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FIBRIN GLUE AS AN ANTIFIBROTIC AGENT ON HUMAN TENON FIBROBLAST (HTFS) THROUGH EXTRACELLULAR MATRIX EXPRESSION

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ABSTRACT : Human tenon fibroblast (HTFs) is the key componentof bleb fibrosis,followingthe process of trabeculectomy, andup to today, no ideal agent is capable of its inhibition. Furthermore, fibrin glue (FG) is a biomaterial that shows potentials in wound healing modulation, thus this study was aimed at investigatingitsantifibroticeffect on HTFs from glaucomatous eyes,expressed in term of Collagen 1, Matrix Metalloproteinase – 3 (MMP-3) and Tissue Inhibitor of Metalloproteinase – 2 (TIMP-2).HTF's from glaucomatous eyes were divided into 3 groups,which consists of FBS 2% as the control, MMC (Mitomycin C), and Fibrin Glue (FG) as treatment. Therefore, the experiment was conducted in 7 days, and the expressions of Col-1, MMP-3, and TIMP-2 were assessed with immunofluorescence staining. In addition, the intensity of fluorescence was measured using ImageJ software, and the difference among groups were analyzed using the Oneway ANOVA test, followed by post-hoc, with the consideration of $p<0.05$ as significant. It was established that FG decreased the expression of Col-1and TIMP-2, as well as a subsequent increase in MMP-3. However, MMC was observed to possess the best antifibrotic effect in this study.FG exerts antifibrotic effect through the modulation of extracellular matrix.

Key words : Fibrosis, fibrin glue, mmc, extracellular matrix.

INTRODUCTION

Glaucoma is an eye disorder characterized by optic neuropathy and a typical visual field defect, where an elevation in intraocular pressure is experienced as one of the main risk factors. This was reportedto affect over 60 million people worldwide in 2010 and the statistics is expected to increase to 80 million by 2020. In addition, the initiation of irreversible blindness has been known to cause a significant decrease in quality of life (Yamanaka *et al*, 2015; Stamper *et al*, 2009).

Trabeculectomy is a procedure often performed to reduce intraocular pressure, andthe main cause of this treatment failure with an incidence ranging from 24% - 74% in the 4 years post-surgery, ensues from the formation of excess fibrosis tissue in the bleb area. Furthermore, several modulation strategies for wound healing have been developed in an attempt to inhibit this occurrence, including the administration of antimetabolites, although MMC is the gold standard for the increase in success rate. In addition, there are several complications related to its administration, encompassing the manifestation of hypotonia, bleb leakage and infection, as well as endophthalmitis, with failure frequency ranging from 23% -51% within 5 years (Komaratih, 2018; Seet *et al*, 2011; Yamanaka *et al*, 2015; Masoumpour *et al*, 2016; Radcliffe, 2010).

Conversely, fibrin glue has been identified as a potential biomaterial for application as a growth factor source, as well as a regulator for proteolysis and antiproteolysis in wound healing. This is known to be biocompatible, biodegradable, non-toxic to the surface of the eyeball, easily and cheaply isolated from peripheral blood, and it is alsopossibly applied autologously (Garfias *et al*, 2012; Holan *et al*, 2015; Li *et al*, 2012; Tabele *et al*, 2012; Gasparroto *et al*, 2014).

The extracellular pathway responsible for the degradation of collagen fibrilers is mediated by the protease enzyme and followed by intracellular lysosomal degradation, thus, the process is initiated through the introduction of specific units by MMP enzymes, encompassing MMP-1, MMP-8, MMP-13 and MMP-14. Furthermore, metalloproteinase matrix inhibitors that consist of TIMP-1, -2, -3, and -4 are ascribed as glycoproteins with the capacity to maintain the balance

of extracellular matrix, which is subsequently remodelled and turned over by MMP (Seet *et al*, 2017; Wang *et al*, 2017; Toiviaien *et al*, 2015; McKleroy *et al*, 2013).

In vitro studies were conducted in cultures of HTFs, as wound healing model post-trabeculectomy. Therefore, the antifibrotic activity of FG was determined with the expression of Col-1, MMP-3 and TIMP-2, and the outcome was compared with MMC as "gold standard".

