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FIBRIN GLUE (FG) ATTENUATES FIBROSIS ON HUMAN TENON'S FIBROBLASTS (HTFS) OF GLAUCOMATOUS EYES : COMPARISON WITH MITOMYCIN C

Yuyun Rindiastuti[1], Evelyn Komaratih*[2], Helen Susilowati[1], Hendrian D. Soebagyo[2] and Fedik A. Rantam[1]

[1]Stem Cell Research and Development Center, Universitas Airlangga, Surabaya, Indonesia.

[2]Department of Ophthalmology, Faculty of Medicine, Universitas Airlangga/ Dr. Soetomo General Academic Hospital, Surabaya.

e-mail : risetpublikasi@gmail.com

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ABSTRACT : Human tenon's fibroblasts (HTFs) are major cells that contribute to the fibrotic response that usually occurs after trabeculectomy. The activation of HTFs into myofibroblast contributes to the disturbance of the extracellular matrix (ECM) remodeling. Excessive ECM deposition, mainly collagen type I, with cell contraction is the major hallmark of bleb fibrosis. HTFs were isolated from the tenon's tissue of a glaucomatous patient. HTFs were divided into 3 groups which consisted of FBS 2% control group, MMC and FG treated group. This study investigated the effects of FG on cell viability, cell migration, cell contractility, collagen synthesis and degradation in HTFs. Cell viability was determined by MTT-assay, while collagen synthesis and degradation were determined by Sirius red binding assay. Cell migration was carried out by in vitro scratch assay, and cell contractility was analyzed by fibroblast populated-collagen gel assay. The differences in cell viability, cell contractility, collagen synthesis and degradation among the various groups were analyzed using One-way ANOVA or Kruskal Wallis test, followed by a posthoc test with 95% confidence interval ($p<0.05$). FG significantly decreased collagen synthesis (31.31±2.91ug/mL) in line with the induction of collagen degradation (85.50±3.16ug/mL) in HTFs when compared to FBS 2% control. While FG inhibits cell contraction while maintaining cell viability in HTFs, MMC provides a better antifibrotic effect in HTFs. FG may inhibit fibrosis formation of HTFs by inhibiting collagen synthesis and cell contraction while inducing collagen degradation. FG might have an antifibrotic effect on HTFs through extracellular matrix remodeling.

Key words : Fibrin glue, tenon's fibroblast, fibrosis, mitomycin C.

INTRODUCTION

It is estimated that more than 60.5 million people worldwide are threatened by glaucoma and this number is predicted to increase to 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. The use of medication that lowers intraocular pressure is the first choice in glaucoma treatment, including agents that reduce aqueous humor production or promote outflow. When this method fails to control IOP, surgical intervention is suggested including laser, filtration surgery, and tube shunt surgery (Masompour *et al*, 2016; Cordeiro *et al*, 2000). Trabeculectomy is the most common surgical intervention in developing countries. A healing response is the critical determinant factor in final IOP after trabeculectomy. Subconjunctival fibrosis is the main cause of failure in glaucoma filtration surgery and it accounts for about 24% to 74% of the failures in the 4 years after surgery (Masompour *et al*, 2016; Lama and Fechtner, 2003).

The human Tenon's fibroblasts (HTFs) are considered the major cell types that contribute to the fibrotic response triggered after trabeculectomy. Upon TGF-â stimulation, fibroblasts are activated and undergo phenotypic transition into myofibroblasts, which are the key effector cells in fibrotic states. TGF-β promotes matrix preservation and deposition by enhancing matrix protein synthesis and by altering the balance between matrix-preserving and matrix-degrading signals (Seet *et al*, 2012; Wang *et al*, 2017). Excessive ECM deposition, mainly collagen type I, with cell contraction is the major hallmark of bleb fibrosis. Several treatments have been developed to successfully modulate subconjunctival fibrosis after glaucoma filtration surgery. Antimetabolite agents, such as mitomycin-C (MMC) and 5-fluorouracil, inhibit fibroblast function when applied locally. However, because of their nonspecific mechanisms of action, these

*Corresponding author.

