ORIGINAL ARTICLE (EXPERIMENTAL STUDY)

Chitosan and nanochitosan haemostatic agents attenuate haemorrhagic coagulopathy and fibrinolysis: a rabbit study

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Abstract

Background. Uncontrolled haemorrhage is the leading cause of potentially survivable death in combat casualties.

Aim. To compare the effects of a chitosan-based and a nanochitosan-based haemostatic agent with a reference agent on coagulation, fibrinolysis, platelets and angiogenic growth factors in an experimental haemorrhage model.

Methods. Thirty adult rabbits (2.1–2.3 kg) were randomly allocated to five groups (n = 6): intact, untreated haemorrhage (control), reference agent, chitosan and nanochitosan. Blood loss, bleeding time, prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), international normalised ratio (INR), fibrinogen, D-dimer, platelet count, vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) were measured 1, 3, 12, 24 and 72 h after injury.

Results. In untreated animals, blood loss was 8.27–8.50 mL and bleeding time 19.8–23.6 min. Haemorrhage prolonged PT (12.83 to 16.55 s) and APTT (36.83 to 64.5 s), increased D-dimer more than fivefold (0.25 to 1.33 µg/mL) and reduced platelets (333.3 to 237.2 × 10^3/µL) by 72 h. All agents attenuated these changes, most markedly nanochitosan: PT was 9.4% and 11.0% shorter than in controls at 1 and 72 h, APTT 12.2% and 12.7% shorter, D-dimer 2.2-fold lower (0.47 and 0.60 vs. 1.03 and 1.33 µg/mL) and platelets higher (278.5 and 280.0 vs. 249.0 and 237.2 × 10^3/µL). Haemorrhage increased VEGF (97.5 to 189.7 ng/mL) and TGF-β (72.7 to 267.5 pg/mL); the rise was smallest with nanochitosan (152.8 ng/mL and 238.5 pg/mL at 72 h).

Conclusion. In this rabbit model, nanochitosan attenuated haemorrhage-associated coagulopathy, hyperfibrinolysis and thrombocytopenia more effectively than conventional chitosan or the reference agent. Confirmation of local haemostatic efficacy and thrombotic safety in standardised large-animal models is required before clinical use.

Keywords: *Blood Coagulation; Chitosan; Hemorrhage; Hemostatics; Nanoparticles; Wounds and Injuries.*

MAIN TEXT

Introduction

Haemorrhage accounts for about 90% of potentially survivable deaths on the battlefield [1] and for 30–40% of all trauma mortality [2]. Bleeding is aggravated by acute traumatic coagulopathy, which is present on admission in about one-quarter of severely injured patients and is associated with higher mortality [3, 4]; hyperfibrinolysis and consumption of platelets and coagulation factors are central to its development [3]. Early control of external bleeding at the point of injury is therefore a priority of combat casualty care [5, 6].

Topical haemostatic dressings are a key element of this strategy [7]. Chitosan, a cationic polysaccharide derived from chitin, has been used in military dressings since the first chitosan bandages were deployed in Iraq and Afghanistan [8], and current chitosan gauzes show efficacy comparable to kaolin-impregnated Combat Gauze in preclinical and field studies [7]. Its positively charged amino groups bind negatively charged erythrocyte and platelet membranes, promoting aggregation and formation of a mucoadhesive seal that works largely independently of the coagulation cascade [9–12]. Reducing chitosan to the nanoscale increases surface area, wound contact and interaction with blood cells, and nanostructured chitosan materials have shown faster clotting and greater thrombin generation than conventional formulations in vitro and in small-animal models [13–17].

Most studies of haemostatic agents report local endpoints such as blood loss and time to haemostasis. Much less is known about how these agents influence the systemic response to haemorrhage, including coagulation parameters, fibrinolysis, platelet consumption and growth factors that govern subsequent tissue repair. The aim of this study was to compare the time course of these systemic markers after experimental haemorrhage treated with a chitosan-based agent, a nanochitosan-based agent and a reference agent.

