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Original Article

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Abstract

Fibrin glue has been widely used for controlling hemorrhage and sealing tissue in surgery. The purpose of this study was to evaluate the hemostatic efficacy of sheep-derived fibrin glue. Six Marino sheep were used to collect concentrate fibrinogen preparation by the ammonium sulfate precipitation method. Crossbred pigs (n=6) were used to evaluate hemostatic efficacy by monitoring whole blood clotting time by the glass slide method, bleeding time and bleeding quantity during liver biopsy. Mean whole blood clotting time on a glass slide started at 201.1±90.47 and 4.43±3.73 seconds, and was completed at 447.83±63.77 and 31.93±4.28 seconds in control and treatment groups respectively. Two-sited open liver biopsies were performed, each biopsy site was assigned as either control or treatment groups. One ml of sheep-derived fibrin glue on the bleeding surface was applied at the treatment sited group. Bleeding quantity was estimated by the increased weight of filter paper after blood straining. Mean bleeding quantity was 0.94±0.38 and 0.1±0.12 g, and mean bleeding time was 175.18±11.80 and 68.08±28.84 seconds in control and treatment sites, respectively. Whole blood clotting time, bleeding time, and bleeding quantity were significantly less in the treatment-sited group, compared to the control-sited group (p<0.01). In conclusion sheep-derived fibrin glue could be applied as an effective hemostatic agent to control hemorrhage after liver biopsy in swine.

Keywords: fibrinogen, liver biopsy, sheep-derived fibrin glue, swine, thrombin

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Introduction

Liver biopsy is the gold standard for diagnosis and prognosis in liver disease (Watson and Bunch, 2009). There are many indications for liver biopsy such as evaluation of abnormal liver function, alteration of liver structure, confirmation or staging of neoplastic diseases, and assessment of disease progression (Vasanjee et al., 2006). Bleeding is one of the most common and serious complications (0.32% - 0.35%), the next complication is death after liver biopsy (0.11%) (Alkozai et al., 2009; Al-Ghamdi, 2011).

Fibrin glue was first used as a topical hemostatic agent in 1960 and developed for commercial products in late 1970 (Tabélé et al., 2012). Two major components of fibrin glue are fibrinogen and thrombin.After combining these two components, fibrinogen will be converted into fibrin clot, which is the final product of the coagulation cascade, by thrombin in the presence of calcium ion (de Boer et al., 2012; Tabélé et al., 2012). In general, fibrin glue is a natural product with great biocompatibility, non toxicity to tissues, does not stimulate inflammation nor delay wound healing and degrades by the normal fibrinolytic pathway (Gibble and Ness, 1990; Radosevich et al., 1997; Morey et al., 2001; Morikawa, 2001; Aksoy et al., 2009). Presently, fibrin glue has been widely used in a variety of surgical procedures, including plastic surgery, cardiothoracic surgery, vascular surgery, neurosurgery, gastrointestinal surgery, and hepatic surgery (Morikawa, 2001). Several studies have shown that the application of fibrin glue in liver surgery is effective in reducing the bleeding quantity and hemostatic time (Wheaton et al., 1994; Davidson et al., 2000; Paulson et al., 2000; Karpelowsky et al., 2006; Taha et al., 2006).

The two basic categories of fibrin glue are commercial fibrin glue and in-house fibrin glue. Methods for producing concentrated fibrinogen involve cryoprecipitation and chemical precipitation methods such as precipitation by ethanol, polyethylene glycol and ammonium sulfate (Silver et al., 1995[a], Wang et al., 1995). Several studies have indicated ammonium sulfate precipitation as yielding higher fibrinogen concentration, and therefore higher bonding strength of fibrin glue (Siedentop et al., 1985; Silver et al., 1995[a]). The advantages of using in-house fibrin glue include reducing the risk of disease transmission, immune reaction, and cost of surgery (Tabélé et al., 2012). However, the limitations of using in-house fibrin glue are the low concentrations of fibrinogen due to blood collection from animals with low body weight, with coagulopathy, or with liver disease and especially for the use in urgent surgery procedures (Gibble and Ness, 1990; Wheaton et al., 1994). Until the present time, the use of commercial fibrin glue has not been a mainstay of common practice in veterinary surgery due to economic concern.