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

Fibrin glue (FG) is a biological tissue adhesive which imitates the final stages of the coagulation cascade when a solution of human fibrinogen is activated by thrombin. The adhesive, sealing, and hemostatic properties of FG are particularly useful in many kinds of surgery. Fibrin glue is used for conjunctival closure in strabismus, vitrectomy, and trabeculectomy surgeries. Fibrin glue is highly biocompatible as well as completely biodegradable and has shown good results with little toxicity for use on the ocular surface (Tabele *et al*, 2012; Sakarya *et al*, 2011). Currently, there is no defined agent for blocking either vascular leakage or coagulation, which are the first steps in wound healing following trabeculectomy surgery. Sakarya *et al* (2011) hypothesized that application of subconjunctival FG following conventional trabeculectomy would create a biological spacer that facilitated hemostasis and humor aqueous outflow. Moreover, growth factors and cytokines released from FG may modulate wound healing by controlling hemostasis, inflammation, fibroblast proliferation, and regulate fibrosis formation in a time-dependent manner as its fibrin clot degrade (Tabele *et al*, 2012; Sakarya *et al*, 2011; Jackson, 2001). A study conducted by Komaratih *et al* (2018) revealed that transplantation of FG itself or FG encapsulated limbal mesenchymal stem cells had a similar effect in reducing bleb fibrosis area in rabbit's eye. However, the role of the antifibrosis effects from FG remained unknown (Komaratih *et al*, 2018). In this study, we created self-made fibrin glue generated from the mixture of PRP derived fibrinogen and PPP derived thrombin to analyze its anti-fibrosis effect on HTFs in glaucomatous eyes. We describe the properties of these FG treated HTFs in assays for proliferation, cell migration, collagen deposition and degradation and collagen gel contraction. We compared these effects against MMC as the 'gold standard'.

MATERIALS AND METHODS

The main reagents included Type I collagenase (Roche USA), alpha modified eagle medium (á-MEM, Gibco-Life Technologies, USA), nonessential amino acid (NEAA, Sigma, USA), Trypsin (Gibco - Life Technologies, USA), fetal bovine serum (FBS, Biowest, USA), basic fibroblast growth factor (bFGF, Gibco- Life Technologies, USA), Insulin-transferin-selenium (ITS, Gibco- Life Technologies, USA), rat tail collagen type I, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphyniltetrazolium bromide (MTT; Gibco- Life Technologies, USA), anticoagulant citrate phosphate dextrose acid (CPDA), $CaCl_2$ (sigma). Primary FITC antibodies for vimentin and secondary antibodies were purchased from BIOSS (USA).

METHODS

Study design

This is an *in vitro* study of HTFs in the glaucomatous eye that was conducted at the Stem Cell research and development center, Universitas Airlangga. All experiments conformed to the local ethics review board from the dr. Soetomo Hospital. Human tenon's fibroblast and peripheral blood was obtained from the same subject. HTFs culture was divided into 3 groups which consisted of a control group treated with 2% FBS in culture media, MMC treated group and FG treated group. Cell viability was evaluated after the 3rd day following treatment. Wound scratch assay was performed for each group and cell migration rate, collagen deposition, and degradation assays, and cell contractility were all measured 7 days after treatment.

Fibrin glue preparation

Fibrin glue was generated according to the protocol established by Komaratih *et al* (2018). Nine milliliters of blood was withdrawn from the peripheral vein using a sterile syringe containing 1 ml CPDA. Following gentle agitation, the blood was placed into a sterile tube at -4°C overnight. As the plasma separated from the erythrocyte, the blood was further centrifuged at 40 g for 10 minutes to obtain a workable amount of plasma. The plasma was stored at -20°C for 24 hours and then centrifuged at 6500 g for 5 minutes at 4°C. Following centrifugation, 2/3 parts of the plasma was removed, 1/3 parts as platelet rich plasma (PRP) was 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 PRP with 10% $CaCl_2$. Fibrin glue was then generated by mixing fibrinogen and thrombin (Komaratih *et al*, 2018).

