

ORIGINAL RESEARCH

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Investigation of the efficacy of a novel topical hemostatic agent: Experimental rat study**Ismail Altintop, Mehmet Tatli***Kayseri Training and Research Hospital, Department of Emergency Medicine, Kayseri, Turkey*Received 29 August 2018; Accepted 20 September 2018
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Abstract

Uncontrollable bleeding constitutes nearly half of all deaths in the military field while in hospitals and the second cause of deaths due to trauma patient. It is important to stop bleeding early regardless of its size, cause. The inadequacy of hemostasis can lead to various bleeding complications so hemostatic agents are widely used all over the world. In our study, naturally occurring diatomite components from algae fossils were obtained through various processes as a topical hemostatic agent with hemostatic effect. The in vivo hemostatic effects of processed diatomite (PD) components is compared with the product commonly used as hemostatic agent chitosan (CeloxR) in this rat study. A randomized and controlled animal experimental study. The study was performed in the Experimental Research Center and 22 male Wistar albino rats. Rats were divided into 3 groups as follows: the control group (n=6), direct gauze compression was applied to the bleeding area without medication; the PD group (n=8), direct compression was applied with PD powder; and the chitosan group (n=8), direct compression was applied with chitosan powder. PD and chitosan groups were found to have a significantly lower bleeding rate (6 (100%)) than the control group (1 (12.5%) and 4 (50%) respectively). The mean time to stop bleeding in the control group was 305.00 ± 15.34 sec., when it is 78.75 ± 22.32 sec., in the PD group, and chitosan group was determined as 101,25 ± 39,07 seconds. As a result, The PD is a useful topical agent because of its superior hemostatic effects and usefulness. PD demonstrated a more potent hemostatic efficacy than Chitosan due to its higher unique surface area. Therefore, the PD administered onto the wound could terminate bleeding.

Keywords: Diatomite, algae fossils, chitosan, hemostatic agent**Introduction**

Diatomite is a sedimentary rock with a fossil character formed by the accumulation of silicic crusts of diatoms, which are water algae from the class of algae. It is a very small protoplasm that is settled in a silty crust or a shell that the diatom has obtained from the surrounding water. About 16,000 different varieties of freshwater diatom, are developing at sea or brackish waters [1]. The dead shells of the dead diatoms accumulate to form diatomite deposits. Al₂O₃, CaO, Fe₂O₃, MgO, K₂O, Na₂O, P₂O₅, TiO₂ are also present to form a large proportion of the diatomite forming compounds [2]. The proportions of the compounds may vary slightly relative to the region in which the diatomite is located [3]. Diatomite skeletons are called frustules. Diatom frustules are formed by single-celled photosynthetic algae, mostly amorphous silica, dispersed in large aquatic environments [1]. The diatomite is characterized by a specific surface area of about 200 m²/g [1]. Sizes range from 2 nanometers to 2 mm. Diatomite may allow

ion exchange as well as active barrier function [4]. It is especially successful in removing polluted water from micropores by filtration [5]. The water is cleaned by microparticles from nitrogen, phosphorus and organic residues [6]. One of the most critical features of diatomite is its high liquid absorption capacity. It has been known for many years that it absorbs more than twice the liquid of its particular weight [7,8]. Diatomite is commercially used in the form of filter, air filter, water filter for reasons of low density, high visibility, high absorption capacity and chemical inertness [9]. Diatomite is inert to many chemicals and is affected only by strong bases at high temperatures and HF (hydrofluoric acid) and sulfuric acid at elevated temperatures [10]. The most important features of diatomite are; nano-sized porous structure, high surface area relative to volume, lack of dissolution characteristics compared to body fluids, natural and non-toxic properties.

Chitosan is a non-toxic biological polysaccharide polymer of deacetylated chitin [5]. It was approved by the United State Food and Drug Administration in June 2006. The positive loaded NH₃⁺ groups interact with negative loaded thrombocytes and erythrocytes, attaching them with an ionic bond [11,12]. This induces the aggregation of thrombocytes in the constitution of thrombus. In vitro studies show its advantageous effects in wound healing on

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the activation of polymorphonuclear neutrophils, macrophages, and fibroblasts [13]. Chitosan possesses antimicrobial function versus fungi and gram-positive and gram-negative bacteria [14]. Celox® is a topical compound of chitosan. Chitosan is used as a hemostatic agent in over the world [15].

