Original Article

Comparison of TGF-β and Type 1 Collagen Expression between Platelet Rich Fibrin Membrane and Conjunctival Autograft Treatment after Conjunctival Excision(An Experimental Animal Model Study)

PJO – Official Journal of Ophthalmological Society of Pakistan

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ABSTRACT

Purpose: To compare TGF- β and type 1 collagen expression between Platelet rich fibrin (PRF) membrane and conjunctival autograft treatment after conjunctival excision.

Study Design: Experimental animal study.

Place and Duration of study: Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia from October 24, 2022 to November, 19, 2022.

Methods: Twenty New Zealand white rabbits were randomly assigned to either the PRF group or the conjunctival autograft group. A 5x5 mm excision was made in the temporal quadrant of the right eye of each rabbit. In the first group, the conjunctival defect was closed using a PRF membrane, while in the second group, closure was done with a conjunctival autograft from the superior quadrant of the same eye. After 14 days, all rabbits were terminated and enucleated. An immunohistochemical study of conjunctival tissue was conducted to assess TGF-β and type 1 collagen expression, and results were statistically analyzed.

Results: An independent T-Test revealed that PRF membrane group exhibited higher TGF-β expression compared to the conjunctival autograft group (p = 0.000), with a mean TGF-β expression of 9.02 in the PRF membrane group and 5.76 in the conjunctival autograft group. Conversely, type 1 collagen expression was found to be higher in the conjunctival autograft group compared to the PRF membrane group (p = 0.032), with a mean type 1 collagen expression of 9.22 in the conjunctival autograft group and 6.92 in the PRF membrane group.

Conclusion: TGF-β expression was higher in the PRF group and type 1 collagen expression was higher in the conjunctival autograft group.

Key Words: PRF, Conjunctival Autograft, TGF-β, Type 1 Collagen, SDGs Goal, Good Health and Well-Being.

How to Cite this Article: Sasono W, Karisma NA, Fatmariyanti S, Legowo D, Notobroto HB. Comparison of TGF-β and Type 1 Collagen Expression between Platelet Rich Fibrin Membrane and Conjunctival Autograft Treatment after Conjunctival Excision (An Experimental Animal Model Study). 2024;40(3):246-256.
Doi:10.36351/pjo.v40i3.1791

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Received: January 02, 2024
Accepted: June 22, 2024

INTRODUCTION

To guarantee the integrity of the ocular surface and theclarity of cornea, conjunctiva must be in good health. Conjunctival integrity is affected by conditions like Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and cicatricial pemphigoid. Around 77% of patients with SJS or TEN will develop an eye problem during the acute stage. Corneal and

conjunctival damage and pseudo membrane and Symblepharon formation will ensue from this inflammatory state.[1] Trauma and surgery can also harm conjunctiva. Ocular trauma is reported to affect between 10 to 20.5 people per 100,000 annually.[2] Simple closure sutures can be used to treat a tiny conjunctival defect. However, it cannot be used to treat a wide bilateral deficiency in larger defects where other more sophisticated procedures become mandatory.[3-5] Conjunctival damage is usually overlooked, but treating a large conjunctival defect is necessary to preserve the health of the ocular surface. Bilateral conjunctival destruction typically develops necessitating the use of amnioticmembrane transplantation to restore the conjunctival tissue.[6]

There are several techniques which are available as an option to treat conjunctival defect, each with its own advantages and disadvantages. The commonly used techniques to treat conjunctival defect are amniotic membrane transplantation, conjunctival autograft, and PRF membrane graft.

Conjunctival autograft is a technique which is most commonly used to treat conjunctival defect after pterygium surgery, however it can also be used to treat conjunctival defect due to trauma.[7] The advantages of conjunctival autograft include; easy to perform and artificial membrane is not required to close the defect. However conjunctival autograft impose iatrogenic damage to the conjunctiva at donor site.[8]

Another technique to treat conjunctival defect is amniotic membrane transplantation (AMT). This method uses human amniotic membrane that has been treated to serve as a material for the reconstruction of ocular surface. The risk of spreading diseases, less transparency, changes in quality and stability of membrane, and a poor mechanical strength are some of the drawbacks. The availability of sufficient medical resources and personnel to handle amniotic membrane processing makes this situation achievable in industrialized nations where AMT is a very dependable and widely utilized technology. However, amniotic membrane is rarely used in underdeveloped nations like Indonesia, particularly in areas with limited medical facilities. Taking into account the geographic circumstances and the poor economic conditions in the outlying districts, majority of the patients cannot be referred to more advanced medical facilities. Use of amniotic membrane in peripheral locations is difficult for this reason, hence most ophthalmologists need to use alternative procedures to treat conjunctival defect.

