A COMPARATIVE STUDY ON THE THERAPEUTIC POTENTIAL OF OCULAR AND NON-OCULAR STEM CELL SECRETOME ON ALKALI-INDUCED LIMBAL STEM CELL NICHE DAMAGE

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ABSTRACT : The study of secretome from stem cells has become a promising area of research in limbal stem cell deficiency (LSCD). This study aimed to investigate the therapeutic potential of secretome from AD-MSCs and LSCs in a culture of alkali-damaged LSCs as a model of limbal niche damage. Secretome was derived from 4 passages of AD-MSCs and LSCs under serum deprivation conditions. LSCs were damaged using 6,25mM NaOH to generate a limbal niche damage model and were then divided into 5 groups consisting of a control group, as well as 50% AD-MSCs, 25% AD-MSCs, 50% LSCs and 25% LSCs as the treated groups. After 48 hours of treatment, cell proliferation was determined by MTT-assay and cell migration was determined by in vitro scratch assay. VEGF supernatant was measured to analyze anti-angiogenic properties. A concentration of EGF and HGF in secretome was measured using ELISA. The data were analyzed using Oneway ANOVA dan post-hoc Tukey. Secretome derived from AD-MSCs and LSCs successfully promoted the proliferation and migration of LSCs, compared to the control, with the highest proliferation and migration rate seen in the 50% LSC secretome group ((94.38±2.51%, *P* 0.000; 68.52 ± 3.51%, *P* 0.000, respectively). EGF (1216.67 pg/mL) and HGF (2943.33 pg/mL) levels were higher in LSC secretome compared to the secretome of AD-MSCs. A 50% AD-MSC secretome with a level of VEGF supernatant at 65 pg/mL showed better anti-angiogenic activity. Secretome from ocular and non-ocular stem cells demonstrated clear therapeutic benefits inlimbal niche damage.

Key words : Limbal stem cell deficiency, limbal stem cell, mesenchymal stem cell, ocular surface regeneration.

INTRODUCTION

Damage to limbal stem cells (LSCs) and their niche leads to limbal stem cell deficiency (LSCD) and is most frequently related to severe chemical burns. This condition results in corneal opacification and neovascularization, leading to vision loss (Sejpal *et al*, 2013; Qihua *et al*, 2018). The therapeutic strategy for LSCD depends on the severity and can range from conservative to invasive treatment. In cases of severe LSCD with more than 75% damage to the LSC niche, limbal stem cell transplantation is the treatment of choice. While the specific properties of the limbal niche have not yet been fully characterized, it is likely to include both cellular and extracellular components. Interaction between the production of growth and cellular factors plays an important role in maintaining the limbal niche. Hence, introducing an external growth factor in LSCD cases should be considered one alternative for ocular surface regeneration.

Mesenchymal stem cell (MSC) populations have a multi-lineage differentiation capacity and a regenerative potential across many tissues and organs. Limbal stem cells (LSCs) and adipose derived-mesenchymal stem cells (AD-MSCs) can also differentiate into corneal epithelial-like cells in certain culture conditions. However, availability and cultivation of healthy donor LSCs remains a challenge. Consequently, other sources of stem cells with a similar potential to LSCs warrant exploration (Aslan *et al*, 2012;

Haagdorens *et al*, 2016; Qihua *et al*, 2018). Ma *et al* (2006) were the first to expand and transplant bone marrow MSCs within human amniotic membrane (HAM) in order to repair the ocular surface of rats with LSCD (Ma *et al*, 2006). Stem cells exert their therapeutic potential through homing-direct differentiation mechanisms and paracrine effects (Aslan *et al*, 2012; Haagdorens *et al*, 2016). Mesenchymal stem cells secrete various bioactive factors, known as secretome, in their conditioned media. The secretome from stem cells contains anti-inflammatory, growth and immunomodulatory factors, as well as cytokines, which are all thought to contribute to ocular surface regeneration. The use of secretome has many advantages compared to a stem cell itself, namely a lack of immune rejection, manufacturable potential and convenient transport (Chuang *et al*, 2012; Wuchter *et al*, 2014). Consequently, MSC secretome can be a useful as an alternative cell-free based therapy for ocular surface regeneration in LSCD. Previous studies have demonstrated the essential role of secretome derived from limbal fibroblasts in regulating epithelial proliferation, differentiation, and wound healing. Indeed, the cell-free nature of MSC secretome has gained particular interest with respect to its safety (Amirjamshidi *et al*, 2011; Saharadze *et al*, 2019).

