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FIBRIN GLUE (FG) ENCAPSULATED LIMBAL MESENCHYMAL STEM CELLS (LMSCS) DECREASE BLEB FIBROSIS AREA AFTER TRABECULECTOMY THROUGH TGF-β AND MMP-9 MODULATION

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Abstract– Trabeculectomy is the most common surgical strategy in glaucoma. Postoperative scar remains the leading cause of failure. MSCs hold therapeutic promise for bleb fibrosis, yet the precise mechanisms underlying tissue level changes remain unclear. We hypothesize that fibrin glue encapsulated LMSCs favorably inhibit bleb fibrosis. LMSCs were isolated from rabbit corneoscleral rim by enzymatic digestion method. This was animal study including 4 groups with 10 rabbits in each group. The group including no trabeculectomy and trabeculectomy group, further divided in trabeculectomy only, with subconjunctival FG implantation, and FG encapsulated LMSCs transplantation group. Bleb fibrosis area, expression of TGF-β and MMP-9 immunohistochemistry were measured after 21 days. MANOVA and regression analysis were analyzed. Mean fibrosis area of FG (3.67 ± 0.9%) and FG encapsulated LMSCs group (3.69 ± 0.8%) were statistically different compared to trabeculectomy only group (28.6 ± 10.9%; p=0.00). TGF-β was significantly decrease in FG encapsulated LMSCs group (1.56 ± 0.5) compared to trabeculectomy only group (2.2 ± 0.5; p=0.019). There was strong correlation between TGF-β (β=2.03; p=0.002) and MMP-9 (â=-1.17 p=0.02) with decreasing fibrosis area in FG encapsulated LMSCs group. FG encapsulated LMSCs had a favorable effect on ECM regulation as well as suppression of TGF-â signaling and MMP-9 as negative regulatory of fibrosis in subconjunctival bleb area. Paracrine signaling on modulation of TGF-β polarity and MMP-9 activity may contribute to bleb fibrosis inhibition. These data provide insight into the regenerative effects of LMSCs and provide a foundation for clinical application. FG encapsulated LMSCs decrease bleb fibrosis area through TGF-β and MMP-9 signaling modulation

INTRODUCTION

Glaucoma is the second leading cause of blindness in the world. It was estimated that more than 60 million people worldwide would suffer from glaucoma in 2010and it will increase to approximately 80 million by 2020. As glaucoma is often associated with high intraocular pressure (IOP). The most widely performed procedure to reduce intraocular pressure is trabeculectomy, which is used tocreate a channel between the anterior chamber of the eye and the subconjunctival space.In trabeculectomy, the bleb is formed in the sub-Tenon's space. Formation of a functioning filtering bleb is the cardinal sign of a successful filtering glaucoma surgery. Anincomplete wound healing at the site of filtering surgery is necessary, which is against most other surgeries thatcomplete healing and restoration of normal architecture of the incised tissue would be a preferred outcome (Seet *et al.,* 2011; Yamanaka *et al.,* 2015; Masoumpour *et al.,* 2016; Radcliffe, 2010).

The failure of glaucoma filtration surgery is usually due to excessive wound healing under the conjunctiva. Thisexuberant scarring and resultant obstruction are generally resulted from the healing response to the surgical injury.Therefore, wound healing suppression is necessary to prevent obstruction of the created channel. So far, many effortshave been made to control the process of wound healing and improve the outcome of filtering surgeries. The use ofanti-fibrotic agents [5-

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fluorouracil (5-FU)] and Mitomycin C (MMC) have become a commonpractice to increase the success rates of filtering surgeries, and the advantages offered by these agents are accompaniedby unique complications such as blebitis, endophthalmitis, and late bleb leakage (Masoumpour *et al.,* 2016; Radcliffe, 2010).

