

EFFECTS OF ANKAFERD BLOOD STOPPER-REINFORCED PLATELET-RICH FIBRIN MEMBRANE ON GUIDED TISSUE REGENERATION IN EXPERIMENTAL BONE DEFECTS

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ABSTRACT

Background and Aim: To evaluate the effects of Ankaferd Blood Stopper added platelet-rich fibrin administered in cranial bone defects created in rabbits via histomorphometric assessment.

Materials and Method: Four circular 5 mm defects were created in the crania of 16 New Zealand rabbits. Each defect in each animal received one of four treatments: no treatment (EC group), platelet-rich fibrin administration (PRF group), Ankaferd Blood Stopper added platelet-rich fibrin administration (PRF + ABS group) and collagen membrane administration (CM group). Histomorphometric assessment was conducted at 4 and 8 weeks after surgery.

Results: Between-group comparisons of the new bone area revealed significant differences between the PRF + ABS group and the remaining three groups at 4 weeks. The new bone area was significantly larger at 8 weeks than at 4 weeks in all groups.

Conclusion: The use of Ankaferd Blood Stopper in conjunction with platelet-rich fibrin improves bone healing, strengthens the membrane property of platelet-rich fibrin and promotes better ossification.

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Keywords: Ankaferd Blood Stopper, Guided Bone Regeneration, Membrane, Platelet-Rich Fibrin

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ANKAFERD BLOOD STOPPER İLE GÜÇLENDİRİLMİŞ PLATELETTEN ZENGİN FİBRİN MEMBRANIN DENEYSEL KEMİK DEFİKTLERİNDE YÖNLENDİRİLMİŞ DOKU REJENERASYONU ÜZERİNE ETKİLERİ

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ÖZ

Amaç: Ankaferd Blood Stopper ile güçlendirilmiş trombositten zengin fibrinin tavşanlar üzerinde oluşturulan kalvaryal kemik defektleri üzerindeki etkilerinin histomorfometrik yöntemler ile değerlendirmek.

Gereç ve Yöntem: 16 Yeni Zelanda tavşanı kafatasında, dörder adet 5 mm çapında dairesel kemik defekti oluşturuldu. Her bir hayvandaki dört farklı defekt bölgesine, dört farklı tedaviden biri uygulandı: tedavi yok (EC grubu), trombositten zengin fibrin uygulaması (PRF grubu), Ankaferd Blood Stopper ile beraber trombositten zengin fibrin uygulaması (PRF + ABS grubu) ve kolajen membran uygulaması (CM grubu). Histomorfometrik değerlendirme cerrahiden 4 ve 8 hafta sonra yapıldı.

Bulgular: Yeni kemik alanının gruplar arası karşılaştırmalarında, 4.hafta örneklerinde PRF + ABS grubu ile kalan üç grup arasında anlamlı fark olduğu görüldü. Yeni kemik alanının, tüm gruplarda 8.hafta örneklerinde, 4.hafta örneklerine göre anlamlı derecede yüksek olduğu görüldü.

Sonuç: Ankaferd Blood Stopper'ın trombositten zengin fibrin ile birlikte kullanılması; kemik iyileşmesini arttırmakta, trombositten zengin fibrinin membran özelliğini güçlendirmekte ve daha iyi bir kemikleşme sağlamaktadır.

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Anahtar Kelimeler: Ankaferd Blood Stopper, Yönlendirilmiş Kemik Rejenerasyonu, Membran, Trombositten Zengin Fibrin

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INTRODUCTION

Guided tissue regeneration (GTR) is a widely used method to repair bone defects due to pathological lesions or to increase bone volume before dental implants.¹ Barrier membranes used in GTR are of vital importance for appropriate bone formation. Membranes protect the site of defect until the completion of bone tissue development by preventing soft tissues from growing towards the inside of the defect. Membranes used in GTR need to have various characteristics to ensure maximum bone regeneration. The most important ones include being biocompatible, correct hardness to maintain defect space, ability to block epithelial cell migration and the ability to be resorbed after completion of bone regeneration.² Despite a number of membranes with substantial success being frequently used today, these do not make an economical treatment alternative for every patient.

