Functional effect of hemostatic pad based on a collagen with chitosan by addition of batroxobin

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Functional effect of hemostatic pad based on a collagen with chitosan by addition of batroxobin

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This certifies that the Master's Thesis
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설레임과 두려움을 안고 시작했던 석사학위 과정을 마무리 지으며 지난 시간을 되돌아 볼 수 있게 되었습니다. 수많은 시행 착오를 통해 그 노력들이 더해져 비로소 논문이 완성되어져 가는 과정은 저에게 학문의 길 뿐만 아니라 나 자신을 다듬을 수 있는 수양의 시간이었습니다. 이러한 결실을 맺기까지 제 부족한 역량을 이끌어 주신 많은 분들께 이 시간을 통해 감사의 마음을 전하고자 합니다.

먼저, 처음 뵈었을 때부터 지금까지 늘 제가 할 수 있는 이상의 것을 펼칠 수 있도록 응원 해주시고, 연구자로서의 소양 뿐만 아니라 연구, 실험을 하는 것에 대한 즐거움을 일깨워 주신 박종철 지도 교수님께 진심으로 감사 드립니다. 교수님의 가르침 덕분에 자신감을 가지고 첫 발을 내딛을 수 있었으며 지금의 모습으로 성장할 수 있었습니다. 다시 한 번 감사 드립니다. 그리고 자문 심사 때부터 뵐 때마다 늘 긴장하는 저에게 많은 독려와 세심한 조언 아끼지 않으셨던 나동균 교수님, 바쁘신 와중에도 시간을 내어 제 연구에 많은 관심을 가져 주시고 조언해주신 김철훈 교수님께도 깊은 감사의 말씀을 드리겠습니다. 교수님들의 지도와 격려 덕분에 부족한 제가 석사학위를 무사히 마칠 수 있었습니다.

처음에는 그저 무서운 언니였지만 늘 제가 해낼 수 있도록 이끌어 주며 많은 조언 아낌없이 주는 지금은 없어서는 안 될 내 정신적 지주 미희 언니, 늘 실수하고 덜렁대서 다치는 저를 친언니처럼 곁을 내주고 잘못까지 안아준 내 러블리 사수 병주 언니, 얼굴만 봐도 웃음이 날만큼 재미있고 늘 모두를 세심하게 아우르는 내 장꾸 큰 언니 현숙 언니, 그렇게 낯을 가려 차가운 얼굴로 날 보더니 이젠 나만 보면 웃고 또 선배로서는 늘 내게 따뜻한 격려 해주는 내 이쁜 째끼 민아, 내가 매일 놀리고 장난쳐도 돌아서면 또 받아주는 늘 내가 기댈 수 있는 따뜻하고 착한 내 이쁜 혜찌 혜진, 힘들 일을 할 때에 늘 묵묵하게 도와주고 지원해주는 민성오빠, 대형오빠 그리고 멀리 있지만 늘 반갑게 맞아주시는 태화 언니까지. 낯설고 어려웠던 실험실 생활에 늘 많이 아껴주고 응원해준 우리 연구실 식구들에게도 너무나도 감사 드립니다.

마지막으로 아직까지도 공부하겠다고 자기 욕심 부리는 철 없는 다 큰 딸을 항상 응원해주시고 묵묵히 뒷바라지 해주시는 또 늘 내가 최고라고 말해주며 내가 원하는 건 세상 어떤 것이라도 가져다 주실 것 같은 슈퍼맨 같은 세상에 둘도 없는 내 사랑하는 아빠,
외동인 나에게 친구 같이 또 언니 같이, 태어나 지금껏 한 번도 외롭다 느낀 적 없을 만큼 큰 사랑 주고도 늘 더 주지 못해서 마음 아파하고 나보다 나를 더 많이 사랑해주시는 언제 어디서나 내가 가장 반짝거릴 수 있도록 빛을 만들어주는 세상에 둘도 없는 내 사랑하는 엄마,
내 어떠한 선택도 늘 믿고 스스로 이겨 나갈 수 있도록 묵묵히 기다려주시고 또 이끌어 주시는 죽을 때 까지 영원한 내 편인 부모님 덕분에 제가 여기까지 올 수 있었습니다. 세상 어떤 수식어로도 그 사랑 보답할 수 없을 것 같습니다. 앞으로도 언제 어디서든 항상 열심히 하고, 어디에 내놓아도 가슴 뿌듯한 늘 부모님께 자랑스러운 딸이 되겠습니다. 항상 감사하고 많이 사랑합니다.

앞으로의 길에서도 늘 겸손하고 많은 이들에게 도움이 될 수 있는 사람이 되겠습니다. 이 지면 안에 다 열거 하지 못하였지만, 그 동안 도움주신 많은 분들께 다시 한 번 깊은 감사의 말씀을 전합니다.

2015년 12월
선경미 드림

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Functional effect of hemostatic pad based on a collagen with chitosan by addition of batroxobin

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Uncontrolled excessive bleeding in daily life, whether due to sudden accident, surgical operations, or combat may cause infectious complications that may lead to death by shock. Therefore, topical hemostats and sealants are required to assist effective treatment, arrest bleeding, and stabilize the casualty before definitive care.

This study use collagen and chitosan, which are both natural materials and are one of the most widely used hemostats these days. The materials are role in platelet activation, adhesion and aggregation. The use of chitosan helps the blood coagulation as it combines with red blood cells. However, these materials have been reported to lack sufficient hemostatic effect in a timely manner.

Hence, in this research, a novel pad made a faster and more effective sponge type hemostats by using the recombinant batroxobin, a venomous component from the snake *Bothrops atrox moojeni*, which transforms the

fibrinogen to fibrin in the process of hemostasis; it expected that recombinant batroxobin would support natural substances to achieve a synergetic effect of hemostasis performances. First, collagen and chitosan were dissolved with batroxobin in acetic acid for fabrication of a novel hemostatic pad and then characteristic analysis using Scanning Electron Microscope. Fabricated sponge groups were collagen-only, chitosan-only, collagen with chitosan, collagen or chitosan containing recombinant batroxobin 1, 2 and 3 BU/well for *in vitro* studies, collagen or chitosan containing recombinant batroxobin 3 and 5 BU/well for *in vivo* studies and collagen with chitosan containing recombinant batroxobin 1, 2, 3 and 5 BU/well. Fibrinogen assay and platelet activation assays represented respective hemostats mechanism, even combination of the materials. Whole blood clotting assay and animal experiments investigated blood coagulation activities in the injury sites. In addition, H&E and PTAH staining methods for histological analysis. Through the experimentations result, a novel hemostatic pad confirmed that the substances maintained to their hemostatic properties in the pad, also coagulation studies proved pad effectively reduced blood loss and shortened bleeding time by addition of batroxobin.

In conclusion, the novel hemostatic pad is reasonable that an enormous potential for excessive bleeding injury and improve effects of natural substance hemostatic pad when it is cooperated with recombinant batroxobin.

Key words : hemostasis, hemostatic pad, collagen, chitosan, recombinant batroxobin

Functional effect of hemostatic pad based on a collagen with chitosan by addition of batroxobin

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I. INTRODUCTION

1. Hemostasis

Excessive hemorrhage occurs from serious accidents, preoperative measures for injury, and in warfare.[1,2] Hemostasis can be divided into primary and secondary procedures as followed Figure 1. In primary hemostasis, extrinsic pathway, through tissue damage, can activate tissue factor (TF) and then TF and factor VII (tissue factor complex) may directly lead to generation of factor X. Along with extrinsic pathway, intrinsic pathway occurs by Hageman factor (factor XII) activation in the primary hemostasis process. Subsequently, platelet factor (PF-3) and clotting factor (factors VIII and IX) are precursor forms of cofactors for factor IX activation. Activated factor XII may directly initiate blood coagulation with factor VII.[3-6] Finally, secondary hemostasis, in other words blood coagulation cascade, is generated by factor X. The activation of factor X is catalyzed by factors IXa and VIIa. In blood coagulation cascade, factor X induces the production of prothrombinase. Prothrombinase triggers conversion of prothrombin to

thrombin. Fibrinogen transformation to fibrin occurs by means of generated thrombin. The created fibrin assists blood clotting, in addition to generating platelets through the primary process; activation and adhesion also help to make a hemostatic plug in conjunction with them.[6,7]

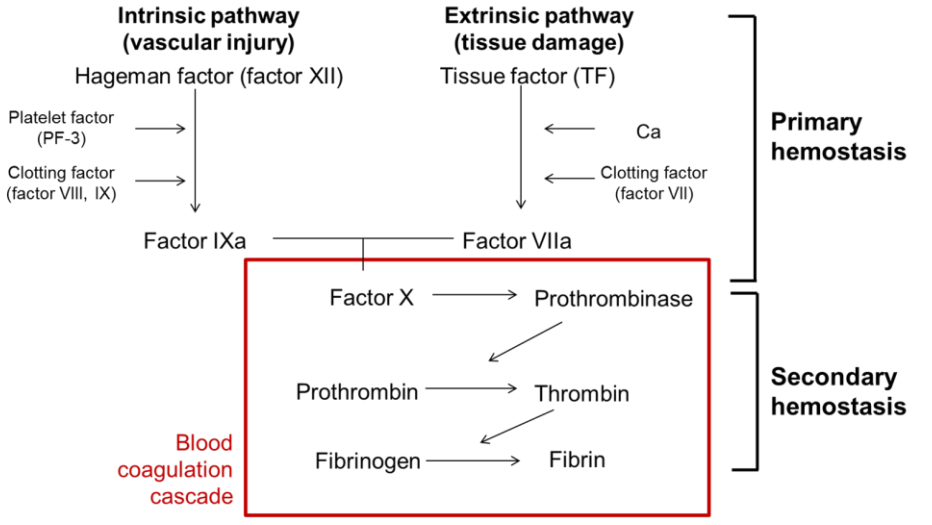

Figure 1. The process of hemostasis. Following damage to the blood vessel wall and tissue factor (TF) come into the contact with coagulation factors in the blood. TF in complex with factor VIIa and factor XII in complex with factor IXa activates minute amounts of factor X. Factor X activates prothrombin into thrombin which activates platelets. Additional Thrombin converts fibrinogen to monomeric fibrin that polymerizes to fibrin fibers.

2. Natural substances as hemostatic agents

Among the many natural substances, this study focused on collagen and chitosan use for hemostatic efficacy. Generally, they are widely used as biomaterials, preoperative products, and for wound healing. Recent research has identified the substances as a promising hemostatic agent due to their potential bioactivity in hemostasis.

