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Antifibrotic Effects of Limbal Mesenchymal Stem Cells-Conditioned Media (LMSCs-CM) on Human Tenon's Fibroblasts (HTFs) in Glaucomatous Eyes: Comparison with Mitomycin C

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Abstract | **Original Research Article**

Human tenon's fibroblasts (HTFs) is the key effector of bleb fibrosis after trabeculectomy. Excessive fibroblast proliferation and differentiation into myofibroblast contribute to disturbance of extracellular matrix remodeling, result in irreversible fibrosis. In this study we investigated the effect of LMSCs-CM on cell viability, cell migration, cell contractility, collagen synthesis and degradation in HTFs. HTFs from glaucomatous eye were divided into 3 groups consist of FBS 2% control group, MMC, and LMSCs-CM for 7 days. The cell viability was determined by MTT-assay while collagen synthesis and degradation were determined by Sirius red binding assay. Cell migration was determined by in vitro scratch assay and cell contractility was analyzed by fibroblast populated-collagen gel assay. The differences of cell viability, cell contractility, collagen synthesis and degradation among groups were analyzed using Oneway ANOVA or Kruskal Wallis test followed by post-hoc test with 95% confidence interval ($p<0.05$). The results showed that LMSCs-CM inhibited collagen synthesis and induced collagen degradation in HTFS. LMSCs-CM inhibit cell contraction, while maintain cell viability in HTFs. However, MMC provides better antifibrotic effect on HTFs. In conclusion, LMSCs-CM might have antifibrotic effect on HTFs.

Keywords: Tenon's fibroblast, limbal stem cell, fibrosis, collagen, MMC.

INTRODUCTION

It was estimated that more than 60 million people worldwide suffer from glaucoma in 2010, and this population will increase to approximately 80 million by 2020 [1]. Trabeculectomy is still regarded as the "gold standard" for glaucoma filtration surgery (GFS. Trabeculectomy differs from most surgical procedures in that inhibition of wound healing is desirable to achieve surgical success. Formation of scar tissue in the bleb is the main complication which obstructs the outflow of the aqueous humor and consequently leads to the failure of the surgery [1,2]. Therefore, wound healing modulation in the bleb is mandatory to improve the outcome of the surgery. Mitomycin C (MMC) improved success rate of trabeculectomy, yet it is still far from ideal. Nonspecific cytotoxic effects of MMC is associated with sight-threatening side effects and a pathology characterized by extensive cell death. Hence, the search for alternate, safer and more targeted anti-fibrotic agents and strategies is important to increase surgical success and the lifespan of GFS in patients [2-4].

The human Tenon's fibroblasts (HTFs) are considered the major cell type contributing to the fibrotic response after trabeculectomy. Upon TGF-β stimulation, fibroblasts are activated and undergo phenotypic transition into myofibroblasts, the key effector cells in fibrotic states. In addition to its role in myofibroblast differentiation, TGF-β promotes matrix preservation and deposition by enhancing matrix protein synthesis and by altering the balance between matrix-preserving and matrix-degrading signals [5,6]. Excessive expression of collagen type I is the major hallmark of bleb fibrosis, therefore collagen deposition is commonly used to evaluate surgical failure in trabeculectomy [5].

Mesenchymal stem cells (MSCs) from different origins and limbal MSCs (LMSCs) share

comparable immunoregulatory properties in vitro. However, LMSCs showed higher anti-inflammatory properties for ocular surface wound healing compared to adipose derived-mesenchymal stem cells (ADSC) [7]. Given the immunomodulatory and trophic properties of MSCs, they have become attractive candidates for the treatment of fibrosis in a variety of models. Various studies on stem cellconditioned media (CM) showed that the secreted factor within its conditioned media alone without the stem cell itself may cause tissue repair in various conditions that involved tissue/organ damage [8,9]. Secretome containing CM are easily manufactured and no need to match the donor and the recipient to avoid rejection problems as it is devoid of cells. Therefore, stem cell-derived conditioned media have a promising prospect to be produced as pharmaceuticals for regenerative medicine [10,11].