MATERIALS AND METHODS

The main reagents required include alpha modified eagle medium (α-MEM, Gibco-Life Technologies, USA), non-essential 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), anticoagulant citrate phosphate acid dextrose, CaCl2 (Sigma). In addition, primary FITC antibodies required for vimentin immunochemistry was purchased from BIOSS (USA).An *in vitro* studywas conducted at the Stem Cell research and Development Center, and theprocesswas aproved by the institutionalethical boardof Dr. Soetomo General Academic Hospital,both in Universitas Airlangga. In addition, the HTFs cultures were divided into three groups, consisting of the control(FBS 2%), and the treatmentof MMC and FG. Therefore, the expression of Col-1, MMP-3 and TIMP-2 were required in the determination of antifibrotic effects, which were analyzed after 7 days of culture.

HTFs isolation

Tenon biopsies isolated from 2 female patients (n=2) aged 25-40 years, diagnosed with advanced open-angle glaucoma,and based on the following inclusion criteria (1) therapy with antiglaucoma drugs, (2) has no previous surgical therapy, and (3) were undergoing trabeculectomy. In addition, the isolation of cells was conducted in accordance with themodified protocol established by Przekora *et al* (2017). This required washing the tissue using PBS, cutting into small pieces, and placing in a petri dish, followed by the addition of 3ml culture media (α-MEM, 1% penicillin streptomycin, 1% amphotericin B, 1% NEAA, 5% FBS, 5ug/mL ITS and 5ng/mL bFGF). Furthermore, the mixture was cultured for 7 days, harvested as well as passaged, andthe confirmation for the fibroblast phenotype present involved the characterization of cells at passage 3 for the expression of vimentin FITC antibody, followed by DAPI counterstaining. Subsequently, stained cells were visualized with the flourescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus) and positive expressions were identified by virtue of the glowing green coloration.

Fibrin glue production

Fibrin glue was isolated through a technique developed by Komaratih *et al* (2018). This element is known to originate from the centrifugation of peripheral blood with anticoagulants, where 10 ml of the sample was aseptically collected from the cubitti vein, using a sterile syringe containing CPDA at a 9: 1 ratio. Furthermore, the placement into a sterile centrifuge tube, followed by storage at 4^{o}C for 24 hours in an upright position facilitated the separation of plasma and erythriocytes, as the centrifugation ensued at a speed 40g for 10 minutes. This was conducted to promote the concentration of plasma, which was stored in a sterile centrifuge tube, reserved at -20^{o}C for 24 hours, and then centrifuged at 4^{o}C, with a speed 6500g for 4 minutes. Subsequently, 2/3 of the upper plasma was removed and 1/3 of the platelete rich plasma (PRP) was stored in a sterile micro tube, to be prepared as a thrombin making material, while the buffy coat pellet was stored in a similar containment,but at -30^{o}C, in order to serve as a component of fibrinogen. In addition, thrombin was created by mixing the PRP components with 10% $CaCl_2$, andfibrin glue involvedits combination with fibrinogen at a ratio of 1: 1.

Immunoflourescence staining of COL-1, MMP-3 and TIMP-2

After 7 days of treatment, each group was tested with immunofluorescence, in order to measure the expression level of Col-1, MMP-3, TIMP-2. Therefore, the cultured media was aspirated, and fixation performed with 3% formaldehyde for 15 minutes at room temperature, followed by a 4 times wash with PBS, drying, and blocking with PBS containing 1% serum for 15 minutes at room temperature, then the 4 times washingand subsequent drying commenced again. Furthermore, antibodies Col-1, MMP-3, TIMP-2 were added to every cell culture well, according to the group, which was then incubated at -4° C overnight, followed by PBS washing and DAPI counterstaining. Therefore, the results were observed with a fluorescent microscope at 200x magnification, andthe expression level was analyzed using ImageJ software. This was expressed in corrected total cell fluorescence (CTCF), which was determined using the formula: Integrated Density - (Area of selected cell X Mean fluorescence of background readings).

