Original Research

A FIRST STEP TO NOVEL APPROACH FOR TREATING ALKALI INJURY OF THE CORNEA: EFFECT OF PLATELET RICH FIBRIN LYSATES ON CULTURED RABBIT (*Oryctolagus cuniculus*) LIMBAL STEM CELL PROLIFERATION EXPOSED TO SODIUM HYDROXIDE

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ABSTRACT

Chemical injuries of the eye produce extensive damage to the ocular surface and limbal stem cells, resulting in permanent unilateral or bilateral visual impairment. Alkali injuries occur more frequently than acid injuries. Platelets are a rich source of potential wound healing, promoting polypeptide growth factors. This study aimed to investigate the effect of platelet-rich fibrin (PRF) lysates on limbal stem cell proliferation, which was exposed to sodium hydroxide that resembled limbal stem cell deficiency due to chemical trauma. Confluent rabbit (Oryctolagus cuniculus) limbal stem cells wounded using 20µL of 0.00625 M sodium hydroxide (pH 13) were treated with platelet-rich fibrin lysates (PRF) (0, 5, and 10%). PRF lysates were prepared from peripheral rabbit blood according to Choukroun's method without using anticoagulant and foreign factors for platelet activation. The proliferation of limbal stem cells was measured by a 3-(4,5-dimethylthiazol-2-yl)-2.50 diphenyl tetrazolium bromide (MTT) colorimetric assay at 24, 48, and 72 hours after exposure to sodium hydroxide. Proliferation significantly increased limbal stem cells with PRF lysates 5% ($p<0.01$) and 10% ($p<0.01$) group compared with the control (PRF 0%). There was no significant difference between PRF lysates of 5% and 10% ($p>0.01$). The highest proliferation of limbal stem cells was found in the PRF lysates 5% group after 48 hours (100.24%). PRF stimulated limbal stem cell proliferation in chemical trauma caused by the sodium hydroxide model. PRF repaired the limbal stem cell niche and influenced the limbal stemness. The present findings warrant further research on PRF as a novel alternative treatment for limbal stem cell deficiency.

Keywords: *Platelet-rich fibrin lysates; limbal stem cells; limbal stem cells deficiency; proliferation; sodium hydroxide*

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Highlights:

1. Sodium hydroxide causing platelet rich fibrin stimulates limbal stem cell proliferation in chemical trauma.
2. Limbal stem cell niche and influences limbal stemness was repaired by Platelet Rich Fibrin.

INTRODUCTION

Limbal stem cell deficiency is related to the destruction of limbal stem cells or insufficient stromal microenvironment to support stem cell function. Limbal stem cell deficiency is characterized by intrusion of conjunctival epithelial cells, which have goblet cells on the cornea, neovascularization, chronic inflammation, and persistent epithelial defect. The most common etiology of limbal stem cell deficiency is chemical trauma. According to several studies, the ratio of the relative frequencies of acids and alkalis as the causative agents of chemical injury ranges from 1:1 to 1:4. Currently, no medical treatment is available to restore limbal stem cells after acute inflammation and inciting event control (Anderson et al. 2001, Kocaba et al. 2016).

Regeneration of limbal stem cells is regulated by microenvironment/niche through interaction between mesenchymal cells, cytokine, oxygen, nutrient, and growth factor. The growth factor is important in maintaining the deficiency of limbal stem cells and promoting the proliferation of limbal stem cells. Platelet is the main source of growth factors which has a role in tissue regenerate-platelet-rich fibrin. It is a new revolution in the platelet therapy concept (Castro-Muñozledo 2015, Freire et al. 2014, Naik et al. 2013).

Platelet-rich fibrin was first published in France by Choukroun et al. study in 2001 (Naik et al. 2013). Platelet-rich fibrin becomes more frequently applied in order to support the wound healing process because it has many advantages compared to the PRP, such as the simple and effective collecting process, no added substances from the outside to activate the platelets, arrested platelets into the fibrin network, and support for hemostasis, cell migration, and proliferation. Platelet-rich fibrin is obtained from the blood without anticoagulant and centrifuged just once to obtain the PRF matrix. The platelets in the fibrin fiber network were activated to release growth factors without additional components, such as bovine thrombin and calcium chloride (Nguyen et al. 2016).

