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Histological evaluation of Algan Hemostatic Agent's effect in a rat experimental spleen injury model

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Abstract

Uncontrolled hemorrhage may result from injuries to the parenchym and splenic capsule. Hemostatic material applications are among the methods used to prevent spleen parenchymal hemorrhage. Algan Hemostatic Agent (AHA) is a standardized mixture of six distinct herbs that are capable of hemostasis, either individually or in combination. Aim of this study was to investigate efficiency of AHA in bleeding control in experimental spleen injury model, and to evaluate its histopathological effects and IL-1β, TNF-α, and Bax expressions in tissue. Twenty-four Wistar albino female rats, aged 10-12 weeks, were randomly separated into three groups (n=8); control, AHA-liquid, and AHA-powder. After surgery physiological saline, liquid and powder were applied to the injured area for twenty seconds. On the tenth postoperative day all rats' spleens were removed for histopathological and immunohistochemical analysis. The AHA-liquid group demonstrated more efficacy in controlling hemorrhage than the AHA powder group after both the initial and subsequent applications. Parenchyma of the spleen was intact, and a thin capsule was detected in the liquid group. In the powder group, thick granulation tissue was observed along with acute lymphocyte and neutrophil infiltration. Expressions of IL-1 β and TNF-α were mild in control and AHA-liquid groups and intense in AHA-powder groups. Similar Bax protein expression was detected in all groups. Current study demonstrated that liquid form of AHA was more effective in reducing local bleeding and inflammation in spleen tissue. Therefore, liquid form could be preferred in animal experiments and clinics as a rapid, safe, and effective agent for organ injury.

Keywords: Algan Hemostatic Agent, splenectomy, histopathology, immunohistochemistry, bleeding, rat

1. Introduction

Uncontrolled bleeding resulting from trauma is the leading cause of preventable death, as reported by the World Health Organization (1,2). It is critical to stop bleeding in the early stages to prevent life-threatening complications (3,4). Splenic injuries are the second most common type of injury resulting from abdominal trauma and are the most prevalent preventable cause of mortality (5). Bleeding, which can be caused by penetrating trauma or spleen surgery, is one of the most prevalent causes of splenectomy today, which is essential for the treatment of spleen injuries. However, it is quite challenging to control bleeding in partial splenectomy (6). Studies have been conducted to prevent splenic bleeding after trauma and to partially preserve the spleen (7, 8). Nonsurgical interventions for solid organ damage have attracted more attention because they function more effectively than expected. Natural hemostatic agents, such as herb, wax, and grease, have been utilized for years for ceasing severe hemorrhaging (9, 10). Numerous hemostatic agents (bovine collagen, bovine thrombin, autologous plasma, fibrin adhesive, etc.) are currently available, and their importance is unavoidable (11, 12). There are two main types of topical hemostatic agents: physical agents that use passive substrate to promote hemostasis (10, 13, 14), and biologic agents that increase coagulation at the bleeding site (15). These agents have risk factors such as edema, hypersensitivity, coagulopathy, and allergies (16). Despite these products and significant medical advancements, there is still no ideal product for hemostasis, so effective hemostatic solutions are required. Algan Hemostatic Agent (AHA) is contained in gel, liquid, powder, and foam formulations and is derived from a standardized mixture of six separate herbs (15). AHA powder is an absorbable starch-based agent of class III and AHA liquid is a class III hemostatic agent (Certificate numbers:1783-MDD-213 and 1783-MDD-196, respectively). Each herb in AHA is capable of hemostasis, either individually or in combination. It provides hemostasis by forming thick polymeric networks that serve as a physical barrier in the area of application and prevent blood loss by entrapping blood and its components within these networks.

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All biocompatibility and hemodynamic tests, including sensitivity, cytotoxicity, and irritation, have validated the safety as well as efficacy of AHA as a hemostatic agent. According to the available literature, AHA is the first and only approved herbal product that contains no additives. Moreover, it is an easy-to-use, low-cost product that does not require special storage conditions (16-20).