3D culture was reported to enhance the anti-fibrotic potentials of MSCs. Hence, we conducted in vitro study to investigate anti-fibrotic effect of 3D LMSCs-CM in HTFS of glaucomatous eye and examined parameters important for wound repair and fibrosis. In this study, we describe the properties of these LMSCs-CM treated HTFs in assays for proliferation, cell migration, collagen deposition and degradation, and collagen gel contraction. We compared these effects against MMC-treated counterparts as the 'gold standard'.

MATERIALS AND METHODS

Materials

The main reagents included Type I collagenase (Roche USA), 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), rat tail collagen type I (Gibco- Life Technologies, USA), anticoagulant citrate phosphate acid dextrose, $CaCl_2$ (sigma). Primary FITC antibodies for immunochemistry to CD73, CD90, CD105, CD45, vimentin, and secondary antibodies were purchased from BIOSS (USA).

Methods

Study Design

This is an in vitro study in HTFs of glaucomatous eye, conducted in Stem Cell research and development center, Universitas Airlangga. All experiments conformed to local ethics review board, dr. Soetomo Hospital. HTFs culture were divided into 3 groups consist of control group treated with 2% FBS in culture media, MMC treated group, and LMSCs-CM treated group. Cells viability was evaluated at 3rd day after treatment. Wound scratch assay was performed for each group, cell migration rate, collagen deposition and degradation assays, and cell contractility were measured in 7th day after treatment.

LMSCs Isolation and Characterization

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, Universitas Airlangga. Cells isolation was conducted according to protocol established by Chen *et al.* with our modification. In brief, the tissues were incubated in 0.2mg/mL of collagenase a type I in α-MEM for 30 minutes at 37^{o}C with gentle agitation. Cells suspension were collected and cultured in complete media containing α-MEM, 1% penicillin-streptomycin, 1% amphotericin B, 1% NEAA, 5ng/mL bFGF, and 10% FBS). After 5 to 7 days of culture, colonies of LMCs were harvested and passaged. To confirm the mesenchymal origin phenotype, LMSCs were characterized at passage 3 for the expression of the mesenchymal markers to CD90, CD73, CD105, and CD45 FITC antibody (BIOSS, USA). Stained cells were visualized under the fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus) [12].

Generation of LMSCs-CM

LMSCs of P4 with good growth status were collected by trypsin digestion, and 3D spheroids were generated according to the previous report protocol established by Rangarunlert *et al*. Briefly, MSCs were prepared as 7.5×10^5/mL cell suspensions. 30 μL of cell solution per drop (containing about 25,000 cells) was prepared onto the culture plate. Cell drops were cultured inversely for 24 hours in an incubator at 37∘C with 5% CO2. To obtain LMSCs-CM the plate were re-inversed and floating colonies of 3D spheroid were cultured in complete culture media with 2% FBS for 24 hours. Conditioned media were collected and filtered using Millipore filter 0.45um [13].

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, had no previous surgical therapy, and were undergoing trabeculectomy. Cells isolation was conducted according to protocol established by Przekora *et al*. 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 left to air dry for up to 1 min to attach to the well bottom. Then the tissues were 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, 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 the fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus) [14].

Cell viability, collagen degradation, and collagen deposition

HTFs of P4 were seeded in 96 multiwell plates with $2.5x10^4$ cells/well with complete culture media for 24 hours. Treatment with 2% FBS as a control group, MMC, LMSCs-CM were given in each group for 7 days. Cell viability was determined by 3,-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay according to method used by Freshney. Collagen degradation and deposition were determined using Sirius red binding assay according to method used by Heng *et al.* and Taskiran *et al.* respectively. Concentration of type I collagen degradation and deposition were determined according to standard curve established from rat tail collagen type I [15-17].