Platelet-rich fibrin (PRF) is a widely used successful endogenous coagulation system product.^{3,4} PRF is made of a fibrin network that contains cytokines and glycoproteins.⁵ The primary advantage of PRF is that the gelation procedure does not require the addition of a product of animal origin. However, PRF acts as a source of growth factors to facilitate tissue repair and regeneration. Moreover, PRF is used as a resorbable barrier membrane through its compressed membranous form as part of GTR, since it chemically contains biopolymer fibrin.⁶ However, growth factors that are released from PRF membranes accelerate tissue regeneration. In addition, it has been observed that PRF membranes act as a matrix for the growth of periosteal cells that contribute to bone repair.⁵ However, PRF membrane is not able to preserve the space required for tissue regeneration long enough, since it is resorbed within 2 weeks.⁷ Because it is well known that the stability of polymer-based materials is usually controlled by the modification of the density of cross-links between polymer fibres, another alternative to improve this disadvantage is to strengthen the fibrin webs in the PRF membrane. Also, resistance is ensured against decomposition due to enzymatic reactions by using methods to increase these cross-links, despite some loss of bioactivity.⁸

Ankaferd Blood Stopper® (ABS) (Ankaferd Saglik Urunleri A.Ş., İstanbul, Turkey) is a substance of herbal origin that has been used for centuries as a haemostatic agent. ABS is produced from a mixture of plants in standard ratios. These plants are *Urtica dioica* (6.0 g/100 mL), *Vitis vinifera*

(8.0 mg/100 mL), *Alpinia officinarum* (7.0 mg/100 mL), *Thymus vulgaris* (5.0 mg/100 mL) and *Glycyrrhiza glabra* (7.0 mg/100 mL).⁹ Each of these plants has various effects on cellular proliferation, vascular dynamics, angiogenesis, blood cells, cell mediators and endothelium.¹⁰ Studies in recent years have shown the influences of ABS on bone recovery.^{11,12}

Although its mechanism of action is not clearly established, overall haemostatic and biochemical tests indicate that ABS rapidly develops a protein web after coming into contact with blood or serum. Studies demonstrate that this web is formed by its interaction with the proteins in the blood, primarily fibrinogen, and that live red blood cells are aggregated on this web. It has been found to ensure haemostasis without affecting any coagulation factors. The most important benefit of ABS compared to other antihaemorrhagic agents is its positive effects on wound healing. This is an important advantage, considering the negative effects of haemostatic agents on wound healing.¹³

The objective of this study is to investigate whether ABS and PRF membranes, which have been widely used in many fields in recent years, together form a stronger and more successful membrane in terms of wound healing and to examine its effects on bone healing using histomorphometric methods.

MATERIALS AND METHODS

Experimental Model

This study was approved by the Hacettepe University Animal Experimentation Local Ethics Committee on 09/02/2016 [GM1] under decision No. 2016/02-03. The study was conducted on 16 adult male New Zealand rabbits with an average body weight of 3500 g. The subjects were housed in appropriate cages at 22 ± 2 °C, ensuring 12 hours of light and 12 hours of darkness. The animals were transferred to the laboratory environment where the experiments would take place at least 1 week before the surgical intervention to provide adequate sanitary conditions, to prevent infections, for accommodation to the new environment and for general health status control. The subjects were fed with standard laboratory animal food and water. Each animal was kept in a separate cage to ensure easy access to water and food, adequate moving space and a stress-free ambiance.

Four groups were designated for each experimental animal with two being in the right calvaria and two in the left calvaria. The groups were:

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- Collagen membrane (CM) group
- Empty control (EC) group
- PRF membrane (PRF) group
- PRF membrane + ABS (PRF + ABS) group

The duration of the study was selected as 4 and 8 weeks. The CM and EC groups and the PRF + ABS and PRF groups were assigned to the left and right sides of the calvaria, respectively, in each experimental animal when designing the groups. In addition, the PRF and CM groups were left to the posterior area, while the ABS + PRF and EC groups were assigned to the anterior area, in order to ensure standardisation and to prevent potential confusions.

Preparation of PRF Membrane and ABS Solution

PRF obtained from 5–7 mL of blood drawn from the central ear artery centrifuged at 3000 rpm for 10 minutes was transformed into a membrane by leaching the serum using the unique PRF kit and was saved to cover the defect area. For the PRF + ABS group, PRF that was turned into a membrane was kept in a preconditioned 20% ABS solution, containing 4 mL of ABS and 16 mL of sterile saline, for 5 minutes and prepared for placement to the site of operation.

Surgical Procedure

Ketamine hydrochloride (Alfamine, Alfasan, The Netherlands) 35 mg/kg and xylazine hydrochloride (Alfazyne, Alfasan, The Netherlands) 2.5 mg/kg were administered via the intramuscular route to each experimental animal for anaesthesia. The intervention site was cleaned using povidone iodine (Batticon, Adeka, Turkey), after shaving the hair in the right and left mandible. Local anaesthetic solution (1 mL) (Ultracain D-S Forte, Sanofi Aventis, Turkey) was infiltrated to the respective site for haemostatic control. The bone surface was exposed by an ~3 cm full-thickness incision that goes down to the periosteum on the middle calvarium line along the linea media using a No. 15 scalpel. Two bone osteotomies were performed on the right side of the linea media and two on the left side of the linea media, making a total of four and leaving at least 3 mm of distance between, using a trepan bur with an outer diameter of 5 mm and inner diameter of 4 mm under sterile saline cooling without damaging the dura (Figure 1).

