

# Determination of the Effects of Ankaferd Wound Dressing on the Wound Healing Process in Rats

**Short title: Ankaferd added dressing affect wound**

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## ABSTRACT

The effects of a composite nanofiber wound dressing material consisting of a Poly Vinyl Alcohol and Poly Vinyl Pyrrolidone polymer mixture with a hemostatic agent doped with Ankaferd Blood Stopper on the healing of experimentally induced dermal wound in rats were examined. Rats were divided into 4 groups (n: 6). Histological material was examined on the tissues taken from the wound site, whereas Total Antioxidant Status, Total Oxidant Status, and Oxidative Stress Index analyses were performed on the blood samples taken from the cardia. The material that was produced had hydrophilic properties, and both the ABS doped and undoped forms of the material positively affected wound healing. In the histopathological examinations, macroscopic evaluations revealed a statistically significant difference between the groups in terms of wound diameter, reepithelization, and inflammation formation ( $p: 0.019$ ). In parallel with wound healing and histological outcomes, TAS values increased in the ABS doped groups, and TOS and OSI values decreased in the wound dressing groups ( $p<0.05$ ). It was concluded that the ABS doped dressing did not have a negative effect on wound healing, it accelerated healing, and it could be used effectively and safely to treat skin injuries. However, further studies are needed to evaluate the clinical and histopathological benefits and potential adverse effects of wound dressings produced by using ABS doped polymers on wound healing.

**Keywords:** Ankaferd, Nanofiber, Wound, Rat, and Oxidative stress index.

## INTRODUCTION

A skin wound is an injury that compromises the integrity of the epidermis as a physical barrier, hence disrupting its normal anatomical composition and physiology (1). Today, millions of people are injured due to various causes, and many hemostatic agents are used to stop bleeding, with different levels of action and duration of stopping bleeding. The Ankaferd Blooding Stopper (ABS), which is used after surgery and is effective in many cases such as bleeding gums, is an antibacterial hemostatic agent without additives consisting of various ratios of dried roots and leaves of *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*, which are plants that are used in traditional Turkish medicine (2-4).

In the intermediate recovery step, hemostasis is a natural process consisting of the inflammation, proliferation, and maturation stages (5, 6). In this process, the general characteristics of the injured tissue should be known well, and tissue-specific treatment methods should be applied. The aim of these methods is to create an ideal environment for epithelial formation in the injured area by stimulating the inflammatory cells, platelets, and extracellular matrix involved in wound healing (5). Wound dressings are among the treatment methods that can create the ideal environment for proliferation by mimicking the extracellular matrix, protect the wound against microorganisms and infections, and contribute to the healing process.

Today, with the discovery of new-generation biopolymers and the development of novel production techniques, modern wound dressings are produced as an alternative to conventional wound dressings (7). Poly Vinyl Alcohol (PVA) and Poly Vinyl Pyrrolidone (PVP), which are non-toxic, bio-based, renewable, and sustainable polymers, are used in biomedical, pharmaceutical, and regenerative medicine (8). They can be converted into biocompatible and biodegradable, water-retaining, water-soluble, and nanofiber forms (9). As natural synthetic

polymers, PVA and PVP are easy to process because they have controllable physical properties and mechanical strength, and so, they are widely used for wound dressings (10-12). Electrospinning, which is a nanofiber production technique is a preferred method aimed at producing nano-diameter fibers by the electric field effect of polymer-based gels, and it is preferred because fibers that are one hundred times smaller (with an average radius of 10 nm-500 nm) can be produced compared to those produced by the classical method (13-15).

It is stated that the products that used in treatments with experimental practices reduce oxidative stress (TOS/TAS=OSI) by increasing the total antioxidant status (TAS) (16, 17). OSI analysis is used to determine oxidative stress levels as an easy, precise, automated, and inexpensive method.

Although various polymers and methods are used in nanofiber production, there is no study in the literature in which ABS doped PVA and PVP polymers were produced by the electrospinning method. The aim of study is to investigate the healing process and biochemical effects of a medical textile product containing an ABS-added polymer which can stop bleeding and heal wounds fast in a rat model with experimentally induced wounds. Scanning Electron Microscope (SEM) and Fourier Transform Infrared Spectrophotometry (FTIR) analyses were carried out to characterize ABS doped and non doped ABS polymers in the production of the material.

## **MATERIALS AND METHODS**

The study was started by obtaining the approval of the ethics committee of Selcuk University, Experimental Medicine Research and Application Center (decision date: 25.01.2019 and decision number: 2019-2). All chemical materials that were used in the study were supplied by Sigma and Merck.

