

Comparison of α -Smooth Muscle Actin (α -SMA) and Collagen Type III Expression after Conjunctival Excision Between Platelet-Rich Fibrin (PRF) Membrane and Conjunctival Autograft Treatment: An Animal Model Experimental Study on *Oryctolagus cuniculus*



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ABSTRACT

Purpose: To evaluate the impact of Platelet-Rich Fibrin (PRF) membrane and conjunctival autografts on α -Smooth Muscle Actin (α -SMA) and type III collagen expression following conjunctival excision in *Oryctolagus cuniculus*.

Study Design: Experimental study with a randomized post-test only design.

Place and Duration of Study: Airlangga University, Surabaya in November 2022.

Methods: Twenty rabbit eyes were categorized into two groups: those sutured with PRF membrane and those sutured with conjunctival autografts after temporal conjunctival excision. The study assessed α -SMA and type III collagen expression through the Immunoreactive Score (IRS) method. Data analysis is conducted using appropriate statistical methods, such as the t-test or Mann-Whitney U test, to compare the expression of α -SMA and collagen type III between the two groups.

Results: The data for each sample represented the average IRS value observed at 200x magnification. In the conjunctival autograft group, the mean IRS of α -SMA expression was 5.52 ± 0.84 , significantly higher than the PRF membrane group (2.34 ± 0.34) with $p < 0.05$. Similarly, the conjunctival autograft group showed a higher mean IRS of type III collagen (3.87 ± 0.25) compared to the PRF membrane group (2.29 ± 0.31) ($p < 0.05$).

Conclusion: Variations in α -SMA and type III collagen expression exist between the PRF membrane and conjunctival autograft groups in the rabbit corneal model, suggesting difference in their efficacy for conjunctival defect repair.

Key Words: α -Smooth Muscle Actin, type III collagen, Platelet-Rich Fibrin, autograft, conjunctiva.

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INTRODUCTION

The ocular surface, comprising the eyelids, tear film, cornea, sclera, and conjunctiva, is essential for maintaining eye health. Diseases and surgeries, such as Stevens Johnson Syndrome (SJS), Toxic Epidermal Necrolysis (TEN), and ocular surface surgeries, can

result in conjunctival disorders and defects. Trauma, chemical burns, and scar tissue may cause conjunctival defects, necessitating closure methods ranging from simple techniques for small defects to the use of artificial membranes or grafts for larger ones.¹⁻⁴

Trauma affects approximately 55 million individuals daily, with conjunctival injuries being common among males aged 20 to 40 working in industrial or chemical settings. In Indonesia, ocular trauma cases in children primarily result from blunt trauma, thermal injuries, sharp trauma, and chemical exposure. Pterygium incidence varies globally, while SJS and TEN cases often require bilateral conjunctival reconstruction or amniotic membrane transplantation.⁵⁻⁸

Autograft conjunctiva is commonly employed but has limitations for large defects and subsequent surgeries. Amniotic membrane transplantation (AMT) is effective but complex and costly. Platelet-Rich Fibrin (PRF) membrane emerges as an alternative, being autologous, cost-effective, and easy to prepare. PRF's potential for ocular surface reconstruction has been explored in various medical fields, but comprehensive studies comparing its effectiveness are limited.^{9,10}

This study aims to investigate the impact of PRF membrane and autograft conjunctiva on α -Smooth Muscle Actin (α -SMA) and type III collagen expression following conjunctival excision in New Zealand white rabbits. Addressing a gap in Indonesian literature, this research compares PRF membrane and autograft conjunctiva in conjunctival wound healing, offering insights for ocular surface reconstruction.

