Damanhour Journal of Veterinary Sciences

Journal homepage: www.damanhourjvetsci.com

E-ISSN 2636-3003 | ISSN 2636-2996

Preparation of fibrin glue from catfish blood and its application

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A B S T R A C T

Fibrin glue was prepared from catfish (Clarias lazera) blood by mixing the coagulation factors Fish fibrinogen, prothrombin extracted from fish blood, and thromboplastin was prepared from fish muscles. The present study was conducted in 15 adult male New Zealand white rabbits divided into three equal groups (n = 5); Skin wounds group, Second-degree skin burn group, and bleeding ear vein group to evaluate the healing potential of the prepared fibrin glue. The same animal was used as self-control for each treatment. Rabbits were anesthetized with xylazine hydrochloride 5 mg/kg and Ketamine hydrochloride 35 mg/kg BW I/M. In the first group, dorsal skin incisions were created. In the second group, second-degree skin burns were induced. Wounds were treated with fibrin glue and/or saline as control. In the third group, hemostasis of the bleeding ear vessels was performed by applying a gauze pad soaked with fibrin glue in order to be compared with gauze of 0.9% normal saline. The biopsies were taken on the 7th day in the first group and on the 10th day in the second group. Wound healing was monitored through macroscopic and histological examination. Treatment with fibrin glue increased the re-epithelialization and the regeneration of the epidermal granular layer in both skin incisions and skin burns. In the third group, the mean bleeding time was reduced in treated-fibrin glue to 13.8 seconds compared with average 28 seconds in controls. It is suggested that fish fibrin glue could be used for wound and burn healing, as well as for hemostasis purposes.

Keywords: Catfish blood, fibrinogen, healing wound, prothrombin

.1. Introduction

Fibrin glue is a model of the final step of blood coagulation (Halil et al., 2010). Mainly, it is consisting of two-component materials; fibrinogen and thrombin, in the presence of small amounts of calcium and factor XIII, the thrombin converts fibrinogen into insoluble fibrin, the final stable form of the agent. Fibrin glue is a biological glue made from blood coagulation proteins, which is commonly used in a variety of clinical and veterinary settings where soft tissue disruption due to surgery, trauma, or other injury cannot be repaired by sutures, such as burns, and where sealing of the wound site is needed to prevent fluid leakage and promote wound repair (Martinowitz and Saltz, 1996). Control of hemorrhage is the initial step in healing wounds, while care and having a widely deployable bandage to staunch blood loss will decrease the loss of life (Champion et al. 2003). In the multiple animal studies, the fibrin bandages have proven to significantly decrease blood loss during hemorrhage in various conditions (Larson et al., 1995). Fibrin glue not only causes hemostasis but also enhances healing; the presence of stabilized fibrin structure stimulates the growth of fibroblasts.

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P ISSN: 2636-3003 EISSN: 2636-2996
DOI:10.5455/DJVS.22432

Received: November 15, 2018; Received in revised form: December 22, 2018; accepted: December 28, 2018.

This combined effect on wound healing is essential for the fibroblastic action (Kollias and Fox, 1996; Trombelli et al., 1996). Fibrin glue became the first modern era biomaterial approved as a hemostat, sealant, and adhesive by the Food and Drug Administration in 1998. Fibrin glue derived from mammals or human was still subject to the same limitations, such as availability, cost, and possible transmission of pathogens (Uibo et al., 2009). In this study, we have attempted to avoid these problems through the use of fibrinogen and thrombin derived from fish blood. Several studies demonstrate advantages of fish fibrin glue, fish fibrin may be safer than mammalian fibrin for uses (Wolf, 1988), In addition to the rapid blood clotting of fish compared to mammals (Laidmae et al., 2006), also showed antibodies formed at low titers that recognized when exposed to salmon fibrin glue (Kathleen et al., 2006). Therefore, the major purpose in this study was to investigate the possibility of using catfish derived clotting elements to constitute safer fibrin glue that can be used for surgical medical purposes and has the advantage of short time application plus avoiding blood-borne diseases.

