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EVALUATION OF BIOCOMPATIBILITY AND PRELIMINARY HEMOSTATIC EFFICACY OF A CHITOSAN-BASED HEMOSTATIC SPONGE (FIELD HEMOSTATIC SPONGE) IN EXPERIMENTAL MODELS

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Summary

This study evaluated the biocompatibility and preliminary hemostatic efficacy of FHS, a chitosan-based material developed in Vietnam. Assessments included *in vitro* cytotoxicity, hemolysis, acute systemic toxicity, and *in vivo* hemostatic performance in mice. Cytotoxicity was evaluated on L929 fibroblasts using the MTT assay after exposure to FHS extracts in DMEM; Axiostat extract served as a reference. Acute systemic toxicity was assessed in mice via intraperitoneal injection of FHS extract with 72-hour observation. Hemolytic activity was examined using rabbit blood, with PBS and distilled water as negative and positive controls; hemolysis was measured at 545 nm and erythrocyte morphology was observed by SEM. Hemostatic efficacy was evaluated using a mouse tail amputation model based on bleeding time and blood loss. Results showed FHS was non-cytotoxic (85.32–89.47% viability), caused no acute toxicity, and had low hemolysis (1.83%) with normal erythrocyte morphology. It reduced bleeding time (60.6%) and blood loss (65.3%). No significant differences were found compared to Axiostat. FHS shows good biocompatibility and potential for preclinical and clinical hemostatic applications.

Keywords: *Chitosan-based hemostatic sponge; in vitro cytotoxicity; in vivo; Acute systemic toxicity; Hemolysis; Mouse tail amputation model.*

1. Introduction

Bleeding from trauma or surgical procedures is a leading cause of complications and mortality, especially in emergencies. Rapid, effective, and safe hemostasis remains a critical requirement in medical practice. In recent years, biologically derived hemostatic materials, particularly chitosan, have attracted significant attention due to their good biocompatibility, biodegradability, and potential to support coagulation [1],[2]. Chitosan-based field hemostatic sponges (FHS), a porous material first developed at the Vietnam–Russia Tropical Center [3], show potential for biomedical applications. However, systematic studies on the biocompatibility and hemostatic efficacy of this material in experimental models are still lacking. Therefore, this study was conducted to comprehensively evaluate the biocompatibility and preliminary hemostatic efficacy of FHS as a scientific basis for further research and applications. The specific objectives were to assess *in vitro* cytotoxicity and hemolysis, acute systemic toxicity, and preliminary hemostatic performance using a mouse tail amputation model.

2. Study Subjects and Methods

2.1. Materials, Subjects, and Research Equipment

2.1.1. Test Samples:



Fig. 1. Axiostat and FHS

FHS is a chitosan sponge (Degree of Deacetylation 73.6–87.7%) developed at the Vietnam–Russia Tropical Center, meeting CSQP: 2024/NĐVN [3], for topical hemostasis (8 × 8 cm, 2.8–3.4 g, sterile-packed). *Axiostat*® (India, 8 × 8 cm) was used as reference (Fig. 1). *Extracts* were prepared per ISO [4] (0.2 g/mL in 0.9% NaCl, 37°C, 24 h), filtered, and used fresh or stored at 2-8°C.

2.1.2. Experimental Animals:

White *Swiss* mice (20.0 ± 2.0 g) were used for acute systemic toxicity and preliminary hemostatic tests (Healthy mice of both sexes, with females confirmed to be non-pregnant). Whole blood from healthy adult white rabbits (2.8–3.5 kg) was used for hemolysis assays. Animals supplied by the Laboratory Animal Supply Unit of Vietnam Military Medical University were acclimatized under laboratory conditions for at least one week before the study, with free access to standard laboratory animal feed and boiled, cooled water. All animal procedures were approved by the Institutional Animal Care and Use Committee of Vietnam Military Medical University (approval No. IACUC-2007/23, dated July 20, 2023).

