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RESEARCH ARTICLE

Toxicity and hemostatic potential of poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine] based hemostatic material on albino rabbits

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Abstract

The present study was designed to evaluate the hemostatic potential of poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine]-based hemostatic dressing material on albino rabbits. In vitro cytotoxicity study of poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine]-based hemostatic dressing samples was carried out with L929 cells, and the cytotoxic potential was evaluated at the end of 24 h. The skin irritation was carried out in albino rabbits. Extract of the material was applied topically and irritation response was evaluated up to 72 h. The hemostatic study was initiated in rabbits after general anesthesia with a mixture of ketamine and xylazine. Using a sharp surgical blade, a 1.0 cm longitudinal incision was made on the right (test) and left (control) marginal ear arteries. Through the resultant jet spray of blood, the right 1.0 cm long wound was immediately covered with a 2×2 cm^2 piece of test material (poly [ß-(1,4)-2-amino-2-deoxy-D glucosamine] of known weight (w1). Similarly the left wound (1.0 cm length) was covered with commercially-available bandage (control) of known weight (w2). Direct pressure was applied for 2 min and then the samples were removed and weighed immediately (w3 for test and w4 for control) after hemostasis. Blood loss (w3–w1 for the Test and w4–w2 for control) was calculated from the materials weight before and after absorbing blood. The result of the study indicated that the indigenously developed material has local biological activity in the form of hemostatic action and, together with its ability to activate macrophages, resulted in wound healing applications. Hence, the present study concluded that the poly [ß-(1,4)-2-amino-2-deoxy-D glucosamine]-based hemostatic dressing material is non-toxic, non-skin irritant, and has better hemostatic potential than a commercially available material with enhanced hemostatic capabilities for various wound dressing.

Keywords: *Haemostatic; wound healing; skin irritation; cytotoxicity; SEM*

Introduction

Accidents and trauma-related injuries account for ~ 10% of deaths per year (Kumar et al. 2008; Institute of Road Traffic Education 2008). Uncontrolled bleeding from wound sites is a major cause of these preventable deaths. From a fatal wound; ~ 40 ml/min of blood is lost and; if it continues for 20 min; the victim dies of hemorrhagic shock. Bleeding to death before reaching a medical facility is common to battlefield injuries as well. Very often victims bleed to death due to lack of pre-hospital care; time; and distance to reach a hospital. Due to the absence of external hemostatic products; the primary intervention to stop bleeding still remains to be cotton gauze using pressure.

Hemostasis is the body's normal physiological response for the prevention and stopping of bleeding or hemorrhage by a mechanism involving blocking of any vascular break and thereby helps to ensure blood fluidity and blood vessel integrity. Abnormalities in hemostasis can result in bleeding and, with the advent of advanced hemostatic agents, traumatic hemorrhage can now be better controlled. It would be desirable that such hemostatic wound dressings also provide a barrier to the access of bacteria and be removable with a minimal trauma to the underlying neoepithelium and clot. Exsanguinating hemorrhage remains the most common cause of battlefield death after traumatic injury. The general practice is to compress extremity hemorrhage

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by applying gauze bandages to the wound. There have been many advances recently in hemostasis research that have stimulated interest in the potential for a field dressing that employs an additional hemostatic mechanism to combat this second most common cause of death from civilian trauma (Bellamy 1984; Sauaia et al. 1995; Peng and Shek 2009).

Winter initiated the concept of an active involvement of a wound dressing in establishing and maintaining of an optimal environment for wound repair. This awareness resulted in the development of wound dressings from traditional passive materials to functionally active dressings (Winter 1962). It was reported that chitin and chitosan accelerate wound healing, and remedies using chitin and chitosan in wound treatments have already been in the market (Pruden et al. 1970; Fukasawa et al. 1992; Shigemasa and Minami 1995; Rao and Sharma 1997; Xie et al. 2008). Chitosan, having hydrogel-forming properties, have been considered to be advantageous in their application as a wound dressing material (Coln et al. 1983; Muzzarelli 2002; Purna and Babu 2000; Garner and Brown 2002; Masayuki et al. 2002; Eduardo et al. 2008). It has been widely used around the world for biomedical applications due to its unique characteristics such as polycationic nature, biodegradability, bioadhesiveness, and non-toxicity.

