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Functional improvement of hemostatic dressing by addition of recombinant batroxobin

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ABSTRACT

Although a number of natural materials have been used as hemostatic agents, many substances do not act quickly enough. Here, we created a novel dressings using collagen and chitosan with recombinant batroxobin (r-Bat) to promote faster and more effective hemostasis. We hypothesized that r-Bat would promote synergetic blood coagulation because it contains a blood coagulation active site different than those of collagen and chitosan. Our results suggest that each substances can maintain hemostatic properties while in the mixed dressings and that our novel hemostatic dressings promotes potent control of bleeding, as demonstrated by a whole blood assay and rat hemorrhage model. In a rat femoral artery model, the scaffold with a high r-Bat concentration more rapidly controlled excessive bleeding. This novel dressings has enormous possible for rapidly controlling bleeding and it improves upon the effect of collagen and chitosan used alone. Our novel r-Bat dressings is a possible candidate for improving preoperative care and displays promising properties as an absorbable agent in hemostasis.

Statement of Significance

Despite the excellent hemostatic properties of collagen and chitosan pads, they reported to brittle behavior and lack sufficient hemostatic effect within relevant time. Therefore, we created a novel pad using collagen and chitosan with recombinant batroxobin (r-Bat). r-Bat acts as a thrombin-like enzyme in the coagulation cascade. Specifically, r-Bat, in contrast to thrombin, only splits fibrinopeptide A off and does not influence other hemostatic factors or cells, which makes it clinically useful as a stable hemostatic agent. Also the materials in the pad have synergetic effect because they have different hemostatic mechanisms in the coagulation cascade. This report propose the novel hemostatic pad isreasonable that a great potential for excessive bleeding injury and improve effects of natural substance hemostatic pad.

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1. Introduction

Excessive hemorrhaging can result from serious accidents, preoperative measures for injury, and a variety of other reasons encountered in daily life [1,2]. Advanced topical hemostatic agents including bandages, fibrin glue, liquids, powder, gels, and scaffolding (also referred to as dressings) are used to control hemorrhages. Scaffolding used in conjunction with a number of agents can assist with effective control of bleeding while reducing or even stopping oozing after the hemostasis process. Furthermore, the dressing covers the injury site to prevent complications from infection. Many types of natural substances are used as scaffold dressing biomaterials in preoperative products and wound healing. Research suggests that collagen and chitosan are promising agents for use as hemostatic dressings due to their potential bioactivity in hemostasis.

Collagen, the main component of extracellular matrix, is used for a variety of applications in tissue engineering due to its high biocompatibility. Collagen is also known to enable platelet aggregation and releasing hemostatic factors that promote coagulation and formation of granulation tissue after blood coagulation [3–7].

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In blood coagulation, collagen induces platelet adhesion and assists in the formation of blebs with strong procoagulant activity [8,9]. In addition, collagen is extensively used in medical applications because of its low disease transmission hazard [4,10–12].

Chitosan displays antimicrobial activity and promotes tissue growth to facilitate wound healing. Chitosan is used as a sealant for wound puncture sites, in wound post-treatment care, and as a carrier for drug delivery [2,13–16]. In hemostasis, chitosan accelerates tissue regeneration, fibroblast synthesis of collagen, and coagulation by inducing activation and aggregation of platelets; chitosan also aids in aggregation of erythrocytes for formation of the hemostatic plug [17–19]. Moreover, chitosan nonspecifically binds to cell membranes, which assists in rapid hemostasis [7].

In present work, we prepared snake venom component, Batroxobin, that used in combination with collagen and chitosan for more faster and effective control of bleeding within relevant time. The components functions as fibrinogen clot enzymes, plasminogen activators, prothrombin activators, factor X activators, or hemorrhagins [20–23]. One venom component, from the snake *Bothrops atrox moojeni*, acts as a thrombin-like enzyme in the coagulation cascade. Generally, cleavage and removal of fibrinopeptides A (α chain) and B (β chain) from fibrinogen are catalyzed by thrombin. The cleaved fibrinopeptides polymerize, forming a fibrin polymer that is the main functional element of the hemostatic plug [24,25]. The cleaved fibrinogen α chain initiates non-covalent combination to form a fibrin clot [26,27]. In contrast to thrombin, batroxobin only splits fibrinopeptide A (α chain) off and does not affect polymerization in crosslinking networks of fibrin [23,28]. Also batroxobin does not influence other hemostatic factors or cells, which makes it clinically useful as a stable hemostatic agent [27]. But unfortunately, n-Bat is expensive and in short supply making it difficult to commercialize. Therefore we prepared recombinant batroxobin (r-Bat), obtained from cDNA expressed in Pichia pastoris, is cost effective and can be mass-produced [28].

Therefore, in this study, we prepared a novel collagen and chitosan hemostatic dressing containing r-Bat. Because each of the materials used in the dressing involve different hemostatic mechanisms within the coagulation cascade, the dressing was predicted to be effective at controlling bleeding. Also, incorporation of r-Bat was predicted to provide a synergetic effect to natural substances for induce rapid hemostasis. Experiments performed here in an animal model confirm the efficacy of this novel hemostatic dressing. The dressing should be a definite improve control of excessive hemorrhage.