Therefore in this study, in-house fibrin glue was developed from concentrated sheep-derived fibrinogen by the ammonium sulfate precipitation method, which can be an alternative method to control hemorrhage. Moreover, sheep-derived fibrin glue can be produced from plasma as the byproduct of sheep blood agar production. The objective of this study was to evaluate the hemostatic efficacy of sheep-derived fibrin glue. Whole blood clotting time by glass slide method, bleeding time and bleeding quantity during the liver biopsy in swine were selected to determine the efficacy of sheep-derived fibrin glue.

Materials and Methods

Animals: This study was approved by the Institutional Animal Care and Use Committee (IACUC), Chulalongkorn University (Approval No. 13310065)

Six healthy Marino sheep 30–50 kg., were used for sheep-derived fibrin glue preparation. Approximately 90 ml of blood was collected from the jugular vein into a tube containing 10 ml of anticoagulant (3.7% w/v sodium citrate) and then gently mixed immediately. Six healthy crossbred pigs 18–22 kg., were used to evaluate hemostatic efficacy by liver biopsy (n=6).

Experimental design

***Experiment 1*:** a glass slide method was used to evaluate whole blood clotting time. Forty microliters (µl) of blood were collected from each pig and then each sample was divided into control (n=6) and treatment (n=6) groups with 20 µl of blood per each sample.

Experiment 2: Bleeding time and bleeding quantity following by liver biopsy were used to evaluate hemostatic efficacy.Two open liver biopsy sites were performed using endoscopic cup biopsy forceps. The biopsy sites of each pig were divided into control (n=6) and treatment (n=6) groups.

Fibrin glue preparation: Sheep blood samples were centrifuged at 4°C 600*g*for 20 minutes to obtain plasma and all plasma samples were pooled. Concentrated fibrinogen was prepared by the ammonium sulfate precipitation method. Saturated ammonium sulfate (80 gram in distilled water 100 ml) was added into pool plasma at a ratio of 1:5 to plasma and then centrifuged at 4°C 1600*g* for 5 minutes. Supernatants were discarded and pellets were resuspended in distilled water at a ratio of 1:10 to plasma and then centrifuged at 4°C 1600*g* for 5 minutes. Soluble protein was collected as fibrinogen-rich fraction protein (Silver et al., 1995[b])(Fig 1).

Lyophilized commercial bovine thrombin, 1000 International Unit (IU) per vial (EMD Millipore Inc., SDG, USA), was used in this study. Thrombin solution was generated by adding 1 ml calcium chloride at a concentration of 40 mmol/L in order to make the final concentration of 1000 IU/ml of thrombin.

Concentration of fibrinogen: Fibrinogen concentration was measured by the heat precipitation method (Millar et al., 1971). One hundred microliters of plasma were heated for 3 minutes in a water bath (56 ± 1°C) and then centrifuged at room temperature at 2000 RPM for 3 minutes. The supernatant was evaluated for its protein concentration (g/dl) as fibrinogen-free fraction protein (Protein 1) by a refractometer. Fibrinogen-rich fraction protein (Protein 2), which is the final result of the

ammonium sulfate precipitation method, was measured for its concentration (g/dl) by refractometer. Fibrinogen concentration (mg/dl) was calculated as the difference between the protein 2 and protein 1 multiply with 1000 (fibrinogen concentration (mg/dl) = (Protein 2 – Protein 1) x 1000) (Millar et al., 1971)

