

07. The Effect of Limbal Mesenchymal

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The Effect of Limbal Mesenchymal Stem Cell Secretome on Corneal Expression of Interleukin-1 in Experimental Model of Corneal Alkali Injury

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Abstract

Background: To investigate the effect of limbal mesenchymal stem cells secretome (L-MSCS) on corneal expression of Interleukin-1 (IL-1) in alkali ocular chemical injury animal model.

Methods: Secretome was collected from limbal mesenchymal stem cells of corneoscleral tissue of healthy male rabbit. Rabbits were exposed to NaOH. The research subjects consisted of 3 groups, normal control group, Balance saline solution (BSS) group, and L-MSCS group. The level of corneal expression of IL-1 by immunohistochemical staining were analyzed.

Results: The mean level of IL-1 expression with immunoreactive score (IRS) was 3.2 in normal group, 5.4 in BSS group, and 5.1 in L-MSCS group after seven days administration of topical instillation. The level of IL-1 expression in L-MSCS group was lower than BSS group. There was no difference in IL-1 expression between L-MSCS group and the BSS group ($p > 0.05$) but a significant difference was found between normal group and BSS group, normal group and L-MSCS group, and between the three groups ($p < 0.05$).

Conclusions: Topical instillation of L-MSCS has a potential effect on decrease the level of IL-1 expression in the 7th day of chemical ocular injury animal model even though the difference was not statistically significant.

Keywords: Limbal mesenchymal stem cell; secretome; Interleukin-1; corneal alkali injury; corneal wound healing

1. Introduction

Chemical ocular injury is an ocular emergency that requires early immediate and intensive treatment so that the risk of complications can be reduced. Persistent ulcers, slow process of epithelialization, corneal perforation, and angiogenesis are the most common complications due to inflammation, neovascularization, and corneal conjunctivalization post traumatic of alkali injury. Approximately 11.5% - 22.1% of all eye trauma are chemical injuries. Males are more susceptible which is around 73.5% with the majority of age are 20-40 years (Eslani et al., 2014; Baradaran-Rafi et al., 2017; Zhao et al., 2010; Alan, 2011; Ke et al., 2015).

In alkali ocular injury there is damage to the corneal epithelium and an increase in proinflammatory cytokines such as interleukin-1 (IL-1) which the main cytokine on corneal wound healing. Therefore, the reduction of IL-1 expression is very important to prevent further complications such as corneal perforation.

The cornea is a transparent and avascular tissue, so that nutrients and oxygenation of the cornea are obtained by diffusion through the aqueous humor, tear film, and limbal blood vessels. The success of the healing process mainly depends on the process of cell migration and proliferation which is controlled by the release of growth factors in the wound area (Diegelmann & Evans, 2010; Klenker et al., 2017; Dinc E et al., 2020).

The main goals of chemical ocular injury therapy are to accelerate epithelialization, reduce inflammation, promote repair and prevent complications. In the acute phase of chemical injury, anti-inflammatory, anti-angiogenic therapy and improvement of corneal epithelial healing are important aspects in the treatment and prognosis of chemical trauma. Limbal Mesenchymal Stem Cells Secretome (L-MSCS) have been identified as a source of growth factors, which have significant potential to reduce proinflammatory cytokines and repair-regenerate damaged tissue. Beside that, the study by Komaratih et al., regarding the administration of topical instillation to corneal L-MSC with alkali injury in vitro showed that adipose MSC decreased Vascular Endothelial Growth Factor (VEGF) which exhibited anti-angiogenic activity (Klenker et al., 2017; Dinc E et al., 2020; Bermudez et al., 2016; Bermudez et al., 2015; Komaratih et al., 2019). The aim of this study is to analyze role of L-MSCS on corneal IL-1 expression in experimental model of alkali injury which exposure by NaOH on day 7.