In vitro scratch assay

HTFs were seeded into wells of six-well culture dishes and allowed to attach and grow to confluence. The cells were then scratched wounded by a pipette tip and washed with medium to remove loose or dead cells. Treatment with 2% FBS as a control group, MMC, and LMSCs-CM were given in each group for 7 days. The wound at the same spot, marked by a reference line drawn on the outside of the dish, was photographed at different time points for a period of 3^{rd} and 7^{th} day after treatment with a phase-contrast microscope equipped with a camera. Two different fields of each scratch wound were photographed. Each condition was performed in triplicate. The wound widths were analyzed as cell migration rate with Image J software. The data were expressed as averages of the percentage width of the wounds at the measured time point over the original width at time 0 hr

Cell contraction assay

To assess the influence of LMSCs-CM on HTFs contraction, we measured the contraction of HTFs-seeded collagen gels. First, HTFs were resuspended at a density of $3x10^5$ cells/mL with complete culture media. Each gel was made from dialyzed collagen 0.1% acetic acid and mixed gently. Sodium hydroxide (0.1 M) was then added to the gel to return the solution to physiological pH and to precipitate the collagen. Thirty microliters of HTFs (at $3x10^5$ cells/mL) were then seeded into the neutralized gel and resuspended briefly. This was added to a well of 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 collagen coated dish were taken with a digital camera. The images were then measured and assessed using ImageJ software by comparing cell-gel contractile area with total area [17].

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

Identification of LMSCs and Generation of LMSCs-CM

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 and P63 according to immunocytochemistry analysis of stem cell-related surface markers. Hanging drop technique successfully generated 3D spheroid colonies for producing conditioned media (Figure 1).

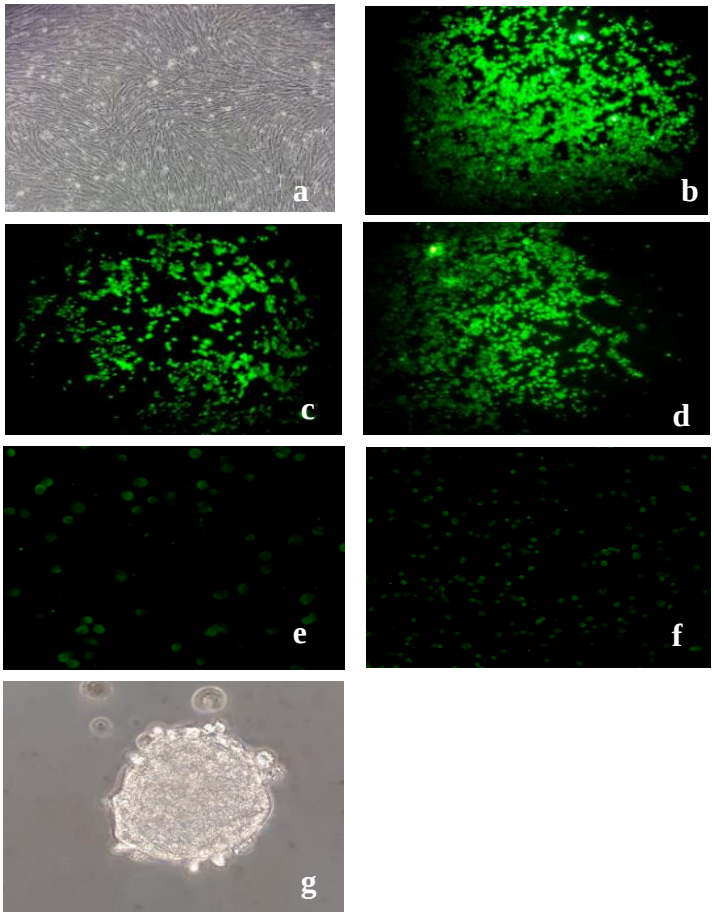

Fig-1: Limbal mesenchymal stem cells, inverted microscope, objective 100x, (a) fibroblast like morphology (phase-contrast), immunocytochemistry positive to (b) CD105 +, (c) CD73 +, (d) CD90 +, negative to (e) CD45, (f) P63 FITC antibody, (g) 3D spheroid formation.