It was reported that the liquid fibrin sealants have been in use for many years in general trauma and neurological surgery (Ochsner et al. 1990; Shaffrey et al. 1990). Similarly, alginates are pro-thrombogenic and have a platelet aggregatory action that can be modified by saturating the material with ionic zinc and optimizing the calcium concentration (Segal et al. 1998). Experimental trials of hemostasis in 2 mm deep surgically created buccal mucosal wounds in anesthetized dogs demonstrated the superior efficacy of alginate dressing compared to standard gauze (Matthew et al. 1994). Clinical trials have shown it to be effective as a hemostat in skin graft donor sites and tooth extraction sockets (Groves and Lawrence 1986). Kheirabadi et al. (2009) determined the efficacy of new hemostatic dressings in a model of extremity arterial hemorrhage in swine and was found that it was an effective dressing in arterial hemorrhage model. Briefly, the animals (swine model) were instrumented with arterial lines, baseline labs were drawn, and a standard wound model was used on each animal. The wound was a 6 mm diameter arteriotomy of the femoral artery. Each animal was allowed to bleed freely for 45 s and the amount of blood lost during this period was recorded. After 45 s the hemostatic agent was applied as directed by manufacturer instructions. Compression was applied for 2 min and then homeostasis was observed for 3 min.

Any material replacing natural tissue or coming into contact with surrounding soft tissues are generally considered to show a more severe inflammatory response. Hence, the biocompatibility of this material has to be evaluated in the soft tissue and cell culture environment as well. In the present study, an effort was made to evaluate the cytotoxic, skin irritation, and hemostatic potential of a newly developed, poly [ß-(1, 4)-2-amino-2-deoxy-D glucosamine]-based hemostatic dressing material.

Materials and methods

Reagents and materials

Chitosan, poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine] (Primex, Haugesund, Norway), Acetic acid (Merck, Mumbai, Maharastra, India), de-ionized water (Aqualab, Castle Hill, Australia), physiological saline (Fresenius Kabi, Tal.Shirur, Pune, India), autoclave (Reliance, Chennai, Tamilnadu, India), glass wares (Borosil, Mumbai, Maharastra, India), surgical blade (Magna Marketing, Panki, Kanpur, India), sterile surgical instruments, ketamine (Neon Laboratory Ltd, Andheri, Maharastra, India), xylaxin (Indian immunologicals Ltd, Hyderabad, Andra Pradesh, India), RPMI 1640 (Himedia, Mumbai, Maharastra, India), and fetal bovine serum (Sigma, St.Louis, MO, USA),

Preparation of test material

Poly [β-(1,4)-2-amino-2-2deoxy-D-glucosamine] is a natural biomaterial, polysachharide, isolated and purified from non-mammalian sources (Axio Biosolutions Pvt. Ltd., Ahmedabad, India). It comes under GRAS (Generally Recognized as Safe) category of USFDA. It has been widely used around the world for biomedical applications due to its unique characteristics such as polycationic nature, biodegradability, bioadhesiveness, and non-toxicity.

The material was prepared by homogenizing GRAS certified Chitosan, poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine] isolated and purified from non-mammalian sources in acetic acid, filtered through sieve mesh, and then lyophilizing in specially designed metal molds to produce a porous, flexible, interconnected dressing which is then cut and vacuum-packed. They are finally sterilized using gamma irradiation up to 15 kGy.

Both test (Batch No. H-061, Axio Biosolutions Pvt. Ltd, India) and control (Batch HC-07-116,07-117-03, Hemcon Inc., Portland, OR, USA) material were porous and are isolated from non-mammalian source.

Animals

New Zealand White rabbits were received from the Division of Laboratory of Animal Sciences, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (Thiruvananthapuram, Kerala, India). Rabbits of either sex weighing between 2000–3000 g from a single colony were used for skin irritation and hemostatic study.

Individual animals were identified with tattoo marks on ears. In addition to this, each animal cage was identified by labels having details such as study number, study name, animal number(s), date of experiment initiation, and experiment completion. All the animals were acclimatized for a period of 5 days before initiation of experiment.

The rationale for the selection of rabbits was mainly due to the availability and the fact that handling is very easy. Historical data related to the pathological and biochemical parameters of this species are well documented.

