

PROTEIN PROFILE IN SELF-MADE FIBRIN GLUE AS PROMISING BIOMATERIAL FOR WOUND HEALING MODULATION AFTER TRABECULECTOMY

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ABSTRACT : Subconjunctival fibrosis is the main cause of failure in trabeculectomy. Application of fibrin glue following conventional trabeculectomy is hypothesized to increase its surgical success rate. This study aimed to investigate the protein profile in self-made fibrin glue which could possibly contribute to wound healing modulation after trabeculectomy. Peripheral blood was obtained from a single donor using citrate phosphate dextrose adenine (CPDA-1) anticoagulant. Fibrin glue was generated from the mixture of fibrinogen and thrombin. Fibrinogen and thrombin were isolated from plasma pellet and calcium chloride (CaCl₂) activated platelet rich plasma (PRP) components using gradient centrifugation alongside the single freeze-thaw cycle technique. Analysis of protein profile was conducted using liquid chromatography – mass spectrometry (LC – MS). Protein identification was obtained from the Universal Protein knowledge base (UniProt). Liquid chromatography – mass spectrometry analysis revealed 139 proteins in the self-made fibrin glue. There were 26 unique proteins related to the wound healing process, including growth factor related proteins, cell adhesion proteins, and platelet related proteins. There were 30 proteins corresponding to extracellular matrix remodeling which consists of 15 proteolysis activation related proteins, 9 proteolysis regulation related proteins, and 6 negative regulation of proteolysis related proteins. Moreover, as it is a biomaterial, our self-made fibrin glue could act as a biological spacer to facilitate aqueous outflow, and its degradation time of 7-14 days could also be taken advantage of. Self-made fibrin glue contains unique proteins which can contribute to wound healing modulation after trabeculectomy.

Key words : Fibrin glue, LC-MS, wound healing, trabeculectomy.

INTRODUCTION

It is estimated that more than 60.5 million people worldwide are threatened by glaucoma and this number is projected to reach 79.6 million by 2020. Glaucoma permanently damages optic neurons, leading to irreversible blindness (Yamanaka *et al*, 2015; Masompour *et al*, 2016). Reducing intraocular pressure (IOP) is the most effective strategy to halt the progression of visual impairment. Medication which lowers intraocular pressure is the most popular method of treatment for glaucoma, including agents that reduce aqueous humor production or promote outflow. Owing to varying intensities of the disease, such agents occasionally fail to control IOP levels, and surgical intervention is then suggested, including laser, filtration surgery, and tube shunt surgery (Masompour *et al*, 2016;

Cardeiro *et al*, 2000).

Trabeculectomy is the most common surgical intervention in developing countries. The patient's healing response is the key determinant factor in final IOP after trabeculectomy. Subconjunctival fibrosis is the main cause of failure in glaucoma filtration surgery, with an occurrence rate of 24% to 74% within 4 years after surgery (Masompour *et al*, 2016; Lama and Fechtner, 2003). Several treatments and surgical approaches have been developed to successfully modulate subconjunctival fibrosis after glaucoma filtration surgery. Steroids and nonsteroidal anti-inflammatory drugs can be applied topically and systemically to reduce inflammation and fibrosis. Antimetabolite agents such as mitomycin-C and 5-fluorouracil inhibit fibroblast function when applied locally. However, because of their nonspecific

mechanisms of action, these agents can cause widespread cell death and apoptosis, potentially resulting in sight-threatening complications such as severe postoperative hypotony, bleb leaks, and endophthalmitis (Yamanaka *et al*, 2015; Masompour *et al*, 2016; Lama and Fechtner, 2003).