This study aims to process diatomite components received directly from nature physically and chemically to obtain the hemostatic effect, to show a hemostatic result of the PD components in vivo comparative studies, and to provide a brand new and unique topical hemostatic agent to the literature.

Materials and Methods

The study was carried out with approval from the local experimental animals ethics committee (Local Ethics Committee number, HADYEK-14.12.2016, 16/52). The study was performed in the Experimental Research Center and 22 male Wistar albino rats weighing 158-215 g were used. The rats were housed in polycarbonate cages (three rats per each cage), on coarse sawdust bedding, under conventional experimental animal housing conditions with controlled temperature (21 ± 2 °C), humidity ($50 \pm 5\%$), air change (12 air change per hour), temperature (12 hours light, 12 hours dark) and ad libitum feed.

Rats (n=22) were divided into 3 groups as follows: the control group (n=6), direct gauze compression was applied to the bleeding area without medication; the PD group (n=8), direct compression was applied with PD powder; and the chitosan group (n=8), direct compression was applied with chitosan powder.

Diatomite compounds have been obtained from the mines located in different cities of Turkey; Kayseri, Niğde and Nevşehir. Diatomite components are separated from impurities by subjecting it to wet milling and separation processes, calcination and after washing with hydrogen peroxide; a topical hemostatic agent has been obtained to have hemostatic effect by electrostatically being loaded with negative ions and used in our study after sterilization in an autoclave.

Observation of the morphology

The surface morphology of the PD microparticles was observed by a scanning electron microscope (SEM). Microparticles loaded aluminium foil was coated with gold metal under vacuum and then examined by SEM (EVOL25, Zeiss, Germany).

Evaluation of the Physical and Chemical Properties of PD.

The elemental composition of the sample material was analyzed by XRF (X-ray fluorescence). X-ray diffraction (AXSD8 Advance, Bruker, USA) was used to illuminate the crystal structure.

Rat femoral artery bleeding tests

Rats were fed with the same amount of food. The rats were randomly selected for groups. Before starting the experiment, ketamine and xylazine hydrochloride were used for anaesthesia. Right inguinal regions of the rat were shaved and wiped with oktenisept®. The skin and subcutaneous tissue were dissected to show the femoral artery and vein. Haemorrhage comprised of a complete incision of the femoral artery and vein. One of the researchers was responsible for the dissection. Another researcher was responsible for compression. Other investigator collected the accumulated blood with a gauze by pressing. The gauze was moved

out and instantly the homeostatic material was applied (Celox® or PD). In all applications, a standard weight of 200 gr was put on the bleeding area. At this time, the clock was started. The first check was taken at the 60th second. After every 30 seconds, the bleeding was checked. If the bleeding had stopped, it was recorded as bleeding stopping time (BST). if not, compression was continued. Eventually, if the bleeding had stopped, it was recorded as a BST.

Rats were evaluated simultaneously whether shock occurred after bleeding, the shell formed on the wound(scaffold), cleaning the material on wound easily or not and finally whether bleeding continued after removing the scab or not. Pictures were shown and taken during the experiment.

Histopathologic evaluation

The study included formalin-fixed and paraffin wax embedded specimens from vessels of rats in all experimental groups. For routine histology, dissected wound area tissues removed from the rats and fixed in 10% formaldehyde. Then, dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin. 5µm thick paraffin sections were cut from each specimen. After deparaffinization and rehydration, all sections were stained with Masson's trichrome. Morphological evaluation of vessels, photographs were taken with a photomicroscope (Olympus BX-51, Tokyo, Japan).

Statistical analysis

Statistical analysis was carried out with SPSS 22.0 for Windows. Continuous variables were expressed as mean±standard(mean±SD) deviation. Categorical parameters were given as numbers (n) and percentages (%). Fisher's exact test was used for comparison of categorical variables. All calculations were compared with each other by means of a one-way ANOVA (post-Hoc test Tukey) with the paired sample t-test. The statistical significance level (p) was set at <0.05.