Material and Methods

Animals and ethics

The study was performed on 30 clinically healthy adult rabbits (*Oryctolagus cuniculus*) weighing 2.1–2.3 kg. Before the experiment the animals were quarantined for 14 days, during which their general condition was monitored, and only healthy animals were included. They were housed under standard vivarium conditions (20–22 °C, relative humidity 50–60%, 12/12 h light–dark cycle) with an identical standard diet and free access to water. The study is reported with reference to the ARRIVE 2.0 guidelines [18].

Experimental groups and haemorrhage model

Animals were randomly allocated to five groups (n = 6 each): (1) intact animals without injury, which provided baseline physiological values; (2) control—experimental haemorrhage

without treatment, reflecting the natural course of the process; (3) reference group—haemorrhage treated with standard drug haemostatic therapy; (4) chitosan group—haemorrhage treated with a micronised chitosan-based agent; and (5) nanochitosan group—haemorrhage treated with a nanochitosan-based agent. In all injured groups, haemorrhage was induced with the same standardised surgical technique, and the biological material obtained during the experiment was used for laboratory analysis.

Outcome measures

Measurements were made 1, 3, 12, 24 and 72 h after injury. Blood loss was determined gravimetrically: blood flowing from the wound was collected with sterile cotton swabs until haemostasis, the swabs were weighed before and after use on an electronic balance (accuracy ±0.01 g), and 1 g was taken as 1 mL of blood. Bleeding time was measured with a stopwatch from injury to complete haemostasis, and the blood-loss index was calculated as blood loss divided by body weight (mL/kg). Body weight was measured on an electronic laboratory balance (accuracy ±1 g) in fasting animals at rest. PT, APTT and TT were determined in citrated plasma; INR was calculated as $(PT_{patient}/PT_{normal})^{ISI}$; fibrinogen was measured by the Clauss method and D-dimer by an immunoturbidimetric assay. Platelets were counted with an impedance haematology analyser (Mindray BC-2800, China). Plasma VEGF (ng/mL) and TGF-β (pg/mL) were measured by solid-phase sandwich enzyme-linked immunosorbent assay, with optical density read at 450 nm (Mindray MR-96A, China) and concentrations calculated from calibration curves. Skin and bone samples were stained with haematoxylin and eosin and examined at ×100 magnification.

Statistical analysis

Data are presented as mean ± standard error of the mean (M ± m). Statistical analysis was performed with Microsoft Excel and STATISTICA 10 (StatSoft, USA). Homogeneity of variance was checked with Fisher's F test and the shape of the distribution with the excess (kurtosis) criterion; differences between groups and between time points were assessed with Student's t test. Pearson's or Spearman's correlation coefficients were used depending on the distribution. $p < 0.05$ was considered significant.

Results

Haemorrhage model in untreated animals

In the untreated control group, blood loss was stable at 8.27–8.50 mL and bleeding time ranged from 19.8 to 23.6 min, with the longest bleeding time at 12 h; the blood-loss index peaked at 24 h (4.04 ± 0.06 mL/kg) (Table 1). Body weight increased transiently by 2.3% at 1 h, then fell by 3.2% at 24 h and recovered partly (−2.3%) by 72 h compared with intact animals. Haemorrhage caused a sustained hypocoagulable shift with consumption: PT rose from 12.83 ± 0.60 s to 15.58 ±

0.05 s at 1 h and 16.55 ± 0.04 s at 72 h, APTT from 36.83 ± 1.08 s to 60.17 ± 0.31 s and 64.5 ± 0.45 s, and INR from 1.07 ± 0.03 to 1.30. Thrombin time shortened from 14.0 ± 0.58 s to 12.53–12.90 s and fibrinogen increased by 21.3% at 1 h (3.82 ± 0.03 g/L) before declining to 3.55 ± 0.02 g/L at 72 h. D-dimer increased from 0.25 ± 0.04 µg/mL to 1.03 ± 0.02 µg/mL at 1 h and 1.33 ± 0.03 µg/mL at 72 h, and the platelet count fell from 333.3 ± 8.8 to 249.0 ± 1.0 and 237.2 ± 0.6 × 10 [3]/µL. VEGF and TGF-β rose progressively throughout the observation period (Table 1). All changes from intact values were statistically significant ($p < 0.01$).

