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Original Article

Effect of Platelet Rich Fibrin Membrane (PRF) or Conjunctival Autograft on VEGF Expression and Microvascular Density Post Conjunctival Excision

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ABSTRACT

The conjunctiva plays a crucial role in maintaining eye health, especially the cornea. Preserving conjunctival tissue integrity is essential to prevent a range of eye disorders, from mild to severe, including blindness. Techniques commonly used for repairing conjunctival damage include autografts, amniotic membrane transplantation, oral mucosal grafts, and PRF membrane. However, it is unclear if PRF membrane grafts have different effects on VEGF expression and angiogenesis compared to conjunctival autografts. This study employs a true experimental design, utilizing a randomized post-test only two-group design, to compare the impact of PRF membrane transplantation and conjunctival autografting on VEGF expression and angiogenesis subsequent to conjunctival excision in adult New Zealand white rabbits. Twenty adult male rabbits were randomly split into two categories: the autograft cohort and the PRF membrane cohort. Conjunctival excisions were performed on the temporal conjunctival aspect of the right eye in each rabbit. In the first group, the conjunctival defect was repaired through conjunctival autografting, while in the second group, the defect was closed using a PRF membrane. After 14 days following treatment, all samples were collected, and the right eyes that had undergone treatment were enucleated. The conjunctival tissues from the treated areas were then processed for histopathological examination. Immunohistochemical and Hematoxylin and Eosin (HE) staining were conducted on each sample. The independent t-test used for statistical analysis demonstrated a notably elevated VEGF expression level in the PRF membrane group in comparison to the conjunctival autograft group ($p<0.05$). In addition, a significant variance in angiogenesis was evident between the PRF membrane and the conjunctival autograft groups ($p<0.05$).

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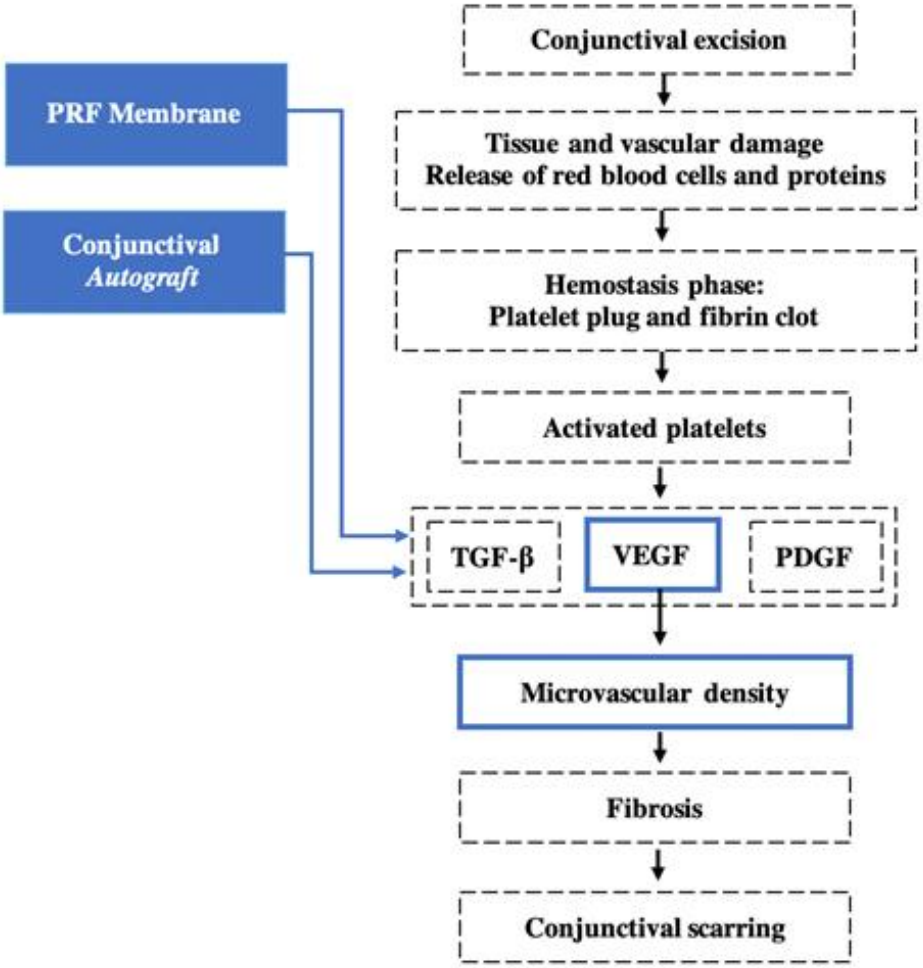
GRAPHICAL ABSTRACT