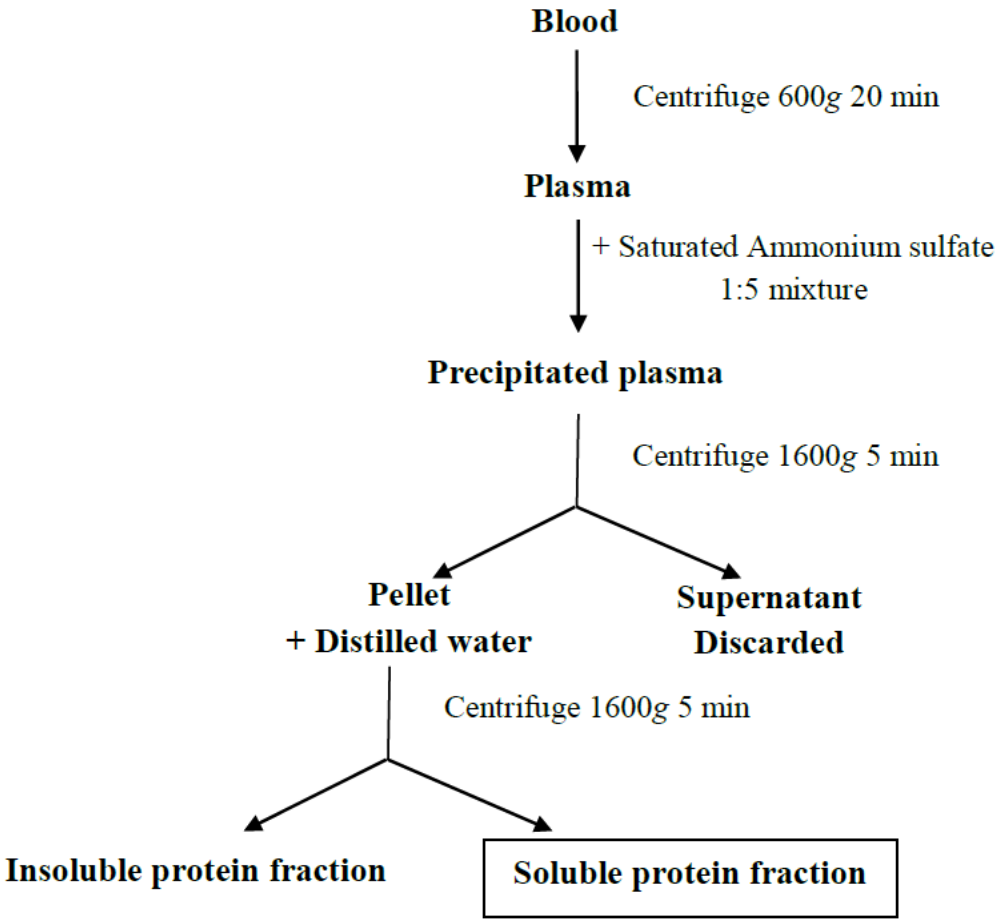

Figure 1 Concentrated fibrinogen preparation by the ammonium sulfate precipitation method (Modified from Silver et al., 1995[b])

Whole blood clotting time: Twenty microliters of blood were dropped onto a glass slide with either 20 µl of 0.9% saline (w/v), or 20 µl of sheep-derive fibrin glue in the control and treatment groups respectively. The clotting time was determined as the time from the blood dropping onto the glass slide until blood clotting was first mentioned (second) (Gross et al., 1929).

Liver biopsy: Liver biopsies were performed under general anesthesia. The animals were premedicated and inducted with 2.2 mg/kg of ketamine (Calypsol®; Gedene Richter Inc., Budapest, Hungary), 2.2 mg/kg of xylazine (Seton®; Calier Inc., Barcelona, Spain) and 4.4 mg/kg of zolazepam-tiletamine (Zoletil®; Virbac Inc., Texas, USA) intramuscularly, analgesic of meperidine (Demerol®; Hospira, Illinois, USA) intramuscularly (Smith et al., 1997) and antibiotic prophylaxis of amoxicillin-clavulanic acid (Synulox®; Pfizer Inc., NY, USA) 8.75 mg/kg intramuscularly. Anesthesia was maintained with isoflurane and oxygen delivery by endotracheal intubation. The heart rate (BPM), systolic blood pressure (mmHg) and oxygen saturation (%) were monitored during the period of anesthesia. Two sites of liver biopsy were performed at the peripheral area using 5 mm. endoscopic cup biopsy forceps at the left lateral lobe. Two biopsy sites were allocated as either control or treatment groups. Approximately 1 ml of sheep derived fibrin glue was applied onto the bleeding surface in the treatment group. Bleeding quantity was estimated by applying no.1 filter paper (Whatman®; GE Healthcare, Buckinghamshire United Kingdom) without pressure on the biopsy site until hemostasis was completed. The filter papers were weighed before and after blood absorption to measure the amount of blood loss in term of weight. The time that hemostasis accomplished was recorded as bleeding time (second). Suturing of the biopsy site in the control group or reapplying 1 ml of sheep-derived fibrin glue in the treatment group was considered if bleeding continued to occur for more than 3 minutes. After complete hemostasis, the liver and peritoneal cavities were inspected for 30 minutes to assess if there were rebleeding. Then, the animals were euthanized using pentobarbital sodium (Nembutal®; Ceva Santé Animale Inc., Libourne, France) 100 mg/kg intravenously (Shaw and Reilly, 2001).