HTFs, Cell viability and migration

This study successfully established rapid cultivation of HTFs in 14 days with good cell performance as seen in figure 2.

The effect of LMSCs-CM on cell viability of HTFs culture is presented in figure 3. Cell viability of HTFs decreased after MMC treatment (64.38%±3.12%) compared to those with FBS 2% control group (89.14%±8.69%, p=0.000) and LMSCs-CM (77.62%±6.52%, p=0.002). However, there was significant decreased of cell viability in MMC (p=0.001) and LMSCs-CM (p=0.021) compared to control group.

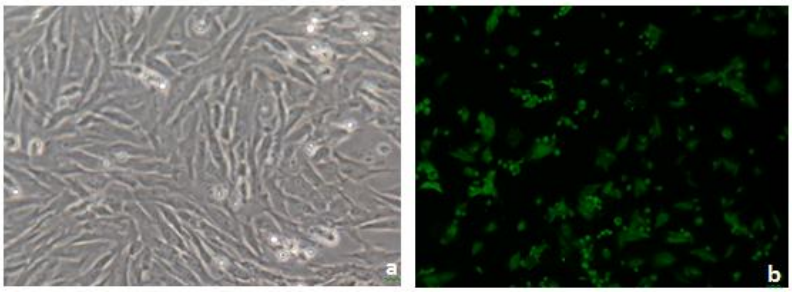

Fig-2: HTFs on passage 4. (a) Fibroblast morphology in phase contrast, 200x, (b) vimentin + of HTFs on passage 3

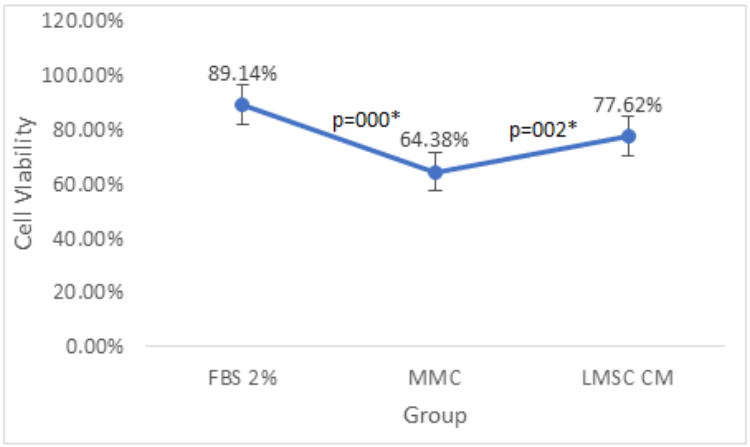

Fig-3: Effect of LMSCs-CM on cell viability of HTFs compared to FBS 2% control group and MMC group (Mann-Whitney, *statistically significant)

MMC decreased cell migration rate on days 3 and 7 compared to FBS 2% control group and LMSCs-CM group (Figure 4). Cell migration rate on days 3 in LMSCs-CM and MMC group were 4.5 fold and 8.5 fold lower than FBS 2% control group, respectively. Cell migration rate tend to increase on days 7 from 1.5 to 3 fold in all groups.