This study aimed to investigate the therapeutic potential of secretome generated from AD-MSCs and LSCs in a culture of alkali-damaged LSCs, as a model of limbal niche damage. The potential effects derived from AD-MSC and LSC secretome are cell proliferation and migration. In addition, concentrations of epidermal growth factor (EGF) and hepatocyte growth factor (HGF) contained within secretome were measured, as they may contribute to the therapeutic potential that secretome has in limbal niche regeneration.

MATERIALS AND METHODS

Materials

The reagents included Type I collagenase (Roche USA), alpha modified eagle medium (á-MEM, Gibco-Life Technologies, USA), nonessential amino acid (NEAA, Sigma, USA), Trypsin-EDTA (Gibco - Life Technologies, USA), fetal bovine serum (FBS, Biowest, USA), NaOH (Gibco-Life Technologies, USA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphyniltetrazolium bromide (MTT; Gibco-Life Technologies, USA). FITC conjugated antibodies for CD 105, CD 90, CD 73, P63 and CD 45 were purchased from BIOSS (USA). Human vascular endothelial growth factor (VEGF), EGF and HGF ELISA kits were obtained from the Bioassay Technology Laboratory in China.

Methods

Study design

This *in vitro* study investigated the potential therapeutic effect of secretome generated from AD-MSCs and LSCs in a culture of LSCs that were damaged with NaOH, as a model of limbal niche damage. All experiments were conducted with approval from the institutional ethics review board at the Universitas Airlangga through the Stem Cell Research and Development Center.

Cell isolation and characterization

Adipose-derived MSC isolation and characterization

Adipose-derived MSCs were isolated from a single, healthy 27 year-old-woman donor under aseptic technique. Following an informed consent, 2×2 cm of adipose tissue was collected. Adipose-derived MSCs were isolated, with modification, using an enzymatic method based on protocol developed by Zhu *et al* (2013). Adipose tissue was briefly minced into approximately 1 cm^3 pieces and then washed with PBS. The tissue was then placed in sterile tubes containing a α-MEM medium with an enzymatic cocktail comprised of 0.075% Collagenase Type I. An incubation period at 37°C lasted for 30-60 minutes. Following incubation, the digested tissues were washed with culture medium, transferred into a new sterile tube and centrifuged at 300 g for 5 min. Supernatant was then discarded and cell pellets were resuspended in a culture medium composed of á-MEM, 1% penicillin-streptomycin, 1% amphotericin B, 1% NEAA, 5% FBS, and 5ng/mL of bFGF. Culture medium was stored in an incubator at 37°C and 5% CO_2 and changed every 3 days until a 90% confluence was reached. It was then passaged with a warm trypsination method. To confirm the MSC's phenotype, cells were monitored for the expression of CD 105, CD 90, CD 73, and CD 45 at the third passage. 5×10^3 cells were then cultured in 96 multiwell plates until an 80% confluence was reached. The cells were then fixated with 10% formaldehyde for 15 minutes. Fixated cells were each stained with FITC conjugated antibody and incubated for 60 minutes at 37°C. This was followed by an overnight incubation at 4°C. Stained cells were then washed 3 times using PBS tween 0.2%, followed by a DAPI counterstain and visualized under inverted fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus).

Limbal stem cell isolation and characterization

Corneoscleral tissue from healthy male rabbits was obtained from the Stem Cell Research and Development

Center, at the Universitas Airlangga. Cell isolation was conducted according to protocol established by Komaratih *et al* (2019). In short, the tissues were incubated in 0.2mg/mL collagenase type I and á-MEM for 30 minutes at 37ºC with gentle agitation. Cell suspensons were collected and then cultured in a complete medium 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 LSCs were harvested and passaged. To confirm the LSC's phenotype, cells were observed at passage 3 for the expression of p63. 5×10^3 cells were then cultured in 96 multiwell plates until 80% confluence was reached. The cells were then fixated with 10% formaldehyde for 15 minutes and then each stained with FITC conjugated antibody and incubated for 60 minutes at 37ºC. An overnight incubation at 4ºC followed. Stained cells were washed 3 times using PBS tween 0.2%, followed by a DAPI counterstain and visualized under inverted fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus).