Statistical analysis: Fibrinogen concentration was described as descriptive analysis. Paired t-test was used to evaluate the statistical significance of whole blood clotting time on a glass slide, bleeding time and

bleeding quantity between control and treatment groups.

Results

The fibrinogen concentration was 3000 mg/dl (30mg/ml) after precipitation, which was 7.5 times higher compared to the concentration before precipitation.

***Experiment 1*:** There was statistically significant difference in whole blood clotting time between control and treatment groups (p<0.01)(Table1) (Fig 2A).

Table 1 Whole blood clotting time (mean±SD) in control and treatment groups

Groups	Start clot (seconds)	Complete clot (seconds)
Control	201.1±90.47[a]	447.83±63.77[b]
Treatment	4.43±3.73[a]	31.93±4.28[b]

Same superscript letters in the same column represent statistically significant (p < 0.01)

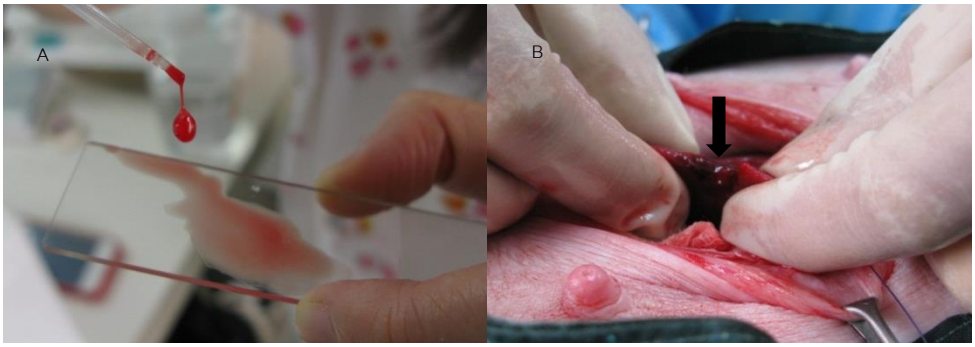

Figure 2 Complete blood clot on glass slide and fibrin clot on bleeding surface (Fig. 2a) whole blood clotting was performed after binding blood with fibrin glue on a glass slide in the treatment group (Fig. 2b) fibrin clot (arrow) was covered on bleeding surface after applied sheep-derive fibrin glue for controlling hemorrhage in the treatment group

***Experiment 2*:** After the liver biopsy, bleeding time and bleeding quantity in the treatment group were significantly lower compared with the control group (p<0.01) (Table 2,3). In the control group, suturing was required to achieve hemostasis in 5/6 animals (83.33%). Fibrin clot was presented on the bleeding surface in all animals in treatment groups (Fig 2B). All animals in treatment groups a had lower bleeding time and bleeding quantity compared with animals in the control group (100%). Rebleeding did not occur in all animals.

