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The second step in vitro trial of Ankaferd® Bloodstopper®: comparison with other hemostatic agents

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Aim: To investigate the efficacy of Ankaferd Bloodstopper (ABS) and compare ABS with the other hemostatic agents still used in urologic surgery.

Materials and methods: Forty Wistar rats were divided into 5 groups: Group T (traditional), partial nephrectomy (PN) with hilar control as per the conventional technique; Group G (Glubran 2), conventional PN followed by the application of Glubran 2; Group F (FloSeal), FloSeal application to the resected area of the kidney; Group C (Celox), Celox was applied; Group A (ABS), Ankaferd was used. Warm ischemia time (WIT) and hemostasis time (HT) were recorded and histopathologic features were compared among the groups.

Results: In Group A, a significant decrease in WIT was detected while the difference was significant ($P < 0.001$). Statistical analysis confirmed that the evaluation of HT (in seconds) returned similar results to those seen in WITs among all groups. In Group A, decreased HT was confirmed in comparison to Group T while HT increased in comparison to the other groups (G, C, and F) ($P < 0.001$). Increased fibrosis and adhesion were shown in Group F while significant erythrocyte aggregation and microvascular proliferation were observed in Groups G, F, and A ($P < 0.001$).

Conclusion: A novel hemostatic agent, ABS, is as effective as other licensed hemostatic agents with comparable WIT and HT and better results in terms of histopathologic findings.

Key words: Hemostatic agents, hemostasis, comparison, partial nephrectomy

Ankaferd bloodstopper ikinci basamak in vitro çalışması: Diğer hemostatik ajanlarla karşılaştırma

Amaç: Bu çalışmada Ankaferd BloodStopper'ın etkinliği diğer hemostatik ajanlar ile karşılaştırılarak yapılmaya çalışılmıştır.

Yöntem ve gereç: Kırk Wistar rat beş gruba ayrıldı. Bu gruplar: T grubu (traditional), hilar kontrol ile parsiyel nefrektomi yapılan, G grubu (Glubran 2) konvansiyonel parsiyel nefrektomi sonrası Glubran 2 uygulanan, F grubu eksize edilen böbrek alanına FloSeal uygulanan, C grubu hemostatik ajan olarak Celox kullanılan, A grubu ise yeni bir hemostatik ajan olan Ankaferd (Ankaferd-ABS) kullanılan gruplar olarak belirlendi. Sıcak iskemi süreleri ve hemostaz zamanları kayıt edildi. Gruplar arasındaki histopatolojik özellikler karşılaştırıldı. Istatistiksel analiz için Kruskal-Wallis ve Mann-Whitney U testleri kullanıldı.

Bulgular: A grubunda ölçülen sıcak iskemi süresi anlamlı derecede düşük olarak saptanırken diğer gruplarla fark anlamlı idi ($P < 0,001$). A grubunda T grubuyla karşılaştırıldığında azalan hemostaz zamanın diğer gruplarla karşılaştırıldığında artmış olduğu belirlendi ($P < 0,001$). A grubunda diğer gruplarla karşılaştırıldığında anlamlı derecede fibrozis, adhezyon ve kalsifikasyon oluşmadığı gözlendi. F grubunda fibrozis ve adhezyonun artmış olduğu gösterildi. A, F ve G gruplarında eritrosit agregasyonu ve mikrovasküler proliferasyonun anlamlı olarak yüksek olduğu gözlendi ($P < 0,001$).

Sonuç: Yeni çıkan bir hemostatik ajan olan Ankaferd, diğer lisanslı hemostatik ajanlar kadar etkilidir, sıcak iskemi süresi ve hemostaz süreleri için karşılaştırılabilir ve histopatolojik olarak da iyi sonuçlara sahiptir.

Anahtar sözcükler: Hemostatik ajanlar; hemoztaz; karşılaştırma, parsiyel nefrektomi

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Introduction

In recent literature, many technical methods have been introduced for controlling bleeding during the partial nephrectomy. In our previous study, we demonstrated the efficacy of Ankaferd Bloodstopper (ABS) in a rat partial nephrectomy model (1). However, the most important factor for preventing renal parenchymal damage during partial nephrectomy is to decrease the warm ischemia time (WIT). In addition, new emphasis is being given to the importance of speedy hemostasis in partial nephrectomy. According to the urology guidelines of AUA and EAU, this surgery should be chosen, particularly for especially small renal tumors of less than 7 cm (2). In the conventional technique, either laparoscopically or open, hilar vascular control, repair of the collecting system, blood vessels, and capsular closure with Surgicel® bolsters are required (3).