Statistical analysis

Statistical analysis was performed using SPSS version 19.0 software, and the differences influorescence

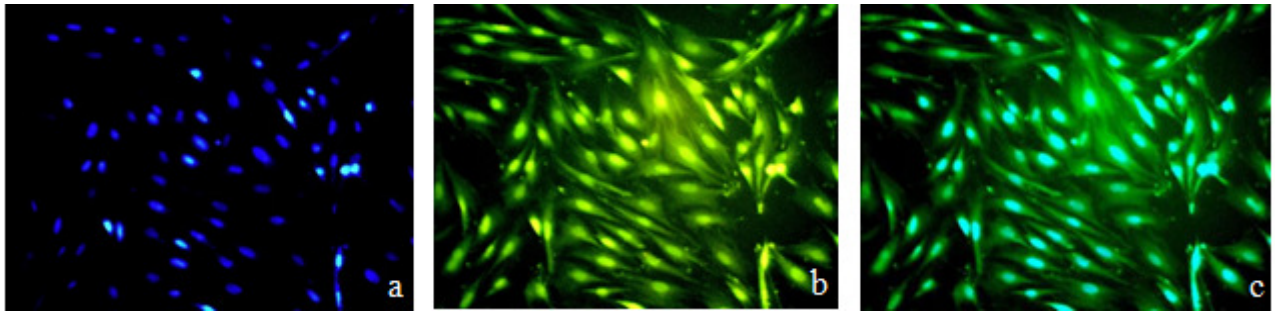

Fig. 1 : HTFs were stained with Vimentin FITC conjugated antibody. (a) cells were stained with DAPI, (b) FITC labelled Vimentin; (c) merge of section (a) and (b) (Inverted Fluorescence Microscope, 200x magnification).