First, collagen is the main component of the extracellular matrix, which is the triple helix protein structure. The collagen enables platelet aggregation and release of hemostatic factor for promoting coagulation and granulation tissue after blood coagulation.[8-10] Moreover, collagen, and thereby the adherent platelets, assists in the formation of blebs with strong procoagulant activity; it is also extensively used in medical applications because of its low hazard of disease transmission.[9,11-13]

Chitosan has been found to promote tissue growth and antimicrobial effects to advance wound healing. Chitosan is also an invaluable substance in biomaterials as a sealant for wound puncture sites, wound care, and drug delivery.[2,14-17] In hemostasis, chitosan accelerates coagulation by activation and aggregation of platelets; it also involves the aggregation of erythrocyte.[18-20] On the other hand chitosan nonspecific binding to cell membranes for assist of rapid hemostasis.[20]

3. Batroxobin

Snake venoms consist of various components that have efficacy as coagulation agents. Generally, the components serve functions as fibrinogen clot enzyme, plasminogen activator, prothrombin activator, factor X activator, and hemorrhagins.[21-24] One of the components, natural batroxobin, from the snake *Bothrops atrox moojeni*, acts as a thrombin-like enzyme in coagulation cascade. As shown in Figure 2, 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.[25,26] The cleaved fibrinogen α chain initiates non-covalent combination to form fibrin clot.[27,28] In contrast to thrombin, batroxobin only splits off fibrinopeptide A and does not affect polymerization for crosslinking networks of fibrin.[21,29] In addition, batroxobin does not influence other hemostatic factor and cells. Because of this property, batroxobin can be used clinically as a stable hemostatic agent.[28] Even though natural batroxobin has positive properties, it hard to use in commercialized product because of its high cost and short supply. For that reason, recombinant batroxobin was used for this study, from its cDNA expressed in Pichia pastoris. The recombinant batroxobin is more cost effective and can be mass produced. In addition, the recombinant batroxobin can used in acidic environments because it is not sensitive to pH level.

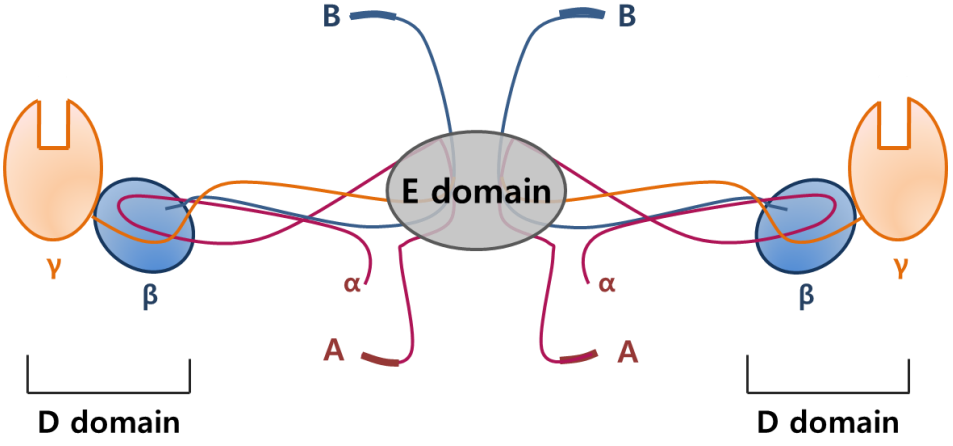

Figure 2. Schematic structure domains of fibrinogen and fibrin. fibrinogen convert fibrin to associate with D and E domains. D domain combines non-covalently with the specific binding site in the E domain. α and β chain is participate in fibrin polymerization through binding interactions.

4. Hemostatic pad

When uncontrolled bleeding occurs due to complicated situations, advanced topical hemostatic agents are utilized to control hemorrhage. For that reason, many types of hemostatic agent have been used for effective control of excessive bleeding such as bandage, fibrin glue, liquid, powder, gel, and pad. One of the hemostats types is the pad (also referred to as scaffold), that can not only assist with effective control of bleeding but also stop oozing after the hemostasis process. In addition, the pad type covers the injury site to inhibit infection complications. Therefore, in this study, pads made by accreted materials in different mechanisms are used for more rapid and effective control of hemostasis. The three of mechanisms are role in hemostasis as described Figure 3.

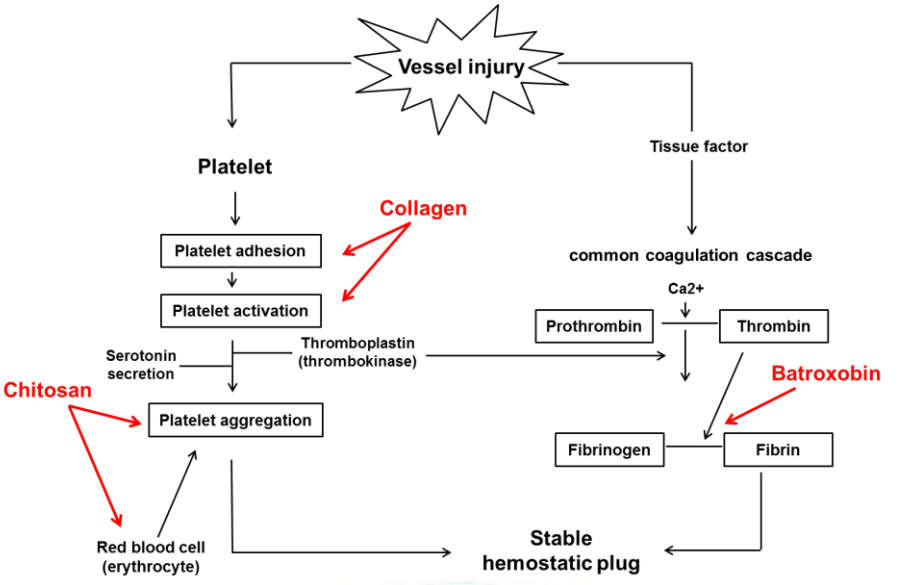

Figure 3. The three of hemostatic agent mechanism in coagulation. Collagen is role of platelet adhesion and activation in hemostasis, also chitosan is role in hemostasis like collagen. In addition, chitosan is aggregation with erythrocyte for blood clot. Batroxobin assist of fibrinogen convert to fibrin as thrombin.

5. Objective of the study

The present study sought to fabricate a novel hemostatic pad based on collagen and chitosan containing recombinant batroxobin. Batroxobin was expected to assist collagen and chitosan for a synergetic effect on rapid hemostasis. The mechanisms of respective components were evaluated by fibrinogen assay and platelet activation assay. A whole blood clot assay confirmed the blood clot ability and effect of batroxobin. Furthermore, animal model experiments provided a basis for confirming the efficacy of the novel pad.

II. MATERIALS AND METHODS

1. Materials

Bovine collagen type I fiber and Chitosan powder from shrimp shell (low viscosity) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Recombinant batroxobin (r-Batroxobin) solution (recombinant batroxobin unit (BU/ml)) dissolved in phosphate buffered saline (PBS) was kindly donated by Biobud (Soengnam-si, Korea). Cyanmethemoglobin Standard Solution (Stanbio Laboratory, Boerne, TX, USA), equivalent to 180 mg/ml of hemoglobin, was diluted in prepared Drabkin's reagent for determinate concentrations of hemoglobin. All other reagent used were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Grade 1 filter paper (4.25 cm in diameter), purchased from Whatman (Kent, UK), was used for animal experiments. 4 mm biopsy punch purchased from Jeungdo bio&plant (Seoul, Korea).

2. Fabrication of hemostatic pad

A 1 % (w/v) collagen or chitosan powder was dissolved in 0.5 M acetic acid, pH 5.5. In addition, a blend of collagen and chitosan was prepared by through mixing of collagen and chitosan in 0.5 M acetic acid in the ratio of 1:1. The solutions were then stirred at 4°C for 24 hr to obtain a homogenous solution. r-Batroxobin solution was added to each at the 23rd hour of mixing, and was then continuously stirred for 1 hr. r-Batroxobin 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 done to remove bubbles using a centrifuge at 3,000 rpm for 10 min at 4℃. Each solution was loaded on 24 well plate and frozen in a -78℃ deep freezer for 48 hr. Finally, the frozen solution was lyophilized for 48 hr using a -50℃ freeze-dryer (Daeiltech, Korea). All the samples were stored at 4℃ before experiment use.

3. Characterization of the hemostatic pad

Each sample was fixed with 2 % Glutaraldehyde-Paraformaldehyde in 0.1 M PBS, pH 7.4, for 2 hr and washed three times for 30 min (90 min total) in 0.1 M PBS. Each was post-fixed with 1 % Osmium Tetroxide (OsO_4) dissolved in 0.1 M PBS for 2 hr and dehydrated in ascending gradual series (50–100 %) of ethanol and infiltrated with the Iso amyl acetate and subjected to Critical Point Dryer (HCP-2, Hitachi, Tokyo, Japan). They were coated with gold by ion sputter (IB-3, Eiko, kobe, Japan) 6 mA for 6 min and morphology of hemostatic pads were examined and photographed using Field Emission Scanning Electron Microscopy (FE SEM S-800, Hitachi, Tokyo, Japan) at an acceleration emission voltage of 20 kV. The average pore sizes of pads were determined from measurements on a typical FE SEM image.

4. *In vitro* studies

A. Fibrinogen clotting assay

Firstly, 20 mM Trisma base prepared by dissolved in distilled water with a pH of 7.5 and dissolved fibrinogen solution of 10 mg/ml in 0.9 % warm saline. To execute fibrinogen conversion to fibrin by batroxobin released from the hemostatic pad, 400 μL of 20 mM Tris-HCl, pH 7.5, was added to the hemostatic pads containing r-Batroxobin 1, 2 and 3 BU/well and incubated for 10 min at 37℃. After incubation, the buffer solution releasing batroxobin was collected. The buffer solution mixed with 10 mg/ml fibrinogen solution and incubated at 37℃ for 10 min. Respective concentrations of r-Batroxobin reacted with fibrinogen and fibrin formation were confirmed by turbidity of the released solutions. The turbidity was detected at 405 nm by automatic microplate spectrophotometer.

B. Platelet activation assay

Sprague-Dawley (SD) rat, weight 350–400 g, blood was collected using a syringe containing 0.109 M sodium citrate in a ratio of 4:1. To isolate high concentrations of platelet solution, whole blood was centrifuged at 2,500 rpm for 5 min, and platelet rich plasma (PRP) was separated from the red blood cell and further centrifuged at 2,500 rpm for 5 min to obtained pellets of rich platelets. The pellet was diluted with PBS in a 1:4 ratio and was added each hemostatic pad, r-Batroxobin 0, 1, 2 and 3 BU/well. After incubation at 37℃ for 20 min, each hemostatic pad were removed and fixed with 2 % Glutaraldehyde-Paraformaldehyde in 0.1 M PBS, pH 7.4. Activated platelets were observed under FE SEM.