2. Material and Method

2.1. Limbal mesenchymal stem cell (L-MSC) secretome

The cell was cultured at the stem cell laboratory, Universitas Airlangga. To obtain secretome, 5 x 10³ cells/well of L-MSC was treated for 24 hours using media composed of α -MEM, 1% NEAA, 1% penicillin-streptomycin, 1% amphotericin B, and 2 % FBS). The secretome was collected and filtered using 0.45 μ m millipore, the osmolarity and acidity were adjusted for 270-300 mOsm/L and 7.2-7.5, respectively. The secretome was packaged into a single dose application in a sterile Eppendorf tube and stored at - 20oC until further application.

2.2. Animal model of alkali injury

A corneal alkali injury was made in the right eye of each rabbit. Each rabbit was anesthetized by intramuscular injection of xylazine-ketamine (ketamine 40 mg/ml, xylazine 20 mg/ml). A piece of Whatman filter paper (7 mm diameter) soaked in 1N NaOH was applied to the center of the cornea for 30 seconds. The cornea was rinsed with 30 ml of saline for 30 seconds after exposure of NaOH.

2.3. Animal model treatment

Twenty one of New Zealand white rabbits were randomly divided into three groups. The first group (normal control group) was the group without NaOH exposure and therapy. The second group (NaOH + BSS) was the group with exposure to NaOH and balance saline solution (BSS) eye drops. The third group (NaOH + limbal MSC secretome) was the group with exposure to NaOH and limbal MSC secretome eye drops. BSS and limbal MSC secretome eye drops were administered every 8 hours for 7 days. The degree of clinical corneal neovascularization, histopathological neovascular cell count were measured 7 days after treatment

2.4. Evaluation of Corneal IL-1 Expression

Enucleation was performed on the 7th day after exposure to NaOH and eyeballs were stored in formalin. The paraffin-blocked tissue was cut with a thickness of 3 μ m and placed on a glass object and then incubated

at 45°C for one night. The preparations were paraffinized and washed with running water, then incubated with 3% H₂O₂ for 3 minutes and washed with running water. The preparations were incubated with citrate buffer PH 6 at 95°C for 45 minutes, then cooled for 30 minutes and washed with PBS twice for 3-5 minutes. The preparations were then incubated with blocking serum for 15 minutes and drained. Immunohistochemical staining was performed with IL-1 antibody. The dried preparations were incubated with primary and secondary antibody. The stained tissue was observed using a light microscope with a magnification of 400x and images were taken. The level of IL-1 expression was evaluated with immunoreactive score (IRS) which divided into four groups. There are score 0-1 (negative expression), score 2-3 (weak positive), score 4-8 (moderate positive), score 9-12 (strong positive).

2.5. Statistical analysis

The data was analyzed using SPSS where $p < 0.05$ was considered to be statistically significant. Statistical measures are presented in a descriptive table (e.g median, interquartile deviation, mean and standard deviation). Statistical analysis for the level of IL-1 expression between 3 groups were analyzed with Kruskal Wallis while between 2 groups were analyzed with Mann Whitney.

3. Results

The mean level of IL-1 expression by immunohistochemistry showed in the normal control group that was 3,2; the group which given with NaOH and BSS was 5,4 while in the group NaOH + L-MSCS was 5,1. The mean data of IL-1 expression is shown in Figure 1.

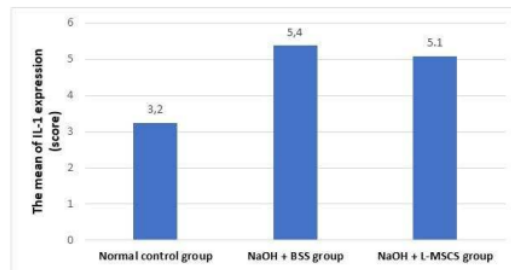


Figure 1. The difference of IL-1 expressions between three groups

From the statistical examination using the Kruskal-Wallis test shown in table 1, there was a significant difference in IL-1 expression between the normal group, BSS, and L-MSCS group ($p = 0.034$). By using the Mann-Whitney test, if using of $\alpha = 5\%$, there was a significant difference between the level of IL-1 expression between the normal group and the BSS group ($p = 0.013$) while there was no significant difference between the group with L-MSCS group and the normal group ($p = 0.055$) and between L-MSCS group and the BSS group ($p = 0.848$). This indicates that the expression level of IL-1 in the group with topical instillation of L-MSCS was lower than the group with topical BSS and shows a decrease although the difference is not significant.