2.1.3. Equipment and Chemicals:

Equipment included CO_2 incubator, inverted microscope, SEM, refrigerated centrifuge, cold storage, liquid nitrogen, cell culture system, cell counter, ELISA reader, standard surgical tools, and other lab devices. Key reagents included DMEM, PBS, trypsin–EDTA, FBS, glutamine, penicillin, DMSO, MTT, L929 cells, saline, distilled water, and other consumables.

2.2. Research Methods

2.2.1. *In vitro* cytotoxicity evaluation of FHS:

Cytotoxicity was evaluated on L929 fibroblasts (ATCC® CCL-1™) using the MTT assay per ISO 10993-5 [5] and Ran Chen [6] to assess cell death and growth inhibition [7]. Cells (10^3/well) in supplemented DMEM were incubated with FHS extract for 24 -72h; negative control: DMEM, reference: Axiostat; Each group was repeated five times. Cell viability was measured at 570 nm and calculated as $(OD_t/OD_\beta) \times 100$, where OD_t and OD_β are mean optical densities of the test sample and negative control, respectively; >70% indicated non-cytotoxicity [5].

2.2.2. Acute systemic toxicity evaluation of FHS in mice:

According to ISO 10993-11 [8], acute systemic toxicity testing evaluates potential adverse effects following exposure to a test material and/or its extract for ≤24 h; this refers to the exposure period, not the subsequent observation period. A limit test was conducted in 10 white mice given a single intraperitoneal injection of undiluted FHS extract at an injection volume of 50 mL/kg body weight, in accordance with ISO 10993-11 [8]. The experimental procedure was adapted from Leilei Sun [9]. Clinical signs and behavior were assessed at 4, 24, 48, and 72 h after injection [8,9].2.2.3. Hemolysis evaluation of FHS:

Hemolysis was assessed by RBC membrane damage and Hb release [10],[11]. Anticoagulated rabbit blood (3.8% sodium citrate) was used with four groups: FHS and Axiostat (1×1 cm + 10 mL 0.9% NaCl), positive control (water), and negative control (PBS). After 30 min at 37°C, 0.2 mL of 2.25× diluted blood was added and incubated for 60 min; supernatants were centrifuged, absorbance measured at 545 nm, and samples observed by SEM (n = 10). Hemolysis (%) = (Dtn–Dca)/(Dcd–Dca) × 100%, where Dtn, Dca, and Dcd represent test, negative (0%), and positive (100%) controls [9]. SEM assessed RBC morphology and membrane integrity.

2.2.4. Preliminary hemostatic evaluation using a mouse tail amputation model:

Hemostatic efficacy was assessed using a mouse tail amputation model reflecting vasoconstriction, platelet aggregation, and coagulation [12],[13]. Mice were randomly allocated to three groups (n = 10 per group): medical gauze, FHS, and Axiostat as the

reference. The sample size was selected based on a similar study [14], considering biological variability and the 3Rs principle to ensure reliable results while minimizing unnecessary animal use [15]. After anesthesia, tails were disinfected (70% ethanol), transected 1 cm from the tip, immersed in 0.9% NaCl at 37°C, and the first blood drop was removed. A 0.5 × 0.5 cm material was applied and gently pressed, and blood was absorbed with pre-weighed gauze. Bleeding time (cessation ≥10 s; >6 min = failure) and blood loss (gauze weight difference) were recorded and compared with control and reference groups. After initial hemostasis was achieved, the wound was monitored for 30 min for rebleeding, followed by 24-h post-procedure monitoring. To minimize operator-related bias, experiments were performed by two trained operators using standardized, gentle pressure throughout.

2.3. Data analysis: Experiments had 5–10 independent replicates. Data were analyzed with Excel 2010 and SPSS 20.0, presented as mean ± SD or %. $p < 0.05$ was considered significant. Between-group comparisons were performed using Welch's independent-samples t-test.

