Evaluation of hemostatic effects of an adhesive with recombinant batroxobin

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Evaluation of hemostatic effects of an adhesive with recombinant batroxobin

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ABSTRACT

Evaluation of hemostatic effects of an adhesive with recombinant batroxobin

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Uncontrolled bleeding can cause serious complications in surgical procedures, traumatic injuries on battle fields and in daily life. Excessive blood loss could ultimately threaten human life. Therefore, studies concerning hemostatic materials and their therapeutic solutions have been undertaken to arrest bleeding within a relevant time to minimize blood loss. This research focused on a topical hemostatic adhesive which is composed of powdery, biodegradable adhesive and recombinant batroxobin. Batroxobin is derived from the venom of a snake, *Bothrops atrox moojeni,* and has an active property for hemostasis.

In the blood coagulation system, batroxobin converts fibrinogen to fibrin, which was represented by a fibrinogen clotting assay with the batroxobin-containing adhesive. Also, whole blood obtained from an SD rat was examined to determine whether it could form clots on the hemostatic adhesive. Through

in vivo experiments, activities of the adhesive on the bleeding sites of the animals were evaluated with thirty-two SD rats and thirty-two ICR mice. The animals were placed into four groups (n=8). One group was left untreated and the other groups were allocated adhesives containing 0, 30 or 50 NIH U/ml batroxobin respectively. To compare the amount of blood loss, the liver wound model was applied. The result was histologically analyzed by H&E and PTAH staining methods. The femoral artery injury model of rats was applied for analysis of the hemostasis times and patterns.

The results of the liver model indicated that blood loss was reduced with the treatment of those mice that were allocated adhesive containing increasing concentrations of batroxobin. The time required for hemostasis was also significantly decreased with application of the adhesive compared to the control. The 50 NIH U/ml batroxobin-containing adhesive was remarkably effective for reduction of coagulation time. Also, the addition of batroxobin could decrease the occurrence of re-bleeding during the hemostatic procedure with the adhesive. The biodegradable adhesive played a role as the first mechanical barrier against bleeding; while batroxobin was directly involved in formation of blood clots to arrest bleeding. Hence, the adhesive promoted hemostasis successfully in both types of bleeding injuries with an increase in the concentration of batroxobin.

In conclusion, an adhesive containing the appropriate concentration of batroxobin would be effective in controlling bleeding with respect to the amount of blood loss and the blood arresting time.

Key words: hemostasis, batroxobin, hemostatic adhesive, bleeding injury

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I. INTRODUCTION

1. Blood coagulation

Rapid hemostasis is critical for injuries accompanied by excessive bleeding provoked in war time, pre and post operations and daily life. In fact, bleeding inappropriately dealt with may threaten life and finally cause death [1, 2, 3]. Therefore, the studies about effective hemostatic agents with proper blood clotting time have been conducted. During the process of hemostasis, blood coagulation is triggered to form fibrin clots, and ultimately arrest bleeding. The blood coagulation system is stimulated by vascular injuries [4]. Once the blood vessel is damaged, vasoconstriction is initially occurred to reduce the blood flow, and then the key factor of blood coagulation, thrombin, activates platelets resulting in recruitment of platelets to the injury sites. The recruited platelets are aggregated to form loose platelet plug as they interact with fibrinogen platelet receptors. The platelet plug which is induced by conversion of fibrinogen to a fibrin mesh is at first weakly structured, but cross-linking polymerization of fibrin is progressed to have stable networks [4, 5, 6].

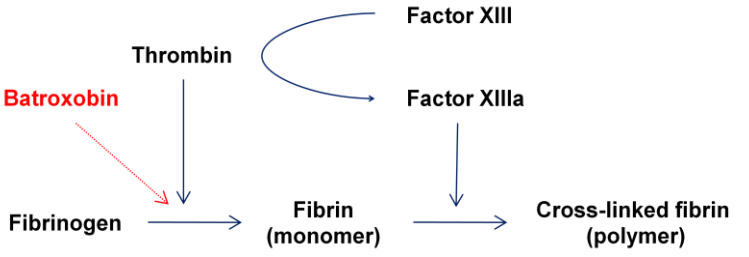

Figure 1. Fibrin formation in blood coagulation cascade. At the end of blood coagulation cascade, thrombin acts for conversion of fibrinogen to fibrin, and also activates coagulation factor XIII which initiates polymerization of fibrin. The role of batroxobin in this system is the same as that of thrombin but not involved in the formation of cross linked fibrin.

2. Batroxobin

Batroxobin is a venomous component of a snake, *Bothrops atrox moojeni*, which acts as a thrombin-like enzyme on hemostasis system. In blood coagulation cascade, batroxobin plays a role of serine protease and is involved in fibrinogen conversion. As described in figure 2, D domain and E domain are existed in fibrinogen and interact with each other to form fibrin networks. The segments of fibrinogen α chains are non-covalently associated with a specific binding site of the E domain, but β segments covalently interact with the E domain. During this process, batroxobin cleaves only the fibrinogen α chain unlike thrombin acts on cleavage sites in both fibrinogen α and β chains [7, 8]. As a result of cleavage of fibrinogen by either batroxobin or thrombin, fibrinopeptides are subsequently split off, and interact non-covalently each other to form fibrin web [8, 9, 10]. The loosely formed fibrin clots in this process are soluble and not stabilized yet to withstand fibrinolysis, but the blood coagulation system driven with thrombin is progressed to polymerize the fibrin clots. On the other hand, batroxobin does not affect the formation of crosslinking networks of fibrin by polymerization. It is because the coagulation factor XIII involved in stabilization of fibrin clots is activated only by thrombin but not by batroxobin. Also, other clotting factors and cells are not influenced by batroxobin in contrast to thrombin[9], and it would be a positive property of batroxobin in therapeutic uses. The hemsotatic effect of natural batroxobin has been already known and indeed commercialized in the global market place of hemostatic agents. For example, a Swiss company, Pentapharm has been producing a drug based on batroxobin, and also similarly other types of enzymatic venoms from snakes are also existed in the market, Ancrod from Knoll in USA and Bothropase from Ravizza in Italy are representative examples of the products derived from the natural snake venom. However, natural sources often face challenges of high cost and shortage in supply. Recombinant batroxobin rewarding these drawbacks was used for this research, which its cDNA was expressed in *Pichia pastoris*. The recombinant batroxobin is functionally characterized and more cost effective, and mass production is also available [10].

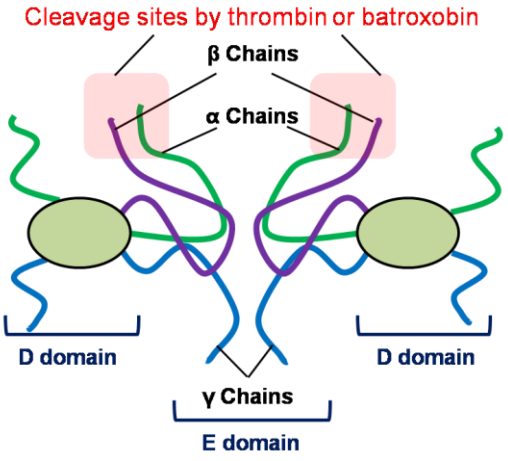

Figure 2. Cleavage of fibrinogen. Fibrinogen conversion to fibrin is conducted by association of its D and E domains. Cleavage of α chain of fibrinogens initiates that the binding pocket of the D domain combines non-covalently with the specific binding site in the E domain. Then, the binding site in the β segment of the D domain is interacted with the E domain for polymerization.

3. Biodegradable adhesive

A biodegradable adhesive (LYDEX) has been studied by Kyoto University as an alternative adhesive for clinical usage, which compensates the defects of biodegradability and infection in conventional medical adhesives such as aldehyde based adhesive and fibrin glue [11]. LYDEX is composed of aldehyded dextran and ε-poly (L-lysine) with crosslinking networks, which forms Schiff base, and is characterized by high bonding strength, high flexibility, low toxicity and no risk of infection [11, 12, 13]. The adhesive formed by in-situ gelling is applied to sealing air leakage after lung surgery and keratoplasty [13, 14].

