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INTRACAMERAL INJECTION OF LIMBAL MESENCHYMAL STEM CELLS SECRETOME ALLEVIATE INFLAMMATION WITH DELAYED STRUCTURAL RECOVERY ON CORNEAL ENDOTHELIAL CELLS IN PHACOEMULSIFIED RABBIT EYES

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ABSTRACT : The objective of this research is to investigate the effects of intracameral injection of limbalmesenchymal stem cells (LMSCs) secretomeon corneal endothelial cells density and caspase-3 expression after exposure to phacoemulsification ultrasound energy in rabbits. Twenty-four New Zealand white rabbits (n = 24 eyes) were randomly divided into 2 groups and exposed to phaco ultrasound. The control group was treated with intracameral injection of balanced salt solution (BSS), while intracameral injection of LMSCs secretome was utilized on the treatment group. Thereafter, the endothelial cells density was evaluated with specular microscopy before ultrasound exposure on the 3rd daywith the corneaused to evaluate theexpression of *caspase-3* by immunohistochemistry staining. There were no signs of inflammation in both groups. However, exposure of ultrasound energy significantly reduced the corneal endothelial cell density in the control and treatment groups (-93.69 ± 139.04; p = 0.04 and -119.31 ± 85.41, p = 0.001, respectively). Furthermore, the corneal endothelial cells density (p = 0.592) and caspase 3 expression after treatment for 3 days were indifferent in treatment group compared to control with p = 0.625; 2.17 ± 0.777 and 1.17 ± 0.322, respectively. Intracameral injection of LMSCs secretome tends to alleviate ocular inflammation, improved the profile of endothelial cells density and corneal caspase-3 expression in rabbit eyes after exposure to ultrasound energy-phacoemulsification.

Key words : Limbal mesenchymal stem cells secretome, endhotelial cells density, phacoemulsification, caspase-3.

INTRODUCTION

Most cataract extraction are currently performed with phacoemulsification techniques which uses ultrasound energy to fragment and emulsify the lens. With advances in the development of surgical devices and techniques, the safety and efficacy of phacoemulsification has rapidly increased. However, its use has been expanded to include cataracts with hard nucleus, thereby, leading to the need for greater ultrasound energy.This results in greater mechanical and thermal risk of corneal endothelial damage which ultimately triggers cell death through the apoptotic pathway. Some studies report that the endothelial cells loss of between 8.0% and 16.7% has a side effect of phacoemulsification. Damage and loss of corneal endothelial cells at some point tends to disrupte the function of the pump and tight junction which causes corneal edema and irreversible damage (Geffen *et al*, 2008; Bonnano, 2012; Takahashi, 2016; Alarfaj, 2017).

Corneal transplantation is currently the only effective treatment for corneal endotheliopathy due to its low regeneration ability. A national bullous keratopathy survey in Japan reported that cataract surgery was the most common cause of penetrating keratoplasty (PK) by 24.2%. Similarly, various other studies showed cataract surgery was ranked second after Fuchs endothelium dystrophy, thereby, making it the biggest cause of secondary endothelial damage. Keratoplasty has several disadvantages including limited corneal donors and the high level of postoperative endothelial cell damage at 49%. (Zavala *et al*, 2013; Zavala *et al*, 2017).

There are various *in vitro* studies aimed at triggering endothelial cell proliferation which showeda promising result. One of such methods is the secretion factors from stem cells also known as conditioned media, secretome, microvesicles or exosome which are expected to provide new therapeutic alternatives for endothelial damage. Furthermore, the stem cell conditioned mediumswhich

have been studied have same benefits. Various studies regarding the secretion factors from stem cells showed that it has the ability to repair various damaged tissue or organ. (Zavala *et al*, 2013; Pawitan, 2014).

The mesenchymal stem cells secrete bioactive factors in conditioned medium contains, growth nutrients, as well as proinflammatory and anti-inflammatory cytokines popullary known as secretome. One of the secreted growth factors is fibroblast growth factor-2 (FGF-2) with various effects on corneal endothelium, include triggering cell proliferation, endothelial migration and morphological changes to mesenchymal or endothelium transition (EnMT). In addition, FGF-2 also inhibites apoptosis through the Phosphoinositide-3-kinase (PI3-K) pathway. Corneal limbal is source of mesenchymal stem cell, LMSCs secretome in the corneal endothelium capabel of improving cell density, while preventingthe apoptosis of damaged corneal endothelial cells (Bonnano, 2012; Pawitan, 2014; Vizoso *et al*, 2017).