Many growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF-β are released from PRF. Recently, studies have demonstrated that PRF has a very significant slow, sustained release of key growth factors for at least one week and up to 28 days, which means that PRF could stimulate its environment for a significant time during wound healing (Wu et al. 2012). Platelet-rich fibrin is applied in many clinical trials, but the mechanism of PRF to wound healing has not been studied much. This research was conducted to evaluate the effect of PRF on the proliferation in cultures of rabbit limbal stem cells exposed to sodium hydroxide.

MATERIALS AND METHODS

This study was an in vitro experimental study in cultured rabbit limbal stem cells exposed to natrium hydroxide and treated with platelet lysates. All rabbits were cared for following the procedures and designs approved by the animal experimentation ethics (no.771-KE) committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia. This study aimed to investigate the difference in limbal stem cell proliferation exposed to natrium hydroxide resembling the limbal stem cell deficiency model caused by chemical trauma. This study was a true experimental study using a posttest-only control group design due to the presence of intervention, control, replication, randomization, and observation after intervention only. Experimental units were divided into three different intervention groups which were then evaluated 24, 48, and 72 hours after the intervention. First was the control group without PRF, the second was the intervention group with PRF of 5%, and the third was the intervention group with PRF of 10%. The independent variables of this study were PRF lysates and observation time. The dependent variable of this study was limbal stem cell proliferation.

RESULTS

This study used passage 5 for limbal stem cell culture with viable cell count 77% (1,62 x 10^6 cell/mL) and total cell count 2.09 x 10^6 cell/mL. Cell surface marker identification used CD45, p63, CD 73, CD 90, and CD 105 in the population of limbal stem cells isolated from the limbus. CD 73, CD 90, and CD 105 were positively expressed on limbal stem cell culture and negatively expressed for CD 45. It indicated that the limbal stem cell culture population was true limbal mesenchymal stem cell. p63 was also positively expressed, showing that the population was a limbal epithelial stem cell. The ratio of limbal mesenchymal and limbal epithelial stem cells was 1: 1.005. Natrium hydroxide exposure used 20 µl of 0.00625 M in every well and caused less than 50% limbal stem cell damage.

PRF effect was evaluated by assessing cell viability using an MTT assay. MTT assay result was a percentage of the viable cell. Proliferation was highest in group II (PRF 5% intervention) in 48 hours of observation. Mean of viable cells percentage on control group in 24, 48, 72 hours observation were a (79.73 $\pm$ 14.23)%, (74.60 $\pm$ 25.00)%, and (62.7 $\pm$ 12.40)%. Mean of viable cells percentage on group with PRF 5% in 24, 48, 72 hours observation were (90.74 $\pm$ 6.22)%, (100.24 $\pm$ 14.88)%, and (93.61 $\pm$ 13.35)%, respectively. Mean of viable cells percentage on group with PRF 10% in 24, 48, and 72 hours observation were respectively (89.17 $\pm$ 8.42)%, (100.17 $\pm$ 19.72)%, and (92.50 $\pm$ 16.30). There was a significant difference between group II (PRF 5%) and group III compared with group I (control) ($p<0.01$). There was no significant difference between group II (PRF 5%) and group III (PRF 10%). Statistically, there was no significant difference between the time of observations ($p>0.01$).

Table 1. Percentage of viable cells among various groups

Group	Time (hour)	n	Percentage of the viable cell (%)	
			Min.	Max.
Group 1 (control)	24	8	60.58	103.31
	48	8	14.55	89.85
	72	8	47.38	78.02
	Total	24	14.55	103.31
Group II (PRF 5%)	24	8	70.21	120.53
	48	8	77.00	115.24
	72	8	77.68	116.41
	Total	24	70.21	120.53
Group III (PRF 10%)	24	8	78.22	102.37
	48	8	80.88	134.02
	72	8	66.91	116.61
	Total	24	66.91	134.02
Total	24	24	60.58	120.53
	48	24	14.55	134.02
	72	24	47.38	116.61
	Total	72	14.55	134.02