Introduction

The conjunctiva is a highly intricate tissue located on the eye's surface, playing a central role in upholding the eye's overall health. Its functions are intertwined with those of essential ocular components such as the cornea, tear film, nasolacrimal system, and eyelids. Comprising epithelial layers, the conjunctiva is responsible for upholding the delicate equilibrium of the tear film. Diseases affecting the conjunctiva exhibit a wide spectrum in terms of their etiology, clinical manifestations, and recuperation processes. Minor injuries or defects in the conjunctiva can often heal naturally. However, in cases of extensive damage caused by factors like burns, thermal trauma, or acute chemical injuries, the reconstructive treatment of the conjunctiva becomes imperative. Replacement tissues are utilized to achieve optimal outcomes in the healing process [1, 2]. Several techniques have been developed to enhance the healing of conjunctival defects, including amniotic membrane transplantation, mucosal membrane transplantation, and conjunctival autograft. Amniotic membrane transplantation, while effective, has certain drawbacks, including the potential for disease transmission, reduced transparency, logistical challenges in availability and preparation, and higher costs. Conjunctival autograft, in particular, has gained popularity due to its ease of implementation, lower rates of inflammation, cost-effectiveness, and superior outcomes. Platelet-Rich Fibrin (PRF) membrane has emerged as a promising option.

PRF membrane contains an array of matrix proteins and growth factors critical for wound healing, including Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), and Transforming Growth Factor (TGF-). These growth factors are gradually released over a period of 7 to 28 days. In ophthalmology, specifically in the context of conjunctival defects, PRF has shown significant potential in promoting conjunctival regeneration and wound healing and demonstrated excellent healing outcomes in experimental rabbits after pterygium excision following PRF application. Moreover, PRF's application can mitigate the formation of minimal scar tissue by modulating the TGF- expression and inhibiting excessive expression [3-5].

Materials and Methods

Ethical approval

This study has been approved by the Ethics Commission of the Faculty of Veterinary Medicine, University Airlangga (2.KEH.171.12.2022).

Experimental animals

The experimental unit in this study consisted of New Zealand White rabbits (Oryctolagus cuniculus). Inclusion criteria for selecting these experimental units were as follows: rabbits of the New Zealand White breed, aged between 4 to 6 months, weighing between 3,000 grams and 3,500 grams, male gender, and in good overall health with both their body and eyes in a healthy condition. Exclusion criteria were applied to any rabbits that were diagnosed by a veterinarian with pre-existing diseases affecting their body or eyes that could potentially transmit disease during the research. The allocation of subjects to treatment groups was done simply and randomly using a lottery system. Any rabbits that became ill, died, or experienced complications such as scleral perforation, vitreous prolapse, infection, or bleeding during and after the operation were removed from the study.

Procedure of creating PRF membrane

The preparation of PRF membranes is a crucial step before the surgical procedure begins. To create a PRF membrane, a 5 mL blood sample is taken from the auricular vein of a rabbit, without any anticoagulant, and placed into a glass-coated tube. The sample is then immediately subjected to centrifugation at 2,700 rpm for 12 minutes using a centrifuge machine. After centrifugation, the tube's contents separate into three distinct layers: the top layer (supernatant) contains platelet-poor plasma (PPP), the middle layer is a fibrin clot that forms the basis of PRF membrane, and the bottom layer consists of red blood cells. The liquid PPP layer is carefully extracted using a pipette. The fibrin clot in the middle layer is separated from the solid red blood cell layer using forceps. The isolated fibrin clot is then placed into a PRF box and gently compressed. This compression process serves two purposes: It removes any remaining fluid within the fibrin clot and transforms the dense fibrin clot, initially tube-shaped, into a thin sheet suitable for use as a membrane. This membrane can be sutured to close the conjunctival defect during the surgical procedure.

