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Efficacy and Safety of Novel Chitosan Hydrogel As Haemostat In Rat Neurosurgical Model

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ABSTRACT

Introduction: Bleeding in neurosurgery presents a significant challenge due to the brain's dense network of blood vessels. This project aimed to design a biocompatible and biodegradable hemostatic agent based on chitosan hydrogel to rectify disadvantages such as neural compression for use in the control of bleeding in nerve injuries.

Methods: The injury was made in the lambda region, which has a dense network of blood vessels. Using an electric drill, a hole with a diameter of 2 mm was created. Bleeding in control group was examined by a sterile gauze pad. In other groups, hydrogel dressing and commercial hemostasis such as surgical were used to control bleeding. The main parameters including volume of blood lost and the time of the bleeding, were measured.

Results: The results demonstrated that chitosan similar to surgical, has an improved hemostatic property. In addition, chitosan hydrogel was non-cytotoxic, obtaining cell viability values ≥ 89% for the L929 cells.

Conclusion: The results of the present study indicate that the safety and enhanced efficacy of chitosan-based hemostats, make them a potentially suitable option for use in neurological surgery.

Keywords:
Haemostasis, Chitosan; Neurosurgery; Bleeding; Cytotoxicity.

INTRODUCTION

Bleeding in neurosurgery is a critical challenge due to the brain's dense network of blood vessels. During surgical procedures, any inadvertent damage to these vessels can lead to significant blood loss, complicating the procedure and affecting patient outcomes (1). To manage this risk, multiple methods have been developed in order to promote a proper hemostasis. The methods are usually grouped into three categories including thermal, biochemical and mechanical procedures (2). Although, the bipolar coagulator as a fundamental thermal procedure is effective for coagulating blood vessels and used in many surgical fields but thermal injury to surrounding tissue can still occur and result in damage to critical neurovascular structures (3). Over the years, several chemical, biological and combined materials such as microfibrillar collagen (4), fibrin sealants (5), oxidized cellulose (6), gel foam (7) and Tranexamic acid (8) have been proposed as novel hemostatic agents. The hemostatic agents help accelerate and reinforce natural clot formation. Despite their effectiveness in promoting hemostasis, various reports indicate that these agents may induce a granulomatous reaction and are prone to swelling during the postoperative period. Therefore, caution must be exercised when applying them in bony canals or near cranial nerves (9).

Various mechanical devices, such as bone wax and gelatin sponges, are widely used in neurosurgery with proven effectiveness. Bone wax acts purely mechanically by occluding transected vessels, allowing for rapid cessation of bleeding. Despite its wide application, it is firmly hydrophobic and is not metabolized, causing many complications such as infection, inhibition of osteogenesis, granuloma formation, and allergic reactions (10, 11). Gelatin sponges are absorbable porcine gelatin, widely used in many neurosurgical fields. Gelatin sponges absorb

blood at approximately 45 times their own weight. Due to their high absorption capability, they are indicated in both cranial and spinal surgical procedures to facilitate the control of capillary, venous, and arteriolar hemorrhage (12). However, it has been reported that gelatin sponges may be related to the obstruction of neural tissues. This was reported to be caused by the osmotic expansion of the absorbable gelatin sponge within the enclosed space, including the spinal cord (13, 14).

As mentioned, despite the existence of various types of hemostatic agents in the field of neurosurgery, the need for an ideal hemostatic agent is still observed due to disadvantages such as swelling, obstruction of neural tissues, or allergic reactions. Chitosan, a natural polysaccharide derived from chitin, has been recognized as a versatile and effective biomedical material. Chitin is one of the most abundant biopolymers after cellulose. Found abundantly in the exoskeleton of crustaceans such as shrimp and crab, chitin is converted to chitosan through a deacetylation process (15-17). The history of chitin and chitosan dates back to the 19th century. For the first time in 1811, a French scientist named Braconot extracted chitin from mushrooms. In 1859, the German chemist Rudolf Friedrich first described the deacetylation process of chitin, which converts it into chitosan. This process involves removing acetyl groups from chitin in the presence of potassium hydroxide, resulting in a more soluble and versatile polymer. The name "chitosan" was coined later, reflecting its chitinous origins (18).