Secretome production

1×10^6 AD-MSCs and LSCs obtained at passage 4 were cultured in medium for 24 hours in 100mm diameter culture dishes to reach a confluence of 80%. One day later, the cells were treated using medium composed of α-MEM, 1% penicillin-streptomycin, 1% amphotericin B, 1% NEAA and 2% FBS. Cell secretome collected after 48 hours was then filtered using a 0.45 um Millipore and stored at -80ºC until further use.

Alkali-induced limbal niche damage

5×10^3 cells/well of LSCs were cultured in 96 multiwell plates for 24 hours in a complete culture medium until 80% confluence was reached. One day later, the cells were destroyed using 20ul of 6.25mM NaOH for 1 minute, followed by three repetitions of a PBS washing until greater than 50% damage to LSCs was achieved.

Cell proliferation

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphyniltetrazolium bromide assay (MTT assay) was conducted to determine the effect that AD-MSC and LSC secretome had on cell proliferation following alkali-induced LSC damage. This assay was performed according to methods put forth by Riss *et al* (2004), with eight replications for each group. Finally, after damaging LSCs using NaOH, the cells were treated for 48 hours in each of the following five culture media: complete, supplemented with 25% AD-MSC secretome, supplemented with 50% AD-MSC secretome, supplemented with 25% LSC secretome, and finally, supplemented with 50% LSC secretome. After 48 hours

of incubation, 10 µl of 0.45mg/mL of tetrazolium solution was added to each well for a further incubation time of 4 hours at 37ºC. Following incubation, 100 µl of solubilization solution was added to dissolve the formazan crystals. The plates were then immediately analyzed using a microplate reader at an absorbance of 570nm.

In vitro scratch assay

3×10^5 LSCs/well were seeded into six-well culture dishes, allowed to attach, and grown to confluence. The cells were then scratch wounded by yellow pipette tips and washed with medium to remove loose or dead cells. Cells were then treated with each of the five culture media (as discussed above) for 48 hours. Cell migration rates were analyzed according to Freshney (2000). Each scratch wound, marked by a reference line drawn on the outside of the dish, was photographed at varying time periods with a phase-contrast microscope equipped with a camera for 48 hours after treatment. Each scratch wound was photographed over two different fields for a total of four replications. The wound closure area denoted the cell migration rate, and was analyzed with Image J software. The data expressed reflected average percentages of wound closure areas at measured time points over the original width, starting from hour zero (Freshney, 2000).

Growth factor measurement

Growth factor measurement was conducted to determine concentrations of EGF and HGF contained in the secretome of AD-MSCs and LSCs. The concentration of VEGF supernatant after cell treatment was measured to determine the anti-angiogenic activity of secretome derived from AD-MSCs and LSCs in an alkali-induced limbal niche damage model. An enzyme-linked immunosorbent assay was performed according to Bioassay Technology Laboratory's protocol. In short, samples were collected and centrifuged at 3000rpm for 20 minutes. 40 µl of supernatant was placed into ELISA well plates. Biotinylated antibody and streptavidin HRP were added, then samples were incubated at 37ºC for 60 minutes. Samples were then washed 3x with washing buffer, solutions A and B were added, followed by an incubation at 37ºC for 10 minutes. Stop solution was then added and the plates were read immediately using a microplate reader at an absorbance of 450nm.

Statistical analysis

The data of cell proliferation and migration were presented as mean ± standard deviation. T cell proliferation and migration difference between groups was analyzed with ANOVA and Post-hoc Tukey using SPSS software, version 19.0, where *P* < 0.05 was considered to be statistically significant.

RESULTS

Cell characterization

AD-MSCs and LSCs were successfully isolated, with observations of fibroblast-like morphology appearances beginning at 72 hours. Cell characterization at the third passage indicated a AD-MSC population with positive stains for CD 105, CD 90, and CD 73 FITC conjugated antibody, but negative stain for CD 45 antibody. The population of LSCs stained positive for p63 antibody, as depicted in Fig. 1.