METHODS

This is an experimental study using animals with the pre and post test control group design used to investigate the effect of LMSCs secretome on corneal endothelium cells density. Similarly, the post test is designed to evaluate the expression of caspase-3 in corneal endothelium after exposure to ultrasound energy and phacoemulsification. This study was conducted at the Center For Stem Cell Research and Development, Airlangga University in May 2019, with the animal protocol approved by the institutional review board and ethics committees of the Veterinary Faculty, Airlangga University. The inclusion criteria were New Zealand male white rabbits aged 12-18 months weighing 2.5 - 3.5 kg, while theexclusion criteria include animals declared by veterinarians to posses unhealthy eye conditions with transmitable diseases. Furthermore, the drop out criteria conssits of unhealthy, dead, corneal perforation, vitreous prolapse, infected, and bleeding rabbits during and after surgery.Twenty-four New Zealand white rabbits (24 eyes) were randomly divided into 2 groups. The control group was intracamerally injected with0.2ml BSS, while 0.2ml LMSCs secretome was injected in the treatment group.

Surgical procedures

Twenty four rabbits met the inclusion criteria (n=24 eyes), withanterior segment examination carried out using a handheld slit lamp. The rabbits were divided into two groups of 12 with anesthesia corneal endothelial injury model performed using the modified technique from Nemet *et al* (2007) with a NIDEK CV 9000R phacoemulsification machine. The disinfection of eyelid werecarried out with 10% povidone-iodine. The field of operation was narrowed with sterile ducts and eye speculum mounted. The eyeball was disinfected with Povidone Iodine 5% and irrigated with BSS.

Endothelial injury model using phacoemulsification ultrasound exposure controlled variable parameters as follows: aspiration flow rate 25 ml/s, 70% power and 90cm bottle height using a single operator. The incision used were according to the 2.2 mm phacotip, with its position beveled up in the center of the anterior chamber without touching the endothelium and anterior lens capsule. The power was turned on and alternated every 10 seconds for 2.5 minutes (total time 5 minutes). The corneal incision wound was closed with stromal hydration and its impermeability checked. In the control group, intracameral injection of 0.2 ml BSS was administered, and0.2ml LMSCs secretome produced and stored at -80°C by Komaratih *et al* (2019) used in the treatment group.

Clinical examinations were performed preoperatively and postoperatively on days 1, 2 and 3. The examination consisted of slit-lamp to check corneal clarity and intraocular inflammation. Endothelial cell density were examined using specular microscopy (CEM-530, Nidek Co., Ltd) on anesthetized rabbit at preoperative and postoperative day 3. Furthermore, all rabbits received topical tetracaine hydrochloride and levofloxacin every 3 hours (Neo Levo; Rohto Lab, Tokyo) on their right eyes during follow-up.

Also, each group was sacrificed with decapitation on the third postoperative 3. The rabbit eyes were enucleated, with blades and scissors used to remove their corneas which was further fixed in a 10% buffered formaldehyde (pH 7.0) for histology examination using caspase-3 antibody (bs-0087R, BIOSS, USA).

Histopathology

The rabbit corneal specimens were embedded in paraffin, sectioned at 4 ìm and stained with caspase-3 antibody (bs-0087R, BIOSS, USA) using standard techniques from the pathology anatomy laboratory of Dr. Soetomo General Hospital. The number of positively stained endothelial cells was assessed using a 40× objective and counted on 3 adjacent sections.

Statistical analysis

Statistical analysis was performed using the statistical package IBM SPSS Statistics for Windows (Version 19.0; IBM Corp., Armonk, NY, USA), whilecomparisons of the mean baseline values of the ECD between the two groups were performed using the independent t-test.

There was a significant difference in the preoperative and postoperative ECD between the two groups, with its density change assessed at postoperative time point using paired t-test. Furthermore, the positively stained endothelial cell counts from the histology sections were also compared using mann whitneytest with thedata presented as mean ± SD. The significance was set at $p < 0.05$ for this study.

RESULTS

Before surgery, axial length was measured with ultrasound biomicroscopy. Table 1 shows the average length of rabbit's axial in the control group was 15.41 mm and 15.83 mm in the treatment group. The data were normally distributed in the two groups (p control = 0.391 and p treatment = 0.851, $p > 0.05$) without differences ($p = 0.077$, $p > 0.05$) (Table 1).