Chitin and chitosan, as natural amino polysaccharides with a unique structure and multi-purpose properties, are widely used in medicine and industry. Chitosan possesses the characteristics of natural biopolymers, such as biocompatibility, biodegradability, and antibacterial activity, making it an attractive option for medical applications. Additionally, the mechanical properties and high adhesion of chitosan to biological tissues have garnered attention for its effectiveness in hemostasis and wound healing (19).

One of the key features of chitosan is its ability to enhance the blood coagulation process. Chitosan promotes coagulation by forming a network of fibrin and platelets (20). Additionally, chitosan can inhibit fibrin-decomposing enzymes, which reinforce blood clots and reduce bleeding time (21). These properties make it an appealing substance for the production of a wide range of hemostats such as hemostatic dressings, hemostatic sprays, and surgical adhesives. Hemostatic dressings are widely used in the control of external bleeding. By making direct contact with the wound surface, these dressings advance the coagulation process and prevent bleeding. Studies have shown that the use of chitosan dressings, compared to traditional dressings, significantly reduces bleeding time and accelerates healing wounds (22). Chitosan sprays are especially useful in cases where wound access is difficult, such as internal injuries or deep wounds. Chitosan adhesives are another important form of the polymer. Due to their strong adhesive properties, they are used in various surgeries to close wounds and prevent bleeding (23). The current project aims to design a biocompatible and biodegradable hemostatic agent without disadvantages such as excessive swelling for use in the control of bleeding in nerve injuries. After successful synthesis, in vitro and in vivo hemostasis and cytotoxicity are also investigated.

MATERIALS AND METHODS

Materials

Chitosan (molecular weight 1100 kDa, deacetylation degree 75%) and calcium chloride dihydrate with a purity of 98.0% was purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium supplemented with L-glutamine (4 mM), penicillin (50 units/ml), streptomycin (50 mg/ml Sigma-Aldrich,) and 10% (v/v) fetal bovine serum (Invitrogen, CA, USA) was used for culturing cells.

Twenty four adult Wistar rats weighing between 200-250g were randomly selected from Royan animal research center. The rats were randomized into 3 experimental groups of eight rats. They were kept at a constant temperature (22±1°C) under a 12h light/dark cycle. All experiments were approved by the Institutional Animal Care and Use Committee of Tarbiat Modares University of Medical Sciences.

Synthesis of Hydrogel Dressings

Our paste/hydrogel hemostatic agents were typically prepared as follows: Quaternized N-substituted carboxymethyl chitosan (N-substituted CMCh) derivatives are dissolved in 20

mM calcium buffer to achieve a 40% w/w concentration. The solution was mixed for 10 minutes to form a homogeneous polymer. Finally, the solution was filled into test tubes (inner diameter 20 mm) and subjected to gamma irradiation at 25 kGy for 8 hours using a 60 Co facility. The morphology of the hydrogels was observed with a Scanning Electron Microscope (FSEM- HITACHI S-4160, Japan) at an accelerating voltage of 1 kV.

Solubility/ Swelling of Hydrogel Dressings

This test is based on EN 13726-1 to determine the physical properties of hydrogel dressings in the presence of a copious quantity of exudate. Hydrogel is introduced into a stoppered flask containing phosphate-buffered saline (PBS) and allowed to stand for 2 hours, after which it is inspected, and an assessment is made to determine whether the dressing is soluble, dispersible (remains in two distinct phases), or if the dressing retains its original structure. For the swelling test, the hydrogel sample was immersed in distilled water for 2 hours, and then the swelling ratio was determined according to the equation WS-WD/WD×100, where WS and WD refer to the weight of the gel after and before swelling, respectively.

Hemolytic Properties of Hydrogel Dressings

This procedure provides a protocol for assessing the hemolytic properties of the dressing. The test method was based on ISO 10993-4 as follows: For complete lysis, blood samples were lysed with water as the positive control, while the negative control consisted of blood incubated in PBS alone. Commercial hemostatic samples made from cellulose and composite materials, such as kaolin and chitosan, were selected for comparison. An extract of 30 mg from each sample was prepared according to ISO 10993-12, and 500 microliters of blood was added to each tube. The tubes remained in a suitable test tube rack for at least 3 hours at 37±2°C in a water bath. The absorbance of the solution was read with a spectrophotometer at a wavelength of 540 nm. The percent hemolysis is calculated by correcting for the background from the blank sample:

$$\text{Hemolysis\%} = \frac{\text{Sample Absorbance - Blank Absorbance}}{\text{Test Blood Absorbance - Blank Absorbance}}$$