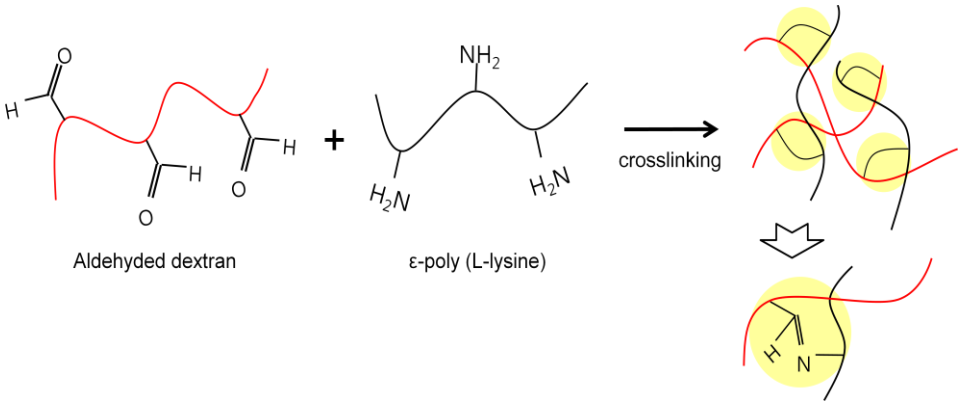

Figure 3. Chemical structure of LYDEX. LYDEX is formulated by crosslinking of aldehyded dextran and ε-poly (L-lysine) [13].

4. Hemostatic adhesives

For controlling hemorrhage, many kinds of material were investigated and used in various bleeding occasions. However, commercially used hemostatic agents could not be satisfied with some of ideal conditions such as safety, rapid and effective reactivity, easy and comfortable use, cost effectiveness, and approvability [15, 16]. For example, animal derived agents like thrombin, fibrinogen and collagen are effective hemostatic agents; but they can cause foreign body reaction, and also have a risk of disease transmission when it is applied to human [17, 18, 19]. Cellulose, cyanoacrylates and other synthetic materials were alternatively used but still problematic because of one or more of disadvantages such as their poor effectiveness, low degradability in vivo, high cost and some allergic reactions [18, 20]. The form of the materials also critically affects their performance and use, and thus a type of products is selectively applied in regards to severity, location, shape and size of bleeding injury. The adhesives in liquid form are usually able to use for relatively large wound but difficult to arrest severe bleeding [17]. By contrast, powdered products are advantageous for hemostasis in irregular surfaces of wound and portability [17]. As various types of the material functioning hemostasis are existed with different characteristics, some agents are combined to improve their hemostatic performance. In this study, biodegradable powdery LYDEX was chosen as a novel base of adhesives and the active agent of blood coagulation, recombinant batroxobin was added with the expectation of enhanced hemostasis.

5. Objectives of the study

The aim of this study is to examine the performance of the hemostatic adhesive composed of recombinant batroxobin and LYDEX, and finally estimate the potential for development to be a novel hemostatic adhesive and application to patients. The blood clotting ability of the batroxobin-LYDEX adhesive was identified by cytotoxicity test, fibrinogen clotting assay and whole blood clotting assay. Furthermore, two animal models were adopted to verify the rapid and effective hemostatic functions of the adhesive in hemorrhage. The liver wound model provided changes of total blood loss with applications of batroxobin and LYDEX. Also, excessive and continuous bleeding condition was induced by the femoral artery wound model to investigate the progression of hemostasis. The hemostatic effects of the adhesive were verified by histological analysis.

II. MATERIALS AND METHODS

1. Materials

Recombinant batroxobin dissolved in phosphate buffered saline (PBS) and human fibrinogen were kindly donated by Biobud (Korea). Active solutions containing batroxobin were prepared with various concentrations required for *in vitro* and *in vivo* experiments, and they are reconstituted with calcium chloride to give the final concentration of 20 mM [21]. LYDEX providing a mechanical template was also generously supplied by Prof. S.H. Hyon, Kyoto University in the form of powder. The fibrinogen stock solution of 25 mg/ml was made by dilution in distilled water. Drabkin's reagent was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), and reconstituted according to the manufacturer's preparation instructions. Cyanmethemoglobin Standard Solution (Stanbio laboratory, Boerne, TX, USA) which is equivalent to 180 mg/ml of hemoglobin was diluted in prepared Drabkin's solution for determination of hemoglobin concentration. Grade1 filter paper (4.25 cm in diameter) was used for animal experiments, which was purchased from Whatman (Kent,UK).

2. Cells and cell culture conditions

L-929 cells derived from murine fibroblasts (American Type Culture Collection, VA, USA) were cultured in Eagle's Minimum Essential Medium (MEM, Welgene, Korea) constituted by 90% of 1.5 g/L sodium bicarbonate, 0.1 mM non-essesntial amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum. Cells were maintained at 37 ℃ in a 5% CO_2 atmospheric incubator.

3. *In vitro* studies

A. Preparation of the batroxobin-LYDEX adhesive

The batroxbin-LYDEX gel was formulated in 24-well plates as the 150 μl active solution was added to LYDEX (50 μg) for each well. Powdery LYDEX was evenly spread on the surface of the well by tapping the side of the plate, and the active solution was carefully dropped on it to be participated in the formation of the gel. For *in vitro* studies, the content of batroxobin in the solution was prepared in 0, 5, 10 and 15 NIH U/ml.

B. Cytotoxicity of batroxobin

(1) Batroxobin treatment

Cytotoxicity of batroxobin used in this study was examined in triplicate with L-929 cells. The toxicity was investigated in the range of concentration from 0 NIH U/ml to 60 NIH U/ml. For the experiments, the cells were initially seeded at a density of 5 X 10^4 cells/cm^2 and incubated overnight at 37 ℃ in a CO_2 incubator. After the cells were stably attached, the culture media was discarded and PBS was used to wash the cells. Then, the cells seeded in each well were treated with 500 μl of batroxobin solution prepared in 0, 3.75, 7.5, 15, 30 and 60 NIH U/ml. After treatments, the cells were incubated for 24 hr, and the viability was evaluated by MTT assay.

(2) MTT assay

After 24 hr of treatment for cytotoxicity test of batroxobin, the treatment solutions were removed and washed out with PBS. Then, 0.5 mg/ml of a yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT, Amresco, Solon, OH, USA) was added, and incubated at 37 ℃ in the CO_2 incubator for 2 hr. During the incubation period, the purple

formazan salts were formed by reaction with metabolically active cells, and they were dissolved in dimethyl sulfoxide. The dissolved salts were transferred to a 96-well reading plate, and the absorbance was measured at 570 nm by an automatic microplate spectrophotometer (Spectra MAX 3340, Molecular Devices Corp, Sunnyvale, CA, USA).

C. Fibrinogen clotting assay

To evaluate the fibrin formation by batroxobin released out from the gel, 200 µl of 20 mM Tris-HCl (pH 7.5) was added to the prepared gel and incubated for 1, 5 or 10 min at 37 ℃. The buffer solution containing batroxobin was collected at each time point. The fibrinogen (stock prepared in 25 mg/ml) was mixed with the buffer solution to make 0.5 mg/ml fibrinogen, and 2 min was given for the formation of fibrin clots [22, 23]. Batroxobin released out from the LYDEX gel containing different concentrations of batroxobin was reacted with fibrinogen, and consequently fibrin networks were formed. The fibrin formation was verified by turbidity changes of the mixture of fibrinogen and the baroxobin released solution. The turbidity was detected at 405 nm by using the automatic microplate spectrophotometer.

D. Whole blood clotting assay

Whole blood was obtained from 8 week old male Sprague-Dawley (SD) rats. Zoletil (Boehringer Ingelheim Agrovet, Hellerup, Denmark) and Rompun (Bayer, Toronto, Canada) were intraperitoneally administered to the animal for anesthesia. The dosage of the drugs was determined by the weight of the animal with 30 mg/kg for Zoletil and 10 mg/kg for Rompun. The abdominal vena cava of the anesthetized rat was exposed to collect the whole blood using a syringe containing 0.109 M sodium citrate (BD, UK) in a 9:1 ratio. The citrated blood sample was well shaken to prevent coagulation. Blood (200 µl) was then loaded on the batroxobin-LYDEX gel prepared with various concentrations of batroxobin in 0, 5,

10 and 15 NIH U/ml, and the plate was incubated at 37 ℃. After 5 min of incubation, PBS was added to dissolve unstable blood clots. The diluted blood in PBS was then transferred into a 96-well plate to react with drabkin's solution. Prior to measure the light absorbance at 540 nm using a Synergy H4 hybrid reader (Bio tek, Winooski, VT, USA), the solution was stabilized the color with incubation at 37 ℃ for 15 min and protected from light.