METHODS

This research is an experimental study with a randomized post-test only group design to evaluate the expression of α -SMA and collagen type III in *Oryctolagus cuniculus*. The independent variable in this study is the type of graft, while the dependent variables are the expression of α -SMA and collagen type III. The conjunctiva was excised, and the PRF membrane was sutured in the first group, whereas conjunctival autograft was performed in the second group. This study is an experimental research aimed at assessing the impact of platelet-rich fibrin (PRF) membrane and conjunctival autograft on the expression of α -SMA and collagen type III post-conjunctival excision in the *Oryctolagus cuniculus*

model. The research was conducted at the Laboratory of the Airlangga University, Surabaya in November 2022.

The experimental units in this study are New Zealand white rabbits (*Oryctolagus cuniculus*). The inclusion criteria for experimental units are New Zealand rabbits aged 6-9 months, weighing between 3000 g and 3500 g, male, with healthy body and eye conditions. Exclusion criteria comprised of rabbits diagnosed by a veterinarian with diseases in the body or eyes that may potentially transmit diseases during the study. The number of replications is calculated using the Lemeshow formula with 30% dropout factor, the replication used in this study is 10 rabbits in each group. Discontinuation criteria include sick rabbits, deaths, or complications during and after surgery, such as scleral perforation, vitreous prolapse, infection, bleeding during and after surgery. Subject allocation into treatment groups is done through simple random allocation using drawing.¹¹

Rabbit cages were the living space for rabbits during the study, made of iron bars, each cage containing 1 rabbit. The rabbit cage used was a closed system rabbit cage measuring 90 cm \times 60 cm \times 60 cm. Rabbit food and water were provided in equal portions in the morning, afternoon, and evening. Tools used in this study included: data collection sheets, handheld slit lamp, eyelid speculum, caliper, face mask, eye surgery tools for conjunctivoplasty, eye enucleation surgery set, PRF box, Inami operation microscope, centrifuge machine, centrifuge tube, digital camera.

Materials for surgery and anesthesia included: 2% Tetracaine Hydrochloride eye drops, 5% povidone iodine, 0.5% Moxifloxacin antibiotic eye drops (Vigamox; Alcon Lab, Texas), 1cc and 3cc syringes, Vicryl 7.0, Nylon 10.0, conjunctival autograft, 5 ml of New Zealand rabbit blood from the femoral vein, Ketamine Hydrochloride and Xylazine Hydrochloride for anesthesia. Tools and materials for the preparation of specimens and preparations with Hematoxylin Eosin staining, staining holder, EZ Mount, filter paper, tissue, xylene, alcohol (70%, 80%, 96%), Hematoxylin and Eosin stains, sterile aqua, paraffin, and immunohistochemistry examination tools including: TGF β monoclonal antibody reagent, 10% formalin buffer, and alcohol (80%, 90%, 96%, and 99%).

Rabbits that met the inclusion criteria were acclimated for 2 weeks before the study began. Randomization and grouping of rabbits were carried

out using a simple random allocation method, where the result was division of rabbits into two groups: the PRF membrane group and the conjunctival autograft group. Rabbits were anesthetized using Ketamine Hydrochloride and Xylazine Hydrochloride. Eye surgery was performed by excising the conjunctiva and applying the assigned graft. Observations and measurements were conducted postoperatively, focusing on the expression of α -SMA and collagen type III.

Ten eyes were used for each control and treatment group, requiring a total of 20 rabbits. The control group underwent suturing using conjunctival autograft after temporal conjunctival excision, while the treatment group was sutured with PRF membrane after temporal conjunctival excision. In the PRF membrane group, the conjunctival defect was closed by suturing a PRF membrane made from the rabbit's own whole blood. In the conjunctival autograft group, the defect was closed by suturing conjunctival autograft. Treatment was applied only to the right eyes in both the rabbit groups.

Before the study, the anterior ocular segment condition was examined using a portable operation microscope and it was found to be within normal limits. During the observation period, each rabbit received same treatment, including a balanced diet of pellets and mineral water provided in equal portions in the morning, afternoon, and evening. Moxifloxacin 0.5% eye drops (Vigamox; Alcon Lab, Texas) were administered postoperatively by instilling four drops once a day for 14 days in each experimental rabbit to prevent infection. In the PRF membrane group, Moxifloxacin was instilled only in the right eye, while in the conjunctival autograft group, it was instilled in both eyes.