3. Research Results

3.1. In vitro cytotoxicity evaluation results

After 24 h, L929 cells adhered and began proliferating. With an initial seeding of 0.3–0.4 × 10^6 cells/flask, cell coverage reached 25–30%, with the highest proliferation between days 2–5, and ~80% coverage by days 7–8, yielding ~10^6 cells/flask on average. For FHS, cell viability with extract exposure at 24–72 h ranged from 85.32–89.47%, comparable to Axiostat (83.49–92.11%), with no significant difference (p>0.05).

3.2. Acute systemic toxicity in mice

No clinical signs - reduced activity, dyspnea, abdominal pain, body weight loss, neurological or circulatory abnormalities, or death were observed in mice at 4–72 h after FHS extract administration (Fig. 3).

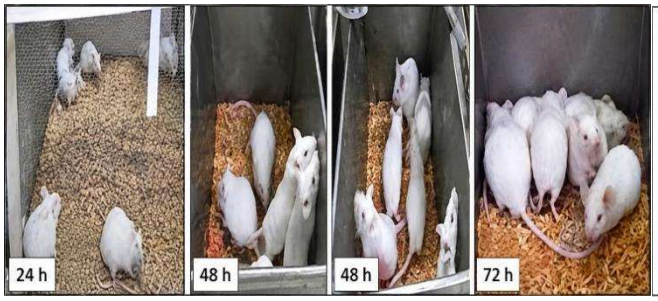


Fig. 2. a, b: L929 cells at seeding (0.3–0.4 × 10^6 cells/flask) covering 75% of the flask surface after 6 days of culture; c: Percentage of viable cells

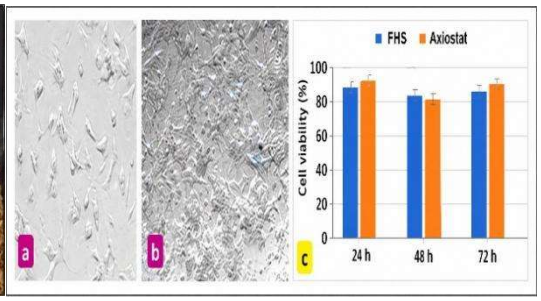


Fig. 3. Images of mice after acute systemic toxicity testing at 24–72 h

3.3. Hemolysis evaluation results of the field hemostatic sponge

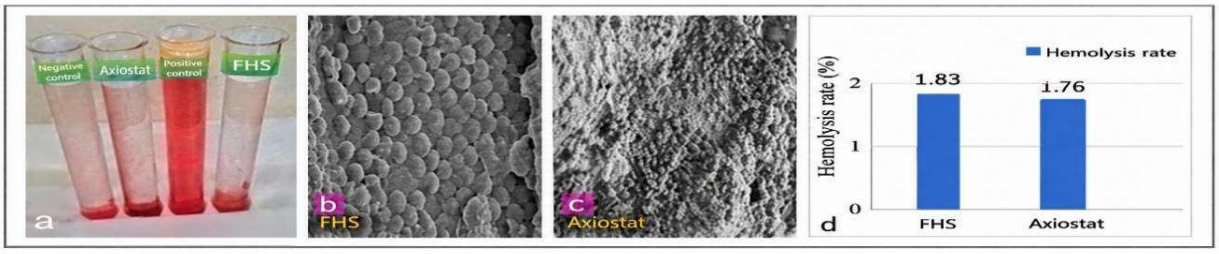


Fig. 4. Hemolysis results. a: Images of control and test groups in the hemolysis assay; SEM morphology of RBCs on b. FHS; c. Axiostat; d. Hemolysis of FHS and Axiostat.

SEM observations (Fig. 4 b, c) show RBCs on FHS and Axiostat retain near-round/disc shapes, uniform size, no spiking or strong shrinkage, no rupture, and no membrane debris, though many RBCs adhere to the material surface. Hemolysis rates of FHS and Axiostat were both <2% (1.83%; 1.76%), with no significant difference (p>0.05).