2. Material and methods

2.1. Chemicals and materials

Pure acetone, $BaCl_2$, absolute ethanol, sodium citrate, NaCl, and sodium EDTA were obtained from El-Nasr Pharmaceutical Chemicals Co., Egypt. Tris base was obtained from El-Gomhoria Pharmaceutical Chemicals Company, Egypt. L-lysine was from Vegan Society Approved, UK.

2.2. Blood collection and plasma preparation

Blood was collected from healthy catfish (*Clarias lazera*) with an average body weight of 1.5–2 kg/fish from a private fish farm in Egypt. The blood samples were taken directly in the fish farm, the blood was collected from the caudal blood vessels of catfish into 20 ml syringe containing 0.5 ml of 540-mM sodium citrate according to Wang et al. (2000). Whole blood was held on icebox until centrifugation at 1,500 g for 20 minutes at 4°C. The plasma was stored at −80°C for at least 2 days.

2.3. Blood collection and plasma preparation

Protein fractionation procedures were done at 4°C unless otherwise indicated. Fibrinogen was purified according to the modified method of Wang et al., (2000), employing precipitation twice by ethanol. After that, 350 ml frozen plasma was thawed slowly for 2 days at 4°C, and then centrifuged at 4,000 rpm for 10 minutes, the supernatant was removed, protein precipitate containing fibrinogen and factor XIII was dissolved, and re-suspended in a re-suspension buffer; (containing 20 mM Tris base, 55 mM sodium citrate, and 27 mM lysine were dissolved in 1 liter distilled water) up to 350 ml for original volume of plasma at pH 6.8, the solution was treated with cool absolute ethanol dropwise with gentle stirring at a final concentration of 10%. The mixture was centrifuged in cooling centrifuge at 4,000 rpm for 20 minutes; the pellets were solubilized in resuspension buffer. A second 10% ethanol precipitation step was carried out. Precipitate collected from ethanol precipitation after cooling centrifugation was re-suspended in the re-suspension buffer and dialyzed versus dialysis buffer (containing 20 mM sodium citrate and 100 mM sodium chloride in 1 liter distilled water) at room temperature for 24 hours. The dialyzed protein was collected and centrifuged for 20 minutes at 4,000 rpm. Extracted fibrinogen was lyophilized in a freeze dryer and stored at 4°C.

2.4. Extraction of catfish prothrombin from blood plasma

Prothrombin was extracted from the plasma using a modification of the methods of Bajaj et al., (1981); Ngai and Chang, (1991) and Michaud et al., (2002). All steps in the protein purification were done at 4°C. One part of 1 M BaCl2 was added to eleven parts of citrated plasma (32 ml of 1 M BaCl2 to 350 ml plasma) to adsorb prothrombin onto barium citrate. The mixture was centrifuged for 30 minutes at 3,600 g. The precipitate was suspended in 32 ml of 0.2 M sodium EDTA at 4°C in which the barium was removed from the barium citrate complex, freeing the prothrombin. The suspension was dialyzed for 40 minutes against dialysis buffer (A); containing 0.02 M sodium EDTA, 0.02 M sodium citrate dissolved in 1 liter of 0.9% sodium chloride. Change buffer (A) replaced to buffer (B); containing 0.02 M sodium citrate dissolved in 1 liter of 0.09% sodium chloride for 5 hours. The sample was centrifuged for 30 minutes at 3,600 g and the precipitate was discarded. 1 M ammonium sulphate was added to the supernatant under gentle stirring to a final concentration of 40% (v/v). The sample was centrifuged for 30 minutes at 3,600 g. 1 M ammonium sulfate was added to the supernatant until 60% (v/v). The mixture was centrifuged for 30 minutes at 3,600 g. The precipitate was dissolved in 20 ml of 0.025 M sodium citrate. The suspension was dialyzed against 0.025 M sodium citrate overnight. The dialyzed sample was centrifuged for 30 minutes at 5,000 g to remove the precipitate. The produced supernatant about 18 ml containing crude prothrombin was used for hydrolyzing (activating) to thrombin.