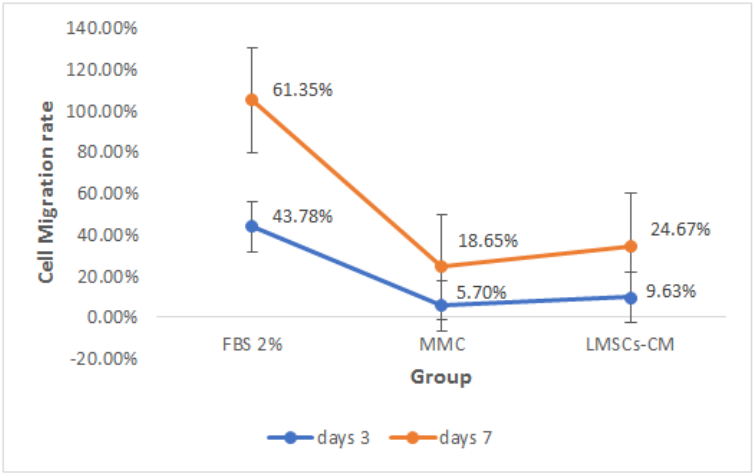

Fig-4: Cell migration rate on days 3 and 7

Collagen Deposition, Degradation, and cell contractility

MMC (19.04±0.54ug/mL) and LMSCs-CM (24.14±3.19ug/mL) were significantly decreased collagen deposition compared to FBS 2% control group (229.0±1.24ug/mL, p=0.001). Yet, collagen deposition in MMC group was significantly lower compared to LMSCs-CM group (p=0.01) (Figure 5).

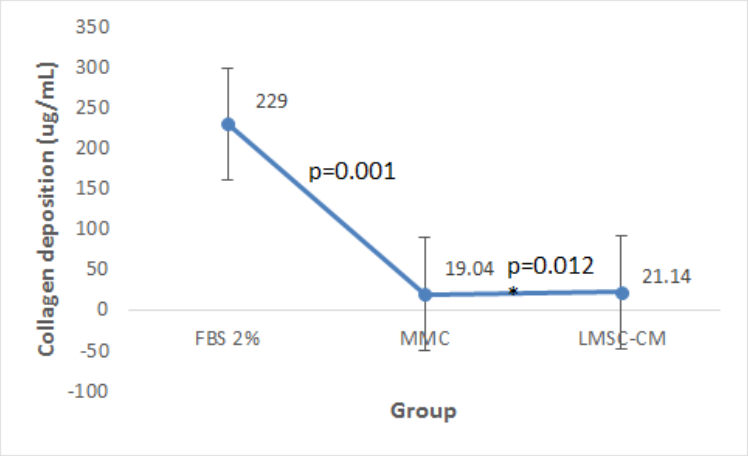

Fig-5: Collagen deposition (Mann-Whitney, * statistically significant).

MMC (96.43±1.04ug/mL) and LMSCs-CM (87.79±1.05ug/mL) were significantly increased collagen degradation compared to FBS 2% control group (19.09±3.1ug/mL, p=0.001). However, collagen degradation in MMC and LMSCs-CM group were not different statistically (p=0.2) (Figure 6).

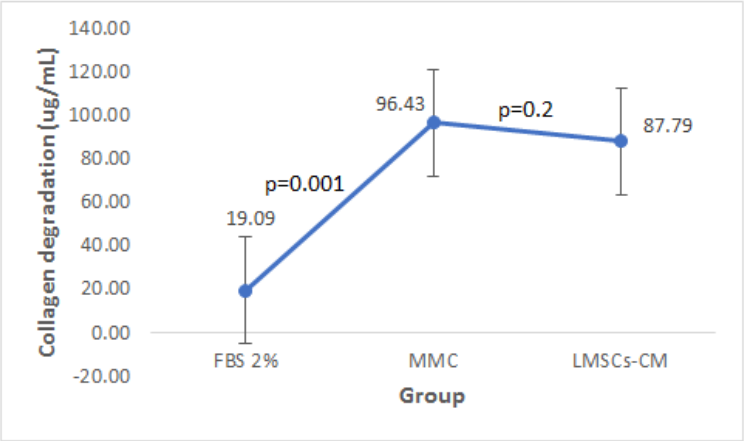

Fig-6: Collagen degradation (Mann-Whitney, * statistically significant)

MMC (31.23±6.96ug/mL) and LMSCs-CM (42.22±7.05ug/mL) were significantly decreased cell contractility compared to FBS 2% control group (69.08±5.23ug/mL, p=0.000). Cell contraction in MMC was significantly lower than LMSCs-CM group (p=0.01) (Figure 7).