4. In vivo studies

A. Animals and preoperative preparation

Two different families of the animal, Imprinting Control Region (ICR) mouse and Sprague-Dawley (SD) rat, were applied for in vivo studies. All animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Yonsei Laboratory Animal Research Center (YLARC) (Permit #: 2013-0017). All mice were maintained in the specific pathogen-free facility of the YLARC.

The liver model was conducted with 7 week old male ICR mice, and thirty-two healthy mice were randomly assigned to one of four groups including no treatment, applications of LYDEX and the active solutions composed of three different concentrations of 0, 30 and 50 NIH U/ml. For the application to the femoral artery model, thirty-two male SD rats at the age of 8 weeks were utilized, and they were divided into four groups in the same way as the liver model. Prior to the experiments, the animals were anesthetized by intraperitoneal injection of Zoletil and Rompun as described above in whole blood clotting assay. After all the experiments have been performed, the animals were euthanized. The parafilm cut in 5 x 5 cm and the weights of filter paper were measured before starting animal experiments.

B. Liver model in ICR mouse

Hemostasis in liver injury model was evaluated by a modified method described by Murakami et al [24]. To start the liver model, the anesthetized ICR mouse was fixed on a cork board, and the liver was exposed to make a pricking wound using a 13G needle. The wound was made in the thickest area in the middle of the left lobe. Immediately after making the injury, LYDEX (5 mg) was applied on the site of bleeding followed by the addition of the active solution (20 µl) containing batroxobin. Then, the pre-weighed filter paper and the parafilm were placed under the lobe. While the blood was absorbed by the filter paper for 1 min, the cork board was tilted at 45 °. The parafilm was used to prevent to absorb other body fluids except blood running out from the wound. The filter paper with blood was measured to evaluate the total amount of blood from bleeding in each treatment group.

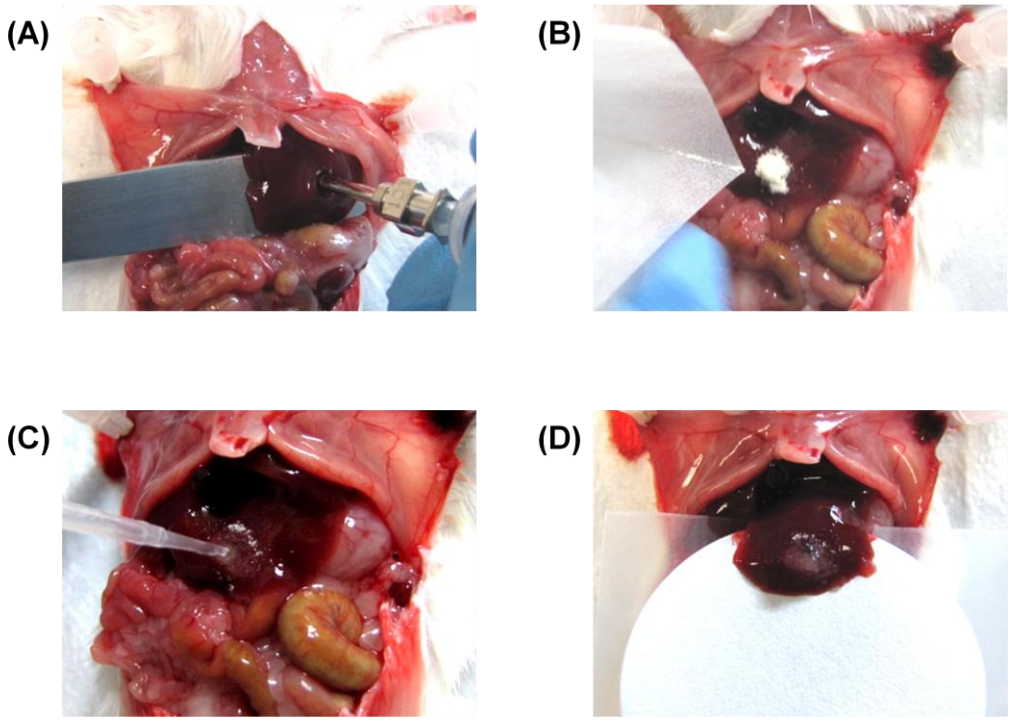

Figure 4. Procedure of liver injury model. (A) The left lobe of the mouse liver was pricked with a 13G needle. (B) The wound site was covered by 5mg LYDEX powder, (C) followed by 20 μl batroxobin solution with $CaCl_2$ (20 mM). (D) Filter paper was placed under the lobe of the liver. Parafilm was also located beneath the filter paper.

C. Femoral artery model in SD rat

The anesthetized SD rat was fixed on a cork board using needles. A right leg of the animal was incised to expose the femoral artery, and then the vessel was cut with a scalpel. After inducing an arterial injury, it was remained without any treatment for 10 sec to give time for blood flowing throughout the injured site. Then, blood and serous fluid were cleaned by gauze. LYDEX (20 mg) was applied on the wound area, followed by the 50 μl active solution prepared in various concentrations of batroxobin. Immediately after the application of the hemostatic agents, the cork board was tilted at about 45 ° and the filter paper was placed below the injured site to absorb blood flowing down. It was carefully performed to avoid that the gel formed by LYDEX and the active solution stuck to the filter paper. The filter paper was replaced every 15 sec with wiping out the blood stained around the wound area to find out accurate time point for hemostasis completion. The experiment was lasted for 5 min after application of the hemostatic agents. The sheets of filter paper with absorbed blood at each time point were weighed in order to compare the amounts of blood from bleeding among different treatment groups. Also, the filter paper absorbing the blood from bleeding was used to evaluate the amount of hemoglobin released out for every 15 sec. The filter paper was dried at 4 ℃ overnight, and cut into 4 pieces to be placed into a test tube. For releasing media, 4 ml saline was added and the blood was released out for 2 hr in a shaking incubator. The solution containing blood was then reacted with drabkin's solution in 37 ℃ for 10 min. After the color of the solution was stabilized, the absorbance was measured at 540 nm by the spectrophotometer.

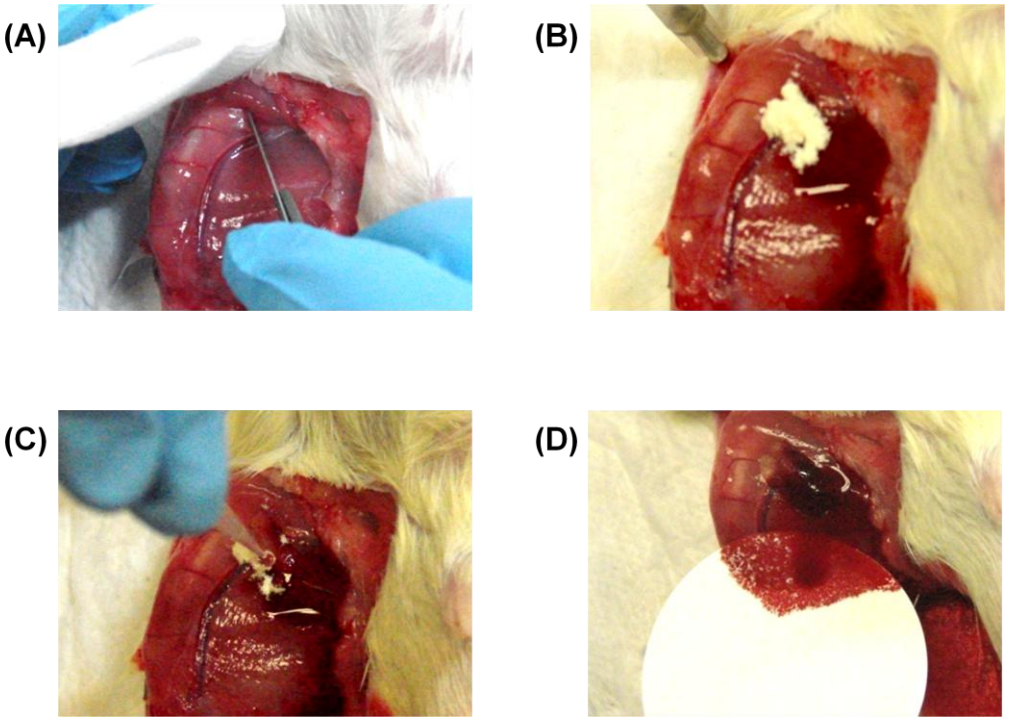

Figure 5. Procedure of femoral artery injury model. (A) The right femoral artery was exposed and cut. (B) 20 mg LYDEX was topically applied to the wound site. (C) The 50 μl active solution containing batroxobin and $CaCl_2$ (20mM) was dropped on LYDEX, and subsequently gel was formed. (D) Filter paper was placed below the injured area.