Recently, the use of hemostatic agents has become more popular, especially in the field of urological surgery. Many materials have been proposed as a way to stop hemorrhage during kidney surgery (4). In particular, an increase in the demand for hemostatic agents has followed an increased demand for laparoscopic kidney surgery (5). The difficulty of hemostasis in laparoscopic partial nephrectomy (LPN) has led to technical concerns over this type of surgery (6). In 1909, Bergel first described the use of dry plasma to facilitate hemostasis (7). Fibrin glues, absorbable fibrin adhesive, synthetic hydrogel polymer, and liquid albumin-indocyanine green solder were subsequently developed to use for the same purpose as a sealing agent (8-11).

ABS is a unique folkloric medicinal plant extract that has historically been used in Turkish traditional medicine. ABS is composed of a mixture of 5 plants, each with some effect on endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics, and cell mediators (12). In this study, we aimed to compare the efficacy of ABS with other hemostatic and sealant agents (Glubran 2, FloSeal, and Celox) that have already been licensed for use in controlling kidney surgery bleeding.

Materials and methods

Study protocol

This study was approved by the local animal review and ethics committee and the research council at Ankara Training and Research Hospital. A total of 40 Wistar rats weighing 200 to 240 g were divided into 5 groups and underwent right lower pole PN. The animals were fed into separate cages in which the temperature was 22 °C. Targeted excised tissue was determined to be approximately 1 cm^2 in each rat. The PN techniques of the groups were standardized and groups were determined according to the type of hemostatic agent used during the operation. Group T (traditional) (GT) underwent right PN with hilar vascular control including intracorporeal suturing of the renal parenchyma and collecting duct. Group G (Glubran 2) (GG) underwent conventional PN with a Glubran 2 application. Group F (Floseal) (GF) received an application of Floseal to the renal parenchyma and collecting duct with hilar control. In Group C (Celox) (GC), the PN and Celox application were performed with hilar control. In Group A (Ankaferd) (GA), Ankaferd was applied to the resected tissue following the PN. In the first month following scarification, the right nephrectomy was performed. Gross specimens and histological sections were blindly evaluated by 2 pathologists (HU, MA). Sections were stained with hematoxylin and eosin.

Surgical procedure

After administering a prophylactic single dose broad-spectrum antibiotics (sulbactam-ampicillin intramuscular), general anesthesia with the combination of ketamine HCl (40 mg/kg, ParkeDavis) and xylazine HCl (10 mg/kg, Bayer, Germany), a midline incision was made in the abdomen after sterilization and draping. The right kidney was completely mobilized. The right renal artery and vein were occluded with a Rommel vascular clamp to achieve hilar control. The lower third of the right kidney was resected in guillotine fashion with a single stroke of an amputating knife. The rats were randomized for surgical technique according to the groups. After the procedure, sponges were used to collect all visible clots and blood, and the kidney was replaced in the renal fossa.

Hemostatic techniques and identification of hemostatic agents

In Group T, bimanual pressure was applied to the amputated renal margin by the surgical assistant. Segmental vessels and the collecting system were repaired with absorbable sutures (3/0 polyglycolic

acid). Neither sponges nor Surgicel® were used. As an alternative to the traditional method, 4 types of hemostatic agents (Glubran 2, Floseal, Celox, and ABS) were used in a similar fashion on the resected bleeding area in order to promote active hemostasis.

Glubran 2: Includes N-butyl-2-cyanoacrylate and methacryloxysulfolane (NBCA MS) as a sealant agent. Application of sealant to the excised renal surface has been designed for 1 cc Glubran 2 dribbled onto the whole bleeding area. The application should be done when the bleeding starts from the tissue. Afterward, the freezing of the agent was observed and the hemostatic effect of Glubran 2 was evaluated.