The impact of various forms of AHA on organs including the liver, kidney, in addition to its effect on wound healing, was examined using a variety of surgical models (15-20). As there is limited literature on the effects of hemostatic agents on tissue and a clinical need for effective hemostatic agents, the purpose of this study was to examine the effects of AHA on tissue in an experimental surgical spleen injury model. The effects of AHA on splenic tissue were investigated on postoperative day 10 using histopathology and the immunohistochemical markers IL-1, TNF- α, and Bax.

2. Material and Methods

2.1. Animals and study design

The animals used in this research were obtained from the Marmara University Medical School Experimental Animal Breeding and Experimental Research Centre, Istanbul, Turkey. And the animal experiments performed for this study were in accordance with the ethical standards that were approved by the Local Animal Experiments Ethics Council of Marmara University (approval number and date: 09.2021mar-11.01.2021).

Twenty-four female 10-12-weeks-old Wistar albino rats weighing between 250-280 g were used in our study and they were randomly separated into three groups of eight animals each. Animal numbers were determined by considering previously published AHA surgical model studies (17). Groups were determined as control (physiological saline solution impregnated gauze), AHA-liquid and AHA-powder. Before surgery, physiological saline (2 ml) was infused into gauze pathches, AHA-liquid (2 ml) was placed in an injector, and AHA-powder (2 g) was ready for use to pour in the bleeding area.

2.2. Surgical procedure, bleeding test and intra abdominal adhesion study

Surgical procedures were performed under general anesthesia with intraperitoneal injections of 100 mg/kg ketamine hydrochloride (Ketalar, Eczacıbaşı, Istanbul, Turkey) and 10 mg/kg xylazine (Rompun, Bayer, Istanbul, Turkey) in all three groups. The depth of anesthesia was determined by monitoring skin/finger pinch responses, palpebral/corneal reflexes, and heart rate, respiratory rate, and other physiologic activities. The anterior abdominal wall furs of all rats were shaved. After disinfection with a 10% povidone-iodine solution, a 3 cm median incision was made (Fig. 1.a) to expose the spleen. A 1 cm spleen injury was performed in all rats (Fig. 1.b), covering the terminal branches of the arteries at the lower pole of the spleen (18). Immediately after the onset of hemorrhage, the sponge soaked with physiological saline was compressed lightly on the injured region for a duration of 20 seconds in the control (Fig. 1.c) group. AHA-liquid was applied to the bleeding area through an injector with light pressure by absorbing the sponge for 20 seconds in the AHA-liquid group (Fig. 1.e). AHA powder was manually applied to the bleeding surface for a duration of 20 seconds, ensuring that no pressure was applied (Fig. 1.g). The timer was started to record the time and the area was checked after 20 seconds. If bleeding did not stop after 20 seconds, the procedure was repeated with the same amount of material and the number and outcomes of the subsequent applications were recorded. Failure to attain hemostasis following the third application was considered as a failed outcome for each group. On day 10 after spleen injury, the anterior abdominal wall of rats was disinfected under anesthesia. The abdomen was opened, and the localization of intra-abdominal adhesion was semiquantitatively assessed according to the Bothin scale (21). Hematoma and fluid collection in the abdomen, if any, were recorded. Animals were euthanized under high anesthesia and the spleens of all rats were removed for histopathological and immunohistochemical studies.

2.3. Histopathology

Spleen tissues from all animals were fixed in a 10% neutral buffered formalin solution at room temperature. Specimens were then processed for dehydration, clearing, and soaking using a tissue processor and embedded in paraffin. 4-5 μm thick sections were cut from the blocks using a microtome (Thermo Shandon Finesse E). Hematoxylin and eosin (H&E) stains were applied for histopathological evaluation. The mounted specimens were then examined, scored, and photographed under a light microscope (Leica DM 2000, Germany) equipped with the LasV 4.10 program. A scoring system from 0 (normal) to 3 (severe) was used for a semi-quantitative assessment of cell necrosis, inflammation status, granulation tissue formation, and residue reaction (17).