C. Whole blood clotting assay

Whole blood was obtained from male SD rats, weight 350–400 g, from the abdominal vena cava using a syringe containing 0.109 M sodium citrate in the ratio of 4:1. For anesthetic, 30 mg/kg Zoletil (Boehringer Ingelheim Agrovet, Hellerup, Denmark) and 10 mg/kg Rompun (Bayer, Toronto, Canada) were administered by intramuscular injection. The abdominal vena cava of the rats were exposed to collect the whole blood using a syringe containing 0.109 M sodium citrate in the ratio of 4:1. Each hemostatic pad, with various concentrations of r-Batroxobin in 0, 1, 2 and 3 BU/well were dipped in 24 well plates containing 400 μL of citrated blood, and then the plate was incubated with shaking at 37℃ for 10 min. After incubation, unstable blood clots were dissolved by addition to PBS. The diluted blood in the PBS reacted with Drabkin's solution in a 1:10 ratio and then gently rinsing 3 times with reagent by pipette. The solutions were mixed well and allowed to stand for 15 min at room temperature. The total hemoglobin concentration (mg/ml) of each hemostatic pad was determined at 540 nm using a Versamax (Molecular devices korea, Okchun, Korea).

5. *In vivo* studies

A. Preparation of animal experiments

(A) Animals

All animal experiments were performed with the "Guide for the Care and Use of Laboratory Animals" and protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the Yonsei Laboratory Animal Research Center (YLARC) (Permit #: 2015-0052). Animals were maintained in the specific pathogen-free facility of the YLARC. SD rats were used for *in vivo* studies. For the femoral artery model, 120 healthy male SD rats, weight 350–400 g, were randomly assigned to experiment groups. The liver model was randomly utilized with 120 healthy male SD rats, weight 230–280 g. Prior to *in vivo* studies, Zoletil and Rompun (as described above) were administered by intramuscular injection for anesthetic in *in vitro* studies.

(B) Treatments

Ten groups assigned to *in vivo* studies, including no treatment, only application of the collagen, chitosan, collagen with chitosan pads and each pad with concentrations of recombinant batroxobin (r-Batroxobin) of 3, 5 BU/well.

B. Blood coagulation in SD rat model

(A) Femoral artery model

Amount of bleeding pattern and hemostasis time was evaluated by femoral artery model as described by You KE et al[30] with a slight modification. Figure 4 images simplify shown that the process of femoral artery experiment. The anesthetized SD rat was fixed on a Styrofoam board using needles and tilted at about 45 degrees. The left leg was incised and muscle fascia removed to expose the femoral artery, and then the vessel was cut using a scalpel. After inflicting the injury, the treatments were applied (as per group) on the arterial wound area. After the application of the pad, filter paper was placed immediately below to absorb the blood. The filter paper was changed every 10 sec, and wet filter paper weight taken to determine total amount of bleeding and accurate times of cessation of bleeding. The experiment for each animal was defined as acute hemostasis bleeding stopped before 15 min.

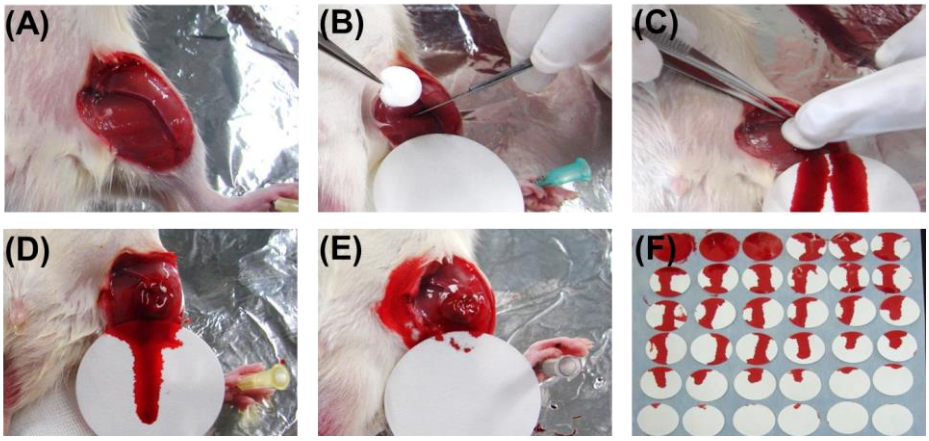

Figure 4. Procedure of the femoral artery model of SD rat. (A) The left femoral artery was exposed and tilted a Styrofoam at about 45 degrees. (B) Femoral artery cut by using a scalpel. (C) Hemostatic pad directly applied on injury and placed filter paper below wound site. (D) Filter paper absorbed blood and replaced every 10 sec. (E) Acute hemostasis time point measured by unwetted paper. (F) Bleeding pattern confirmed to each treatment groups.

(B) Liver model

Prior to the experiments, the anesthetized SD rat was fixed on a Styrofoam board using needles and tilted at about 45 degrees. Figure 5 explained liver model, briefly. After abdominal incision, the left lateral lobe of liver was exposed to make a hole. Then, the 5 x 5 cm^2 para-film was placed under the liver lobe to prevent absorption of blood other than that from the wound site. The wound was made in the middle of lobe using a 4 mm biopsy punch and then free bleeding was allowed for 5 sec. While bleeding was profuse, a hemostatic pad applied on the left liver lobe and filter paper was placed below the liver. The filter paper was replaced every 30 sec and immediately wet filter paper weight measured immediately to determine total amount of bleeding and accurate times of cessation of bleeding. The experiment for each animal was defined as acute hemostasis bleeding stopped before 5 min.

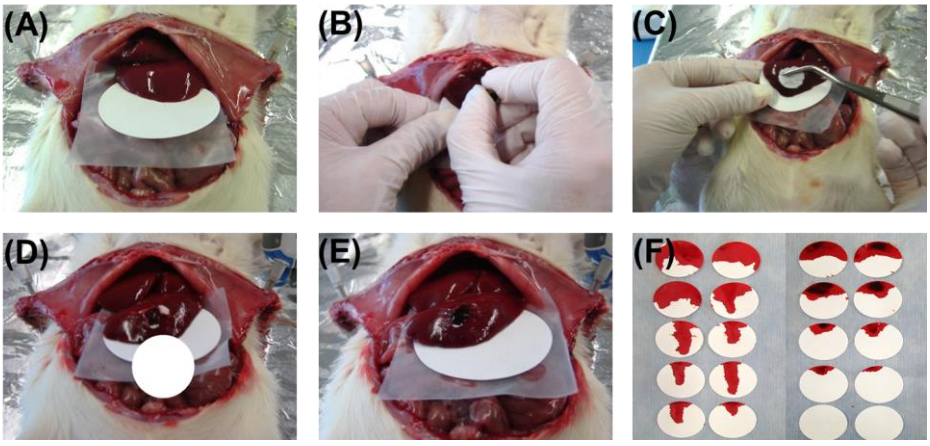

Figure 5. procedure of the liver injury model of SD rat. (A) The left lateral lobe of liver exposed to make a hole and placed parafilm and filter paper under the lobe. (B) A hole made by 4 mm biopsy punch and allowed free bleeding. (C) Hemostatic pad applied on the liver lobe. (D) Filter paper absorbed blood and changed every 30 sec. (E) Acute hemostasis time point measured and confirmed to maintenance of hemostasis for 1 min. (F) Bleeding pattern evaluated each hemostatic pad.

6. Histological analysis

To examine the blood clot formation during in vivo studies, the liver lobe was isolated with wound tissue containing hemostatic pad. The isolated tissue was fixed in 10 % neutral buffered formaldehyde, dehydrated in grading ethanol, and embedded in paraffin. The paraffin block was vertically serially sectioned at 5 μm thickness, and then the tissue of the middle of wound site was found with a hole. The tissue produced were placed on slides glass, which were then de-paraffinized in an oven set at 60℃ and stained with hematoxylin and eosin (H&E) for microscopic examination of blood clot formation. For fibrin clot formation, the tissue slides were de-paraffinized in an oven set at 60℃ and stained with phosphotungstic acid hematoxylin (PTAH). PTAH stain visualized not only fibrin but also muscle dyed blue or bluish purple.

7. Statistical analysis

All results were statistically analyzed by Student t-test using Microsoft Office Excel 2010. Data are expressed as mean ± standard deviation of mean. P values <0.05 were considered statistically significant.

III. RESULTS

1. Characteristics of hemostatic pad

SEM images of the freeze-dried hemostatic pad are presented in Figure 6, indicating a micro-porous top layer. All groups had accurate 3D porous structure and relatively uniform pore-size distribution with inherent interconnectivity. Only collagen treated pad was determined to be 49.01±2.7 μm, whereas collagen with r-Batroxobin groups were determined to be 23.11±1.88 μm without reference to batroxobin concentration. That indicated the diameter of pore size increased after addition of r-Batroxobin to collagen. The chitosan groups showed less perfect shape of porous structure compared to collagen treated groups, but all chitosan treated groups had almost the same size as 55.19±2.69 μm. The collagen with chitosan containing r-Batroxobin groups were well interconnected with a mean pore size of 57.79±6.65, 57.78±5.14, 55.96±5.91, 56.13±4.35 μm, respectively. The images were not changed structure even with concentration of r-Batroxobin treated.