PRF membrane is a membrane which is made from a second-generation blood concentrate which was firstly invented by Choukroun in 2006.[9] PRF membrane contains many growth factors, cytokines, circulating stem cells, and circulating leukocytes which is very useful in conjunctival wound healing. The first-generation membrane before the invention of PRF is autologous fibrin membrane. The first-generation membrane has a fundamental disadvantage of the difficulty to process it due to the need of more sophisticated facilities, complex, and a higher cost. The PRF membrane has a substantial advantage which is very practical, cheaper, and easier to produce since it only needs a centrifuge and a PRF box. This advantage will allow PRF membrane to be widely used in future in many remote and peripheral areas in developing countries such as Indonesia which have limitations in medical facilities.[10]

Several studies revealed that PRF has anti-inflammatory effect and can be a temporary scaffold for fibroblast migration. PRF membrane is also known to contain many important growth factors for conjunctival wound healing such as TGF-β. TGF-β is an important growth factor that can accelerate wound healing.[11] TGF-β can accelerate formation of granulation tissue and thus accelerates the closure of conjunctival defect. TGF-β is also modulates fibroblast and type 1 collagen.

Type 1 collagen is an important component of conjunctival extracellular matrix and it comprises of almost 80-90% of conjunctival tissue. Despite many studies about PRF membrane, there is still limited study which compares the expression of TGF-β and type 1 collagen between PRF membrane and conjunctival autograft treatment after conjunctival excision (conjunctival defect model).[9,11]This study was designed to compare the expression of TGF-β and type 1 collagen between PRF membrane and conjunctival autograft in animal eyes.

METHODS

This study is an animal model study with true experimental post-test only group design. The main purpose of this study is to compare the differences in TGF-β and type 1 collagen expression between conjunctival autograft and PRF membrane in adult

New Zealand white rabbits (Oryctolagus cuniculus) following conjunctival excision. Inclusion criteria for this study are adult New Zealand white rabbits weighing 3000–3500 grams with normal fibrinogen levels (1.66 ± 0.39 g/L) and blood levels of 1.66 ± 0.39 g/L, normal blood platelets (in the range of 390 – 821 × 109/L). The age of rabbits ranged from 4 to 10 months and they were in good physical and ocular health. Each rabbit was carefully selected and examined by a veterinarian. Rabbits which were declared to have a disease or had the potential to transmit the disease during the investigation were excluded. Rabbit was considered as a drop out sample if they got sick or died or had surgical complication like scleral perforation, vitreous prolapse, infection, and hemorrhage both during or after the procedure. The independent variables in this study were the PRF membrane and the conjunctival autograft, while the dependent variable in this study was the expression of TGF-β and type 1 collagen.

A total of 20 male New Zealand rabbits (20 eyes) were randomly divided into two groups with simple random sampling method. The first group underwent conjunctival excision and PRF membrane graft suturing on the right eye conjunctival defect, while the second group underwent conjunctival autograft on the right eye conjunctival defect. The bulbar conjunctiva of 5×5 mm size was excised on the temporal side. The rabbit was firstanesthetized using an injection of ketamine at a dose of 50 mg/kg and xylazine at a dose of 5 mg/kg mixed with 0.5 mL of balanced saline (BSS). Westcott scissors were used to create conjunctival defect and to remove the Tenon's tissue from the temporal interpalpebral bulbar conjunctiva at a distance of 3 mm from the limbus. All rabbits were enucleated under general anesthesia on the fourteenth day following surgery. Hhistopathological examination was performed in the bulbar conjunctiva on the site of the conjunctival defect area by utilizing TGF-β antibodies and collagen type 1 to evaluate the expression of TGF-β and type 1 collagen in conjunctiva.

To create PRF membrane, 5 ml of blood from a rabbit auricular vein was taken and subsequently kept in a glass tube without anticoagulant. The blood sample was quickly centrifuged to form three layers of blood. Failing to do so, would result in blood clot resulting in failure to form three layers of blood after centrifugation process. The blood sample was centrifuged for 12 minutes at the speed of 2700 rpm (Choukroon protocol). Acellular plasma, which is the supernatant from the centrifuge, was at the top, followed by PRF clots in the middle, and red blood cells at the bottom. The blood clot was taken out of the tube following the centrifugation procedure, and the red blood cell component at the bottom of the clot was separated mechanically by using forceps and Wescott scissors. The PRF clot was then compressed using a PRF box to transform it into a membrane.

