Effect of platelet rich fibrin membrane on the expression of smooth muscle actin and transforming growth factor beta in corneal wound healing *Pseudomonas aeruginosa* keratitis patient: a literature review

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### ABSTRACT

One of the most common types of microbial keratitis is bacterial keratitis. The most common cause is *Pseudomonas aeruginosa*, which is more severe and causes local tissue damage. Fibrosis can form and cause vision loss, and perforation and spread to surrounding tissues in severe cases. *Platelet-rich fibrin* (PRF) membrane grafting is a new surgical technique that can optimize wound healing by preventing fibrosis formation by modifying Transforming Growth Factor Beta (TGF-β) expression and suppressing its overexpression. In addition, it preserves the structural integrity of the eye. It promotes the sustained release of growth factors, both of which are necessary for tissue regeneration and the induction of new collagen lamellae production. We suggest that in *Pseudomonas aeruginosa* keratitis, PRF may affect the expression of TGF- and Alpha Smooth Muscle Actin (-SMA). This literature review will reveal more details on how PRF can control inflammation and aid in *Pseudomonas aeruginosa* keratitis wound healing.

### INTRODUCTION

Keratitis is an inflammation of the cornea from various causes and clinical manifestations, due to disruption of defense mechanisms caused by injury or small epithelial defects. Various types of pathogens or environmental influences can cause corneal inflammation. Keratitis is divided into two, namely infectious and non-infectious keratitis. Bacterial keratitis usually develops when the eye's defense mechanisms are compromised. The most common causative microbes include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes*.¹² Initiating the host's innate and adaptive immune responses requires *Pseudomonas aeruginosa*. In a corneal rat model, *Pseudomonas aeruginosa* can increase the expression of caspase-1, TNF, and caspase-3 while decreasing the expression of protein kinase 3 interacting receptors, which are key biomarkers in the mechanism of death of critical epithelial cells.³ With a prevalence of 3.2% in instances that have been reported, turbidity ranks as the sixth most common cause of blindness worldwide. According to the WHO, infectious diseases account for 6 million cases of blindness worldwide.⁴ According to a study conducted in India, macaque infection accounted for 57.52% of cases of cloudiness, with cicatrical neubelea accounting for 42.85% of cases and the central region accounting for 49.35%.⁵ According to a study from Indonesia that was published at Cipto Mangunkusumo Hospital in Jakarta, *Pseudomonas sp.* bacteria accounted for 25% of the bacteria in the culture results, making keratitis the most infectious cause of the disease.⁶

In previous studies, Platelet rich fibrin (PRF), which has many advantages over platelet rich plasma (PRP), includes a simple and effective collection process, arrested platelets are absorbed into the fibrin network, which promotes cell migration, proliferation, hemostasis, and the absence of external activators for platelets. It is being used more frequently to speed up the healing of wounds. An anticoagulant-free blood sample is taken to obtain platelet-rich fibrin, which is subsequently centrifuged once to create the PRF matrix.⁷ Numerous matrix proteins and growth factors, including platelet-derived growth factors (PDGF), vascular endothelial growth factors (VEGF), and transforming growth factors (TGF-β), are present in the PRF membrane and are essential for promoting wound healing.⁸ The fibrosis process in wound healing depends on TGF-β because it promotes fibroblast migration, which is necessary for the production of extracellular matrix. Collagen, the most prevalent substance in the extracellular matrix, myofibroblasts aid in wound healing, and other proteins make up this structure.¹⁰,¹¹ α-SMA, a smooth muscle cell apparatus, is present in myofibroblasts and can be recognized by immunohistochemistry.
staining with α-SMA antibodies. Extracellular matrix, collagen, and myofibroblasts are all deposited in excess in the damaged organs as a result of long-term fibroblast activation. Controlling fibroblast activity, particularly during the proliferative phase, is a crucial method for influencing wound healing results. Therefore, it is crucial to comprehend the impact of Platelet Rich Fibrin Membrane on Alpha Smooth Muscle Actin and Transforming Growth Factor Beta Expression in Pseudomonas Aeruginosa Keratitis.

**Corneal Anatomy and Physiology**

Epithelium, Bowman’s layer, stroma, Descemet’s membrane, and endothelium are the five layers that make up the transparent, avascular cornea (Figure 1). The cornea’s dimensions in adults are 11–12 mm horizontally and 10–11 mm vertically. It is 500–600 μm thick in the middle and gradually gets thicker as it moves outside. The cornea relies on the passage of oxygen and glucose through the tear film to obtain sustenance. Additionally, the limbal circulation delivers oxygen to the peripheral cornea. The cornea has one of the largest densities of nerve endings in the body and is 100 times more sensitive than the conjunctiva. The subepithelial plexus is made up of sensory nerve fibers that branch from the long ciliary nerves. The first branch of the sensory nerve fibers that branch from the eye’s intrinsic conditions, resulting in epithelial defects. Causes include acute abrasion trauma, bullous keratopathy, recurrent corneal erosion, recurrent epithelial damage, retained foreign body, or corneal surgery. In addition, the decreased immunological ability of the host, both locally and systemically, facilitates infection.