There is currently no agent identified to efficiently block vascular leakage and coagulation, which are the first steps of wound healing in trabeculectomy surgery. Fibrin glue is a biological tissue adhesive which imitates the final stages of coagulation cascade, when a solution of human fibrinogen is activated by thrombin. The adhesive, sealing and hemostatic properties of fibrin glue make it particularly applicable in many kinds of surgery (Sakarya *et al*, 2011; Tabele *et al*, 2012; Hermeto *et al*, 2012; Daglioglu *et al*, 2018). Fibrin glue is used for conjunctival closure in strabismus, vitrectomy, and trabeculectomy surgeries. We hypothesized that the application of subconjunctival fibrin glue following conventional trabeculectomy would create a biological spacer which facilitates hemostasis and humor aqueous outflow. Moreover, growth factors and cytokines released from fibrin glue may modulate wound healing by controlling hemostasis, inflammation, fibroblast proliferation, and regulate fibrosis formation in a time-dependent manner as its fibrin clot degrades. In this study, we created self-made fibrin glue generated from a mixture of fibrinogen and thrombin rich in platelet rich plasma (PRP) to modulate wound healing after trabeculectomy (Cavichilo *et al*, 2013; Lee *et al*, 2008). We conducted LC-MS based proteomics analysis of our fibrin glue in comparison to thrombin and plasma to identify proteins that may play a role in wound healing modulation after trabeculectomy.

MATERIALS AND METHODS

Materials

Materials using in this study were CPDA-1 (Sigma), sequencing-grade trifluoroacetic acid (TFA-Sigma), HPLC-grade acetonitrile and HPLC grade water from Fisher Scientific.

Methods

Study design

This was laboratory study with a single human subject as a blood donor for fibrin glue isolation. This study was conducted under ethical approval from Institutional Ethics, Universitas Airlangga. The study was conducted from October to November 2017 in Stem Cell Research and Development Center, Universitas Airlangga.

Fibrin glue, thrombin and plasma preparation

Fibrin glue was generated according to Lee *et al* (2008) with our modification (Lee *et al*, 2008). Nine milliliters of peripheral vein blood was withdrawn using sterile syringe containing 1 ml CPDA-1. Following gentle agitation, the blood was placed overnight in a sterile tube in -4°C . As the plasma separated from erythrocyte, the blood was further centrifuged at 40 g for 10 minutes so that it could be obtained. The plasma was stored in -20°C for 24 hours and then centrifuged at 6500 g for 5 minutes, 4°C . Following centrifugation, 2/3 parts of plasma were collected for further analysis, 1/3 parts of concentrated plasma (PRP) were stored to prepare thrombin, and pellets were collected and stored at -30°C as the fibrinogen component for fibrin glue. Thrombin was isolated by mixing 1/3 parts of concentrated plasma with 10% CaCl_2 . Fibrin glue was generated by mixing fibrinogen and thrombin. Fibrin glue, thrombin, and remaining plasma were stored at -20°C until further analysis.

Liquid chromatography-Mass Spectrometry

Fibrin glue, thrombin, and plasma were digested with TPCK-treated trypsin for 16-20 hours at 37°C with a substrate-to-enzyme ratio of 50:1 (w/w) in 50 mM ammonium bicarbonate buffer solution at pH 8.5 (the pH of the buffer solution was adjusted with 1 M ammonium hydroxide). The digested material was loaded to the LC column with TFA/H₂O as solvent A and 3 mM TFA/CH₃CN as solvent B. Then, the final sample was analyzed for protein identification using LC-MS/MS analysis based on company protocols (Fig. 1). Detected proteins were analyzed according to UNIPROT website (Geyer *et al*, 2018; Gautam *et al*, 2013; Yu *et al*, 2010).

RESULTS

We conducted liquid chromatography on each sample as a separation method to reduce highly abundant proteins such as albumin. Mass spectrometry was performed to detect large amounts of proteins in single analysis. We identified 93 proteins from plasma, 110 proteins from thrombin, and 139 proteins from fibrin glue (Table 1). Out of the 139 proteins found in fibrin glue, we identified 26 unique proteins that may positively contribute to the wound healing process after trabeculectomy (Table 2).

Among the 26 unique proteins, there were 6 proteins, including IGF-1, HGF, HGF-like protein, platelet factor-4, coagulation factor V and coagulation factor X, that indicated the therapeutic potential of fibrin glue in wound healing modulation after trabeculectomy. LC-MS study revealed 15 proteins related to the regulation of proteolysis as depicted in Table 3.