2.5. Tissue thromboplastin preparation

Tissue thromboplastin powder was prepared according to Barrow et al. (1996). 0.5 kg fresh catfish muscles were homogenized in excess of pure acetone, and then centrifuged at 3,000 rpm for 5 minutes, the supernatant was removed. The slurry precipitate was allowed to dry under vacuum to produce catfish tissue powder, which is dissolved in saline 0.9% at range 1 tissue and 3 saline before use.

2.6. Surgical applications

2.6.1. Animal care and general Surgical Procedures

A total of 15 adult male New Zealand rabbits weighing about 1.5 kg were purchased from a rabbitary in Shatby Agriculture Faculty, Egypt and were housed at the Department of Physiology in the Faculty of Veterinary Medicine at Damanhour University, Damanhour-Egypt. In general, rabbits were housed randomly in plastic cages and divided into three experimental groups for different surgical applications to be carried out; five rabbits for each application to evaluate the wound-healing potency on using fish fibrin glue. The rabbits were supplied with food and water ad libitum. The same animal was used as self-control for each treatment; Photography was applied to monitor the healing process for macroscopic and histological analysis. Rabbits were anesthetized with xylazine hydrochloride 5 mg/kg and then 2 minutes later, Ketamine hydrochloride 35 mg/kg body weight was intramuscularly injected which provided approximately 30–45 minutes of anesthesia according to Jahanmehr et al. (2004).

2.6.2. First application (skin wounds group)

Two of identical full thickness dermal wounds about 1.5 cm-long incisions were surgically created on the dorsal skin surface in each anesthetized animal. There was enough distance between wounds. All wounds were quickly fixed by surgical suture. The first wound was washed with saline and considered as control wound. The second wound was treated with fibrin glue. After 7 days (Halil et al., 2010), all animals were anesthetized again and biopsies were taken.

2.6.3. Second application (Second-degree skin burn group)

Two rounded areas each of 2 cm diameter of the shaved dorsal skin were exposed to water at 95°C for 10 seconds according to Maximiano et al. (2009) to induce second-degree skin burn in each animal. There was enough distance between wounds. After 2 hours, damaged tissue was removed. The first burn was treated with Vaseline as control; the second burn was treated with fibrin glue. Finally, all wounds were covered with an elastic bandage. After 10 days, full thickness skin biopsies were collected for histological processing.

2.6.4. Third application (bleeding ear vein group)

In this experiment, two punctures were made on small ear vessels**.** After 2 seconds, experimental gauze soaked with saline (control) or fibrin glue was applied and with manual pressure over the puncture for 5 seconds. The bleeding time was measured and recorded by a digital stopwatch every 5 seconds till complete hemostasis.

2.7. Histological Examinations

The biopsies of skin samples were fixed in a 10% buffered formaldehyde solution and then processed for histological examination. Paraffin sections of 4 µm were prepared and stained by hematoxylin and eosin stain (H & E) according to Bancroft and Gamble (2008).

2.8. Statistical Analyses

Data of third application (differences between bleeding times) were processed using unpaired student *t*-test way with SPSS software version 20.0 for Windows (SPSS Inc., Chicago, IL, USA) (SPSS, 2009).

3. Results

3.1. Clotting factors content

Fibrinogen separated from catfish blood plasma was found to be around 600 mg/dl plasma. The solution contained prothrombin extracted from 350 ml plasma after purification and various stages of dialysis was about 18 ml. Furthermore, 0.5 kg fresh catfish muscles produced 80 g catfish tissue powder, which was used as a source of thromboplastin for activation of prothrombin to thrombin.

3.2. Formation of fibrin glue

This was done by mixing 0.3 parts of prothrombin and 0.3 part of thromboplastin solution in the presence 0.15 part of 1 M calcium chloride added to one part of fibrinogen concentrate (19 mg/ml in saline). Clotting occurs within 7 seconds (Fig.1).

3.3. Macroscopic observations

In the first application, the healing pattern observed after 7 days showed that topical application of fibrin glue improved wound healing pattern. The initial length of incision wound was decreased to 0.8 cm compared with 1.3 lengths in the control (Fig. 2). Also, in the second application, the wounds treated with fibrin glue were decreased to average 1 cm diameter compared with 1.8 cm diameter in the control (Fig. 5).