In order to assess the TGF and type 1 collagen expression, a veterinary pathologist used a light microscope (Nikon H600L microscope; 300 megapixels DS Fi2 camera) to collect all the data at 400× and 1000× magnification. To ascertain the expression of TGF-β and type 1 collagen in the conjunctival healing region, histopathological analysis was performed. This method was used in a blinded fashion by a veterinary pathologist. The slides with serial numbers were used to minimize bias. According to the modified Remmele method, the TGF-expression score and type 1 collagen were calculated. The Remmele scale index (Immuno Reactive Score/IRS) was created by multiplying the percentage score of immunoreactivity positive cells or areas by the color intensity score of immunoreactive cells. The details of the average IRS value seen in 5 (five) Fields of View at 400× magnification served as the data for each sample.

Shapiro-Wilk normality test was carried out and revealed that data were normally distributed on all groups ($p > 0.05$). Independent T-Test were performed to compare the TGF-β and type 1 collagen expression between conjunctival autograft and PRF membrane group. The data were examined and processed by utilizing SPSS version 26 (IBM corporation, New York, NY, USA).

The anterior segment of 20 male New Zealand rabbits (20 eyes) that satisfied the inclusion criteria was examined with a portable slit lamp. Two groups of ten rabbits each were formed from the group of rabbits. Up to 10 rabbits in group 1 had their right eye's conjunctival defect repaired using a PRF membrane graft created from their own whole blood sample, while in group 2, the conjunctival defect in the right eye was repaired using the conjunctiva autograft technique.

RESULTS

All rabbits had minimum of 5ml of their blood drawn

for fibrinogen and blood platelet examination prior to treatment. The average level of fibrinogen was 330.18 mg/dL, with a coefficient of variation of 65.24%, and the data was normally distributed ($p > 0.05$). Table 1 shows analysis of fibrinogen.

Using the Lemeshow calculation formula, an estimate of the number of replications revealed that n = 7 was the number of replications. The number of replications we employed in this investigation, with a dropout factor of 30%, was 10 samples. A handheld slit lamp was used for evaluation, and it was discovered that on the seventh day, the vicryl suture was not visible in the PRF group. The edge of the PRF membrane transitions into the surrounding conjunctiva in a smooth, non-jagged fashion. It was also discovered that there was neovascularization across the PRF membrane. On the other hand, on day 14, both groups displayed fine tissue with vascularity in the defectlocation that mirrored the conjunctival tissue all around it (Figure 1). There was no complication during the observation period.

Table 1: *Blood platelets and fibrinogen levels in rabbit samples.*

Variable	Result				
	n	Mean	Standard Deviation	Minimum	Maximums
Fibrinogen	20	305.6 mg/dL	127.8	119.7 mg/dL	561.7 mg/dL
Thrombocyte	20	328.0 $\times 10^3/\mu L$	50.1	$235 \times 10^3/\mu L$	$417 \times 10^3/\mu L$