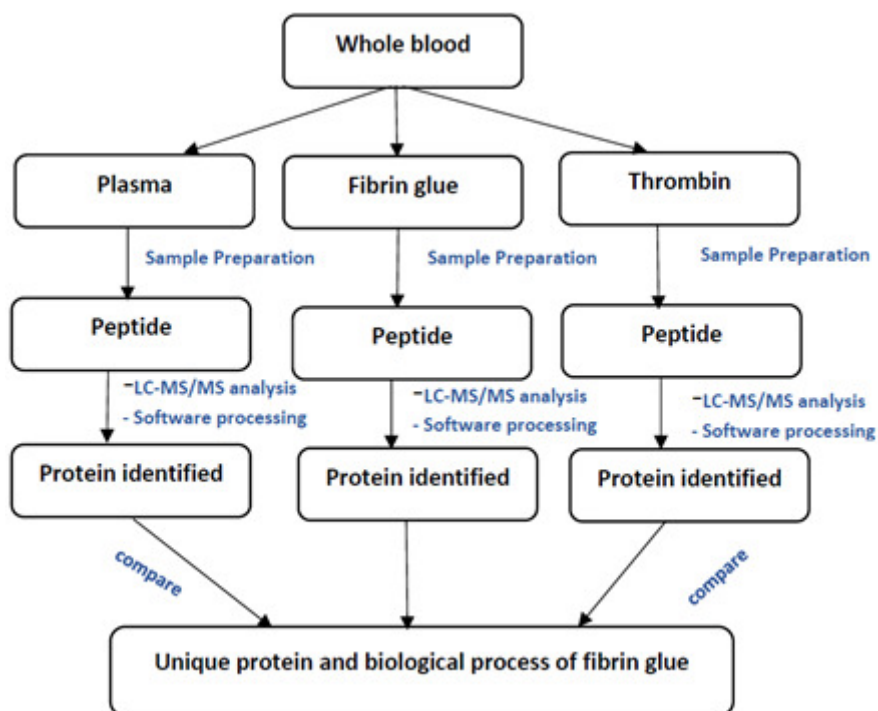


Fig. 1 : Study flowchart.

Table 1 : Protein identified in each sample.

Unique Protein Plasma	Unique Protein Fibrin glue		Unique Protein in thrombin
E3 ubiquitin-protein ligase TRIM17	insulin-like growth factor I	Hepatocyte growth factor activator	Complement factor H-related protein 1
Isoform 2 of L-selectin	Coagulation factor V	Hepatocyte growth factor-like protein	Putative macrophage stimulating 1-like protein
	Tetranectin	Selenoprotein P	Ig kappa chain V-II region TEW
	Ig gamma-3 chain C region	Hemoglobin subunit delta	Protein SZT2
	Fermitin family homolog 3	Ig heavy chain V-III region POM	Serum amyloid A-1 protein
	Myosin light polypeptide 6	Ig heavy chain V-III region POM	Klotho
	Integrin alpha-IIb	Complement factor D	
	C4b-binding protein beta chain	Coagulation factor X	
	Mannan-binding lectin serine protease 2	Sex hormone-binding globulin	
	Pleckstrin	Mediator of RNA polymerase II transcription subunit 13-like	
	Pyruvate kinase PKM	Carboxypeptidase B2	
	Alpha-enolase	Extracellular matrix protein 1	

DISCUSSION

Wound healing after trabeculectomy consists of overlapping phases, including blood coagulation, fibroblast proliferation, migration, differentiation and remodeling. Differing from the healing process of other tissues, the natural wound healing process should be preserved for successful bleb formation. Regulation of the blood coagulation phase is critical and determines the outcome

of wound healing. Currently there is no agent identified for blocking vascular leakage and coagulation, which are the first steps to successful wound healing in trabeculectomy surgery (Sakarya *et al*, 2011; Kahook and Noecker, 2006; Wang *et al*, 2017). Blood-derived topical hemostatic products are used in surgery because of their unique biological and physical advantages, in comparison to weaker synthetic products. They possess

Table 2 : Unique proteins identified in fibrin glue.