Isolation of HTFs

The Tenon's biopsies were collected from 2 female patients (n = 2) aged 25-40 years, who had been diagnosed with advanced open-angle glaucoma, while having no previous surgical therapy, and were undergoing trabeculectomy. The isolation of cells was conducted according to the protocol established by Przekora *et al* (2017) with our modification. The tissues were washed twice with PBS and cut into 2 pieces using a sterile scalpel. These were placed in separate wells of a 12-

well plate using light pressure and were left to air dry for up to 1 min while being attached to the well bottom. The tissues were then cultured in media containing α-MEM, 1% penicillin-streptomycin, 1% amphotericin B, 1% NEAA, 5 % FBS, 5ug/mL ITS and 5ng/mL bFGF. After 5 to 7 days of culture, the cells were harvested and passaged. To confirm the fibroblast phenotype, cells were characterized at passage 3 for the expression of vimentin FITC antibody. Stained cells were visualized under a fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus).

Cell viability, collagen degradation and collagen deposition

HTFs of P4 were seeded in a 96 multi-well plate with 5×10^3 cells/well, with a complete culture media for 24 hours. Treatment with 2% FBS as a control group, as well as MMC and FG were given to each group. Cell viability was determined after 3 days of treatment by 3,-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay, according to the method utilized by Freshney (2000). Collagen degradation and deposition were determined after 7 days of treatment using a Sirius red binding assay, following the method used by Heng *et al* (2006) and Taskiran *et al* (1999), respectively. The concentration of type I collagen degradation and deposition were determined according to the standard curve established from rat tail collagen type I (Freshney, 2000; Heng *et al*, 2006; Taskiran *et al*, 1999).

***In vitro* scratch assay**

HTFs at 3×10^5 cells/well were seeded into six-well culture dishes and allowed to attach and grow to confluence. The cells were then scratched wounded using a yellow pipette tip and washed with a medium to remove loose or dead cells. Treatment with 2% FBS was done as a control group, while MMC and FG were given to each group for 7 days. Cell migration rates were analyzed according to Freshney (2000). The wound at the same spot, marked by a reference line drawn on the outside of the dish, was photographed at different time points on the 3rd and 7th day after treatment. This was done with a phase-contrast microscope equipped with a camera. Two different fields of each scratch wound were photographed and each condition was performed in triplicate. The wound closure area was analyzed as a cell migration rate with Image J software. The data was expressed as averages of the percentage wound closure area at the measured time point over the original width at time 0 hour (Freshney, 2000).

Cell contraction assay

To assess the influence of FG on HTFs contraction, we measured the contraction of HTFs-seeded collagen gels. First, HTFs were resuspended at a density of 3×10^5 cells/mL with a complete culture media. Each gel was made from dialyzed collagen in 0.1% acetic acid and gently mixed. Sodium hydroxide (0.1 M) was then added to the gel to return the solution to a physiological pH and to precipitate the collagen. Thirty microliters of HTFs (at 3×10^5 cells/mL) were then seeded into the neutralized gel and resuspended briefly. This was then added to a well from a 12-well plate and incubated at 37°C for 24 hours. Each treatment was then given into each collagen seeded HTFs. The fibroblast-populated collagen gels were incubated for 7 days. The images of contractile cells grown over a collagen-coated dish were taken with a digital camera. The images were then measured and assessed using ImageJ software by comparing the cell-gel contractile area with the total area (Freshney, 2000).

Statistical analysis

Statistical analysis was performed using SPSS version 19.0 software. One-way ANOVA or Kruskal Wallis test and post-hoc test with 95% confidence interval ($p<0.05$) was used to determine the statistical significance of differences of cell viability, collagen degradation and deposition and cell contraction among HTFs after treatment.

RESULTS

Human tenon's fibroblast isolation

This study successfully isolated HTFs in a relatively short time (an average of 7 days). This was required to reach the first monolayer at 90% confluency. Supplementation with bFGF and NEAA were able to promote cell proliferation while maintaining the cell's phenotype and viability. Immunofluorescent staining of vimentin, a specific marker of fibroblast, revealed that near 100% of the cells after passage-2 were vimentin-positive (Fig. 1).