D. Histological analysis

To examine the blood clot formation for the *in vivo* experiment, the liver wound was isolated with surrounding tissue at the final stage of the experiment of the liver wound model. The isolated tissue was fixed in 10% formalin and embedded in paraffin. The paraffin block was vertically cut into sections in 4 μm thickness to observe clot formation around the layers of tissue and adhesives in the middle of the wound area. Then, the tissue slides were deparaffinized in an oven set at 60 ℃ and stained by hematoxylin and eosin (H&E) or phosphotungstic acid hematoxyin (PTAH) [25]. H&E staining was performed to examine grossly the blood clot formation as erythrocytes and fibrin is not distinguishable. For PTAH staining, the tissue sample was fixed in potassium dichromate for 30 min after passing through alcohol and xylene [26]. Also, the treatment duration of the PTAH stain solution was carefully adjusted to one hour and also the next step of dehydration with alcohol was shortly processed to avoid staining of erythrocyte but to dye only fibrin blue. After two different staining procedures were completed, fibrin formation on the tissue was observed and obtained images by an inverted microscope (IX5, Olympus, Japan) equipped with a DP 71 digital camera (Olympus).

5. Statistical analysis

All results were statistically analyzed by Student t-test using Microsoft Office Excel 2007. Data are expressed as mean ± standard error of mean. P values <0.05 were considered statistically significant.

III. RESULTS

1. In vitro studies

A. Cytotoxicity test of batroxobin

Prior to all other experiments, the hemostatically active agent, recombinant batroxobin was examined whether it would affect the viability of L-929 cells. As a result, the viability of the cell was almost not influenced by treatment of batroxobin in this range of concentration from 3.75 NIH U/ml to 60 NIH U/ml compared to the control (0 NIH U/ml). Also, the cell morphology was not changed even with the highest concentration of batroxobin (60 NIH U/ml) treated (data not shown).

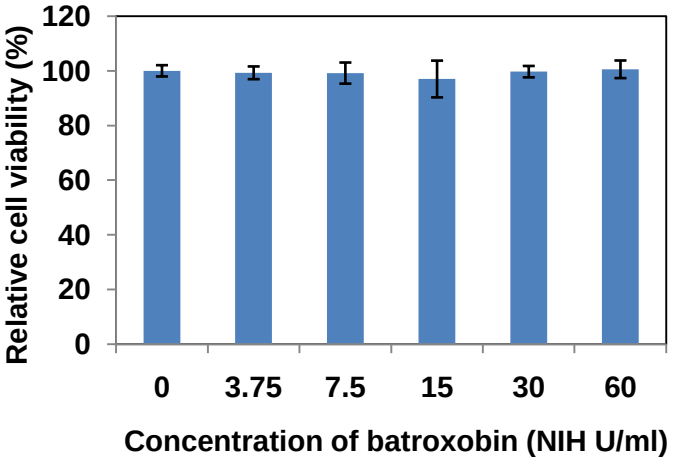

Figure 6. Cytotoxicity of batroxobin. MTT assay was performed to evaluate the cell viability of L-929 after treatment of batroxobin concentrated from 0 to 60 NIH U/ml. The viability was not affected by batroxobin in this range of concentration.

B. Fibrinogen clotting assay

As the concentration of batroxobin in the gel was increased, the turbidity of the solution was elevated at every time point examined. This indicates that batroxobin was involved in fibrinogen conversion. In addition, the fibrin formation was incubation time dependently increased in all the experimental groups. When the incubation time was given for 1 min, the degrees of fibrin formation were not apparently different in the groups treated three different concentrations of batroxobin, but the batroxobin not containing group showed distinctively low degree of fibrinogen conversion throughout the experimental time. The group of batroxobin treated with 15 NIH U/ml was significantly increased in turbidity by 10 min of the incubation time compared to the other two groups with treatment of batroxobin.

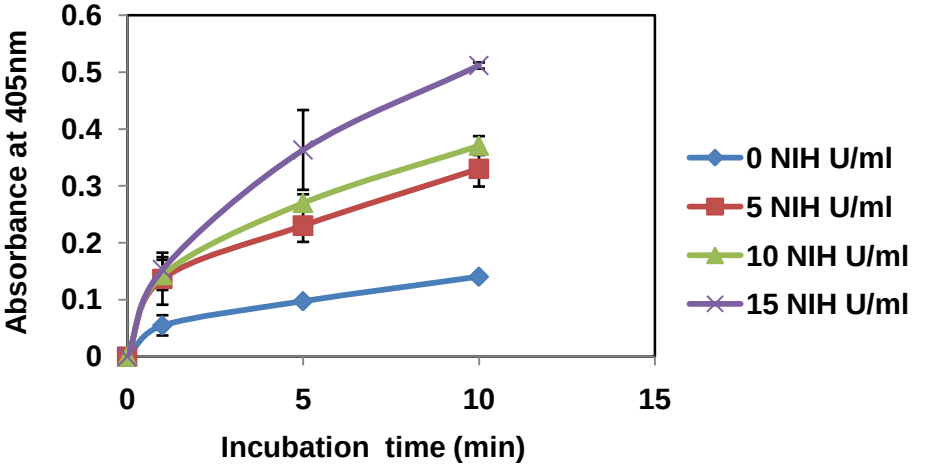

Figure 7. Fibrinogen clot formation. The fibrin net work formation was initiated by the addition of fibrinogen into the solution containing batroxobin from the batroxobin-LYDEX gel. The degree of fibrin formation was spectrophotometrically measured at 405 nm. The fibrin network was increasingly formed with increasing duration of incubation. Also, the formation of fibrin clot was influenced by the concentration of batroxobin.

C. Whole blood clotting assay

Based on the result of fibrinogen clotting assay, fibrin conversion within whole blood was examined. After discarding blood not involved in clot formation, blood clots were remained on the surface of the gel and it was presented darkly in figure 8. Blood clots were formed in different degrees on the batroxobin-LYDEX gel containing various concentrations of batroxobin. With the batroxobin mixed in the gel, the formation of blood clot was apparently increased compared to it in the control group without batroxobin, and more clots were formed with the addition of increasing concentrations of batroxobin. The effect of the blood clot formation was significantly magnified when 15 NIH U/ml of baroxobin was treated.

Figure 8. Photographic images of whole blood clot formation on batroxobin-LYDEX gel. Once the blood contacted to the batroxobin-LYDEX gel, batroxobin and $CaCl_2$ started to be released and reacted with the blood on the gel to form blood clots. With increasing concentrations of batroxobin, more clots were observed on the surface of the gel.

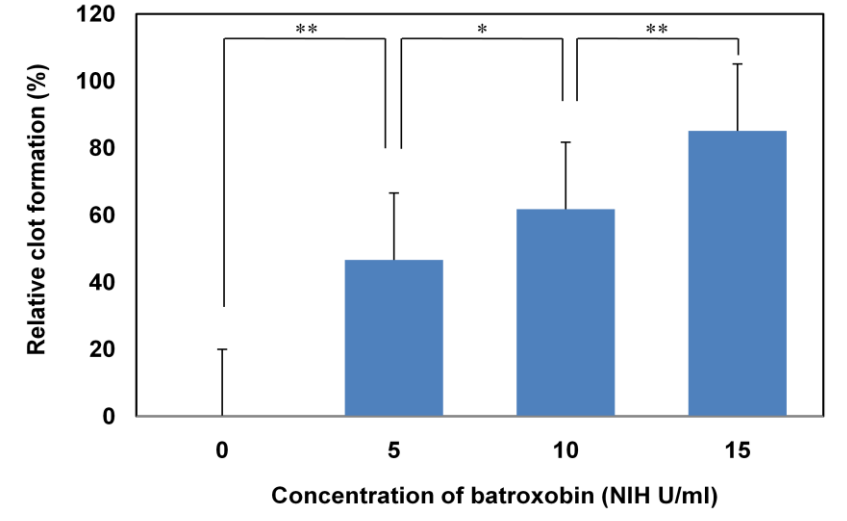

Figure 9. Whole blood clot formation on the batroxobin-LYDEX gel. The formation of clot was expressed in percentaage of the control. The amount of clot was increased with concentration of batroxobin. On the gel containing 15 NIH U/ml batroxobin, blood clots were remarkably more formed than the clots on the 10 NIH U/ml batroxobin-LYDEX gel (*$p<0.05$, **$p<0.005$).