FloSeal: This gelatin matrix hemostatic sealant was applied to the resected area with its own application device. The sponge embedded with isotonic NaCl was used for manual compression to promote active hemostasis.

Celox: A new chitosan granular dressing (CELOX [CX], SAM Medical Products, Newport, OR, USA) that reports success in controlling hemorrhage. This agent is a fine granular product that works by interacting directly with red blood cells and platelets to form a cross-linked barrier clot, independent of native factors. Celox was applied by pouring the contents of one package into the wound.

Ankaferd® BloodStopper®: ABS is a hemostatic agent including unique folkloric medicinal plant extracts that has historically been used in Turkish traditional medicine. A dosage of 2 cc of the injectable form of ABS was applied to the amputated renal margin slowly until the bleeding stopped. Afterwards, a compress was applied to the resected surface for 2-3 min. Immediate application of the product to the bleeding area is the major factor for effective hemostasis with Ankaferd.

Objective parameters

WIT was measured from the initial occlusion of the hilar vessels until the final release of the right renal vessels. Hemostasis time (HT) was determined from the first observation of bleeding, through the application of the hemostatic agent until active hemostasis. Urine extravasation, adherence to adjacent organs and infection at the operated renal margin were evaluated. Pathological specimens were evaluated with emphasis on the presence or absence of giant cell reaction, intestinal metaplasia, acute inflammation, foreign material reaction, fibrosis, adhesions, necrosis, fistula, erythrocyte aggregation, microvascular proliferation, fibroblastic activation, siderophages, glomerular necrosis, and calcification. Our team used the pathological scoring system detailed in our previous study.

Statistical analysis

Results were analyzed with SPSS® 15.0 for Windows®. Definitive statistics were determined as the mean ± standard deviation (SD), minimum, maximum, and percent. Kruskal-Wallis and Mann-Whitney U-tests were used to evaluate significance among the groups. The post hoc Bonferroni test was also used to correct the significance level in subgroup comparisons. P value was determined significant at < 0.05.

Results

A total of 40 open right lower-pole partial nephrectomies were performed and major bleeding was induced in each case. The rats used for this experimental study had similar morphometric characteristics in body and kidney shape. The mean kidney size was 2 × 2.5 × 0.5 cm. The resected lower pole kidney tissues were also similar in size and shape, approximately 1 cm^2. All animals survived during the operation and postoperative period.

Operative findings

Warm ischemia time (WIT) (in seconds) was determined for the groups as follows: Group T, 150.4 (SD: 10.2); Group G, 43.3 (SD: 1.7); Group F, 52.1 (SD: 1.7); Group C, 66.6 (SD: 2.2); and Group A, 81.5 (SD: 6.5). Among the groups, there were significant differences (95% CI) ($P < 0.001$) (Figure 1A). In Group A, a significant decrease in WIT was detected while the difference compared with the other groups was also significant ($P < 0.001$). The evaluation of hemostasis time (HT) (in seconds) among the groups yielded results statistically similar to those of the WITs. Results for HT were as follows: Group T, 140.1 (SD: 10.2); Group G, 32.9 (SD: 1.2); Group F, 40.9 (SD: 1.1); Group C, 55.8 (SD: 1.8); and Group A, 70.1 (SD: 6.6), and were detected with significant differences (95% CI) ($P < 0.001$) (Figure 1B). In Group A,

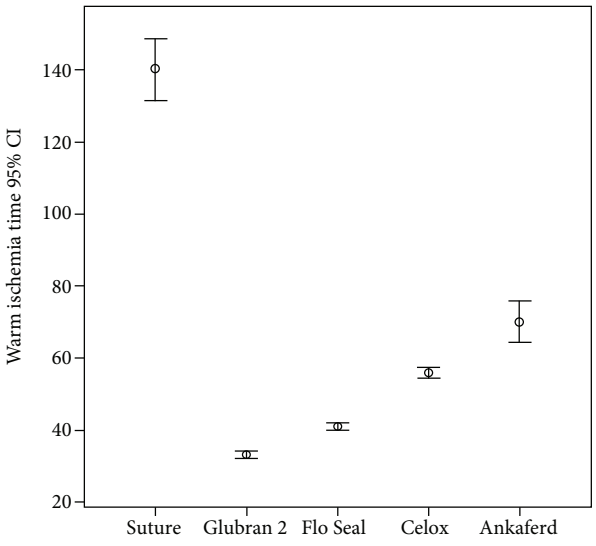

Figure 1. Warm ischemia times (WITs) of the groups.