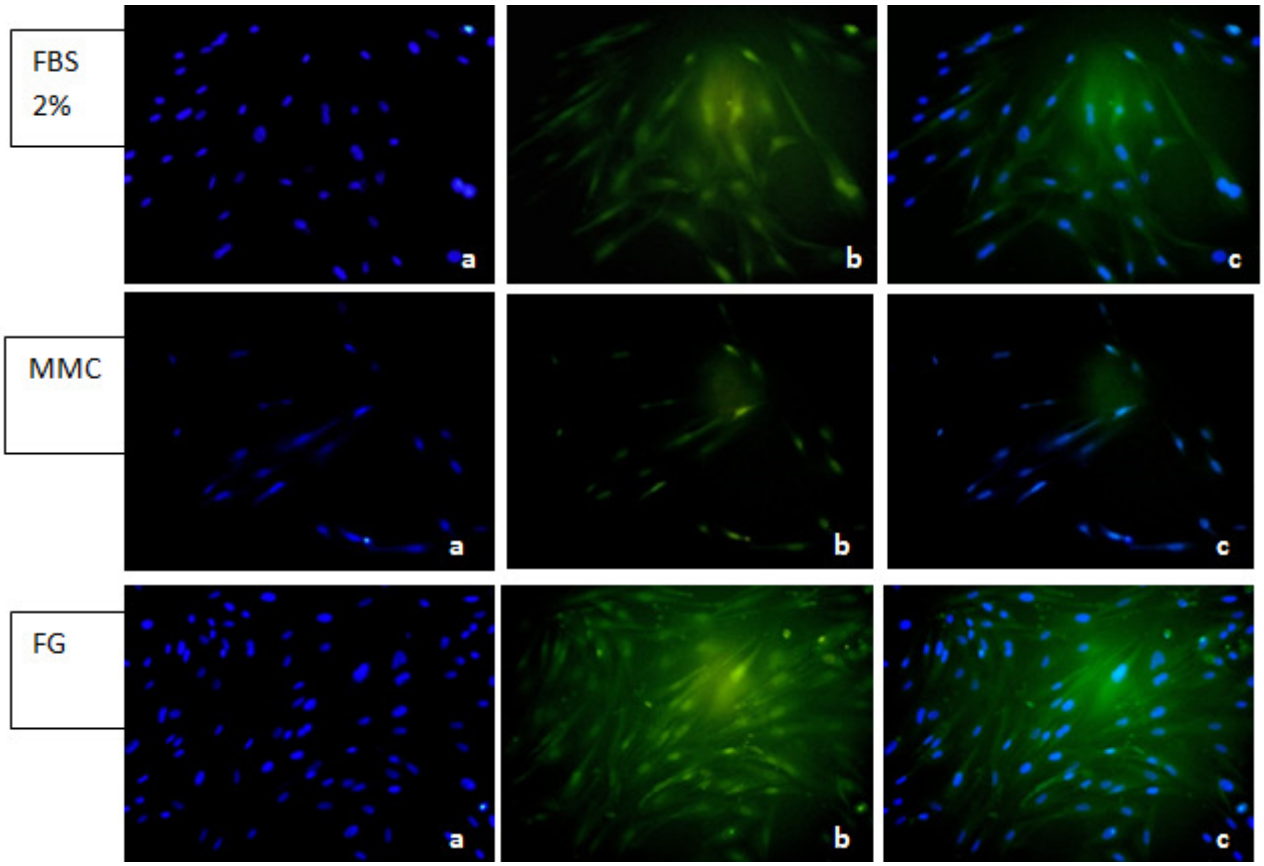

Fig. 2 : HTFs stained with FITC Antibody labelled Col-1 on group FBS2%, MMC and FG. (a) were stained with DAPI; (b) Flourescein; (c) a merge of (a) and (b) (Inverted Fluorescence Microscope, 200x magnification).

expression of Col-1, MMP-3 and TIMP-2 amongst groups were evaluatedusing the oneway ANOVA and post-hoc tests with $p<0.05$ used to determine the significance level.

RESULTS AND DISCUSSION

HTFs were isolated from the tenon capsule tissue collected from a 39 years old female patient, diagnosed with primary open-angle glaucoma, and evaluated to have met the inclusion criteria. Furthermore, the tenon fibroblast cells were isolated with the explant technique, which is capable of initiating sprouting on the 3rd day post-culture and 90-100% cell confluence on the 7th day. In addition,, the cultures were characterized using vimentin antibodies to confirm fibroblast phenotype at passage 3 (Fig. 1).

Expression of Col-1, MMP-3 and TIMP-2 with Immunocytochemistry stained

After 7 days of treatment, each group was stained with Col-1 FITC antibody, and then visualized under the flourescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus), in order to measure the level of Col-1 expression (Fig. 2). Furthermore, the valueobtainedconcurrently decreased in the MMC treatment group (0.41×10^6 pixel; p=0.000), compare to the FBS 2% control (1.52×10^6 pixel) and FG treatment (0.78×10^6 pixel; p=0.001) (Fig. 5).