In Vitro Analysis of Red Blood Cell Adhesion

To investigate the adhesion of red blood cells to the dressing, 100 mg of hydrogel was placed in a glass tube. Next, 200 microliters of fresh blood containing an anticoagulant were added, and the tube was incubated at 37 ºC for 1 hour. To remove any non-adherent red blood cells, the dressing was washed three times with PBS solution. The adhesion of the red blood cells was then examined using a scanning electron microscope (SEM).

Cytotoxicity Assay

Cell culture: L929 mouse fibroblasts were obtained from the National Cell Bank of Iran. Dulbecco's Modified Eagle's Medium supplemented with L-glutamine (4 mM), penicillin (50 units/ml), streptomycin (50 mg/ml, Sigma-Aldrich), and 10% (v/v) fetal bovine serum (Invitrogen, CA, USA) was used for culturing cells in a 5% CO2 humidified incubator at 37 ºC.

MTT assay: Methyl thiazolyl diphenyl-tetrazolium (MTT) reagent was used to detect the cytotoxicity of the hydrogel dressing. Test sample extract was prepared in 100 µl treatment medium based on ISO 10993-12. 1×10^4 cells/well were seeded in a 96-well microtiter plate and cultured overnight in a 5% CO2 humidified incubator. Different concentrations of the sample extract (100%, 50%, 25%) were added to the cells. After 24 and 48 h of incubation, the medium was replaced with DMEM medium plus 10% (v/v) MTT reagent (5 mg/ml). After 4 h of incubation, the medium/MTT was removed, and 100 µl of DMSO was added to dissolve the formazan crystals. The plates were read in a microplate reader (ELx800, Biotek, USA) at 570 nm.

Hemostasis in Rat Model

Twenty-four healthy Wistar rats, weighing between 200-250 g, were obtained from the Royan Institute. The rats were specific pathogen-free and housed in a controlled environment (21°C, 12 h day/night cycle). Additionally, the rats had sufficient access to food and water. Ketamine and xylazine, in a ratio of 5:1, were used as anesthetics. All experiments were approved by the Institutional Animal Care and Use Committee of Tarbiat Modares University of Medical Sciences. The injury was made in the lambda region, which is the intersection of the transverse and sagittal sinuses. This region has a

dense network of blood vessels that supply the brain. After administering anesthesia, the animal's head was shaved and secured in a prone position using standard stereotactic instruments. The scalp was then removed, and the lambda area was identified. An electric drill was used to create a hole with a diameter of 2 mm. Bleeding in the control group was examined using a sterile gauze pad. In the other groups, hydrogel dressing and commercial hemostatic agents such as Surgicel were used to control bleeding. The main parameters measured included the volume of blood lost and the duration of the bleeding.

RESULTS

Solubility/ Swelling of hydrogel dressings

Given the significant volume of bleeding in neurosurgery, it is crucial to preserve the integrity of hemostasis during the procedure. Hemostasis should not be disrupted or rapidly separated at the bleeding site. In fact, hemostats must prevent hemorrhage while maintaining visibility of the operative field for up to one hour during surgery. This test is based on EN 13726-1 to determine the dispersion/solubility of the hydrogel in the presence of PBS buffer for two hours. As shown in Figure 1 (A, B), the hydrogel did not dissolve and retained its original structure. The degree of swelling of the gel was less than 10%, which reduces the risk of obstruction of the neural tissues.