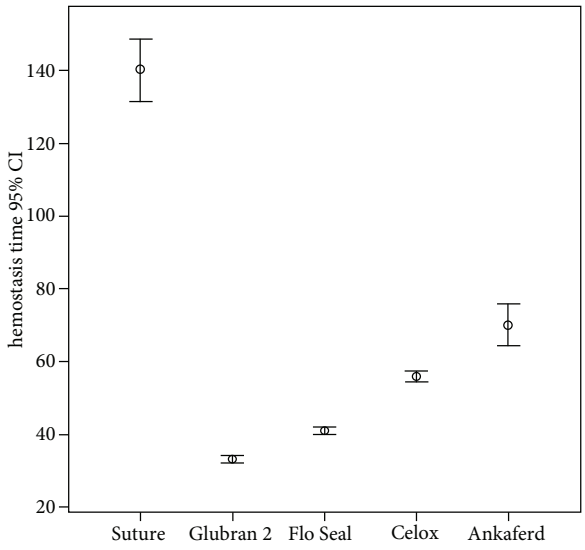

Figure 2. Hemostasis times (HTs) of the groups.

decreased HT was confirmed when compared with Group T while increased HT was detected in comparison with the other groups (G, C, and F) ($P < 0.001$). Hemostatic efficacy was observed macroscopically for each rat in the groups and the macroscopic appearance of the kidneys following application of each hemostatic agent is shown in Figure 2 (A-E). Determination of the surgical effect of hemostatic agents during the operation is shown in the Table. Blood loss could not be evaluated objectively due to the small kidney size and resected tissue. There were no significant intraoperative complications.

Scarification findings

For each group, macroscopic examination was performed. In Group T, increased adherence to the adjacent tissue was observed and the resected renal surfaces showed irregular shape and hardness. In Group G, the appearance of Glubran was observed on the resected area and minimal adhesion occurred. In Group F, good biocompatibility of FloSeal and restoration of resected tissue were observed with increased perirenal adhesion. In Group C, the resorption of Celox and absence of tissue reaction were confirmed. In Group A, ABS kidneys were all in good condition, especially in the resected area, although redness and gelatinous, wealthy tissue were observed in the transected kidney. No hematoma, urinoma, or urine leakage was seen in any of the groups.

Histopathologic findings of specimens

Fibrosis, adhesion, and calcification were not significantly demonstrated in Group A compared with the other groups ($P < 0.001$). Increased fibrosis

Table. The determination of surgical effect of hemostatic agents during the operation.

Hemostatic Agent	Hemostatic Effect (macro) onto the resected kidney area
Glubran 2	Easy application, no need for compression, very adhesive to adjacent tissue, one thin layer provides the haemostatic effect
FloSeal	Special device used, compression required
Celox	Safe for adjacent tissue, easy and generous application, granular fashion, compression required
Ankaferd BloodStopper	The formation of aggregate (protein network), ruddy surface and compression generally required

and adhesion were shown in Group F. The differences regarding glomerular necrosis, calcification, fibrosis, giant cell reaction, adhesion, and tubular thyroidization were confirmed significantly compared with Group T ($P < 0.001$). There was no difference in acute inflammation and fibroblast activation ($P = 0.2$). Erythrocyte aggregation and microvascular proliferation were observed to be significantly higher in Groups G, F, and A ($P < 0.001$) (Figure 3A, 3B, 3C).

Discussion

Hemostasis is one of the most important technical objectives in open partial nephrectomies for solid renal masses smaller than 4 cm. In fact, this has become the standard of care even in the face of a normal contralateral kidney (13). Recently, the use of

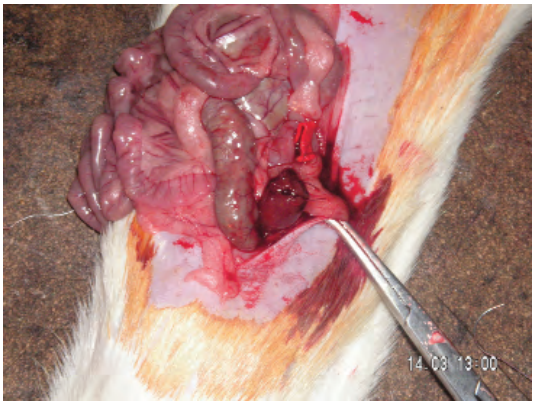

Figure 3A. Macroview of the kidneys following traditional partial nephrectomy with suture closure.