Animal husbandry and welfare

All animals were handled humanely, without causing pain or distress, and with due care for their welfare. The care and management of the animals will comply with the regulations of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Government of India. All the animal experiments were carried out after prior approval from Institutional Animal Ethics Committee and in accordance with approved institutional protocol.

Animals were maintained in a controlled environment with a temperature 22 ± 3°C, humidity of 30–70%, and a light/dark cycle of 12 h. The animals were provided with commercially available feed and aqua guard filtered fresh drinking water, ad libitum.

Methodology

Scanning electron microscopy (SEM)

The test sample poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine] was placed in the holder of a SEM microscope (ESEM EDAX XL-30) after processing for Scanning Electron Microscopy. The electron beam of 30 KV was bombarded under vacuum with a pressure of ~ 0.8 Torr. The detector used was a BSE (Back Scattered Electron) detector. Surface as well as vertical sections of 100 µm were imaged at 100×, 33×, 150×, and 250× magnifications. The pore diameter was measured with the help of inbuilt software.

In vitro cytotoxicity study

In vitro cytotoxicity studies of poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine]-based hemostatic dressing samples was carried out with the L929 (mouse fibroblast subcutaneous connective tissue) cell line procured from the National Centre for Cell Sciences (Pune, India). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, pencillin (100 IU/ml), and streptomycin (100 mg/ml). The culture was incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide (24–26 h) with a medium change at an interval of 3 days.

The extract of poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine] was prepared by incubating the material with physiological saline at 37°C for 24 h and diluted with medium containing serum to get an extraction ratio of 1.25 cm^2/ml. Phenol as negative and high density polyethylene (USP grade) as negative controls were used for the study. The culture medium and the specimen were removed and the cell cytotoxicity was evaluated qualitatively. The qualitative evaluation of cytotoxicity involves examining the cells microscopically to assess for changes in general morphology, vacuolization, detachment, cell lysis, and membrane. The change from normal cell morphology was recorded as none, slight, moderate, and severe, depending upon the extent of cell damage.

Skin irritation study

Healthy, smooth skinned rabbits with a body weight range between 2000–3000 g were used for the study. The fur on the back of the animal (dorsal side) was clipped closely and the skin ensured to be free of mechanical trauma or signs of irritation and disinfected with 70% alcohol. The test material, poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine]-based hemostatic sponge (6 cm^2) was moistened slightly with physiological saline and applied topically on the upper left side of the animal. Similarly, a sterile gauze piece (6 cm^2) moistened with physiological saline was applied on the lower left side of the animal. Application sites were covered with an occlusive bandage for 4 h. All the applied sites were observed at 1, 24, 48, and 72 h after removal of patches for the evidence of any tissue reactions. Grading of tissue reaction for erythema and edema was done and the primary irritation index was calculated by adding the scores of each animal and dividing the total by the number of animals (ISO 10993-10, 2002).

Evaluation of hemostatic potential

After general anesthesia with a mixture of ketamine and xylazine, six healthy, adult rabbits (2000–3000 g) were shaved and disinfected on both ears. Using a longitudinal incision, 1.0 cm wound segments were made on the right and left marginal ear arteries. Through the resultant jet spray of blood, the right wound were immediately covered with 2 × 2 cm^2 piece of poly [ß-(1,4)-2-amino-2-deoxy-D glucosamine]-based dressing of known weight (w1).

Similarly the left wound (1 cm) was covered with commercially-available bandage (control) of known weight (w2). Direct pressure was applied for 2 min and then the samples were removed and weighed immediately (w3 for test and w4 for control) after hemostasis. All the blood that oozed from the initial scissors cut on the arteries surfaces were absorbed by both types of materials (test and control). Hemostasis was regarded as complete when bleeding was no longer observed in the wound site. The time taken for hemostasis was noted. Blood loss (w3–w1 for the test and w4–w2 for control) were calculated from the materials weights before and after absorbing blood. The following parameters were evaluated from the study

1. Adherence strength of the dressing;
2. Time taken for complete hemostasis;
3. Survival of the animal and body weight;
4. Observation of re-bleeding;
5. Time taken for re-bleeding;
6. Absorption of blood by the dressing;
7. Pre-treatment blood loss; and
8. Animal blood pressure after experiment (Table 1).