Results

Characterization of Diatomite

In our preliminary studies, surface area measurements were performed on the diatomite-2 components. BET results show a surface area of 160.385 m² / g. Diatomite-2 samples were used in our studies. Diatomite-2 microcapsules have an average length of 77 µm (Pa2), width average 19 µm (PaR2) and capsules consist of an average of 6-12 layers (Pa2). There is microcapsule frame average 30 column (PaR2) similar structure and 20 units in each skeleton column (Da1) quadreal pore (Da2). The average distance between skeleton columns is 1.45 µm, the number of pores reaches approximately to 1200 in each layer in the form of sieves, the average pores are in the range of 20-600 nm and expected to react as essential barriers for microorganisms (Figure 1).

X-ray fluorescence analysis

Diatomite compounds obtained from two different regions were examined by X-Ray Fluorescence (XRF) and their preliminary results were shown (Table 1) in order to investigate the properties of the compounds in the PD composition. The diatomites examined as diatomite-1 and diatomite-2 since they contain different proportions of the components according to the regions in which they are obtained. Al₂O₃ (4,42 %), Fe₂O₃ (5,50%), CaO (2,52 %) compounds are found in diatomite-2 composition.

Animal study findings for hemostasis

There was no statistically significant difference between control and Chitosan and PD groups weighing before and after treatment ($p > 0.05$). There was a statistically significant difference in comparison of mean bleeding time and weight differences (Line 3, $p < 0.05$) between control and study groups (Table 2). According to the multiple comparison tests (Tukey) comparisons of the control group and study group by weight difference and bleeding time variables there were significant differences between the control group and Chitosan and PD groups separately ($p < 0.05$). Both PD and Chitosan group had significantly high Scaffold formation rates respectively (3 (37.5%)), (7(87.5%)) where the control group had no scaffold observed (0 (0%)) ($p < 0.05$). Likewise both PD (7 (87.5%)) and Chitosan (6 (75%)) were cleaned on wound significantly better than the control group (6 (100%)), ($p < 0.05$).

While comparing the bleeding occurrence after scaffold formation; PD the Chitosan group, respectively 1 (12.5%)) and 4 (50%) rats had bleeding, but all the rats had bleeding in the control group (6 (100%)) ($p < 0.05$).

Among PD and Chitosan group only 1 rat(s) emerged shock after bleeding in the group (1 (12.5%)), (1 (12.5%)), respectively, the rats emerging shock after bleeding in the control group were 3 (50%)and that was found to be significantly different ($p > 0.05$).

In the control group, the mean time to stop bleeding was 305.00 ± 15.34 seconds, in the PD group, 78.75 ± 22.32 seconds and chitosan group was $101,25 \pm 39,07$ seconds (Table 3).

Chitosan and PD were given a comparative box plot chart

according to the control group (Figure 2).

When the weight difference after bleeding was examined, it was determined that the control group was significantly different from PD and chitosan group ($p < 0.05$).

At the end of the study, the rats were sacrificed by cervical dislocation method under ketamine/xylazine anaesthesia.

Histopathological findings

For histological evaluation, the Masson's trichrome staining technique was used to stain the structure of the vessels. Histological sections of the treated and nontreated femoral vessels are shown in Figure 3. All picture shows a cross-section of femoral vessels following complete vessel dissection. The general architecture of the femoral vessels was structurally standard in all groups. Bleeding zones outside of the vein were noted in the control group where nothing was applied. It was observed that these bleeding areas were not observed in the treated groups and the endothelium, media and adventitia were not affected by the treatment of the blood vessel walls. The smooth muscle contraction is evident by the circular appearance of the vessel and by dense folding of the internal elastic lamina in Figure 3B, treated with PD.

Examination of scaffold scab with SEM

The scaffold images taken from the wound examined by SEM analysis are shown in figure 4.

In our first laboratory review, the interaction of the absorbance properties of PD with whole blood and plasma is shown in figure 5.