Table 1. Statistical analysis of IL-1 expressions between three groups

Groups	N	IL-1 expression (score)			p
		Median \pm IQD	Min	Max	
Normal control	7	3,60 \pm 1,30 ^a	1,40	5,40	0,034*
NaOH + BSS	7	5,20 \pm 0,70 ^b	4,00	7,00	
NaOH + L-MSCS	7	5,60 \pm 1,30 ^b	2,60	7,40	

*p<0,05 = significant

^{ab} Different superscripts indicate there is differences between groups

A representative image of the IL-1 assay in the normal control group, BSS and the L-MSCS group is shown in Figure 2. IL-1 expression is indicated by the brownish color of the corneal cells and corneal tissue.

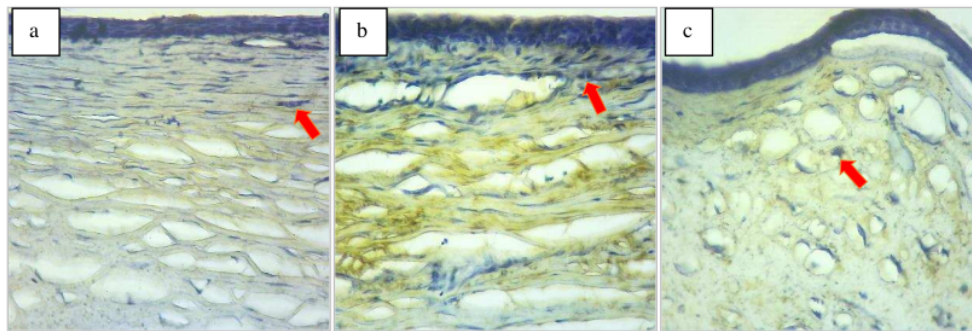


Figure 2. Comparison of representative histopathological features of IL-1 expression with immunohistochemical staining in central cornea between normal control group (a), NaOH + BSS group (b) and NaOH + L-MSCS (c), red arrows represent cells expressing IL-1 with magnification 400x

4. Discussion

IL-1 is a multipotent cytokine with a variety of activities, including critical mediation of the acute-phase wound healing response, chemotactic effects and inflammatory cell activation, and stimulation of neovascularization. IL-1 was expressed by corneal epithelial cells in alkali ocular injury on days 2, 5, 7, and 14 days after trauma. IL-1 also induces corneal stromal cells to express keratocyte growth factor (KGF) so that the production of IL-1 in basal epithelial cells during the early stages of alkali ocular injury cause corneal stromal damage and induced to secretion of KGF (Sotozono et al., 2010; Wilson, 2021).

In addition to the acute phase of corneal injury, IL-1 cytokines also modulate the proliferative phase of the apoptotic response of keratocytes and myofibroblasts. IL-1 also regulates the expression of HGF and KGF produced by corneal keratocytes and fibroblasts that also play a role in corneal epithelial cell healing, as well

as the expression of collagenases and metalloproteinases required for breakdown and remodeling of the stromal matrix.

In measuring the level of IL-1 expression, as measured by the IRS score, there was no significant difference after topical instillation of L-MSCS group and the BSS group. This is in contrast to a previous study by Dinc et al., (2020) which stated that the IL-1 scores significantly decreased in the group which given with bone marrow and adipose MSCS when compared to the alkali control group while the IL-1 scores significantly increased both in the epithelial and stromal layers in the alkali control group without any treatment when compared to the normal group.