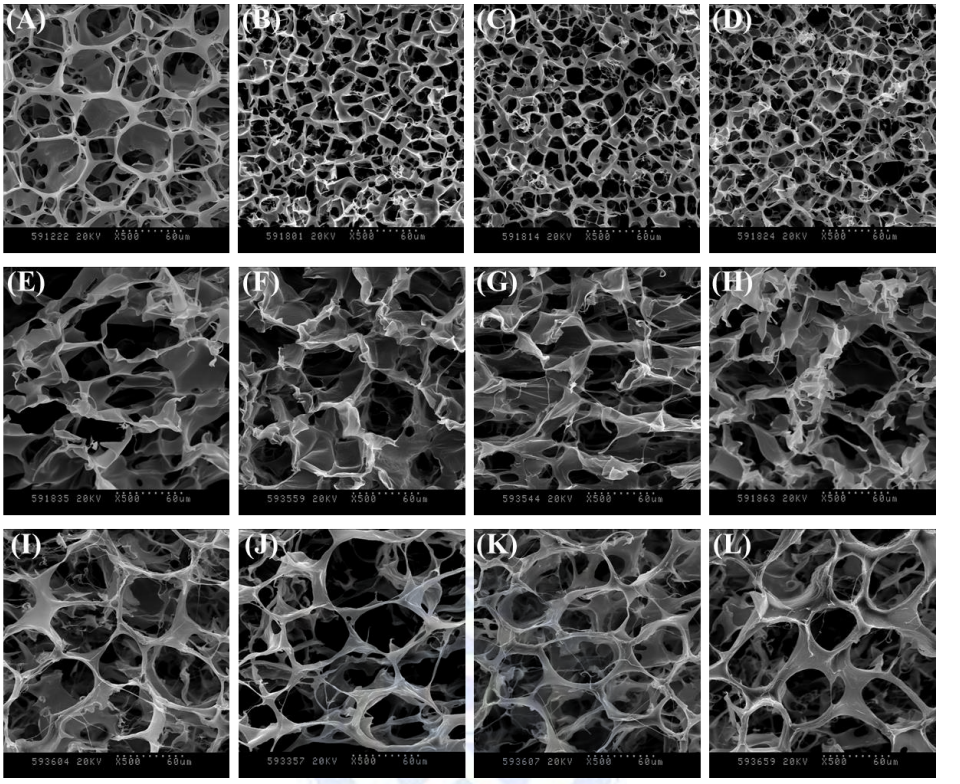

Figure 6. SEM micrographs of the surface of the hemostatic pad on (A) Only collagen, (B) Collagen containing r-Batroxobin 1 BU/well, (C) Collagen containing r-Batroxobin 2 BU/well, (D) Collagen containing r-Batroxobin 3 BU/well, (E) Only chitosan, (F) Chitosan containing r-Batroxobin 1 BU/well, (G) Chitosan containing r-Batroxobin 2 BU/well, (H) Chitosan containing r-Batroxobin 3 BU/well, (I) Collagen with chitosan, (J) Collagen with chitosan containing r-Batroxobin 1 BU/well, (K) Collagen with chitosan containing r-Batroxobin 2 BU/well, (L) Collagen with chitosan containing r-Batroxobin 3 BU/well. Scale bar=50μm.

2. *In vitro* studies

A. Fibrinogen clotting assay

The turbidity of the solution was increased as shown in Figure 7. The more r-Batroxobin concentration was increased, the more fibrin polymerization increased compared with only collagen or chitosan pads. In the collagen treated groups, collagen with r-Batroxobin 2 BU/well was significantly increased in turbidity compared to the collagen-only pad, whereas chitosan groups showed no difference in turbidity with different concentrations of r-Batroxobin without chitosan-only treated pad. As the concentration of r-Batroxobin increased, the collagen with chitosan groups also increased fibrin turbidity.

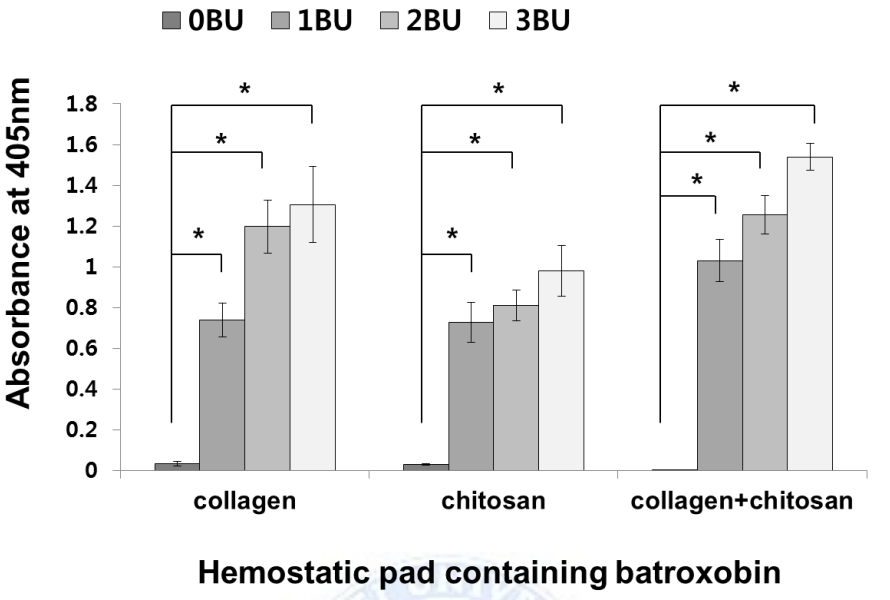

Figure 7. Measurement of fibrin turbidity. Fibrinogen conversion to fibrin was formation by buffer solution containing r-Bat 1, 2 and 3 BU/well. The degree of fibrin formation was measured at 405nm using spectrometer. * $P < 0.05$ compared to control(non-containing r-Batroxobin) treated group.

B. Platelet activation assay

As Figure 8 shows, each hemostatic pad incubated with rich platelets were fixed and viewed under the SEM. Activated platelets were shown with pseudopod formation in all treated groups, but low numbers of activated platelets were observed in collagen or chitosan pads containing r-Batroxobin 0, 1, 2 and 3 BU/well. In contrast, higher numbers of activated platelets were generated in collagen with chitosan pads containing r-Batroxobin 0, 1, 2 and 3 BU/well, and significantly more platelet aggregation adhered onto the groups.

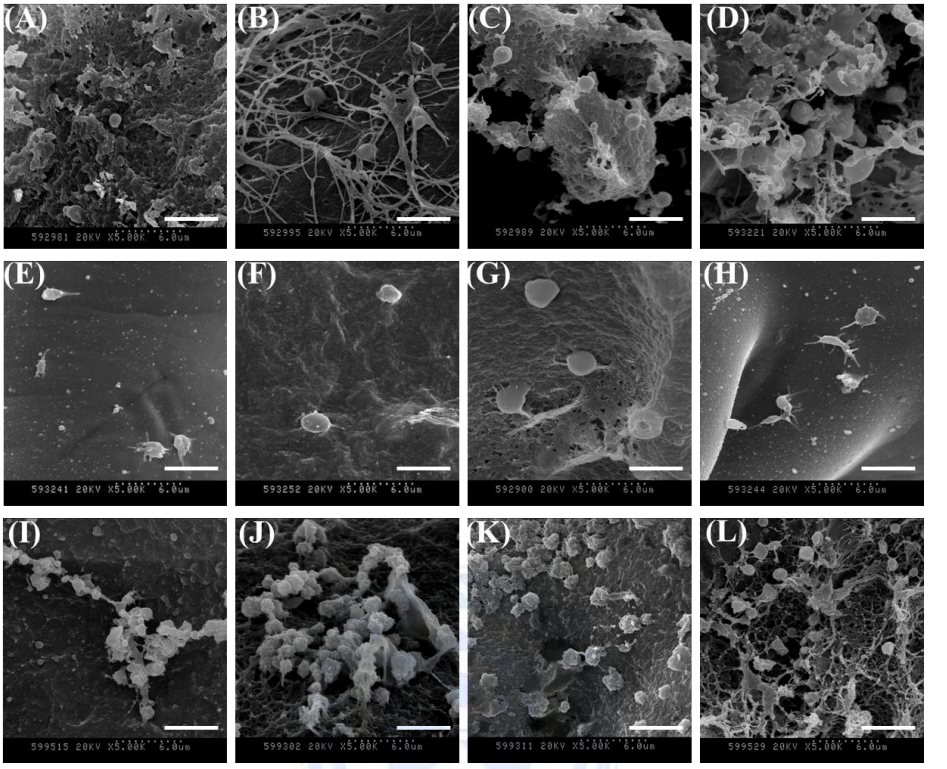

Figure 8. SEM images of the platelet activation of the hemostatic pad on (A) Only collagen, (B) Collagen containing r-Batroxobin 1 BU/well, (C) Collagen containing r-Batroxobin 2 BU/well, (D) Collagen containing r-Batroxobin 3 BU/well, (E) Only chitosan, (F) Chitosan containing r-Batroxobin 1 BU/well, (G) Chitosan containing r-Batroxobin 2 BU/well, (H) Chitosan containing r-Batroxobin 3 BU/well, (I) Collagen with chitosan, (J) Collagen with chitosan containing r-Batroxobin 1 BU/well, (K) Collagen with chitosan containing r-Batroxobin 2 BU/well, (L) Collagen with chitosan containing r-Batroxobin 3 BU/well. Scale bar=50μm.

C. Whole blood clotting assay

For the investigation of blood clot formation in composite sponges, observation macroscopy use by photographic images and measurement of hemoglobin concentration in unformed clot blood. As the r-Batroxobin concentration was increased, clot formation was gradually elevated along with darkness of non-trapped clots, as shown in Figure 9. After measuring hemoglobin concentration, the degrees of clot formation was estimated with back calculation method and then converted into percentages. A higher absorbance degree of hemoglobin indicated slower formation rate.[11] In figure 10, about 50 % of clot formation was found in collagen- or chitosan-only treated groups compared to untreated group, and more clots were formed with the increasing concentrations of r-Batroxobin. With inclusion of 2 BU/well to hemostatic pad, the percentage of clot formation rapidly increased and an almost-completely formed clot was seen in the hemostatic pad containing 3 BU/well. In addition, collagen with chitosan groups observed entirely formed clots by addition of 2 BU/well of r-Batroxobin as collagen or chitosan containing r-Batroxobin 3 BU/well.

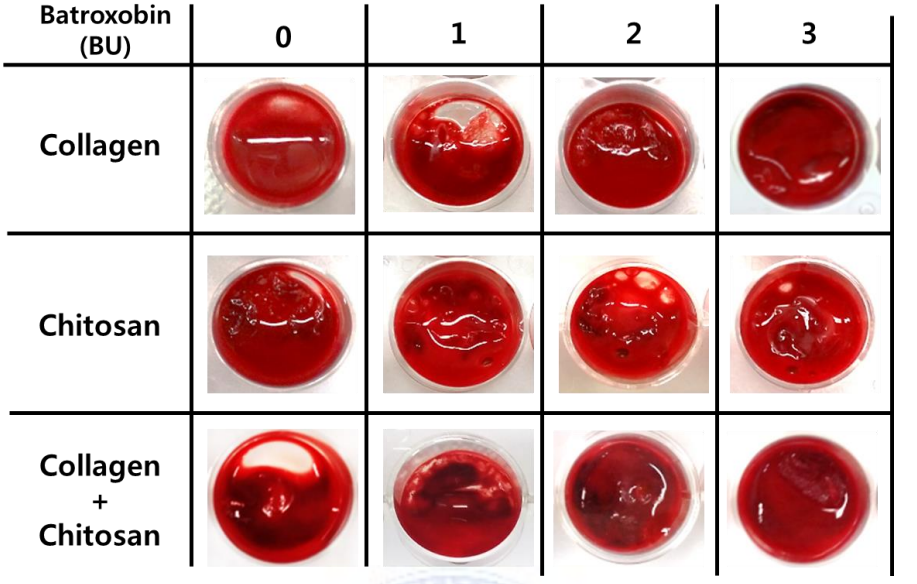

Figure 9. Macroscopy of whole blood clot formation on hemostatic pad containing r-Batroxobin. Immediately incubated the scaffold with r-Batroxobin 0, 1, 2 and 3 BU/well, clots take a photograph tilted at 30 degree. clots were expended through increasing concentrations of r-Batroxobin.