Table 1. Bleeding, coagulation and growth factor parameters in intact animals and in untreated controls after haemorrhage (mean ± SEM; n = 6 per group).

Parameter	Intact	1 h	3 h	12 h	24 h	72 h
Blood loss, mL	—	8.27 ± 0.14	8.45 ± 0.16	8.50 ± 0.15	8.48 ± 0.17	8.30 ± 0.13
Bleeding time, min	—	20.5 ± 0.43	21.8 ± 0.50	23.6 ± 0.55	22.9 ± 0.48	19.8 ± 0.40
Blood-loss index, mL/kg	—	3.74 ± 0.05	3.84 ± 0.06	3.95 ± 0.07	4.04 ± 0.06	3.92 ± 0.05
PT, s	12.83 ± 0.60	15.58 ± 0.05	15.77 ± 0.06	16.05 ± 0.04	16.25 ± 0.04	16.55 ± 0.04
APTT, s	36.83 ± 1.08	60.17 ± 0.31	61.50 ± 0.43	62.58 ± 0.42	63.50 ± 0.43	64.50 ± 0.45
TT, s	14.00 ± 0.58	12.53 ± 0.04	12.60 ± 0.03	12.72 ± 0.02	12.82 ± 0.03	12.90 ± 0.05
INR	1.07 ± 0.03	1.24 ± 0.02	1.22 ± 0.02	1.23 ± 0.05	1.30 ± 0.01	1.30
Fibrinogen, g/L	3.15 ± 0.10	3.82 ± 0.03	3.88 ± 0.02	3.75 ± 0.02	3.65 ± 0.02	3.55 ± 0.02
D-dimer, µg/mL	0.25 ± 0.04	1.03 ± 0.02	1.09 ± 0.01	1.17 ± 0.02	1.25 ± 0.03	1.33 ± 0.03
Platelets, $\times 10^3$/µL	333.3 ± 8.8	249.0 ± 1.0	249.0 ± 0.7	246.3 ± 0.9	241.7 ± 0.7	237.2 ± 0.6
VEGF, ng/mL	97.5	149.7	158.8	170.8	179.7	189.7
TGF-β, pg/mL	72.7	227.5	235.0	247.5	257.5	267.5

All coagulation values in controls differed from intact values ($p < 0.01$). VEGF and TGF-β are group means. APTT = activated partial thromboplastin time; INR = international normalised ratio; PT = prothrombin time; TT = thrombin time.

Effects of haemostatic agents on bleeding and coagulation

All three agents shortened PT, APTT and TT and lowered INR compared with untreated controls at every time point, and nanochitosan produced the largest effect (Table 2; Figures 1 and 2). At 1 h, PT was 5.52%, 6.49% and 9.38% shorter than in controls with the reference agent, chitosan and nanochitosan, and at 72 h 6.47%, 7.86% and 11.00% shorter, respectively; the only

exception was nanochitosan at 24 h (4.49%). The APTT reduction with nanochitosan (12.20% at 1 h and 12.67% at 72 h) was about twice that with the reference agent and chitosan (5.5–6.8%). TT was 5.6–6.1% shorter with the reference agent, 7.9–8.9% with chitosan and 11.4–11.8% with nanochitosan than in controls. INR remained stable at 1.10 throughout with nanochitosan, 9.8–15.4% below control values. None of the agents restored PT or APTT to intact values within 72 h. Fibrinogen was higher than in controls in all treated groups, with the smallest increase in the nanochitosan group (4.00 g/L at 72 h vs. 4.22 g/L with chitosan and 4.40 g/L with the reference agent).

Table 2. Coagulation parameters in the treated groups after haemorrhage (group means).