A)

B)

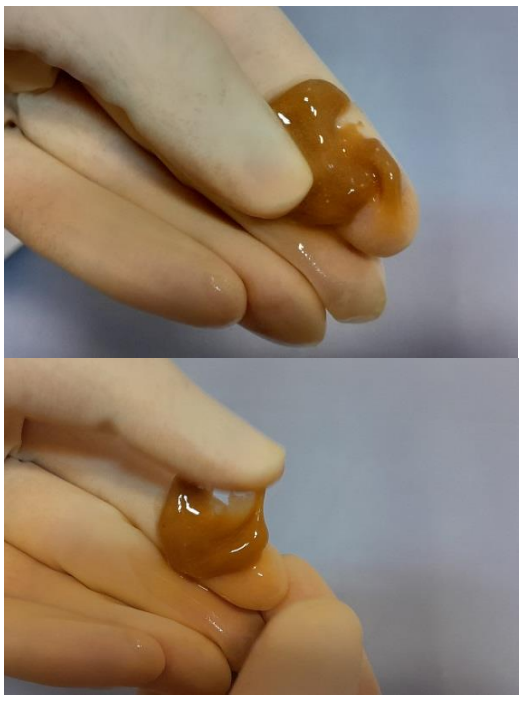


Figure 1: Gel dressing before (A) and after in the PBS solution for 2 hours (B)

Red blood cells adhesion and hemolytic properties of hydrogel dressings

As seen in the scanning electron microscope images in Figure 2A, the red blood cells are significantly adhered to the chitosan hydrogel. As shown in Figure 2B, the hemostatic cellulose (Asa Pharma) exhibited the highest hemolysis at 12% compared to other hemostatic materials. The hydrogel also demonstrated approximately 4.5 to 5.2% hemolysis, which is similar to the chitosan-based hemostatic agents. This indicates that hemostatic agents based on chitosan have better blood compatibility compared to cellulosic materials.

Cytotoxicity assay

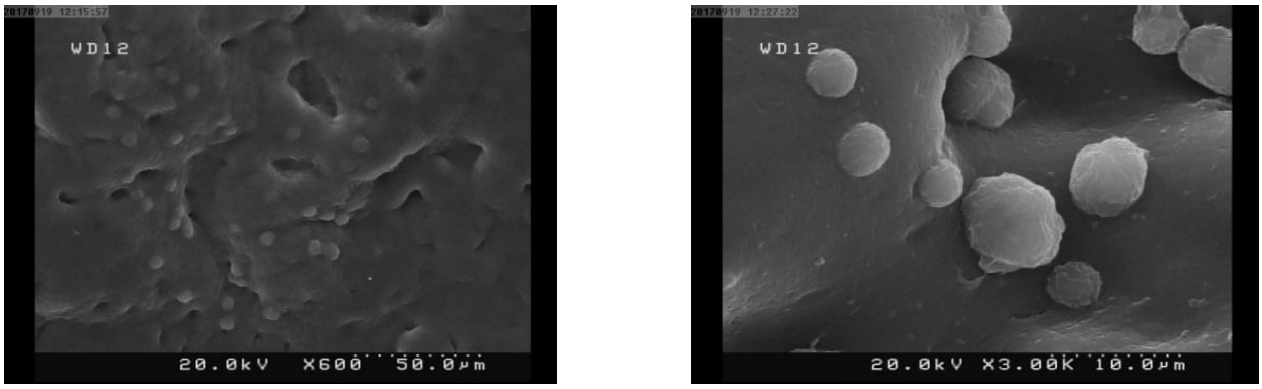
The most important parameter of an ideal biological material is its non-toxicity. To investigate this parameter, cell toxicity is assessed by calculating the viability percentage of living cells using the colorimetric method, which is based on the reduction of the MTT reagent. The results presented in Figure 3 indicate that the viability of the hydrogel at various concentrations after 24 and 48 hours is higher than 89%, demonstrating good biocompatibility. These results align with previous studies regarding the non-toxicity of the natural polymer chitosan. Furthermore, the statistical analysis indicates that there is no significant difference between the control group and the chitosan hydrogel.