2.4. Immunohistochemistry

Five μm thick sections from all groups were deparaffinized, rehydrated with ethanol series and rinsed in distilled water and washed in phosphate buffered saline (PBS). Citrate buffer (pH: 6.0) heating was used for antigen retrieval until reaching sub-boil temperature. Endogenous peroxidase was blocked in 3% hydrogen peroxide for 20 minutes. Ultra V Block (Cat. no: TA-125-UB, Thermo Scientific, USA) was dropped for non-specific binding inhibition and samples were incubated at room temperature for seven minutes. Then, the slides were incubated with primary antibodies of IL-1β (Cat. no: sc-52012, Santa Cruz, USA), TNF-α (Cat. no: 52B83, Santa Cruz, USA) and Bax (Cat. no: sc-7480, Santa Cruz, USA) at a dilution of 1:150 overnight at +4°C. After rinsing with PBS, sections were incubated for 20 minutes at room temperature with biotinylated goat anti-polyvalent (Cat. no: TP-125-BN, Thermo Scientific, USA) and streptavidin peroxidase (Cat. No.: TS -125- HR, Thermo Scientific, USA), respectively. DAB (Cat. no: TA-

125-HD, Thermo Scientific, USA) was used for the chromogenic reaction and the samples were observed under the microscope until the reaction developed. The samples were rinsed with distilled water to stop the reaction. All slides were counterstained with Mayer's hematoxylin and mounted with entellan. Sections were examined and micrographs were captured with a light microscope (Zeiss Axio Scope.A1, Jena, Germany) Photomicrographs were analyzed for immunointensity using the Image J program.

2.5. Quantification of immunointensity

Samples obtained from the spleen section of each animal in each group were analyzed for IL-1β, TNF-α and Bax immunointensity in photomicrographs taken under the 20X objective as described in the previous study.[22] For this purpose, the total area of the tissue was measured and the measurements of DAB positive areas were performed in the same section area. The collected pixel data were converted into percentage values, and the results were then statistically analyzed.

2.6. Statistical analysis

The data for the present study were analyzed using Statistical Package for the Social Sciences (SPSS) software, version 22.0 (Chicago, IL). Categorical variables were analyzed utilizing the Fisher exact test (if necessary) and the Pearson Chi-squared test. Kruskal Wallis variance analysis was employed to compare the scores of the three groups. When differences were identified, this group was determined using the Mann Whitney test with p values subjected to Finner adjustment. In accordance with the type of the data, descriptive statistics (median, [Q1:p25 Q3:p75]) or frequency distribution (n and%), were employed to summarize the demographic information. To analyze immunointensity results, the non-parametric Kruskal-Wallis test was used considering the number of groups and sample size. Tamhane's T2 test was used for the multiple comparisons. The results were expressed as mean ± standard deviation (SD), and $p<0.05$ was considered significant for all analyses.

3. Results

3.1. Bleeding test and intra abdominal adhesion results

The efficacy of the initial 20-second administration of AHA liquid (Fig. 1f) and AHA powder (Fig. 1h) (62.5% and 50%, respectively) were statistically significantly higher than the results of the control group (Fig. 1d) ($p<0.001$). Moreover, when the AHA groups were compared, bleeding control success rates of the first application results of AHA-liquid were significantly higher than those of AHA-powder (Table 1). The success rates of AHA liquid (100%) and AHA powder (75%) during the second 20-second application were significantly higher than the results of the control group's second application ($p=0.002$) (Table 1). Upon group-wide evaluation of success rates, the 100% success rate for hemorrhage control in AHA liquid and powder applications was significantly higher than that of the control group (0%) ($p=0.001$). Within the control group, three consecutive 20-second applications were unable to stop the bleeding of eight rats (Table 1).