The expression of MMP-3 was elevated at the MMC

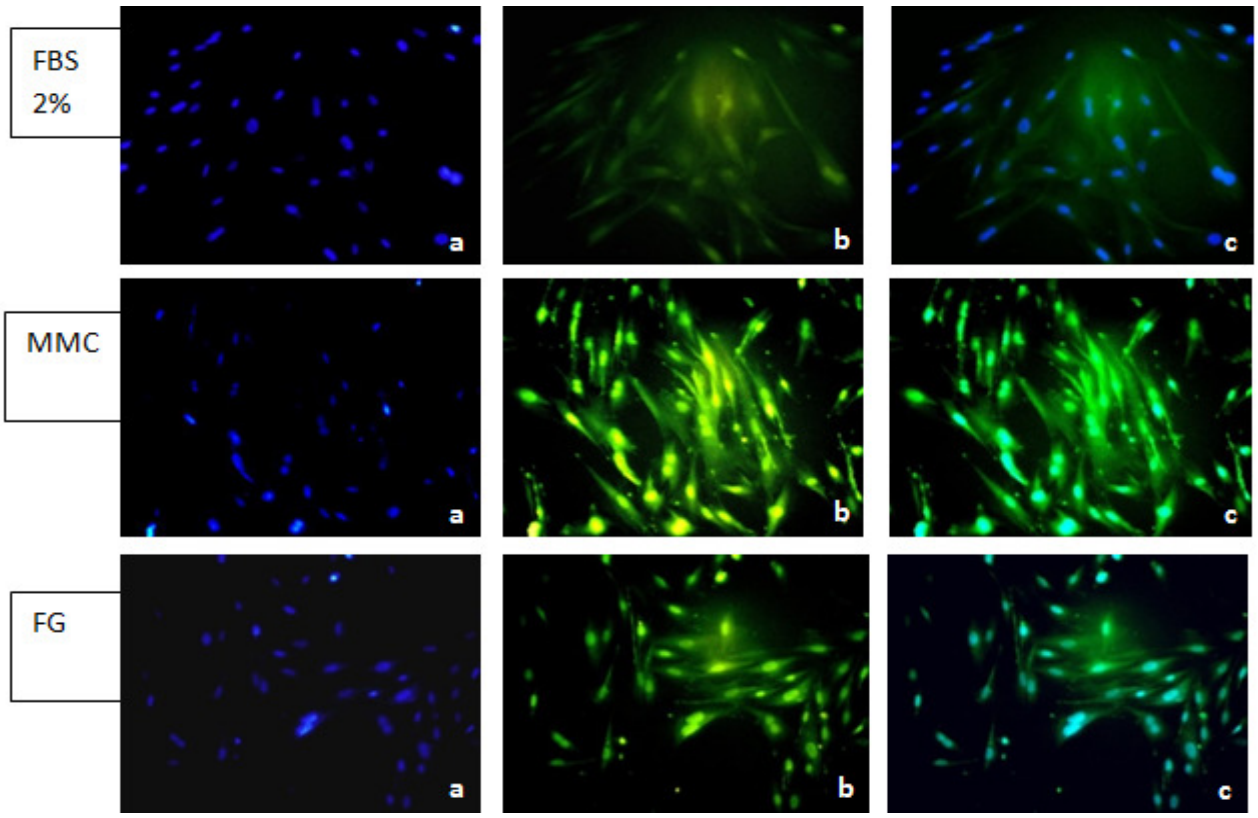

Fig. 3 : HTFs stained with FITC Antibody labelled MMP-3 on group FBS2%, MMC and FG. (a) stained with DAPI; (b) FITC labelled MMP-3; (c) a merge of (a) and (b) (Inverted Fluorescence Microscope, 200x magnification).