The experimental animals underwent a 7-day adaptation to the new environment. During this period, all rabbits received the same general care. Each experimental rabbit was labeled and housed individually in a room with windows and an exhaust fan to maintain air circulation. Cage hygiene and rabbit food were provided in a balanced manner to

both treatment groups to enhance the likelihood of experimental rabbit survival until the termination phase. Rabbit evaluation using a portable operation microscope was performed to assess the condition of anterior segment and conjunctiva after temporal excision.

Before suturing the PRF membrane, blood was drawn from the experimental rabbits in the treatment group, specifically 5 cc from the rabbit's auricular vein, and collected in a glass-layered tube without anticoagulant. The formed PRF membrane, transparent and slightly thicker than the conjunctiva, was the middle layer known as the fibrin clot. The formed fibrin clot was gently compressed to remove any remaining fluid, resulting in a thin PRF sheet used to cover the conjunctival defect.

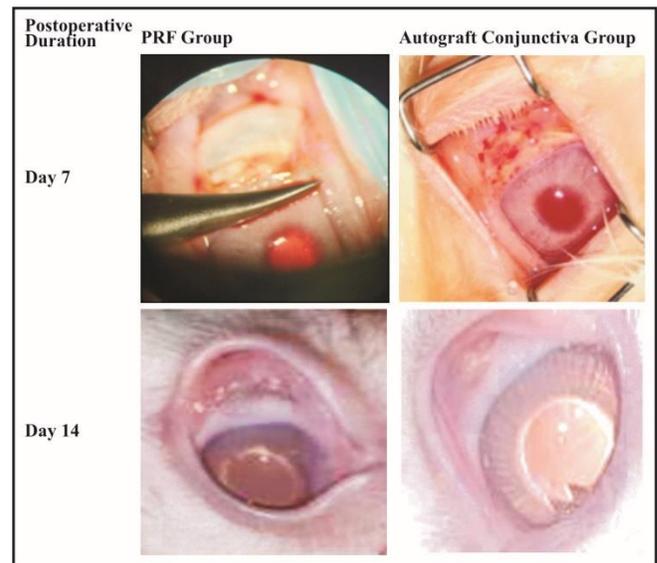


Figure 1: Results of postoperative conjunctival defect evaluation on days 7 and 14.

During the 14-day observation period, the experimental rabbits showed excellent conditions, active movements and stable body weight. Examination using a portable operation microscope revealed that in the treatment group, suture threads were still visible on day 14. A smooth transition was

Table1: Blood examination results for rabbits: platelet count and fibrinogen.

| Variable Examined | n | Mean | Results | | |
|-------------------|----|---------------------------------|--------------------|-------------------------------|-------------------------------|
| | | | Standard Deviation | Minimum | Maximum |
| Fibrinogen | 20 | 305.6 mg/dL | 127.8 | 119.7 mg/dL | 561.7 mg/dL |
| Platelets | 20 | 328.0 $\times 10^3/\mu\text{L}$ | 50.1 | 235 $\times 10^3/\mu\text{L}$ | 417 $\times 10^3/\mu\text{L}$ |

observed without serrations at the edge of the PRF membrane. In both groups, fine tissue resembling conjunctival tissue was observed in the defect area. No secondary infections were detected during the observation period.

The expression of α -SMA (Figure 2) was examined using immunohistochemical staining (IHC) with α -SMA antibodies. IHC staining was performed using an indirect method, where the monoclonal antibody used to detect a marker was its secondary antibody. This histopathological examination aimed to determine the expression in the conjunctival defect healing area in both the PRF membrane and conjunctival autograft groups. The α -SMA expression assessment used the Remmele scale index (Immuno Reactive Score/IRS). The α -SMA expression was the result of multiplying the percentage score of immunoreactive positive cells or areas with the color intensity score on immunoreactive cells (Table 2). The data for each sample represented the average IRS value observed at 200x magnification.