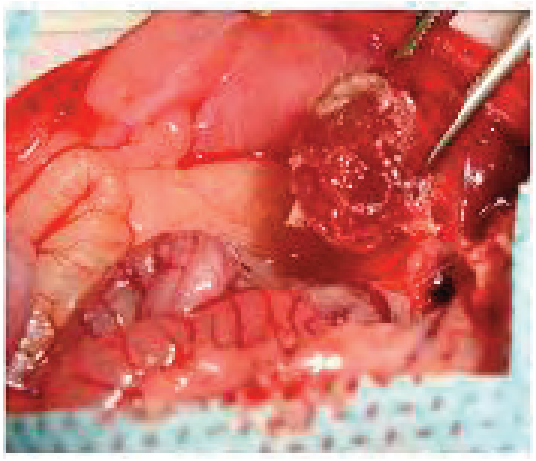

Figure 3B. Macroview of the kidneys following application of Glubran 2 onto the resected kidney surface.

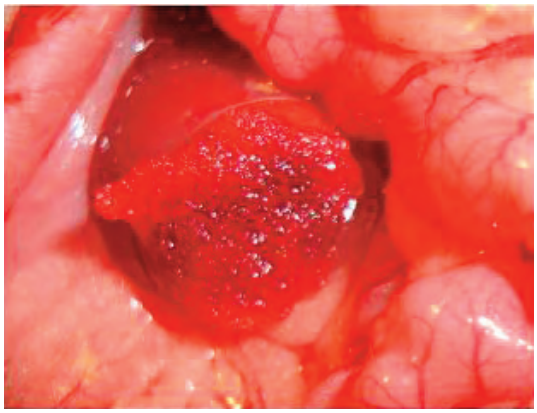

Figure 3C. Macroview of the kidneys following application of FloSeal onto the resected kidney surface.

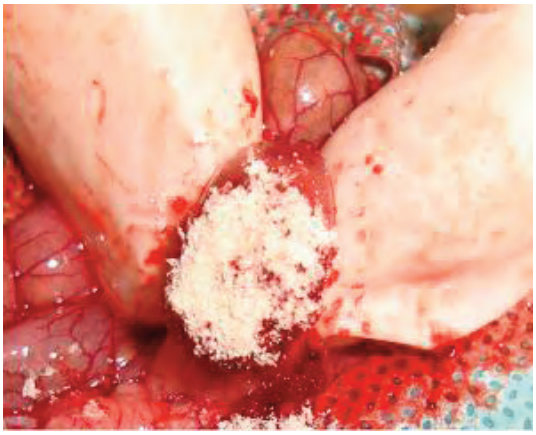

Figure 3D. Macroview of the kidneys following application of Celox onto the resected kidney surface.

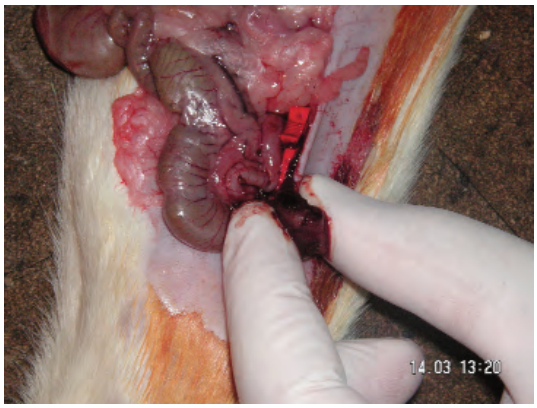

Figure 3E. Macroview of the kidneys following application of Ankaferd onto the resected kidney surface.

