increases, it is indispensible that clinicians continue to analytically evaluate it to upgrade the effectiveness, the limits of indications and the safety with its complications.

To our knowledge, this is the first study reporting the effects of ABS in an experimental random skin flap study with histopathological evidence. In conclusion, despite its efficacy in surgical hemostasis, our results show that ABS use should be cautious, particularly with marginally perfused tissues such as random based skin flaps.

Conflict of Interest: There is no conflict of interest among the authors.

Disclosure: The authors have no financial interest in any of the products or devices mentioned in this article.

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