Hemostasis in Rat Model

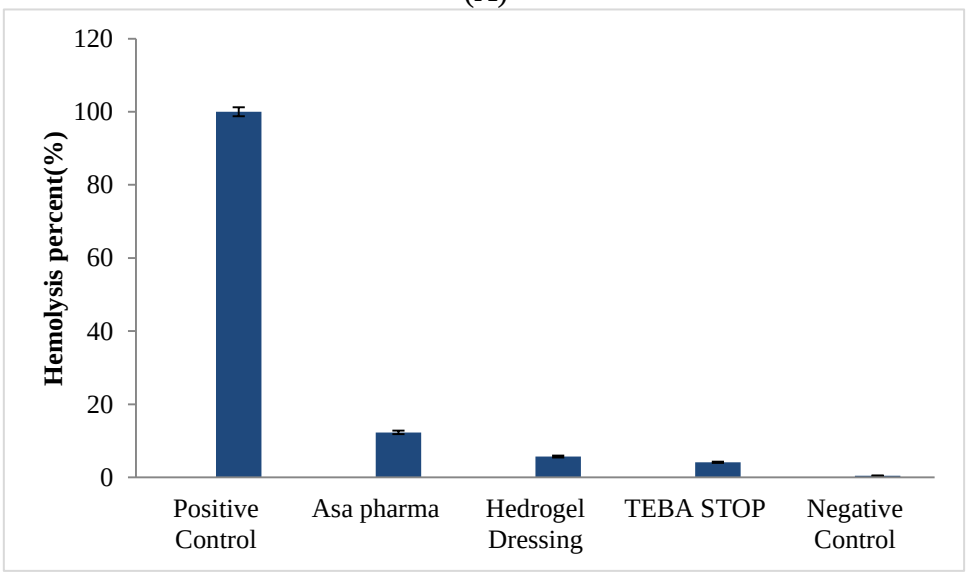
To evaluate the hemostatic efficiency of chitosan hydrogel, an injury was made in the lambda region, which has a dense network of blood vessels. The volume and time of blood loss were recorded as key parameters. As shown in graph 4B, the control group exhibited a blood loss of 1200 microliters over 179 seconds, while Surgicel and chitosan hydrogel resulted in blood losses of 400 and 100 microliters within 58 and 36 seconds, respectively.

DISCUSSION

Hemostasis is a critical step in every surgical procedure. In particular, in neurosurgery, effective hemostasis can significantly lower the risk of perioperative complications. Over the years, various materials and medical devices have been developed to ensure and improve proper hemostasis. Bone wax, gelatin sponge, fibrin sealant, oxidized cellulose, microfibrillar collagen, gel foam, tranexamic acid, and platelet-rich



(A)



(B)

Figure 2: Scanning electron microscope images of red blood cells adhered to the chitosan hydrogel (A) and the hemolysis graph f hemostatic materials(B).