Statistical analysis

All the results were expressed as mean ± SE. The total variation and difference among means were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc analysis. A *p*-value less than 0.05 were considered significant.

Results

Scanning electron microscopy

The SEM pictures of test material indicated that the pore size ranges from 30–180 µm. A cross-section shows the

Table 1. Hemostatic potential of test and control material.

Parameters	Test ($M \pm$ SE)	Control ($M \pm$ SE)
No. of animals	6	
Average weight of material (g)	0.1762 ± 0.0079	0.3175 ± 0.0134
Average weight of material with blood (g)	0.4472 ± 0.1190[a]	0.3922 ± 0.0147[b]
Blood absorbed (g)	0.2710 ± 0.1220	0.0747 ± 0.0108
Time taken for complete hemostasis (s)	207.50 ± 11.23	463.33 ± 99.26[c]
Number of vessels lacerated	1	1
Adherence strength of the dressing	Moderate	High
Time taken for hemostasis	2 mts	2 mts
Pre-treatment blood loss	0–1 drop	0–3 drops
Observation of re-bleeding	6–11 min	60 min later
Duration of re-bleeding (s)	30–60	30
Health of animal	Normal	
Survival of the animal	Yes	
Body weight at acclimatization (g)	2230.00 ± 160.89	
Body weight at experiment day (g)	2267.50 ± 162.23	
Body weight at end of experiments (g)	2279.33 ± 165.50	
Blood pressure after the experiment (mmHg)	105.4 ± 4.2/84.6 ± 3.0	
Hematological parameters of animals ($M \pm$ SE)		
Hemoglobin (g/dL)	12.95 ± 0.34	
RBC count ($\times 10^6$ per mm^3)	6.63 ± 0.41	
Total count ($\times 10^3$ per mm^3)	6.67 ± 0.32	
Clotting time (min)	0.279 ± 0.114	
Neutrophils (%)	40.83 ± 1.66	
Eosinophils (%)	2.67 ± 0.33	
Basophils (%)	3.00 ± 0.58	
Monocytes (%)	1.67 ± 0.56	
Lymphocytes (%)	51.83 ± 1.74	

[a] Statistical significance: Average weight of test material compared with average weight of test material with blood ($p < 0.05$).
[b] Average weight of control material compared with average weight of control material with blood ($p < 0.01$).
[c] Time taken for complete haemostasis of test material compared with control material ($p < 0.05$).

honeycomb structure, ensuring the interconnected pores with adequate pore size. This structure is formed due to the lyophilization process and helps in the absorption of blood (Figure 1). Figure 2 indicates the holistic view showing the clear interconnected pore structure.

In vitro cytotoxicity studies

Extract was prepared by incubating the test material with physiological saline at 37 ± 2°C for 24–26 h and diluting with medium containing serum to get an extraction ratio of 1.25 cm^2/ml. The extract was incubated after dilution with the L-929 cell lines for ~ 24–26 h and examined periodically. Along with this phenol was used as a positive control and high density poly ethylene as a negative control, respectively.

The results of the in vitro cell culture cytotoxicity revealed that the material, poly [ß-(1,4)-2-amino-2-deoxy-D glucosamine] was non-cytotoxic to L929 cell lines and suggested that no toxic leachables were leached out from the material to damage the tested cells. The cellular responses to high density polyethylene and negative controls were scored as 0