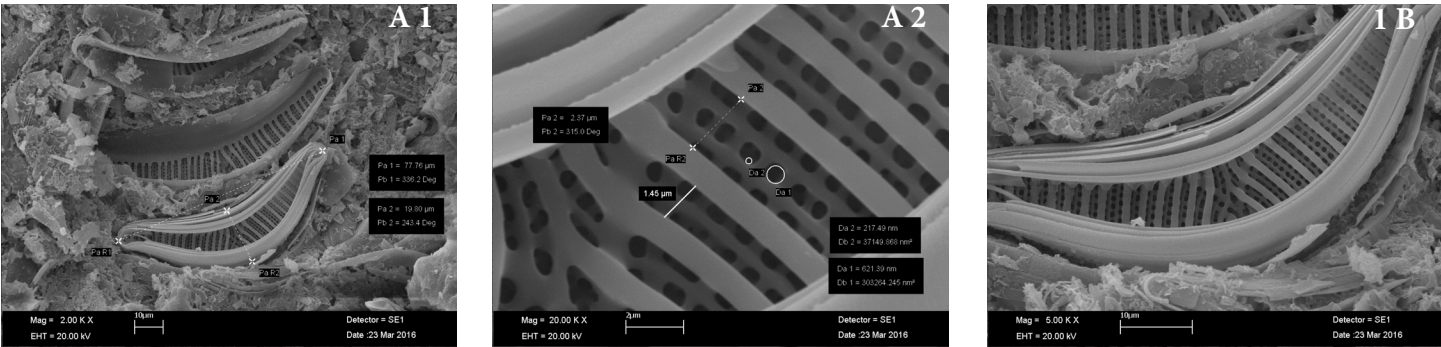


Figure 1. A) Diatomite-2 The appearance of microcapsules. Pa 1(Microcapsule long axis size), Pa 2 (Microcapsule short axis size and capsule layers image). Diatomite-2 Microcapsule inner wall structure measurements. Da1 (Micro capsule nanometer pore size), Da2 (Microcapsule small pore size). Pa2 (Dimension between skeleton columns). 1B) Diatomite microcapsule inner layers image.

Table 1. The distribution of XRF values of diatomite compounds obtained from two different regions

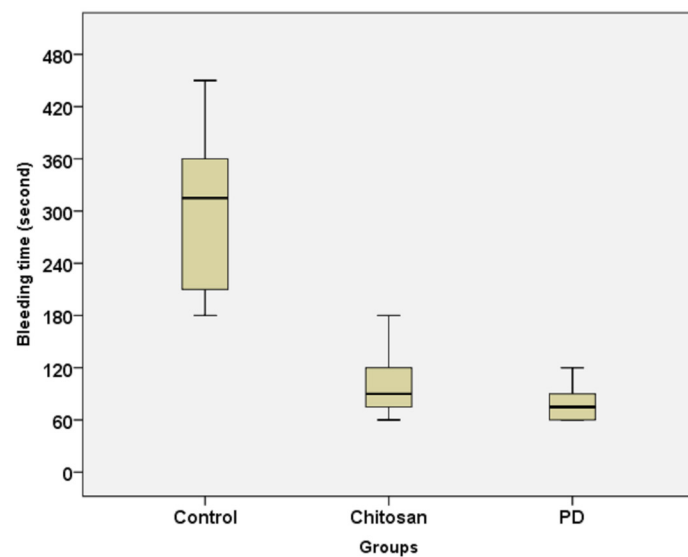
Name of compound	XRF Results	
	Diatomite-1 (%)	Diatomite-2 (%)
Al2O3	3,02	4,42
Fe2O3	2,76	5,50
CaO	1,52	2,52