Modulation wound healing after trabeculectomy is desirable. Mesenchymal stem cells (MSCs) showed antifibrosis effect on many tissues such as skin, liver, and lung through paracrine pathway. Adipose-derived stem cell (ADSC) therapy may be a hopeful method of preventing fibrosis through decreasing inflammation, inhibiting TGF-β1, and favoring tissue regeneration at the wound site (Zhang *et al.,* 2016; Usunier *et al.,* 2014). Limbal mesenchymal stem cells (LMSCs) govern limbal niche along with cytokines and growth factors to maintain limbal epithelial stem cells that responsible for corneal epithelial regeneration. It was reported that MSCs isolated from ocular tissue give better result in corneal regeneration compared to MScs from other tissues of origin. Scaffold has been proven as ideal tools as cells delivery system through maintaining cell engraftment and survival. Fibrin glue is highly biocompatible and biodegradable that has revealed good result for ocular surface application such as conjunctival closure in strabismus surgery, vitrectomy, and trabeculectomy. Thus, fibrin glue is one of the ideal tools for cell delivery (Garfias *et al.,* 2012; Holan *et al.,* 2015; Li *et al.,* 2012; Tabele *et al.,* 2012).

Our investigations through thepresent study show the antifibrosis effect of subconjunctival injection of fibrin glue encapsulated LMSCs after trabeculectomy in the rabbit's eye. Labelled LMSCs were successfully engraft within bleb area along with fibrin glue degradation. Fibrin glue encapsulated LMSCs decreased bleb fibrosis area via TGF-β and MMP-9 modulation.

MATERIALS AND METHODS

Materials

The main reagents included Type I collagenase (Roche USA), dispase (Roche, USA), alpha modified eagle medium (β-MEM, Gibco-Life Technologies, USA), non essential amino acid (NEAA, Sigma, USA), Trypsin (Gibco - Life Technologies, USA), fetal bovine serum (FBS, Biowest, USA), anticoagulant citrate phosphate acid dextrose, $CaCl_2$ (sigma), PKH26 for cell labelling (sigma). Primary FITC antibodies for immunochemistry to CD73, CD90, CD105, CD45 and secondary antibodies were purchased from BIOSS (USA).

METHODS

Study design

This is an animal study in New Zealand white rabbit, conducted in Stem Cell research and development center, Universitas Airlangga from April to September 2018. All experiments conformed to local animal ethics review board, Univeritas Airlangga. There were 4 groups consist of control group without trabeculectomy, trabeculectomy group, trabeculectomy with FG group, and trabeculectomy with FG encapsulated LMSCs group with 10 rabbit's eyes in each group. Cells engraftment was evaluated at 7^{th}, 14^{th}, and 21^{th} day after trabeculectomy in trabeculectomy with FG encapsulated LMSCs eyes group. Bleb fibrosis area, TGF-β, and MMP-9 immunohistochemistry from histological section of conjunctiva-scleralbleb areas were observed at 21^{th} days after operation.

Primary LMSC Culture

Rabbit corneoscleral tissues of healthy male rabbit was obtained from Stem cell research and development center, Universitas Airlangga. The experimental protocol was evaluated and exempted by the Local Review Board, UniversitasAirlangga. Stem cell culture was conducted in Stem cell research and development center, Universitas Airlangga. In brief, the tissues were incubated in 2.4 U/mL of Dispase II in alpha modified Eagle's medium (α-MEM) for 30 minutes. The tissues then incubated in 0.2 mg/mL of collagenase A type I in α-MEM for overnight at 37°C. Cells suspension were collected and cultured in complete media containing α-MEM, 1% penicillin-streptomycin, 1% amphotericin B, 1% NEAA, and 10% fetal bovine serum (FBS). After 5 to 7 days of culture, colonies of LMCs were harvested and passaged. To confirm the mesenchymal origin phenotype, LMCs were characterized at passage 3 for the expression of the mesenchymal markers to CD90, CD73, CD105, and CD45 FITC antibody (BIOSS, USA). $0.5x10^4$ cells/ul were grown in immunostaining chamber for overnight and fixed for 5 min in methanol at –10 °C. After fixation, the methanol was removed and desiccated. Cells were incubated for 20 min with

blocking serum and washed 3 times in PBS and then incubated for 1 h with primary antibody for CD73, CD90, CD105 and CD45. After washing for 5 min in PBS, the cells were incubated for 45 min with a secondary antibody and washed 3 times in PBS. After washing, the cells were mounted with mounting medium and visualized under the fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus) (Chen *et al*., 2011).