### ***Preparation of nanofibers***

In this study, nanofibers were prepared to form a homogeneous wound dressing surface and provide a longer-lasting effect by the slow release of the drug additive. Additionally, a control group was formed to examine the effects of the nanofiber surface with and without the drug additive and show the effects of the drug. The electrospinning process was carried out for nanofiber wound dressing production (12, 18, 19). Sterile gauze was used for fiber collection, and two different solutions were used as polymer solutions (9). First, 10% by mass PVA (Mw: 72,000) in pure water was obtained by stirring at a temperature of 70°C for 1 hour. Then, 10% PVP (Mw: 58,000) in ethanol was prepared with a magnetic stirrer at room temperature for 30 minutes. These two polymer solutions were mixed at room temperature at a ratio of 3/4 PVA and 1/4 PVP respectively to obtain a carrier polymer solution. 10 ml of the carrier polymer solution was made nanofiber without any additive. Taking 10 ml of the same polymeric solution, 1 ml of Ankaferd was added and nanofiber was produced. The undoped fiber was collected on sterile gauze with a rotary collector at a speed of 400 rpm under the influence of an electric field of 1.7 kV/cm at a flow rate of 1 ml/h. The ABS doped fiber was collected with a rotary collector at a speed of 400 rpm under an electric field of 1.9 kV/cm at a flow rate of 0.6 ml/h.

### ***Experiments on material characterization***

The experiments in this context were carried out at Necmettin Erbakan University, Science and Technology Research and Application Center (BITAM).

### ***Scanning Electron Microscopy (SEM) micrographs of polymers***

A scanning electron microscope (SEM) was used to examine the morphology of the produced materials. The surface morphologies of the polymers were examined at magnification rates of 1,000 and 5,000.

### ***Fourier Transform Infrared (FTIR) spectrophotometry analysis***

FTIR analyses were performed to determine the interaction of the drug mixture and the polymer structure (20).

### ***Animal materials and treatments***

Twelve-week-old female Wistar albino rats with an average weight of approximately 295 g were used in the study. Female rats were preferred because the skin of females is thinner than that of males, and the lack of estrogen in males would affect cutaneous wound healing negatively (21, 22). The rats were housed without any water and feed restriction at room temperature in a 24-h light-dark cycle (23). The study was planned to include a total of 24 rats, with four groups (n:6 each) (Table 1).

After the groups were formed, the rats were administered 70 mg/kg Ketamine and 10 mg/kg Xylazine through the intraperitoneal (i.p.) route (24, 25). In each rat whose back area was shaved, a wound with a diameter of 15 mm was created using a biopsy needle. Group I was not treated. Group II received a single dose of local ABS in spray form. ABS doped wound dressing was used for group III, non doped ABS wound dressing was used for group IV (Figure 1), and the study completed on day 14 (26).

### ***Histological evaluation***

The skin tissue samples were embedded in paraffin after routine tissue processing steps, and the samples were fixated in 10% formaldehyde for histological analyses (27). Sections of paraffin blocks at a thickness of 6 µm each were stained with Hematoxylin-Eosin to determine their general histological structures (28). The sections were examined at 40x magnification under a light microscope equipped with a digital camera (Nikon Eclipse, E-400 equipped with Nikon DS Camera Control Unit DS-L1 with DS Camera Head DS-5M), and digital images of relevant areas were taken (29-32).

### ***Scoring evaluation scale***

Wound scoring was made as 0 (no inflammation), 1 (mild inflammation), 2 (moderate inflammation), and 3 (severe inflammation). Wound diameter was recorded in millimeters (mm) (25, 26). The scoring process was performed according to the evaluation scale shown in Table 2.

#### **Biochemical analysis**

On the last day of the study, 1.5 ml of blood was taken from each rat intracardially. After the blood samples were centrifuged for 3000 cycles at +4°C, for 10 min, the serum parts were separated. TAS and TOS measurements of the sera were performed using commercial kits (Rel Assay Diagnostics). In a spectrophotometer (Biochrom Libra S22), the absorbances of the samples were measured at 660 nm, and OSI was determined according to the standard reference (33, 34).

#### **Statistical analysis**

The Statistical Package for the Social Sciences (SPSS, version 21.0, IBM Corporation, Armonk, NY) software was used for all statistical analyses. One-way analysis of variance (ANOVA) and the least significant difference (LSD) method were used in the pair wise comparisons ( $p < 0.05$ ). Statistical analysis was performed based on the 14th day in the scoring of the wound diameters.

### **RESULTS**

#### **Materials and characterization**

##### **Scanning Electron Microscopy (SEM) micrographs of polymers**

Using the SEM images, the average fiber diameters were calculated with the help of the "Fiji ImageJ for Windows V 1.8.0" program from NIH images, and the plots shown in Figure 3 were obtained. The fiber diameters ranged from 400 nm to 2  $\mu$ m. The average diameter of the unadulterated fibers was 1.066  $\mu$ m, and the average diameter of the doped fibers was 0.974  $\mu$ m. The determined average diameter values were sufficient to provide permeability (35-37). While a mesh structure was detected at the points where the fibers overlapped on the surfaces of the doped fibers, no droplet or clump-like formation was observed on the surfaces (Figure 2).