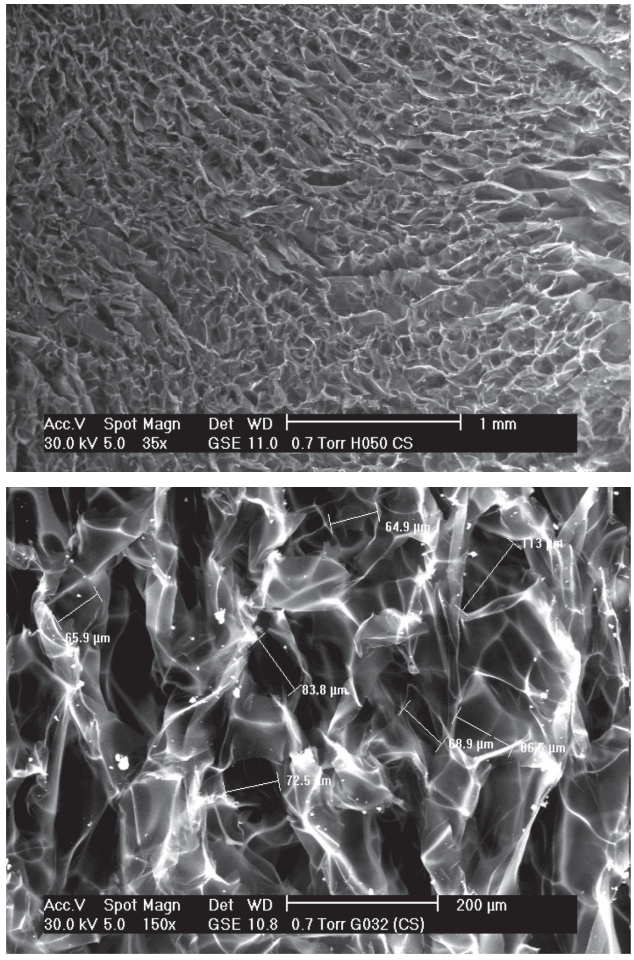

Figure 1. SEM image of the surface of test material.

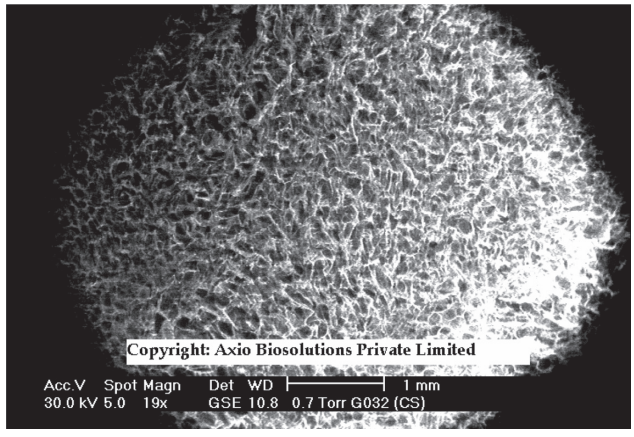

Figure 2. Holistic view showing the clear interconnected pore structure.

(non-cytotoxic) response, while the positive control scored 3, which is severely cytotoxic [data not shown] (Cytotoxicity scale: 0: no cytotoxicity, 1: slight, 2: mild, 3: moderate, and 4: severe (ISO10993-5, 1999).

Skin irritation

The general physical conditions of all the experimental animals were normal. The body weight and feed intake were normal and none of the animals showed any abnormality

or behavioral changes during the experimental period. The grading of erythema and edema of test and control sites for all animals at 24, 48, and 72 h were done at each observation period and recorded as shown in Table 2. The severity of mean scoring is negligible, slight, moderate, and severe when the mean score is 0–0.4, 0.5–1.9, 2–4.9, and 5–8, respectively. It was found that none of the animals showed any adverse skin reaction at any of the experimental time period. The primary irritation index was calculated by adding the scores of each animal and dividing the total by the number of animals and the score was 0.

Hemostatic potential

Hemostasis was regarded as complete when bleeding was no longer observed in the wound site. No clinical abnormalities observed in any of animals throughout the experiment period. The blood loss was calculated from the materials weights before and after absorbing blood (Table 1). The mean blood absorption capacity of the test material H-061 was found to be 0.2719 g, and that of the control HC was 0.0747 g. It was found that the average time taken for complete hemostasis was 207.5 s for the test material and 463.33 s for the control material HC (Table 1). Re-bleeding was observed in two animals treated with the test material H-061 (Rabbit ID-212 for 30 s duration and Rabbit ID 249 for 60 s duration) and in one animal treated with the control material HC (Rabbit ID-255 for 30 s duration).

The average body weight of the animals on the day of experiment was 2267 g and at the end of observation day was 2279 g. The blood pressure of the experimental animals after the experiment was also found to be normal. Pre-treatment hematologic data was taken from all the experimental animals and was under normal range, as mentioned in Table 1.

Discussion

The development of actively hemostatic wound dressings for use in severe trauma remains a major public-health and military goal. However, although some manufacturers claim that existing dressings activate platelets and/or blood coagulation, mechanistic evidence is often lacking (Jesty et al. 2009). The preparation of in situ-forming hydrogels, composed of oxidized dextran and amine-containing polymers, for their potential use as a wound dressing to promote blood clotting, was reported by Peng and Shek (2009). In the present study we report the hemostatic effect of poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine]-based material.