2. *In vivo* studies

A. Liver model in ICR mouse

The difference of total blood loss among various treatment conditions was investigated by the pricking wound model of ICR mouse. When the bleeding sites were covered with the gel containing batroxobin, the amount of blood loss in these treatment groups was reduced compared to that in the LYDEX only treated group and more obviously to that in the group without any treatment. The amount of blood in the untreated group was measured with average 210.3 mg, but the application of LYDEX led to quarterly decrease it despite of no addition of batroxobin. This phenomenon was more clearly shown with treatment of 50 NIH U/ml batroxobin with LYDEX powder. Although the outcome of the 30 NIH U/ml batroxobin containing adhesive was comparable to that when the batroxobin not containing solution was applied to the wound site, 50 NIH U/ml batroxobin represented much better hemostatic performance than the group treated with LYDEX only.

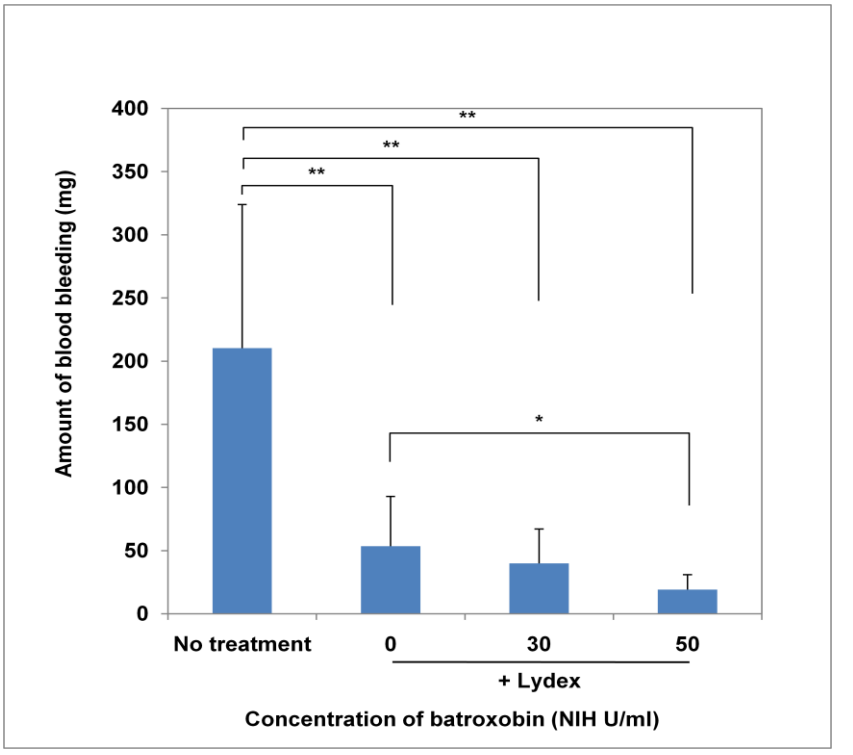

Figure 10. Total blood loss in liver wound model. Hemorrhage induced by liver incision was controlled by different treatment conditions. The amounts of blood from bleeding in the groups treated by LYDEX-based adhesives were reduced half compared to it in the untreatment group. The concentration of batroxobin in the gel also positively affected the reduction in blood loss.

B. Femoral artery model in SD rat

The hemostatic effects in this model were analyzed in two different ways. The amount of blood from bleeding was directly obtained as its increased weight of filter paper after blood absorption was measured. Additionally, the concentration of hemoglobin in the blood was evaluated by an indirect method. The curves which are fitting on the bar graphs indicate the tendency of the hemostasis, and the intersection point of the curve and the x-axis means the time when the hemostasis is completed (Figure 12 and Figure 14). The hemostatic time shown in Table1 was calculated from the equation of the curve fitting on the bar graph in figure 12. According to the figure 12, the curve on the graph of all groups is approaching to the x-axis. Put differently, the hemostasis was progressed as time went by even in the untreated group. However, when the wound was remained without any medications, bleeding was not finished over 5 min. In the same manner, the group treated with LYDEX gel not containing batroxobin could not stop bleeding, but less amount of blood was detected all throughout the period of experimental time compared to that in the group without any treatment. Moreover, the addition of batroxobin with the adhesive was effective to shorten hemostasis time. Figure 12 (C) and Table 1 show that the hemostasis was accomplished within 245.7 sec with the application of the 30 NIH U/ml batroxobin-LYDEX gel, and the increased concentration of batroxobin with 50 NIH U/ml reduced the time for hemostasis by 174.2 sec (Figure 12 (D) and Table 1). Although it was observed that bleeding was tended to stop after treatment, bleeding was restarted in some subjects. If the decreasing amount of blood was turned to be increased again with time progress, it was regarded as re-bleeding and counted the number of subjects undergone re-bleeding. The phenomenon was observed in the groups applied with LYDEX, but the untreated group was exempted for the analysis. As a result, half of the subjects were experienced re-bleeding when the adhesive without batroxobin was applied, but the addition of batroxobin with increasing concentrations positively affected to decline the recurrence of bleeding. The gel with 50 NIH U/ml batroxobin could

result in reduction of re-bleeding up to 25% (Figure 13). In figure 14, the concentration of hemoglobin existed in the blood from wound area is represented. Corresponding to the results from the evaluation of the weight of blood, the duration when the concentration of hemoglobin was reached zero was reduced with applications of the adhesive containing increasing concentrations of batroxobin. The time taken for hemostasis was estimated by the concentration of hemoglobin, and it was slightly shorter than the time obtained from the change in the weight of blood. Reduction of hemostasis time with the 50 NIH U/ml batroxobin-containing adhesive was apparent with detection of the concentration of hemoglobin as shown in other *in vivo* tests.

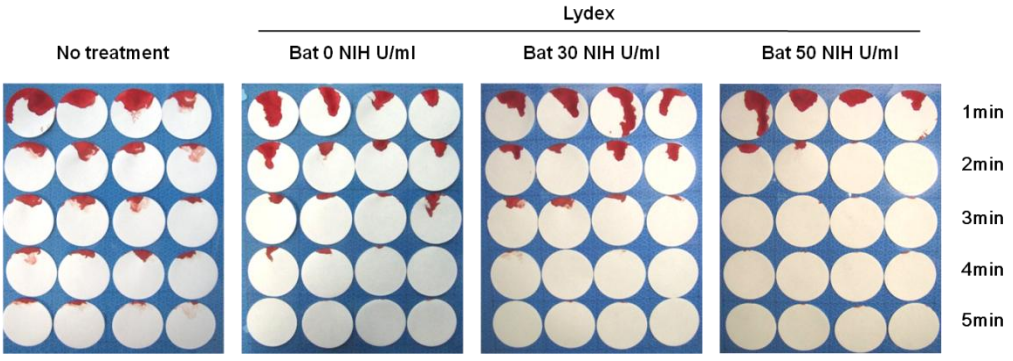

Figure 11. Photographic images of blood absorbed in filter paper in liver wound model. The images show blood blotting on filter paper with replacement every 15 sec. The filter paper was arrayed right and down towards with direction of time progress. The application of LYDEX and increasing concentration of batroxobin induced the reduction in the hemostatic time. This is represented by that the filter paper without any blood stain was found at early time points after treatments.