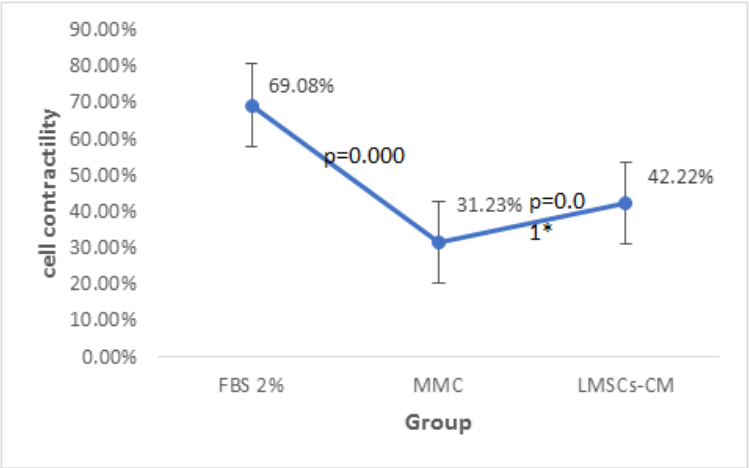

Fig-7: Cell contractility (Tukey HSD, * statistically significant)

DISCUSSION

Fibroblast is the main effector in bleb fibrosis after trabeculectomy. Fibroblast stimulated by macrophages, neutrophils, and inflammatory cytokines are involved in complex processes, leading to further extracellular matrix (ECM) secretion and remodeling. Any overwhelming activity within this process can cause overstimulation of fibroblasts, formation and accumulation of extraneous ECM. Matrix metalloproteinases (MMPs) contribute in ECM remodeling and the subsequent matrix contraction in scar formation. The unrestrained proliferation of fibroblasts induces a strong inflammatory reaction with abundant ECM, mainly type I collagen formation [14, 18]. MMC still proven as gold standard in trabeculectomy to halt bleb fibrosis.

This study indicates that MMC leads the antifibrosis effect in HTFs since that agent successfully decrease cell viability and inhibit cell migration in wound scratch assay. Seet *et al.* showed that MMC was far more effective in effecting this inhibition compared to secreted protein acid rich in cysteine (SPARC) silencing HTFs model. MMC affects HTFs by inhibiting cell proliferation, inducing widespread apoptosis, increasing susceptibility to T cell-mediated lysis, reducing the production of pro-fibrotic genes and proteins and reducing cell migration. MMC treated HTFs inhibit type I collagen deposition in accordance with induction of collagen degradation as the main fibrosis related ECM compared to FBS 2% control group and LMSCs-CM group. Moreover, MMC successfully inhibited type I collagen deposition compared to LMSCs-CM group. MMC-treated HTFs were severely limited in the ability to contract the collagen gel compared to FBS 2% control group and LMSCs-CM group. It has been proposed that MMC improves surgical outcome at least in part through the induction of apoptosis, which leads to early termination of the wound healing response [1,2,4,19].

We generated LMSCs-CM from cultured of 3D spheroid colonies to improve antifibrosis secreted factor. Three-dimensional (3D) cell culture has been reported to increase the therapeutic potentials of MSCs. MSCs have been reported to have antifibrotic properties by paracrine secretions of cytokines, such as insulin growth factor 1 (IGF-1), interleukin-6 (IL-6), and hepatocyte growth factor (HGF) [20,21]. Aggregation of MSCs into 3D spheroids increased the expression of the anti-inflammatory protein TNFα stimulated gene/protein 6 (TSG-6), as well as their paracrine secretion of angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiogenin [13,22]. *In vitro* studies indicated conditioned medium of 3D cultured MSCs protected hepatocytes from cell injury and apoptosis more effectively than 2D cultured cells [22]. CM provides key advantages over stem-cell based applications related to safety of immune compatibility, tumorigenicity, emboli formation and the transmission of infection, stable in preservation, economical, and more practical in clinic [22,23].