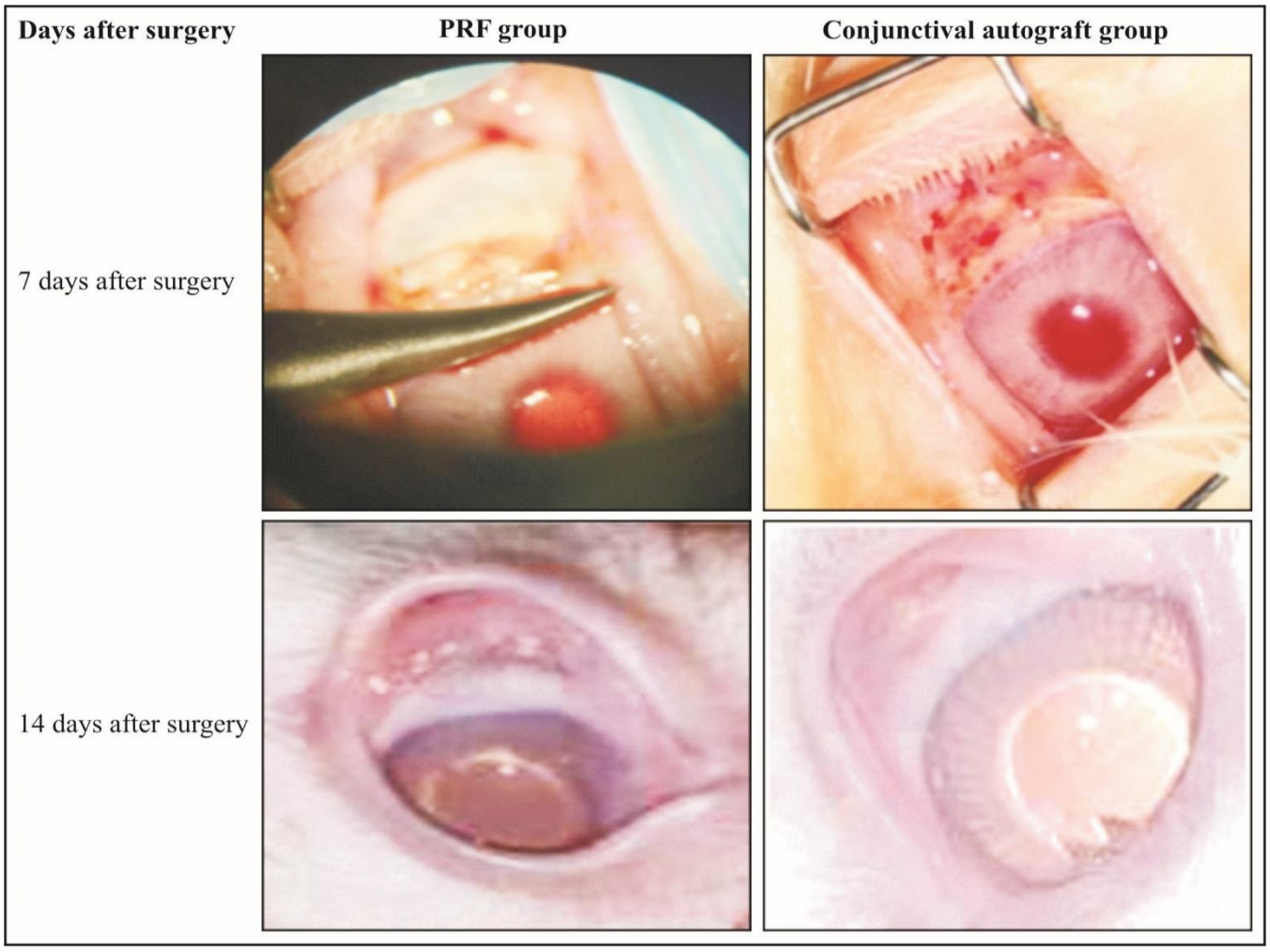

Figure 1: On days 7 and 14, conjunctival defects following excision are examined. On the seventh day, the vicryl sutures were no longer evident in the PRF group and the PRF membrane and surrounding conjunctiva transitioned smoothly.

The brown chromogen color in the microscope field of view served as a marker for TGF-β expression and became the basis for the measurement of TGF-β expression (IRS/Remelle Index). The TGF-β looked more brownish and had a darker color on the PRF membrane group (1000× magnification) in comparison with conjunctival autograft group (Figure 2). Thisindicated a stronger TGF-β expression in the PRF membrane group compared with conjunctival autograft group.

According to table 2, the mean level of TGF-β expression in the PRF membrane group was 9.02/field of view with a standard deviation of 1.43, whereas the mean level was 5.76/field of view with a standard deviation of 1.84 in the conjunctival autograft group. The PRF membrane and conjunctival autograft group had significantly different levels of TGF-β expression according to the Independent T-Test ($p = 0.000^*$; $\alpha<0.05$). A higher TGF-β expression was found in the PRF membrane group compared with conjunctival autograft group.