##### **Fourier Transform Infrared Spectrophotometry (FTIR) analysis**

In the FTIR spectra of the PVA-PVP polymer mixture, an O-H stretching vibration peak was seen at approximately 3290  $\text{cm}^{-1}$ . The spectrum band corresponded to a C-H stretch that occurred at a peak of asymmetrical stretching vibration of approximately 2862  $\text{cm}^{-1}$  (35, 37). PVA and PVP had C=O groups showing a vibration band at about 1716  $\text{cm}^{-1}$ . The C-N stretching vibration peak of the amine structure was seen at 1242  $\text{cm}^{-1}$ . The spectrum bands for C-O groups, which are acetyl groups, appeared at 1100  $\text{cm}^{-1}$ . The plane for the C-H bend was outside the rings and formed the absorption band of approximately 720  $\text{cm}^{-1}$  (36). In the case of the ABS doped fibers, a soft formation of around 1645  $\text{cm}^{-1}$  was observed instead of the peak loss at 1716  $\text{cm}^{-1}$ . Here, the oxygen band in the C=O group was transformed into a C=C stretching vibration by a free reaction. It was concluded that an oxidation reaction occurred by the binding of the oxygen atoms in the ABS additive (Figure 4).

#### **Body weights**

It was determined that there was no statistically significant difference between the mean body weights of the rats in the groups ( $p$ : 0.643). The statistics of the test were found as  $F_{23}$ : 0.497 ( $p > 0.05$ ) (Table 3).

#### **Wound scores**

There was no loss of animal in the groups. In the evaluations of the median scores on the last day of the study, the average wound diameter was found to be reduced by half compared to the first day of the study in the rats in group I, while the recovery in group II was more limited. In group III, where the best healing process was observed, it was seen that almost all wounds were closed, and there was even hair growth in the injured area. It was determined that the recovery rate in group IV was faster than groups I. and II. (Table 4). In the Tukey and Duncan tests, the test statistics were found to be  $p$ : 0.019,  $df$ : 23, and  $F$ : 4.144. When the wound diameters were compared, it was seen that the 1st and 4th groups were nearly similar. It was observed that the wound diameter in the second group did not decrease. It was determined that the wound diameter in the 3rd group decreased statistically significantly (Wound diameter: Group II>I>V>III;  $p < 0.05$ ).

There was no significant difference among the groups in the macroscopic wound measurements performed on the third day (Figure 5,  $p > 0.05$ ). In the evaluation made on the seventh day, brown colored scab formation was observed in all groups. It was determined that the wound healing rate was the highest in group III, and the healing rate in group IV was higher than those in groups I and II ( $p < 0.05$ ). In the histopathological evaluations, reepithelization was found to occur at a high rate in groups III and IV, at a moderate rate in group I, and at the minimum rate in group II ( $p < 0.05$ ). On the fourteenth day, the wounds were completely closed in all rats in group III, while the wounds of the rats in group IV were substantially closed. It was seen that the wounds were partially closed in group I, and healing was more limited in group II (Figure 5). In summary, reepithelization occurred at the maximum level in group III, at a high level in group IV, at a moderate level in group I, and at the minimum level in group II (Reepithelization rate: Group III>IV>I>II;  $p < 0.05$ ).

In groups III and IV, it was observed that the borders of the epidermis, dermis, and hypodermis showed normal morphological features, and the densities of connective tissue and collagen in the dermis layer were sufficient, whereas the density of collagen in the other groups was insufficient. Wound healing was seen to be characterized

by a decrease in the number of neutrophils and new vascularization (Figure 6). It can be stated that the ABS dopped wound dressing accelerated healing, while the non dopped ABS wound dressing had a limited effect on wound healing (Figure 2). Additionally, it was determined that the wound healing rate in the group that was administered local ABS to the injured area was slower than that in the control group.

#### **Biochemical analysis**

The level of oxidation (TOS) was the highest in the control group. ABS was found to reduce oxidation (Table 5). It was determined that the application of wound dressing with the ABS additive caused a significant decrease in TOS values (Table 5). In parallel with these results, the level of antioxidants (TAS) increased in groups IV and II, but the highest increase was in group III ( $p < 0.05$ ). (The OSI ranking: Group III > IV > II > I).

#### **DISCUSSION**

Injuries caused by many factors such as traumas, surgical operations, and burns can lead to serious health problems, ranging from disability to death. Wound dressing is a practical process that allows the wound to heal in a short time under hygienic conditions (38). If there is bleeding in the injured area, wound dressings containing hemostatic additive agents may be preferred (39). In this study, the effects of wound dressings containing nanofibers with the doping of ABS, which is a hemostatic agent, on wound healing were evaluated in macroscopic, histopathological, and biochemical terms.

