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RESEARCH ARTICLE

Hybrid nanocomposite of Ag-exchanged zeolite improve hemostatic efficiency in a lethal rabbit model of external bleeding

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ABSTRACT

Uncontrolled bleeding contributes significantly to civilian suffering and battlefield fatalities. In this work, we created a nano compound hemostat based on natural zeolite (clinoptilolite) and evaluated it in a deadly rabbit model of external bleeding versus controls.

Methods:

In 25 mature male New Zealand white rabbits, a modified complicated groin injury was produced aseptically. Each animal was randomly assigned to one of five groups, including Control, quikclot, Zeolite, Ag-Zeolite, and Ag-Zeolite/Nano chitosan. Within three minutes of damage, hemostasis

Three and 180 minutes following the use of hemostatic drugs, hemostasis and survival monitored, respectively. The wound repair characteristics of the surviving animals were assessed. Tukey's multiple comparisons were used after the analysis of variance (ANOVA) test to determine how the groups differed from one another.

Results:

Comparing the Ag-zeolite/Nano chitosan product to the control and quikclot groups, it took less time for hemostasis to occur. Compared to the control and quikclot groups, who received treatment with Ag-zeolite/Nano chitosan, respectively, there was no mortality in this group. While the majority of the rabbits in the quickclot group had severe dead soft tissue, all of the animals that survived in the Ag-zeolite/Nano chitosan group displayed good healing properties.

Conclusions:

The application of the new nanocomposite hemostat has shown good healing qualities, can control bleeding efficiently, and significantly lower mortality from a deadly groin wound.

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INTRODUCTION

Hemorrhagic shock due to traumatic injury, is life threating for the wounded in combat and accidents, especially when the direct pressure failed or a tourniquet could not be ` receives medical attention in a hospital. The military or emergency medical settings are where these deaths occur most frequently. Therefore, a person's likelihood of life before the arrival of trained emergency responders can significantly change with early and successful bleeding control(1-3). With this in mind, the major objective in emergencies should be the swift creation of a successful hemostasis, primarily in its topical form, in cases when a patient has endured trauma and its ensuing bleeding. There have been various topical hemostatic medicines produced during the past 20 years and utilized to reduce bleeding, notably in the context of combat (4). A granular synthetic zeolite hemostat is one of the

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most alluring antihemorrhagic agents (commercial name Quikclot). The U.S. government has authorized it, only for external hemorrhage stops that are resistant to treatment with other methods.

Synthetic zeolite, which does not occur naturally, is the major ingredient in Quikclot. Natural zeolites, however, have some significant benefits over their synthetic counterparts. First off, natural zeolites are produced at a significantly lower cost than synthetic zeolites. Because they are widespread on earth. Second, natural zeolites are good prospects for biomedical applications such as antibacterial medicines, anticancer treatment, preservative medium (5, 6).due to their stable physicochemical properties in biological settings. In addition, several problems stem from the usage of a quick clot in medicine. First, when a quick clot comes into touch with blood, it undergoes an exothermic reaction that causes a rapid rise in temperature that injures and destroys tissues. Second, after application, it might be challenging to completely remove the agent from the site, and a reaction to the foreign body could result in the development of inflammatory granulomas or abscesses. One method of reducing the heat of hydration is the exchange of cations for calcium. However, the strength of new clots was lowered by the exchange of calcium ions, which decreased the hemostatic effectiveness of modified zeolite (7-10).

The conflict between heat and hemostatic effectiveness must be resolved. To do this, we used a nano form of chitosan to increase the clots' tensile strength after the calcium ions were replenished. Chitosan, an organic polysaccharide that carries positively charged ions, draws negatively charged blood cells called erythrocytes to the location of the lesion and causes powerful blood clots to form. Hemostasis is caused by the blood clots accumulating on the open wound. Additionally, chitosan is antimicrobial to some bacterial strains. Chitosan is employed in several medical equipment and health care products because of its biodegradability and biological features, including hemostatic activity, antibacterial activity, and the capacity to speed up wound healing (11-15).

In this work, we produced a new nanocomposite hemostat and examined its hemostatic effectiveness and wound-healing capabilities in a fatal external bleeding. The hemostat is made of modified natural zeolite (clinoptilolite) and nano chitosan.