- In the CM group, the defect cavity was irrigated with saline followed by covering the defect site with collagen membrane rehydrated with saline.
- In the EC group, no intervention was made after irrigating the defect cavity with saline.

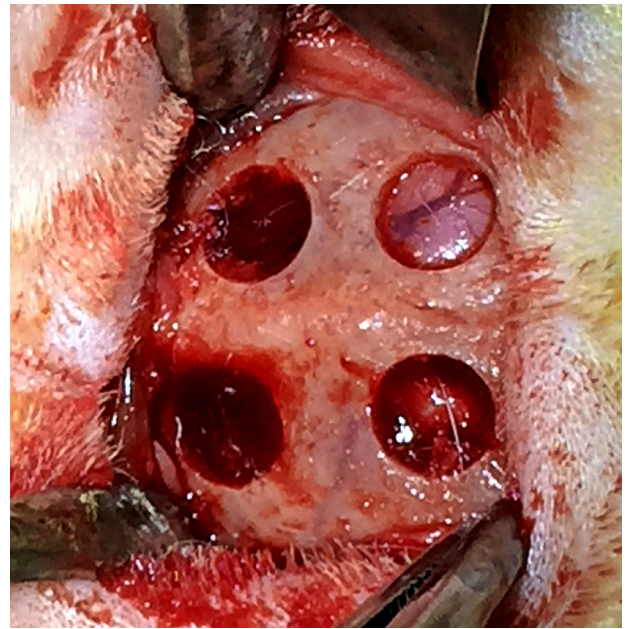


Figure 1. Four bone defects measuring 5 mm in diameter were created of the calvaria

- In the PRF group, the defect site was covered with PRF membrane.
- In the PRF + ABS group, the defect cavity was covered with ABS loaded PRF membrane (Figure 2).

Finally, the skin and subcutaneous tissues were primarily closed using a resorbable 16 mm 3/8 cutting edge 4.0 polyglactin suture (Coated Vicryl, Ethicon, Johnson & Johnson, Belgium). Wound closure spray (Opsite, Smith & Nephew, Canada) was administered on the stitch sites to prevent postoperative infection.

In the postoperative period, the subjects were given meloxicam 1 mg/kg (Maxicam X4, Sanovel, Turkey) for analgesia and antibiotic therapy with enrofloxacin 2.5 mg/kg intramuscular for 5 days (Baytril-K 5%, Bayer, US). Each animal was kept in a separate cage during the trial under a cycle of 12 hours of light and 12 hours of darkness. The average ambient temperature was 22–24 °C with a humidity of 55–70%. Wound health was regularly examined, while adequate food and water were supplied.

Tissue Processing

One-half of the animals were sacrificed at the end of 4 weeks and the rest at the end of the 8th week using lethal doses of intramuscular xylazine HCl (Alfazyne, Alfasan, The Netherlands) 30 mg/kg and ketamine HCl (Alfamine, Alfasan, The Netherlands) 70 mg/kg. The defective sites from each rabbit were identified and removed as a block from the

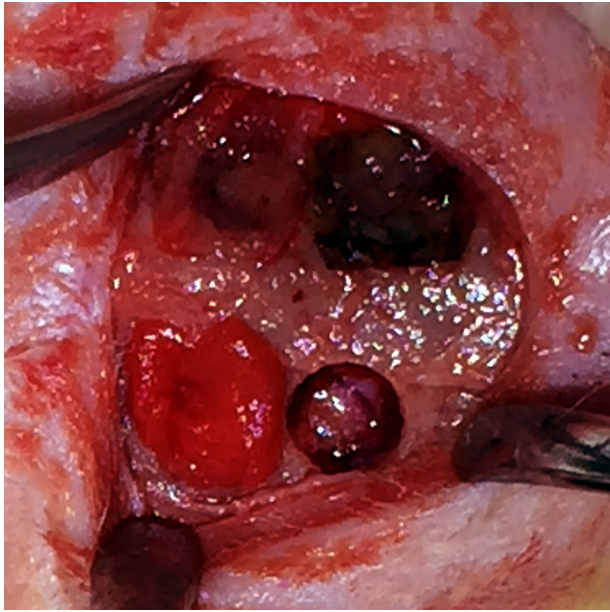


Figure 2. The defect areas were covered with various membranes (collagen, PRF, ABS+PRF)

cranium together with a certain amount of surrounding intact bone tissue. This was followed by the fixation of the samples for each rabbit separated by subgroups in 10% buffered formaldehyde for 48 hours.