Hydrophilicity is a sought-after feature for wound dressing materials (35, 36, 40-42). PVA and PVP are biocompatible polymers that have biodegradability and non-toxic properties (9). It was stated that the addition of silver nano particles into PVA and PVP had a notable effect on wound healing in New Zealand white rabbits (14). It was determined that the wound dressing in this study was sufficient to provide the permeability of fiber diameters. Moreover, no droplet or lump-like formation was observed on the surface. Due to these properties, it is thought that the substance produced in this study would be suitable for use and may be a preferred method in case of injuries.

In the recovery process, wound diameter was examined for reepithelization and inflammation formation. In the measurements made on the last day of the study, it was seen that the mean wound diameter in the ABS dopped wound dressing group was much smaller than that in the control group, whereas the mean wound diameter in the local ABS-treated group was higher than that in the control group. This situation was interpreted as that the ABS dopped wound dressing was effective in healing, and the local ABS application slowed down healing.

There are many studies in the relevant literature investigating the effects of ABS on wound healing. It was reported that ABS is an agent that accelerates recovery, and it is effective in accelerating the wound healing process in periodontal treatment (43). Similarly, it was stated that ABS is effective in trauma-related soft tissue defects that cannot be repaired by primary closure (44). ABS was observed to accelerate the healing process in bone defects created in the rat tibia (45). It was shown that ABS applied to wounds on the back skin of rats accelerated healing (46). According to a similar study, ABS speeds up wound healing in rats in the early period (47). ABS was also reported to be effective in healing full-thickness skin wounds in rats (39). It was seen to provide healing even in second-degree burns (4). In a similar study, ABS was shown to have a positive effect on wound healing in rats (27). It was also stated that ABS has a positive effect on wound healing in diabetic rats (48). In contrast with the aforementioned results, ABS was not found to be effective in preventing postoperative intra-abdominal adhesions, and its use could be harmful because it caused abdominal organ damage (49). In the same study, it was stated that ABS did not prevent adhesions, but it even increased them on a macroscopic level. Our findings supported previous results, and the ABS dopped dressings in this study were effective in reducing the wound size from 15x15 mm to 1x1 mm, thus contributing to healing. Although our results were similar to the results of other studies in the literature in which the positive effects of ABS on wound healing have been stated (26, 27), they differed from some others showing that ABS is not effective alone, and it even slows healing. It was thought that the amount of ABS used in each study, the diameter of the wound that is created, and the age and sex of the animals may be different. The cycle occurring in females may have affected the results of our study by affecting the wound healing process as well as every other process in the body.

Reepithelization is a stage of healing that occurs during the proliferation phase, and it is regulated by growth factors. Measuring the level of reepithelization is a frequently used method for the histopathological evaluation of the healing process (44, 45). It was stated that the level of epithelialization in the tibia tissue of the rat group in which ABS was used in the treatment was higher than in the control group (44). ABS is effective in full-thickness wound healing by accelerating proliferation in the epithelial tissues of rats (39). In a similar study, it was reported that ABS, which was used for treating rat palatal mucosal injuries, increased the level of reepithelization (50). Another similar study revealed that ABS contributed to the formation of epithelialization in a full-thickness skin wound model in rats (26). According to our results, the rate of reepithelization was the highest in the ABS dopped dressing group (III), and it was limited in the local ABS group (I) (Figure 5, Table 4). It was considered that brown-colored scab formation, which was observed more prominently in the ABS dopped dressing group than in the other groups, occurred because of reepithelization and was caused by the formation of an encapsulated protein network on the wound area. Consistent with our results, scab formation was observed in the wound area in similar studies using ABS (51-53).

Inflammation is a reaction developed by the body in cases of tissue damage, exposure to certain chemicals, and infectious diseases. This situation, in which the substances taking part in the immune system are directed to the damaged tissue, is an important parameter used in evaluating wound healing (26). While inflammation is not observed in the normal healing process, its severity may increase in necrotic conditions (53). In wound treatment, interventions that prevent inflammation are preferred. The use of ABS prevented inflammation and necrosis in experimentally created bone defects in the tibia of rats (45). It was reported that ABS did not cause inflammation in experimentally induced full-thickness skin wound healing in rats (39). According to another study, ABS prevented inflammation by inhibiting collagen destruction during the recovery period (53, 54). It was demonstrated that ABS reduced inflammation in a wound model created in rats (27). Likewise, it was reported that the use of ABS in rats with colonic anastomosis induced collagen formation and increased anastomosis (55). In a study that was conducted to prevent postoperative intra-abdominal adhesions using ABS, an increase in inflammatory reactions to fibrosis was observed in groups using ABS (49). In the evaluation made with the available data, it was determined that inflammation did not occur in any animal in our study, and this situation was compatible with most other studies in the literature.