Fibrin Glue Preparation

Autologous fibrin glue contains the mixture of fibrinogen and thrombin. Rabbit ear blood were collected in sterile tube containing CPDA anticoagulant. The blood was centrifuged at 40g for 10 minutes to separate plasma and red blood cell components. Blood plasma was collected and stored at -80^{o}C refrigerator for overnight. In other day, thawed plasma was centrifuged at 6000g for 10 minutes in -4^{o}C. Fibrinogen was collected from buffy coat interface, whereas thrombin was obtained from the mixture of 1/3 parts of plasma above the buffy coat with calcium chloride ($CaCl_2$) (Tabele *et al*., 2012).

Encapsulation of LMSC in Fibrin Glue

Amount of 5x10^3 LMSCs were labelled using PKH26. Cells were suspended in 50 mL of fibrin glue by mixing thoroughly. The mixture of cells and fibrin glue were stored in sterile tube at 37^{o}C incubator for further application.

Glaucoma filtration surgery in rabbits

To anaesthetize the rabbits, Ketamine (50 mg/kg) and Xylazine (10 mg/kg) were given by intramuscular injections. A fornix based conjunctival flap was then raised and blunt dissection of subconjunctival area was performed. Following this, a partial thickness scleral tunnel was created with a crescent blade starting 2 mm behind the limbus and continuing until the blade was visible in the anterior chamber. The scleral tunnel was closed with releasable suture using 10-0 nylon suture. In trabeculectomy with FG encapsulated LMSCs group, five thousand LMSCs in 50 mL of fibrin glue were slowly and carefully injected into subconjunctival space with a 29-G needle. Similarly, 50 mL of fibrin glue was injected into each space in the same way in trabeculectomy with FG encapsulated LMSCs group. The conjunctival incision then was closed with two interrupted sutures as well as with a central mattress type 10-0 nylon.

Histological analysis

Conjunctiva-scleral Bleb areas were harvested for histologic detection 21 days after the operation. They were bisected and immediately fixed in 10 % formalin. For fibrosis area evaluation, histological sections were stained with hematoxylin and eosin, examined under microscope (Nikon Eclipse E400; Nikon, Tokyo, Japan), and qualified with Image J software. Tracing of LMSCs engraftment were determined by frozen sectioned tissue analysis in 7, 14, and 21 days after operation.

Immunohistochemistry

Bleb areas were examined for the expression of MMP-9 dan TGF-β. The sections were fixed in 4% paraformaldehyde for 30 minutes in room temperature. The sections were incubated in 20 min with blocking serum and washed 3 times in PBS and then incubated for 1 h with primary antibody for TGF-β and MMP-9 FITC antibody. After washing for 5 min in PBS, the sections were incubated for 45 min with a secondary antibody and washed 3 times in PBS. After washing, the cells were mounted with mounting medium and visualized under the fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus).

Statistical analysis

Percent of fibrosis area, cells positive for MMP-9 and TGF-â expression were presented in mean ± SD. The differences of percent of fibrosis area, cells positive for MMP-9 and TGF-β expression were analyzed by MANOVA test. Regression analysis were conducted to determine the contribution of for MMP-9 and TGF-β expression to bleb fibrosis area (SPSS 19 version).

