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Original Research Article

Fibrin Glue Maintain Limbal Mesenchymal Stem Cells Survival: A Novel Cell Based Therapy Strategy for Modulating Wound Healing After Trabeculectomy

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Abstract

Aim: To investigate the potential capacity of limbal mesenchymal stem cells (MSCs) incorporated in fibrin glue as cell delivery system in modulating wound healing after trabeculectomy. ***Methods:*** Limbal MSCs were obtained from rabbit corneoscleral tissue. MSCs adhesion on fibrin glue derived from the mixture of fibrinogen and thrombin in concentration 1:1 and 1: 0.5 were observed 2 hours after cells seeding. Cell proliferation was assayed by modified tetrazolium method (MTT assay) on day 3. Cells adhesion and viability were analyzed using independent t test (SPSS 19 version). Preliminary study in animal model was conducted in 6 rabbit eyes to observe the role of fibrin glue as cell delivery system. MSCs were labelled using PKH26 prior to subconjunctival transplantation following common trabeculectomy procedure on rabbit eyes. Two eyes were enucleated on day 7, 14, and 21 to obtain conjunctival tissue of trabeculectomy site. Frozen sectioned specimen of conjunctival tissue was observed under fluorescence microscope to analyze cells engraftment and survival. ***Results:*** Isolated cells from corneoscleral tissue showed MSCs as they were positive for CD73,CD90,CD105, and negative for CD45. There were no significant differences of cells adhesion (p=0.3) and viability (p=0.2) between fibrin glue composed of fibrinogen:thrombin 1:1 and 1:0.5. Cells engraftment and survival were observed during experimental periods on day 7,14, and 21. Cells began to migrate on day 21 as the time of fibrin glue degradation. Combined MSCs and fibrin glue may facilitate wound healing modulation after trabeculectomy. MSCs may release antifibrosis factors slowly as gradual degradation of fibrin glue. Moreover, fibrin glue properties may promote cells engraftment and survival. ***Conclusion:*** Combination of fibrin glue and MSCs may be an alternative for modulating wound healing after trabeculectomy

Keywords: mesenchymal stem cells, limbal stromal cells, fibrin glue, trabeculectomy, wound healing, bleb failure.

INTRODUCTION

It is estimated that more than 60.5 million people worldwide are threatened by glaucoma and this number tend to reach 79.6 million by 2020. Glaucoma permanently damages optic neurons, leading to irreversible blindness. Reducing intraocular pressure is the most effective strategy to halt the progression of visual impairement. Medication lowering intraocular pressure is the first choice in glaucoma including agents that reduce aqueous humor production or promote outflow. While this agent fail to control IOP, surgical intervention is suggested including laser, filtration surgery, and tube shunt surgery. There is similar effect in lowering IOP between trabeculectomy and tube shunt surgery . Trabeculectomy is the most common surgical intervention in developing countries. Healing response is the critical determinant factor in final IOP after trabeculectomy. Tissue fibrosis resulting from impaired wound healing response is the main cause of bleb failure [1-3].

Modulation wound healing after trabeculectomy is desirable. Several strategies have been developed to modulate subconjunctival scarring after trabeculectomy. Topically or systemically steroid or nonsteroid antiinflammatory drugs has been applied to modulate inflammation and fibrosis. Whereas, topically antimetabolites such as mitomycin C or 5-fluorouracil inhibit fibroblast function and mitosis activity. However, its nonspecific antimetabolites properties can cause cells apoptosis and destruct cells microenvironment resulting in sight threatening complication such as hypotony, bleb leaks, and endophthalmitis [2, 3].

Biodegradable implants can be an alternative to prevent subconjunctival fibrosis after trabeculectomy. Amniotic membrane transplantation showed favourable effect in bleb survival. Mesenchymal portion of amniotic membrane exerts bioactive factors that supress transforming growth factor β (TGF- β) as its antifibrosis effect. Mesenchymal stem cells (MSCs) showed antifibrosis effect on many tissue such as skin, liver, and lung through paracrine pathway. Limbal mesenchymal stem cells govern limbal niche along with cytokines dan 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 toos for cell delivery, such as keratonocytes and retinal progenitor cells (RPCs) [4-6].