OSI is a measure of oxidative stress status. Oxidative stress occurs due to the overproduction of reactive oxygen species or when antioxidant protection is insufficient (56). It has been reported that increased OSI values in many conditions such as diabetes, wounds, burns, and aging can be reduced by using the antioxidant properties of various herbal extracts (17, 57-67). Our results showed that ABS, which is an herbal product, is effective against oxidative stress that occurs during the wound healing process, in line with the literature. The use of only dressing and ABS alone corresponded to a reduction in the increased stress by 1.5 times ( $p < 0.05$ ). It was determined that the ABS dopped dressing increased the TAS levels by approximately twice, while reducing the OSI values by 1/3 compared to the control (Table 5).

## CONCLUSION

The results of this study showed that ABS alone did not contribute to wound healing, and the ABS dopped wound dressing provided rapid and uneventful healing in the injured area. It was determined that local ABS application did not shorten the wound healing duration, and the ABS dopped nanofiber dressing provided healing in the injured area in a much shorter time compared to the control group. Since it does not cause inflammation, it can be stated that ABS is a hemostatic agent with antiseptic and antimicrobial properties. Our results were interpreted as that ABS dopped dressings can be used safely, do not have any negative effects on wound healing, and can be an effective and safe product for use by shortening the healing process in skin wounds.

## Thanks

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## Conflicts of interest

The authors declare that there are no conflicts of interest in the study.

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## Tables

**Table 1. Groups**

Groups (n=6)
Group I. Control
Group II. ABS local application
Group III. ABS dopped wound dressing
Group IV. ABS non dopped wound dressing

**Table 2. Scoring evaluation scale.**

Ulcer (unit)	Reepithelization (unit)	Inflammation (unit)
0: None	0: None	0: None
1: At 1/3 of the wound site	1: At 1/3 of the wound site	1: Lightweight
2: At 2/3 of the wound site	2: At 2/3 of the wound site	2: Medium
3: The entire wound site	3: The entire wound site	3: Severe

**Table 3. Average body weights of rats**

Groups (n=6)	Average body weight (gr) ± Standard deviation (SD)		
	First day	Day three	Last day
Group I.	283 ± 4.60	288 ± 4.58	270 ± 4.30
Group II.	297 ± 3.34	295 ± 1.12	295 ± 4.39
Group III.	286 ± 2.24	287 ± 5.31	267 ± 1.66
Group IV.	292 ± 2.38	292 ± 3.39	287 ± 6.42

**Table 4. Values obtained from wound scores on different days of the study**

Group (n=6)	Day	Ulcer (mm)*	Reepithelization (mm)*	Inflammation (unit)*	Wound diameter (mm)
Group I.	Day 0	3	0	0	15x15
	Day 3	3	0	0	14x14
	Day 7	2	2	0	10x10
	Day 14	2	2	0	7x7
Group II.	Day 0	3	0	0	15x15
	Day 3	3	0	0	12x12
	Day 7	2	1	0	11x11
	Day 14	2	1	0	10x10
Group III.	Day 0	3	0	0	15x15