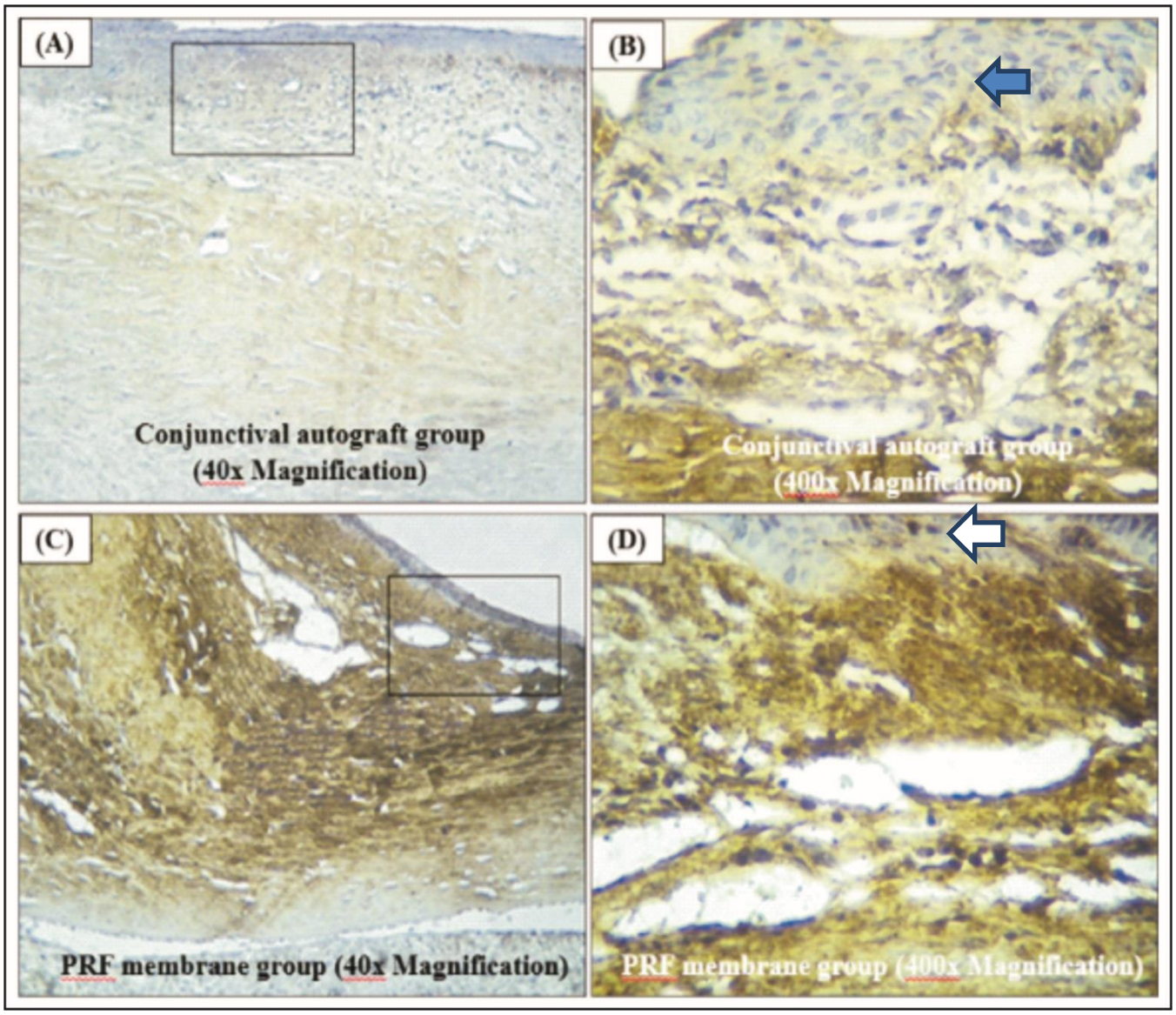

Figure 2: Comparison of TGF-β expression under microscopic view between conjunctival autograft and PRF membrane group. (A) Conjunctival autograft group with 40x magnification (B) Conjunctival autograft group with 400x Magnification. TGF-β expression was weaker and marked by a lighter brown chromogen colour (blue arrow) in conjunctival autograft group compared with PRF membrane group. (C) PRF Membrane group with 40× Magnification. (D) PRF Membrane group with 400x Magnification. TGF-β expression was stronger and marked by a darker brown chromogen colour (white arrow) in PRF membrane group compared with conjunctival autograft group.

Table 2: *Comparison of TGF-β expression between conjunctival autograft and PRF membrane group.*

Group	TGF-β Ekspression					
	n	Mean	Standard Deviation	Minimum	Maximum	*P*
Autograft	10	5.76	1.43	3.0	8.2	0.000
PRF	10	9.02	1.84	6.4	12.0	