Aim of this study is investigate the potential capacity of MSCs incorporated in fibrin glue as cells delivery system in modulating wound healing after trabeculectomy. In this present study, we incorporated limbal MSCs in fibrin glue to observed cells adhesion and viability invitro. Preliminary study in rabbit eyes was conducted to observe the potential of fibrin glue as cells delivery system. Labelled MSCs were transplanted with fibrin glue into subconjunctival space of rabbit eyes to analyzed cells engraftment and survival invivo.

MATERIALS AND METHODS

Materials

The main reagents included Type I collagenase (Roche USA), dispase (Roche, USA), low glucose dulbelco's modified eagle medium (DMEM, Gibco-Life Technologies, USA), non essential amino acid (NEAA, Sigma, USA), Trypsin (Gibco - Life Technologies, USA), fetal bovine serum (FBS, Biowest, USA), anticoagulant citrate acid dextrose, CaCl2 (sigma), PKH26 for cell labelling (sigma). Primary antibodies for immunochemistry to CD73, CD90, CD105, CD45 and secondary antibodies were purchased from BD (Cambridge, UK), MTT tetrazoluim assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Roche, Indianapolis, IN).

Methods

Study design

This research consists of invitro study and preliminary study in animal model. Invitro study was conducted in MSCs population seeded in fibrin glue obtained from single rabbit donor. There were two groups consist of MSCs seeded in fibrin glue derived from the mixture of fibrinogen and thrombin in concentration 1:1 and 1:0.5. Preliminary study to observe the potential of fibrin glue as cells delivery system was conducted in 6 rabbit eyes. Labelled MSCs in fibrin glue were transplanted into subconjunctival space following trabeculectomy. Every 2 eyes were enucleated on day 7, 14, and 21 to observe cells engraftment.

Primary MSCs isolation and culture

Limbal MSCs were isolated according to protocol developed by Li et al (2012) with our modifications [7]. Corneoscleral rims were obtained from healthy male rabbit at the age of 3 months. Ethical clearance was obtained from local animal ethical review board before collecting the sample. Corneoscleral rim tissue was removed from a sterile phosphate buffer saline (PBS) solution with Penicillin (200 units/ml) & Streptomycin (200ug/ml) in a laminar flow clean bench and washed twice with PBS. This tissue was cut into small pieces of about 2mmx2mm, the pieces were washed to remove blood. Pieces of tissue were transferred into sterile tube containing 2mg/ml dispase in DMEM and digested at 37^{o}Cfor 30 minutes. This was followed by centrifugation at 250g for 5 min. After the supernatant was removed, the pieces of tissue was resuspended with 0.2mg/ml collagenase I in DMEM and further digested for 16-18 hours at 37^{o}C until completely digested. The digestion reaction was stopped by adding DMEM with 10 % FBS. The pellet was centrifuged at 250g for 10 min, washed with culture media, and centrifuged twice. Cells were then completely resuspended and transferred into a 60 mm culture dish to be incubated at 37^{o}C under 5% CO_2 and saturated humidity. When primary cells reached 80 % confluence, they was treated with 0.25 % trypsin (with 0.02 % EDTA) and subcultured into new culture dishes.

Cells characterization using immufluorescence

Mesenchymal stem cells were characterized using immunofluorescence staining of CD73, CD90, CD105 and CD45 molecules from the second passage. $0.5x10^4$ 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).

Fibrin glue preparation

Fibrin glue was generated according to Lee *et al*.(2008) with our modification [17]. Nine mililiters blood from single rabbit ear vein were withdrawn using

sterile syringe containing 1 ml citrate acid dextrose. Following gentle agitation, the blood were placed into sterile tube in -4^{0}C for overnight. As the plasma were separated from erytrocyte, the blood were further centrifuged at 40 g for 10 minutes to obtain amount of plasma. Plasma were stored in -20^{o}C for 24 hours and then centrifuged at 6500 g for 5 minutes, 4^{0}C. Following centrifugation, 2/3 parts of plasma were removed, 1/3 were stored to prepare thrombin, and pellets were collected and stored in in -30^{0}C as fibrinogen component for fibrin glue. Thrombin were isolated by mixing 1/3 parts of concentrated plasma with 10% $CaCl_2$. Fibrin glue were generated by mixing fibrinogen and thrombin.