Histomorphometric analysis

Histomorphometric analysis was performed by an examiner who was blinded to the identity of samples. The samples were decalcified in 10% acetylsalicylic acid solution renewed every 3 days and controlled for 21 days. Dehydration was accomplished by passing the tissues rinsed with distilled water through the alcohol series. Then, these were bundled into paraffin blocks after transparency with the xylene series. All the blocks were turned into transverse serial cross-sections of 4 to 6 μm thickness with a sampling rate of 1/20 and placed on glass slides for assessment of histological structure. The cross-sections were deparaffinised overnight in a 60 °C incubator with xylene followed by dehydration and stained with haematoxylin and eosin (H & E). Each stained cross-section was closed with Canada balsam. Five individual photos of each cross-section were captured under $\times 100$ magnification. New bone trabeculae and soft tissue areas that filled in the defect site in each photo were measured and calculated in μm^2 .

Statistical analysis

The data from the study were analysed using the SPSS 18.0 (SPSS Inc., Chicago, IL) statistical package software.

Kruskal Wallis test was used to compare the ossification rates of the subjects in different groups. A significant difference was found in the results of the analysis and Mann Whitney test with Bonferroni correction was used for pairwise comparison analysis. In each group, the comparison of the 4th week and 8th week data was analyzed by Wilcoxon test. Statistical significance for type 1 error level (α) values that were smaller than 0.05 were considered statistically significant.

RESULTS

Almost all of the histological preparations from the groups showed that the site of defect consisted of new bone trabeculae and loose collagenised connective tissue that were bound together at 4 weeks. It was noted that the connective tissue acquired a more cellular collagenised character towards the centre of the defect. New bone production was found to be primarily advancing in a centripetal manner from the edge of the defect to the centre by periosteal activation. This centripetal trend was more prominent in the PRF + ABS group. New bone trabeculae were taking form as a result of the activation of damaged periosteum, with most parts localised at the defect margins. In histological preparations of the 8th week, the general histological view was similar to the 4 week samples of the same group, except for the higher number of bone trabeculae. No bone production was observed that filled the whole defect site, although bone production was increased in the central parts (Figure 3).

Regenerative potential of the used protocol was evaluated with the help of histomorphometric analysis and measuring the amount of new bone formation. The percentage of newly formed bone at 4 and 8 weeks in various groups was shown (Table 1).

Measurements were reported as a median and interquartile range. Quantitative results of the histomorphometric analysis of bone defects showed the percentage of osteogenesis at 8 weeks in all groups to be higher than that at 4 weeks. The highest rate of osteogenesis was observed in the PRF + ABS group after 8 weeks (34.42%), while the lowest rate was observed in the EC group after 4 weeks (5.00%). Bone regeneration was higher in the PRF + ABS group compared to the other groups after 4 and 8 weeks. In the intergroup test, the PRF + ABS group had a statistically significant difference compared to the other three groups in terms of the rate of newly formed bone after 4 weeks ($p < 0.05$). The CM, PRF and PRF + ABS groups had statistically

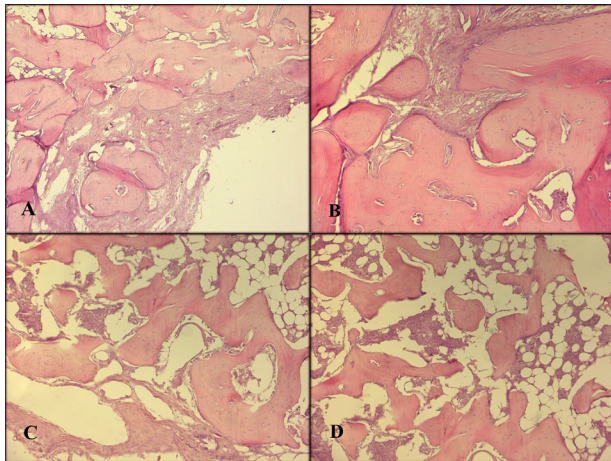


Figure 3. A. At 4 weeks specimens in the EC group (hematoxylin and eosin [H&E], ×40). B. At 8 weeks, new bone trabeculae were observed towards the center of the defect at the continuation of the host bone trabeculae specimen in the CM group (H&E, ×100). C. At 8 weeks, PRF group specimens show anastomosing new bone trabeculae's that start from the periphery, and cellular structured connective tissue (H&E, ×100). D. At 4 weeks, specimens in the PRF+ABS group showed formation of anastomosing new bone trabeculae and cellular structured collagen connective tissue (H&E, ×100).

significant differences compared to EC group after 8 weeks ($p < 0.05$).