3.4. Histological examination

In the first application, the skin wounds after 7 days of treatment with fibrin glue showed that the wound gap is filled with newly formed granulation tissue while its edges possessed massive accumulation of basophilic lymphocytic cells, as well as macrophages (Fig. 4) compared with control which was completely plugged with a fibrous tissue mass invaded with fibroblast and fibrous tissue (Fig. 3). In the second application, the fibrin glue treated wound showed that the dermis is covered by newly formed epidermal tissue indicating recovery from the burn damage (Fig.7) compared with control that showed re-epithelialization with the formation of new amorphous collagen layer (Fig.6).

3.5. Controlling bleeding in the ear blood vessels

In the control group, the bleeding stopped after a minimum of 26 seconds and a maximum of 30 seconds (average 28 seconds). While in the fibrin glue treated group, bleeding was stopped after a minimum of 13 seconds and a maximum of 15 seconds by gauze soaked with fibrin glue; average 13.8 seconds. The results are summarized in Table (1).

Table 1

Bleeding times (seconds) obtained from small vessel in rabbit ears.

	Rabbit Number					Mean±SE
Group	1	2	3	4	5	
control	30	26	27	28	29	28±0.07
Fibrin glue	13	15	14	13	14	13.80±0.037

Values are mean ± standard error of mean.

P<0.01

Figure (1): Fibrin glue formation.

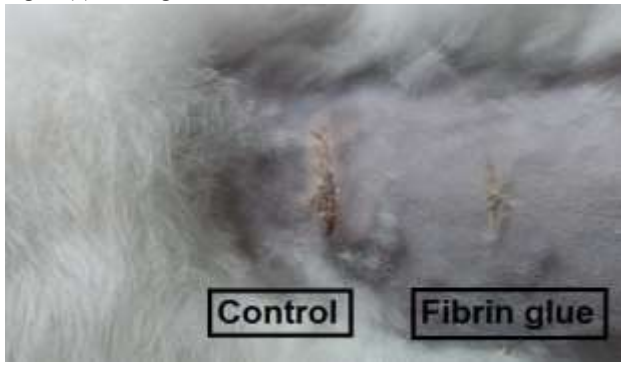

Figure (2): Skin incisions on the back of the rabbit on the 7th day: Saline treated (control) and treated with fibrin glue.

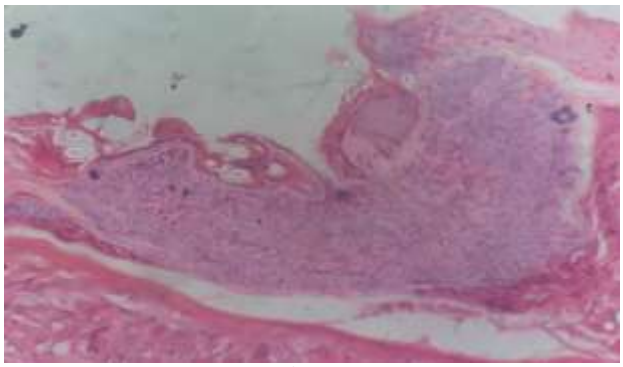

Figure (3): Histological section of the control group. The skin incision of the rabbit tissue on the 7th day. The wound is completely plugged with a fibrous tissue mass invaded with fibroblast and fibrous tissue formation. (H & E, X 40).

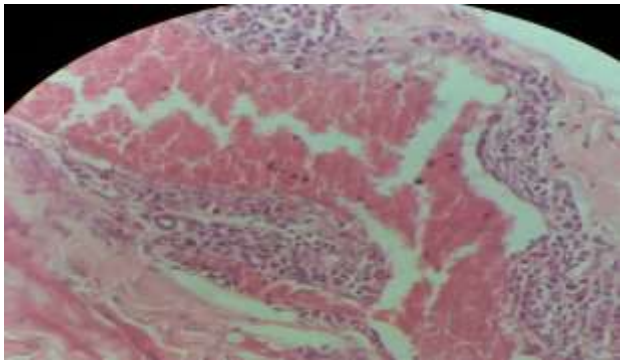

Figure (4): Histological section of the fibrin glue-treated wound site of a rabbit on the 7th day. Devascularization connective tissue with a few cells, rich in collagen fibers is seen under the regenerated epidermis (H & E, X 40).