Viability and Migration of human tenon's fibroblast

The effect of FG on cell viability of HTFs culture is presented in Fig. 2. Cell viability of HTFs decreased after MMC treatment (54.88%±3.02%) compared to those with FBS 2% control group (90.04%±6.79%, p=0.000) and FG (72.40%±2.98%, p=0.002). However, there was a significant decrease in cell viability for MMC (p=0.001) and FG (p=0.003) when compared to the FBS 2% control group (Fig. 2).

MMC decreased cell migration rate on days 3 and 7, as compared to the FBS 2% control group and FG group

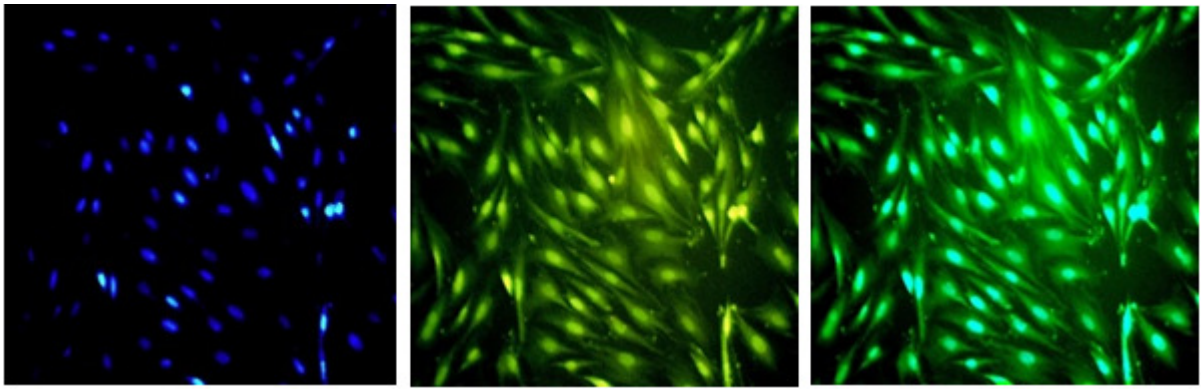


Fig. 1 : The cells were stained with Vimentin FITC conjugated antibody. Left section, cells were stained with DAPI; middle section with FITC labeled CD Vimentin; right section merge of the previous two sections (Inverted Fluorescence Microscope, 200x magnification).

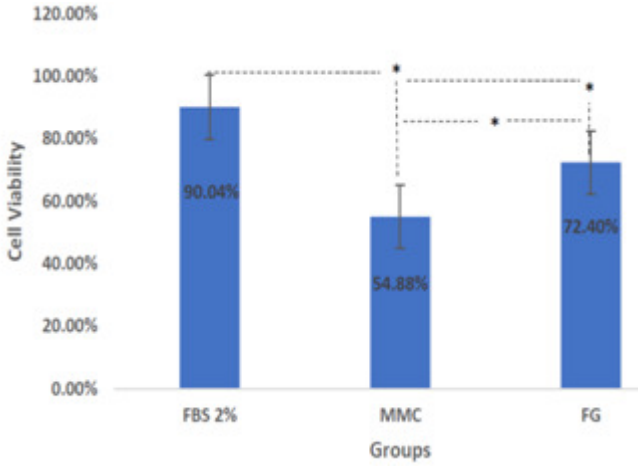


Fig. 2 : Effect of FG on cell viability of HTFs (Kruskal Wallis, Mann-Whitney, *statistically significant).

(Fig. 3). Cell migration rate on days 3 in FG (13.24%) and MMC group (5.70%) were 3.6-fold and 8.3-fold lower than the FBS 2% control group (47.80%), respectively. Cell migration rate tended to increase on day 7 with 29.04%, 18.0%, 72.30% in the FG, MMC, and FBS 2% control group, respectively (Fig. 3).

Collagen deposition, degradation and cell contractility

MMC (18.54±1.54ug/mL) and FG (31.31±2.91ug/mL) had significantly decreased collagen deposition when compared to the FBS 2% control group (159.30±2.94ug/mL, p=0.001). However, MMC group had the greatest effect on reducing collagen deposition compared to the FG group (p=0.01) (Fig. 4).