Table 1. Bleeding test results for all study groups

	Application Results			
	Positive	**Negative**	**Total**	**p**
1st Application (20 seconds)				
AHA-liquid	5 (62.5)	3 (37.5)	8 (100)	0.026
AHA-powder	4 (50)	4 (50)	8 (100)	
Control	0 (0)	8 (100)	8 (100)	
Total	9 (37.5)	15 (62.5)	24 (100)	
2nd Application (20 seconds)				
AHA-liquid	3 (100)	0 (0)	3 (100)	0.002
AHA-powder	3 (75)	1 (25)	4 (100)	
Control	0 (0)	8 (100)	8 (100)	
Total	6 (40)	9 (60)	15 (100)	
3rd Application (20 seconds)				
AHA-liquid	0 (0)	0 (0)	0 (0)	na
AHA-powder	0 (0)	1 (100)	1 (100)	
Control	0 (0)	8 (100)	8 (100)	
Total	0 (0)	8 (100)	8 (100)	
Result				
AHA-liquid	8 (100)	0 (0)	8 (100)	0.001
AHA-powder	8 (100)	0 (0)	8 (100)	
Control	0 (0)	8 (100)	8 (100)	
Total	16 (66.7)	8 (33.3)	24 (100)	

n (%), na: non available

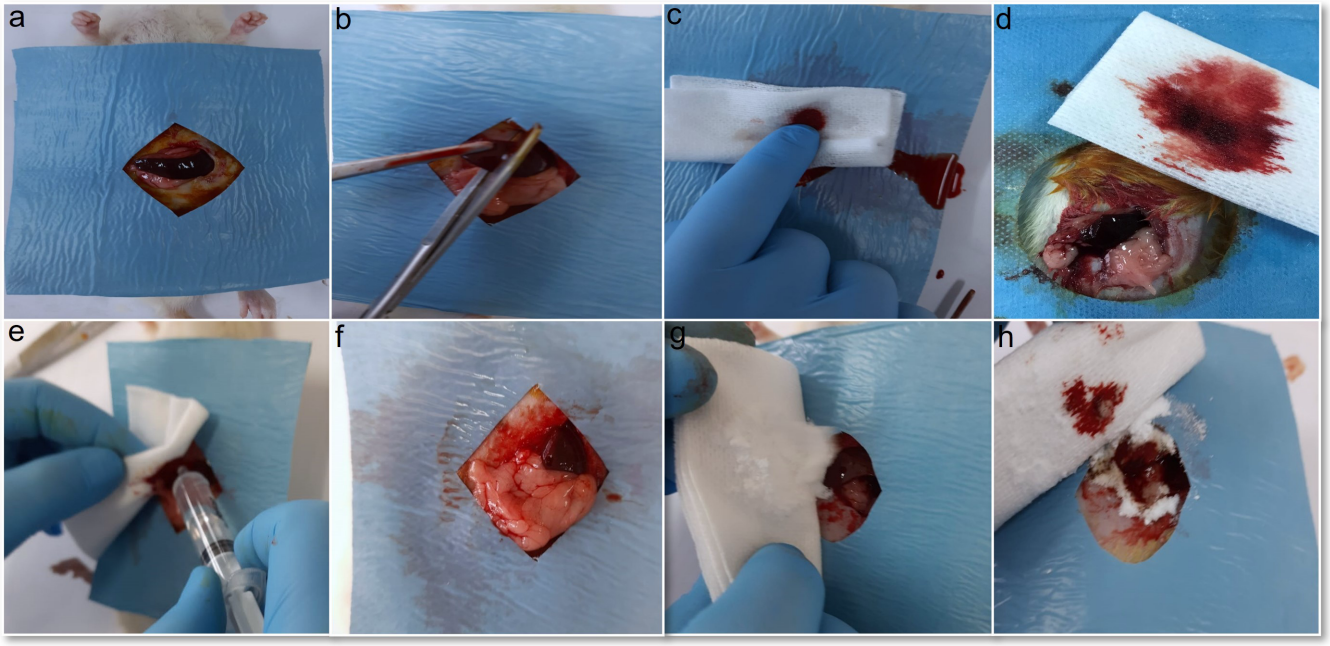

Fig. 1. Removing the spleen from the viscera (a) and removing one third of the spleen for partial splenectomy (b). Serum physiologic (SF) application to injured area (c) and bleeding (d) following removal of saline impregnated gauze in control group. Application of AHA liquid (e) and control of bleeding (f) after application in the AHA-liquid group. AHA powder is applied to the injured area using gauze (g). Control of bleeding (h) after application in the AHA-powder group.