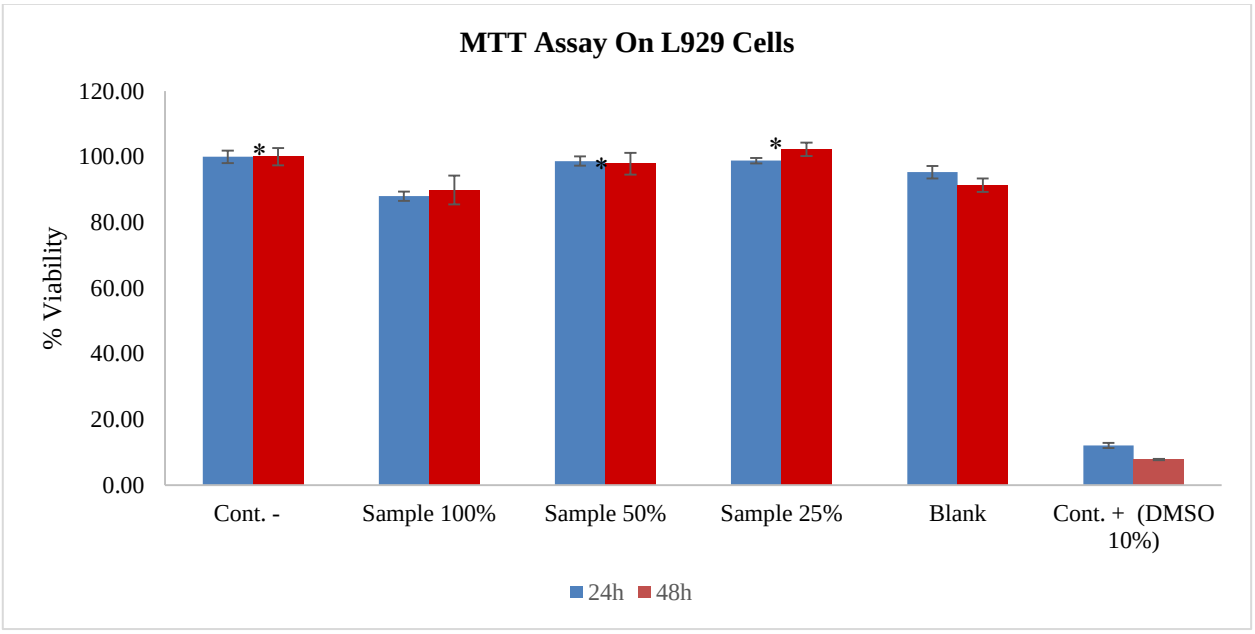
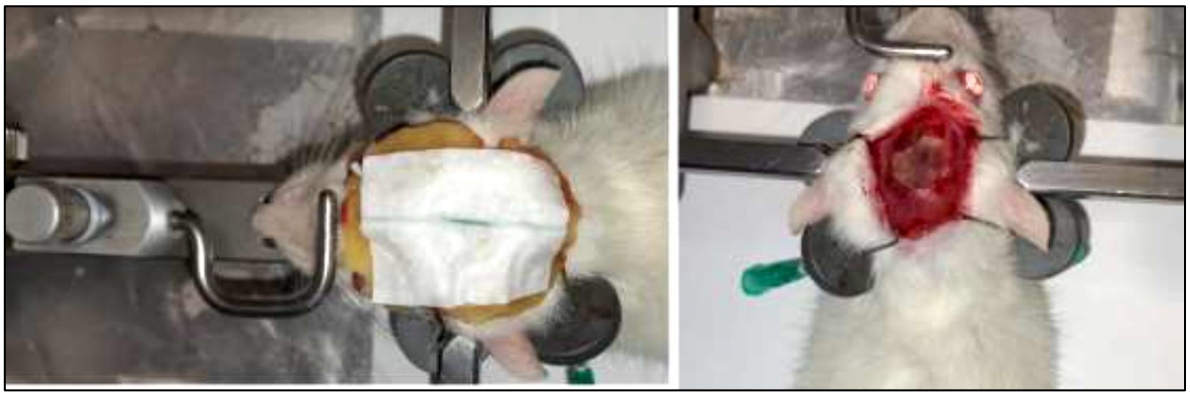
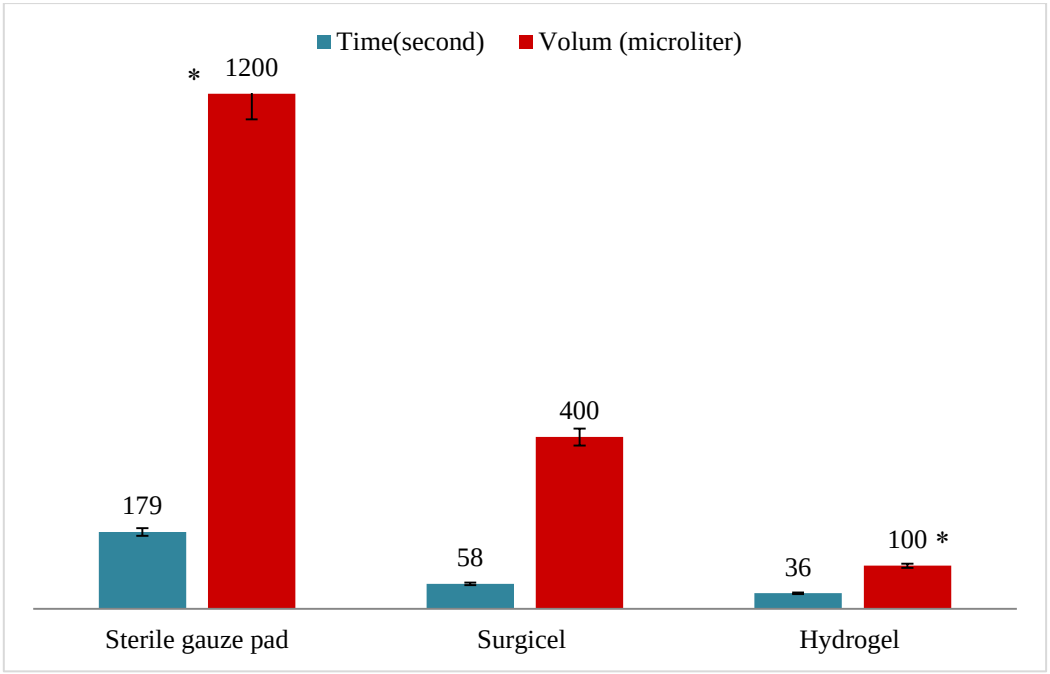