Microbial keratitis is a sight-threatening infection that bacteria, fungi, and parasites can cause. Epithelial defects and injuries are some of the predisposing factors to pathogenic infections in the eye organs. The bacterial species most often the etiology of bacterial keratitis include Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumoniae, and Serratia species. Clinical signs and symptoms of bacterial keratitis include acute pain, redness, photophobia, and corneal ulceration. Keratitis caused by Pseudomonas generally has a more severe presentation than ulcers due to other bacterial species. It is often a challenge in its management, which results in a worse risk of visual prognosis than other bacterial ulcers.

Bacterial keratitis accounts for up to 90% of all cases of microbial keratitis, with Pseudomonas aeruginosa being the most common etiology. Corneal infection by Pseudomonas is frequently associated with contact lens use, and its reported incidence was rare before widespread contact lens use. Apart from Pseudomonas aeruginosa, Streptococcus pneumoniae and Staphylococcus aureus are also the most common etiology of bacterial keratitis.

Elastase A and B, modified elastase, alkaline protease, protease IV, Pseudomonas aeruginosa small protease, and large exoprotease are just a few of the protease enzymes that Pseudomonas aeruginosa can secrete. Some of these enzymes may be involved in the pathogenesis of keratitis. In keratitis, where injection of Weld B into the corneal stroma has been proven to induce considerable corneal injury, metalloproteases, particularly elastase B (Las B) and alkaline protease (AP), play a crucial role. However, protease IV (PIV) also contributes to the pathogenicity of Pseudomonas aeruginosa by degrading several proteins involved in host defense systems, including immunoglobulins, complement proteins, antimicrobial peptides, and surfactants. The primary structural element of the corneal stroma, collagen, can be broken down by Pseudomonas aeruginosa small protease (PASP), leading to corneal obliteration. It has been demonstrated that injecting pure PASP into the rabbit cornea causes epithelial damage and the development of erosional scars that can penetrate the stroma. These results show that PIV participates in the bacterial defense
Figure 2. Schematic diagram of the TGF-β-mediated process of corneal wound healing: (a) Corneal injury causes cytokines, TGF-β and PDGF keratocyte cells to differentiate into myofibroblasts, (b) Corneal lesion healing process is complete and myofibroblast cells experience apoptosis, (c) Myofibroblast cells remodel the extracellular matrix and neovascularization appears.

Mechanism against different host immune components. In contrast, PASP degrades the collagen structural elements of the eye, and both of these mechanisms are involved in the pathogenesis of keratitis.

Alpha Smooth Muscle Actin (α-SMA)

The actin isoform known as alpha-smooth muscle actin (α-SMA), which predominates in vascular smooth muscle cells, is crucial to developing fibrosis. It has also been demonstrated that the fibroblasts of subcutaneous tissue contain α-SMA. Myofibroblast activation is crucial to the fibrotic response because myofibroblasts are a particular type of fibroblast with a distinctive metabolism, shape, and capacity to express α-SMA. Myofibroblasts cease growing and start producing a lot of extracellular component proteins when they are activated. The expression of α-SMA is correlated with the rate of myofibroblast activation.

Myofibroblasts are fibroblast cells that have undergone partial smooth muscle phenotypic differentiation. Myofibroblasts can contract by employing a variety of cytoskeleton proteins, including α-SMA, which is frequently present in smooth muscle cells. By making the edges of the wound constrict, these cells can hasten the healing process of the tissue. Previous research has demonstrated that when subjected to chemicals that elicit smooth muscle contraction, such as adrenaline or angiotensin, granulation tissue taken from wounds can contract in vitro in a manner resembling smooth muscle. After the wound-healing process is complete, myofibroblast cells undergo apoptosis. In some disorders of the fibrotic process, the mechanism of myofibroblast apoptosis is thought to fail to occur, causing myofibroblast persistence, extracellular matrix expansion, and wound contracture. The large contractile force generated by myofibroblasts has an important role in physiological tissue remodeling. Still, it has a negative impact on tissue function when it is generated excessively, for example, in cases of hypertrophic fibrosis. Potential therapeutic targets for modulating myofibroblast function include α-SMA and myofibroblast cytoskeleton indicators, membrane surface proteins, and extracellular matrix. These unique biological traits and variables can govern fibroblast development. Actin is a globular structural protein that forms actin filaments when it polymerizes in a helical configuration. The cytoskeleton, a three-dimensional network found inside eukaryotic cells, is made up of actin filaments. Actin filaments are involved in the mechanical support of the cell, cell shape determination, and cell movement. Actin participates in the mechanism of muscle contraction in muscle cells along with myosin. The majority of actin in the cytoplasm is coupled to ATP. However, actin can also bind to ADP.