In the control group, adhesion was observed between the wound site and the omentum (62.5%) and the spleen incision line (12.5%), however in two animals (25%) adhesion was not seen. The locations and degree of adhesion in the AHA-liquid group were similar to the control. In the powder group, there was no adhesion in one animal (12.5%), but there was adhesion between the spleen and liver (50%) and the spleen and intestine (37.5%). There was no noticeable difference between the experimental and control groups' results when they were compared.

3.2. Histopathological examination results

In the histopathological evaluation, a regenerated thin capsule on the injured superficial area was overlying the intact spleen parenchyma and mild inflammation was detected in the peripheral adipose tissue (Fig. 2.a-b, Table 2) of the control group. Mesothelial cell proliferation was seen on the surface of the capsule and a newly formed thin capsule consisting of connective tissue was observed beneath the mesothelial cells. Mild inflammation was detected by lymphocyte infiltration in the peripheral adipose tissue. Spleen parenchyma was intact and granulation tissue was not detected in the AHA-liquid group (Fig. 2.c-d, Table 2). Thick superficial granulation tissue was distinct and the underlying spleen parenchyma was intact (Fig. 2.e-f) in the AHA-powder group. In addition, acute severe inflammation with neutrophil and lymphocytes was observed in peripheral adipose tissue in the adherence area of AHA powder group (Table 2). Necrosis was not detected among study groups (Fig. 2.a-f, Table 2). Inflammation in peripheral adipose tissue status was significantly increased in AHA-powder group than liquid and control groups ($p<0.001$). The formation of granulation tissue was significantly higher in the AHA-powder group compared to the other groups ($p<0.001$). Regarding the residue (foreign body) reaction, there was no statistically significant difference between study groups ($p=0.368$) (Table 2).

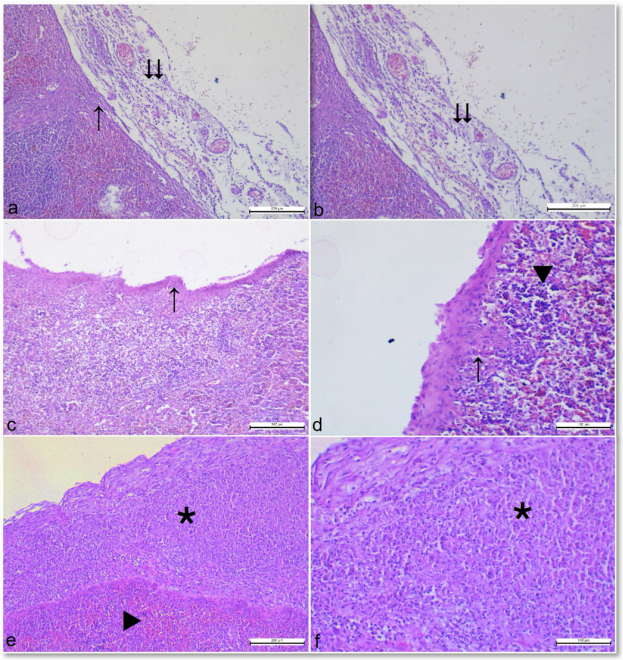

Fig. 2. Photomicrograph is showing the thin capsule (□) formation and mild inflammation (□) in peripheral tissue (a) and the higher magnification of thin capsule (b) on the surface of the spleen in the control group. Thin regenerated capsule (□) formation and underlying intact spleen parenchyma (▼) are seen with lower (c) and higher (d) magnification on the surface of spleen in the AHA-liquid group. Thick granulation tissue (□) and intact spleen parenchyma (▼) are observed with lower (e) and higher (f) magnification on the surface of spleen in the AHA-powder group. (Hematoxylin and eosin, Scale bars: a: 500 µm, b,c,e: 200 µm and d,f: 100 µm).