RESULTS

Identification of LMSCs

LMSCs exhibited fibroblast morphology and expanded easily when cultured in regular medium in vitro. They were confirmed positive for CD73, CD90, and CD105 and negative for CD45 according to immunocytochemistry analysis of stem cell-related surface markers (Fig. 1).

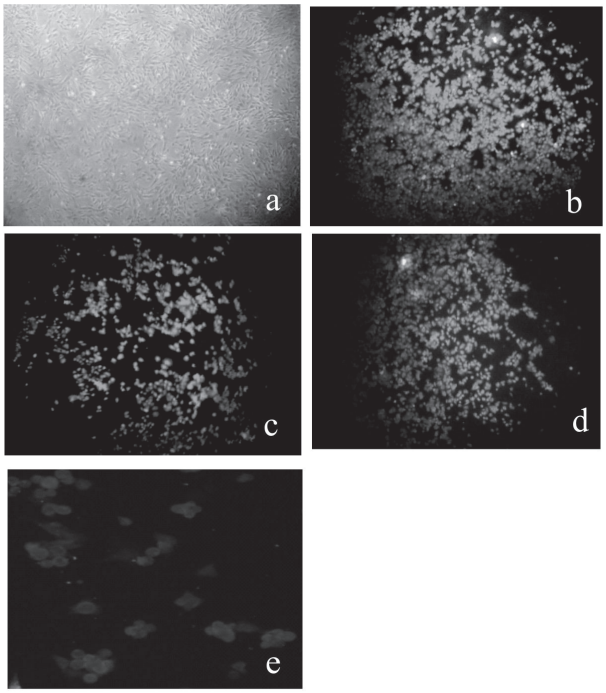

Fig. 1. Limbal mesenchymal stem cells, inverted microscope, objective 100x, (a) fibroblast like morphology, immunocytochemistry to (b) CD105 +, (c) CD73 +, (d) CD90 +, (e) CD45 - FITC antibody.

Fibrin glue encapsulated LMSCs reduce bleb fibrosis area

Both LMSCs encapsulation in fibrin glue (3.69 ± 0.8%) and fibrin glue itself (3.67 ± 0.9%) reduced bleb fibrosis area compared to trabeculectomy only group (28.6 ± 10.9%; p=0.00) (Fig. 2).

Gross examination on postoperative day 21 showed successfully blebs formation in all treatment groups. By contrast, less inflammatory signs were noticed in both LMSCs encapsulation in fibrin glue and fibrin glue group (Fig. 3).

Engraftment of LMSCs were confirmed in the cell injection group at 7, 14, and 21 days after operation. They were detected by frozen sections with a fluorescent microscope. We recorded a large number of green PKH26-labeled cells evenly distributed in the subconjunctival space start form 7th days and tend to migrate over fibrin glue degradation at 21th after cells injection (Fig. 4). Thus, a large number of engrafted cells were confirmed in the LMSCs treated subconjunctival spaces, which probably contributed to the inhibition of fibrosis formation.