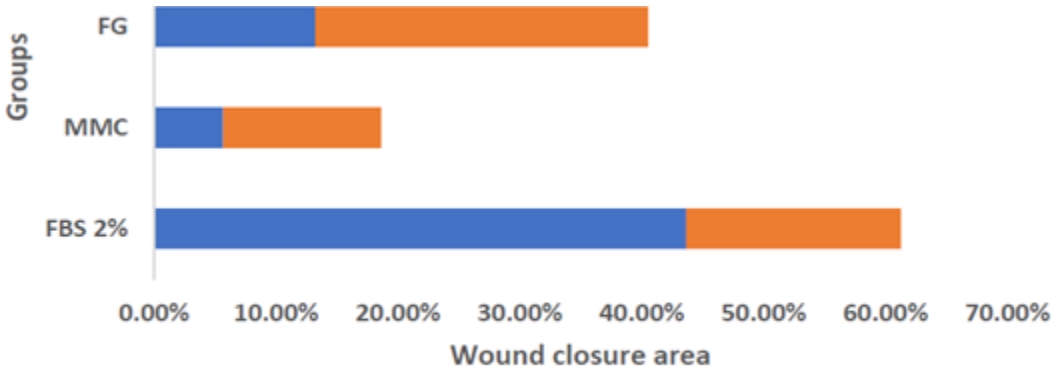
MMC (98.43±2.14ug/mL) and FG (85.50±3.16ug/mL) had significantly increased collagen degradation compared to the FBS 2% group (39.59±2.14ug/mL, p=0.001). Collagen degradation in MMC group was significantly higher compared to the FG group (p=0.03) (Fig. 5).

MMC (29.21±5.96%) and FG (42.48±6.17%) had significantly decreased cell contractility compared to the FBS 2% control group (65.03±4.23%, p=0.000). Cell contraction in MMC was significantly lower than the FG group (p=0.01) (Fig. 6).

DISCUSSION

We successfully generated self-made FG from the mixture of fibrinogen and thrombin-derived PRP using a modified simple cryoprecipitation method to improve antifibrosis secreted factor. Cryoprecipitation is one of the established methods for FG isolation. However, this preparation method remained inconsistent and this may be explained by the quality of the fibrinogen and variation in the additional factors that are present in the fibrinogen solution (Laurens *et al*, 2006; Zhao *et al*, 2008). Our modification in the FG isolation method was aimed at increasing fibrin associated matrix proteins, cytokines, proteases or protease inhibitors that may provide many functions in wound repair. This study revealed that FG was able to maintain HTFs cell viability compared to the MMC group, while FG proved its anti-fibrosis effect on HTFs. Fibrin glue decreased cell migration when compared to the FBS 2% control group, yet MMC had a better inhibitory effect on cell migration. Kim *et al* (2013) showed that fibrin glue provides a natural environment for cells because of its chemotactic, hemostatic, and mitogenic properties. Fibrin glue has been proven as an ideal cell delivery system, to improve cell incorporation and survival at an injured site. It was proposed that FG has no toxic effect on cells and that it may modulate cell activity through a cell-fibrin matrix interaction (Kim *et al*, 2013; Xie *et al*, 2012).

The wound healing consisted of four main phases including vascular leakage–coagulation, inflammatory, proliferative, and remodeling. After injury, blood coagulation takes place and fibrin clots are formed to reduce blood loss. This is followed by an inflammatory phase where neutrophils, macrophages, and lymphocytes are attracted to the region. This leads to a proliferative