Cell proliferation and migration

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphyniltetrazolium bromide assay displaying the highest cell proliferation rate were as follows: those treated with 50% LSC secretome (94.38 ± 2.51%), followed by 50% AD-MSC secretome (92.68 ± 2.01%), 25% AD-MSC secretome (90.96 ± 3.11%), 25% LSC secretome (88.85 ± 2.61%), and finally, complete culture medium, which served as a control (70.57 ± 2.57%) (Fig. 2). A significant difference was found between the control group and all of the other groups (ANOVA, post-hoc Tukey, P 0.000), whereas differences between secretome groups of 50% and 25% concentrations were not significant (ANOVA, post-hoc Tukey, $P > 0.05$).

In vitro scratch assay showed that cells treated with 50% LSC secretome had the most rapid wound closure rate (68.52±3.51%), followed by 50% AD-MSC secretome (67.17±3.51%), 25% AD-MSC secretome (66.62 ± 3.11%), 25% LSC secretome (66.05 ± 2.01%), and finally, a control with complete culture medium (48.07 ± 1.57%) (Fig. 3). The wound closure area of the control group differed significantly compared to other groups, (ANOVA, post-hoc Tukey, $P > 0.000$). However, no differences were found among treatment groups, (ANOVA, post-hoc Tukey, $P > 0.05$).

EGF, HGF and VEGF concentration

An enzyme-linked immunosorbent assay showed EGF concentrations of 183.33 pg/mL and 1216.67 pg/mL in secretome derived from AD-MSCs and LSCs, respectively. The concentration found in LSC secretome was 6.6x higher than in AD-MSC secretome. HGF concentration in AD-MSC secretome was 261.33 pg/mL, whereas HGF concentration in LSC secretome was 11.26x higher, at 2943.33 pg/mL. The secretome derived from AD-MSCs and LSCs at different concentrations was found to inhibit VEGF secretion at varying levels. In the 50% and 25% AD-MSC secretome treated group, concentrations of 65 pg/mL and 115 pg/mL, respectively, inhibited VEGF secretion. And in the 50% and 25% LSC secretome treated group , concentrations of 250 pg/mL and 265 pg/mL, respectively, inhibited VEGF secretion as compared to the control group, which had a concentration of 785 pg/mL (Fig. 4).

DISCUSSION

This study found that stem cells of both ocular and non-ocular origin have a similar effect in regenerating LSC niche. However, the highest cell proliferation rate was obtained in the 50% LSC secretome group. In cell concentrations showing more than 50% alkali-induced damage, 50% LSC secretome was able to stimulate cell reconstitution at a rapid rate. The ability of secretome in promoting the proliferation rate was comparable to its migration rate induction, as shown in areas of wound closure. This is in line with a mouse model study conducted by Amirjamshidi *et al* (2011), which demonstrated that secretome derived from limbal fibroblasts had therapeutic benefits, such as the promotion of limbal stem cell proliferation and differentiation into corneal epithelial cells (Amirjamshidi *et al*, 2011). Conversely, secretome derived from skin fibroblasts failed to promote corneal epithelial growth in a mouse model of LSCD (Notara *et al*, 2011; Amirjamshidi *et al*, 2011).

