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ANTIFIBROSIS EFFECT OF FIBRIN GLUE ON TGF-â AND á-SMA EXPRESSION IN HUMAN TENON FIBROBLAST, AS WOUND HEALING MODEL AFTER TRABECULECTOMY : AN *IN VITRO* STUDY

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ABSTRACT : Fibrin glue (FG), which has already been known for its vital function as tissue adhesive, has lately been found with another important role in reducing inflammation and fibrosis especially in patients with glaucoma, who have gone through trabeculectomy. This study aimed at investigating the capability of fibrin glue in reducing profibrotic factors such as TGF-β and α-SMA at the early stage of fibrosis after trabeculectomy. This study used Human Tenon Fibroblasts (HTFs), as the key effector of bleb fibrosis after trabeculectomy. These were isolated from tenon tissues in eyes with glaucoma. These HTFs were cultured and divided into 3 groups made up of FBS 2% control group, MMCp and FG groups. Then, after 7 days of culture, TGF-β and α-SMA expression were assessed by immunofluorescence staining and its intensity was measured using ImageJ software. The results between groups were analyzed using Kruskal-Wallis or Brown forsythe test followed by post-hoc test with 95% confidence interval (p<0.05). FG successfully decreased the expressions of TGF-â and α-SMA, which has a key role in fibrosis with mean expression intensity of 93.68±3.96% for TGF-β and 142.97±11.03% for α-SMA. The results showed that FG has a role in fibrosis inhibition considering the fact that it decreases profibrotic factors. However, MMC has the best antifibrotic effect among the experimental groups. FG plays a role as an antifibrotic agent in HTFs by reducing the profibrotic factors.

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

INTRODUCTION

Glaucoma is known worldwide as the leading cause of irreversible blindness. It is reported that over 60.5 million people were with glaucoma worldwide in 2010, however, this figure is expected to increase to 79.6 million by 2020 (Quigley and Broman, 2006). Intraocular pressure reduction remains the main treatment method for this disease. In a situation whereby medical therapy is not effective, glaucoma is corrected through surgery, such as trabeculectomy. However, trabeculectomy comes with bleb fibrosis, which is a major disadvantage of this method. This bleb fibrosis usually begins with the expression of profibrotic factors. Also, TGF-β is one of fibrotic factor with an important role in the process. TGF-β induces fibroblast transdifferentiation into myofibroblast, a cell which produces extracellular matrix causing wound contraction and thereby resulting in fibrosis. According to Yamanaka *et al* (2015) the major cell type playing this role in glaucomatous eyes,is Human Tenon Fibroblasts (HTFs).

Fibrin glue (FG) is a biological tissue adhesive made up of fibrinogen, fibronectin, plasminogen, factor XIII, aprotinin and thrombin. Although, FG is already used as adjunctive therapy in trabeculectomy, glaucoma drainage device implantation, bleb repair and tube erosion, it has the capacity to be applied directly to the scleral flap or combined with suture. Sullivan *et al* (1996) reported the succesful use of FG in trabeculectomy involving a case series and found that blebs were good with no excessive inflamation sign. Also, FG encapsulated limbal mesemchymal stem cell has effect in reducing bleb fibrosis area in rabbits' eyes (Lu *et al*, 2018; Komaratih *et al*, 2018). Therefore, this study aims at analyzing FG antifibrotic effect on HFTs of glaucoma patient. Research was conducted on its role in early fibrosisprocess by assessing TGF-β and α-SMA expression, which was then compared with MMC, a well-known adjuvant therapy in trabeculectomy.

MATERIALS AND METHODS

Materials

The main reagents included alpha modified eagle medium (α-MEM) from Gibco-Life Technologies, USA; non-essential amino acid (NEAA) from Sigma, USA; Trypsin from Gibco-Life Technologies, USA); fetal bovine serum (FBS) from Biowest, USA; basic fibroblast growth factor (bFGF)from Gibco-Life Technologies, USA;and Insulin-transferin-selenium (ITS) from Gibco-Life Technologies, USA.Also, Primary FITC antibodies for immunochemistry to vimentin and TGF-β from BIOSS, USA and Primary FITC conjugated antibodies for immunochemistry to á-SMAfrom Santa Cruz, USA.

Methods

Study design

This is an *in vitro* study involving the HTFs of patients with glaucoma, conducted in Stem Cell Research and Development Center, Universitas Airlangga. All experimental procedures conformed to local ethics review board, dr. Soetomo Hospital. Also, the HTFs culture was divided into 3 groups consisting of control treated with 2% FBS in culture media, MMC treated, and FG treated groups. Then, the TGF-β and α-SMA expression levelswere measured on the 7th day after treatment.