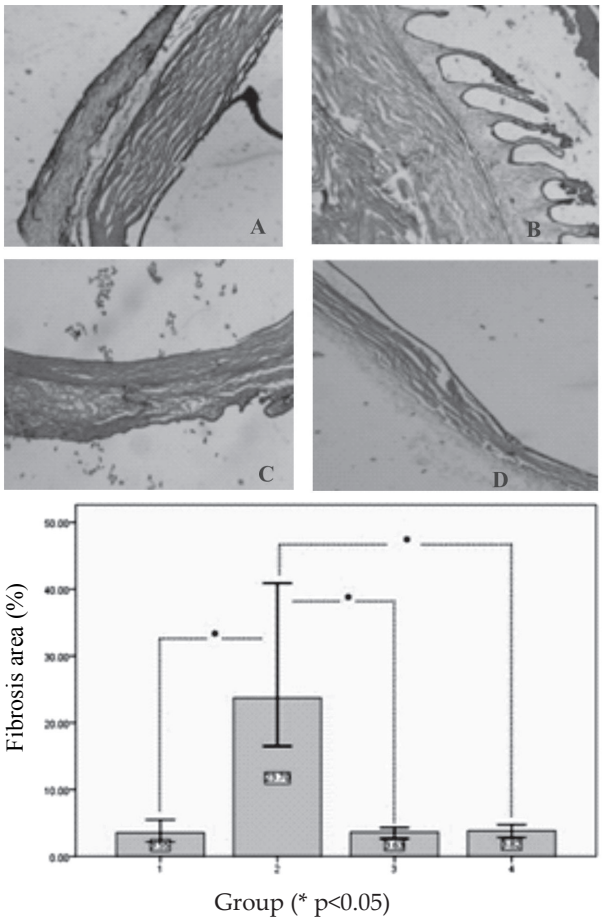

Fig. 2. Microscopic findings of HE stained tissues around bleb area (objective 400x), arrow: fibrosis area. (a) control group, without trabeculectomy, (b) trabeculectomy group, (c) trabeculectomy with FG group, (d) trabeculectomy with FG encapsulated LMSCs group, (e) graph showing mean fibrosis area,1: control group, without trabeculectomy, 2: trabeculectomy group, 3: trabeculectomy with FG group, 4: trabeculectomy with FG encapsulated LMSCs group.

Fibrin glue encapsulated LMSCs reduce TGF-β expression

Immunohistochemistry analysis revealed that TGF-β expression was significantly decrease in FG encapsulated LMSCs group (1.56 ± 0.5) compared to trabeculectomy only group (2.2 ± 0.5; p=0.019) (Fig. 5).

Regression analysis showed strong correlation between TGF-β (β=2.03; p=0.002) and reduced bleb fibrosis area in FG encapsulated LMSCs group. LMSCs might contribute to TGF-β inhibition in association with reducing bleb fibrosis formation.

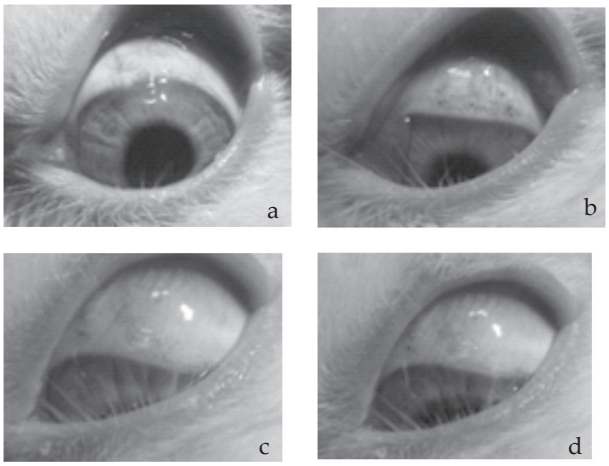

Fig. 3. Gross examination of bleb morphology, black arrow: bleb area, (a) ocular surface of control group, without trabeculectomy, (b) marked inflammation around bleb in trabeculectomy group, (c, d) minimal inflammation in FG group and FG encapsulated LMSCs group.

Fibrin glue encapsulated LMSCs modulate proteolysis activity of MMP-9

Expression of MMP-9 in FG encapsulated LMSCs group (3.60 ± 0.95) was not different statistically compared to control (3.33 ± 1.19) and trabeculectomy group (4.22 ± 1.40) (Fig 6). By contrast, regression analysis revealed significant contribution of FG encapsulated LMSCs to proteolysis activity of MMP-9 in fibrosis formation (β=-1.17 p=0.02).