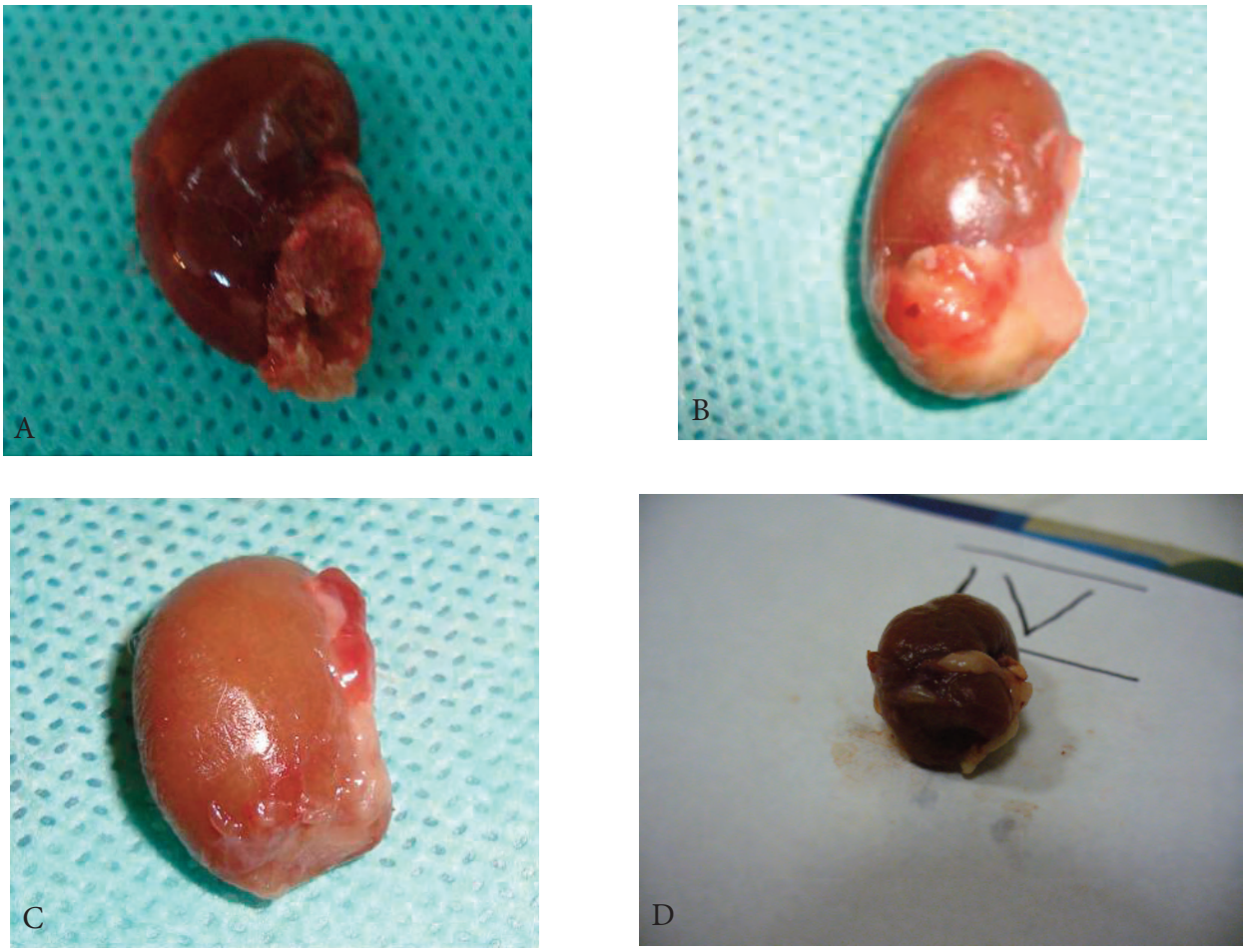

Figure 4. A. The appearance of Glubran on the resected area, including minimal adhesion.
B. Good biocompatibility of FloSeal and restoration of resected tissue were observed with increased peri-renal adhesion.
C. Resorption of Celox and absence of tissue reaction were confirmed.
D. ABS kidneys, were all in good condition especially in the resected area, some redness and gelatinous, wealthy tissue.

laparoscopic techniques in PN has increased the likelihood of hemostasis within a suitable WIT to help preserve renal function. Hemostasis by suturing often requires longer warm ischemia time (14). Most investigators limited reconstruction time to 30 min, while some felt that it could be extended to as long as 1 h (1). Bleeding and ischemic renal damage due to prolonged warm ischemia periods are the most important complications following the surgery (1). In order to decrease WIT and PNT, various tissue sealants, and hemostatic agents have been developed to replace tissue suturing.