Figure (5): Photographs of macroscopic appearances of the second-degree burn with a 2cm diameter that were Vaseline-treated (control) or treated with fibrin glue on the 10th day.

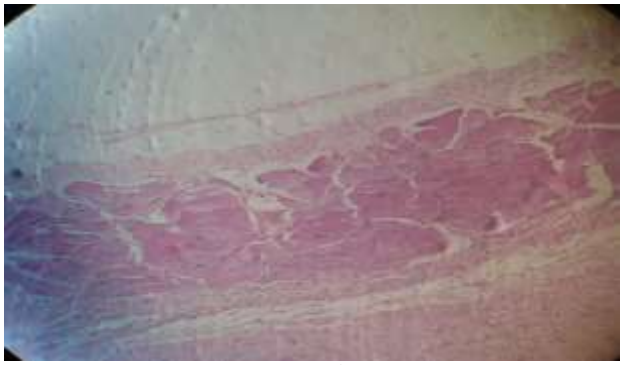

Figure (6): Microscopic examination of the skin. Second-degree burn area taken from the control group (Vaseline-treated) on the 10th day. The re-epithelialization can be observed with a formation of new amorphous collagen layer indicating the beginning of scar formation and healing of the skin tissue. (H & E, X 40).

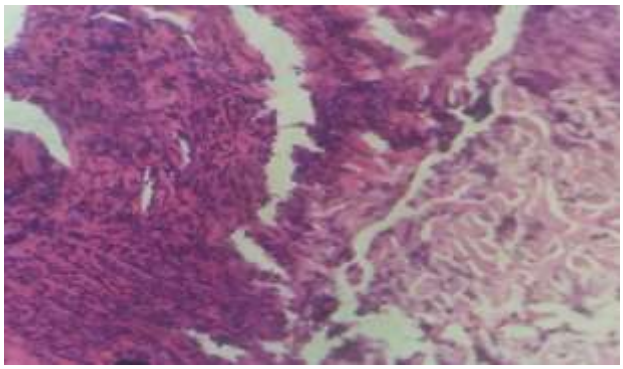

Figure (7): Microscopic examination of the second-degree burn area treated by fibrin glue for 10 days. The dermis is covered by newly formed epidermal tissue indicating recovery from the burn damage. (H & E, X 40).

4. Discussion

The major purpose of this study was to investigate the possibility of using catfish derived clotting elements to constitute safer fibrin glue that can be used for medical purposes in wounds healing. Fish blood proteins are a potentially safer, equally effective, and less costly alternative to human or other mammalian blood proteins.

In the first application, all skin wound group treated with fibrin glue showed more rapid healing than the control group without scar formation compared with control, the control wounds were characterized by an increase in scar formation in the healing area. That is clear in the healing of control skin wound incision that was accompanied by the formation of fibrous connective tissue leading to scar remodeling at the site of the incision. In contrast, the fibrin glue-treated wounds had aggregations of granulocyte cells without fibrous tissue formation and the wound defect was completely plugged, also the repaired tissue architecture has been studied histologically, our results agree with results obtained by Cortan et al.(1999) who reported that fibroblast formation is the hallmark of the healing process. Also, our results showed that the skin wound healing process is explained as stimulation of fibroblastic cell proliferation, consecutive tissue synthesis and collagen fibers were seen under the regenerated epidermis. Michel and Harmand (1990) demonstrated that the effect of fibrin glue containing thrombin and calcium stimulates fibroblast proliferation and collagen synthesis *in vitro*. Hashimoto et al. (1992) obtained a much better result in comparison with the control group, by using fibrin glue experimentally in the incisional repair of mongrel dogs. Additional studies on fibrin glue, including fibroblastic growth factor and endothelial cell growth factor, revealed that wound healing in the presence of these materials was more successful than the controls (Stanojkovi et la., 2008; Li et al., 2007). Also, Trombelli et al. (1996) demonstrated that fibrin glue not only causes hemostasis but also enhances healing; the presence of stabilized fibrin structure stimulates the growth of fibroblasts.