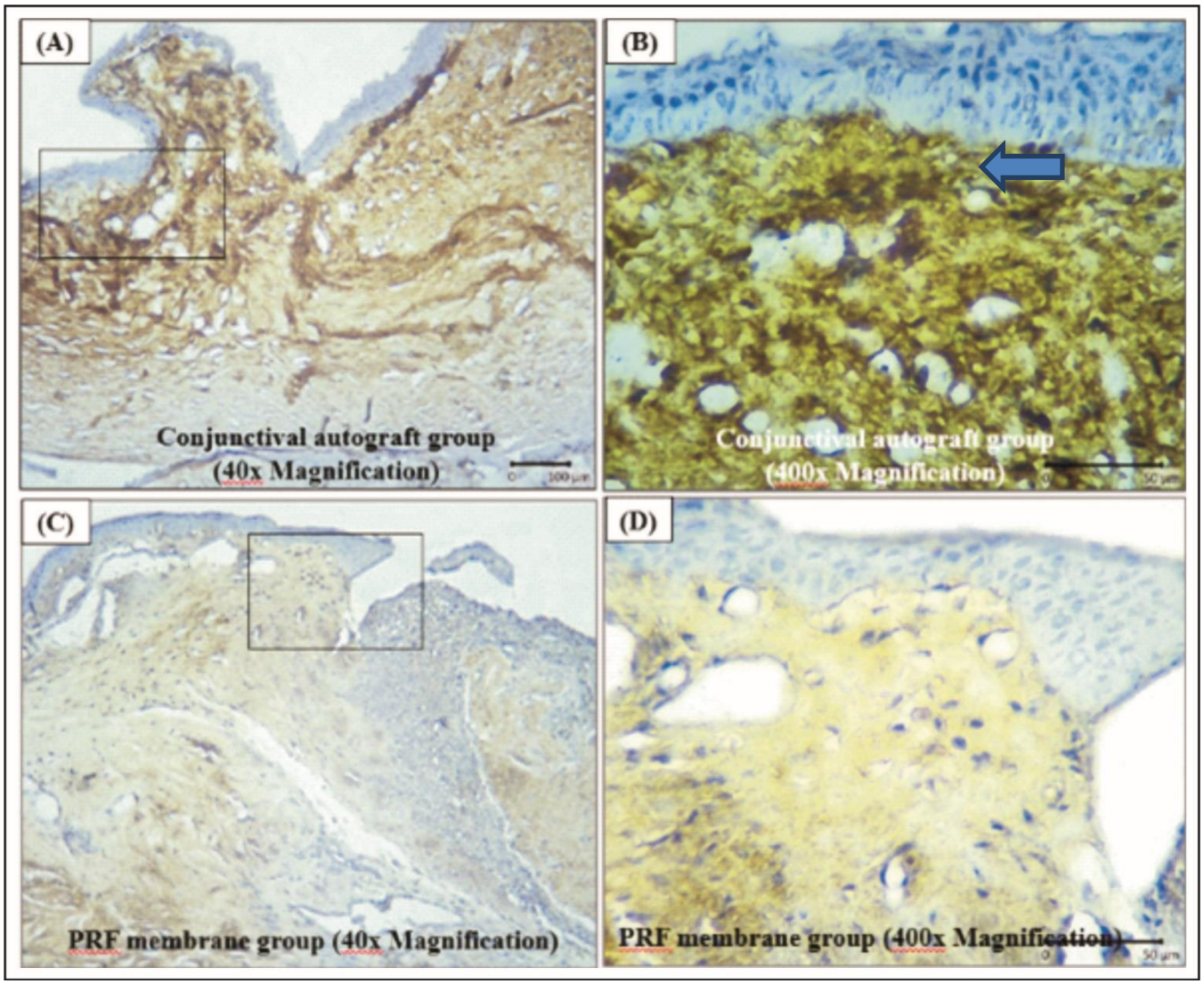

Figure 3: Comparison of type 1 collagen expression under microscopic view between conjunctival autograft and PRF membrane group. (A) Conjunctival autograft group with 40x magnification (B) Conjunctival autograft group with 400× Magnification. Type 1 collagen expression was stronger and marked by a darker brown chromogen colour in conjunctival autograft group compared with PRF membrane group. (C) PRF Membrane group with 40x Magnification. (D) PRF Membrane group with 400× Magnification. Type 1 collagen expression was weaker and marked by a lighter brown chromogen colour in PRF membrane group compared with conjunctival autograft group.

Table 3: *Comparison of type 1 collagen expression between conjunctival autograft and PRF membrane group.*

Group	Type 1 Collagen Expression					
	n	Mean	Standard Deviation	Minimum	Maximum	*p*
Autograft	10	9.22	2.48	5.2	12.0	0.032
PRF	10	6.92	1.90	4.4	10.0	

Type 1 collagen expression between PRF membrane group compared with conjunctival autograft group

Type 1 collagen is one of the most predominant components which compose conjunctival extracellular matrix. Similar to how TGF-β expression is determined, the IRS index is used to determine the level of type 1 collagen expression by analyzing the brown chromogen colour under a microscope. Type 1 collagen expression had a darker color on the conjunctival autograft group (Figure 3). This indicated a stronger type 1 collagen expression in the conjunctival autograft group compared with PRF membrane group. The PRF membrane and conjunctival autograft group had significantly different levels of type 1 collagen expression according to the Independent T-Test ($p = 0.032^*$; $\alpha<0.05$). A higher type 1 collagen expression was found in the conjunctival group compared with PRF membrane group.

DISCUSSION

Conjunctival wound healing is a complex process which involves many cytokines, growth factors, and proteases which interact to regulate important phases of wound healing. TGF-β is one of the main components of fibrotic responses involved in wound healing.[12]It stimulates fibroblast migration andsynthesizes conjunctival extracellular matrix. As a result, TGF-β is known to have an important role in many diseases with overproduction of fibrosis tissue. TGF-β expression is different in each conjunctival wound healing process. It is increased in the inflammatory phase, early remodeling phase, and middle remodeling phase. In the late remodeling phase, TGF-β expression decreases so that it reduces fibrosis minimizing the scar tissue formation.[13]