Table 2. Comparison of Control, Chitosan and PD groups

Continues	Groups			p
	Control (n=6)	Chitosan (n=8)	PD (n=8)	
Variables	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	
First weight	189.0 $\pm$ 15.3	187.8 $\pm$ 17.0	188.8 $\pm$ 16.4	0.988
Last weight	184.0 $\pm$ 14.4	184.9 $\pm$ 16.9	186.1 $\pm$ 15.9	0.970
Weight difference	-5.0 $\pm$ 1.3 ^a	-2.9 $\pm$ 1.1 ^b	-2.7 $\pm$ 1.0 ^b	0.003
Bleeding time	305.0 $\pm$ 99.3 ^a	101.3 $\pm$ 39.1 ^b	78.8 $\pm$ 22.3 ^b	<0.001
Categoric Variables	n(%)	n(%)	n(%)	p
Scaffold formation				
Complete	0(0) ^a	6(75) ^b	7(87.5) ^b	0.002
Weak	6(100)	2(25)	1(12.5)	
Cleaning from wound				
Easy	6(100) ^a	3(37.5) ^b	7(87.5) ^{ab}	0.038
Difficult	0(0)	5(62.5)	1(87.5)	
Bleeding after scaffold				
+	3(50)	1(12.5)	1(12.5)	0.250
-	3(50)	7(87.5)	7(87.5)	

PD; Processed Diatomite, Data n(%), mean $\pm$ SD or average

According to multiple comparison tests (Tukey), groups with different superscript letters were found to have statistically significant differences.

**Table 3.** BT in the rat femoral artery model

Treatment (mean)	Time(s)(mean)
No treatment group	305,00
Chitosan group	101,25
PD group	78,75
s. (second)	

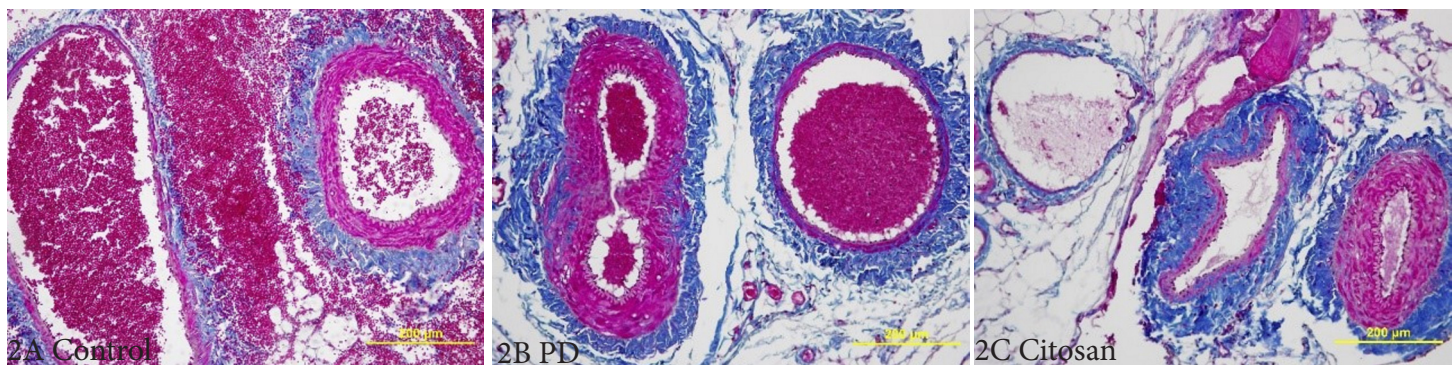
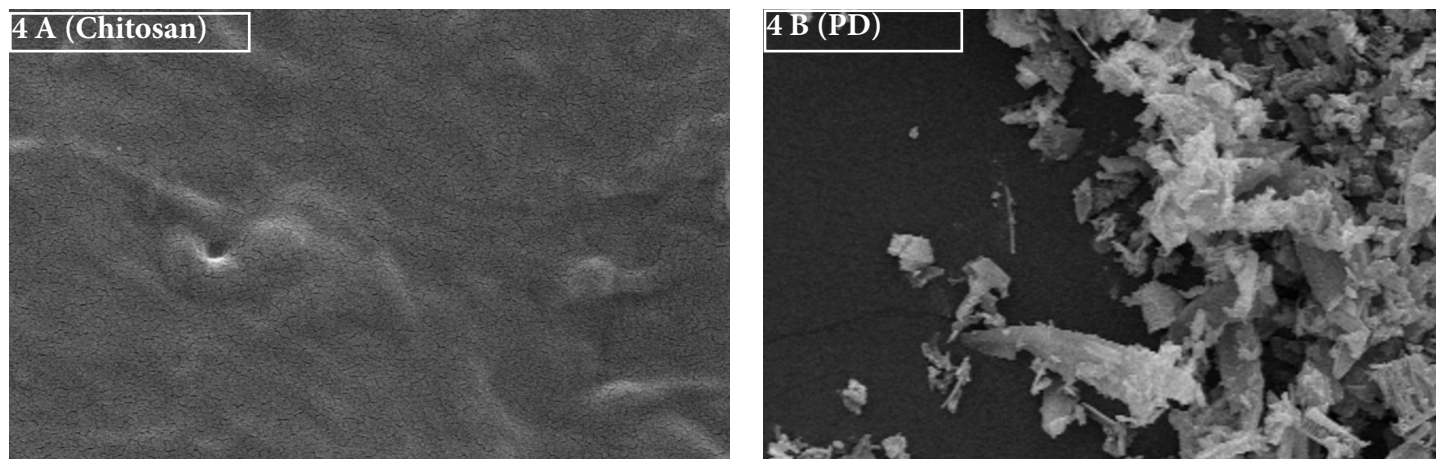
Figure 2. Distribution of control, chitosan and PD groups (box plot graph)