MSCs adhesion and viability test in fibrin glue

To determine whether MSCs were capable of surviving in fibrin glue, MSCs incorporated into fibrin glue were prepared at a final concentration of 1x10^4 cells/200ul/well and incubated in a 37^{0}C, CO_2 incubator. We compared MSCs cultured in fibrin glue derived from the mixture of fibrinogen and thrombin in concentration 1:1 and 1:0.5. On days 0 (2 h after cells seeding), cell supernatans were removed from each well and cells were analyzed under inverted microscope to determine the number of adhered cells in percent confluency. Cells viability was assessed by using MTT tetrazoluim assay according to the manufacturer's instructions. Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm .

MSCs engraftment and survival invivo

We conducted invivo preliminary study in rabbit eyes to determine engraftment and survival of MSCs incorporated in fibrin glue. MSCs were labelled with PKH26 before mixed with fibrin glue derived from the mixture of fibrinogen and thrombin in concentration 1:1. Cells were suspended in diluent C solution and PKH26 and incubated for 10 minutes in room temperature. The labelling reaction was halted by addition of equal volume of complete culture medium. The labelled cells were suspended in 50 ul fibrin glue immediately before cells transplantation. A total 5x10^5 cells in fibrin glue were delivered into subconjunctival space of 6 rabbit eyes after conducted common trabeculectomy procedure. Cells engraftment and survival were observed in every two rabbit eyes on day 7, 14, and 21 after transplantation. In each observation day, two rabbits were terminated and enucleated eyes were sent to pathology anatomy department to perform frozen section. The frozen sectioned speciment of conjunctival tissues were observed under fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus).

Statistical analysis

Percent of cells adhesion and proliferation were presented in mean ± SD. The differences of cells adhesion and viability between MSCs incorporated in fibrin glue derived from fibrinogen and thrombin in concentration 1:1 and 1:0.5 were analyzed by independent t test (SPSS 19 version).

RESULTS

Cells Isolation and Culture

In this study, cells isolation was performed using enzyme digestion method. First, tissue fragments were treated with dispase to remove epithelial component, then collagenase was added to digest the tissue matrix completely. A total 8x10^5 cells/ml with 98% viability were obtained from this isolation method. The cells were attached well in its first 24 hours and colony unit forming fibroblast began to observe as seen in figure 1.

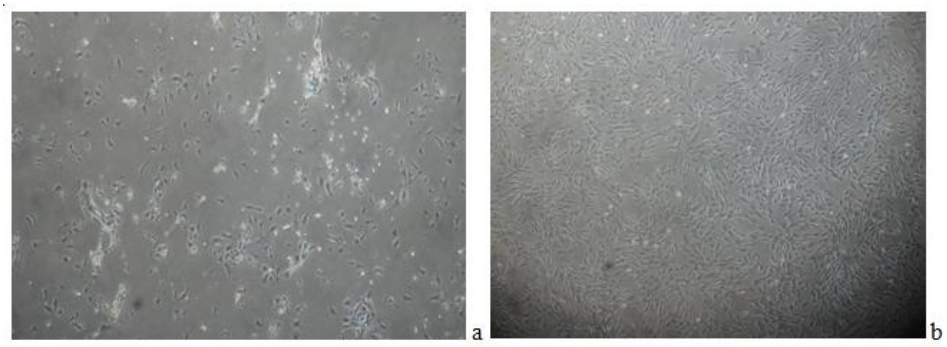

Fig-1: Primary cells isolation from corneoscleral rim tissue (x100). A: 24 hours after cells seeding showed round shape single cell. B: Colony forming unit fibloblast with 80% confluency on day 7

After 7 days, the number of cells with colony forming unit fibroblast morphology increase and 80% of confluency was observed and subcultured. The cell number was an average of 2x10^6 cells/ml at the begining of the first passage. After the first passage, the cell number increased rapidly compared to primary cells, reaching 3x10^6 cells/ml on day 6 of the first passage. The fibroblastic morphology was maintained through repeated subculture procedure until 5th passase without any specific stimulation.

Cells Characterization

Limbal MSCs have specific surface markers similar to MSCs from other sources such as bone

marrow MSCs. In this study, expression of CD73, CD90, CD105, and lack expression of CD45 were used to identify MSCs. The result of immunofluorescence staining revealed that this cultured cells were positive for CD73, CD90, CD105 but negative for CD45 as marker for hematopoietic stem cells (figure 2).