Furthermore, there were no complications during the phacoemulsification procedure and incision wound impermeable with the anterior chamber was properly formulated after the procedure.

Endothelial Cell Density (ECD) Post Phacoemulsification Exposure

There were no differnces in the evaluation time between the control and treatment groups (p day 0 = 0.11 and p day 3 = 0.072, $p > 0.05$). However, in the control group, there was a cell density of 2730.47 ± 139.72 on the first day, which was significantly different from day 3 by 2636.78 ± 209.81 ($p = 0.04$, $p > 0.05$). In the treatment group, the mean density of day 0 at 2582.86 ± 273.05 also showed a significant difference on day-3 with a density of 2463.56 ± 237.81 ($p = 0.001$, $p < 0.05$) (Table 2).

Each control and treatment group showed a decrease in endothelial cell density on day 3. Furthermore, the change in density in the control group was -93.69 ± 139.04 and the treatment group was 11119.31 ± 85.41, with no significant differences between these two groups ($p = 0.592$, $p > 0.05$).

Expression of caspase-3 in corneal endothelial cells

The examination of caspase-3 expression was carried out by immunohistochemical staining using antibodies. It was calculated based on the number of cells from 3 visual fields under light microscope with 400 times magnification which appears brown in cytoplasm (Fig. 2).

Table 3 shows the mean expression of caspase-3 endothelial cells in each group with no statistical difference between the 2 groups. In the control group its expression was 1.17 ± 0.322, while in the treatment group it was 2.17 ± 0.777 ($p = 0.625$, $p > 0.05$).

Table 1 : Axial Length Distribution.

Group	n	Axial Length				p
		$\bar{x}$	SD	Min	Max	
Control	12	15,41	0,60	14,61	16,79	0,077
Treatment	12	15,83	0,52	14,97	16,58	

DISCUSSION

This is the first study to evaluate the effect of intracameral injection of LMSC secretome to the corneal endothelial damage model. It was induced by phacoemulsification energy with endothelial cell loss and apoptosis assessed by density and caspase-3 expression, respectively. In addition, various methods have been used to induce corneal endothelial cell injury, which includes chemical, cryoinjury and mechanical injuries. The main advantage of the injury model of this study its similarity to what patients experience clinically. This phacoemulsification model also has the potential to be modified depending on the desired level. Rabbit's eye possess similar anatomy to the human eye, its corneal endothelium has a fairly high proliferation ability which diminishes with age. In addition, a fairly low proliferation capacity is shown in those over 12 months. Valdez-Garcia *et al* (2015) showed the highest mitotic corneal endothelial cell activity of rabbits at 3 months with 36 hours post-trauma. 12 months old rabbits, showed decreased mitotic activity and 18 months showed no mitotic activity in 72 hours post-trauma event. However, the difficulty faced is that the size of the eyeball is relatively smaller and the lens consumes more space in the eyeball which makes the anterior chamber narrower. (Davis, 1929; Gozkir *et al*, 1997).

Preoperative examination include anterior segment, axial length and endothelial cell density. In the anterior segment examination using a portable slit lamp, all rabbits were found in good condition. Axial length was measured to determine the bottle height for phacoemulsification machine. The mean in the control group was 15.41 mm, and 15.83 in the treatment group. The data were normally distributed (p control = 0.391 and p treatment = 0.851, $p > 0.05$) with no differences between the two groups ($p = 0.077$, $p > 0.05$). This is also consistent with the results of research by Bozkir *et al* (1997) where the average length of the rabbit eyeball was 15.12 ± 0.51 mm. Furthermore, the bottle height for phacoemulsification was set the same for each sample as high as 90 cm due to the homogenous data obtained.

Since the introduction of phaco-emulsification, significant improvement has been made to minimize corneal endothelial cell loss. Their reversible damages of the corneal endothelial are the available option used to