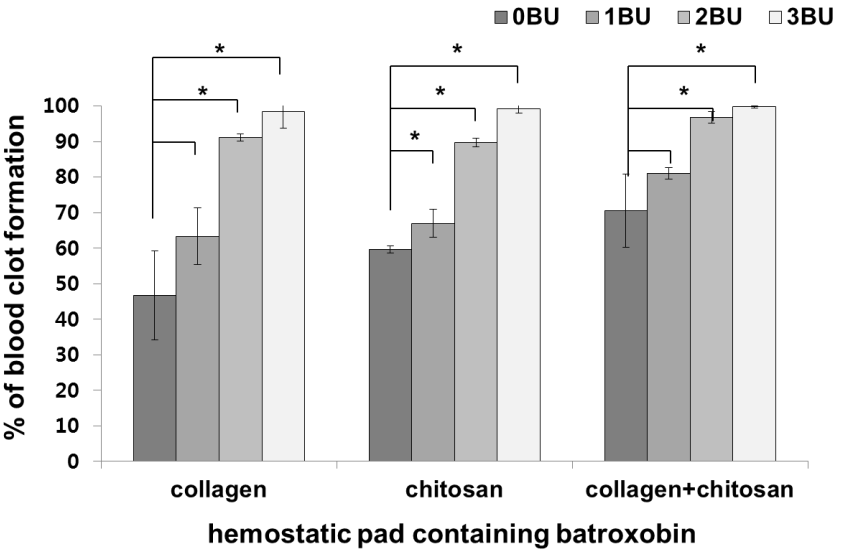

Figure 10. Whole blood clot formation on hemostatic pad containing r-Batroxobin. The degree of clot formation was represented to percentage of the control. Formed clotting was increased with r-Batroxobin and notably on the collagen or chitosan treated containing 3 BU/well r-Batroxobin and collagen with chitosan containing 2, 3 BU/well r-Batroxobin were completely formed than other treated groups. * $P < 0.05$ compared to control(non-containing r-Batroxobin) treated group.

3. *In vivo* studies

A. Blood coagulation in femoral artery model

The amount of blood loss was analyzed by measurement of the accumulated weight of blood absorbed by the filter paper, and accurate bleeding cessation time was recorded once filter papers absorbed no blood. In Figure 11, the graphs confirm the tendency of the hemostasis over 15 min, and the curves fitting on the bar indicate the pattern of hemostasis at each time point. Additionally, the total amount of bleeding out, first-bleeding, and re-bleeding were compared with each group calculated from the equation of the bleeding stop point in Figure 12 and Table 1. The reduction in blood bleeding out with time point is shown in Figure 11 but complete hemostasis was not achieved by 15 min, after which the injury remained untreated. In contrast, hemostatic pad was hemostasis time was sharply reduced compared with untreated group within 30 sec, even all treated groups were not equal. Moreover, increased r-Batroxobin concentration effectively lessened hemostasis time point and showed a remarkably reduced amount of re-bleeding out. Through Figure 12A, all treated groups blood loss amounts were small compared with the control (untreatment) group; also chitosan and collagen with chitosan containing 5 BU/well yielded more diminished amounts of blood. In the first-bleeding comparison graph in Figure 12B, all groups were similar in blood loss except the chitosan containing 5 BU/well. In addition, the amount of re-bleeding treatment containing r-Batroxobin groups were distinguished from those treated without r-Batroxobin. In fact, re-bleeding was almost non-existing when treated containing 5 BU/well, which reduced re-bleeding up to 0.1g compared with other groups in Figure 12C. Table 1 shows the hemostasis time and generated re-bleeding time. With increased concentration of r-Batroxobin or collagen treated with chitosan, re-bleeding time was delayed and blood loss reduced.

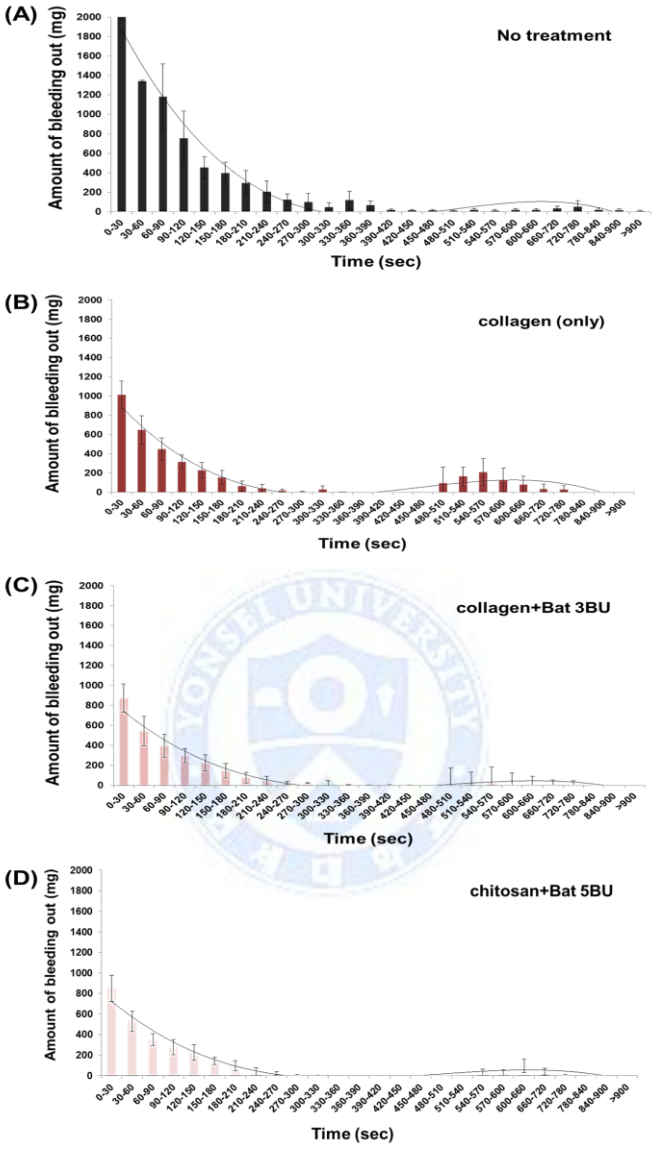

Figure 11. Measurement of amount of blood from femoral artery injury model. The graphs observed tendency of the hemostasis with 15 min and the curves indicated the pattern of hemostasis each time point. (A) The untreated group. Bleeding could not stopped until 15 min. (B) Collagen-only, (C) Collagen containing r-Batroxobin 3 BU/well, (D) Collagen containing r-Batroxobin 5 BU/well.

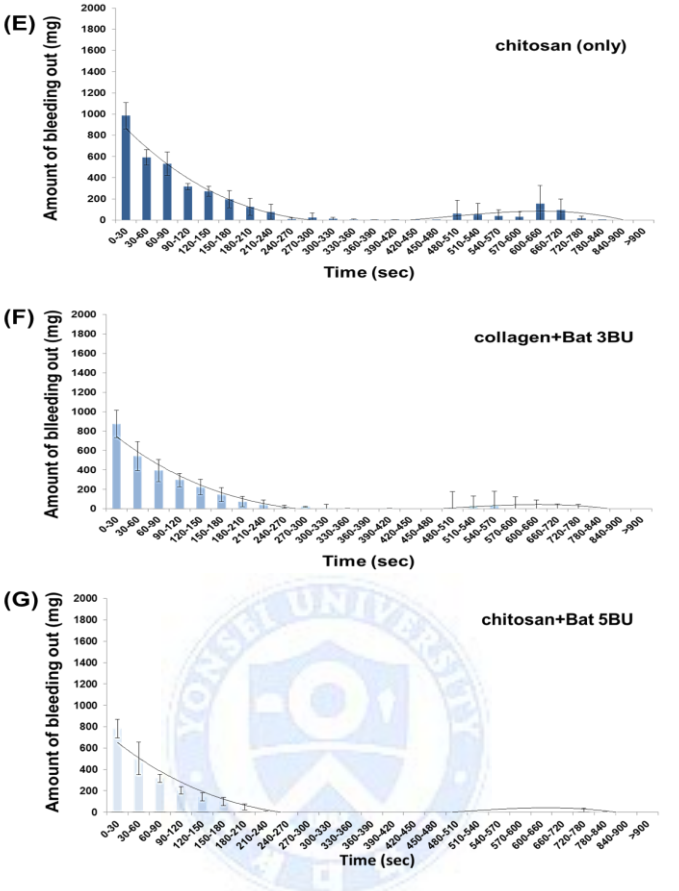

Figure 11. (continued) (E) Chitosan-only, (F) chitosan containing r-Batroxobin 3 BU/well, (G) chitosan containing r-Batroxobin 5 BU/well.

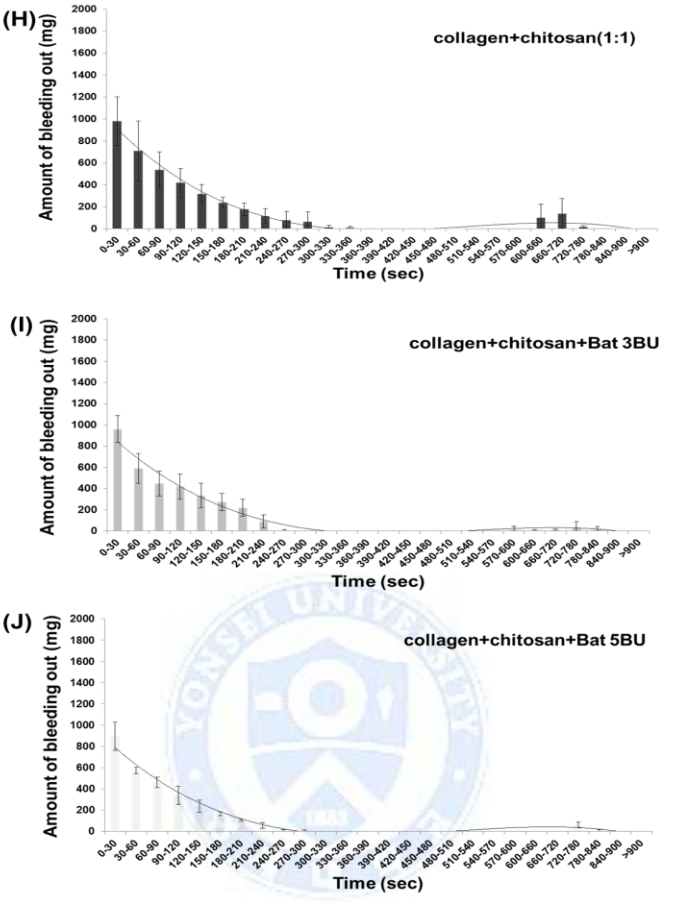

Figure 11. (continued) (H) Collagen with chitosan, (I) Collagen with chitosan containing r-Batroxobin 3 BU/well, (J) Collagen with chitosan containing r-Batroxobin 5 BU/well.