In this study, although there was a decrease in the IL-1 expression score, there was no significant difference between the limbus SPM metabolite group and the BSS group. This could be due to the fact that in this study enucleation and evaluation of IL-1 was carried out on the 7th day after chemical trauma. As is known, the 7th day of the corneal healing process is still the beginning of the proliferation phase so that proinflammatory cytokines such as IL-1 have not decreased much. The IL-1 cytokine will decrease to normal levels again on day 14 (Sotozono et al., 2010).

Within one hour after trauma to the corneal epithelium, large numbers of immune cells migrate into the corneal stroma from the limbal vessels. These cells are attracted into the stroma by IL-1 and TNF- which are released by injured epithelial cells through chemotaxis processes and cascade effects known as cytokine networks. After that, these two cytokines bind to receptors on keratocytes as the beginning of the apoptotic process in the anterior stroma, namely in the proliferative phase. This can be reason for the decrease in IL-1 which has not been significant in this study (Wilson, 2021).

Research by Wilson & Esposito, (2010) reported that IL-1 is also expressed by healthy corneal epithelial cells and released into tears and corneal stroma after trauma. Sotozono et al., (2010) also reported that in healthy corneas it is still possible to detect IL-1 on keratocytes using immunohistochemistry. IL-1 in small amounts will keep cells alive but in moderate amounts this cytokine stimulates apoptosis of these cells. This causes that in this study IL-1 expression was obtained with a score of 3.2 (weak positive) in the normal group without any treatment. There was still a significant difference between the normal group and the group with limbus SPM metabolites because on the 7th post-traumatic day IL-1 expression had not decreased to normal levels because according to research by Sotozono et al., (2010) it was stated that IL-1 levels decreased to normal on the 14th post-traumatic day.

The role of limbus SPM metabolites in decreasing IL-1 expression is the content of growth factors in it, especially EGF and HGF. HGF can inhibit the expression of TNF- α , IL-1, and IL-6, as well as the infiltration of inflammatory cells, resulting in an immune modulating effect and the expression of proinflammatory cytokines such as IL-1, IL-6, and TNF- α can be decreased. EGF itself is one of the growth factors that play an important role in the regeneration of corneal epithelial cells and the wound healing process (Park et al., 2020; Dinc et al., 2020).

Research by Oh et al., (2012) stated that the immunosuppressant mechanism mediated by SPM has not been fully elucidated but SPM has been shown to inhibit T cell function and decrease the expression of IL-1 and TNF- cytokines. SPM metabolites contain many proinflammatory cytokines and increase the secretion of IL-10 from dendritic cells.

Another important cytokine that affects the structure of the epithelium and stroma besides IL-1 is IL-6. IL-6 is known to have a dual role in the inflammatory process. IL-6 acts as a proinflammatory cytokine. This is known from the high expression of IL-6 on day 7 after the cornea is exposed to alkaline chemical trauma and causes infiltration by neutrophils which cause stromal melting of the cornea. On the other hand, IL-6 is also an important immunoregulatory cytokine that controls the development of dendritic cells. The role of SPM metabolites in reducing IL-6 cytokines is evident from a significant decrease in IL-6 levels in corneal epithelial cell cultures that were damaged by alkaline chemical trauma on day 7 so that it can be an answer in

this study why the decrease in IL-1 cytokine expression caused not significant but followed by improved histopathological changes. This can be due to the presence of the IL-6 cytokine pathway which may decrease so that it affects the process of corneal tissue repair (Sotozono et al., 2010; Oh et al., 2012).

5. Conclusion

In conclusion, topical instillation of L-MSCS has a potential effect on decrease the level of IL-1 expression in the 7th day of chemical ocular injury animal model even though the difference was not statistically significant.

6. Acknowledgements

None

7. Conflict of Interest

Nul

8. Ethical Standard

Ethical approval was obtained from Institutional Ethical Committee of Faculty of Veterinary medicine, Universitas Airlangga (No: 2.KE.114.09.2021). All Procedures were performed with ethical standards.

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