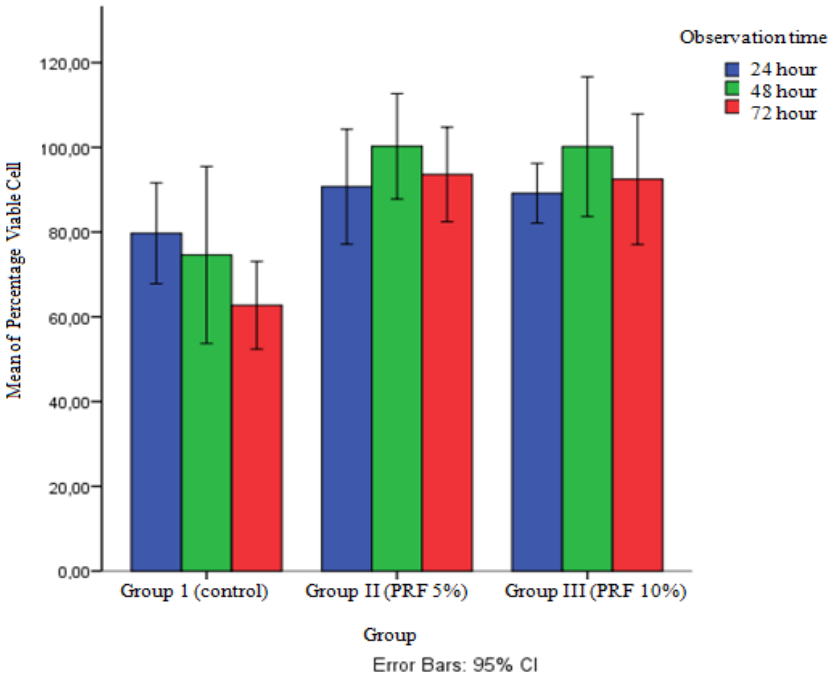

Figure 1. Mean of viable percentage cells among various groups

Note: The results are expressed as the mean of percentage viable cell + SD. The results had statistically significant differences concerning control ($p<0.01$) (two-way ANOVA), and significant differences concerning control ($p<0.01$) (Post Hoc Tukey HSD)

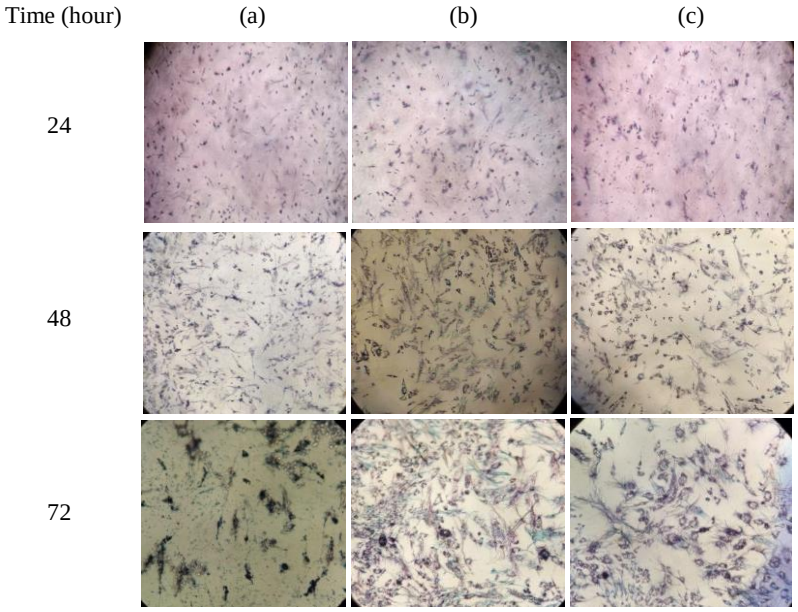

Figure 2. Limbal stem cell was exposed to sodium hydroxide and incubated for 24, 48, and 72 hours. Limbal stem cell was exposed to sodium hydroxide (a), sodium hydroxide and treated by PRF 5% (b), to sodium hydroxide and treated by PRF 10% (c).

DISCUSSION

Homeostasis of corneal epithelium is essential for maintaining a healthy ocular surface and for corneal transparency and accurate vision. Continuous renewal epithelium is provided by a population of adult stem/progenitor cells residing in the limbus, the transitional zone between the vascular conjunctiva and the avascular transparent cornea. Limbal stem cells require a particular environment to retain their stem cell properties (Sacchetti et al. 2018). The environment is provided by the stem cell niche in which signaling from adjacent cells, as well as properties of the basal membrane, are believed to play a role in the maintenance of their 'stemness'.