3.4. Hemostatic efficacy in the mouse tail amputation model

Table 1. Bleeding time and blood loss in the mouse tail amputation model

Hemostatic material	**Bleeding time** (s)		**Blood loss** (mg)	
	Mean ± SD	% reduction	Mean ± SD	% reduction
Gauze	98.7 ± 6.7		95.8 ± 7.4	
FHS	38.9 ± 7.3[a]	60.6	33.2 ± 5.6[a]	65.3
Axiostat	40.1 ± 7.2[a]	59.4	33.9 ± 6.6[a]	64.6
	*Note: [a] p <0.001 vs. gauze			

Results in Table 1 show FHS reduced bleeding time to 38.9 s (60.6%) and blood loss to 33.2 mg (65.3%) versus gauze (p < 0.001). Values were comparable to Axiostat (p >0.05).

4. Discussion

4.1. In vitro cytotoxicity evaluation of FHS on L929 cells

L929 cells grew stably, adhered well, and reached suitable density (Fig. 2 a, b). Exposure to FHS extract maintained cell viability >85% at 24–72 h, exceeding the 70% threshold of ISO 10993-5 [5], indicating non-cytotoxicity (Fig. 2c) and comparable to Axiostat (p>0.05). Similar to previous studies on chitosan-based materials showing >85–90% cell viability [6],[9],[16], FHS demonstrates good *in vitro* cytocompatibility, and it is non-cytotoxic.

4.2. Acute systemic toxicity of FHS in mice

No mortality or abnormal clinical signs were observed in mice at 4, 24, 48, and 72 h (Fig. 3). According to ISO 10993-11 [8], FHS shows no acute systemic toxicity. Recent studies also report chitosan-based materials as non-toxic in mice [17], and a systematic review indicates chitosan nanoparticles have low acute toxicity, with LD_{50} typically > 5000 mg/kg [18].

4.3. Hemolysis evaluation of field hemostatic sponge

FHS showed a hemolysis rate of 1.83% (<2%), meeting the non-hemolytic criterion of ISO 10993-4 [10]. SEM images revealed normal RBC morphology with uniform size and no deformation or rupture (Fig. 4). Although RBC adhesion to the material surface was observed, no morphological damage occurred, indicating no adverse effect on cell integrity.

Compared to Axiostat, hemolysis was similar (1.83% vs. 1.76%, p > 0.05) with no morphological differences. Previous chitosan-based materials, despite variations in form and composition, including $CaCO_3$ [19] and sodium hyaluronate [6], consistently showed low hemolysis (<5%) using *in vitro* Hb release assays, meeting ISO standards. These results confirm that FHS has good blood compatibility for hemostatic applications.

4.4. Primary hemostatic efficacy in the mouse tail amputation model

The mouse tail amputation model is a simple, low-cost *in vivo* method. FHS reduced bleeding time by 60.6% and blood loss by 65.3% vs. gauze (Table 1), consistent with chitosan-based materials reducing time from 168 s to 67 s and blood loss from 175 mg to 100 mg [14]. The effect is due to chitosan's rapid blood absorption, cell/protein concentration, and porous scaffold stabilizing clots [20]. Efficacy was comparable to Axiostat, showing high potential for local bleeding control.

5. Conclusion

FHS is non-cytotoxic *in vitro* shows no acute systemic toxicity in mice, and is non-hemolytic, with cell and blood compatibility comparable to Axiostat, meeting ISO 10993-4, -5, and -11 standards. In the mouse tail amputation model, FHS significantly reduced bleeding time and blood loss, with efficacy similar to Axiostat, consistent with the hemostatic mechanism of chitosan-based materials. FHS demonstrates good biocompatibility and effective local hemostasis, supporting further investigation of its hemostatic performance in larger animal models and preclinical studies.

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