In the second application, it was observed that healing signs of the skin lesions indicated by shrinkage of burn size and subside of inflammatory reactions (redness, swelling, and blistering) were much less in fibrin glue treated burn lesions compared with the control group. This gross observation coincides with the results of histological examination of sections take after 10 days. Stone et al. (2000) and other studies demonstrated that the healing time was defined as the time at which the dressing could be removed without the need for soaking and without causing bleeding. In our study, after the dressings were removed, the entire group of rabbits showed more healed areas under the fibrin glue in comparison with control Vaseline lesions which could be due to the mechanical and biological properties of fibrin glue. These clinical observations coincide with the results of macroscopic or microscopic findings, our results are better than Moghaddam et al. (2013) results in which using of fibrin glue in combination with cultured autologous keratinocyte for treatment of the third-degree burns can accelerate inflammatory response and fibrotic tissue formation but did not have any promotive effect on re-epithelialization which is the most important aim of wound healing.

In the present work, fibrinogen separated from catfish blood plasma was found to be around 6 mg/ml plasma compared to a salmon range of 1.5 to 6 mg/ml, while the human was 1.4–4 mg/ml (Wang et al. 2000), which implies that fish blood is a good and reliable source of fibrinogen extraction. In this study, hemostatic experimental gauze soaked in fibrin glue was evaluated as a possible alternative to gauze soaked with saline. The hemostatic efficacy of the prepared fibrin glue was assessed using the bleeding rabbit vein procedure. The bleeding time was 28 seconds by using the standard gauze, while on using fibrin glue was more reduced to be about 13 seconds. The results obtained in the present work demonstrate that fibrin glue is highly effective in controlling hemorrhage in severe rabbit ear bleeding as compared to saline. Our results better than Jahanmehr et al. (2004) which was 19 seconds with mammals fibrin glue sealant, while in this study could be because we used fish fibrin glue. .The hemostatic activity of fish derived fibrin glue can be due to the rapid

formation of fibrin network clot. Data from several animal models of vascular, parenchymal, and mucous membrane bleeding have shown promising results in achieving hemostasis with and without the presence of coagulopathy (Gustafson et al., 2007). However, our experimental study demonstrates that the fibrin glue is effective as hemostatic aid in blood vessel injury, as well as in tissues oozing blood which are impossible or impractical to ligate. This conclusion comes along with previous studies which indicated that fibrin glue has been widely used in surgical procedures over the last several decades, although reference shows the growth in their use has been hampered by the unavailability. Fibrin glues may be used as adhesives or sealants to prevent leakage, and some data support their contribution to wound healing or as a controlled release system (Martinowitz and Spotnitz, 1997; Alving et al., 1995). Fibrin sealants are composed primarily of fibrinogen and thrombin. Some commercial glue available in Europe, and now one in the USA, has also incorporated an anti-fibrinolytic agent to prolong the life of the polymerized clot (Wang et al., 2000).

Until recently, no commercial sources of fibrin sealants were available, leading many investigators to use cryoprecipitate from either the patient or blood bank sources as the origin of the fibrinogen and bovine thrombin to catalyze clot formation. Two areas of concern raised with such materials are the dangers of infection by bacteria, viruses, or prions derived from the blood source. The blood of farmed, domesticated stocks of fish offers an unexplored, innovative source of clotting factors free of mammalian viruses and prions. The rapid blood clotting in most bony fishes including catfish has been well known for many years. The mechanism is a cascade of clotting factors ending in thrombin converting fibrinogen to fibrin similar to that in mammals. The low ambient temperature and the relatively remote phylogeny of fishes are strong barriers to trans-species infectious agents that could be a danger to humans or animals.

5. Conclusion

This study demonstrated that catfish fibrin glue was successfully used to promote healing of skin incisions and burns. Furthermore, the control of local bleeding to reduce blood loss in using gauze soaked is more effective than standard gauze in stopping of hemorrhage. Therefore, this study suggests that fish fibrin glue could be used for wound and burn healing, as well as for hemostasis purposes.

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