Table 2. Histopathological score results for all study groups

	AHA-liquid	AHA-powder	Control	p
Necrosis	0 [0 0]	0 [0 0]	0 [0 0]	na
Inflammation in peripheral adipose tissue	[b]1 [1 1]	[b]2 [2 3]	[a]1 [1 1]	<0.001
Formation of granulation tissue	[a]0 [0 1]	[a]2 [2 2]	[b]0 [0 0]	<0.001
Residue (foreign body) reaction	0 [0 0]	0 [0 0]	0 [0 0]	0.368

Median and quartile values M[Q1 Q3] are utilized to represent quantitative variable values. Distinct superscript letters within each row symbolize statistically significant differences (p<0.05) as determined by the Mann Whitney test, utilizing Finner adjustment for p-values. na: non available. AHA: Algan hemostatic agent.

3.3. Immunohistochemistry results

Immunohistochemical evaluation of all groups revealed varying degrees of IL-1 β, TNF-α, and Bax immunopositivity (Fig. 3). In all three groups, incised tissue regions, granulation tissue, and parenchymal areas showed immunopositivity. While inflammatory IL -1β and TNF-α immunopositivity was intense in the granulation region, apoptotic Bax protein expression was diffuse throughout the tissue. When all the tissues were examined, it was observed that the distribution of immunopositivity was similar in the three groups. According to statistical analysis, the distribution of IL-1 β immunointesity was 15.55 ± 2.32 % in the control group, 15.70 ± 1.68 % in the AHA-liquid group, and 16.96 ± 1.27 % in the AHA-powder group. These values were found to be similar in all groups (p>0.05). TNF-α analysis revealed 12.04 ± 1.64 % in the control group, 12.46 ± 2.19 % in the AHA-liquid group, and 12.29 ± 1.54 % in the AHA-powder group. TNF-α levels did not differ between groups in terms of distribution (p>0.05). Bax immunointensity distribution analysis revealed 13.04 ± 3.18 % in the control group, 13.25 ± 3.29 % in the AHA-liquid group, and 12.71 ± 4.40 % in the AHA-powder group (Table 3). Statistical analysis revealed similar apoptotic Bax protein expression in all groups (p>0.05). Graphical demonstration of statistical analysis was presented in Fig. 4.

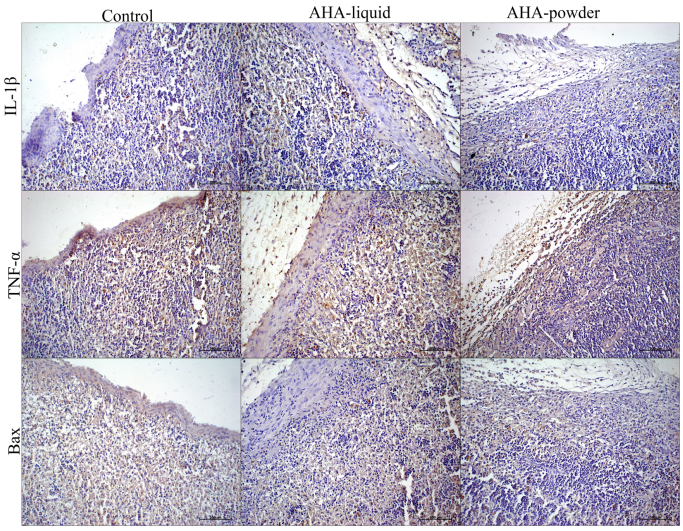

Fig. 3. Representative microscopic images of IL-1β, TNF-α, and Bax immunohistochemically stained samples in the control (SF), AHA-liquid, and AHA-powder groups. Staining: IL-1β, TNF-α ve Bax immunohistochemistry. Scale bars: 20 µm.