Figure 3: Histology of the untreated, and treated with PD (B); Kito (3C) femoral vessels following complete vessel dissection. (3A) Control femoral blood vessels were cut and left bleeding for 3 seconds. Free flowing blood in the control vessel formed a detached clot during sample fixation. (3B) Treatment with PD caused complete occlusion of the vessel and termination of hemorrhage within a few seconds. (3C) Construction is evident by round shape of the vessel and folding in the internal elastic lamina Star; bleeding area, Arrow; internal elastic lamina. Masson's trichrome, original magnification x20.

Table 4. Bleeding time

Bleeding Time (m)	First n(%)	Second n(%)	Third n(%)	Fourth n(%)	Fifth n(%)	Sixth n(%)	Seventh n(%)	Unsuccessful n(%)
Control	0(0)	0(0)	1(16,6)	1(16,6)	1(16,6)	2(33,3)	1(16,6)	0
Chitosan	2(25)	3(37,5)	3(37,5)	0	0	0	0	0
PD	4(50)	3(37,5)	1(12,5)	0	0	0	0	0

The data are expressed as number (n) and percentage(%). m. (minute)

**Figure 4.** SEM image of PD and chitosan nanoparticle**Figure 5.** Interaction of the absorbance properties of PD with whole blood and plasma

Discussion

One of the most critical features of diatomite is its high liquid absorption capacity. It has been known for many years that it absorbs more than twice the liquid of its own weight. The results of our SEM analyses indicated that PD is such a candidate of a good hemostatic agent having a high absorbent capacity with poriferous structure in nano size and having a high structure are more than its volume. In a study performed by Zhang et al. diatomite is used as a carrier for gastrointestinal nano prednisone and mesalamine have been found and successfully tested experimentally [16]. Most importantly, in this study, colon cancer cells (Caco-2, HT-29 and HCT-116) were accepted as non-toxic by cell viability test. It was also determined by Terracciano and colleagues that a nano-carrier was a potential drug carrier studied in different cell lines, and one of the most important points was a positive result in cytotoxicity tests [17]. This nanocarrier feature can be used to increase the bleed stopper feature when needed.

Aluminium sulphate and ferric sulphate are hemostatic effective agents commonly used throughout the world to stop bleeding [18,19]. Al_2O_3 , Fe_2O_3 , CaO compounds are found in diatomite-2 composition.

Hemostatic agents are mainly used to stop venous and small arterial bleedings. We used a hemostatic agent for significant rat arterial and venous bleedings model. Moreover, also we showed their efficiency even in arterial and venous bleedings model. In our study, in the PD group, the bleeding stopped in 87,5% of the rats. In 87,5% of the control group, the bleeding did not finish in the first 4 min, but in the PD group, the bleeding stopped in the first 3 min in all rats. Dikme O et al. used locally oxytocellulose powder as a bleeding stopper in rats and they performed their work by exploring the bleeding stop time at 0,1,2,3,4 minutes [20]. In

our study, the femoral artery and venous haemorrhage model was planned similarly to the research performed with Crofton et al. [21]. Crofton et al. measured the bleeding time by applying chitosan directly to the hemorrhagic area in powder form [21].