MATERIAL AND METHODS

Materials

Silicic acid, Sodium aluminate, sodium hydroxide, acetic acid, Sodium tripolyphosphate, and $AgNO_3$ were obtained from Merck co. Chitosan (medium molecular weight) was obtained from Aldrich.

Synthesis of modified Zeolite

A solution of $AgNO_3$ (0.01 M) was made a n d infused to the zeolite suspension at a ratio of 10: 90 (wt.%) and mixed continuously, ionic exchange process was done at 70°C for 24 hours; in the end, the particles were separated by centrifuge, washed to remove additional Ag ions, and dried at room temperature. The particle size of zeolite was about 500- 1500 nm.

Synthesis of nano chitosan

Sodium tripolyphosphate (STPP) was used to synthesize the chitosan nanoparticles in dilute solution as a cross-linker. So, first of all 0.5 g chitosan was dissolved in 30 ml acetic acid (0.5% dilute), then 150 ml deionized water was added. 150 ml of the STPP (2 mg/ml) was slowly dropped into the as prepared chitosan solution (180 ml). After stirring (1000 rpm/min) for 20 min, a milky emulsion of nano chitosan was obtained. The chitosan is in nanometer scale with the thickness of 10-50 nm.

Preparation of hybrid composite of nano chitosan/ modified zeolite:

100 g zeolite containing Ag ions was mixed with 300 ml of nano chitosan emulsion was ultrasounded and then stirred at 30 °C for 48 hours. The suspension was centrifuged and dried at room temperature. All of the prepared materials were sterilized for further using.

Animals and Grouping

In this investigation, 25 mature male New Zealand white rabbits weighing 2-2.5 kg were employed. The animals were housed in conditions of 223 °C, 605% humidity, and a 12-hour light/ dark cycle for two weeks before the research. Intramuscular injections of Xylazine (5 mg kg^{-1}; Alfasan, Netherlands) and Ketamine (40 mg kg^{-1}) were used to put rabbits to sleep (Alfasan, Netherlands). The University of Tabriz Research Ethics Committee authorized all animal studies (Act No.d/9661/43).

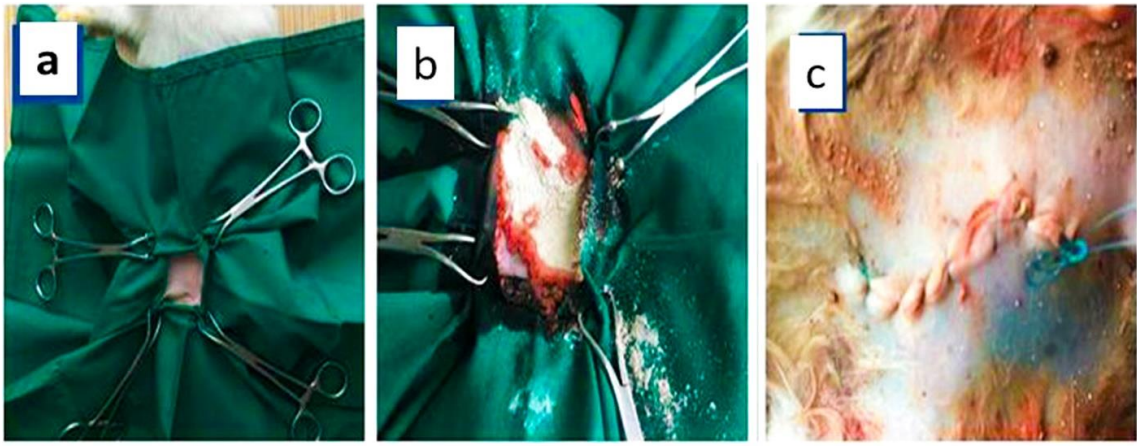

Fig. 1 Illustrates the surgical procedure. a.Induction of uncontrolled bleeding. b. Addition of hemostatic agent to the wounded site c. wound suturing