In this study, concentrations of EGF and HGF in secretome generated from LSCs was much higher than that generated from AD-MSCs. Yet, cell proliferation and migration effects did not differ. Alkali injury is the highest cause of destruction in LSCs population, compromising not only the stem cell population, but also its microenvironment. Theoretically, it is possible to restore stem cell function, both by expanding its population through modulations in the microenvironment, and by inducing transient amplifying cell (TACs) mitosis with the use of appropriate growth factors (Solomon *et al*, 2002; Shimmura *et al*, 2008; Kolli *et al*, 2008; Basu *et al*, 2012). A variety of growth factors play key roles in limbal stem cell regeneration. Insulin like growth factor (IGF-1) that rapidly up-regulates following limbal stem cell damage, enhances the expression of IGF receptors and promotes the differentiation of LSCs into corneal epithelial cells. In contrast, EGF and basic fibroblast growth factor (bFGF), which are also produced by damage to corneal epithelium, support limbal cell proliferation without any effect on their differentiation (Trosan *et al*, 2012; Svobodova *et al*, 2012; Rama *et al*, 2017). In healthy limbal niche, HGF is produced by limbal fibroblasts, which cause LSC proliferation, migration, and differentiation into corneal epithelial cells (Ebrahimi *et al*, 2008). Studies have shown that the amniotic membrane contains high levels of EGF, KGF, HGF, TGF (tumor growth factor), and bFGF, which are all potentially involved in epithelial–
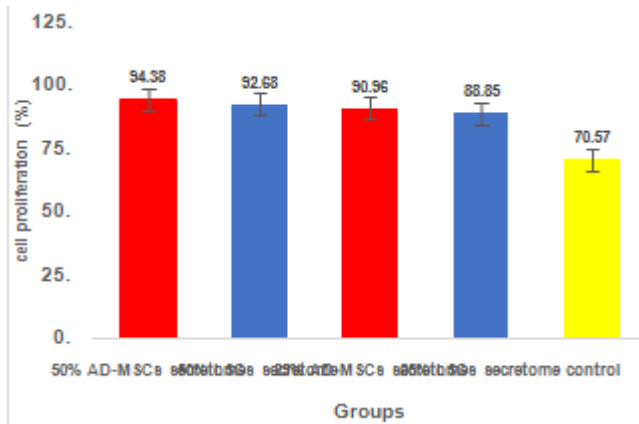
Fig. 1 : Cell proliferation rate among groups.

However, the availability of healthy donor limbal tissue remains a problem. Therefore, it is necessary to optimize the production of secretome from a non-ocular stem cell origin (Jeng *et al*, 2011; Notara *et al*, 2011). In this study, lower concentrations of EGF and HGF, as found in AD-MSC secretome, more effectively stimulated cell proliferation and migration compared to the lower concentrations of LSC secretome. However, the overall effect was similar. Adipose-derived MSCs proliferated faster than adult MSCs from other origins, and displayed later senescence compared to bone marrow MSCs. It was shown that AD-MSCs cultured in corneal epithelial
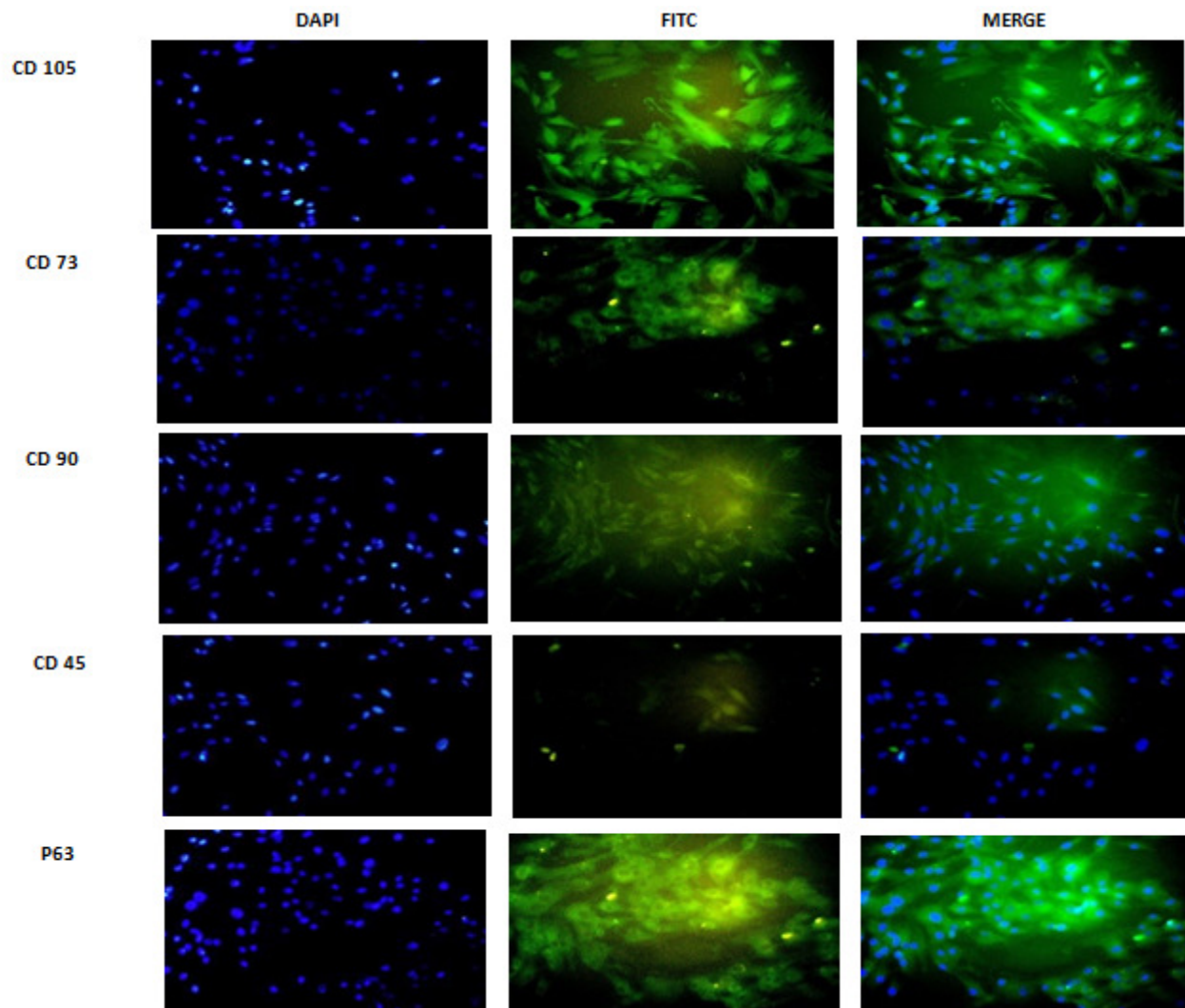