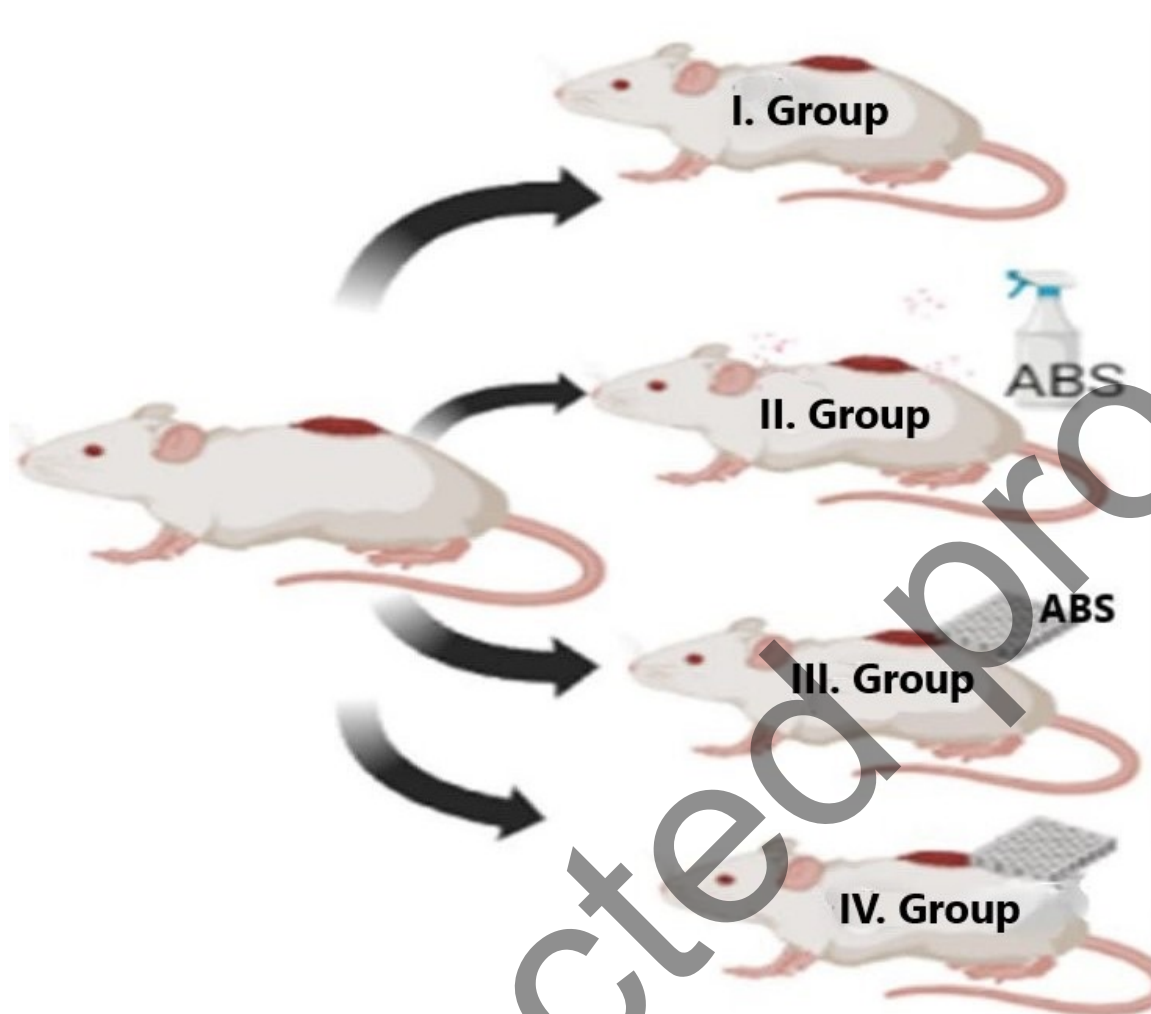
	Day 3	2	1	0	10x10
	Day 7	1	2	0	6x6
	Day 14	0	3	0	1x1
Group IV.	Day 0	3	0	0	15x15
	Day 3	2	1	0	10x10
	Day 7	2	2	0	7x7
	Day 14	1	2	0	6x6

\*Scoring was made according to the "Scoring Evaluation Scale" specified in Table 2.

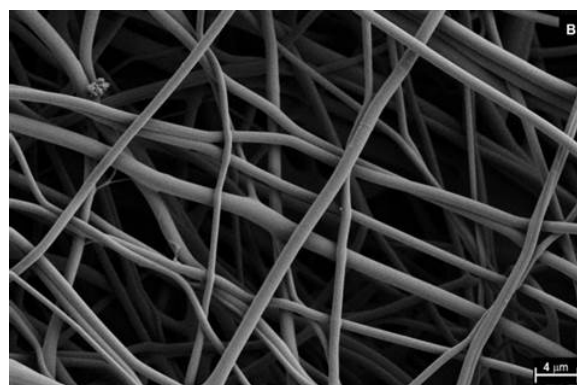
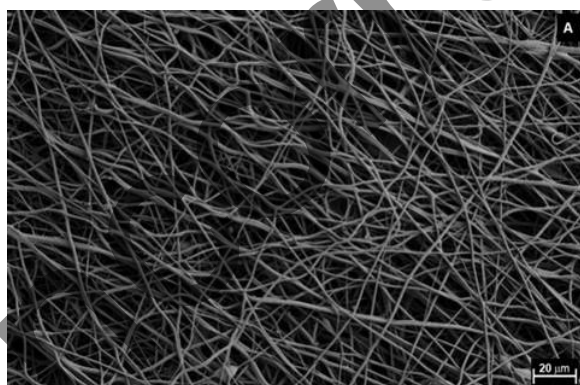
**Table 5.** Comparison of serum TAS, TOS and OSI values between groups