Studies examining the hemostatic effect of ABS on renal surgery or renal tissue damage have been performed previously (15). We confirmed that ABS facilitated effective hemostasis with a decrease in partial nephrectomy time and warm ischemia time in various partial nephrectomy models (1). In addition, erythrocyte aggregation, which is a main action mechanism of ABS, and protein network formation were demonstrated previously in renal hemorrhage as in our recent study (1). The protein network induced by ABS is formed rapidly (<1 s); however, blood cells, particularly erythrocytes, also participate in protein network formation. It was shown that ABS-induced protein networks were capable of regulating further coagulation and hemostatic reactions. Hence, regular hemostatic processes were spared during formation

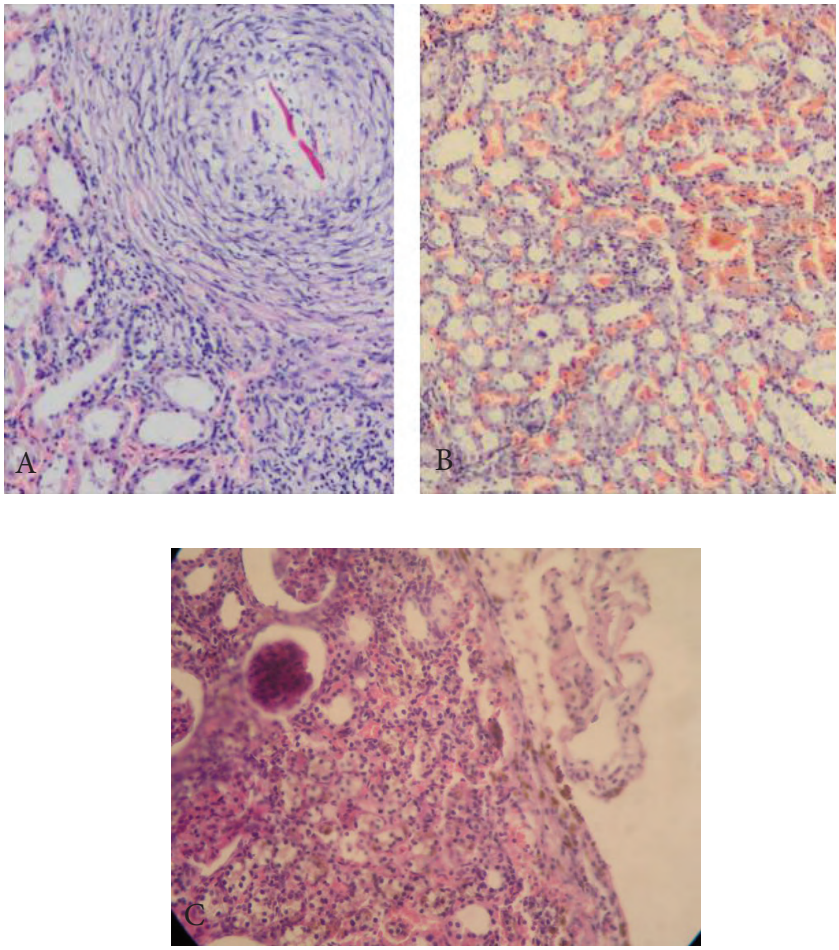

Figure 5. A, B. Erythrocyte aggregation and microvascular proliferation in Group F and Group G. C. Erythrocyte aggregation and microvascular proliferation in Group A.

of the protein network, with the blood clotting process being driven by protein agglutination (12). The main target of the experimental preliminary studies regarding the efficacy of ABS on parenchymal organ hemorrhage is to adopt the use of ABS in human organs. We demonstrated the efficacy and safety of ABS in a renal trauma model (15); however, decreased hemorrhage was demonstrated in radical prostatectomy with the application of an ABS tampon, 2.5 × 7 cm, as well (16). In one article, ABS was successfully used in a case with upper gastrointestinal bleeding, while, in another, therapeutic potential for the management of hemorrhage in open heart surgery was confirmed (1). Additionally, the Phase 1 study stressed the safety and applicability of ABS for use in humans.