Parameter / group	1 h	3 h	12 h	24 h	72 h
PT, s (control: 15.58–16.55)					
Reference agent	14.72	14.92	15.10	15.28	15.48
Chitosan	14.57	14.65	14.85	15.05	15.25
Nanochitosan	14.12	14.22	14.33	15.52	14.73
APTT, s (control: 60.17–64.50)					
Reference agent	56.83	57.67	58.67	59.67	60.67
Chitosan	56.08	57.83	58.83	59.83	60.83
Nanochitosan	52.83	53.33	54.33	55.33	56.33
TT, s (control: 12.53–12.90)					
Reference agent	11.77	11.85	11.97	12.07	12.18
Chitosan	11.42	11.58	11.67	11.80	11.88
Nanochitosan	11.08	11.13	11.22	11.33	11.43
INR (control: 1.22–1.30)					
Reference agent	1.21	1.09	1.16	1.17	1.20
Chitosan	1.10	1.10	1.12	1.15	1.17
Nanochitosan	1.10	1.10	1.10	1.10	1.10
Fibrinogen, g/L (control: 3.55–3.88)					
Reference agent	4.12	4.20	4.30	4.28	4.40
Chitosan	4.27	4.30	4.23	4.33	4.22
Nanochitosan	4.02	4.15	4.00	4.10	4.00

Values are group means (n = 6). Intact values: PT 12.83 s, APTT 36.83 s, TT 14.00 s, INR 1.07, fibrinogen 3.15 g/L. Abbreviations as in Table 1.

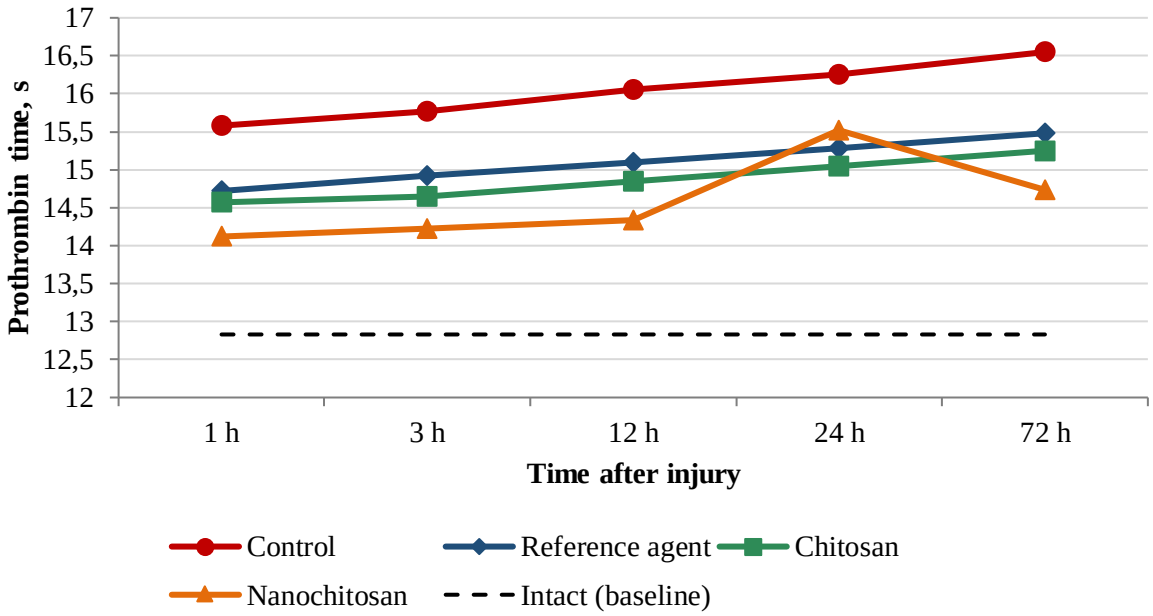


Figure 1. Prothrombin time after haemorrhage in untreated controls and in groups treated with the reference agent, chitosan and nanochitosan (group means; n = 6 per group). The black dashed line shows the intact baseline.