Insulin-like growth factor I	Tropomyosin alpha-4 chain
Coagulation factor V	Hepatocyte growth factor activator
Tetranectin	Hepatocyte growth factor-like protein
Ig gamma-3 chain C region	Selenoprotein P
Fermitin family homolog 3	Hemoglobin subunit delta
Myosin light polypeptide 6	Ig heavy chain V-III region POM
Integrin alpha-IIb	Ig heavy chain V-III region POM
C4b-binding protein beta chain	Complement factor D
Mannan-binding lectin serine protease 2	Coagulation factor X
Pleckstrin	Sex hormone-binding globulin
Pyruvate kinase PKM	mediator of RNA polymerase II transcription subunit 13-like
alpha-enolase	Carboxypeptidase B2
Isoform SERCA3E of Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	Extracellular matrix protein 1
Talin-1	Mannan-binding lectin serine protease 2

Table 3 : Protein related to regulatory of proteolysis.

Mannan-binding lectin serine protease 2
Coagulation factor X
Complement factor D
Alpha-1-antichymotrypsin
Immunoglobulin kappa variable 2D-28
Immunoglobulin heavy variable 3-23
Immunoglobulin kappa constant
Immunoglobulin heavy constant gamma 3
Tetranectin
Complement component C8 beta chain
Complement component C8 gamma chain
Apolipoprotein(a)
Variant surface glycoprotein AnTaT 1.1
C4b-binding protein beta chain
Hepatocyte growth factor-like protein

tissue sealing and hemostatic properties. Some of these products can stimulate cell growth and differentiation, which would have advantageous for clinical use and cell therapy (Burnouf *et al*, 2009; Stachowicz *et al*, 2017). Fibrin glue is a biological tissue adhesive which imitates the final stages of coagulation cascade, when a solution of human fibrinogen is activated by thrombin which then modulates wound healing after trabeculectomy (Sakarya *et al*, 2011; Lee *et al*, 2008).

We successfully generated fibrin glue from the mixture of fibrinogen and thrombin rich in PRP that we can call platelet fibrin glue or platelet glue. Burnouf *et al* revealed that biomaterial platelet glue has a tensile strength intermediate between that of platelet glue and fibrin sealant and contains platelet growth factors. It was found that, compared to platelet gel, significant

advantages of platelet glue include its higher level of mechanical strength and adhesive properties, which owes to the formation of a strong fibrin clot upon activation by thrombin (Burnouf *et al*, 2009). Liquid chromatography-mass spectrometry study successfully identified certain proteins in our self-made fibrin glue which may contribute to wound healing after trabeculectomy. Coagulation factor V and X may control hemostasis just after fibrin glue application by initiating blood coagulation within surgical spaces. The formation of a blood clot is the initial mechanism of natural wound closure. Once the coagulation cascade is triggered, activated factor X selectively hydrolyzes prothrombin to thrombin. In the presence of thrombin, fibrinogen is converted to fibrin. Thrombin also activates factor XIII, which stabilizes the clot (Eby *et al*, 2001; Aslam *et al*, 2017). Fibrin glue may possibly be able to coat the opposing faces of tenon-scleral surfaces and scleral-scleral surfaces to halt vascular leakage. Since, it inhibits both hemorrhage and vascular leakage, we could expect less postoperative inflammation. It was in accordance with a study conducted by Hermetoetl *al* and Dagliolu *et al* that both revealed the occurrence of less inflammation in skin wound healing and colonic anastomose after fibrin glue administration (Hermeto *et al*, 2012; Daglioglu *et al*, 2018). Additionally, it is well known that subconjunctival hemorrhage after glaucoma is associated closely with failed bleb and increased subconjunctival fibrosis. Since there is no vascular leakage and hemorrhage from the opposing surfaces, aqueous humor escapes freely from the anterior chamber into the fibrin glue coated scleral flap-scleral bed and tenon-scleral interface. Our previous

study showed that fibrin glue, both in its original form and as a stem cell carrier, was able to reduce the area of bleb fibrosis after trabeculectomy in a rabbit eye model. In such study, we observed that fibrin glue degradation takes 14-21 days in subconjunctival bleb space. (Komaratih *et al*, 2018).