Isolation of HTFs

The tenon tissue were collected from 2 female patients (n = 2) aged 25-40 years, who had been diagnosed with advanced open-angle glaucoma, but with no previous surgical therapy and undergoing trabeculectomy. The cells isolation process was conducted in line withthe protocol established by Przekora *et al* (2017) although with some modifications. The tissues were washed twice with PBS and cut into 2 pieces using a sterile scalpel. These were placed with light pressure in two separate 12-well plates and left to air dry for about 1 min to attach to the well bottom. Then, the tissues were cultured in media containing á-MEM, 1% penicillin-streptomycin, 1% amphotericin B, 1% NEAA, 5% FBS, 5ug/mL ITS and 5ng/mL bFGF. These cells were harvested and passaged after 5 to 7 days of culture. Also, in order to confirm the fibroblast phenotype, the cells were characterized at passage 3 for the expression of vimentin FITC antibody then counterstained with DAPI. The stained cells were visualized under flourescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus), with positive expressions characterized by glowing green cells.

Fibrin Glue preparation

The fibrin glue was generated in accordance to the protocol established by Komaratih *et al* (2018). This involved taking 9 ml blood from the vein of glaucoma patient using sterile syringe containing 1 ml CPDA. After gentle agitation, the blood was placed into sterile tube at -4^{0}C overnight. Then, the plasma were separated from erythrocyte through centrifugation for about 10 minutes to obtain certain amount of the plasma. These plasma were stored at -20^{o}C for 24 hours and then 6500 g were further centrifuged for 5 minutes at 4^{0}C. Through, these centrifugation processes, 2/3 parts of the plasma were removed, while 1/3 parts (PPP) were stored to prepare thrombin, and pellets (PRP) were collected and stored at -30^{0}C as fibrinogen component for fibrin glue. The thrombin were isolated by mixing 1/3 parts (PPP) of concentrated plasma with 10% $CaCl_2$. Then, the fibrin glue were generated by mixing fibrinogen and thrombin (Komaratih *et al*, 2018).

In vitro wound healing after trabeculectomy model

HTFs at 3 × 10^5 cells/well were seeded into eight-well culture dishes, allowed to attach and grow to confluence. Then, the cells were scratched wounded yellow with pipette tip and washed with medium to remove loose or dead cells. The control group was treated with 2% FBS, while the other treatment groups were given MMC and FG for 7 days.

Immunofluorescence staining of TGF-â and á-SMA

After 7 days of treatment, each group was tested for immunofluorescence to measure the level of expression of TGF-â and á-SMA. Each group was added aspirated culture media, then subjected to fixation with 3% formaldehyde for 15 minutes at room temperature. Then, it was washed with PBS 4 times, dried, blocked with PBS containing 1% serum for 15 minutes at room temperature. The cell cultures were added with antibodies TGF-β and α-SMA to each well according to the group, incubated at -4°C overnight followed by PBS washing and DAPI counterstaining. The results were viewed with a fluorescent microscope at 200x magnification. The expression level was analyzed using ImageJ software and expressed in corrected total cell fluorescence (CTCF) determined using this formula: Integrated Density - (Area of selected cell X Mean fluorescence of background readings)

Statistical analysis

The statistical analysis was performed using SPSSversion 19.0 software. Kruskal-Wallis or Brown forsythe test was performed followed with post-hoc test which was used to determine the statistical significance differences of fluorescence expression of TGF-β and α-SMAat $p<0.05$.

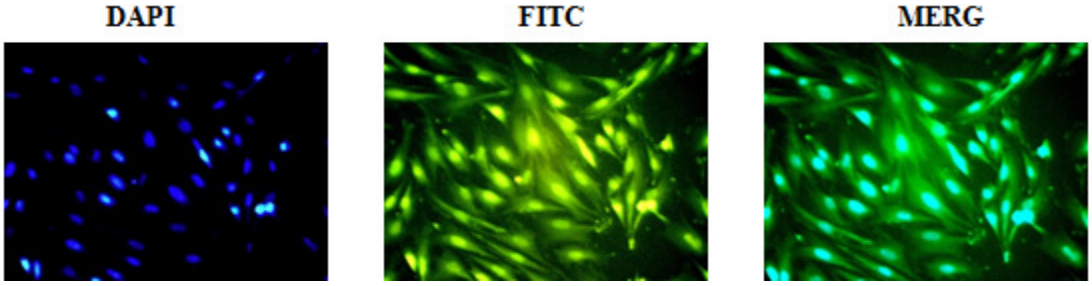

Fig. 1 : HTFs were stained with Vimentin FITC conjugated antibody. Left section, cells were stained with DAPI; middle section with FITC labelled Vimentin; right section merge of the previous two section (Inverted Fluorescence Microscope, 200x magnification).