Table 3. Immunointensity analyses of IL-1β, TNF-α and Bax

	IL-1β Immunointensity (%)	TNF-α Immunointensity (%)	Bax Immunointensity (%)
Control	15.55 ± 2.32	12.04 ± 1.64	13.04 ± 3.18
AHA-liquid	15.70 ± 1.68	12.46 ± 2.19	13.25 ± 3.29
AHA-powder	16.96 ± 1.27	12.29 ± 1.54	12.71 ± 4.40

AHA: Algan hemostatic agent.

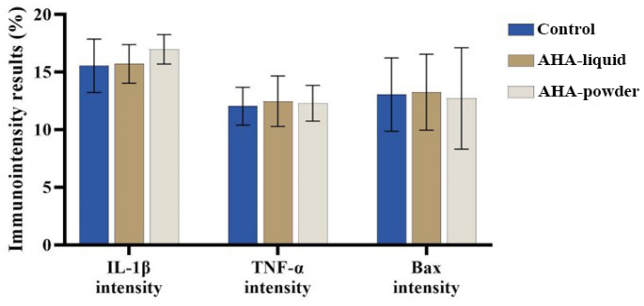

Fig. 4. Graphical demonstration of statistical analyses of IL-1β, TNF-α and Bax antibodies.

4. Discussion

Clinically, partial splenectomy is performed for benign splenic lesions, spleen rupture, and various splenic injuries. Injuries to the splenic capsule and parenchym during surgery or organ transplantation can cause uncontrolled bleeding (23). Many methods have been tried for preventing spleen parenchymal bleeding, and hemostatic material applications are among these methods (24, 25). Local hemostatic agents are utilized as part of this treatment, which is determined by the general condition and severity of the patient's injury (26). In this study, we evaluated the efficacy of the liquid and powder formulations of Algan Hemostatic Agent (AHA) in rat model of spleen injury, as well as its histopathological and immunohistochemical effects and intraabdominal adhesion outcomes on

postoperative tenth day. According to the results of our study, liquid form was more effective than powder form in the first twenty seconds of application and in the second application, bleeding was ceased in all animals in the liquid group whereas hemorrhage continued in one rat in the powder group, which did not achieve success even in the third application. In contrast to our study design, Midi et al. were investigated the effects of Algan hemostatic agent in a splenectomy model using liquid, gel, and powder forms of AHA. It was reported that the AHA groups had the shortest hemostasis, with the powder group having the shortest bleeding duration, followed by the gel and liquid forms (18). In another study, AHA was tested for hepatectomy hemorrhage, and the AHA powder was found to have the quickest bleeding time in both heparinized and non-heparinized groups. It was reported that the powder form of AHA could stop bleeding in 7 and 4 seconds, respectively (27). We examined the effects of an AHA using two forms, and the bleeding was controlled within 20 seconds, suggesting that the current model might have caused more hemorrhaging than the previous model. The minimum duration was found in liquid rather than powder, indicating that the physical effects of liquid were more potent than those of powder. Similar to our findings, previous studies on AHA in models of renal vein bleeding and liver injury revealed that the liquid form was more efficacious than the powder (17, 21). Based on these findings, the differences in stopping bleeding in various AHA forms were possibly due to the incision area, incision size, amount of bleeding, and structural and physical differences between AHA products. Yavuz et al. compared the efficacy of ankaferd and fibrin glue hemostatic agents in the experimental partial splenectomy model, ankaferd was determined to be effective in ceasing hemorrhage within 10 seconds, while the activities of ankaferd and fibrin glue were similar (28). Autologous fibrin glue (AFG) was evaluated for its efficacy in splenic trauma, and it was reported that the resected splenic surfaces of all animals reached complete hemostasis (8). Calcium alginate was reported to reduce intraoperative blood loss following spleen injury in a splenic laceration model. In addition, the calcium alginate group displayed significantly increased inflammation, vascularization, and fibrosis and also exhibited higher adhesion rate. It was suggested that experimental conditions could be influenced by various factors, including animal weight, practitioner expertise, mechanical disparities, vessel variability, the laboratory environment, and others that impact this disparity (29). In order to avoid complications, it is crucial to avoid peritoneal adhesion during surgical procedures (19). Therefore, the effect of hemostatic compounds on intra abdominal adhesion has also been studied (30-32). Yavuz et al. reported that the lowest adhesion was observed in the sham group, while adhesion was higher in the suture group than in the hemostatic agent groups. In the suture group, adhesions occurred between the omentum and spleen (62.5%), the omentum and other sides (62.5%), the spleen and liver, (75%) and the omentum and any other organs (62.5%) (28). In our study, adhesion was observed in the control group, and the AHA groups revealed similar findings. Although adhesion was observed between the spleen and liver (50%) and the spleen and intestine (37.5%) in the powder group compared to the liquid group, the adhesion scores of the experimental groups were similar. Therefore, AHA had no effect on postoperative intra abdominal adhesion formation,