Statistical analysis was performed using SPSS 26.0. After the α -SMA expression examination by an anatomical pathology expert, the results were analyzed. First, a normality test was performed to determine whether the data distribution was normal. The Shapiro-Wilk normality test was used in this study because the sample size was less than 50. The normality test results for α -SMA expression data in the

conjunctival autograft group showed a p-value of 0.194, and in the PRF membrane group, it showed a p-value of 0.214. Since the p-values for both the autograft and PRF groups were > 0.05 , this indicated a normal data distribution for α -SMA expression in both groups. Because the data distribution in both groups was normal, further statistical analysis was performed using the Independent T-Test.

RESULTS

The Independent T-Test results for α -SMA expression data in the conjunctival autograft and PRF groups showed $p = 0.000$. This indicates a significant difference in α -SMA expression between the autograft and PRF groups ($p < 0.05$) as shown in Table 2.

Table 2 shows a significant difference in α -SMA expression between the autograft and PRF groups ($p = 0.000^*$; $\alpha < 0.05$).

The expression of type III collagen (Figure 3) was examined using immunohistochemical staining with type III collagen antibodies. The expression of type III collagen was calculated based on the number of cells expressing type III collagen out of 100 conjunctival cells around the conjunctival excision area, using a light microscope at 200 times magnification. Cells expressing type III collagen appeared with brown-colored cytoplasm.

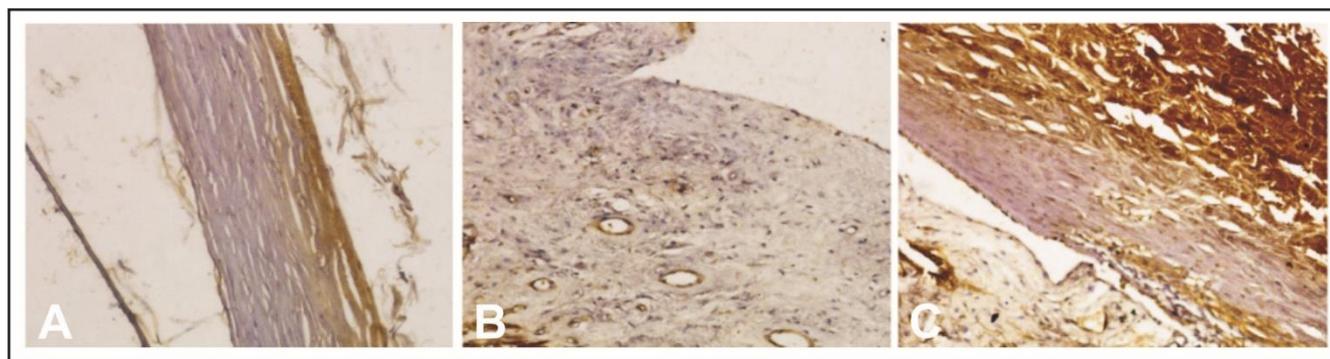


Figure 2: Immunohistochemical staining results for α -SMA: A. α -SMA Without Treatment, B. α -SMA PRF Membrane, C. α -SMA Autograft Conjunctiva. It shows differences in the effects of conjunctival autograft and PRF on α -SMA expression (yellow to brown chromogen color) post-temporal conjunctival excision. In the above slide, α -SMA expression in the autograft group appears stronger compared to the PRF group (IHC staining; magnification 200x, bar 50 μ m; Eclipse E-i microscope; DS Fi2 300-megapixel camera).