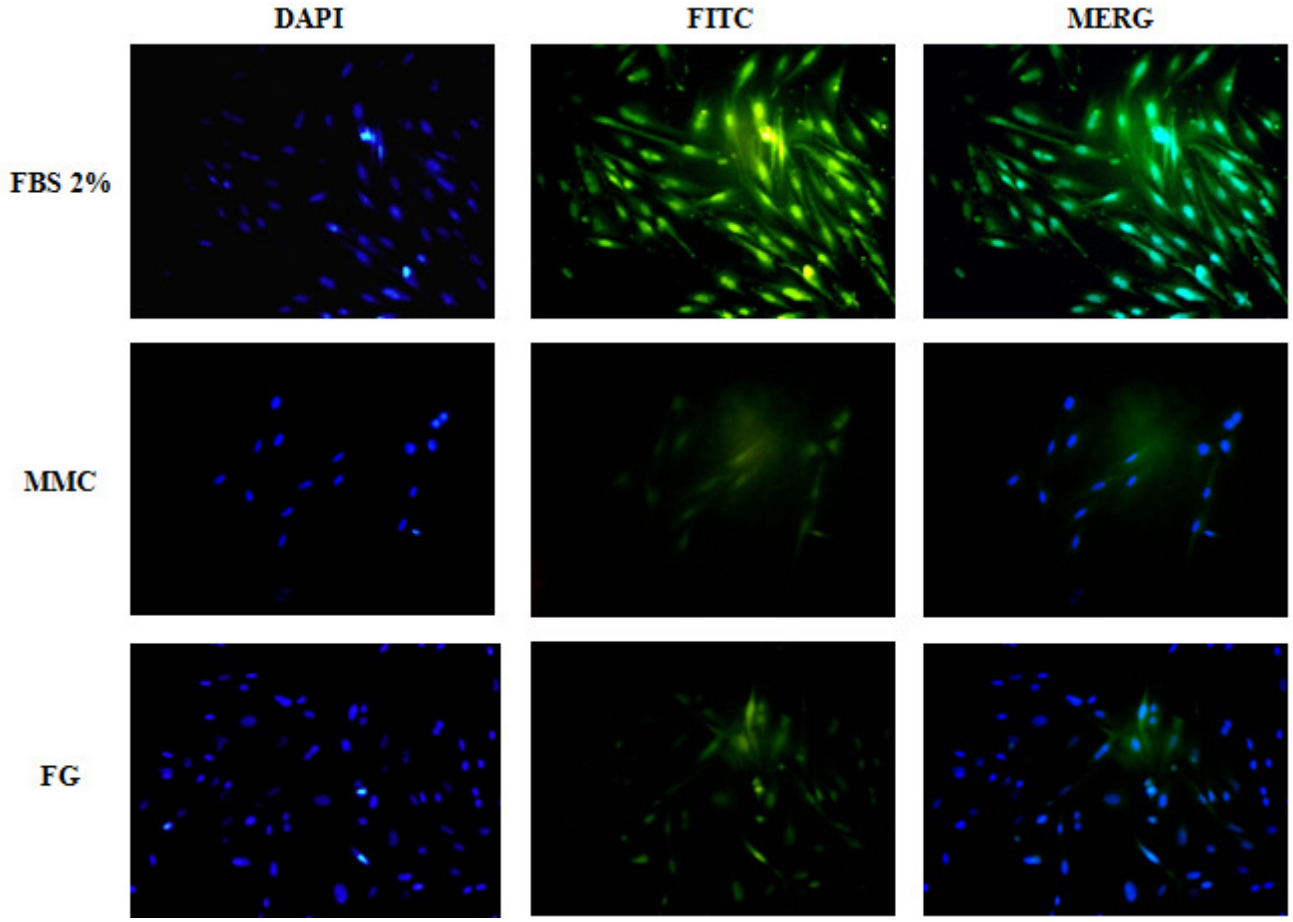

Fig. 2 : HTFs stained with FITC for TGF-â expression on group FBS2%, MMC and FG. Left section, cells stained with DAPI; middle section with FITC labelled TGF-â; right section merge of the previous two section (Inverted Fluorescence Microscope, 200x magnification).