Table 2 Bleeding time (mean±SD) after liver biopsy in control and treatment groups

Groups	Bleeding time (seconds)
Control	175.18±11.8[a]
Treatment	68.08±28.84[a]

Same superscript letters in the same column represent statistically significant (p < 0.01)

Table 3 Bleeding quantity (mean±SD) after liver biopsy in control and treatment groups

Groups	Bleeding quantity (ml)
Control	0.94±0.38[a]
Treatment	0.1±0.12[a]

Same superscript letters in the same column represent statistically significant (p < 0.01)

Discussion

Liver biopsy is an important procedure for the diagnosis, treatment options and prognosis of liver disease in patients. In normal animals, liver biopsy is generally safe and can be accomplished with minimal blood loss (Vasnjee et al., 2006; Rothuizen and Twedt, 2009). However, patients with liver disease that may require liver biopsy as a diasnostic procedure are at a higher risk of bleeding due to the coexistent of coagulopathy (Paulson et al., 2000). Several topical haemostatic agents are used in liver surgery to reduce hemorrhage. The ideal hemostatic agent should be efficient in achieving fast and durable hemostasis as well as not causing adverse effects. In addition, it should be cost efficient and user friendly (Moench et al., 2010). In general, fibrin glue has been widely used in liver surgery to reduce hemostatic time and bleeding quantity (de Boer et al., 2012). The bonding strength of fibrin glue and the patrient's hemostatic time are related to fibrinogen and thrombin concentrations respectively (Durham et al., 1987; Harris et al., 1988; Wheaton et al., 1994; Kheirabadi et al., 2001; MacGillivray, 2003). Fibrinogen concentrations in cryoprecipitated plasma are approximately 20 mg/ml (Dresdale et al., 1985) and in chemical precipitated

plasma range from 13-57 mg/ml (Park and Cha 1993; Kjaergard and Weis-Fogh 1994), similar to this study result, fibrinogen concentration was 30 mg/ml after ammonium sulfate precipitation. Although the concentration was lower than commercial fibrin glue, we believe that the bonding strength of the sheep-derived fibrin glue we prepared was adequate to be used in liver biopsy protocols because there was no rebleeding occurring in any animal. In addition, previous studies were accomplished using lower fibrinogen concentration (22±0.7 mg/ml and 10.72 mg/ml) as hemostatic agent in liver biopsy models (Wheaton et al., 1994; Davidson et al., 2000). The advantages of chemical precipitation over cryoprecipitation are its convenience and the rapid method to concentrate of the fibrinogen (Silver et al., 1995[a]). Thrombin concentration of in-house fibrin glue normally ranges from 500-1000 IU/ml (Wheaton et al., 1994). Since the bleeding time is perhaps as important as bleeding quantity,we decided to use a high concentration of thrombin (1000 mg/ml) to rapidly reduce the bleeding time. The mean bleeding time was significantly shorter in the sheep-derived fibrin glue treatment group than control group. Mean bleeding quantity in the treatment group was 0.1±0.12 g. This showed up as the same direction of results from the previous study, using commercial fibrin glue as a/the hemostatic agent after tru-cut liver biopsy in swine, in which mean bleeding quantity was 0.1 ml (Paulson et al., 2000). However, bleeding quantity after using tru-cut liver biopsy is slightly higher than using endoscopic cup biopsy forceps in normal animals (Vasanjee et al., 2006). The conversion of fibrinogen to fibrin is caused by thrombin in the presence of calcium ions. The concentration of calcium chloride in in-house fibrin glue varied from 20-160 mmol/L (Wang et al., 1995). In this study, 40mmol/L calcium chloride was used due to the report of the previous study showing high bonding strength and rapid formation of fibrin glue after 20-40 mmol/L of calcium chloride was added (Wang et al., 1995). Moreover, this concentration was similar to concentration in commercial fibrin glue (Tisseel®; Baxter, Illinois, USA). Unlike most commercial fibrin glue, the antifibrinolytic agent such as aprotinin was not added in this study due to several studies suggesting that aprotinin is not a necessary component because fibrinolysis of glue before complete hemostasis has not been reported (Durham et al., 1987; Gibble and Ness 1990; Wheaton et al., 1995). Moreover, some present commercial fibrin glue products also does not contain aprotinin as its composition (Quixil®; OMRIX Biopharmaceuticals, Kiryat Ono, Israel). However, antifibrinolytic agent should be added when using fibrin glue at high levels fibrinolysin organs such as the lung, kidney, prostatic gland, and uterus, because long-term bond strength is required (Gibble and Ness 1990) and tranexamic acid is commonly used as an antifibrinolytic agent in in-house fibrin glue (Radosevich et al., 1997).