Fig. 2 : AD-MSCs P-3 were positive for CD 90, CD 105, CD 73 and negative for CD 45' LSCs were positive for p63. Left section: DAPI; middle section: FITC labelled antibody; right section: a merge of both previous sections (Inverted Fluorescence Microscope, at 200x magnification).

stromal interactions of the human ocular surface. These include epithelialization, modulation of proliferation, and differentiation of stromal fibroblasts (Anderson *et al*, 2001; Grueterich *et al*, 2002; Gomes *et al*, 2005; Sangwan *et al*, 2007).

cell conditioned medium up-regulated markers for corneal epithelial-like cells. Moreover, application of AD-MSCs to rabbit corneal surfaces injured by alkali-burn showed enhanced epithelial healing. Hence, AD-MSCs are a viable, alternative tissue-engineered cell source for
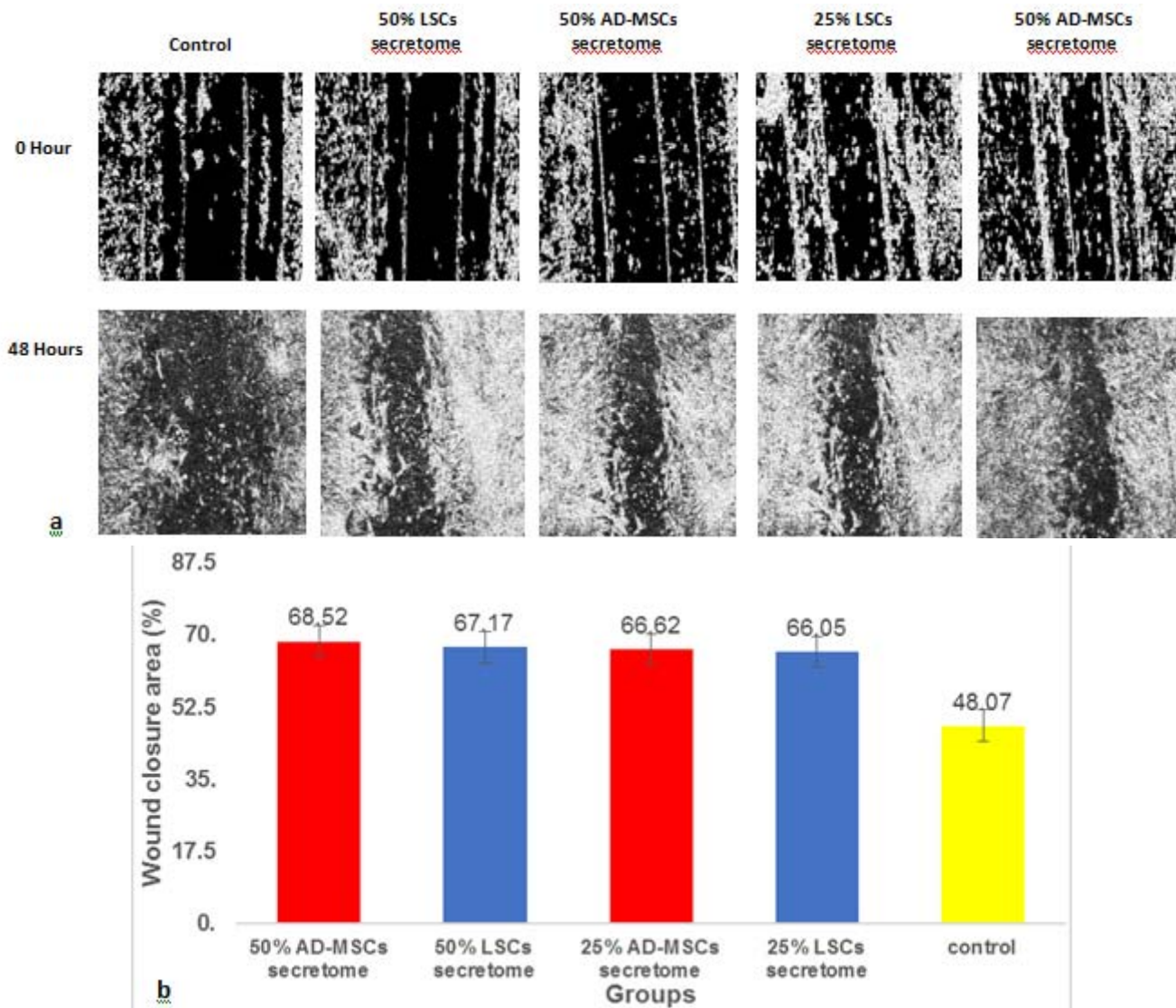
Fig. 3 : Cell migration analysis using *in vitro* scratch assay. (a) photograph of *in vitro* scratched cells at 0 hour (above image) and wound closure area at 48 hours (bottom image) (microscope inverted, 100x magnification, Image process by Image J). (b) graphic showing the percent of wound closure area at 48 hours.

treating corneal epithelial defects and reconstructing the ocular surface (Gu *et al*, 2009; Calonge *et al*, 2019; Kern *et al*, 2006; lan *et al*, 2012; Ma *et al*, 2013; Alio *et al*, 2017; Alio *et al*, 2019). Zeppieri *et al* (2017) reported that topical application of AD-MSCs promoted corneal epithelial wound healing and attenuated inflammation in laser-induced corneal wounds of mice (Zeppieri *et al*, 2017). Holan *et al* (2015) demonstrated that the therapeutic effect of BM-MSCs on the healing of alkali-injured corneal surfaces in rabbits was comparable to that of tissue-specific LSCs. Furthermore, they suggest that extra-ocular stem cells can be used for ocular surface regeneration in cases where autologous LSCs are difficult to obtain (Holan *et al*, 2015).