This study indicates that LMSCs-CM was able to maintain HTFs cell viability compared to MMC group, while LMSCs-CM proven its antifibrosis effect on HTFs. LMSCs-CM decrease cell migration compared to FBS 2% control, yet MMC has better inhibitory effect on cell migration. Usunier *et al.* demonstrated that systemic murine MSC administration attenuated fibrosis in a bleomycin-induced lung injury model. In that study, MSCs synergistically reduced TGFβ-induced fibroblast proliferation and myofibroblast differentiation [11,19].

LMSCs-CM significantly inhibit collagen synthesis in line with induction of collagen degradation in HTFs. This study was consistent with Arti *et al.* which reported that less ECM was produced by fibroblasts after exposure to MSC-conditioned medium, and transplantation of rat MSCs into infarcted rat myocardium attenuated the fibrotic response as assessed by Sirius red staining. Several factors could be responsible for these anti-fibrotic effects, like HGF, bFGF, IGF-1, and adrenomedullin (ADM) [24]. Li *et al.* showed that ADM secretion by MSCs was associated with inhibition of cardiac fibroblast proliferation and collagen I synthesis [25]. Conversely, hepatocyte growth factor (HGF) has been shown to be antifibrotic. Exogenous HGF enhanced histological and functional healing in both acute and more chronic vocal fold fibrosis in canine model [26]. Recently, Qi *et al.* highlighted the importance of TSG-6 (TNF-stimulated gene 6) in the antifibrotic effect of MSCs. In addition to suppressing the secretion of TNF-α by activated macrophages, this protein induces a change in the TGF-β1/TGF-β3 balance, from a profibrotic high ratio to an antifibrotic low ratio [27-29]. These results are confirmed in a coculture model in which recombinant TSG-6 partially reproduced the effects of MSCs [10,29,30]. In different fibrosis models, a decreased expression and concentration of collagen, the main component of the ECM, were found after MSC transplantation. This effect is also obtained after transplanting microvesicles or exosomes secreted into an MSC culture, suggesting a paracrine control of MSCs on ECM degradation [31,32].

Inhibition of collagen deposition and collagen degradation stimulation by treated HTFs with LMSCs-CM indicates that those secreted factors might have role in balance of ECM reconstitution through proteolytic and anti-proteolytic enzyme activity. This study is in accordance with Ishikane *et al.* they showed that after MSC transplantation, the increased expression of MMP-2, MMP-9, MMP-13, and MMP-14 has been observed in several fibrosis models. Following the addition of MSC-CM to a culture of heart fibroblasts, an increase in the activity of MMP-2 and MMP-9 was

found [33]. MSCs seem to have a repressive effect on the expression of TIMPs such as TIMP-1. A reduction in the concentration of TIMP 1 to 4 was shown after MSC transplantation. In an *in vitro* model, a decrease in the expression of TIMP-2 was observed, suggesting that MSCs have a paracrine effect [32-34].

As limitation, this study did not conduct proteomic analysis of LMSCs-CM to determine secreted antifibrosis factor. Moreover, the expression of pro-fibrosis cytokines within HTFs were not observed, hence we could not convince the mechanism governing the antifibrosis effects LMSCs-CM.

CONCLUSION

3D spheroid generated LMSCs-CM exerts antifibrosis effect on HTFs of glaucomatous eye. Yet, MMC provides better inhibitory effect on fibrosis in HTFs. This study might provide further insight for application of LMSC as antifibrosis in trabeculectomy.

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