DISCUSSION

In this study, a calvarial defect model of 5 mm diameter was used to evaluate the biological activity of the PRF + ABS membrane in adult rabbits. Rabbit was chosen as the experimental animal because of having a sufficient amount of blood to obtain PRF. The topic of defect size using a rabbit calvarial defect model is still controversial in the literature.

Despite the fact that critical size defect (CSD) dimensions defined for rabbit calvaria are a circle of 15 mm diameter, a defect of 5 mm diameter is shown in this and similar studies to be sufficient to demonstrate bone regeneration. CSD is the size that cannot heal for the lifetime of the animal without treatment. This and other studies have shown that defects of 5 mm that form the control group do not show complete healing without treatment.¹⁴⁻¹⁶ In addition, this model allows the creation of four groups in a single experimental animal, so that individual differences are avoided and the number of experimental animals used is minimised. For all these reasons, in this study, 5 mm wide calvarial defects of rabbits were placed, instead of creating four 15 mm diameter defects.

PRF is a material that lacks exogenous factors developed to improve the utilisation efficacy of platelet-rich plasma (PRP[GM1]). One advantage of PRF is that it contributes to regeneration by harbouring growth factors, as in PRP. In addition, fibrin and fibrinogen found in PRF preparations also function as a scaffold and adhesive. Lately, some clinical investigators have recommended that the use of PRF in membrane form in a clinical environment as an alternative to commercially available GTR barrier membranes. However, results of these investigations were not as expected because of the rapid resorption of PRF membrane.^{5,17}

A barrier is generally needed to stay at the respective site at least 3–4 weeks, in order to protect the implant site from soft tissue integration. Resorbable membranes produced from synthetic polymers have a resorption duration as long as 12 months, while collagen membranes are more rapidly resorbed and stay stable for approximately 16–38 weeks without degradation. Collagen membranes without cross-links lose structural integrity within 7 days.¹⁸ Kawase

Table 1. Percentage of osteogenesis in different groups at 4 and 8 weeks

Grup	4 weeks %bone formation	8 weeks %bone formation	p value
EC	5.00 (3.25-8.40)	11.90 (7.53-15.20)	<0.001*
CM	12.50 (8.10-18.72)	33.03 (22.89-41.52)	<0.001*
PRF	15.25 (10.02-18.28)	28.20 (27.92-34.56)	<0.001*
PRF+ABS	20.85 (16.40-25.20)	34.42 (25.21-44.50)	<0.001*
p value	0.024*	0.019*	
Post Hoc	1-2 / 1-3 / 1-4	1-2 / 1-3 / 1-4	

* $p < 0.05$

et al.⁷ showed in their study that PRF membranes lose integrity within 1–2 weeks, similar to non-cross-linked collagen membranes, and claimed that PRF may be used as an optimal GTR membrane by increasing the number of cross-links between fibrin tips, resulting in a structure more resistant to resorption. As a haemostatic agent, ABS is known to provide coagulation formation via fibrin cross-links. In addition to these properties, its positive effects on hard and soft tissue have been demonstrated, though the exact mechanism remains to be clarified.¹⁰

In this study, administration of ABS used together with PRF membrane was investigated, which is an autogenous product that accelerates healing by virtue of the inherent growth factors but exhibits negative effects alone. Both 4 and 8 week results showed that PRF + ABS use generated the most favourable effect on new bone formation (21.94% at 4 weeks and 35.23% at 8 weeks). This is caused by the favourable effects of ABS on healing as well as the stronger mechanic characteristics of PRF, owing to the increased number of fibrin cross-links with a prolonged resorption time. New bone percentages in the EC group were significantly lower at 4 and 8 weeks compared to other groups. This result demonstrated once again the need for a membrane for proper GTR.

CONCLUSION

Many ideas have been put forward since PRF membrane possesses sufficient biological properties that can safely be used in GTR. However, the physical properties of this membrane are shown to be insufficient to prevent soft tissue integration. Strengthening the physical properties of PRF will make it more commonly used as a very beneficial, economical endogenous membrane in GTR. In this study, ABS was used to leverage the physical and mechanical properties of PRF membrane and to make use of the factors it contains that positively influence healing. In conclusion, further studies are needed on the physical and mechanical properties of PRF membrane.

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