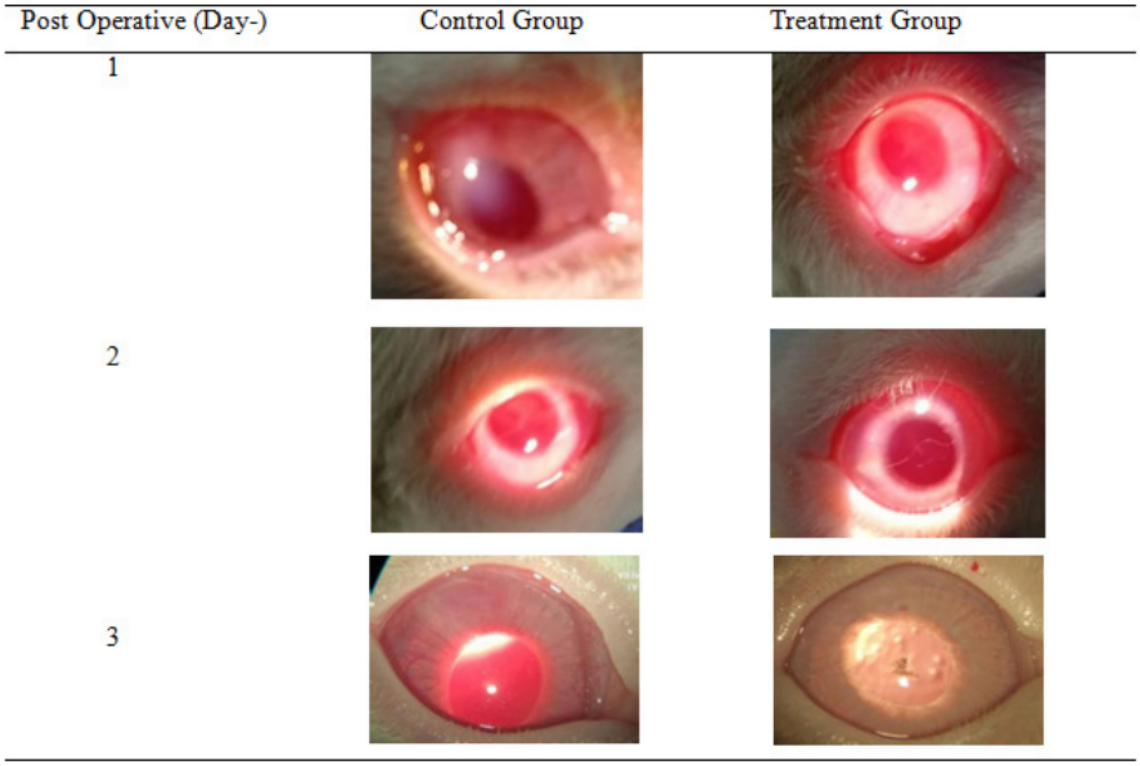

Fig. 1 : Anterior segment evaluation on post operative day-1, 2 and 3.

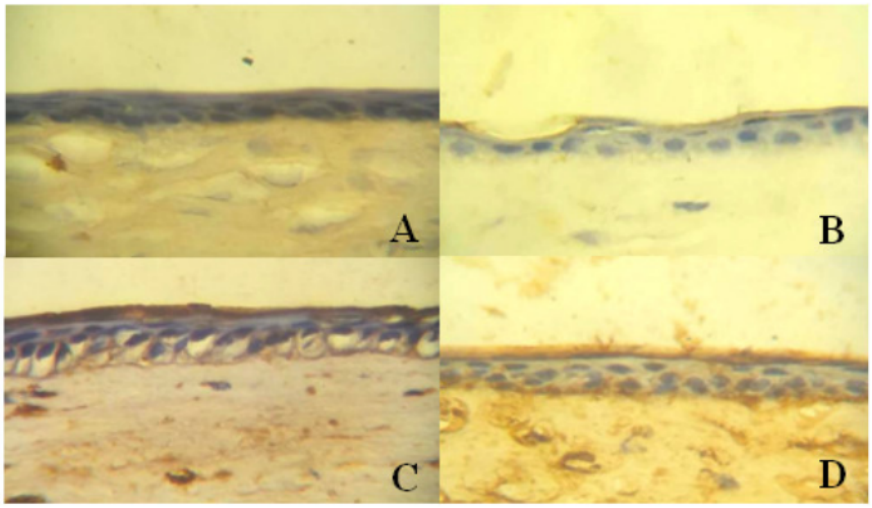

Fig. 2 : Caspase-3 antibody IHC examination, seen with a light microscope 400 times magnification in the control group (A and B) and the treatment group (C and D).

treat the transplantation dysfunction. Possible alternative therapies are conducted using strategies to save endothelial cells which are partially damaged from apoptosis and spurs regeneration. Endothelial cells do not proliferate, though its has the ability to conduct this process with a retained G1 cycle phase due to contact inhibition, TGF-â in aquos humor lacks effective stimulation from growth factors. Corneal mesenchymal stem cells have been shown to have a positive effect in triggering proliferation with the ability to inhibit corneal endothelial cell loss in invitro and exvivo studies. According to previous studies, the therapeutic effect of mesenchymal stromal cells is mediated through secreted factors. Mesenchymal stem cell conditioned media containing secretion factors from

Table 2 : ECD distribution.