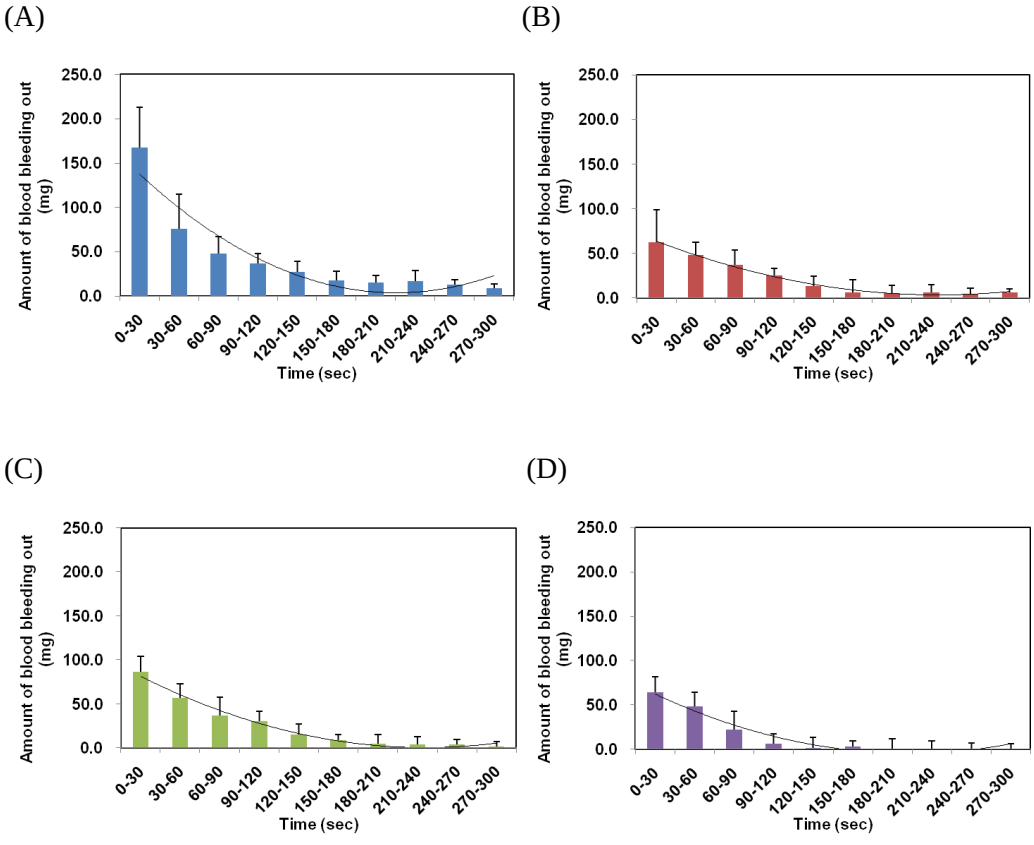

Figure 12. Amount of blood from bleeding in liver wound model. The curve was drawn to fit on the bar graph and its intersection point with the x-axis indicates the time for complete hemostasis. (A) The untreated group. Bleeding was not controlled up to 5 min. The amount of blood flowing out for initial 30 sec was relatively higher than others. (B) The LYDEX gel treated group without batroxobin. The blood was still releasing at 5 min but the curve shifts down from it of the control group. (C) The group treated with the 30 NIH U/ml batroxobin-LYDEX adhesive. Bleeding was arrested at approx 240 sec. (D) The group treated with the 50 NIH U/ml batroxobin-LYDEX adhesive. Hemostatsis was completed between 180 and 210 sec.

Table1. Hemostasis time with various treatments in femoral artery model

Treatment (NIH U/ml)	Time (sec)
No treatment	>300
LYDEX + Batroxobin 0	>300
LYDEX + Batroxobin 30	245.7
LYDEX + Batroxobin 50	174.2

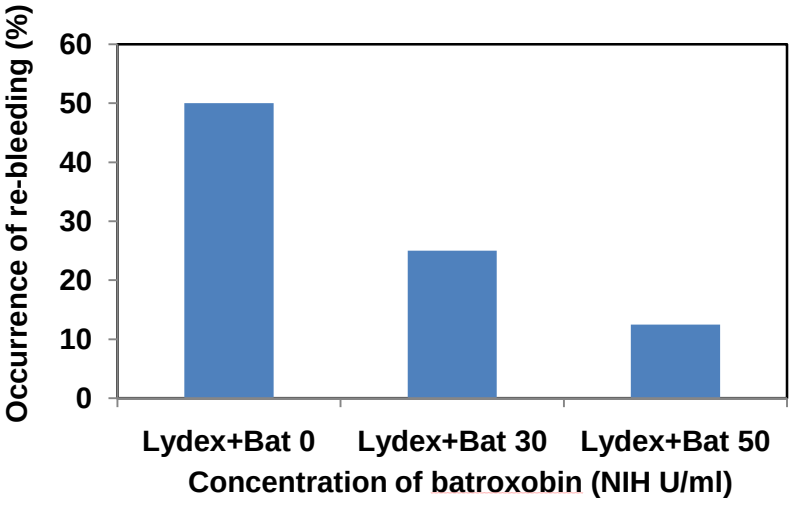

Figure 13. Occurrence of re-bleeding with application of the batroxobin-LYDEX adhesive. Although bleeding was arrested with the LYDEX-based adhesive, the blood was oozed out again. The subjects related in this phenomenon were counted and represented in percentage of out of total subject for each group. Without batroxobin, half of subjects were undergone re-bleeding but its occurrence was proportionally reduced with the addition of batroxobin.

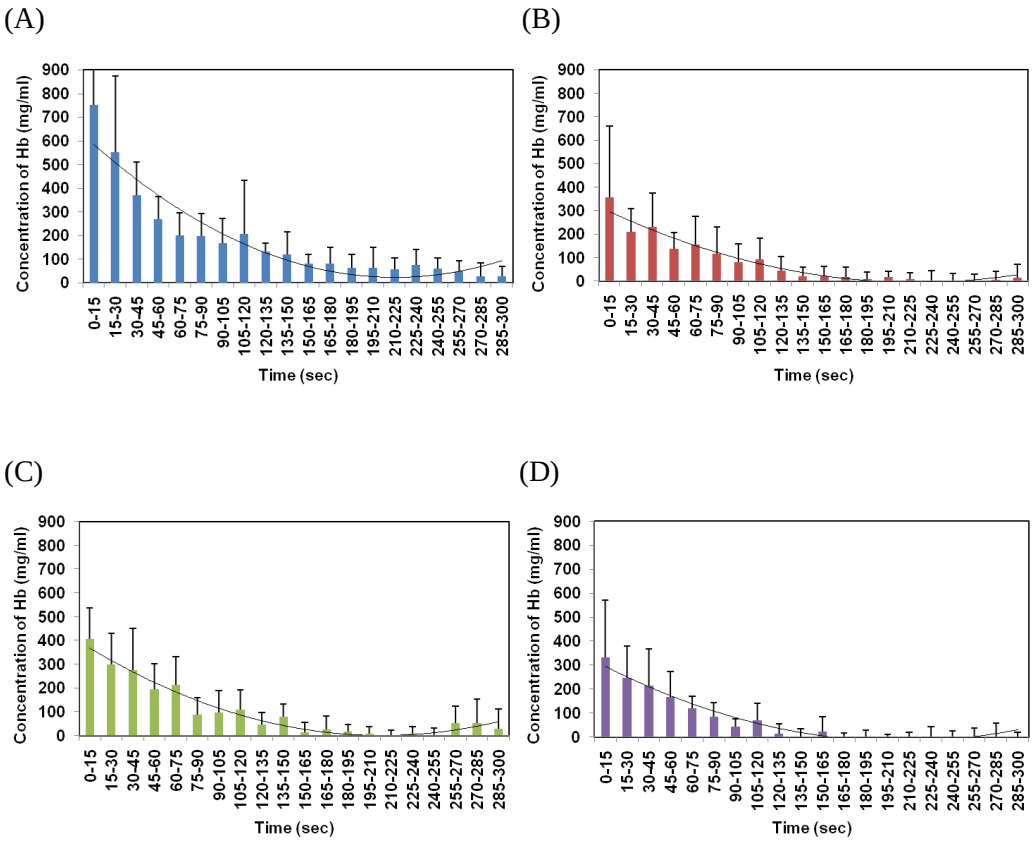

Figure 14. Concentration of hemoglobin in femoral artery injury model. The blood absorbed in filter paper was released in saline to detect the concentration of haemoglobin. From the concentration of haemoglobin, the hemostatic time was found out. The time to stop bleeding was reduced with the adhesive similarly to the result from weight of blood, but it was shortly estimated due to its detection limit. (A) The untreated group. (B) The LYDEX gel treated group without batroxobin. (C) The group treated with the 30 NIH U/ml batroxobin-LYDEX adhesive. (D) The group treated with the 50 NIH U/ml batroxobin-LYDEX adhesive.