Induction of uncontrolled bleeding

25 rabbits were aseptically injured in the groin, resulting in an uncontrolled hemorrhage (16). All rabbits were randomized to be treated with either the medical gauze compression (n = 5), 10 g of Quickclot granules (n = 5) and 10 g of zeolite (n =5), Ag-zeolite (n =5), Ag-zeolite/Nano chitosan (n =5), the hemostatic agents were immediately poured into the wound, then three pieces of gauze were used to pack the wound over the location of the vascular damage then manual pressure was administered for three minutes on all animals. When using only medical gauze, three pieces of gauze were needed. The hemostasis was evaluated after the gauze removal, and no more treatment was given, even if the hemorrhage resumed. If there was no seeping of any type after 20 seconds after ocular inspection by two different laboratory assistants, the hemostasis was deemed to be complete. Three hours after the cut was made, mortality was seen. If the wound site began to bleed throughout this time, no additional therapeutic agent would be provided. Incisions were cleansed with saline. Finally, stitches were used to seal the incision (Fig. 1). After one week of survival, the animals' wound healing conditions were assessed. The animals were given a regular meal throughout this time, and they had unrestricted access to water.

Blood Loss measurement

The blood discharged around the dressings was gently suctioned into a collecting tube. blood loss was continuously recorded. Before application, the medical gauze and treatment agents were all weighed; after the trial, the difference was interpreted as blood loss (7).

Exothermic Reaction measurement

By monitoring the rise in water's temperature after being mixed with the novel hemostatic agents, the heat releasing of the therapeutic agents was examined in vitro. For this purpose, 10 g of Ag-zeolite/Nano chitosan and quick clot were introduced individually to a falcon tube 50ml inserted with a thermometer, followed immediately by 10 mL of DI water. The thermometer's maximum reading was noted (6).

Macroscopic and Histologic Examination

On day 7, a macroscopic examination of the wounds was recorded, and tissue samples were also collected that day. In a formalin solution with a buffer, the tissues were fixed after harvesting. An expert pathologist who was unaware of the samples' group assignment examined the samples histologically and evaluated the sections qualitatively for morphologic alterations.

Statistical Analysis

The statistical analysis was carried out using Graph Pad Prism, Version 5.05 (Graph Pad software, sndiego, USA). The differences among the groups were analyzed using the analysis of variance (ANOVA) test followed by Tukey's multiple comparisons. Results were expressed as mean of standard deviation. In all analyses, $p<.05$ was considered to be statistically significant.

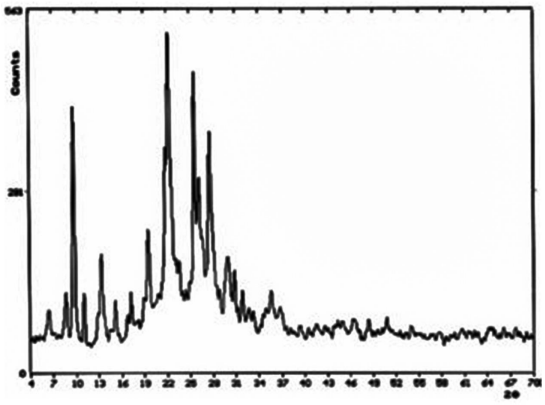

Fig.2 XRD of nano chitosan- Ag zeolite hybrid composite

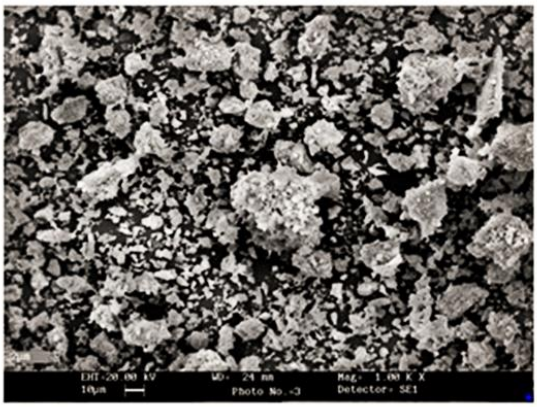

Fig.3 SEM image of nano chitosan- Agzeolite hybrid composite

Table. 1 Post-injury clotting percent, mortality and wound infection differences of the Study groups.

Groups	Complete hemostasis in 3 Min%	Mortality%	Wound infection
Control	0	80	++
Quick clot	80	10	++
Zeolite	40	20	+
Ag-Zeolite	20	40	-
Ag-Zeolite/nano chitosan	100	0	-

RESULTS AND DISCUSSION

Fig. 2 shows XRD patterns of the hybrid composites of nano chitosan/zeolite. As the amounts of Ag were very low, so the main peaks were belonging to zeolite. The presence of chitosan causes some defects in crystallinity of the zeolite which is appeared around 2θ=16-36.