Statistical analysis

The VEGF expression score data were evaluated using the modified Remmele method (Novak et al., 2007). In this technique, the Remmele scale index, also recognized as the Immuno Reactive Score (IRS), is determined by the multiplication of the percentage score of cells or regions displaying positive immunoreactivity with the color intensity score. For the angiogenesis data related to the granulation network following conjunctival excision in this study, it represents the average quantity of microvessels, including arterioles precapillaries, capillary vessels, and post-capillary venules, observed across five different fields of view (LP) under 400x magnification. The data for each sample were derived by calculating the average IRS value observed across five Fields of View (LP) under 400x magnification. This observation was facilitated using a standard Nikon Eclipse E-i light microscope equipped with a DS Fi2 300-megapixel digital camera and Nikon Image System image processing software. Following

this, the acquired data underwent statistical analysis using SPSS for Windows.

The Shapiro-Wilk normality test is used to determine whether the data is normally distributed or not. Since both groups have normally distributed data, we conducted an independent t-test to compare their average VEGF expression. This test is used to determine if there is a significant difference in mean VEGF expression between the two unpaired groups. To use this parametric test, certain conditions must be met, including normally distributed data and working with numeric data on an interval or ratio scale. To check for homogeneity, we used Levene's Test for equality of variances.

Results and Discussion

The VEGF expression score data was assessed using the modified Remmele method [6-8] (Figure 1). In this method, the Remmele scale index, also known as the Immunoreactive Score (IRS), is computed by the multiplication of the percentage score of positively immunoreactive cells or regions by the color intensity score within these cells or immunoreactive regions (refer to Table 1). For each specimen, the IRS value denotes the mean IRS observed across 5 (five) Fields of View (LP) under 400x magnification. Utilizing a Nikon Eclipse E-i standard light microscope, which was outfitted with a 300-megapixel DS Fi2 digital camera, this examination was carried out. The subsequent image processing was done employing Nikkon Image System image processing software.