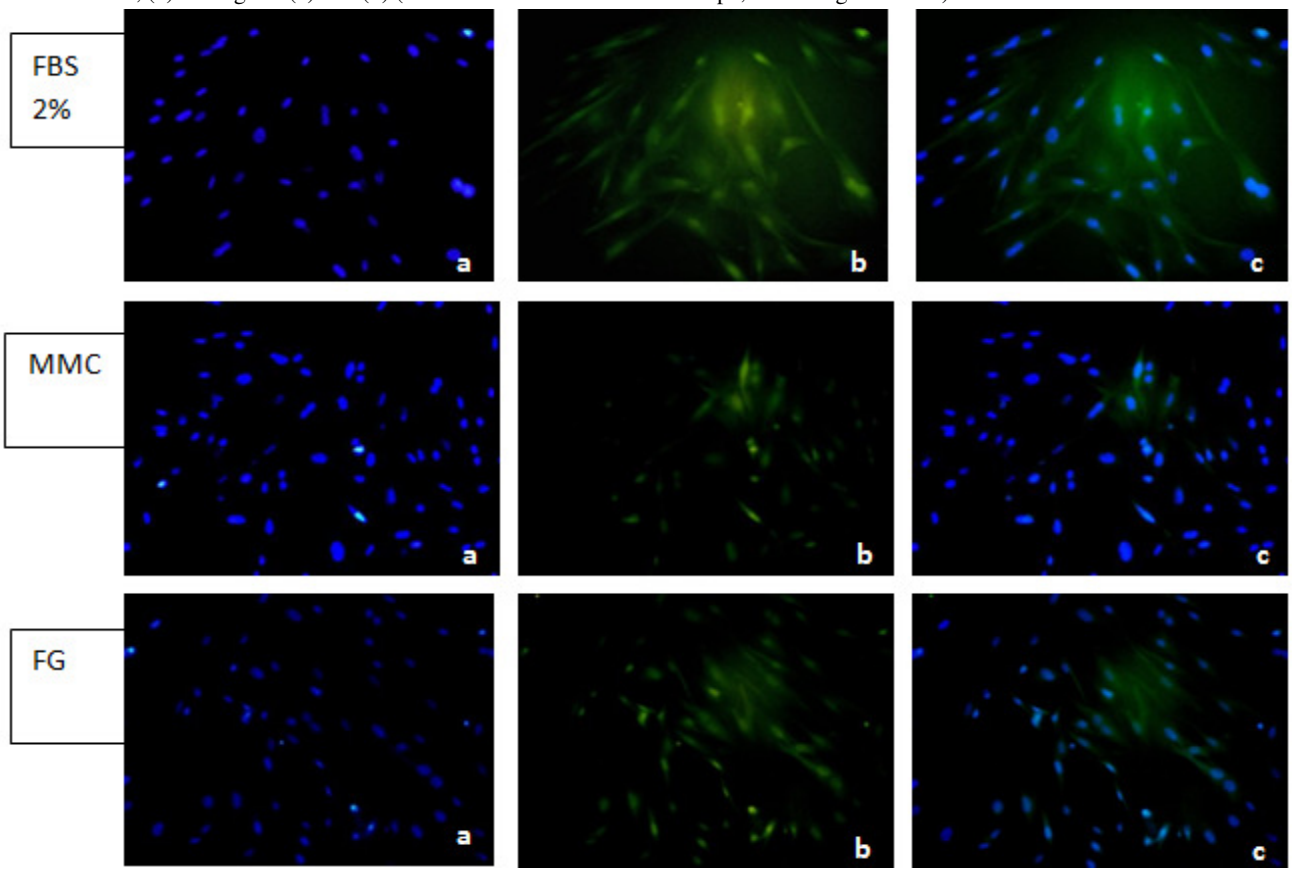

Fig. 4 : HTFs stained with FITC Antibody labelled TIMP-2 on group FBS2%, MMC and FG. (a) stained with DAPI; (b) FITC labelled MMP-3; (c) a merge of (a) and (b) (Inverted Fluorescence Microscope, 200x magnification). (Inverted Fluorescence Microscope, 200x magnification).

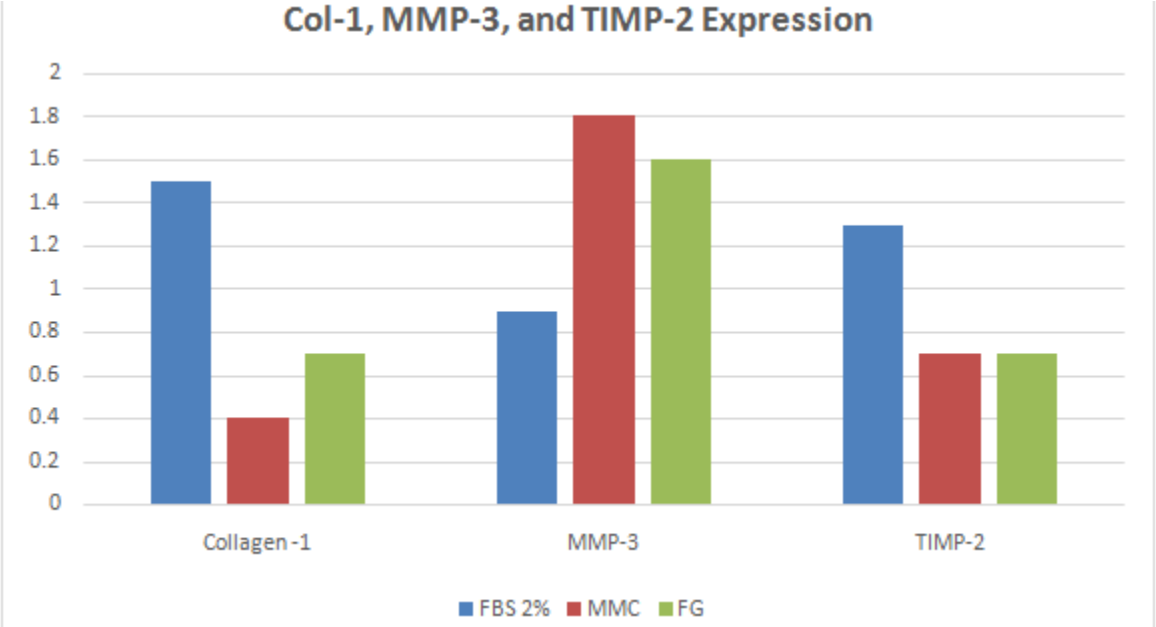

Fig. 5 : Collagen-1, MMP-3 and TIMP-2 Expression among groups.

treatment group (1.87 × 10^6 pixel) in contrastwith the control (0.91 × 10^6 pixel) and FG treatment (1.67 × 10^6 pixel) (Fig. 3). However, an increase in theexpression of MMP-3 were not statistically different between MMC (p=0.09) and FG treatment (p = 0.011) (Fig. 5).