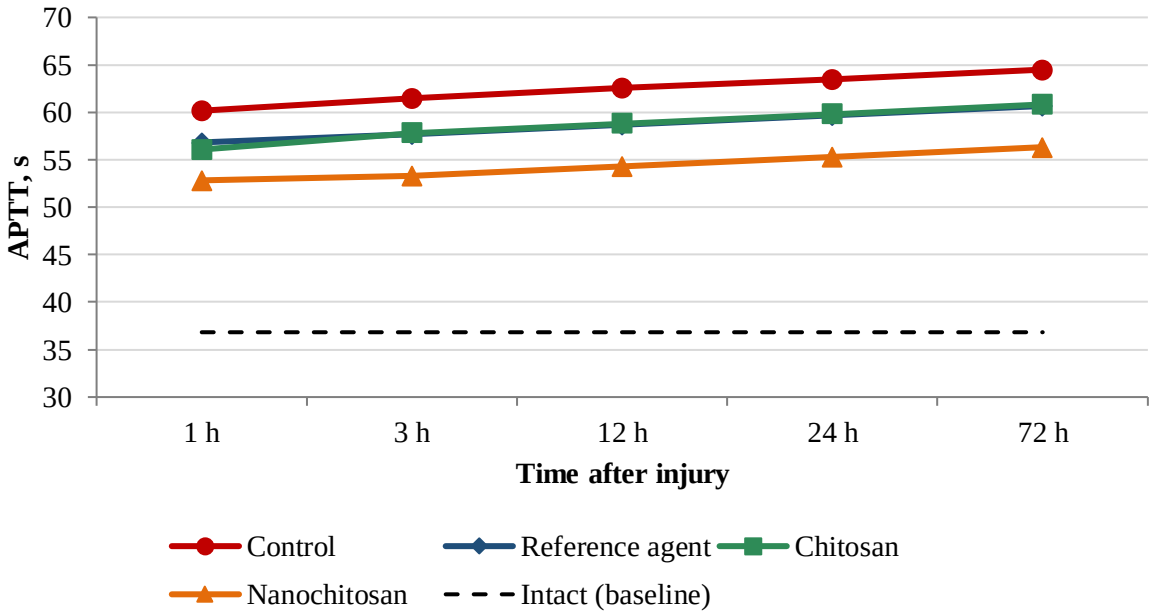


Figure 2. Activated partial thromboplastin time after haemorrhage in untreated controls and treated groups (group means; n = 6 per group). The black dashed line shows the intact baseline.

Fibrinolysis and platelets

All agents reduced D-dimer and preserved platelet counts compared with untreated controls (Table 3; Figure 3). With nanochitosan, D-dimer was 2.19-fold lower than in controls at 1 h (0.47 ± 0.02 vs. 1.03 ± 0.02 µg/mL) and 2.22-fold lower at 72 h (0.60 ± 0.04 vs. 1.33 ± 0.03 µg/mL), compared with 0.95 ± 0.05 µg/mL with chitosan and 1.13 ± 0.02 µg/mL with the reference agent at 72 h. Nanochitosan reduced D-dimer 1.4–2.2 times more effectively than chitosan and 1.7–2.5 times more effectively than the reference agent. The platelet count at 72 h was 280.0 ± 0.5 ×

10 [3]/μL with nanochitosan, 277.2 ± 1.2 with chitosan and 260.5 ± 0.6 with the reference agent, compared with 237.2 ± 0.6 in controls, but remained below the intact value (333.3 ± 8.8). Differences from intact animals and untreated controls were significant ($p \leq 0.001$).

Table 3. D-dimer and platelet count after haemorrhage in all groups (mean ± SEM; n = 6 per group).