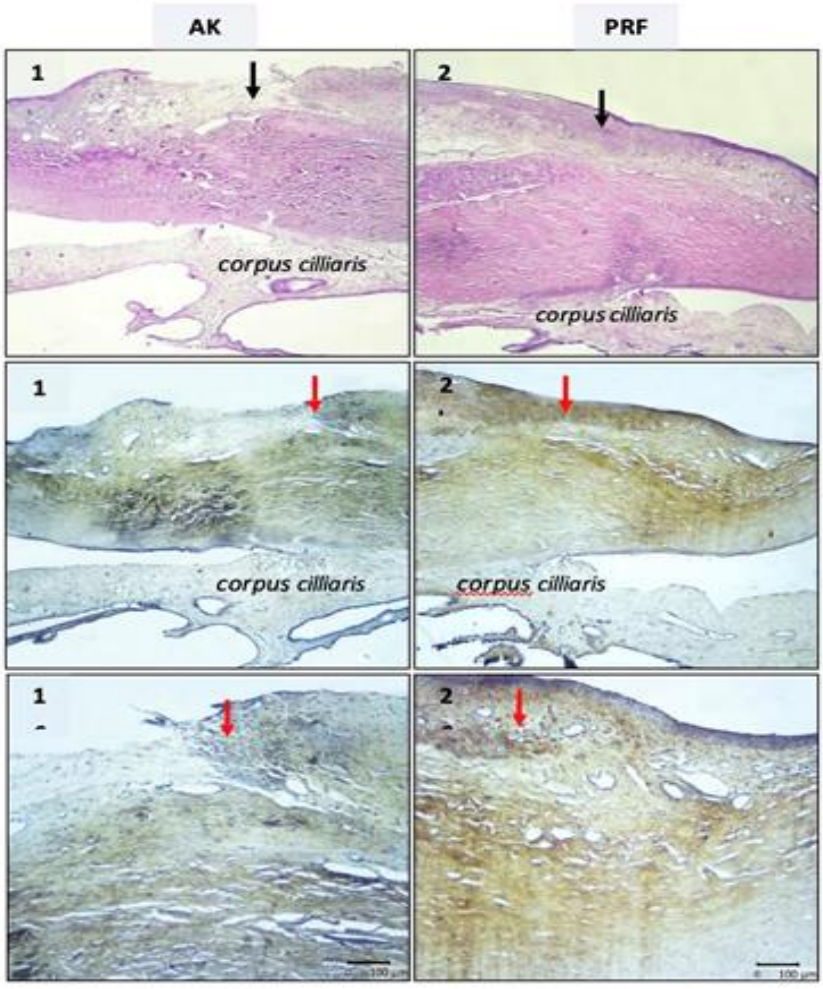


Figure 1: Differences in VEGF expression between treatment groups: namely the group given conjunctival autograft (AK) (slides 1a, 1b, and 1c) and platelet rich fibrin (PRF) (slides 2a, 2b, and 2c) in bulbar conjunctival excision wounds (black arrow). In the slide above, it can be seen that the expression of VEGF in the PRF group appears stronger (overexpression) as indicated by a corrosive brown to dark brown color (red arrow) compared to conjunctival autograft administration (slides 1a and 2a) (HE staining, 40x understanding: slide 1b, 1c 2b, and 2c immuno histochemical staining, 100x magnification; Eclipse E-i microscope; 300 megapixel DS Fi2 camera)

Table 1: Immunoreactive Score (IRS) on the semiquantitative S scale, determined by the multiplication of the percentage score of positive cells (A) by the intensity score for the color reaction (B), represented by the formula IRS = (A x B)

A	B
Score 0: no positive cells	Score 0: no color reaction
Score 1: Positive cells ≤ 10%	Score 1: Low color intensity
Score 2: Positive cells between 11% - 50%	Score 2: Moderate color intensity
Score 3: Positive cells between 51% - 80%	Score 3: Strong color intensity
Score 4: Positive cells more than 80%	

Table 2: Data distribution of VEGF expression per LP

Group	VEGF expression per field of view					
	n	Average	SD	Minimum	Maximum	p-value (1-tailed)
Autograft	10	7.40	2.57	3.2	11.4	0.0015 *
PRF	10	10.56	1.48	6.4	12.0	

*Description: *(significance = 0.0015, α < 0.05); SD: default deviation; and LP: field view)*

The Shapiro-Wilk normality test indicates that the data on VEGF expression in both the autograft and PRF groups follows a normal distribution ($p > 0.05$). Since both groups exhibit normally distributed data, we proceeded to conduct an independent t-test to compare the intermediate VEGF expression between the autograft and PRF groups. To ascertain whether homogeneity is exhibited or not, the nature of the data was investigated using the Levene's Test for equality of variances. In case the outcomes of the Levene's Test for equality of variances reveal non-significance ($p > 0.05$), the data is deemed to be homogeneous. In our case, the results of the Levene's Test for equality of variances resulted in a significance level of 0.067 ($p > 0.05$), indicating that the VEGF expression data indeed demonstrates homogeneity.

Table 2 presents the VEGF expression data, revealing that the PRF group exhibited a notably higher average expression with a mean of 10.56 ± 1.48/LP, while the autograft group had a lower average expression of 7.40 ± 2.57/LP. According to the analysis through independent t-test, the results highlight a notable and statistically significant dissimilarity in VEGF expression between the autograft and PRF groups ($p = 0.003^*$; $\alpha < 0.05$). The decision based on the independent t-test hinges on the significance level (Sig.) of the resulting one-tailed test. In this instance, the independent t-test yielded a significant p-value of 0.003 (<0.05), conclusively demonstrating a noteworthy distinction in VEGF expression between the autograft conjunctiva group and the PRF group. Notably, VEGF expression was significantly higher in the PRF group (mean expression 10.56/LP) compared to the autograft conjunctiva group (mean expression 7.40/LP). Moving on to the angiogenesis data pertaining to the granulation network following conjunctival excision in the study, this data represents the average quantity of microvascular structures, including arterioles precapillaries, capillary vessels, and post-capillary venules, observed in five fields of view (LP) under 400x magnification (Figure 2). The Shapiro-Wilk normality test revealed a normal distribution of data in vascular density for both the autograft and PRF groups ($p > 0.05$). Since both groups displayed normally distributed data, a comparison of vascular density between the autograft and PRF groups was conducted using the independent t-test. The Levene's Test for equality of variances produced a non-significant outcome with a significance level of 0.873 ($p > 0.05$), signifying that the vascular density data exhibits homogeneity.