For FDA (Food and Drug Administration) regulation, a hemostatic agent is defined as a device intended to produce hemostasis by accelerating the clotting process of blood. Broadly, these agents can be thought of as topical hemostatics, anti-fibrinolytics, fibrin sealants, and matrix hemostats (17). The best evidence for hemostatic use in urology comes from renal surgery (17). In one prospectively randomized study, 21 pigs with complex grade 4 renal injuries were treated with FloSeal with and without preliminary renal artery occlusion and conventional suture with gelatin foam bolster (17). FloSeal significantly decreased blood loss and time to hemostasis as we demonstrated the significant less WIT and HT in Group F. However, the histopathologic effect of FloSeal on the renal tissue is still being debated in the

literature. In particular, the application of FloSeal in laparoscopic partial nephrectomies with significant less blood loss and warm ischemia time was successfully shown by Bak et al. (14). Additionally, Gill et al. reported significantly fewer overall complications (16% vs. 37%) in laparoscopic partial nephrectomies using Floseal compared with a suture/Surgicell bolster (18). Glubran® 2 (N-butyl-2-cyanoacrylate) is a synthetic sealant that contains monomers that polymerize after contact with tissue, forming an adhesive layer with high resistance (19). A potential advantage of synthetic sealants is that they are not antigenic and carry no risk of viral infections (19). The use of cyanoacrylate in the field of urology has been achieved. As we demonstrated, the efficacy rates of Glubran 2 application in laparoscopic partial nephrectomies with small and wide resection were 67% and 80%, respectively (20). In our practice, Glubran 2 offered easy preparation and application with no need for compression and was observed to promote significant hemostasis. Another novel hemostatic agent, Celox®, was applied for the first time in our rat model according to Pubmed research. The chitosan dressing is a fairly rigid wafer that forms a mucoadhesive physical barrier at the site of injury or bleeding (21). According to manufacturers, it is reportedly nonallergenic, nonexothermic, able to function in a hypotermic environment, and low in cost (21). We demonstrated parallel histopathologic findings with complete resorption and without host reaction in the renal surface. In another study, it was demonstrated that Celox® improved hemorrhage control and survival and was determined to be a viable option for the treatment of severe hemorrhage (21).

In this study, ABS was compared to licensed hemostatic agents that provided effective hemostasis in partial nephrectomy in evidence based medicine. The best WIT and HT was demonstrated with Glubran® 2 while the histopathologic and macroscopic findings showed the adhesion and remnants of the agent after 1 month. Despite histopathologically significant evidence of fibrosis and adhesion, definite improvements in HT and WIT were shown with FloSeal; moreover, this agent has an internationally acknowledged hemostatic effect in both open and laparoscopic partial nephrectomies. Excellent renal tissue surface and biocompatibility were observed in Celox with significant effects on WIT and HT. As for the final product, ABS demonstrated acceptable hemostatic effect and significantly decreased WIT and HTs with significant histopathologic findings, without fibrosis, adhesion, or host reaction. The exact mechanism of erythrocyte aggregation for hemostatic action was significantly confirmed in ABS group. Generally, each hemostatic agent provided the predominant effect when compared with the traditional suture technique in partial nephrectomy. The unclear dose interval for the best hemostasis with ABS and small size of rat kidneys were the limitations of this study.

In this study, we aimed to compare the histopathologic effects of hemostatic agents. In our opinion, these agents should be considered not only for their hemostatic properties, but also with regard to the histopathologic effects on renal tissue.

Conclusion

This experimental study is the first trial that compared the ABS with other hemostatic agents licensed for internal use (FloSeal, Celox, and Glubran 2). It has been demonstrated that ABS stopped the bleeding and decreased WIT and HT, while more familiar findings were detected histopathologically. However, not only the decrease in WIT and HT, but also histopathologic biocompatibility should be stressed as the one of the major criteria for becoming an optimal hemostatic agent. We think that more advanced experimental and clinical trials will improve and clarify the optimal efficacy of ABS in renal surgery.

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