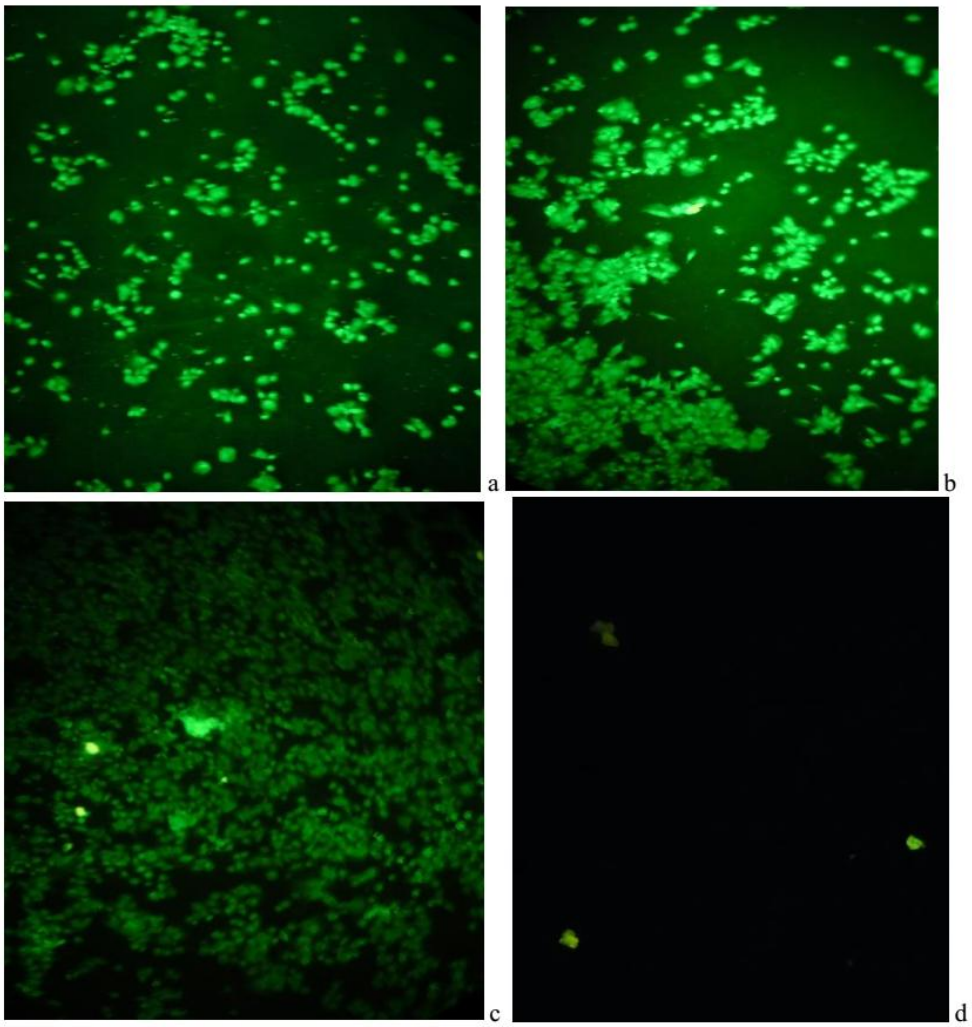

Fig-2: MSCs immunofluorescence A: CD73, B: CD90, C: CD105, D: CD45

MSCs adhesion, viability and Proliferation in fibrin glue

The adhesion capacity of MSCs grown in fibrin glue with composition of fibrinogen and thrombin with concentration of 1:1 and 1:0.5 were analyzed on day 0, 2 hours after cells seeding. There were no significant differences of attached cells between groups (p=0.3) (table 1). The cells from each group shared similar morphology as single cell formation on day 0 and fibroblastic formation from day 1. MSCs were evenly distributed in the fibrin glue and maintained fibroblastic formation throughout the experimental periods. The proliferation rate increased dramatically after day 1 and reached 90% confluency on day 3. Proliferation assay was performen on the 3rd day of experiment. Cells viability were calculated with the formula (OD of experimental group-OD of media)/(OD of control group-OD of media). There were no significant different of cells viability between groups from MTT assay (p=0.2) indicating capacity of fibrin glue in maintaing MSCs survival (table 1). However, MSCs grown in fibrin glue with composition of fibrinogen and thrombin at concentration of 1:0.5 showed the highest cell viability indicates higher proliferation capacity.