The scanning electron micrograph of nano chitosan- Ag/zeolite hybrid composite is shown in Fig. 3, which shows that chitosan is in nanometer scale with the thickness of 35-45 nm and, some small particles, that these particles gather together and forming zeolite with a diameter of about 6 mm.

Exothermic Reaction

In the exothermic experiment, Ag-zeolite/Nano chitosan and quick clot both released heat. The Ag-zeolite/Nano chitosan group's temperature increase is 7.3 0.2 °C, whereas Quick clot's temperature increase reaches a maximum of 45.2 0.7 °C. When applying the quikclot granules to the medical gauze in the animal experiment, we can notice significant amount of heat release, but in the Ag-zeolite/nano chitosan group there was no obvious temperature change.

Mortality

The mortality rate in the different study groups is shown in Table.1. The complex groin injury resulted in 80% mortality in the control group. The mortality rate decreased to 10 % by quickclot application. Addition of Ag-zeolite/nano chitosan hemostat offered a statistically significant (p = 0.001*vs.* Control group, p =0.044. *vs.* quikclot group) advantage by decreasing the mortality rate to zero. These results demonstrate that the Ag-zeolite/nano chitosan is more effective than quikclot granules in surviving the animals from lethal external bleeding. However, there is no significant difference between Ag-zeolite/nano chitosan group and quikclot group ($p>0.05$).

Hemostasis

In the animal experiment, there were significant variations in the hemostatic agents applied (p=0.08). Within three minutes of damage, the new nanocomposite induced full hemostasis and decreased the bleeding time in this study's treated animals. In the control group, none of the animals reach hemostasis, compared to 80% in the quickclot group. Complete hemostasis, however, did not occur in any of the animals in the control

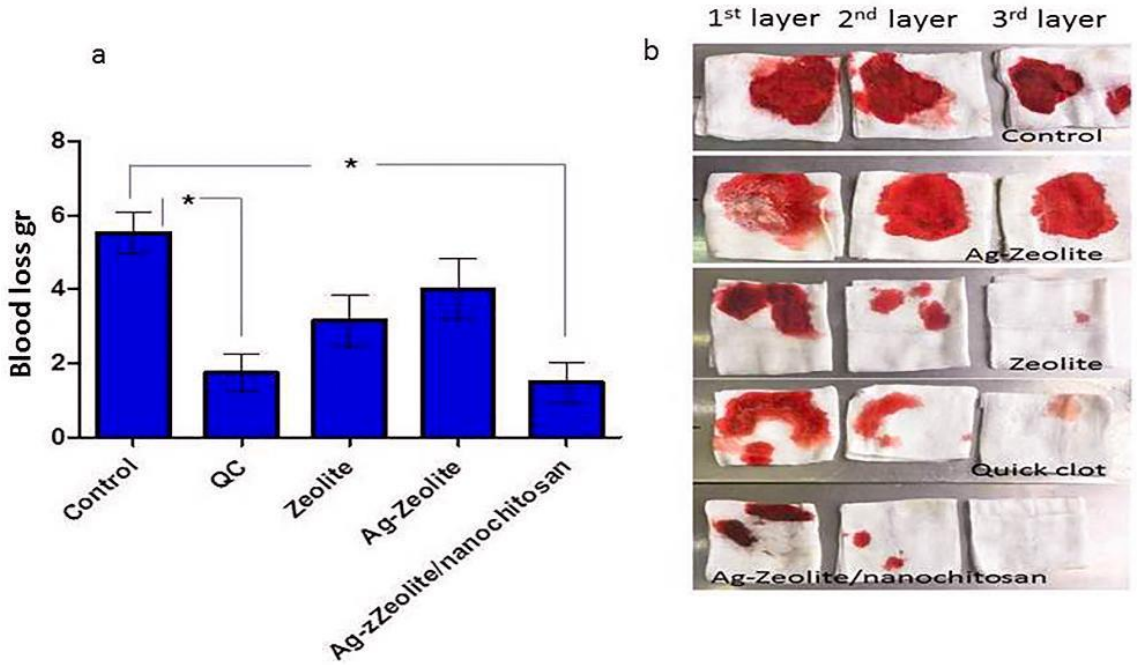

Fig. 4 demonstrates amount of blood loss(gr) in different groups of study.
a. *indicate significant differences between the groups. Data presented as group means ± SEM. *p <0.05
b.Photographic examples showing comparison of blood loss of the study groups

group throughout this time. Furthermore, as all patients in the nano composite group survived, it may be concluded that early hemostasis was able to stop excessive bleeding and save lives.