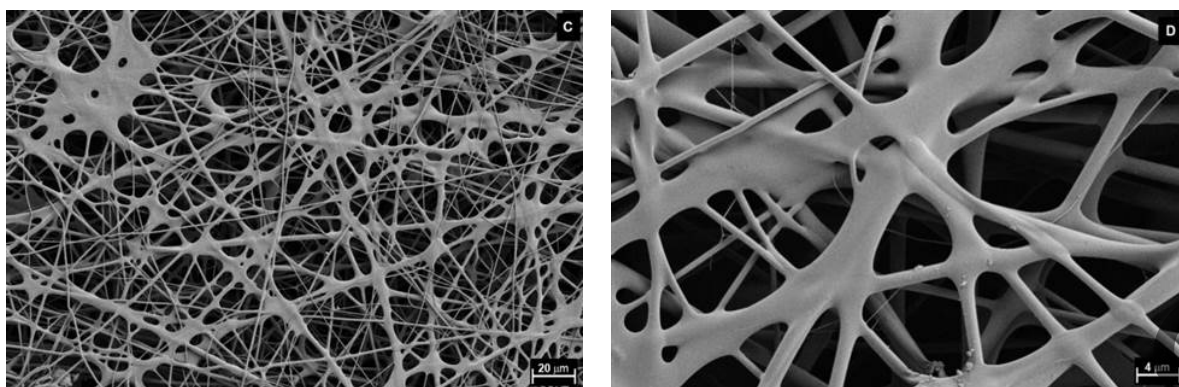
Groups/ Parameters	TAS (mmol Trolox Equiv/L) *Avg. $\pm$ S.D.	TOS ( $\mu$ mol H <sub>2</sub> O <sub>2</sub> Equiv/L) *Avg. $\pm$ S.D.	OSI (Arbitrary Unit) *Avg. $\pm$ S.D.	<i>p</i>
Group I	1.01 $\pm$ 0.05a	13.21 $\pm$ 1.80a	1.31 $\pm$ 0.07a	0.000
Group II	1.50 $\pm$ 0.15b	13.00 $\pm$ 2.00b	0.87 $\pm$ 0.14b	
Group III	2.75 $\pm$ 0.01c	11.10 $\pm$ 2.18c	0.40 $\pm$ 0.13c	
Group IV	1.65 $\pm$ 0.20b	12.50 $\pm$ 1.25b	0.76 $\pm$ 0.13b	

\*There are significant differences between different letters in the same column ( $p < 0.05$ ). Avg.:Average, S.D: Given as standard deviation.

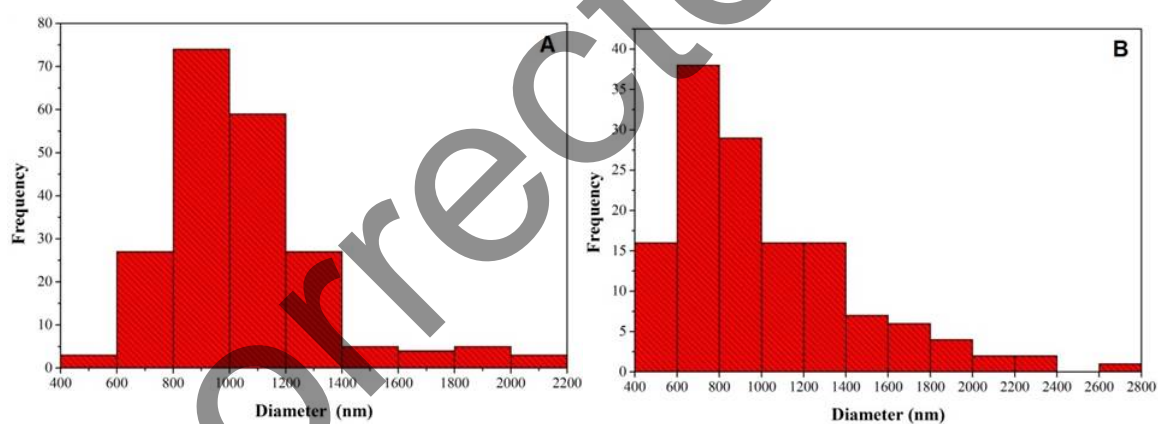


**Figure 1 . Stages of wound formation and treatment in rats**

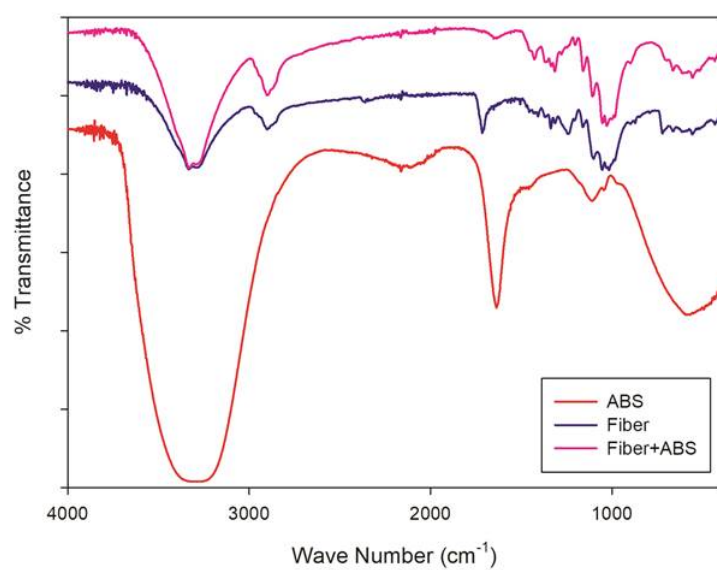




**Figure 2.** Scanning Electron Microscopy (SEM) micrographs of polymers, **A.** ABS non doped (1000 X), **B.** ABS non doped (5000 X), **C.** ABS doped (1000 X) , **D.** ABS doped (5000X)



**Figure 3.** **A.** ABS non doped and **B.** ABS doped wound dressing fiber diameters (nm). Average values of ABS doped (A) and ABS non doped (B) fibers made with "Fiji ImageJ Program" depending on the density of the polymer.