DISCUSSION

Wound healing is a complex process that consists of three phases including inflammatory, cellular proliferation, and remodeling phases (Masompour*et al.,* 2016; Schultz *et al.,* 2011; Wang *et al.,* 2013; Kwak, 2013). Fibrosis formation can occur as a result of an abnormality in the process. Many studies demonstrate MSCs have an antifibrosis effect by means of favoring wound healing during the process. Various researches on the effect of MSCs in fibrosis was previously done in animal models or cells (Kotani *et al.,* 2017; Vasandan *et al.*,2016; Usunier *et al.,* 2014). A treatment of injecting adipose derived mesenchymal stem cells (ADSCs) on injured vocal folds has been performed by one group, which demonstrated the ability of ADSCs to prevent vocal fold atrophy and scarring related to their multipotential ability in the regeneration of injured vocal folds. Another group studied the effects of bone marrow mesenchymal stem cells (BMSCs) therapy on improvement in ventricular remodeling and cardiac function with smaller infarct size and less scar formation antifibrosis (Zhang *et al.,* 2016; Usunier *et al.,* 2014).

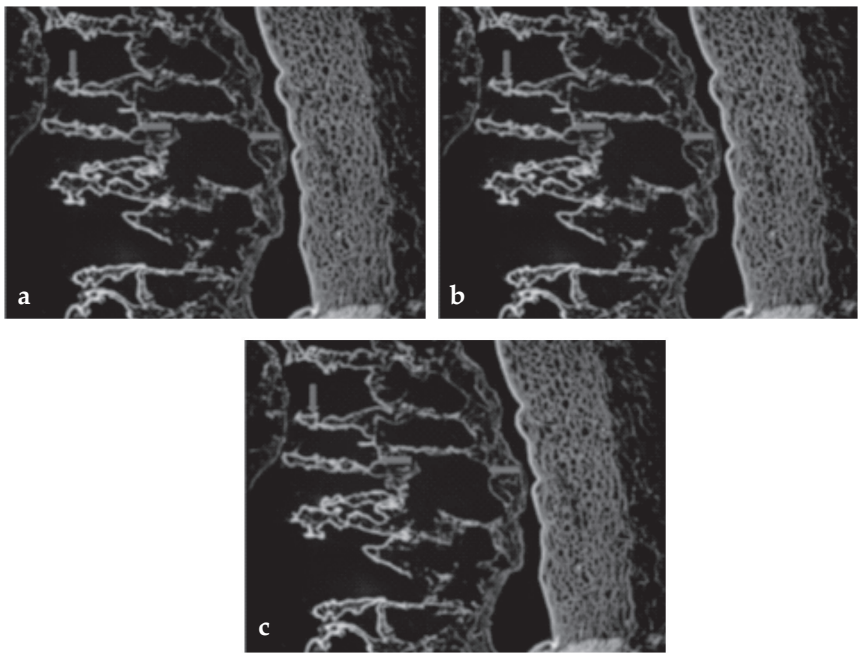

Fig. 4. PKH26 labelled LMSCs in fibrin glue within subconjunctival space (Fluorescent microscope, objective 400x). (a, b) LMSCs (blue arrow) trapped within fibrils structures of fibrin glue (red arrow) on 7th dan 14th days, (c) LMSCs start to migrate as fibrin glue degraded on 21th day.