The molecular weight of α-SMA is 40 kD. The filamentous polymer made...
up of G-actin subunits (microfilaments) is known as F-actin, and each actin component is referred to as globular actin. With a diameter of 7 nm, microfilaments are the cytoskeleton's tiniest element. Actin filaments are polar, like microtubules, with a positive end growing quickly and a negative one growing slowly. Dissociation of the negative end of ADP-actin drives the exchange of ADP-actin bonds for ATP-actin bonds when ADP-actin levels rise. The critical function that this quick bond turnover plays in cell migration.\(^\text{20}\)

When the epithelial barrier is compromised, keratocytes are the first part of the corneal stroma that infectious microorganisms can come into contact with. Within myofibroblasts, these cells can differentiate and express α-SMA. The research by Pergolizzi et al. showed ex vivo keratitis models of -SMA expression. Inflamed corneal samples from another investigation by Spurlin and Lwigale also contained more stromal cells that were positive for α-SMA.\(^\text{21,22}\) The expression of -SMA was found to rise during the embryonic corneal healing process, according to Spurlin and Lwigale. Additionally, it has been demonstrated that the corneal stroma expresses α-SMA in neonatal and adult corneal lesions, and this expression lasts for weeks to months.\(^\text{23}\) Pergolizzi et al. demonstrated the presence of α-SMA within the corneal epithelium in a corneal model with herpes simplex virus-1 (HSV-1) infection.\(^\text{24}\)

**Transforming Growth Factor-Beta (TGF-β)**

The three TGF-β isoforms communicate through the TGFBR1, TGFBR2, and TGFBR3 TGF-receptors. One of the TGF-growth factor dimers attaches to TGFBR2, which initiates canonical TGF-signaling. The TGF-β-TGFBR2 complex then recruits and phosphorylates TGFBR1. The downstream modulators SMAD2 and SMAD3 are phosphorylated by the TGFBR2-TGFBR1 complex after that. SMAD2-SMAD3 complex binds to SMAD4 and undergoes translocation to the nucleus to regulate the TGF-gene transcription. On the other hand, the proteasome’s SMAD-specific E3 ubiquitin ligase enzyme recruits SMAD7 to the activated TGFBR complex and/or phosphorylates SMAD2/3 to cause its destruction. As a result, the ubiquitin-proteasome pathway modifies the signaling of TGF-β1, TGF-β2, or TGF-β3 whereas SMAD7 inhibits the TGF-β response.\(^\text{25}\)

Several agonist or antagonist molecules have been reported to facilitate or inhibit the binding of activated TGF-β1, TGF-β2, and TGF-β3 utilizing their receptor ensembles. For instance, depending on the growth factor involved and whether other growth factors are present in the environment, KCP/CRIM2, CHRDL1, and BMPER/CV-2 can either behave as agonists or antagonists. Other modulators, including follistatin (FST), FSTL1, BMPER/CV-2, and Lefty, can bind to TGFBR1 or TGFBR2, forming inactive non-signaling complexes that inhibit cell responses to TGF-β1, TGF-β2, and TGF-β3.\(^\text{26}\)

TGF-β1 and TGF-β2 are present in tears, and TGF-β1, TGF-β2, and TGF-β3 the watery humor contain. The ocular surface epithelium naturally produces TGF-β1 and TGF-β3, and these growth factor isotypes may be released during the epithelial cell turnover process, making the corneal epithelium one source of TGF-β in tears. Normal corneal endothelial cells also produce TGF-β1 but not TGF-β2. TGF-β1 or TGF-β2 proteins are produced in the rabbit cornea at low levels. TGF-β2 was not detected despite not being present in the corneal epithelial cells. It was found on the epithelial cell surface of the normal cornea and was also found on the stromal surface shortly after rabbits underwent photorefractive keratectomy (PRK). TGF-β1 and TGF-β2 proteins, however, were found in a variety of stromal cells, including keratocytes, corneal fibroblasts, myofibroblasts, and certain bone marrow-derived cells, after PRK injury to the cornea. The mRNAs for TGF-β1 and TGF-β2 were also elevated in corneal endothelial and epithelial cells that were close to wounds. Human corneal epithelium and stroma were both found to contain TGF-β2 LAP, whereas subepithelial stroma had TGF-β3 LAP.\(^\text{23,24}\)