Blood Loss

The volume of blood loss by the various research groups is shown in Fig. 4. Blood loss in the hemostatic agent groups were less than the control group. With the compound of the Ag-zeolite/nano chitosan group showing the lowest blood loss. However, the control group saw the most blood loss. Even though the novel nano compound can reduce bleeding when compared to quikclot, no discernible difference was seen between the two groups, $p>0.05$. However, a significant difference ($P<0.05$) was seen between the control group and the quickclot treated groups.

Wound Healing

The wound repair abilities of the surviving animals were assessed and compared macroscopically and histologically at 7 days after wounding using Ag-zeolite/Nano chitosan and quikclot treatment. Fig. 5 and Fig. 6 show the results of aforementioned examinations. Hemostasis, inflammation, proliferation, and remodeling are the four overlapping stages of the traditional wound healing process (6-17). The wounds of the rabbits who survived in the Ag-zeolite/Nano chitosan group underwent proliferative phases when the wound edges were pulled together to lessen cut defects. All (5/5) of the Ag-zeolite/Nano chitosan group's surviving rabbits exhibited these contraction characteristics. However, wound healing process was still in the inflammatory stage in the quikclot group. These findings demonstrate that Ag-zeolite/Nano chitosan hemostat is significantly more effective than quikclot at speeding up the healing of wounds.

According to the findings of our investigation, bleeding management with Ag-zeolite/Nano chitosan may drastically lower mortality from 80% to nil in a deadly rabbit model of complicated groin injury ($p = 0.001$). In this work, modified natural zeolite with nano chitosan added to it showed better hemostatic activity. After the nano compound was applied to the area that was actively bleeding, the nano chitosan absorbed the blood's water and quickly produced gels that stuck strongly to the tissue, providing good bleeding control. Gel made of nano chitosan facilitates the removal of zeolite granules from the injured site and reduces the inflammatory response to foreign bodies.

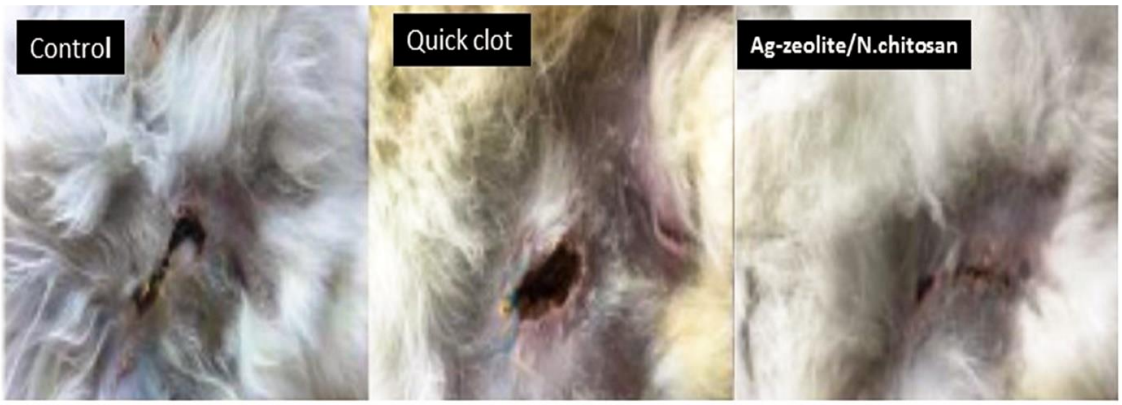

Fig. 5 Macroscopic pictures of wounds of the experimental group on day. There was Superior healing in the nanochitosan/ ag-zeolite group among the other groups.