MSCs are known to have a therapeutic potential for corneal disease treatment because of their potent anti-inflammatory and angiogenic-regulatory properties. Topical and/or sub-conjunctival administration of bone marrow MSCs reduced corneal inflammation and angiogenesis following chemical injuries in murine models as seen through the suppression of inflammatory cell and CD68+ macrophage infiltration into corneas, down-regulation of pro-inflammatory cytokines and pro-angiogenic factors, including VEGF and bFGF (Ye *et al*, 2006; Jiang *et al*, 2010; Roddy *et al*, 2011). For the purposes of this study, AD-MSC secretome proved to be more effective in inhibiting VEGF secretion as compared to LSC secretome, suggesting a more potent anti-angiogenic effect. Angiogenesis is regulated by a variety of pro-angiogenic (VEGF, bFGF) and anti-angiogenic factors (including TSP-1, pigment epithelium-derived factor PEDF). Depending on the tissue microenvironment, MSCs exert pro- and anti-angiogenic functions. However,
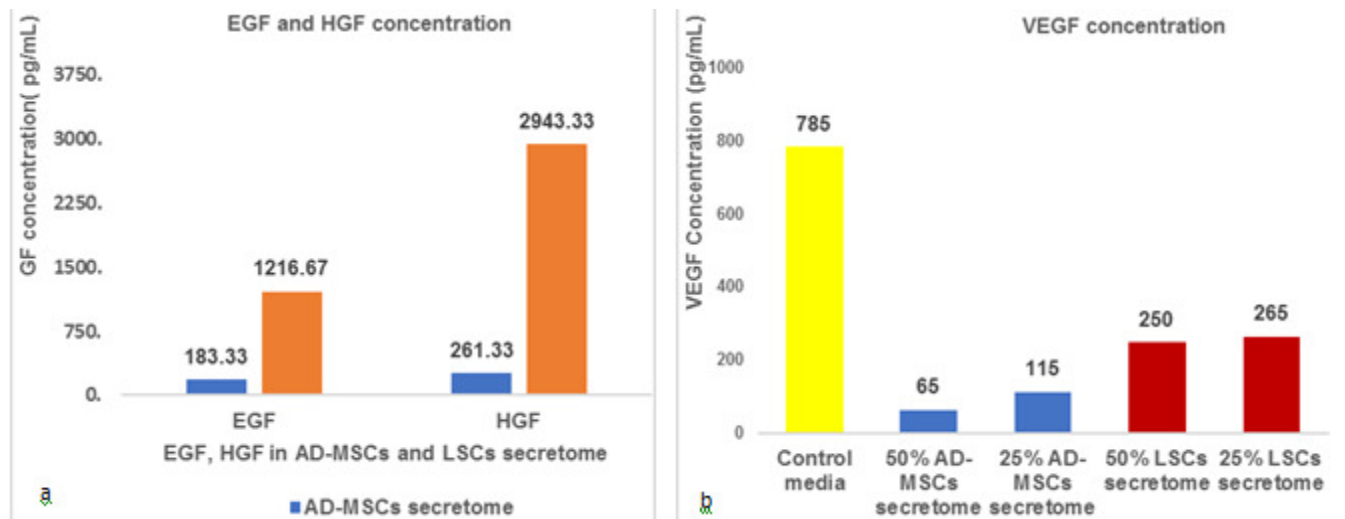
Fig. 4 : Growth factor concentration. (a) concentration of HGF and EGF in secretome derived from AD-MSCs and LSCs. (b) concentration of VEGF in LSCs supernatant after 48 hours of secretome treatment at various concentrations.

MSCs up-regulate TSP-1 to inhibit angiogenesis by disrupting CD47 and VEGF receptor-2 signaling, thereby suppressing the VEGF–Akt–eNOS pathway (Jackson *et al*, 2012; Kaur *et al*, 2010; Bazzazi *et al*, 2018). In a study by Zhang *et al* (2019) intravitreal injection of human umbilical cord MSC-Exo improved hyperglycemia-induced retinal inflammation in diabetic rats. This was achieved by a transference of miR-126, which suppressed HMGB1 (high-mobility group box 1) signaling. In another rat model of blue-light induced retinal damage, intravitreal umbilical cord MSC-Exo injection showed a dose-dependent suppression of choroidal neovascularization by down-regulating VEGFA and inhibiting the NFκB pathway (Zhang *et al*, 2019). In this study, it was shown that AD-MSCs of non-ocular origin could provide an adequate amount of growth factors for limbal niche reconstitution following alkali-induced damage.

CONCLUSION

In summary, this study aimed to determine whether secreted factors from ocular and non-ocular stem cells have a therapeutic benefit in limbal niche damage. Given the relative absence of non-surgical treatments for partial limbal stem cell deficiency, such an approach has a high potential for clinical application. Cell-free based therapy with MSCs or LSCs may translate into relatively safer products due to the low risk of toxicity and immunological rejection. This scope of this study was limited, however, as in vitro examinations lack a full analysis of the cellular and extracellular factors present outside of a LSC niche damage model. Consequently, *in vivo* studies are necessary to further investigate the therapeutic effect that topical application of stem cell secretome has.

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