Table-1: Cell adhesion and viability

Fibrin glue composition	Cell adhesion (% attached cell)		Cell viability %)	
	Mean ± SD	P value	Mean ± SD	P value
Fibrinogen:thrombin (1:1)	74.28 ± 5.3	0.3	99.2 ± 4.56	0.2
Fibrinogen:thrombin (1:0.5)	77.14 ± 4.8		100.9 ± 2.20	

MSCs with fibrin glue engraftment and survival invivo

In this present study, MSCs engrafted and survive successfully as the labelled cells were observed on day 7, 14, and 21 after transplantation (figure 3). On day 7 and 14, cells were observed as a colony trapped within fibrin glue. Cells tend to migrate passed fibrin glue on day 21 as fibrin glue degradation. There may be a complete degradation of fibrin glue as it was no fibriles structure observed. The cells were successfully survive along this observation period, yet we did not determine the cells proliferation invivo.

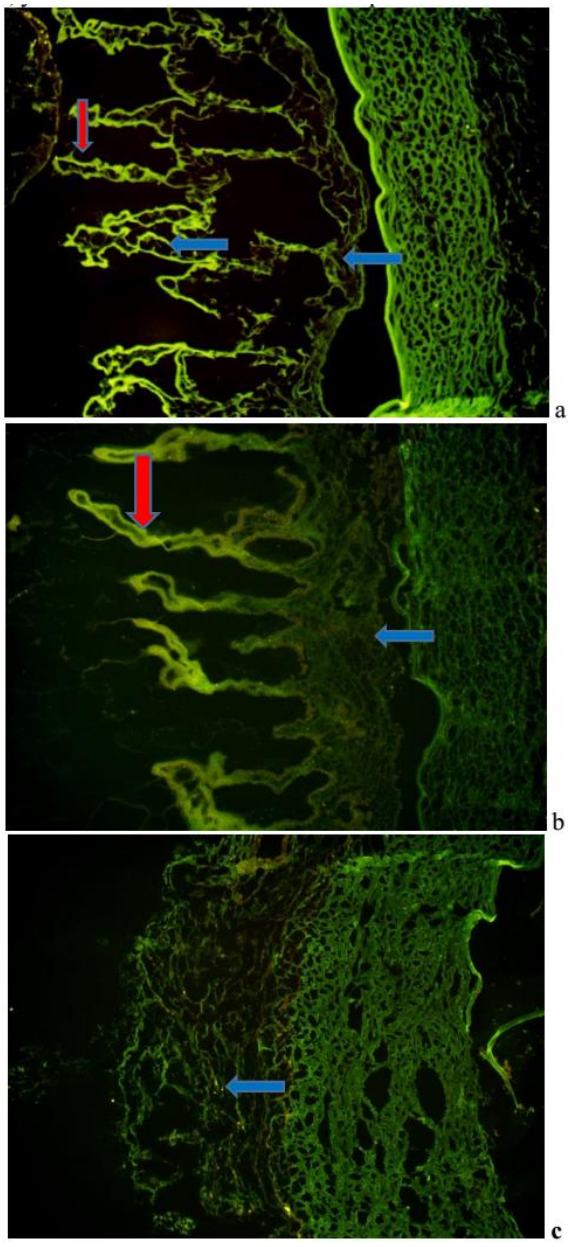

Fig-3: PKH26 labelled MSCs with fibrin glue in subconjunctival space (x400). A, B: MSCs (blue arrow) as cells colony were showed trapped in fibriles stucture of fibrin glue (red arrow) on day 7 and 14. C: MSCs (blue arrow) started to migrate as fibrin glue degraded on day 21

DISCUSSION

In the present study we isolated MSCs from limbal tissue as its unique capacity in preserving corneal regeneration. An isolation protocol was modified from Li et al (2012) for harvesting limbal MSCs from corneoscleral rim tissue. The invitro cultured cells fulfilled ISCT criteria for MSCs, as the cells attached to the culture dish, formed colony forming unit fibroblast, and specific surface marker for MSCs expression (positive for CD73, CD90, CD105, and negative CD45). Antifibrosis function of MSCs has been describes as their immunomodulatory properties to control inflammation response and fibrosis event. Generally, MSCs transplantation reduces the expression and concentration of TGF-β as one of the main targets for antifibrotic therapies. Interestingly, MSCs release exosomes that control extracellular matrix degradation

by modulating concentartion of tissue inhibitor matrix metalloproteinase (TIMP) and matrix metalloproteinase (MMP). It was proven MSCs provides antifibrotic effect through secretion of small molecule, cytokine, and growth factors that act in paracrine event. However, successfull strategy of growth factors delivery in modulating wound healing depends on the capability of local cells to respon the signals. While the local cells population are not sufficient to repair the wound, additional cells must be introduced [7-10].