Group	ECD		p	Density change
	day 0	day 3		
Control	2730.47±139.72 (2464,67 – 2936,33)	2636,78±209,81 (2318,67 – 3053,33)	0,040	-93,69 ± 139,04 (-309,33 – 122,33)
Treatment	2582,86±273,05 (2076,33 – 3034,00)	2463,56 ± 237,81 (2010,00 – 2858,67)	0,001	-119,31 ± 85,41 (-276,00 – 27,00)
P	0,110	0,072		0,592

Table 3 : caspase-3 expression.

Group	n	Caspase-3 expression				p
			SD	Min	Max	
Control	12	1,17	0,322	0	3	0,625
Treatment	12	2,17	0,777	0	7	

stem cells are also expected to have the same effect in terms of triggering proliferation and preventing death.In this study the corneal endothelium cell densities throughout the sample averaged 2656.67 cells/mm^2, with a range between 2076.3 to 3034 cells/mm^2. Sailstad and Peiffer (1981) reported an average rabbit endothelium density of 2998 cells/mm^2, while Morita (1995) reported its densities were between 2180 and 3460 cells per mm^2. In the study by Brambatti, it varied between 2336 and 2771.50 cells per mm^2, while this study showed that the exposure of phacoemulsification ultrasound significantly decreases corneal endothelium cell density. A study by Nemet *et al* (2007) reported a decrease in endothelial cell density up to 551 cells/mm^2, however, in this study the average cell decline was 93.69 cells/mm^2 and 119.31 cells/mm^2 in the control and treatment groups. This difference is mainly due to the varying types of machines used, which showed the safety property of machines against corneal endothelium. Although, it is statistically significant in each group, it seems the damage has not been severe enough to evaluate the therapeutic effect of intracameral LMSCs secretome.

Another factor which influences this study is the measurements, which were limited in the central area, while a research conducted by Kim *et al* (2015) observed changes in corneal endothelial parameters measured in central, superior, temporal, inferior and nasal areas. This showed that corneal endothelial wound healing is mainly observed in the temporal area ie in the primary incision with insignificant changes in the central area do. Usta *et al* (2017) investigated the effect of autologous serum in the anterior chamber of the rabbit's eye after damaging the phacoemulsification ultrasound energy, which showed no significant difference between the treatment and control groups in terms of endothelium cell density on daily measurements fromday 0, 1 and 7. The difficulty in measuring specular microscopy on rabbit eyeballs was one of the causes of corneal measure in the same area being examined due to the absence of visual fixation and a headrest design not suitable for rabbits. Furthermore, this has the potential to cause a shift in the area examined at different times thereby reducing the quality (Sailstad and Peiffer, 1981; Morita, 1995; Albuquerque *et al*, 2015; Coyo *et al*, 2016).

The effect of LMSCs secretome on endothelial cell density and caspase-3 expression has not been examined using in vivo. Although there are positive results associated with the application of LMSCs secretome, there were no significant differences between the groups receiving LMSCs secretome and BSS as a control in terms of changes in endothelial cell density and caspase-3 expression. In this study, LMSCs secretome proved to have safety property and intracamerally utilized to determine any significant inflammation sign during observation. Nakahara *et al* (2013) and Sun *et al* (2017) showed that giving MSCs secretone provides positive results on invitro corneal endothelial cell proliferation. Corneal endothelial cell cultures originating from human donors provided additional bone marrow MSCs secretome which showed positive effects on corneal endothelial culture in terms of maintaining hexagonal cell morphology, maintaining functional proteins through ZO-1 and Na/K-ATPase expression by increasing cell proliferation through Ki-67 expression by 2 times compared to the control Group.

According to a study by Lu *et al* (2010), human corneal endothelial cells in vitro proliferate on day 2, formedin polygonal groups administered with MSCs secretome and is subcultured to passage 6 while maintaining its shape. While in the culture group without media, proliferation occurred on the 3rd day and in the subculture it reached the 2nd passage. Endothelial cells in both groups showed a function phenotype by expressing ZO-1 and Na-K-ATPase. Furthermore, Sun *et al* (2017) made a model of corneal endothelium dysfunction using rabbit eyes scratched with endothelium

and administered with MSCs secretometherapy. On the 7th day, it was cleared and its thickness rapidly decreased. Cell density was 2898 cells/mm^2 in the normal group, 2008 cells/mm2 in the conditioned media group in day-3, and 2654 cells/mm2 in day 7. In contrast, the control group in which the endothelium was damaged had severe edema with the time factor examination noted. Although in the study of Lu *et al* (2010) the endothelial cells was able to show proliferation on day 2, tit wasn't enough to provide significant results on endothelium cell density on day 3. Therefore,an examination with longer time period is needed to ensure endothelial cell proliferation affects cell density.