C. Histological analysis

Histology of the blood clot formation was examined by two different staining methods. H&E staining was able to visualize both fibrin and erythrocytes, yet PTAH staining was specified to dye fibrin. As represented in figure 15, blood clots were stained pink by H&E staining, which was observed in the adhesive. When the liver wound was remained without any treatment (Figure 15 (A) and (E)), pinkish area around the liver tissue was seldom found; but some erythrocytes were observed around the wound site. By contrast, the groups treated with LYDEX show relatively larger pink area, and its intensity was increasingly high with the increasing concentrations of batroxobin. The images from the groups treated with batroxobin (Figure 15 (C), (D), (G) and (H)) include dark pink area those indicted the intensive clot formation with fibrin. However, the group only treated with LYDEX was stained relatively light pink. Therefore, it can be supposed that more blood clots were formed with concentrated batroxobin.

By the PTAH staining method, fibrin was stained blue but erythrocytes were unstained unlike H&E staining. Figure 16 (A) shows that blue fibrin clots were hardly found around wound area as bleeding was uncontrolled in this group. On the other hand, the LYDEX treated groups (Figure 16 (F)-(H)) show the blue area of fibrin clots. However, the area occupied by blue stain in the group without batroxobin (Figure 16 (F)) was smaller than those in the other two groups treated with batroxobin. Even though the blue area was predominantly observed in the groups treated with batroxobin (Figure 16 (G) and (H)), the intensity and the stained area of the 50 NIH U/ml batroxobin treated group were not distinctively different compared to those in the 30 NIH U/ml batroxobin treated group.

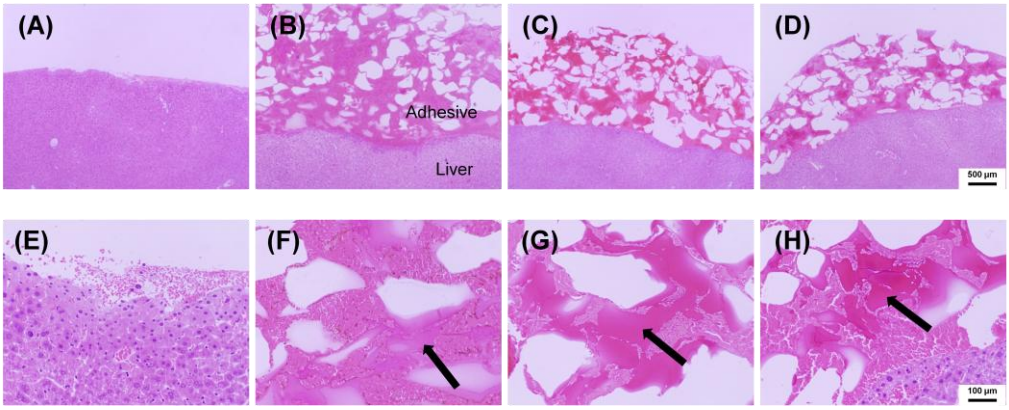

Figure 15. Histological images of the blood clot formation. The liver wound model was histologically analyzed by H&E staining. Images were taken at 40 x magnification ((A)-(D)) and also at 200 x magnification ((E)-(H)). (A), (E): Untreated group, (B), (F): LYDEX & 0 NIH U/ml batroxobin treated group, (C), (G): LYDEX & 30 NIH U/ml batroxobin treated group, (D), (H): LYDEX & 50 NIH U/ml batroxobin treated group. The formation of blood clots was visualized with pink dye (arrow). The blood clots were increasingly formed with batroxobin treatment.

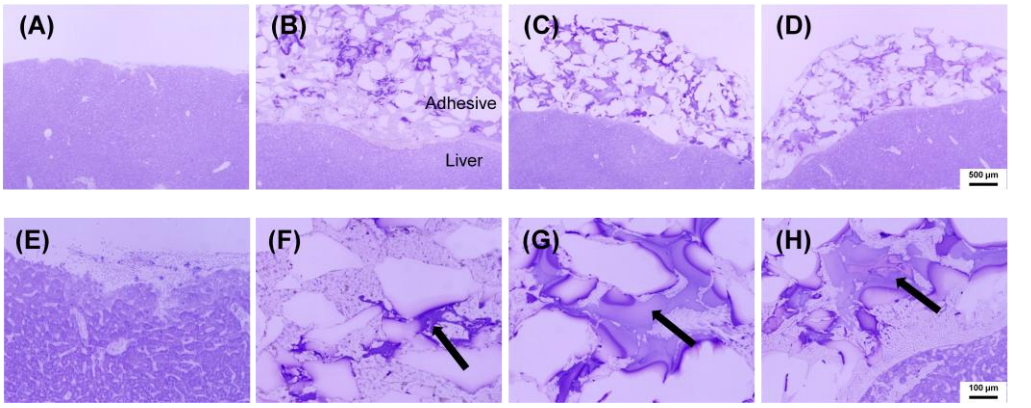

Figure 16. Histological images of the fibrin clot formation. The liver wound model was histologically analyzed by PTAH staining. Images were taken at 40 x magnification ((A)-(D)) and also at 200 x magnification ((E)-(H)). (A), (E): Untreated group, (B), (F): LYDEX & 0 NIH U/ml batroxobin treated group, (C), (G): LYDEX & 30 NIH U/ml batroxobin treated group, (D), (H): LYDEX & 50 NIH U/ml batroxobin treated group. Fibrin was stained blue (arrow). The groups with the application of the adhesive visualize the fibrin clots, and particularly the batroxobin-containing adhesive could enhance the fibrin clots formation.

IV. DISCUSSION

Batroxobin in the LYDEX-based adhesive was effective for hemostasis. This was confirmed through both *in vitro* and *in vivo* experiments. Batroxobin-induced clot formation in whole blood through the conversion of fibrinogen to fibrin; and also the application of the batroxobin-LYDEX adhesive to animal models similarly confirmed the hemostatic effects of the adhesive on a bleeding wound.

In this research, the batroxobin-LYDEX gel was prepared for *in vitro* experiments to verify the effect of batroxobin in the gel for blood coagulation. After the active solution containing batroxobin was dropped onto LYDEX, the solid powdery LYDEX immediately formed a gel under aqueous conditions - as dry LYDEX is liquid absorbable (data not shown). The rapid gelling process of the powdery LYDEX with the fluid blood may be an important criterion for an ideal hemostatic material. From the experiments, it was confirmed that the batroxobin that was released from the gel had not lost its hemostatic property, which involved the conversion of fibrinogen to form blood clots. Both results of the fibrinogen clotting assay and whole blood clotting assay show that the addition of batroxobin can accelerate fibrinogen formation. However, it should be noted that the gel without batroxobin was capable of producing small amounts of clots. The reason for this is that fibrinogen has a high affinity for Ca^{2+} with its three binding sites [27] - the fibrinogen-associated clotting process is initiated with $CaCl_2$ [28]. Therefore, the LYDEX without batroxobin could induce clot formation with $CaCl_2$ in the gel. In figure 8, it can be seen that some clots remained in the grooves on the surface of the gel even after washing with PBS solution. In fact, the gel was fragile under the high pressure of the blood flow, as shown in the *in vivo* result (Figure 13). But the clot formation and hold in the gel would make the adhesive firmer. The rough texture of the adhesive may additionally assist to increase the firmness of the gel with solid clots, and therefore enhance the function of the hemostatic barrier.

In animal experiments, the role of LYDEX in hemostasis was shown and the

batroxobin also effectively worked for reduction of blood loss and shortening of bleeding time with increasing concentrations.

According to the result of the mouse liver model, the amount of total blood loss was significantly decreased with the addition of the 50 NIH U/ml batroxobin solution. As represented in the cytotoxicity test of batroxobin, batroxobin itself is not toxic and does not influence cells and other blood coagulation factors. Therefore, it can be expected that its concentration could be increased to magnify the hemostatic effects to control extremely serious bleeding conditions. Although this study investigated the immediate response of the hemostatic adhesive, low toxicity of the hemostatic adhesive and also its high degradability would support the possibility of long periodic internal use for bleeding organs.