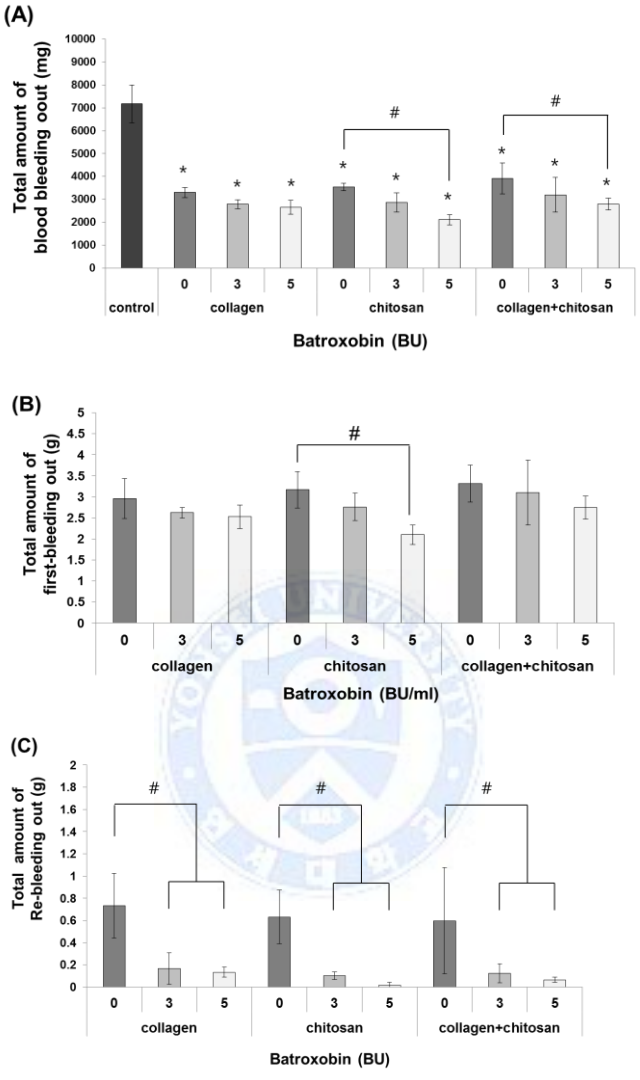

Figure 12. Examination of (A) Total amount of blood from bleeding wound site, (B) First-bleeding and (C) Recurrence bleeding with hemostatic pad. All groups had blood loss which was oozed out again during hemostasis process, even though the scaffold completely covered the bleeding site. * $P < 0.05$ compared to untreated group. # $P < 0.05$ compared to non-containing r-Batroxobin treated group.

Table 1. Hemostasis time, recurrence bleeding time and total amount of blood bleeding out with various treatments in femoral artery model

	Control	Collagen			Chitosan			Collagen+Chitosan (1:1)		
Batroxobin(BU)		0	3	5	0	3	5	0	3	5
Bleeding stop time (sec)	> 900	330	300	270	300	240	240	300	240	240
Re-bleeding time (sec)	-	480	510	570	480	600	780	600	570	780
Total of bleeding out (mg)	7168.4 ±826.1	3301.3 ±224.7	2792.9 ±195.6	2713.3 ±234.4	3538.6 ±157.3	2863.8 ±413.9	2115.2 ±226.7	3912.4 ±679.9	3199.7 ±762.8	2799.9 ±263.2

B. Blood coagulation in liver model

The hemostatic effects on the various treatment groups were examined by measurement of the accumulated weight of blood absorbed on filter paper and accurate bleeding cessation time. After punching a hole in the left liver lobe, were directly covered the wound site with a hemostatic pad to. According to Figure 13, the untreated group bleeding was not controlled within 5 min, but other treated groups with r-Batroxobin stopped bleeding within 3 min. The effect of hemostasis was significantly magnified when scaffold 5 BU/well of r-Batroxobin was included. By comparison with femoral artery model, liver model showed light bleeding site and no recurrence of bleeding. In addition, the gaps of total bleeding of groups were relatively slight between those treated with r-Batroxobin or not. However, blood loss was strikingly reduced in the r-Batroxobin 5 BU/well treated group compared with other treated groups. Figure 14 shows the total amount of bleeding out in the wound site. Prior to femoral artery experiments, when treated with escalated concentration of r-Batroxobin, blood loss in wound site was much reduced.

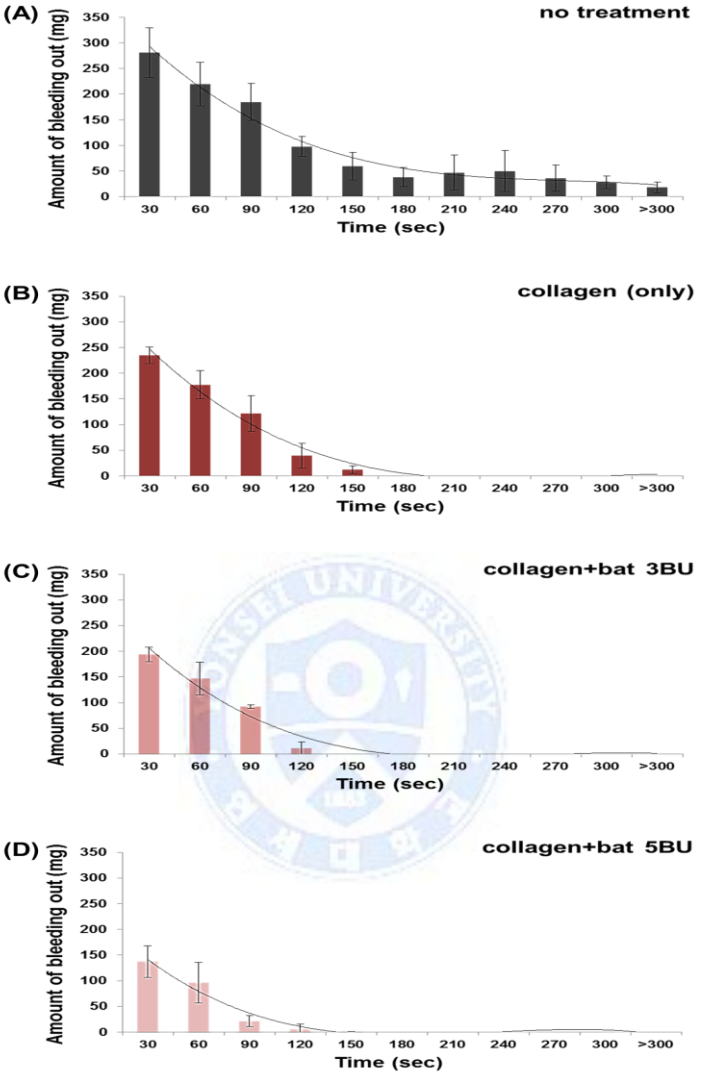

Figure 13. Measurement of amount of blood from liver wound model. The graphs observed tendency of the hemostasis with 5 min and the curves indicated the pattern of hemostasis each time point. (A) The untreated group. Bleeding could not stopped until 15 min. (B) Only collagen, (C) Collagen containing r-Batroxobin 3 BU/well, (D) Collagen containing r-Batroxobin 5 BU/well.

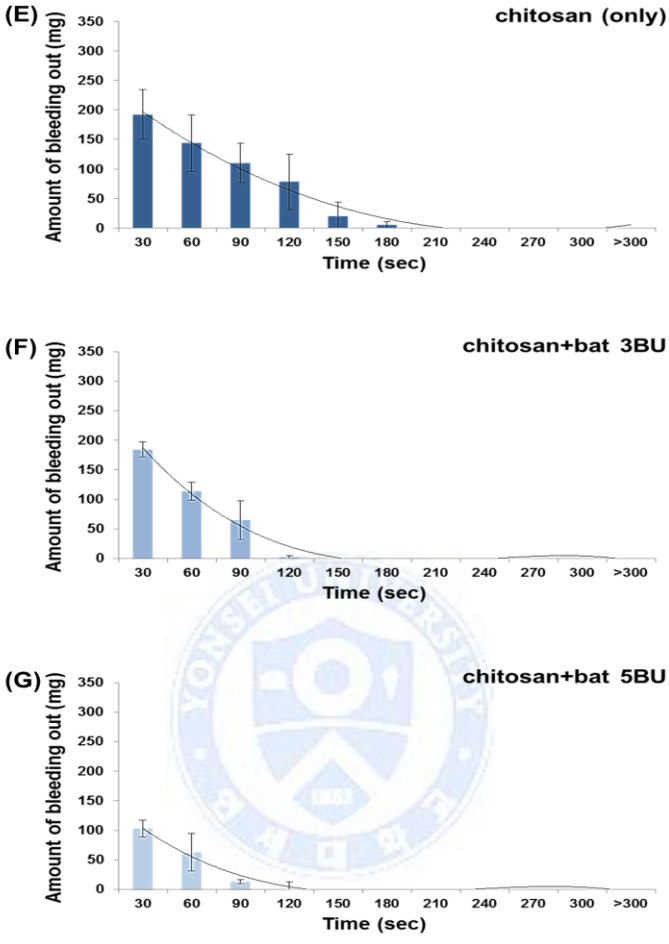

Figure 13. (continued) (E) Only chitosan, (F) chitosan containing r-Batroxobin 3 BU/well, (G) chitosan containing r-Batroxobin 5 BU/well.