Table 2: Distribution data for α -SMA expression.

| Group | n | Mean | SD | Minimum | Maximum | p |
|-----------|----|---------|------|---------|---------|--------|
| Autograft | 10 | 5.52/LP | 0.84 | 3.96 | 6.72 | 0.000* |
| PRF | 10 | 2.34/LP | 0.34 | 1.8 | 2.52 | |

Note: *(significance=0.000, $\alpha < 0.05$); SD: standard deviation; LP: line of sight

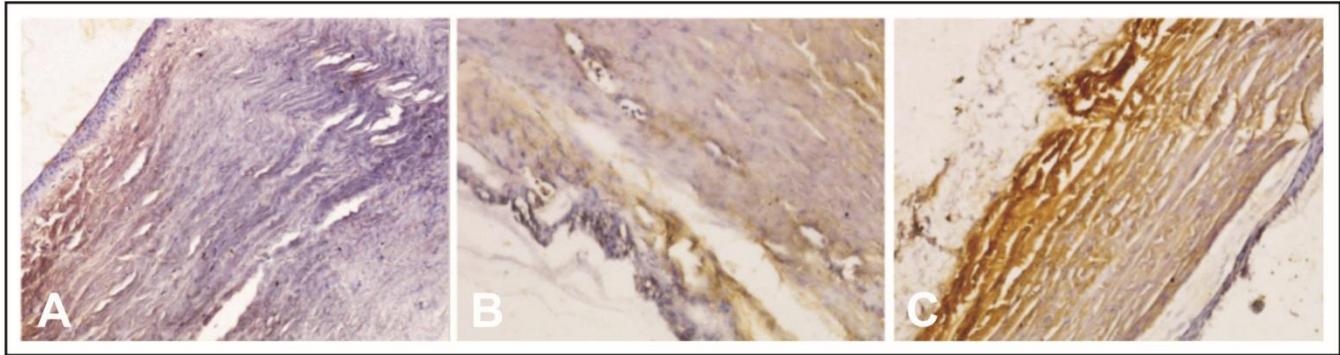


Figure 3: Immunohistochemical staining results for type III collagen: A. Type III Collagen Without Treatment, B. Type III Collagen PRF Membrane, C. Type III Collagen Autograft Conjunctiva. Show differences in the effects of conjunctival autograft and Platelet Rich Fibrin (PRF) on type III collagen expression (yellow to brown chromogen color) post-temporal conjunctival excision. In the above slide, type III collagen expression in the autograft group appears stronger compared to the PRF group (IHC staining; magnification 200x, bar 50 μ m; Eclipse E-i microscope; DS Fi2 300-megapixel camera).

Table3: Distribution data for type III collagen expression.

| Group | N | Mean | SD | Minimum | Maximum | P |
|-----------|----|---------|------|---------|---------|--------|
| Autograft | 10 | 3.87/LP | 0.25 | 3.52 | 4.32 | 0,000* |
| PRF | 10 | 2.29/LP | 0.31 | 1.60 | 2.80 | |

Note: *(significance = 0.032, $\alpha < 0.05$); SD: standard deviation; LP: line of sight

The Shapiro-Wilk normality test results for type III collagen expression data in the autograft group showed a p-value of 0.805, and in the PRF group, it showed a p-value of 0.814. Since the p-values for both the autograft and PRF groups were > 0.05 , this indicated a normal data distribution for type III collagen expression in both groups. Because the data distribution in both groups was normal, further statistical analysis was performed using the Independent T-Test.

The Independent T-Test results for type III collagen expression data in the conjunctival autograft and PRF groups showed $p = 0.000$. This indicates a significant difference in type III collagen expression between the autograft and PRF groups ($p < 0.05$). Type III collagen expression was significantly higher in the conjunctival autograft group (mean expression 3.87/ LP) compared to the PRF group (mean expression 2.29/ LP).