The SEM micrographs display the porous structure of the dressing material. This typical morphology is characterized by a 3D porous and sponge-like network structure with random pore formation. This morphology indicates that the porous structure is generated after the phase separation of the homogeneous chitosan solution during the freeze-drying process. The diameter of the macropores ranges between 47.2–156 μm and not exceeding 200 μm. The cross-sectional micrograph shows the formation pores by polymerization during the freezing process, though the pore size is random. These open pores aid in the process of better absorption of the blood during the application. SEM analysis reveals that the chitosan dressing is structured in a 3D macroporous network that offers good accessibility to the wound and better blood absorption. SEM image indicated the random, cross-linked pore formation in test material. This enables the test material to absorb body fluids like blood and traps the blood cells in it, thus creating a mechanical barrier for hemorrhage.

The biological response of materials and its leachables must be evaluated to determine their suitability for application in physiological systems. The basic in vitro tissue culture study assessed the cell morphology and viability when the cells come in contact with the material or its leachants. The results of the in vitro tissue culture study indicated that the material, poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine], has not adversely affected the general morphology, vacuolization, detachment, cell lysis, and membrane of the fibroblast cells, and confirmed that the material is non-cytotoxic. This suggests that the material did not induce any cytotoxicity just like that of negative control and the positive control showed a severe cytotoxicity.

The requirement of the skin irritation study was non-skin irritant, if the primary irritation index is 0.4 or less. It

Table 2. Scoring of skin irritant study of poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine].

			Observation at		
Sr. no	Animal no	Type of reaction	24 h	48 h	72 h
1	186 ♂	Erythema	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0
		Edema	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0
2	215 ♀	Erythema	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0
		Edema	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0
3	208 ♂	Erythema	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0
		Edema	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0	Test Site: 0 Control site: 0
Primary irritation score				0	
Primary irritation index				0	

was found that the test material, poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine], did not produce any irritation such as erythema, edema or necrosis at the end of 24, 48, or 78 h following the direct application on skin, and confirmed that the tested material is non-skin irritant. The results indicated that the test material did not produce any irritation following the direct application on skin (Table 2).

The hemostatic study was designed to evaluate the hemostatic potential of poly [ß-(1, 4)-2-amino-2-deoxy-D-glucosamine]-based hemostatic dressing (test sample code: H-061) in albino rabbits. All the animals were acclimatized under a controlled environment for 5 days. The health conditions and body weight of the animals were normal in all the days of acclimatization and observation period. Hematological parameters (hemoglobin, RBC count, total count, differential count, and clotting time) were monitored in all the animals and were found to be under normal range.

The results of the study were shown in Table 1. Re-bleeding was observed in three animals. The time taken for complete hemostasis of the test material (207.5 s) was less when compared with the control material (463.33 s). The results indicated that the hemostatic efficiency (absorption capacity) of the test material was higher, when compared to commercially available control material. There was a high adherence strength observed when exposed to control material, whereas the adherence strength of the test material was moderate. The results of the study indicated that the wound dressing material has not interfered with the normal physiological response of hemostasis, emphasizing that the material enhanced the platelet activation and aggregation, coupled with series of enzymatic reactions involving coagulation of proteins and produces fibrin to form a stable hemostatic plug. There was no occurrence of excessive bleeding in any of the experimental animals, which suggested that the adhered material never influenced against the coagulation factors. It was reported that chitosan and chitosan-coated mesoporous silica xerogel bead as a safe hemostatic system for injury (Baldrick 2010; Dai et al. 2010). Similarly, poly [ß-(1,4)-2-amino-2-deoxy-D glucosamine]-based material has local biological activity in the form of hemostatic action and, together with its ability to activate macrophages and cause cytokine stimulation, may result in wound healing applications. Hence, the present study concluded that the indigenously developed poly [ß-(1,4)-2-amino-2-deoxy-D glucosamine]-based hemostatic dressing material is non-toxic, non-skin irritant, and has better hemostatic potential than a commercially available material with enhanced hemostatic capabilities for various wound dressing.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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