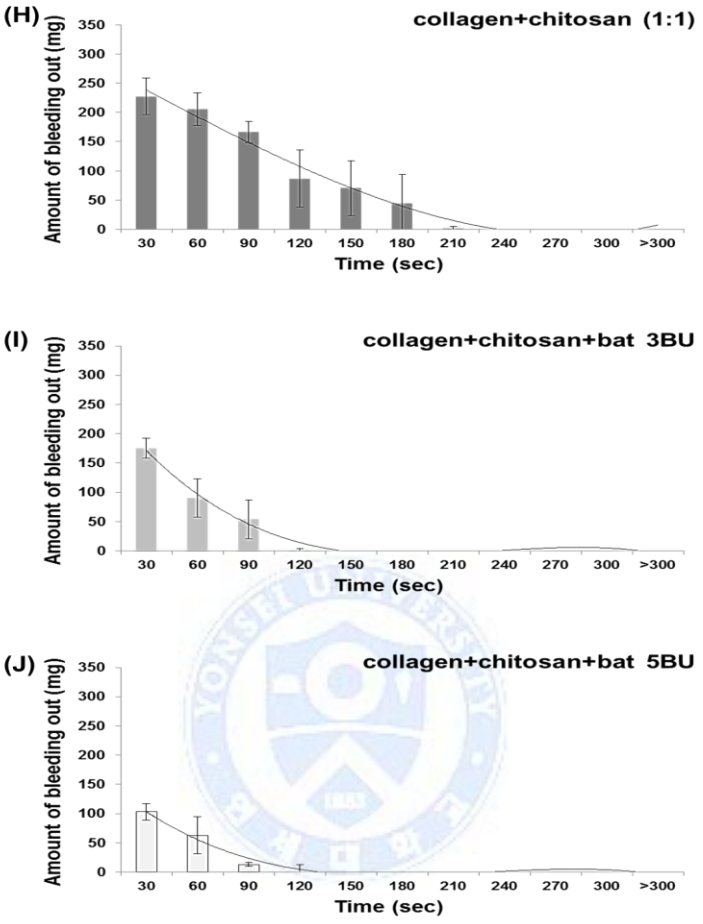

Figure 13. (continued) (H) Collagen with chitosan group, (I) Collagen with chitosan containing r-Batroxobin 3 BU/well, (J) Collagen with chitosan containing r-Batroxobin 5 BU/well.

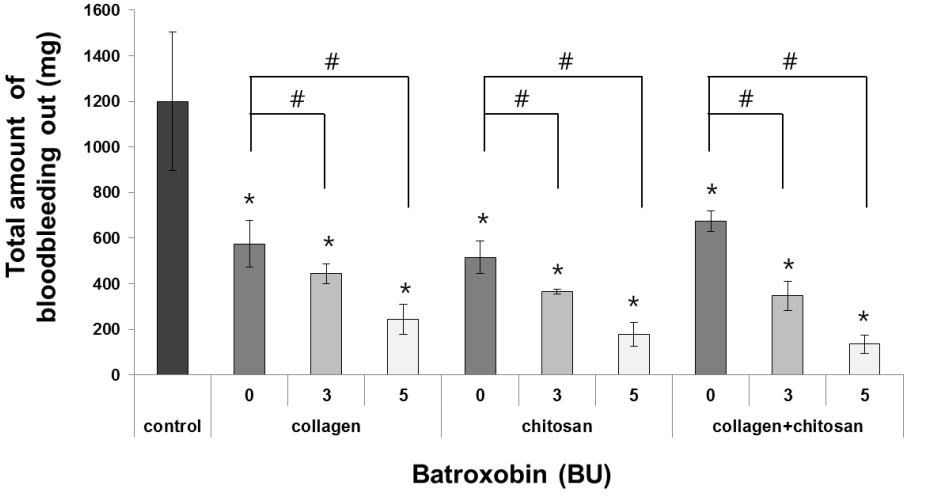

Figure 14. Examination of total blood loss in liver bleeding wound site. The total amount of bleeding out significantly reduced in hemostatic pad containing recombinant batroxobin. * $P < 0.05$ compared to untreated group. # $P < 0.05$ compared to non-containing r-Batroxobin treated group.

4. Histological analysis

Histological results of blood and fibrin clot formation were examined by both H&E staining and PTAH staining. H&E staining allowed visualization of blood clot formation via the use of erythrocyte dye and PTAH staining that was specific to fibrin. As Figure 15 shows, blood clots were stained pinkish red via H&E staining, which was observed in the wound hole of the liver lobe. Figures 15A, 15D and 15G were treated without r-Batroxobin. The images show low concentration of blood clot formation in the areas. In contrast, the more the hemostatic pad increased the r-Batroxobin concentration, the more blood clots formed and its intensity was significantly increased. The results were matched to blood density through by images from Figure 15a to Figure 15i. The images that were treated with r-Batroxobin 5 BU/well include more high dense pinkish red dye than the r-Batroxobin 0 and 3 BU/well.

In another staining method, fibrin was stained bluish purple by PTAH staining. As represented in Figure 16, Figures 16A, 16D and 16G images show that fibrin clots were seldom found around the wound hole with bluish dye. Whereas hemostatic pad containing r-Batroxobin groups were observed with a relatively larger and deep bluish purple area. Especially Figure 16I included a high dense of stained area and dark purple dyed. Therefore, Figures 15 and 16 support that more blood clots and fibrin clots were formed with an increased concentrated r-Batroxobin in the wound area.

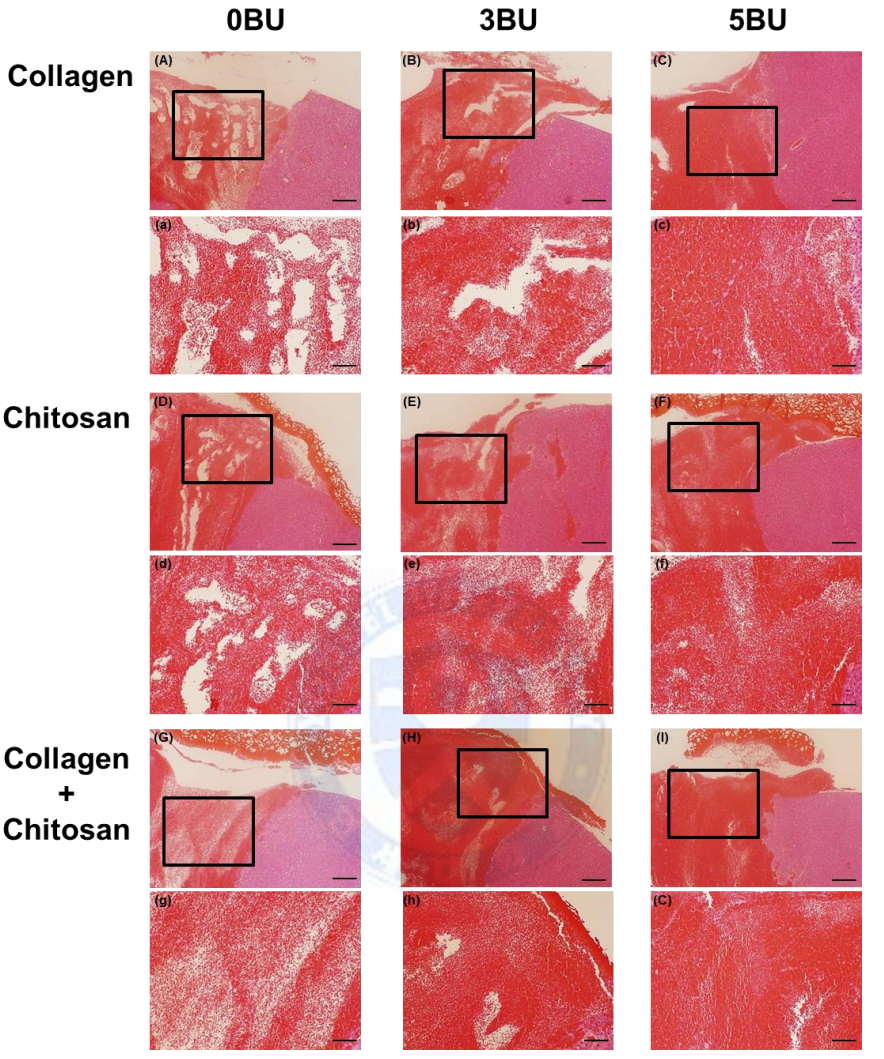

Figure 15. Histological photomicrographs of the liver wound site with blood clot formation. The images were histologically examined by H&E staining. (A)-(I) images were taken at 40x magnification and (a)-(i) images were taken at 100x magnification. Collagen treated group: (A)-(C); Chitosan treated group: (D)-(F); Collagen with Chitosan treated group: (G)-(I); (A), (D) and (G) natural substances with no r-Batroxobin; (B), (E) and (H) natural substances containing r-Batroxobin 3 BU/well; (C), (F) and (I) natural substances containing r-Batroxobin 5 BU/well. 40x magnification bar indicates 500 μm and 100x magnification bar indicates 200 μm.

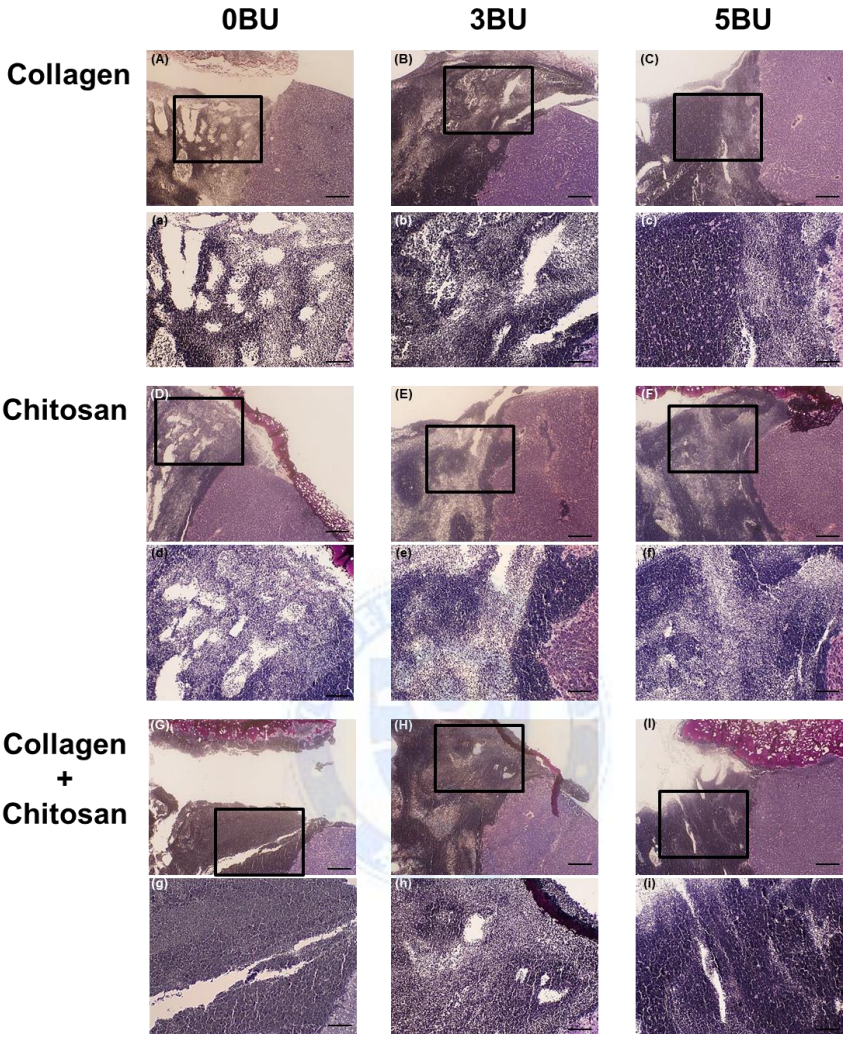

Figure 16. Histological photomicrographs of liver wound site with fibrin clot formation. The images were histologically examined by PTAH staining. (A)-(I) images were taken at 40x magnification and (a)-(i) images were taken at 100x magnification. Collagen treated group: (A)-(C); Chitosan treated group: (D)-(F); Collagen with Chitosan treated group: (G)-(I); (A), (D) and (G) natural substances with no r-Batroxobin; (B), (E) and (H) natural substances containing r-Batroxobin 3 BU/well; (C), (F) and (I) natural substances containing r-Batroxobin 5 BU/well. 40x magnification bar indicates 500 μm and 100x magnification bar indicates 200 μm.