Parameter / group	1 h	3 h	12 h	24 h	72 h
D-dimer, μg/mL (intact: 0.25 ± 0.04)					
Control	1.03 ± 0.02	1.09 ± 0.01	1.17 ± 0.02	1.25 ± 0.03	1.33 ± 0.03
Reference agent	0.89 ± 0.01	1.01 ± 0.01	1.10 ± 0.03	1.10 ± 0.04	1.13 ± 0.02
Chitosan	0.73 ± 0.02	0.80 ± 0.03	0.85 ± 0.03	0.90 ± 0.04	0.95 ± 0.05
Nanochitosan	0.47 ± 0.02	0.40 ± 0.03	0.50 ± 0.01	0.60 ± 0.01	0.60 ± 0.04
Platelets, ×10³/μL (intact: 333.3 ± 8.8)					
Control	249.0 ± 1.0	249.0 ± 0.7	246.3 ± 0.9	241.7 ± 0.7	237.2 ± 0.6
Reference agent	260.2 ± 0.9	255.2 ± 0.7	257.0 ± 0.7	258.2 ± 0.7	260.5 ± 0.6
Chitosan	265.8 ± 1.4	270.0 ± 1.3	272.8 ± 1.1	275.0 ± 1.2	277.2 ± 1.2
Nanochitosan	278.5 ± 1.0	273.0 ± 0.7	276.5 ± 0.7	277.5 ± 0.7	280.0 ± 0.5

All groups differed from intact animals, and all treated groups from untreated controls ($p \leq 0.001$).

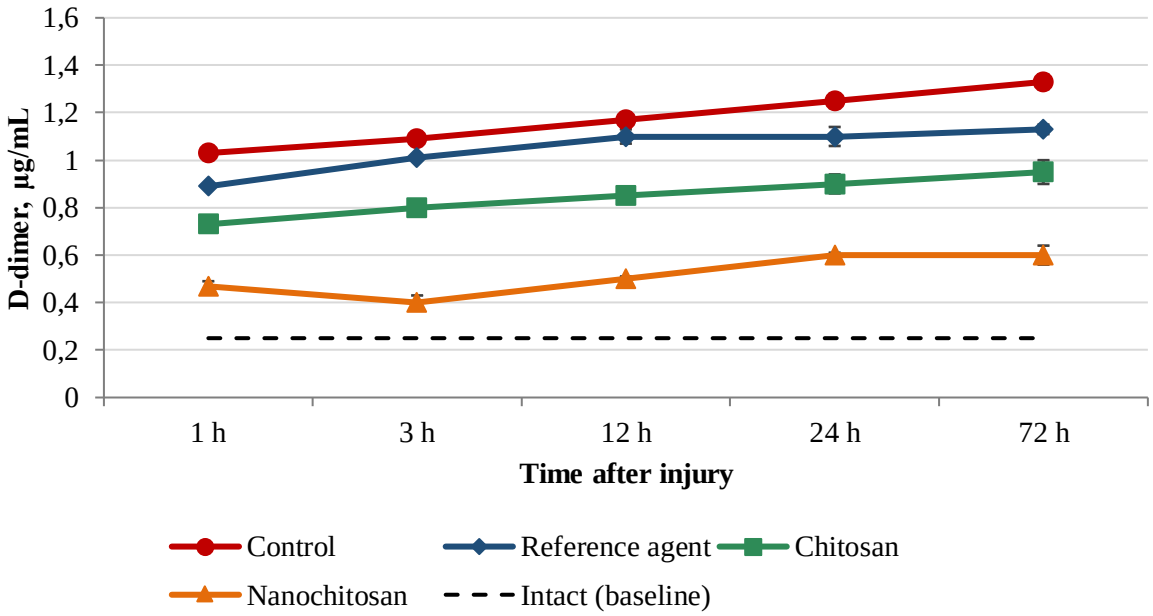


Figure 3. D-dimer concentration after haemorrhage in untreated controls and treated groups (mean ± SEM; n = 6 per group). The black dashed line shows the intact baseline.

Angiogenic and regulatory growth factors

Haemorrhage increased plasma VEGF from 97.5 ng/mL in intact animals to 149.7 ng/mL at 1 h and 189.7 ng/mL at 72 h, and TGF-β from 72.7 pg/mL to 227.5 and 267.5 pg/mL. Both growth factors were lower in all treated groups at every time point, with the lowest values in the nanochitosan group (Figure 4). At 72 h, VEGF was 168.0, 157.5 and 152.8 ng/mL and TGF-β 257.7, 248.5 and 238.5 pg/mL with the reference agent, chitosan and nanochitosan, respectively. Compared with controls, nanochitosan lowered VEGF 1.17–1.28-fold and TGF-β 1.12–1.15-fold. Histological examination of skin and cancellous bone (haematoxylin and eosin, ×100) showed preserved epidermal, dermal and trabecular architecture with zones of endochondral osteogenesis.