MSC and MSCs secretome are known to have positive effects in decreasing cell apoptosis and increasing its survival rate. Dasari *et al* (2007) examined its effect on the regulation of apoptosis in a mouse model of spinal cord injury and analyzed that the administration reduced apoptosis through caspase-3 pathway regulation. Rouhbakhshzaeri *et al* (2018) investigated its effect on endothelial cell loss after phacoemulsification in the exvivo pig eyeshowed that its incubation with MSC-CM significantly reduced the endothelial cell loss rate by an average of 1.29% ± 0.91% compared to the control group with 5.33% ± 3.24% ($p < 0.05$). Studies which stimulate corneal endothelial cells to proliferate are generally invitro and exvivo with the aqueous humor factor ruled out. Conversley, under normal conditions in aquos humor,the TGF-â is insufficient to hold endothelial cells out of the G1 phase. Liu *et al* (2018) examined changes in aqueous humor after phacoemulsification and obtained a significant increase in malondialdehyde, prostaglandin E2 and interleukin-1. Other researchers found that the TGF-â levels increased significantly after the phacoemulsification action. (Zhang *et al*, 2016). Liu (2002) showed a circadian rhythm effect on differences in TGF-â2 and FGF-2 levels in rabbit humor aquos. The total levels of TGF-â2, active TGF-â2 and bFGF in aquos humor changed over a 24-hour period. TGF-â2 and bFGF levels increase between 4am and 8am. Active TGF-â2 levels increase between 8 pm and midnight. The same pattern of change was found when the aquos humor sample was taken in constant dark conditions. In vitreous humor, total TGF-â2 is active, while bFGF remains unchanged for 24 hours. The influence of aqueos humor and circadian rhythm affects the FGF contained in LMSCs secretome, which was analyzed at the end of theprocedure.

Intraocular surgery and anterior segment inflammation was used to reducethe risk associayed with endothelial cell density, which was closely related to changes in the levels of cytokines in aquos humor. A study by Yaguchi *et al* (2017), which analyzed the relationship between endothelial cell density and increased aquos humor cytokines showed that endothelial cell was negatively correlated with increased levels of aquos humor cytokines especially cytokines IL-1á, IL-4, IL-13, MIP -1â, TNF-á and E-selectin. Conversely, the administration of conditioned media which is cytokines rich changes the levels of aquos humor cytokines and affects corneal endothelial cells.The effect depends on the cytokines contents using positive growth factors for corneal endothelium such as FGF-2, which triggers endothelial cells to exit the G1 phase and proliferate. It was observed from invitro studies that the FGF-2 signaling pathway in promoting proliferation is conducted through the phosphorylation of p27 on Ser10, Thr187, PI3-K and ERK1/2 pathways. The PI3K pathway, leads to activation of Akt, which plays an important role in cell survival, inhibits proapoptotic cytokines such as Bcl-2, Bad, Bax, caspase-9 and activation of caspase-3 as an effector of apoptosis. (Jeong *et al*, 2007; Lee *et al*, 2007; Lee *et al*, 2011; Nakahara *et al*, 2013; Zhang *et al*, 2015). The secretomeutilized in this research are expected to posses high FGF-2 content according to research from Bhang *et al* (2014), which showed that using the three-dimensional (3D) spheroid culture method in human adipose stem cells produced conditioned media with VEGF concentrations, FGF-2, as well as HGF and chemokines which were 23 to 27 times higher than the conditioned media produced with conventional monolayer cultures. However, this study failed to take measurements of FGF and other cytokine levels, therefore, further research is needed to ensure the levels are contained and capable ofaffecting corneal endothelial cells.

Its limitation is associated with, the single observation periods on the 3rd day, while theadministration of LMSCs secretome was at the end of the procedure. Specular microscope measurements were carried out in the central area in order not to describe the state of corneal endothelial cell density in other areas. Furthermore, thecytokine level of LMSCs secretome and aquos humor after the procedure were not assessed.

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