Chitosan is used differently to increase this effect with the formation of the natural scaffold at the wound site [22]. It is essential not only to stop the bleeding, but it also forms scaffold s after the bleeding had stopped. The formation of the scaffold with PD was found to be significant in our study. One of the best models for calculating bleeding time in rats is controlling on the femoral artery and vein. Mirzakhani Z et al. found that the lowest bleeding time was 3 sec in different doses in the study of superabsorbent hydrogels [20]. Kyung Eun You et al. found that the best time to bleed was 174.2 sec, during the investigation of a powdery medical adhesive composed of aldehydes dextran and ϵ -poly (L-lysine) [21].

Chitosan-based products have no intrinsic hemostatic properties and are reported to work by electrostatic interaction between negatively charged erythrocyte membranes, platelets, and positively charged chitosan to create a firmly adherent 'gel' over tissues [21]. Extensive research can be applied to investigate whether the PD has these properties or not.

It is important that the hemostatic agents are natural and do not cause histopathological damage in the bleeding area. Histopathological sections with and without PD were investigated. It has been observed that PD has no effect on femoral vessels and is not different from non-administered tissues has been shown that endothelin, media and adventitia are not affected in the area except the bleeding zones. Accordingly, the native structure of PD is shown by the histological study performed.

PD hemostatic agent obtained by treating the components has a high absorption capacity due to the enormous natural porosity surface area. It allows gas exchange in the PD wound site with enhanced blood stopping not toxically compared to the other widely used hemostatic agents and last but not the least it is cleaned quickly and safely from the wound area. Formation of scaffold capacity was shown to be improved by PD also. As a result of all these considerations, we hope it will be a new and unique alternative blood-stopping agent with fundamental properties

In this study, we compared one known homeostatic material to new hemostatic material PD and direct compression without medication. There are not many studies comparing the other hemostatic material with Celox®.

The current hemostatic agents used commercially like Duraseal obtained from synthetic polymers, CoSeal, comprising fibrinogen, fibrin glue, Tisseal, Berilast, Hemaseal and Crosseal, different from those based on bovine albumin and glutaraldehyde (BioGlue) are not natural products. Controversially PD is a natural product. Other Hemostatic agents originated from plants are known as Arista and HemoStase. These agents allow platelets and serum proteins to concentrate where they are applied [24]. They manifest their effect by denaturing protein but cause delayed wound healing, and micro embolisms. These undesired effects were examined in histological sections of PD during our study. It has been determined that there is no toxic injury at the wound site.

In normal conditions, bleeding can be stopped by compression, but finalizing severe haemorrhage is only possible with hemostatic agents. Hemostatic agents are difficult to clean and remove from the wound area [25]. Due to its structural properties, the topical hemostatic agent PD obtained from natural diatomite components had been significantly easier to clean and remove from the wound site than chitosan. The microcapsule property of the PD provided a more significant efficacy in ending bleeding and formation of scaffold than current hemostatic agents. It also provides mechanical support to the fibrinous roof by allowing rapid fluid absorption at the wound site through to its porous structure with large surface area.

Mechanical agents create a physical blockage leading to tamponade. In general, these agents are cost-effective and are often used as first-line modes of treatment. Similar to mechanical agents PD's mineral zeolite acts by absorbing liquid in the wound bed through molecular sieves, thereby increasing the concentration of coagulants to propagate hemostasis. Its activity leads to an exothermic reaction [26].

Conclusion

PD has superior hemostatic agent activity in severe femoral artery haemorrhage or combined arterial/venous hemorrhagic rat model. PD showed a stronger hemostatic activity than Chitosan because of its high specific surface area. Histopathologically, it is important for our study that the tissues are not damaged. More studies are needed on PD.

Limitations

PD, which was used in our study, was our first study and maximum effective products could not be prepared. It is difficult to use as powder. Studies with new innovative applications and varieties are needed. The femoral artery bleeding model in rats is quite difficult. It must be tested in new bleeding models.

Competing interests

The authors declare that they have no competing interest

Financial Disclosure

The financial support for this study was provided by the investigators themselves.

Ethical approval

Before the study, permissions were obtained from local ethical committee.

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