IV. DISCUSSION

Complimentary balance of fibrinolytic pathway and complex interactions with composition in blood vessel is an essential prerequisite for successful hemostasis.[1] Hence, difference mechanisms used are very important and give a more striking effect to blood coagulation in hemostasis. It is known that collagen and chitosan can promote platelet activation and aggregation in intrinsic pathway,[31] also, chitosan induces the blood clot formation as combined with erythrocyte cell.[32] Batroxobin accelerates conversion of fibrinogen to fibrin to help blood clot formation.[21]

In this research, fabrication of the hemostatic pads composed of collagen with chitosan containing various concentrations of recombinant batroxobin was shown, and it demonstrated effects on rapid hemostasis through *in vitro* and *in vivo* studies.

First, when collagen and chitosan fabricated pads with various r-Batroxobin concentrations were used, the sponges were confirmed any properties changes compared to collagen or chitosan only treated pads. The prepared hemostatic pad SEM images showed that the hemostatic pads of collagen and chitosan containing r-Batroxobin 0, 1, 2 and 3 BU/well did not affect micro structural characteristics with retention of the suitable pore size in Figure 6. The difference in pore size between collagen and chitosan was determined by the substances properties. Collagen can be more easily combined with substances other than chitosan powder, so blending with another natural material gives higher porosity in collagen treated groups.[33-36] From the fibrinogen conversion fibrin experiment result, r-Batroxobin maintained their hemostatic mechanism even if mixed with natural substances. As the measurement of fibrin turbidity, r-Batroxobin treated with collagen and with collagen and chitosan groups showed higher turbidity than r-Batroxobin treated with chitosan groups. The reason for collagen-based materials can swell with tissue compression.[11]

Hemostatic pads including collagen had higher-porosity structure, so r-Batroxobin was easily released from the pad. In addition to platelet activation assay, the collagen and chitosan hemostatic mechanism also was not lost when combined with r-Batoxobin. That result was expected as collagen and chitosan used together for increase of coagulation effect. Chitosan binds and aggregates platelets, as well as agglutination of erythrocyte, and collagen affects platelet adhesion and subsequent activation in hemostatic mechanism.[1,19,20,37] In Firgure 8, SEM micrographs also show the precise spread of platelets with protruding pseudopods. In order to achieve the synergetic effect of respective hemostats mechanism, whole blood was kept with the hemostat pad groups. For comparison, a not treated group was used as control. The clot formation is shown in Figure 9, which shows that the group treated with r-Batroxobin 3BU/well had much better blood clot ability compared with other groups. Moreover, collagen with chitosan containing r-Batroxobin 2 BU/well performed equally well in this respect. Chitosan-treated groups had higher percentages of clot than collagen-treated groups. As mentioned above, that was due to chitosan affecting not only platelet aggregation but also binding with red blood cell.[38,39]

A previous study that achieved hemostasis by biodegradable adhesive with batroxobin in different animals,[30,40] it has led to a set of animal experiments to determined that synergetic effect of fabricated hemostatic pad. The efficacy of r-Batroxobin reconfirmed the result of SD rat femoral artery model in Figures 11 and Table 1. According to the result, all groups of hemostatic pad shortened bleeding time lag with increasing concentration of r-Batroxobin compared with collagen-only or chitosan-only treated groups. When the hemostatic pad did not cover bleeding in injury site completely, bleeding ooze occurred because the femoral artery is high blood flow site.[41] However, with increased concentration of r-Batroxobin, recurrence bleeding in the wound site reduced. r-Batroxobin can play an important role of a second barrier for coagulation. During the process of hemostasis in the site, collagen and chitosan cooperated with

r-Batroxobin to rapidly control bleeding. In addition, SD rat puncture model result have been shown to stop bleeding rapidly in hepatic parenchymal as well as exclusive bleeding model. In other liver wound models, the wound was made by a prick.[42] This experiment result concerned higher blood loss because the wound made by a hole in the liver lobe.[43] The hemostats pad containing r-Batroxobin could be completely adhered on the surrounding hepatic tissue and rapidly solidified to assist the bleeding barrier within 30 sec. Figure 14 shows that the amount of total blood loss was conspicuously reduced in the group treated with r-Batroxobin 5 BU/well.

Histological examination of the wound, which was covered with blood clots and fibrin clots, also showed that three substances generated synergetic effects and functionality of r-Batroxobin. In other studies that conformed to histological analysis without any treatment for the wound area, the blood from the bleeding flowed, so clots hardly formed.[9, 30] As Figures 15A(a), 15D(d) and 15G(g), the images show that only collagen or chitosan treated groups rarely exhibited clot formation and not be enough to present upon examination. By contrast, Figures 15B(b)-15I(i) formed blood clots with more dense stained pinkish red by the addition of r-Batroxobin 3 or 5 BU/well. Especially Figures 15C(c), 15F(f) and 15I(i), containing r-Batroxobin 5 BU/well, were definitely deep stained blood clot with high density. The results clearly support that the synergetic effect of respective substances at the hemorrhage site. Moreover the fibrin was selectively stained by PTAH staining method because only H&E staining result was not sufficiently complete for efficacy of r-Batroxobin. Thus, the fibrin formation promoted the entrapped dye with erythrocyte.[40] As H&E images with r-Batroxobin, PTAH staining results also with r-Batroxobin provided an excellent environment for advanced fibrin clots. Furthermore Figures 16H(h) and 16I(i) fully show deep clots formation. As the entrapped erythrocytes were stained with fibrin, the increase in the amount of blood clots might be more highly distinguished through the PTAH staining. This was

already confirmed in the quantitative analysis of the liver wound model. The amount of blood loss was decreased with the treatment of r-Batroxobin.

Overall experimental results support the hypothesis that collagen, chitosan and batroxobin have a synergetic effect on treating an injury site. In addition batroxobin reacted to enhance the hemostatic function of the natural substances pad. r-Batroxobin revealed an excellent hemostatic ability in conjunction with collagen and chitosan at the bleeding site. They also confirmed that early stage hemostasis was accelerated by increasing of r-Batroxobin concentration in an animal model.

V. CONCLUSION

Collagen and chitosan hemostatic pads exhibited analogous hemostatic efficacy and hemostasis success rate in experiments. Furthermore, r-Batroxobin was reconfirmed as having excellent hemostatic properties and offering a stable barrier in excessive bleeding. This is first utility evaluation of collagen and chitosan containing r-Batroxobin for internal clinical use. Based on the result shown above, a novel hemostatic pad has great potential for uncontrolled bleeding injury treatment.

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ABSTRACT(IN KOREAN)

배트록소빈 함유에 의한
콜라겐과 키토산 지혈패드의 기능적 효과

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선 경 미

일상생활에서나 갑작스러운 사고, 수술과정 또는 전쟁 중 제어할 수 없는 과도한 출혈은 감염성 합병증을 초래하여 쇼크에 의해 사망에 이를 수 있게 한다. 그러므로 최종적인 치료 전 급성 출혈을 효과적으로 도우며 환자를 안정에 이르게 하기 위하여 국소 지혈제와 밀폐제를 필요로 하게 된다. 이번 연구에서는 오늘날 지혈제로서 널리 이용되고 있는 천연물질인 콜라겐과 키토산을 사용하였는데, 두 물질은 지혈에서 혈소판의 활성과 응집의 역할을 하게 된다. 또한 키토산은 적혈구와의 결합으로 혈액 응고를 돕기도 한다. 그러나 이 물질들만으로는 적절한 시간 내에 지혈효과를 내기에 충분하지 않다는 많은 연구 결과들이 보고되어져 왔다.

이러한 이유로 본 연구에서는 지혈과정에서 피브리노겐을 피브린으로 전환하여 출혈을 제어하는 기능의 물질로 *Bothrops atrox moojeni* 라는 뱀의 독성 성분 일종인 배트록소빈을 이용하여 더욱 빠르고 효과적인 스펀지 타입의 새로운 지혈제를 만들었으며, 이를 통해 사용된 재조합 배트록소빈이 지혈 기능의 측면에서 천연물질 성분에 시너지 효과를 줄 수 있을 것이라고 기대하였다. 먼저 콜라겐과 키토산을 재조합 배트록소빈과 함께 아세트산에 녹여 새로운 지혈패드를

제작하였고 그의 표면적 특성을 주사전자현미경법을 통해 비교, 확인 하였다. 제작된 스펀지는 콜라겐, 키토산, 콜라겐과 키토산, *in vitro* 실험을 위한 콜라겐 또는 키토산에 재조합 배트록소빈 (1, 2, 3 BU/well)이 포함된 군, *in vivo* 실험을 위한 콜라겐 또는 키토산에 재조합 배트록소빈 (3, 5 BU/well)이 포함된 군, 콜라겐과 키토산에 재조합 배트록소빈(1, 2, 3, 5 BU/well)이 포함된 군으로 나누어 진행하였다. 피브리노겐 응고 분석과 혈소판 활성 분석을 통해 물질들이 혼합되어 있음에도 각각의 지혈 기전을 유지하고 있는지를 확인하였으며, 전혈 응고 분석과 동물실험을 통해서 부상 위치에서 혈액 응고 활성을 보이는 지를 조사하였다. 또한, H&E와 PTAH 염색 방법을 통해 생성된 혈전 생성과 피브린 응집 정도를 조직학적 분석을 통해 확인 하였다. 위와 같은 실험들의 결과를 통해, 새로운 지혈패드가 패드 안에서 각각의 지혈 효과를 유지하고 있음을 확인하고 혈액 응고 실험들을 통해 재조합 배트록소빈의 함유를 통해 출혈시간과 출혈량을 효과적으로 줄인다는 것을 입증하였다.

결론적으로, 본 연구를 통해 제작된 새로운 지혈패드는 과다 출혈 부상에 적용하기에 엄청난 가능성을 가지고 있으며 재조합 배트록소빈과 더해졌을 때 그 천연물질에 효과가 더해졌음을 입증하였다.

핵심되는 말 : 지혈, 지혈패드, 콜라겐, 키토산, 재조합 배트록소빈