Observation	FG	MMC	FBS 2%
Day-3	13.24%	5.70%	47.80%
Day-7	29.04%	18.0%	72.30%

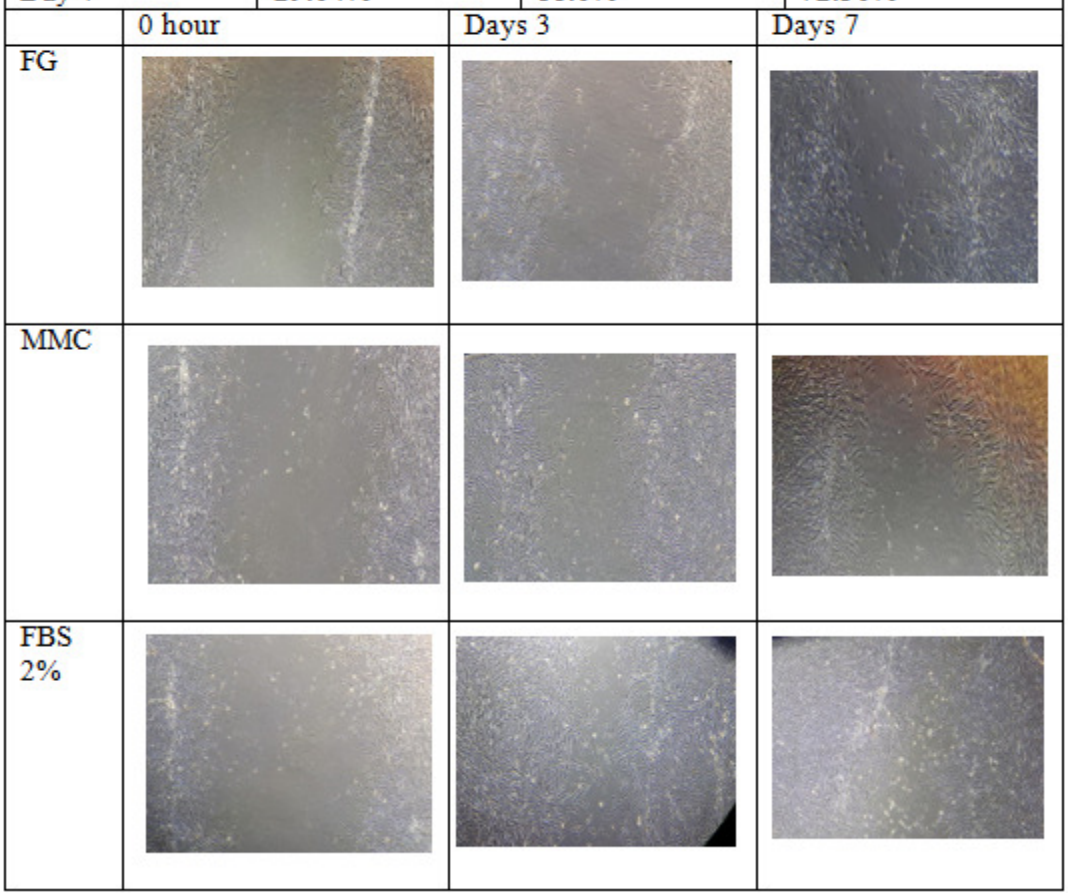


Fig. 3 : Cell migration rate on day- 3 and 7.

phase where fibroblasts migrate into the site of injury and re-epithelialization, angiogenesis and formation of granulation tissue occur. Finally, remodeling of the tissue occurs and involves the formation of scar tissue (Chen *et al*, 2006; Georgoulas *et al*, 2008; Rosentreter *et al*, 2010). Tenon's fibroblast is the key effector in the formation of fibrosis after trabeculectomy. Many different cells take part in the activation of fibroblast into myofibroblast, which is one of the most crucial events in scar formation after trabeculectomy surgery. Myofibroblast initiates extracellular matrix (ECM) secretion and remodeling. The imbalance between proteolysis and anti-proteolysis enzymes leads to excessive ECM deposition, mainly collagen type I. The interaction between collagen type I and myofibroblast contributes to the matrix contraction during scar formation. MMC was still proven o be the "gold standard" in trabeculectomy to halt bleb fibrosis. Our study revealed