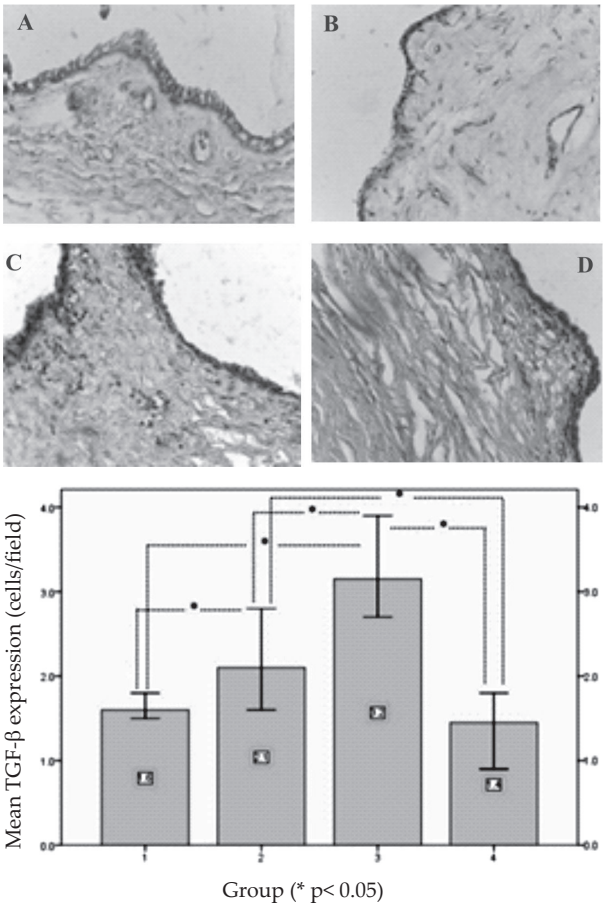

Fig. 5. Expression of TGF-β around bleb area (objective 400x), black arrow: TGF-β positive. (a) control group, without trabeculectomy, (b) trabeculectomy group, (c) trabeculectomy with FG group, (d) trabeculectomy with FG encapsulated LMSCs group, (e) graph showing mean TGF-β expression, 1: control group, without trabeculectomy, 2: trabeculectomy group, 3: trabeculectomy with FG group, 4: trabeculectomy with FG encapsulated LMSCs group.

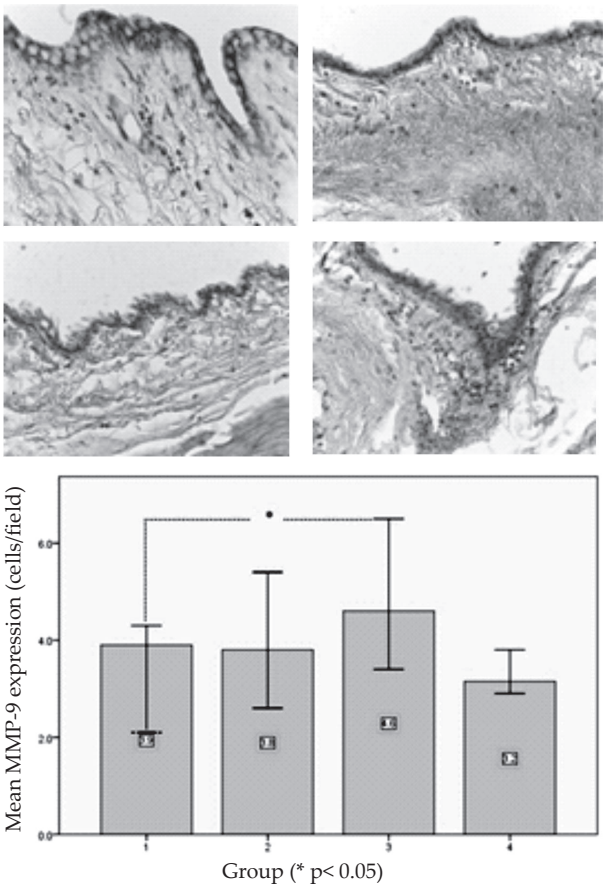

Fig. 6. Expression of MMP-9 around bleb area (objective 400x), black arrow: MMP-9 positive. (a) control group, without trabeculectomy, (b) trabeculectomy group, (c) trabeculectomy with FG group, (d) trabeculectomy with FG encapsulated LMSCs group, (e) graph showing mean TGF-â expression,1: control group, without trabeculectomy, 2: trabeculectomy group, 3: trabeculectomy with FG group, 4: trabeculectomy with FG encapsulated LMSCs group.