Table 3 indicates that vascular density is higher in the PRF group, with an average of 275.42 ± 73.86 per LP, whereas the Autograft group has a lower mean of 219.96 ± 60.26 per LP. The outcomes of the independent t-test analysis reveal a statistically significant escalation in vascular density within the PRF group in comparison to the Autograft conjunctiva group ($p = 0.041^*$; $\alpha < 0.05$).

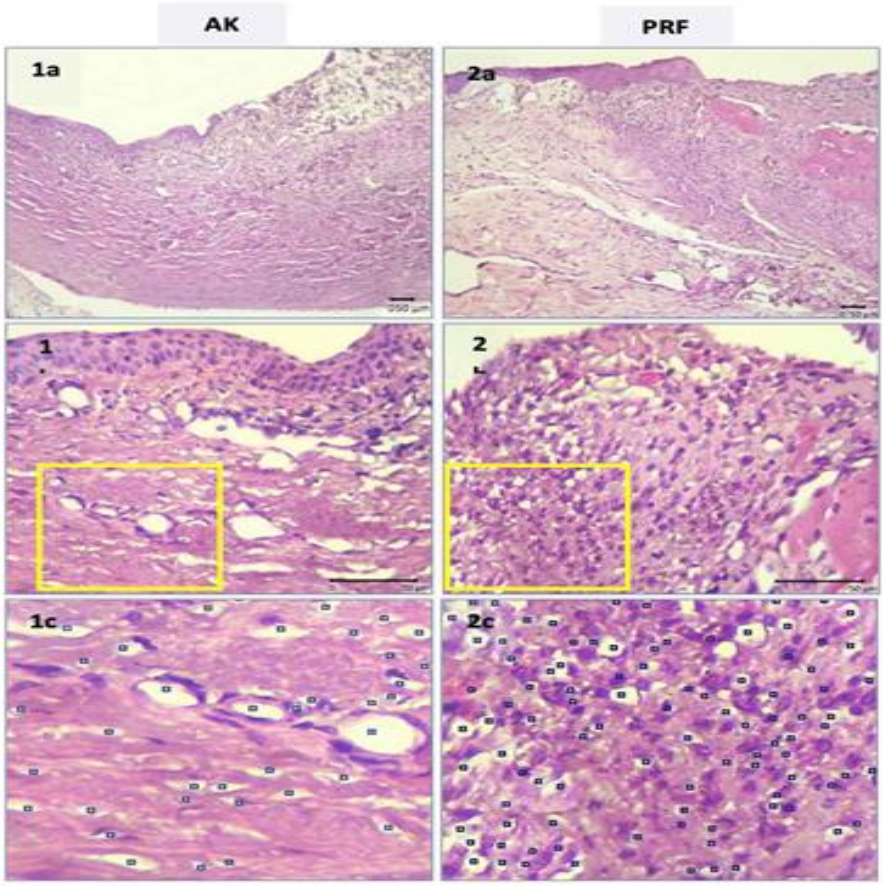


Figure 2: Comparison of the effect of conjunctival autograft (AK) (slide 1a, 1b, and 1c) and platelet rich fibrin (PRF) (slide 2a, 2b, and 2c): On the average number of microvasculature (black box) in bulbar conjunctival excision wounds. In the slide above, it appears that the number of capillaries in the granulation tissue due to PRF administration is relatively greater compared to conjunctival autograft administration, although there is no statistically significant difference (HE staining, slides 1a and 2a, 40x magnification: slides 1b and 2b, 100x magnification, slide 1c and 2c, 400x magnification; Eclipse E-i microscope; DS Fi2 300 megapixel camera)

Table 3: Distribution of vascular density per LP

Group	Density vascular per field of view					
	n	Average	SD	Minimum	Maximum	p-value (1-tailed)
Autograft	10	219.96	60.26	108.2	283.2	0.041*
PRF	10	275.42	73.86	146.6	424.6	

*Description: *(significance = 0.041, α < 0.05); SD: default deviation; and LP: field view)*

The determination made via the independent t-test hinges on the significance level (Sig.) resulting from the one-tailed test. In this particular scenario, the independent t-test produced a significance level of p = 0.041 (<0.05), affirming a statistically significant increase in vascular density in the PRF group in comparison to the Autograft conjunctiva group.