The expression of TIMP-2 decreased in the MMC treatment group (0.72 × 10^6 pixel), comparedwith the FBS 2% (1.36 × 10^6 pixel) and FG (0.73 × 10^6 pixel) (Fig. 4). Statistically a significant decrease was observed on MMC (p=0.04) and FG (p=0.005),in contrast with to FBS 2% control group, although there was no statistically significant between both treatment groups (p=1.000) (Fig. 5).

Tenon's fibroblast cells are the major cells responsible for the initiation and mediation of wound healing and scar tissue formation after trabeculectomy. In addition, the physiological process is implicated in theactivation of tenon fibroblast cells, which initiate the proliferation and formation of excessively accumulated extracellular matrix (ECM) components, leading to the formation of fibrotic scars. Several studies have been identified to focus on the identification of anti-fibrotic agents that are capable of inhibitingthis process and the subsequent ECM production, responsible for the elevation in the success of glaucoma filtration surgery (Przekora *et al*, 2017). Moreover, mitomycin C as a gold standard of antibiotic agent, was used in glaucoma filtration surgery,due to its antiproliferative effects (Seibold *et al*, 2012) and an analysis on the influence of FG on the expression of Col-1, MMP-3 and TIMP-2 was compared with MMC (Przekora *et al*, 2017; Seibold *et al*, 2012).

In addition, the administeration of 0.4mg / mL MMC for 1 minute was identified to possess the greatest effect on the inhibition of fibrosis, with a decrease in the levels of Col-1 and TIMP-2 and elevated MMP-3 in contrast with the control and FG treatment. These results indicated the attainment of fibrosis inhibitory effect on MMC in tenon fibroblast cells by means of a decreasing levels of Col-1 and TIMP-2 expression, as well as an elevation in MMP-3 expression. This was in accordance with a research performed by Seet *et al* (2012), through the provision of 0.4mg/mL MMC for 1 minute, which consequently initiated a decline in the Col-1 expression levels, and an increase in MMP expression levels except MMP-14. The investigationdemonstrated MMC as more effective in the inhibition of fibrosis, in contrast with secreted protein rich acid in cysteine (SPARC) (Seet *et al*, 2012).

Fibrin glue is a potential biomaterial for application as a carrier of growth factors in the process of wound healing, with the ability to regulate the release growth factors. Furthermore, the physically related fibrin and matrix proteins, as well as cytokines, proteases or its inhibitors confer several functions, including angiogenesis, collagen synthesis, wound contractions, and re-epithelialization (Clark, 2003). In this study, autologous FG was isolated from cryoprecipitation technique with CPDA-1 anticoagulant, which is an easyand cost-effective approach. Also, autologous plasma and thrombin were reported to be sources of fibrinogen, in an attempt to reduce immunological reactions (Thorn *et al*, 2004; Mustapha, 2015; Clark, 2003).

Research by Komaratih *et al* (2018) demonstrated that the proteomic analysis of FG shows its content of several proteins that play a role in modulating post-trabeculectomy wound healing,encompassing HGF, HGF-like protein, IGF-1, platelet factor-4, coagulation factor V, and X, tetranectin, Apo-A and extracellular matrix-1 (ECM-1). Therefore, the administration of FG demonstrated a decline in the expression levels of Col-1 and TIMP-2, as well as an increase in MMP-3, in contrastwith the control. This was similar to the study conducted by Komaratih *et al* (2018), which stated that FG confers a beneficial effect on the ECM regulation, consecutively suppressing TGF-β and MMP-9 signaling as a negative regulation of fibrosis in the subconjunctival bleb region. In addition, paracrine signaling in modulating the polarity of TGF-β and activity of MMP-9 also contributes to the inhibition of fibrosis (Komaratih *et al*, 2018).

CONCLUSION

In summary, FG was observed to exert antifibrotic effects on the HTFs of glaucomatous eyes, through ECM modulation. However, MMC currentlydemonstrates better results in the aspect of providing effective result on HTFs, thus, batch to batch variation occur due to the improper optimization of FG isolation.

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Conflict of interest

None.

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