DISCUSSION

Conjunctival wound healing progresses through distinct phases: hemostasis, inflammation, proliferation, and remodeling. Hemostasis initiates injury response, forming fibrin clots and platelet plugs to preserve blood vessel integrity. Platelet activation releases growth factors (PDGF, VEGF, TGF- β , and

interleukins). Inflammation, divided into early and late stages, involves pivotal roles of proinflammatory and anti-inflammatory/pro-angiogenic macrophages. Proliferation facilitates granulation tissue formation characterized by heightened fibroblast activity and angiogenesis, with TGF- β driving fibroblast differentiation into myofibroblasts. Remodeling involves MMP-mediated ECM degradation, collagen type I replacing type III, and myofibroblast reduction through apoptosis.^{12,13}

This study utilized male New Zealand white rabbits (6-9 months, 3–3.5 kg) without eye abnormalities, divided into control (conjunctival autograft suturing) and treatment (Platelet-Rich Fibrin-PRF membrane suturing after conjunctival excision) groups. Both groups underwent temporal bulbar conjunctival excision, with PRF membrane preparation involving rabbit auricular vein blood centrifugation, forming three layers. On day 7, the treatment group exhibited smooth reepithelialization, consistent with previous findings. On day 14, both groups showed smooth tissue with vascularity; however, sample collection issues impacted reliability. Previous studies found PRF membrane promising in pterygium surgery, offering advantages like simplicity, shorter operation times, lower recurrence rates, safety, and minimal complications. PRF advantages include easy preparation, minimal cost, immune system

support, and hemostasis.¹⁴⁻¹⁶ PRF gradual release of autologous growth factors and its role as a growth factor reservoir contribute to wound healing and reduced inflammation. Compared to Amniotic Membrane Transplantation (AMT), PRF offers advantages, including minimal risk of reactions as an allograft. The study reveals a significantly lower average α -SMA expression in the PRF membrane group compared to the conjunctival autograft group, indicating accelerated conjunctival defect healing and reduced fibrosis. The superficial proliferation of cells in conjunctival wound healing involves epithelial formation and cell modification, leading to wound edge reapproximation.¹⁷⁻¹⁹ Granulation tissue forms below the epithelial layer, involving angiogenesis and fibroplasia, with myofibroblasts contributing to wound closure and ECM production. Angiogenesis, driven by VEGF and BFGF, initiates post-wound closure. Platelet rupture, mast cell degranulation and fibrin formation characterize early wound healing, with macrophages playing roles from inflammation to tissue regeneration. PRF's impact on TGF- β levels influences collagen type III production, suggesting a crucial role in the healing process.^{20,21}

PRF membrane application post-ptyerigium excision offers simplicity, shorter operation times, lower recurrence rates, safety, and minimal complications. Collagen type III expression increase in the PRF group aligns with studies showing elevated type III collagen in keloid healing, suggesting a crucial role in the healing process. PRF-induced TGF- β facilitates fibroblast-to-myofibroblast transformation, enhancing ECM filling and collagen production.^{22,24} Controlled collagen synthesis, crucial in fibrotic tissue formation, is regulated by various cytokines, and PRF's TGF- β secretion aids fibroblast differentiation and collagen synthesis. Collagen type III expression significantly surpasses autograft conjunctiva in conjunctival wound healing, indicating potential fibrosis reduction with PRF.²⁵

Limitations of this study include a short 14-day observation period at a single time point, hindering a comprehensive assessment of PRF membrane effects on conjunctival excision healing phases. It focused on α -SMA and type III collagen, neglecting other crucial components in the healing process.

CONCLUSION

The study reveals significant differences in both α -SMA and type III collagen expressions following

conjunctival excision in *Oryctolagus cuniculus*. The autograft group shows notably higher α -SMA and type III collagen expressions compared to the PRF membrane group.

Conflict of Interest: Authors declared no conflict of interest.

Ethical Approval: The study was approved by the Institutional review board/Ethical review board (**No. 2. KEH. 172.12.2022**).

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Author Contribution: All authors contributed equally to the study, from the conceptual framework and data acquisition to data analysis and reporting of the study results through publication.

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