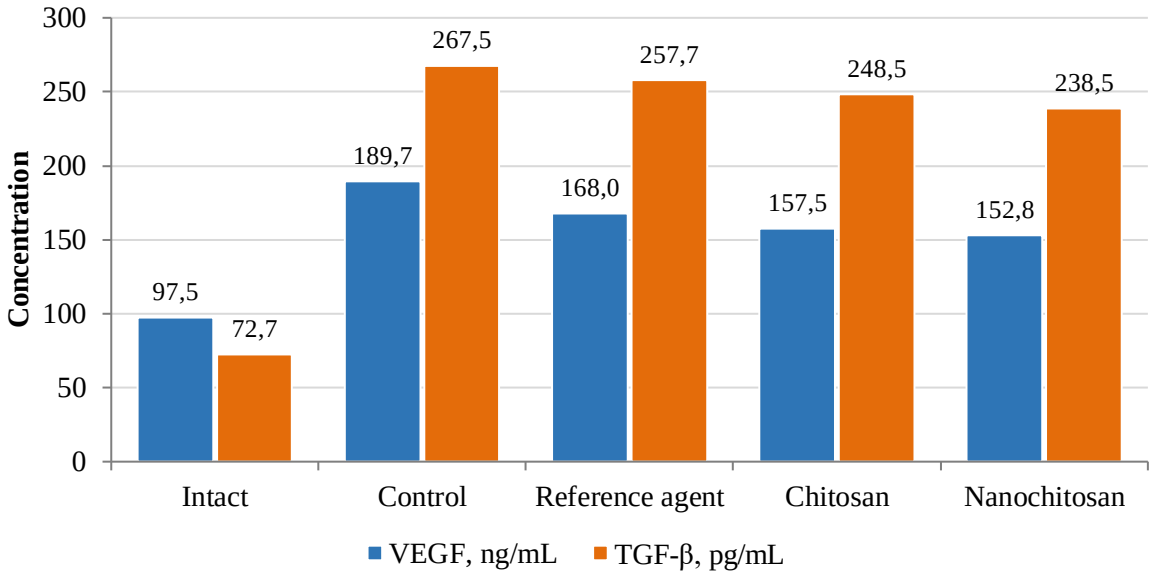


Figure 4. Plasma VEGF and TGF-β 72 h after haemorrhage in intact animals, untreated controls and treated groups (group means; n = 6 per group).

Discussion

This experimental study shows that haemorrhage in rabbits produces a reproducible systemic response: prolongation of PT and APTT, increased INR, a fivefold rise in D-dimer, a fall in platelet count of about 30% and a sustained increase in VEGF and TGF-β. All three haemostatic agents attenuated these changes, and nanochitosan had the largest and most consistent effect on clotting times, D-dimer and platelets.

The pattern in untreated animals resembles the early phase of trauma-induced coagulopathy, in which tissue injury and blood loss activate fibrinolysis and consume platelets and clotting factors [3, 4]. The early increase in fibrinogen and shortening of TT are consistent with an acute-phase response. Because the haemostatic agents were applied topically, their effects on systemic markers are most plausibly explained by faster and more stable local haemostasis, which limits further blood loss, consumption and fibrinolytic activation, rather than by a direct systemic action. This interpretation is consistent with the mechanism of chitosan, which aggregates erythrocytes and platelets through electrostatic interaction and adheres to wet tissue independently of the coagulation cascade [7, 9, 11]; this is also why chitosan dressings are expected to remain effective when the casualty's own clotting is impaired [7, 12]. Without local efficacy endpoints for the treated groups, however, this explanation remains indirect [19, 20].