Histopathological results of this study demonstrated that incision sites in the control and liquid groups began to heal with the formation of a thin capsule. In the control and liquid groups, the injury site and omentum adhesion might have caused mild inflammation. The presence of thick granulation tissue with acute severe inflammation in the AHA-powder group suggested powder remnants might trigger inflammation. In the parenchyma of all postoperative tenth day tissues from all study groups, no continued bleeding was detected. Similar to our results, a study reported that the higher inflammation in suture group and mild in fibrin glue and ankaferd blood stopper groups in splenectomy model (28). In another study, inflammation in the spleen was higher in calcium alginate group (29). Takagi et al. compared Tachosil to two-layered gelatin. Significant invasion of inflammatory lymphocytes was observed in the damaged spleen region of the Tachosil group, whereas no inflammatory alterations were observed in the gelatin groups (33). A study that focused on the role of ankaferd blood stopper (ABS) in cervical inflammation reported lowest inflammatory cell infiltration and mild IL-1 β expression in the ABS treated group (34). This study is the first to investigate IL-1 β, TNF-α and Bax expressions in AHA treated spleen injury. Pro-inflammatory IL-1 β and TNF-α expressions were detected in both the control and AHA-liquid groups, as determined by histopathological scoring. The expression was intense in the granulation tissue of the AHA powder group. Moreover, peripheral adipose tissue positivity might result from material residues or surgical procedures. Necrosis was not detected in the tissues, but apoptosis was detected similarly in all groups via Bax protein expression, indicating that the tissues were in normal apoptosis cycle after spleen injury.

In conclusion, the experimental spleen injury model in the present study demonstrated that AHA is more effective at minimizing local inflammation and preventing hemorrhage when administered in fluid form. Algan functions as a secure, tissue-preserving, and effective agent in bleeding and trauma. Therefore, it has the potential to be utilized in both animal experiments and clinical settings to staunch organ damage in a rapid, and efficient manner. Further study is required to perform comparative analyses of the impacts of Algan and alternative hemostatic agents on tissue using various models.

Conflict of interest

The authors declared no conflict of interest.

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None to declare.

Authors' contributions
Concept: D.Ş.A., U.Ş., K.G., Design: D.Ş.A., U.Ş., K.G., Data Collection or Processing: D.Ş.A., D.M., U.Ş., G.Y., K.G., Analysis or Interpretation: D.Ş.A., D.M., U.Ş., G.Y., K.G., Literature Search: D.Ş.A., D.M., U.Ş., G.Y., K.G., Writing: D.Ş.A., D.M., U.Ş., G.Y., K.G.

Ethical Statement
The animal experiment protocol was approved by the Local Animal Experiments Ethics Council of Marmara University (approval number and date: 09.2021mar-11.01.2021).

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