Further studies on the adverse effect by immunogenicity reaction from using xenogenic products, prevention of biliary leakage, and hemostatic efficacy in coagulopathy animals are required in order to use sheep-derived fibrin glue as the topical hemostatic agent in liver disease patients.

In conclusion, sheep-derived fibrin glue can be used as an effective topical hemostatic agent for controlling hemorrhage following liver biopsy in swine and could further be adapted for liver biopsy procedure in clinically veterinarian practice.

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บทคัดย่อ

ประสิทธิภาพในการห้ามเลือดของกาวไฟบรินแบบเตรียมเองจากเลือดแกะ ในการทำศัลยกรรมตัดชิ้นเนื้อตับเพื่อตรวจในสุกร

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กาวไฟบรินถูกใช้อย่างกว้างขวางในการศัลยกรรมเพื่อช่วยห้ามเลือด และประสานเนื้อเยื่อ การศึกษาครั้งนี้มีจุดประสงค์เพื่อประเมินประสิทธิภาพในการห้ามเลือดของกาวไฟบรินจากเลือดแกะ จากระยะเวลาการแข็งตัวของเลือดบนแผ่นสไลด์ ระยะเวลาตกเลือด และปริมาตรการตกเลือดในระหว่างศัลยกรรมตัดชิ้นเนื้อตับเพื่อตรวจในสุกร แกะจำนวน 6 ตัว ถูกใช้ในการเตรียมไฟบริโนเจนเข้มข้นด้วยวิธีตกตะกอนด้วยแอมโมเนียมซัลเฟต สุกรจำนวน 6 ตัว ถูกใช้ในการศึกษาประสิทธิภาพกาวไฟบรินจากเลือดแกะ โดยค่าเฉลี่ยระยะเวลาที่เลือดเริ่มแข็งตัวบนแผ่นสไลด์เท่ากับ 201.1±90.47 และ 4.43±3.73 วินาที และเลือดแข็งตัวโดยสมบูรณ์ที่ 447.83±63.77 และ 31.93±4.28 วินาที ในกลุ่มควบคุมและกลุ่มทดลองตามลำดับ ทำการศัลยกรรมตัดชิ้นเนื้อตับเพื่อตรวจในสุกรตัวละ 2 ตำแหน่ง โดยเนื้อตับแต่ละตำแหน่งจะถูกแบ่งเป็นกลุ่มควบคุมและกลุ่มทดลอง โดยกลุ่มทดลองได้รับการห้ามเลือดด้วยกาวไฟบรินจากเลือดแกะปริมาตร 1 มิลลิลิตร ประเมินปริมาตรการตกเลือดจากน้ำหนักที่เพิ่มขึ้นของกระดาษกรองจากการซับเลือด ซึ่งพบว่าพบว่ามีค่าเฉลี่ยเท่ากับ 0.94±0.38 กรัม และ 0.1±0.12 กรัม และระยะเวลาเสียเลือดมีค่าเฉลี่ย 175.18±11.80 วินาที และ 68.08±28.84 วินาที ในกลุ่มควบคุมและกลุ่มทดลองตามลำดับ ผลการศึกษาพบว่า ระยะเวลาที่เลือดแข็งตัวบนแผ่นสไลด์ ระยะเวลาตกเลือด และปริมาตรการตกเลือดในระหว่างศัลยกรรมตัดชิ้นเนื้อตับเพื่อตรวจมีความแตกต่างอย่างมีนัยสำคัญระหว่างกลุ่มควบคุมและกลุ่มทดลอง ($p<0.01$) โดยสรุปกาวไฟบรินจากเลือดแกะมีประสิทธิภาพดีในการห้ามเลือดจากการศัลยกรรมตัดชิ้นเนื้อตับเพื่อตรวจในสุกร

คำสำคัญ: ไฟบริโนเจน ศัลยกรรมตัดชิ้นเนื้อตับเพื่อตรวจ กาวไฟบรินจากเลือดแกะ สุกร ทรอมบิน

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