PRF membrane itself is known for its richness in cytokines and growth factors such as TGF-β. In some studies, PRF is shown to have certain unique effects toward TGF-β expression in a conjunctival wound healing model. PRF membrane is proven to cause short term increase in TGF-β expression (3 days and 7 days after wound healing process is started). The increase of TGF-β accelerates cells migration, extracellular matrix production and accelerates overall wound healing. TGF-β expression reduces after the 7th day of wound healing, in conjunction with the absorption and incorporation of PRF membrane. TGF-β expression becomes low after the 28th day of wound healing.[4]Thisprocess of wound healing is different with bare sclera healing of conjunctiva. TGF-β tends to decrease from 3rdto 7th day of conjunctival wound healing, however the expression increases after the 28th day of wound healing process. This condition is followed by increased inflammation and fibrosis until the 28th day. This proves that the increase of TGF-β in the inflammation and proliferation process is beneficial for conjunctival wound healing process.[4]

It was hypothesized that the increase of TGF-β expression in the inflammatory and proliferation process cause a negative-feedback which further decreases TGF-β expression in the remodeling process.[3] Another hypothesis is that because PRF contain important cytokines and growth factors which are speculated to contribute in the regulation of TGF-β expression in each phase of conjunctival wound healing process starting from inflammation process up to remodeling process. Besides, PRF membrane is also known to contain many pluripotent cells which are trapped from blood and those pluripotent cells have ability to regulate growth hormone expression such as TGF-β.[3]

The result of this study revealed there was a different TGF-β expression between conjunctival autograft and PRF membrane group. TGF-β expression was higher in the PRF membrane group compared with conjunctival autograft group. This result is similar to a study by Can et al., which showed that TGF-β expression was significantly higher in the PRF membrane group compared with the bare sclera group (bare sclera group served as the control group in that study). This result convinced us that PRF membrane caused increased TGF-β expression. The TGF-β that is supplied by PRF membrane is considered as exogenous TGF-β because it is not naturally produced in the conjunctiva and might have different actions and different TGF-β isoform compared with those produced by the conjunctiva.[4]

The PRF membrane can maintain 30% of its growth factors for over a week period. Besides, PRF membrane which contain much fibrin makes the remaining growth factors to continue its activity and last longer. After the degradation of fibrin matrix, there is progressive and controlled epithelial factor stimulator. This increases the cell proliferation and adhesion capability. PRF membrane did not only provide growth factors, but it also acted as a scaffold or foundation for epithelial cells which actively

migrate to close the conjunctival defect. The combined mechanical and chemotactic properties of PRF membrane make it a very good medium for the reconstruction of ocular surface especially conjunctiva.[14]

The cellular mechanism of conjunctival wound healing process is influenced by fibroblast and myofibroblast expression.[15] This process is mediated by cytokines and growth factors such as TNF-α, IL-1, and TGF-β. Fibroblast and myofibroblast have an important role in the conjunctival wound healing however it can also cause negative influence if left uncontrolled. Fibroblast and myofibroblast contribute to conjunctival tissue integrity after conjunctival wound healing. However, if this process is uncontrolled, it will cause an overproduction of scar tissue as an aftermath. Thus, the differentiation and degradation of fibroblast and myofibroblast also become important factor in the remodeling process of conjunctival wound healing.[15]

TGF-βis also released by alpha granules present in platelets along with other factors like VEGF, EGF and PDGF.[15] They serve as initiator and controller of conjunctival wound healing including conjunctiva. Those factors along with TGF-β have many important roles such as regulating cell proliferation, extracellular matrix synthesis, angiogenesis, immune response and myofibroblast degradation process. Moreover, TGF-β can induce the conjunctival stromal cells to differentiate into myofibroblast, which is very essential for conjunctival stromal wound healing process.[15] Studies have shown that PRF membrane releases growth factors such as TGF-β, PDGF and VEGF in a stable pattern continuously for 7 days.[16,17,18]

Can et al, utilized PRF membrane to treat descemetocele and corneal ulcer in humans.[4]The result was satisfactory as PRF membrane reduced pain and exhibitedanti-inflammatory effect. However, after few months, the PRF membrane group showed corneal neovascularization. It was thought to be caused by increased effect of VEGF and TGF-β which was released by PRF membrane. Different from the result of conjunctival study, the increase of neovascularization by VEGF and TGF-β seemed to give positive impact for the acceleration of conjunctival defect closure.