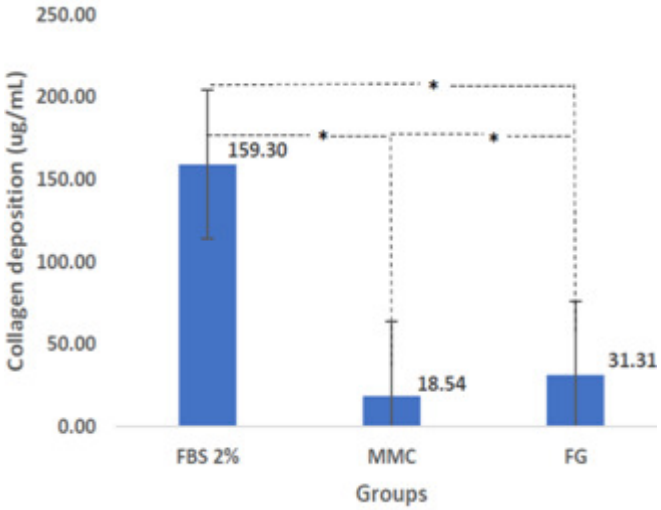


Fig. 4 : Collagen deposition (Kruskal Wallis, Mann-Whitney, * statistically significant).

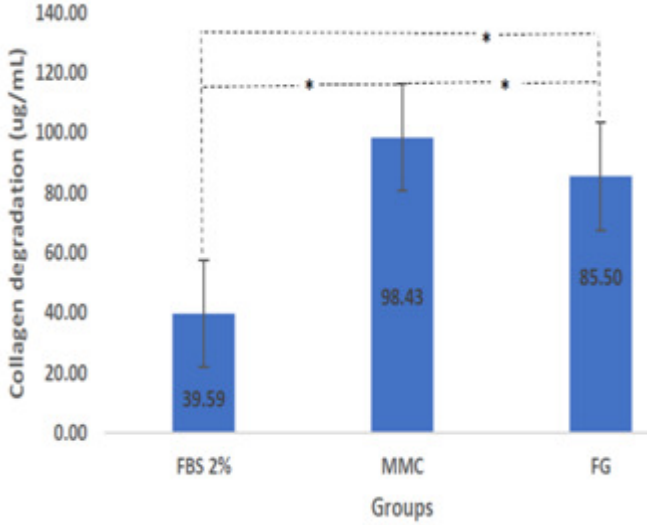


Fig. 5 : Collagen degradation (Kruskal Wallis, Mann-Whitney, * statistically significant).

that MMC had the greatest antifibrosis effect on HTFs since its agent successfully decrease cell viability and inhibited cell migration in the wound scratch assay. However, reconstitution of cell migration appeared after the 7th day, which may indicate that the effect of its agent on cell migration activity is temporary (Arti *et al*, 2014; Mckleroy *et al*, 2013). Seet *et al* (2012) showed that MMC affects HTFs by inhibiting cell proliferation, inducing widespread apoptosis, increasing susceptibility to T cell-mediated lysis and reducing the production of pro-fibrotic genes and proteins. They also reduce cell migration. MMC treated HTFs inhibit type I collagen deposition following the induction of collagen degradation when compared to the FBS 2% control group and the FG treated group. However, MMC remained the best agent to inhibit type I collagen deposition compared to FG. MMC-treated HTFs were severely limited in the

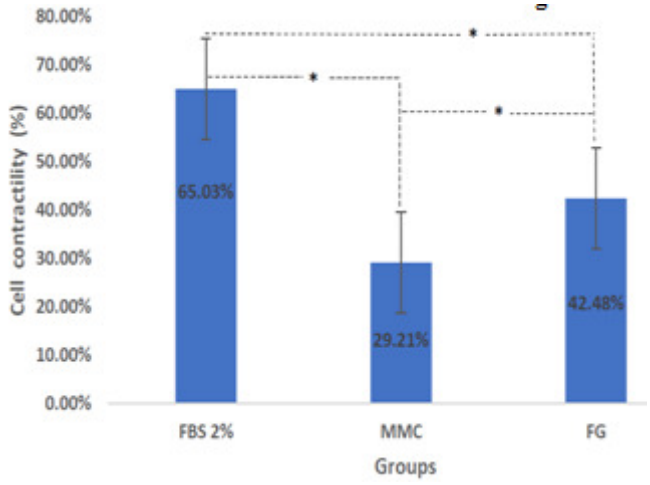


Fig. 6 : Cell contractility (Tukey HSD, * statistically significant).

ability to contract the collagen gel compared to the FBS 2% control group and FG group (Seet *et al*, 2012; Arti *et al*, 2014; Mckleroy *et al*, 2013).