When limbal stem cells are depleted below a certain threshold, a clinical sign of limbal epithelial stem cell deficiency (LSCD) appears, causing gradual vision loss. LSCD occurs due to disease or damage to the limbal stem cell population. Deficiency can arise from injuries, including chemical or thermal burns. In this study, limbal stem cell damage was less than 50% after being exposed to 20 µl of 0.00625 M sodium hydroxide with pH 13 (Kadar et al. 2013). This study differed from the prior one by Pattamatta et al. (2009), which used 0.5 µl of 0.1 M sodium hydroxide. A preliminary study used 0.5 µl of 0.1 M sodium hydroxide, and all limbal stem cell cultures were damaged. The hydroxyl ions rapidly penetrated the eye, causing saponification of cellular membranes with massive cell death and partial hydrolysis of corneal glycosaminoglycans and collagen.

Platelet-rich fibrin described by Choukroun is a second-generation platelet concentrate consisting of a 3-D polymerized autologous fibrin matrix incorporating platelets, growth factors, cytokines, circulating stem cells, and a small number of leukocytes, which have key roles in homeostasis and wound healing. The intrinsic incorporation of these factors within this scaffold allows their progressive and controlled release as the fibrin mesh degrades. PRF releases autologous growth factors gradually, resulting in a stronger and more durable effect of proliferation, differentiation, migration, and matrix synthesis by binding to specific cell-surface-receptor (Cakmak et al. 2017).

In this study, the PRF 5% group had the highest proliferation rate within 48 hours of observation. Increasing proliferation in 48 hours of observation was related to the population doubling time on stem cell culture. The previous study showed that the human corneal epithelial cell line population doubling time was 45 and 42 hours (Fan et al. 2011). The PRF optimum concentration was limited, while studies on prior platelet products PRF had been widely conducted. In the previous study, the optimum concentration of PRP varied from 50% to 10% or less than 1%. Soffer et al. (2003) considered 0.5-1% as the optimum concentration for cellular proliferation and mineralization rates. However, Freire et al. (2014)

found that 50% PRP was the optimum concentration for osteoblast proliferation.

On the other hand, 10% PRP was sufficient to induce marked cell proliferation of MSC derived from adipose tissue. In addition, 5% PL is considered the optimum concentration for MSC and DPSCs proliferation and osteogenesis (Saeed et al. 2017). In this study, the cell proliferation rate was measured by MTT assay. MTT protocol includes a liquid handling step to solubilize formazan precipitates during an assay, thus making the protocols less convenient (Saeed et al. 2017).

In the previous study, the potential function of PRF in wound healing was investigated on Human Gingival Fibroblasts (HGFs) for cell proliferation and migration, which provided evidence for PRF use in periodontitis treatments. The results showed that PRF promoted the proliferation and migration of HGFs (Nguyen et al. 2016).

PRF is rich in various growth factors, including transforming growth factor-beta 1 (TGFβ-1), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF), fibroblast growth factor, epidermal growth factor (EGF), and hepatocyte growth factor. It promotes the movement, proliferation, and differentiation of stem cells, neovascularization, and collagen synthesis. IGF prevents cells from undergoing apoptosis, VEGF stimulates vasculogenesis and angiogenesis, and EGF function in cell proliferation and differentiation (Duan et al. 2017).

In this study, PRF increased limbal epithelial stem cells and limbal mesenchymal stem cells. There was a positive expression of mesenchymal and epithelial surface markers. Cell surface marker identification used CD45, p63, CD 73, CD 90, and CD 105 in the population of limbal stem cells, which were isolated from the limbus. CD 73, CD 90, and CD 105 were positively expressed on limbal stem cell culture and negatively expressed for CD 45. It indicated that the limbal stem cell culture population was a true limbal mesenchymal stem cell. p63 was also positively expressed and it showed that the population was also a true limbal epithelial stem cell. The ratio of limbal mesenchymal stem cell and limbal epithelial stem cell was 1: 1.005 (Mark et al. 2013, Nakatsu et al. 2014). A previous study reported that MSCs supported the survival, growth, and proliferation of various types of cells, such as hematopoietic stem cells, hepatocytes, cardiac progenitor cells, neural stem cells, neurons, and Schwann cells. Hu et al. (2012) demonstrated that MSCs had the same effect on corneal limbal epithelial cells. Several reports previously described the favorable effect of MSC transplantation after ocular surface destruction may be partly attributed to the proliferation of endogenous corneal cells evoked by MSCs.

Strength and limitation

The study addresses a significant clinical problem, which is chemical injuries to the eye resulting in permanent visual impairment. The study investigates a potential novel treatment option using platelet-rich fibrin (PRF) lysates to promote limbal stem cell proliferation and repair the limbal stem cell niche. The study uses a reliable method to assess cell proliferation, the MTT colorimetric assay. The study includes a control group and multiple treatment groups with different concentrations of PRF lysates.The study did not include a detailed analysis of the mechanism by which PRF lysates promote limbal stem cell proliferation. The study did not investigate the long-term effects of PRF lysates on limbal stem cell proliferation or potential adverse effects.