During the process of hemostasis, LYDEX and batroxobin cooperated to effectively control bleeding as they functioned in a timely manner. LYDEX could make the first barrier against bleeding, which was supported by the result of the femoral artery model. The amount of blood that occurred during bleeding for the initial 30 sec (measured in LYDEX treated groups) was almost half that of the group that was not treated. LYDEX might play an important role not only in the early stages but also throughout the process of hemostasis. When the active solution was added to LYDEX, it changed its form into a gel, and consequently held batroxobin in the wound area against the flow of blood. However, in the excessive bleeding model - conducted by the femoral artery wound - some defects of this hemostatic adhesive were seen. In some experimental groups of the model, the adhesive could not withstand the excessive and continuous bleeding, and finally the declining amount of blood from the bleeding increased again as blood oozed out through the crack of the gel. However, the recurrence of bleeding could be reduced by the addition of batroxobin to the adhesive (Figure 13), because batroxobin would make the blood form clots, and the blood clots may block any space for the blood passing through. It would work as a second barrier for hemostasis. In addition, the clots formed between the adhesive and tissue could play a role in increasing the adhesiveness, as the clots

may work as a mediator to connect the topical adhesive to the tissue. One of important properties for an ideal adhesive - the bonding strength of LYDEX to tissue - was investigated by Nakajima (2007). It was found that it has a higher strength than that of commercial fibrin glue [11]. Consequently, the LYDEX-based adhesive can be more strongly supported to prevent bleeding through the gap between the adhesive and the tissue when batroxobin is applied to the blood coagulation process.

A time lag between the results of the direct and indirect methods in the femoral artery model was found. To enhance sensitivity for detection of complete hemostasis time, the concentration of hemoglobin was represented in 15 sec intervals; while the result of weight of blood from bleeding was expressed in 30 sec intervals. However, the hemostasis time obtained from the analysis of the concentration of hemoglobin was shorter than that from the measurement of the weight of blood. It may be due to the relatively high detection limit of hemoglobin and also the incomplete release of blood in saline. However, if the yield of blood release is improved, the method of measuring the concentration of hemoglobin can be useful to quantify the amount of blood from bleeding, because this method is specified to measure only the hemoglobin fraction of the blood contents, i.e. excluding other substances, such as other body fluids and parts of the adhesives. In this study, the hemostasis progress was mainly analyzed by measurement of the weights of the blood from bleeding, and the tendency was verified by both methods.

The functionalities of batroxobin and LYDEX were demonstrated by blood clot staining. In figure 15 ((B)-(D) and (F)-(H)) and figure 16 ((B)-(D) and (F)-(H)), the intensively stained area can be seen, but it was not found in figure 15 (A) and figure 16 (A). Although the fibrin clots were also formed naturally by the physiological response to the bleeding injury, the amount of clots may not be enough to be presented. In other words, the blood clots were formed and held by LYDEX, but without any treatment, the blood from the bleeding flowed, and clots hardly formed. From the histological analysis, the blood clots were observed in larger portion of the adhesive with increasing concentrations of batroxobin. This phenomenon was clearly

shown in the result of H&E staining (figure 15) because the blood could react with batroxobin in the adhesive to form fibrin clots. The overall results of both staining methods were similar but the H&E staining could colorize larger area and more apparently show the differences among different groups compared to PTAH staining. This is because the fibrin was selectively stained by PTAH staining solution, and thus the difference of the fibrin formation between two groups treated with batroxobin may not be visibly detected. On the other hand, H&E staining method was able to dye both fibrin and erythrocytes. During the blood coagulation, the erythrocytes were entrapped by fibrin fibers [29]. Thus, the fibrin formed by batroxobin would promote the entrapment of erythrocytes. Finally, the large amount of blood clots was formed in the groups treated with batroxobin as batroxobin improved production of fibrin entrapping the erythrocytes. Both LYDEX and batroxobin may provide the adhesive environment for erythrocytes, and hence blood clots might be effectively formed. As the entrapped erythrocytes were stained together with fibrin fiber, the increase in the amount of blood clots might be more clearly oberseved through H&E staining. This was already confirmed in the quantitative analysis of the liver wound model. The amount of blood loss was reduced with the treatment of batroxobin.

These observations support that LYDEX mechanically obstructed bleeding, and the active enzymatic source of hemostasis, batoroxin, was then reacted to enhance the hemostatic function of the adhesive. It was also confirmed that in both animal models, hemostasis was accelerated depending on the concentration of the active agent - and also by existence of LYDEX. Therefore, batroxobin and LYDEX might work successfully together during hemostasis with respect to bleeding time and blood loss.

V. CONCLUSION

Batroxobin in the LYDEX-based hemostatic adhesive may be involved in the intrinsic pathway of blood coagulation as it was participated in the conversion of fibrinogen to fibrin. In this study, it was found that hemorrhage caused by injuries in animals was controlled by the hemostatic adhesive. Also, increasing concentrations of batroxobin could improve the hemostatic activity. Therefore, the decreased blood loss and shorter hemostatic time might reduce mortality and any complications induced by uncontrolled bleeding. Eventually, the adhesive consisting of LYDEX and batroxobin would be developed to be a novel hemostatic adhesive for clinical use as it can fulfill requirements of an ideal hemostatic adhesive.

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ABSTRACT (IN KOREAN)

Recombinant batroxobin을 함유한 접착제의 지혈 기능 평가

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유 경 은

일상생활이나 전쟁터에서 발생하는 외상이나 외과적 수술 중에 일어날 수 있는 과도한 출혈은 생명을 위협할 수 있는 상황으로, 출혈량을 최소화하기 위해 적절한 시간 안에 지혈을 하는 것이 중요하다. 따라서 지혈을 위한 여러 가지 재료의 연구가 이루어지고 있다. 이 논문에서는 혈액응고 과정에서 피브리노겐을 피브린으로 전환하여 지혈 기능을 하는 물질인 *Bothrops atrox moojeni* 라는 뱀의 독성분 일종인 배트록소빈을 생분해성 접착제와 함께 사용하였다. 분말형태의 접착제와 재조합 배트록소빈 용액을 혼합하여 젤 형태의 지혈 접착제를 만들어 그 기능에 대해 연구하였다.

이 연구에서 제작된 접착제 속에 들어있는 배트록소빈의 활성은 fibrinogen clotting assay를 통해 확인하였고, SD rat의 전혈을 이용해 배트록소빈의 농도에 따른 혈전 형성 정도를 알아보았다. 또한, SD rat 및 ICR mouse 를 이용해, 배트록소빈을 포함한 접착제를 동물지혈 모델에 적용해 보았다. 각 모델 별로 32마리의 동물을 어떠

한 처치도 하지 않은 대조군 및 배트록소빈 농도 (0, 30, 50 NIH U/ml)에 따른 접착제 적용군을 포함한 네 그룹으로 나누어 실험하였다. 먼저, ICR mouse의 간에 13 G needle로 상처를 내어 흘러나오는 혈액을 여과지로 흡수시켜서 그 무게를 측정하여 출혈량을 계산하였고, 형성된 피브린을 H&E 염색과 PTAH 염색을 통해 관찰하였다. SD rat의 넙다리 동맥을 끊어 흘러나오는 혈액을 15 초 간격으로 여과지로 흡수시켜 무게를 측정해 지혈에 걸리는 시간 및 양상을 확인하였다. 또한, 흡수된 혈액에 포함된 헤모글로빈 농도의 측정을 통해서도 이러한 경향성을 확인하였다.

Mouse의 간 모델에서는 출혈된 혈액의 양이 지혈 접착제를 이용했을 때 대조군에 비해 줄어드는 결과를 얻었고, 이는 배트록소빈의 농도가 증가할수록 그 양이 더 줄어드는 경향을 보였다. 지혈 시간은 대조군에 비해 접착제를 도포했을 때 현저하게 줄어드는 것을 확인하였는데, 특히 50 NIH U/ml의 배트록소빈이 함유된 접착제는 혈액응고 시간을 확연히 줄였다. 접착제를 도포한 실험군의 재출혈 현상은 배트록소빈 농도가 증가할수록 그 발생률이 줄어들었다. 이 실험의 결과로 생분해성 접착제는 출혈에 대한 첫 번째 물리적 장벽의 역할을 하고 배트록소빈은 직접적으로 혈전 형성에 관여함으로써 지혈을 돕는다는 것을 알 수 있었다. 결론적으로, 적합한 농도의 배트록소빈이 포함된 접착제는 출혈량의 감소뿐만 아니라 지혈시간의 단축이라는 측면에서도 출혈을 멈추게 하는 데 효과적으로 작용할 수 있다는 것을 확인하였다.

핵심되는 말 : 지혈, 배트록소빈, 지혈 접착제, 출혈