Fibrin provides a natural environment for cells because of its chemotactic, hemostatic, and mitogenic properties. Fibrin glue has been proven as ideal cells delivery systems to improve cells incorporation and survival in injured area. The main risk of fibrin glue from blood pool is disease or prion transmission. Hence, autologous fibrin glue from a patient's serum can be an alternative. Yet the limitation considering autologus fibrin glue are time to prepare clotted fibrin glue and variation of fibrin glue composition. To ensure the quick and effective clotted formation of fibrin glue, we added thrombin into fibrinogen derived from the mixture of fibrinogen and thrombin in concentration 1:1 and 1:0.5. Our study incorporate MSCs with fibrin glue as an alternative for cells based therapy in modulating wound healing after trabeculectomy. Our results showed that fibrin glue derived from fibrinogen and thrombin in concentration 1:1 and 1:0.5 maintained MSCs survival invitro. Rapid and effective cells adhesion were observed in both of groups indicating fibrin glue as an ideal cells delivery system. In this study we found that fibrin glue derived from 1:1 concentration of fibrinogen and thrombin showed faster clotting. Our result is in conjunction with other study that quickly and effectively fibrin glue were obtained by mixing fibrinogen 60mg/ml with 300 IU/ml thrombin. Study indicated that higher thrombin concentration promoted fibrinogen formation as the source of growth factors and regulatory proteins of fibrosis [7, 10-13].

Yet not significant, fibrin glue promote cells proliferation compared to control group. A variety of growth factors are present in trace amount in the fibrin glue including epidermal groth factors (EGFs), platelet derived growth factor A C B (PDGF-AA,AB, BB), TGF-β1, TGF-β2, insulin-like growth factor 1 and 2 (IGF1 and 2), vascular endothelial growth factor (VEGF), and basic FGF-2 that are released from the platelets upon activation by thrombin. During its experimental periods, fibrin glue did not degrade invitro, as other study stated that fibrin glue degraded 1-2 weeks in vitro [13,14].

In our invivo study, fibrin glue promote cells engfratment and survival during experimental period. Fibrin glue may prolong therapeutic function of MSCs by sustaining microenvironment secretion of growth factors, cytokines, and immunomodulatory factors result in the increase of survival rate. In trabeculectomy surgery, delivering MSCs incorporated in fibrin glue may facilitate aqueous humor to escape freely from the anterior chamber into the fibrin glue coated scleral flap–scleral bed and tenon–scleral interface. In our study, fibrin glue derived from the mixture of fibrinogen and thrombin in concentration 1:1 may facilitated cells migration as fibrin glue started to degrade on day 21. Cytokines and growth factors from MSCs hope to release slowly as the time of fibrin glue degradation. Thus, released bioactive factors combined with fibrin glue properties may modulate wound healing after trabeculectomy and halt fibrosis through paracrine pathway. Other study assumes that first steps of wound healing can be halted for at least 7 days since we have evidence that resorption of fibrin glue takes 7–14 days from anterior chamber [4,5,15,16].

It was proven that fibrin based matrix can induce MSCs differentiation into osteoblast. In the other hand, culture of embryonic stem cells were successfully differentiatiated into RPCs when cultured in fibrin matrix wih RPCs differentiation media. In that study, there was no evidence of osteoblas differentiation along with fibrin matrix reconstitution. In our study, we did not observed effect of fibrin glue on MSCs differentiation. Besides, we did not determine fibrosis event and effect of fibrin glue on MSCs proliferation invivo. Further studies were needed to analyzed antifibrosis effect MSCs with fibrin glue transplantation after trabeculectomy in animal model. However, effect of fibrin glue on MSCs differentiation is still the important issue [5,17].

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