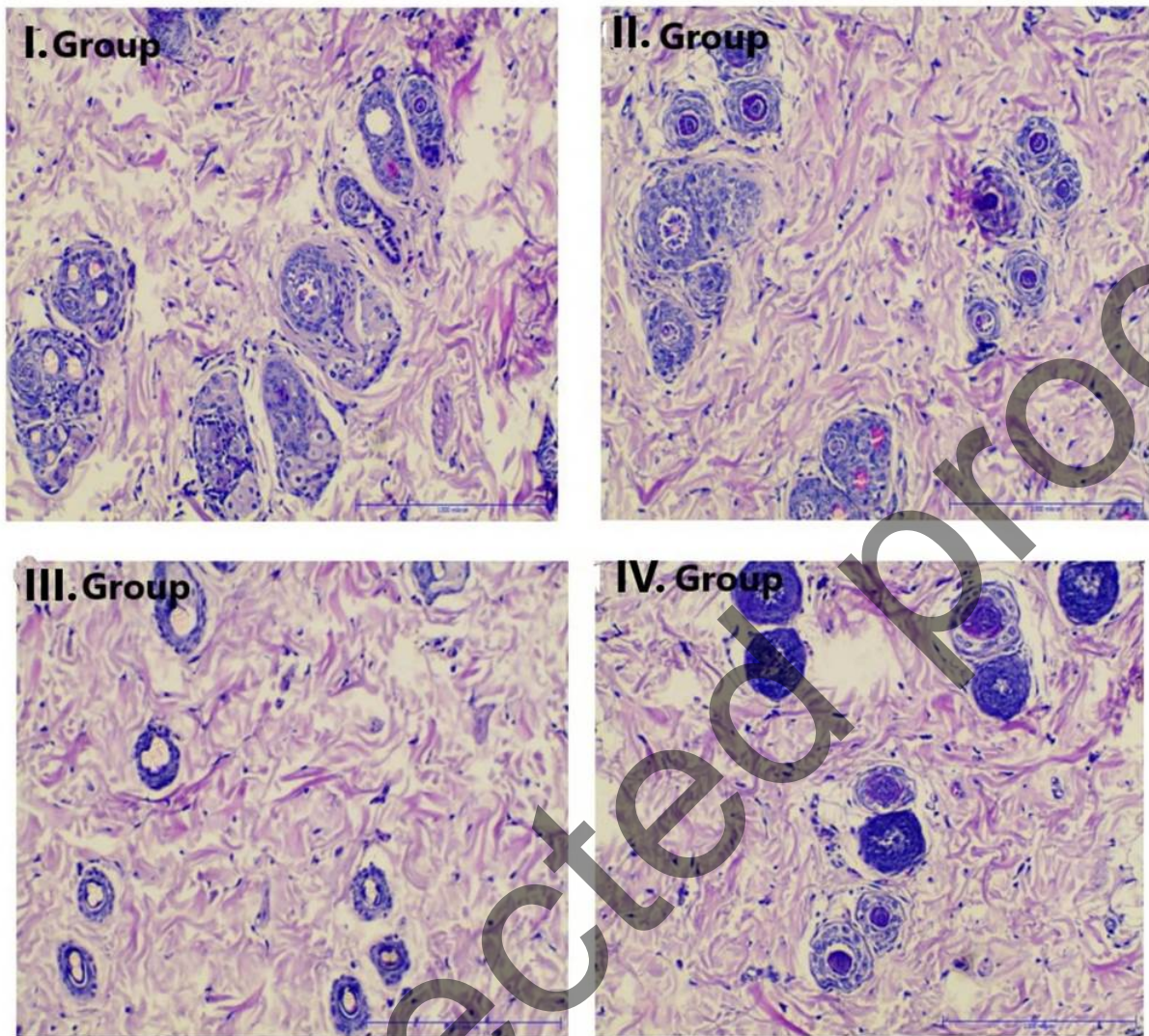


**Figure 4.** FTIR analysis chart



**Figure 5.** The change in wound diameters in the groups determined on the first day (A) and last day (B) of the study





**Figure 6.** On the last day of the study, skin section samples belonging to different groups (H-E staining, scale 100  $\mu$ m X 40).