RESULTS

Isolation and characterization HTFs

The tenon fibroblast cells were isolated through explant technique. The cell culture was supplemented with βFGF and NEAA. Consequently, the cell successfully reached almost 100% confluency in less than 10 days and showed positive expression of vimentin (Fig. 1).

TGF-â and á-SMA expression

Each group was analyzed after 7 days of treatment to measure the level of expression of TGF-β (Fig. 2) and á-SMA (Fig. 3). The level of expression of TGF-â in FBS 2%, MMC and FG groups are 159.80±56.18 pixels, 77.13±15.79 pixels, and 93.69 ± 11.21 pixels, respectively (Table 1). The expression of TGF-β were significantly decreased in MMC and FG groups compared to FBS 2% group (p= 0.002 and p=0.028). However, this decrease in expression of TGF-β was not significantly different between the MMC and FG groups (p=0.05).

Similarly,the expression ofá-SMA in FBS 2%, MMC and FG group are 349.53 ± 66.98 pixels, 102.63 ± 6.62 pixels and 142.97 ± 31.20 pixels respectively (Table 2). In addition, α-SMA expression were significantly

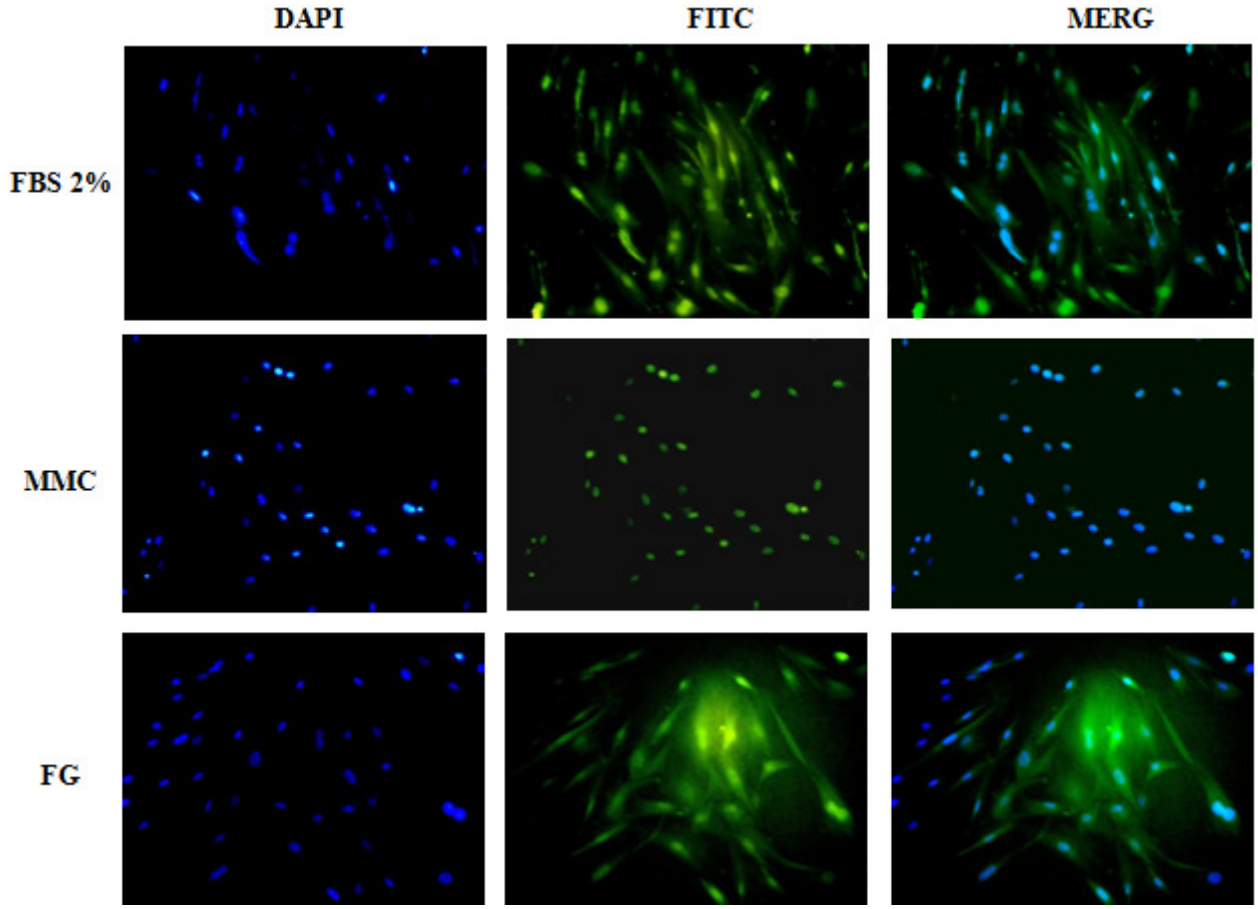

Fig. 3 : HTFs stained with FITC for á-SMA expression on group FBS2%, MMC and FG. Left section, cells were stained with DAPI; middle section with FITC labelled á-SMA; right section merge of the previous two section (Inverted Fluorescence Microscope, 200x magnification).

Table 1 : Mean expression of TGF-â.

Group	N	Mean (pixel)	SD	P (Kruskal-Wallis)
FBS 2%	8	159.80[a]	56.18	0.003
MMC	8	77.13[b]	15.79	
FG	8	93.69[b]	11.21	

Table 2 : Mean expression of á-SMA.

Group	N	Mean (pixel)	SD	P (Brown Forsythe)
FBS 2%	8	349.53[a]	66.98	0.000
MMC	8	102.63[b]	6.62	
FG	8	142.97[c]	31.21	

decreased in MMC and FG groups compared to FBS 2% group ($p<0.05$ for both comparison). However, this decreasing expression of á-SMA was significantly different between the MMC and FG groups ($p=0.032$).

DISCUSSSION

Tenon fibroblast is a cell which plays a vital role in fibrosis formation after trabeculectomy. The fibrosis process begins with excessive expression of profibrotic factors, in which one of the well-known is TGF-β. This induces fibroblast or epitel cells transdifferentiation into myofibroblast, which initiates extracellular matrix (ECM) production and if in excess amount causes fibrosis. The MMC is known as the "gold standard" of adjuvant therapy in trabeculectomy and this study revealed it has the greatest antifibrosis effect on HTFs since it successfully decreased the expressions of both TGF-β and α-SMA (Tables 1 and 2). A study conducted by Szabo *et al* (2019) also showed that MMC reduces á-SMA expression through TGF-β pathway.