The superiority of nanochitosan over conventional chitosan agrees with experimental data showing that nanoparticulate and nanofibrous chitosan increases thrombin generation and clotting efficiency because of its larger specific surface area, porosity and closer contact with blood components [13–15]. In our model, this translated into a halving of D-dimer and the best preservation of platelets, both of which are markers of reduced consumption and fibrinolytic

activation. At the same time, current evidence indicates that most modern haemostatic dressings perform similarly in standardised large-animal models of lethal arterial haemorrhage [21]; differences in systemic markers in a small-animal model therefore need confirmation with clinically relevant endpoints such as immediate haemostasis, re-bleeding, post-treatment blood loss and survival [22, 23].

Safety deserves equal attention. In all treated groups, TT was shorter and fibrinogen higher than in intact animals, indicating a procoagulant shift. Nanochitosan produced the smallest rise in fibrinogen, which may suggest a more balanced response, but thrombotic risk cannot be excluded from these data. Nanoparticles may also enter the circulation from large open wounds. Future studies should include markers of thrombin generation, viscoelastic testing, examination of distal vessels and organs for thrombi, and assessment of particle migration and biodegradation.

The rise in VEGF and TGF-β after haemorrhage reflects the activation of angiogenic and fibrotic repair programmes [24, 25]. The smaller increase in treated animals is consistent with a smaller injury signal. Both factors, however, are required for wound repair: VEGF drives angiogenesis and granulation tissue, and TGF-β coordinates inflammation, matrix deposition and remodelling, with excess TGF-β activity linked to fibrosis and hypertrophic scarring [24, 25]. Chitosan is also known to modulate inflammatory cells and fibroblasts during repair [26]. Lower circulating levels therefore cannot be interpreted as either better or worse healing without wound-level outcomes such as time to closure, granulation, vascular density and scar quality, which should be measured histomorphometrically [27].

From a military perspective, chitosan-based dressings are already part of tactical combat casualty care [7], and a domestically produced nanochitosan agent could improve availability and cost. The present results provide a rationale for further development, but they are a first step [28–30].

Strengths and limitations

Strengths of the study include a five-group design with intact and untreated controls and a reference group, repeated measurements over 72 h, and a broad panel covering coagulation, fibrinolysis, platelets and growth factors. Its limitations are important. Group sizes were small (n = 6), and no a priori sample size calculation was performed. The injury model, the physicochemical characteristics and dose of the chitosan agents and the composition of the standard therapy are described only in general terms, and blinding was not reported. Blood loss and bleeding time are reported for untreated animals only, so local haemostatic efficacy was not compared directly between agents. Repeated blood sampling from the same animals was analysed with t tests without correction for multiple comparisons, and dispersion statistics for treated groups are available only for D-dimer and platelets. The ELISA kits were not specifically validated for rabbit plasma. Finally,

the rabbit model of moderate haemorrhage does not reproduce the lethal junctional or arterial bleeding encountered in combat.

Conclusion

In an experimental rabbit model, haemorrhage caused prolonged clotting times, marked activation of fibrinolysis, thrombocytopenia and a sustained rise in VEGF and TGF-β. Chitosan-based haemostatic agents attenuated these systemic changes, and the nanochitosan formulation was the most effective, halving D-dimer and best preserving platelet counts. These findings justify further evaluation of nanochitosan as a topical haemostatic agent for combat injuries, with local efficacy endpoints, thrombotic safety assessment and testing in standardised large-animal models of lethal haemorrhage.

Additional information

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Conflict of interest. The authors declare no conflict of interest.

Ethical approval. The experimental study is reported with reference to the ARRIVE 2.0 guidelines. The animals were quarantined for 14 days and kept under standard vivarium conditions throughout the experiment.

Consent. Not applicable (animal study).

Author contributions. All authors contributed to the compilation and layout of this material.

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Data availability. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Generative artificial intelligence. During the preparation of this work the authors used Claude (Anthropic, https://claude.ai) in order to translate the dissertation material into English and to structure and edit the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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