Fibrin glue is composed of variable concentrations of thrombin and fibrinogen. Thrombin, as a protease, activates fibrinogen to form fibrin monomers that polymerize to become an unstable fibrin clot. Thrombin activates factor XIII, which then catalyzes the cross-linking between fibrin molecules, increasing the strength of the clot and providing protection from degradation. Clotted FG resembles the extracellular matrix produced during the early phases of wound healing which provides a "pseudomatrix" framework. This may allow the bypassing of much of the inflammatory phase, during which time the matrix would normally be created. This natural covering may help attenuate the inflammatory response which promotes other downstream pathway in a fibrosis event, mainly ECM remodeling. Inhibition of collagen deposition and collagen degradation stimulation by treated HTFs with FG indicates that its secreted factors might have a role in the balance of ECM reconstitution through proteolytic and anti-proteolytic enzyme activity (Sakarya *et al*, 2010; Ruggiero *et al*, 2008; Bahar *et al*, 2006). This result was in line with Sakarya *et al* (2011), who revealed that the mass of fibrin glue itself may contribute to the formation of a successful bleb since we have evidence that subconjunctival placement of a biodegradable implant both helps formation of successful bleb and decreases subconjunctival fibrosis. 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 the tyrosine kinase pathway (Noori *et al*, 2017; Wang *et al*, 2017; Sakarya *et al*, 2011). Hepatocyte growth factor plays an important role in cell survival, regeneration,

antiinflammation 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 trials, the recombinant HGF proteins were able to promote regeneration and inhibit fibrosis progression in amyotrophic lateral sclerosis (Wang *et al*, 2017; Matsumoto *et al*, 2014).

Fibrin glue significantly inhibits collagen synthesis in line with the induction of collagen degradation in HTFs. This study was consistent with Eby *et al* (2001) for the application of fibrin glue after CO_2 laser resurfacing on the skin had diminished the acute and chronic inflammatory response, enhanced neovascularization, and reduced collagen accumulation. Collagen formation is necessary for adequate wound healing (Noori *et al*, 2017; Eby *et al*, 2001). The continuation of the wound healing process is regulated by the cells that are involved in the initial inflammatory response. Therefore, the decrease in collagen production may be a direct result of the diminished inflammatory response noted in the fibrin glue application. Fibrin glue significantly inhibits collagen gel contraction in collagen populated HTFs. This modest decrease in collagen deposition may benefit wound healing by minimizing scar formation and wound contracture (Sakarya *et al*, 2011; Eby *et al*, 2001).

The remarkable advantage of self-made fibrin glue is that it can be obtained at a relatively low-cost while being simple and autologous. It also avoids the potential risks of foreign body reaction and virus infection. A commercially available product of fibrin glue is specifically designed for adhesion of tissues or organs. These findings are somewhat surprising since one would not predict that an agent used to promote fibrotic wound repair would inhibit the fibrotic activity of HTFs. However, the exact mechanism of FG in fibrosis inhibition remains unknown. The critically important message, however, is that although FG has generally been discussed as if it were a single entity, it represents a mixture of fibrinogen and thrombin that may either promote or inhibit wound repair processes. Without precise purification procedures and rigorous quality control of the resulting FG preparation, it would be expected that FG preparation would have variable effects on wound repair. The hope is to develop "specific" preparations of FG that might be most effective for wound repair, especially for fibrosis inhibition.

CONCLUSION

Fibrin glue exerts antifibrosis effects on HTFs of a glaucomatous eye, presumably by inhibiting collagen synthesis and cell contraction following collagen degradation activity. Fibrin glue may regulate proteolysis and anti-proteolysis activity in HTFs culture through an undetermined pathway. There are no toxic effects of FG since it maintains cell viability and migration capacity. However, MMC remains the best agent to inhibit fibrosis on HTFs in this study.

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