2. Materials and methods

2.1. Materials

Collagen type I (from bovine Achilles tendon powder) and chitosan (from shrimp shell, low viscosity) were purchased from Sigma Aldrich (St. Louis, MO, USA). The r-Bat solution (recombinant batroxobin unit [BU/ml dissolved in phosphate buffered saline (PBS)]) was kindly donated by Biobud (Soengnam-si, Republic of Korea). Grade 1 filter paper (4.25 cm in diameter), purchased from Whatman (Kent, UK), was used for animal experiments. A 4-mm biopsy punch was purchased from Jeungdo bio&plant (Seoul, Republic of Korea). All other reagents were purchased from Sigma Aldrich.

2.2. Fabrication of the hemostatic dressings

A 1% (w/v) collagen or chitosan solution was created by dissolving each in 0.5 M acetic acid, pH 5.5. A blend of collagen and chitosan was prepared by mixing collagen and chitosan in 0.5 M acetic acid at a ratio of 1:1. The solutions were stirred at 4 °C for 24 h to obtain homogenous solutions. r-Bat was added to each during the last hour of mixing and continuously stirred for 1 h. r-Bat solutions were prepared with concentrations of 1, 2, and 3 BU/well for *in vitro* and 3 and 5 BU/well for *in vivo* experiments. Degassing was performed to remove bubbles using a centrifuge at 3000 rpm for 10 min at 4 °C. Each solution was loaded onto a 24-well plate and frozen in a −78 °C deep freezer for 48 h. The frozen solution was lyophilized for 48 h using a −50 °C freeze-dryer (Daeiltech, Seoul, Republic of Korea). All samples were stored at 4 °C until use.

2.3. Micro structure examination

The morphology of the hemostatic dressings was examined and photographed using field emission scanning electron microscopy (FE SEM S-800, Hitachi, Tokyo, Japan) at an acceleration emission voltage of 20 kV. Each sample was fixed with 2% glutaraldehyde-paraformaldehyde in 0.1 M PBS at a pH of 7.4 for 2 h and washed three times for 30 min in 0.1 M PBS. Samples were post-fixed with 1% osmium tetroxide (OsO_4) dissolved in 0.1 M PBS for 2 h and dehydrated in an ascending gradual series (50–100%) of ethanol. Samples were then permeabilized with isoamyl acetate and subjected to critical point drying (HCP-2, Hitachi, Tokyo, Japan). The samples were then gold coated by ion sputter (IB-3, Eiko, kobe, Japan) at 6 mA for 6 min. The average pore size of the dressings was determined by measurements performed on a typical FE SEM image.

2.4. In vitro studies

2.4.1. Fibrinogen conversion assay

We prepared a pH 7.5, 20 mM Trisma base in distilled water containing dissolved fibrinogen at 10 mg/ml in 0.9% warm saline. To execute batroxobin-mediated fibrinogen conversion to fibrin, 20-mM Tris-HCl at pH 7.5, was added to the hemostatic dressings containing r-Bat (1, 2, and 3 BU/well) and incubated for 10 min at 37 °C. After incubation, the buffer solution containing released batroxobin was collected. The buffer solution was then mixed with the 10 mg/ml fibrinogen solution and incubated at 37 °C for 10 min. The respective concentrations of r-Bat reacted with fibrinogen. Fibrin formation was confirmed by turbidity of the released solutions. Turbidity was detected at 405 nm on a Versamax spectrophotometer (Molecular Devices Korea, Okchun, Korea).

2.4.2. Platelet activation assay

Sprague-Dawley (SD) rat (weights: 350–400 g) blood was collected using a syringe containing 0.109 M sodium citrate at a ratio of 4:1. To isolate high concentration platelet solutions, whole blood was centrifuged at 2500 rpm for 5 min and the platelet rich plasma (PRP) was separated from the red blood cells and further centrifuged at 2500 rpm for 5 min to obtained pellets of rich platelets. The pellets were diluted with PBS at a 1:4 ratio and added to each dressing containing either r-Bat at 0, 1, 2, or 3 BU/well. After incubation at 37 °C for 20 min, each dressing was removed and fixed with 2% glutaraldehyde-paraformaldehyde in 0.1 M PBS at pH 7.4. Activated platelets were observed under FE SEM.

2.4.3. Whole blood clotting assay

Whole blood was obtained from male SD rats (weights: 350–400 g) from the abdominal vena cava using a syringe containing 0.109 M sodium citrate at a ratio of 4:1. For anesthetic, 30 mg/kg of Zoletil (Boehringer Ingelheim Agrovet, Hellerup, Denmark) and 10 mg/kg of Rompun (Bayer, Toronto, Canada) were administered by intramuscular injection. The abdominal vena cava of the rats were exposed in order to collect whole blood using a syringe

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