Platelet factor-4 facilitates platelet aggregation and platelet alpha granule degradation to release mitogenic growth factors promoting fibroblast activation and proliferation. Fibrin glue releases IGF-1, HGF and HGF-like proteins that play an important role in the proliferation phase by activating tenon fibroblast proliferation and migration. IGF-1 activates fibroblast proliferation through tyrosine kinase pathway (Noori *et al*, 2017; Wang *et al*, 2017). HGF plays an important role in cell survival, regeneration, anti-inflammation and antifibrosis by tyrosine phosphorylation signal activation. Extracellular matrix proteolysis is one of the biological processes governed by HGF action. Matsumoto *et al* (2014) showed that in phase I/II of clinical trial of recombinant HGF proteins were able to promote regeneration and inhibit fibrosis progression in amyotrophic lateral sclerosis. Daglioglu *et al* (2018) showed that fibrin glue was superior to PRP in promoting wound healing on colonic anastomose rat.

The final stages of wound healing in trabeculectomy are epithelization and myofibroblast contraction. In the remodeling phase, fibroblast fabricates the proteolysis enzyme which regulate the degradation and deposition of extracellular matrix (Eby *et al*, 2009; Komaratih *et al*, 2018). Proper control of the remodeling phase is one of the attempts to inhibit bleb fibrosis in post-proliferation phase. It can be deduced that fibrin glue may secrete some proteins contributing to remodeling phase regulation. Some complement factors were secreted by fibrin glue and performs clearance during fibrosis formation through certain catalytic pathway. Indeed, the fibrosis activator TGF- β may activate C3a and C5a cascade that exert fibrosis formation. We assumed that tetranectin released from fibrin glue may regulate cellular response to TGF- β stimulus, fulfilling its role in proteolysis and anti-proteolysis regulation. Regulation of proteolysis plays an important role in successfully bleb formation (Masompour *et al*, 2016; Cordeiro *et al*, 2000).

Apo-A is an auto-proteolysis protein that contributes to extracellular matrix degradation. Extracellular matrix protein 1 regulates cellular inflammatory response and inhibit proteolytic activity of matrix metalloproteinase 9 (MMP9). Regulation of inflammatory response may inhibit inflammatory reaction leading to the activation of fibrosis signaling pathway. Upregulation of MMP9 activity may result in bleb leaks; thus its inhibition is necessary

for successfully bleb formation. Fibrin glue regulates proteolysis proteins, which reveals its potential in fibrosis inhibition. A study that was conducted by Eby *et al* revealed that application of fibrin glue in laser damaged skin result in healing with minimal scarring by modulating the remodeling phase (Eby *et al*, 2009; Lee *et al*, 2008).

Currently the trend of using fibrin glue for commercial purposes is rising. Moreover, fibrin glue can be prepared at a blood transfusion center or from the patients' own blood. Compared to synthetic glues and other biomaterials, they are autologous and can be obtained with low cost. They are physiologically compatible with human tissues, do not cause tissue necrosis or other body reactions and are readily colonized by cells. They are also completely biodegradable in a matter of days to weeks. However, individual batch variation found during fibrin glue isolation requires further optimization (Sakarya *et al*, 2011).

CONCLUSION

Glaucoma filtration surgery is an efficient surgical process widely used for cases of glaucoma in which medication alone is insufficient. Bleb fibrosis is the main cause of surgical failure. Although, the mechanisms of scar tissue formation after the filtering surgery are not fully understood, it is clear that excessive proliferation of Tenon's and conjunctival fibroblasts, differentiation of these fibroblasts into myofibroblasts and uncontrolled production of extracellular matrix play a major role in the process. Fibrin glue may act as biological spacer and regulate blood coagulation, cellular response, inflammatory reaction and proteolysis activity in wound healing after trabeculectomy. Hence, fibrin glue promotes successfully bleb formation for an efficient wound recovery. Clinical study is mandatory to evaluate the potential effects of fibrin glue in wound healing modulation after trabeculectomy.

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