CONCLUSION

PRF promotes increasing proliferation in cultured limbal stem cells exposed to natrium hydroxide that resembled deficient limbal stem cells caused by alkali trauma through repairing niche limbal stem cell conditions. PRF is expected to benefit limbal stem cell regeneration, especially in limbal stem cell deficiency caused by alkali trauma.

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Conflict of interest

None.

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Author contribution

WEP and AM conceptualized, wrote, and revised the manuscript. MT collected and analysis data, finalized the manuscript.

REFERENCES

Anderson D, Ellies P, Pires R, et al (2001). Amniotic membrane transplantation for partial limbal stem cell deficiency. Br. J. Opththalmology 85, 567–575.

Cakmak H, Can G, Can M, et al (2017). A novel graft option after pterygium excision: platelet-rich fibrin for conjunctivoplasty. Eye 31, 1606–1612.

Castro-Muñozledo F (2015). The mammalian limbal stem cell niche: A complex interaction between cells, growth factors and extracellular matrix. In: Biology in Stem Cell Niche. Springer, Switzerland, pp. 23–56.

Duan X, Lin Z, Lin X, et al (2017). Study of platelet-rich fibrin combined with rat periodontal ligament stem cells in periodontal tissue regeneration. J. Cell. Mol. Med. 22, 1047–1055.

Fan T, Xu B, Zhao J, et al (2011). Establishment of an untransfected human corneal epithelial cell line and its biocompability with denuded amniotic membrane. Int. J. Ophthalmol. 4, 228–234.

Freire V, Andollo N, Extebarria J, et al (2014). Corneal wound healing promoted by 3 blood derivatives: An in vitro and in vivo comparative study. Cornea J. 33, 614–620.

Hu N, Zhang Y, Gu H, et al (2012). Effect of bone marrow mesenchymal stem cells on cell proliferation and growth factor expression of limbal epithelial cells in vitro. Ophthalmic Res. 48, 82–88.

Kadar T, Dachier S, Horwitz V, et al (2013). Development of limbal stem cell deficiency following chemical injury-pathogenesis and therapeutic strategies. US Opththalmic Rev. 6, 101–104.

Kocaba V, Damour O, Auxenfans C, et al (2016). Limbal stem cell deficiency management. A review. J. Fr. Ophtalmol. 39, 791–803.

Mark P, Kleinsorge M, Gaebel R, et al (2013). Human mesenchymal stem cells display reduced expression of CD 105 after culture in serum-free medium. Stem Cells Int. 2013, 1–8.

Naik B, Karunakar P, Jayadev M, et al (2013). Role of platelet-rich fibrin in wound healing: A critical review. J. Conserv. Dent. 16, 284–293.

Nakatsu N, González S, Mei H, et al (2014). Human limbal mesenchymal cells support the growth of human corneal epithelial stem/ progenitor cells. Investig. Ophthalmol. Vis. Sci. 55, 6953–6959.

Nguyen H, Nguyen M, Trinh V, et al (2016). Platelet-rich fibrin influences on proliferation and migration of human gingival fibroblast. Int. J. Exp. Dent. Sci. 5, 83–88.

Pattamatta U, Willcox M, Stapleton F, et al (2009). Bovine lactoferrin stimulates human corneal epithelial alkali wound healing in vitro. Investig. Ophthalmol. Vis. Sci. 50, 1636–1643.

Sacchetti M, Rama P, Bruscolini A, et al (2018). Limbal stem cell transplantation: Clinical results, limits, and perspectives. Stem Cells Int. 2018, 1–13.

Saeed M, El-Rahman M, Helal M, et al (2017). Efficacy of human platelerich fibrin exudate vs fetal bovine serum on proliferation and differentiation of dental pulp stem cells. Int. J. Stem Cell 11, 38–47.

Soffer E, Ouhayoun J, Anagnostou F (2003). Fibrin sealants and platelet preparations in bone and periodontal healing. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. 95, 521–528.

Wu C, Lee S, Tsai C, et al (2012). Platelet-rich fibrin increases cell attachment, proliferation and collagen-related protein expression of human osteoblasts. Aust. Dent. J. 57, 207–212.