Figure 3: The viability of the hydrogel at various concentrations after 24 and 48 hours.* $p < 0.05$



(A)



(B)

Figure 4: Photograph of drill site for rat neurosurgical model
(A) and The mean bleeding time and the mean blood loss (B). * $p < 0.05$

plasma have been proposed as the main agents capable of promoting and ensuring good hemostasis in neurosurgical operations. While commercially available haemostats have been approved for use in neurosurgery, there are some caveats to their application in the brain. These include localized neural compression, potential immune reactions, and the risk of thrombus formation. Therefore, there is a need for hemostats that are both effective and safe for use on brain and spinal tissue. Chitosan is a naturally occurring biopolymer that has been shown to accelerate haemostasis. These findings have led to the development of several chitosan-based hemostats, all of which have demonstrated safety for human use in peripheral tissue. While clinical data on the use of chitosan-based haemostats in the brain is limited, various in vivo studies have demonstrated that chitosan is both effective and safe for managing neurosurgical bleeding (24). Brandenberg et al. investigated the efficacy of gel chitosan in small vessel bleeding in the cat brain (24-25). The study showed that using chitosan led to quicker haemostasis compared to standard gauze. All animals survived without any complications or adverse events. The histopathological examination of the brain revealed no inflammation in the nervous tissue (24-25). Sandoval-Sanchez et al. studied the use of chitosan film in duraplasty in a rabbit model (24,26). Chitosan was compared to a collagen matrix, and the results indicated that there were no significant clinical or histological differences between the two groups. In vitro testing showed that chitosan has less swelling than the collagen matrix and reduces the risk of neural compression

(26). Another study conducted by Pogorielov et al. demonstrated that the use of graft chitosan in a rabbit model resulted in reduced inflammation and adhesion formation (27). Rajiv et al. investigated the efficacy and safety of using chitosan gel and gelfoam in managing bleeds in a burr hole neurosurgical sheep model. The study revealed no significant differences in hemostasis time, post-operative edema, or histological examination of the treatment site (28).

This study investigated the hemostatic efficacy and safety of chitosan hydrogel and compared it to the current dressing. The results demonstrate that the hydrogel has an improved hemostatic property compared to the standard dressing. As shown in Figure 2A, chitosan absorbs red blood cells and activates platelets, leading to a concentration of coagulation factors and resulting in clot formation. In addition, the paste/gel-like nature of the chitosan dressing allows it to effectively stop bleeding mechanically, which contributes to its superior performance compared to Surgicel. Furthermore, this unique nature makes the dressing less prone to being washed away, providing better tamponade. While in vivo studies on the use of chitosan-based hemostats in rat neurosurgical models are limited, Crofton et al. investigated and compared the hemostatic efficacy of beta-chitin patches, chitin derivatives, surgical, and FloSeal in cases of rat surgical brain injury (29). Similar to our cell toxicity results, the study demonstrated that chitosan-based haemostats are safe for application to animal central nervous tissue. In their research, no significant difference was found in the macrophage reaction between the beta-chitin patches and the standard options (29.30). The limitation of our study is the lack of further cytotoxicity analyses, such as skin irritation and sensitization, pyrogenicity, genotoxicity, and histological analysis, to find direct and detailed evidence of immune reactions, infection, and inflammation. According to global health protocols, testing is required for all medical devices. Therefore, we will be investigating these tests in our upcoming research.

CONCLUSION

This study investigated the hemostatic efficacy and safety of chitosan hydrogel compared to current dressings. The results demonstrate that the hydrogel exhibits improved hemostatic properties and biocompatibility. Our findings, along with existing literature on chitosan-based hemostats, suggest that chitosan is a superior agent. These results indicate that the safety and enhanced efficacy of chitosan-based hemostats make them a potentially suitable option for use in neurosurgical settings. However, further research is needed to explore the clinical application of chitosan-based hemostats within the central nervous system.

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DECLARATIONS

All authors have participated in the conception and design, as well as the analysis and interpretation of the data. We approve the final version and declare that we have no conflict of interest.

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