This study also revealed that fibrin glue has potential effect as antifibrotic agent considering the fact that it significantly decreasedthe expressions of TGF-β and α-SMA (Tables 1 and 2). This fibrin glue is known to modulate acute or chronic inflammation since it contains sitokin as well as growth factors such as EGF, PDGF, IGF, tetranectin, Apo-A, HGF and FGF. The hepatocyte growth factor playsan important role in cell survival, regeneration, anti-inflammation and antifibrosis by tyrosine phosphorylation signal activation. Also, this HGF governs the extracellular matrix proteolysis, which is a vital biological process. According to Matsumoto *et al* (2014) Recombinant HGF proteins were able to promote

regeneration and inhibit fibrosis progression in amyotrophic lateral sclerosis. In addition, fibrin glue has the capacity to be use as an adjuvant therapy in trabeculectomy or as suture replacement considering the fact that it has tissue binding components. Furthermore, the use of fibrin glue in trabeculectomy has been established in several studies. Sakarya *et al* (2011) revealed that the mass of fibrin glue itself has the capacity of contributing to the formation of successful bleb since it was proven that subconjunctival placement of a biodegradable implant both helps the formation of successful bleb and decreases subconjunctival fibrosis. Another study also revealed that fibrin glue encapsulated limbal mesenchymal stem cells has the capacity to decrease bleb fibrosis area after trabeculectomy through TGF-β and MMP-9 modulation (Komaratih *et al*, 2018).

However, there are some limitations in this study.Proper analysis was not conducted to identify which isoform of TGF-β inhibited by FG and plays a role in fibrosis process. Similarly, the component of the FG was not analyzed in this study, hence, the exact sitokine or growth factor modulating the TGF-β and α-SMA expressions was not known.

CONCLUSION

FG plays the role of an antifibrotic agent in HTFs by reducing the profibrotic factorsin the cells and also has the capacity to be used as trabeculectomy adjuvant. However, MMC has greater effects as antifibrosis agent.

Conflict of interest

There was no recorded conflict of interest in this study.

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