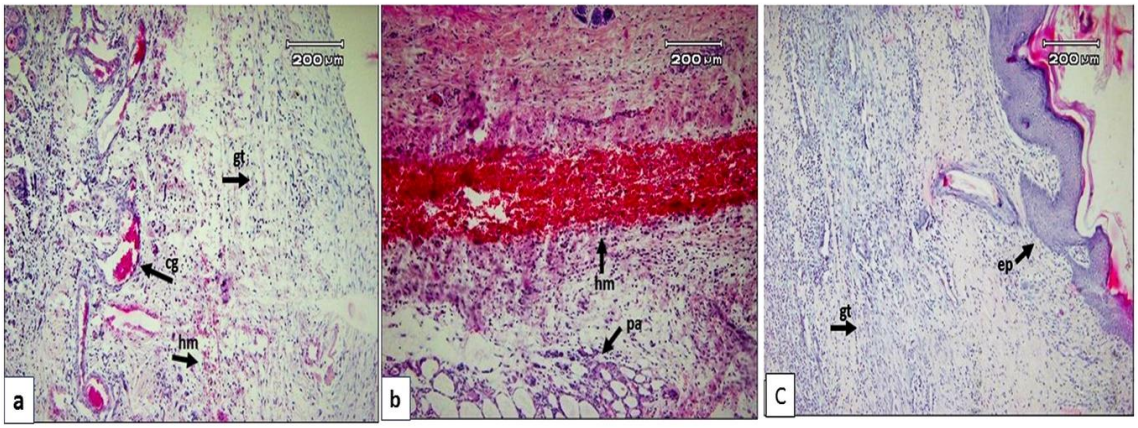

Fig. 6 Histological characteristics of rabbit skin on days 7.

A greater surface area is preferred to encourage contact between chitosan and the platelets, which will improve the hemostatic effects of chitosan-based hemostatic agents. Chitosan can be converted into nanoparticles to accomplish this(18). The findings of the current investigation are consistent with those of other studies(19, 20). However, quick clot decreased mortality from 80% to 10% ($p = 0.044$). Since the quikclot group's hemostatic effectiveness is significantly greater than the control group's (80% vs. zero), it is anticipated that the quikclot group should have a significantly higher survival rate than control group the (90% vs. 20%).

This finding supports the idea that quick stoppage of bleeding is essential for reducing mortality rate in cases of harsh extremities bleedings. Up to 20% more lives can be saved in the field if bleeding is quickly and effectively controlled(21). The use of ordinary gauze decreased the mortality to 40% in prior research, where the model of extensive groin bleeding was meant to represent 100% mortality without any therapy (22). Because of the way that zeolite's porous structure absorbs water and keeps it trapped inside its cavities via hydrogen bonds, which causes heat to be produced when calcium is released into the blood, zeolite tends to lessen bleeding. The following one triggers coagulation factor XIIa, which is the initial stage of the internal coagulation pathway, as a result of the negative charge on the zeolite surface(8, 23). The wound-healing ability of the Ag-zeolite/nano chitosan hemostatic agent has not before been documented. In the current investigation, the Ag-zeolite/Nano chitosan group showed excellent recovery. A week later, necrotic tissues were seen in the rabbits in the quikclot group (3/4), macroscopically. On histological inspection of the repair site treated with Quickclot, it was also

possible to see a superficial infection and edema. The new hemostat treated animals' lack of necrotic tissue might be attributed to silver's antibacterial activity in this investigation. It has been established that silver has antibacterial activity and that it can destroy bacteria, fungi, and certain viruses(16,24). Jiang et al. 2009 also obtained the same outcomes as our study(7). Ion exchange zeolites have excellent antibacterial action in addition to reducing heat generation in the wound region. Thus, heat damage and subsequent infection were decreased in the animal wound sites treated with nano compound. The hemostatic dressing's antibacterial ability is usually valued in battle environments (25, 26).

The whole wound repair process is an intricate chain of actions that starts as soon as an injury occurs and can last anywhere between months and years. This work is in a relatively early stage in proving the efficiency of Ag-zeolite/Nano chitosan. All surviving animals in the Ag-zeolite/nano chitosan group had a high healing ability. The healing activity of chitosan may be related to the wound site's success in healing after being treated with chitosan nano composite. Our findings, which were confirmed by a prior study, showed that chitosan sped up epithelialization and collagen synthesis while promoting wound healing (12, 27).

CONCLUSION

In conclusion, the use of Ag-exchanged zeolite based hemostatic drugs greatly increased early survival in a lethal external bleeding in rabbits. The addition of a nano chitosan substance increased the hemostatic effectiveness of the clots and strengthened their strength. Additionally, they are a great replacement for synthetic zeolite-type hemostatic due to their inexpensive cost, simple production technique, and good bio-compatibility.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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