LMSCs and MSCs have different origins, they share comparable immunoregulatory properties in vitro. For the treatment of ocular surface injuries, both LMSCs and BMMSC or ADCS have been proposed and tested. MSCs proved to be a promising cell type to support the healing of the damaged ocular surface (Zhang *et al.,* 2016; Nakatsu *et al.*, 2014). However, there were limited studies about the antifibrosis effects of MSCs on ocular surface disorder (Holan *et al.,* 2015). The effects of LMSCs on the inhibition of bleb fibrosis formation after trabeculectomy has not been studied. Our study revealed that injection of LMSCs encapsulated in fibrin glue was able to reduce bleb fibrosis area. LMSCs were successfully engrafted by 7th day after injection. Other studies establishing the safety of FG and fibrin-encapsulation have been reported for a range of cell types. Fibrin glue is highly biocompatible, completely biodegradable, and has shown good results with little toxicity. There is no report of increased postoperative inflammation or reaction due to fibrin glue use as noticed in this study (Cavichiolo *et al.,* 2013; Zhao *et al.,* 2008). FG encapsulated LMSCs decreased TGF-β expression that might contribute to fibrosis formation inhibition. However, FG encapsulated LMSCs have no significant effect on MMP-9 expression, yet there was strong correlation between

negative regulation of MMP-9 to fibrosis formation in this group. It has been proven that LMSCs suppress fibrosis by various mechanisms, including reducing the expression of TGF-β1 and collagen and promoting the expression of MMPs, thus accelerating the turnover of the extracellular matrix. Secreted factor of LMSCs contains a lot of growth factors and cytokines, such as IL-10, adrenomedullin, and hepatocyte growth factor (HGF) that may act as antifibrosis factor through paracrine pathway. The cytokine HGF has also proven the ability of inhibiting myofibroblast differentiation which contributes to the limitation of pro-fibrotic functions of myofibroblasts ((Vizoso *et al.,* 2017; Pawitan, 2014; Holan *et al.,* 2015; Amirjamshidi *et al.,* 2011; Holan *et al.,* 2010). Also, MSCs can upregulate inducible nitric oxide when they interact with T cells in a proinflammatory environment which can stop fibrotic tissues from forming (Kotani *et al.,* 2017; Usunier *et al.,* 2014).

This study surprisingly noticed that FG itself was able to decrease fibrosis area formation similar to FG encapsulated LMSCS. In trabeculectomy surgery, after creation of both conjunctival and scleral flap, a thin layer of insoluble fibrin clot is formed by mixing fibrinogen and thrombin components of fibrin glue onto surfaces of the tenon side of conjunctiva. By this way, 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. We assume that first steps of wound healing can be halted for at least 7 days since there was an evidence that resorption of fibrin glue takes 7–14 days from anterior chamber (Jacob dan Nath, 2015; Sakarya *et al.,* 2011; Noori *et al.,* 2017). Fibrin glue may promote less postoperative inflammation since it stops hemorrhage associated with the release of various cytokines which are the main factors of inflammation. We may expect that aqueous humor flows freely for at least 7-14 days long while the resultant fibrin clot degrades physiologically. Fibrin glue itself may contribute formation of successful bleb formation since we found reduced bleb fibrosis area in this study (Nunes *et al.,* 2017; Cavichiolo *et al.,* 2013; Sakarya *et al.,* 2011).

We did not use animal model of glaucoma as the main limitation in this study. Others, this study was not compared to antifibrosis effect of MMC, bleb fibrosis area was not confirmed with certain types of pro-fibrosis extracellular matrix expression, we did not explore other pro and anti-fibrosis cytokines that might contribute to antifibrosis effect of LMSCs.

CONCLUSION

Fibrin glue promote cells engraftment and survival during experimental period. Fibrin glue may prolong therapeutic function of MSCs by sustaining microenvironment secretion of growth factors, cytokines, and immunomodulatory factors. FG encapsulated LMSCs decrease bleb fibrosis area through TGF-β and MMP-9 signaling modulation

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