Based on the findings from our study, the data collected on day 14 indicates a notable increase in VEGF expression within the PRF membrane group when compared to the conjunctival autograft group. This suggests that exogenous VEGF supplied by the applied PRF membrane persists until the 14th day. Furthermore, the higher levels of VEGF expression indicate an accelerated angiogenesis process in the PRF membrane group compared to the conjunctival autograft group [9]. VEGF serves as a robust indicator of angiogenesis occurrence during the conjunctival wound healing process. This, undoubtedly, is a significant benefit specifically associated with the use of the PRF membrane.

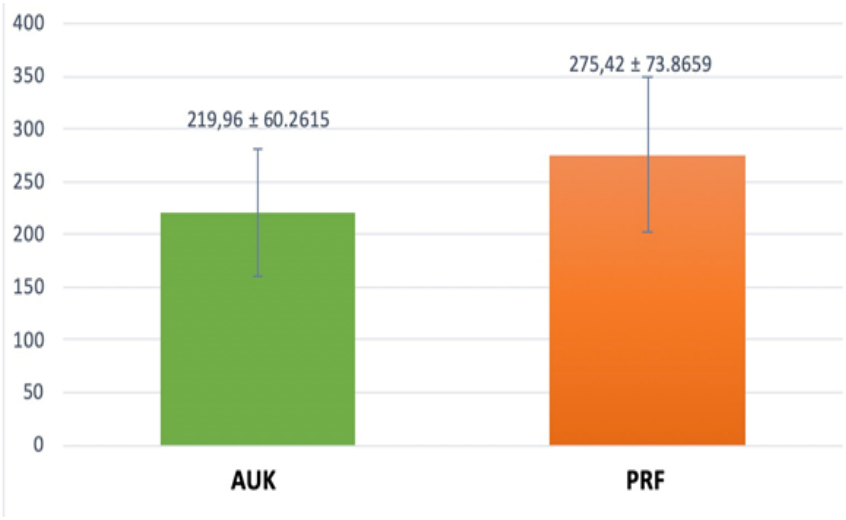


Figure 3: Comparison of mean number of microvascular densities

Angiogenesis is a crucial process in the conjunctival wound healing [10-12], involving the formation of new blood vessels. These blood vessels have a crucial role in transportation of vital extracellular matrix proteins, accelerating the formation of the granulation network, and subsequently playing a part in the remodeling phase of conjunctival wound closure. An early closure of the conjunctival wound is believed to be advantageous for the overall wound healing process as it helps prevent excessive fibrosis during the remodeling phase [3].

The study results unequivocally demonstrate a statistically notable rise in the mean angiogenesis count within the PRF membrane group in comparison to the conjunctival autograft group ($p < 0.05$). This underscores the fact that the heightened VEGF expression in the PRF membrane group is accompanied by a significant increase in angiogenesis [13, 14]. Several hypotheses can be formulated to explain these findings. The first hypothesis relates to the timing of eyeball sampling in rabbits. In our study, the sampling occurred on the 14th day, suggesting that angiogenesis in both the PRF membrane group and the conjunctival autograft group may have reached its peak at this point. In such a scenario, the increased VEGF expression in the PRF membrane group further stimulates the formation of new blood vessels (angiogenesis) [15, 16]. To confirm this hypothesis, additional research is warranted to assess variations in angiogenesis at different time points in a serial fashion [3, 17].

Conclusion

The analysis of immunohistochemistry and statistical data indicated a noteworthy contrast in VEGF expression when comparing the conjunctival autograft group to the PRF group ($p = 0.0015^*$; <0.05). Furthermore, the assessment of angiogenesis revealed a significant disparity in vascular development between the conjunctival autograft and PRF membrane groups ($p = 0.041^*$; <0.05).

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

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