TGF-βin the PRF membrane cause a transient increase of growth factors in the first 7 days of conjunctival wound healing, but after 30 days the expression of these growth factors is decreased. Thus PRF membrane is superior in conjunctival reconstruction compared with corneal procedures done in corneal ulcer and descemetocele.[15]

TGF-β expression starts early and is higher in the PRF membrane. The difference of TGF-β in the early phase and late phase also happened in the PDGF.Theoretically, PDGF and TGF-β are two key mediators in fibrosis and scar tissue production in conjunctival wound healing process.[4] Histopathological analysis with hematoxylin eosin (HE) reveals that on the 28th day, inflammation and scar tissue did not occur in the PRF membrane group. The early conjunctival defect closure, might give signal that the conjunctival wound healing process is almost done thus reducing the signal for subsequent production and expression of TGF-β in the later phase.[4]

The acceleration of conjunctival extracellular matrix formation is an advantage to the overall conjunctival wound healing process. After the conjunctival defect is closed with extracellular matrix, inflammation is decreased and the production of scar tissue was bogged down. Besides providing more exogenous TGF-β, PRF membrane also has a scaffolding advantage which facilitated the conjunctival cells to migrate and closed the conjunctival defect. The role of PRF membrane as a scaffold also contributed in producing the negative feedback which reduce inflammation as a whole after the wound healing was commenced.

Inflammatory mediators such as histamine can stimulate fibroblast to increase proliferation, migration, and collagen production. There is also release of some phagocytic cells such as neutrophil, monocyte, and proteolytic enzyme which support tissue debridement. An activated phagocyte is similar to the activated platelet. Activated phagocyte cells stimulates growth factors such as TGF-β and further unleash, activate, and maintain fibroblast cells. From this explanation we understand that in the inflammatory phase after wound healing process, conjunctival tissue tries to close the wound through increase of inflammation. However, this wound healing closure mechanism, is naturally going to result in the formation of fibrosis or excessive scar tissue. The growth of fibrosis which is formed is very dependent to the wide area of defect, local immune and systemic immune condition which contribute all along the four phases of conjunctival woundhealing.[19,20]

The result of this study showed that there was a significant difference in type 1 collagen expression between PRF membrane and conjunctival autograft group. Type 1 collagen expression was found higher in the conjunctival autograft group compared with PRF membrane group. Type 1 collagen expression signaled the higher inflammation process in the conjunctival autograft group. This was also a sign for an increased proliferation phase. Type 1 collagen in a major contributor for conjunctival extracellular matrix. The increase of type 1 collagen in the inflammation and proliferation is a strong indicator for the overproduction scar tissue at the end of remodelingphase. A lower type 1 collagen expression in PRF membrane had a better ability to prevent fibrosis compared with the conjunctival autograft group.

In many studies, PRF membrane showed many advantages as a unique and special bio membranewhich has scaffolding effect, anti-inflammatory effect, and antibacterial effect.[16] The anti-inflammatory effect of PRF membrane is caused by its ability to modulate M1 macrophage which has proinflammatory property into M2 macrophage which has anti-inflammatory or proresolving property. PRF membrane was also thought to increase the production and activity of M2 macrophage which actively phagocyte and prevent the overproduction of extracellular matrix. M2 macrophage was also thought to have a role in the phagocytosis of the overproduced type 1 collagen and reduced its expression.[2,16]

Type 1 collagen expression in the conjunctival autograft group on the 14th day of conjunctival wound healing means that inflammation and the proliferation process in the conjunctival autograft is higher than the PRF membrane group. This is a major disadvantage for conjunctival autograft technique because it caused an overexpression of type 1 collagen which was higher in the inflammation and proliferative process so it had the tendency to cause a higher scar tissue at the end of remodeling phase. Conjunctival autograft technique is a technique which is mostly performed to close conjunctival defect after pterygium surgery. However, this technique cannot be performed whenever there is a large and bilateral defect such as in the case of mechanical trauma, chemical trauma, after surgery of wide conjunctival tumor, or autoimmune such as SJS and TEN. In this case, PRF membrane is proven to have superiority over the conjunctival autograft since it can be performed in a bilateral and wide conjunctival defect.[17,18] Besides, PRF membrane was also proven to be more superior in suppressing fibrosis which was marked by a lower expression of type 1 collagen in the PRF membrane group.

Research on human eyes should be done in randomized controlled trials to see if similarly suppressing is seen in the humans too.

CONCLUSION

There was a higher expression of TGF-β in the PRF group compared with the conjunctival autograft group. However, type 1 collagen expression was higher in the conjunctival autograft group compared to the PRF group. PRF membrane results in early wound healing and lesser fibrosis at the end in animal study.

Funding: This study was funded by Airlangga University research fund.

Patient's Consent: Researchers followed the guidelines set forth in the Declaration of Helsinki.

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.142.10.2022).**

ACKNOWLEDGMENTS

We would like to express our gratitude to the staff of the Department of Ophthalmology and the Department of Anatomical Pathology, Medical Faculty, Airlangga University, Dr. Soetomo General Academic Hospital, Indonesia. Additionally, we extend our thanks to the staff of the Veterinary Medicine Faculty, Airlangga University, Surabaya, Indonesia.

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