One of the most important growth factors in the cornea’s wound-healing process is TGF-β. A schematic representation of the corneal wound healing process is shown in Figure 2 and 3. As a result of the Bowman’s membrane and corneal lining being torn apart during the corneal injury, the anterior stroma is then exposed to cytokines and other growth factors, including TGF-β and PDGF, which are produced by the corneal epithelial cells, aqueous humor, conjunctiva, and tears. Keratocytes seen in the anterior stroma and/or bone marrow cells develop into myofibroblasts, multiply, and move in the direction of the lesion when TGF-β1 is active. An additional A-domain of fibronectin found in the extracellular matrix effectively maintains TGF-1 levels. The wound is then covered with myofibroblast cells, which start to produce a new extracellular matrix.\(^\text{27}\)

After the corneal injury has healed, myofibroblast cells die due to IL-1-induced apoptosis. Keratocytes can recolonize the anterior stroma thanks to the IL-1 that stromal cells produce. MMP enzymes will subsequently cause myofibroblast cells to reconstruct the extracellular matrix at the wound site. This remodeling of the corneal tissue architecture is crucial for regaining corneal transparency and integrity. In certain pathological conditions, persistent TGF-β levels can potentially cause problems because they can maintain myofibroblast cells that continue to secrete extracellular matrix proteins at excessive levels. This situation can lead to tissue fibrosis and a clouding condition of the cornea, which may persist for a long time even after the myofibroblasts are no longer present at the lesion site. These pathological disorders have a wide range of impacts on visual function, from photophobia and irregular astigmatism to, in extreme cases, total blindness due to corneal fibrosis overlaying the visual axis.\(^\text{28}\)

TGF-β is active after an injury, TGF-β activity appears and is high at 1 hour and 5 days after injury, then begins to decrease on day 7, day 10 and ends on day 14. In the wound healing phase, in the proliferative phase, fibroblasts are converted into myofibroblasts which begin to occur on day 5, then myofibroblasts express α-SMA and reach their peak on day 10.\(^\text{25,26}\)
The Effect of Platelet-Rich Fibrin Membrane in Bacterial Keratitis

In the modern era of regenerative medicine, different kinds of platelet concentrates have been employed to quicken the process of tissue regeneration and wound healing. The processes of homeostasis, angiogenesis, inflammation, and tissue regeneration have all been proven to involve platelets. Platelets, an autologous source, contain more than 1500 bioactive chemicals, including growth factors, immune system messengers, and enzymes. These substances play crucial roles in tissue regeneration and wound healing. A mechanism by which platelet concentrates can promote wound healing is their capacity to provide growth factors at concentrations that are 6–8 times higher than the physiological level required by the body. The release of growth factors by platelets to encourage the development of mesenchymal stem cells and other target cells is another mechanism by which the healing process is influenced.27,28

Platelet concentrates have been employed in regenerative medicine for more than 20 years due to the shown potential of platelets to stimulate tissue regeneration. In order to obtain supraphysiological amounts of growth factors, platelet-rich plasma (PRP) is an autologous concentration of human platelets in plasma. Several steps of centrifugation, the inclusion of non-autologous anticoagulants, and the addition of calcium chloride or bovine thrombin are all required by the PRP process.29 In the maxillofacial field, it was reported in a rabbit experimental animal study, PRP is used in bone healing through a bone graft that is placed on the maxillary bone, which contains lots of platelets can help bone healing by increasing the density of osteoblasts in the maxillary bone graft.30

Platelet-rich fibrin (PRF) was initially made available in France by Choukron et al. in 2001 due to restrictions on the replantation of blood-derived materials. This second-generation platelet concentrate administration seeks to amass platelets, activate the immune system, and release cytokines in the fibrin clot. It is described as an autologous fibrin biomaterial that is leukocyte- and platelet-rich. PRF differs from PRP in a number of ways. Still, it also has a high concentration of leukocytes, which aid in immunity, antibacterial activity, and wound healing. In order to restrict the flow of growth factors into the tissue around the wound during the wound healing process, PRF generates a fibrin-rich network (dense fibrin network) that permits a slower rate of degradation. The release of growth factors from the majority of PRF types can last up to 7 days, and it can even stay longer for some types of PRF; according to research. Standardized production processes, lower prices, and easier manufacturing techniques are further benefits of PRF over PRP. In a 10 mL tube, blood samples were collected without the use of anticoagulants and centrifuged for 10–12 minutes at 2,700–3,000 rpm. Following centrifugation, erythrocytes are found at the tube’s bottom, whereas platelet-poor plasma (PPP) is found at the tube’s top. The PRP clot, which is composed of platelets, leukocytes, and growth factors, is situated in the tube’s center between the PPP layer and the erythrocytes. Taking PRF aims to enable fibrin polymerization and physiological activation of platelets. Platelet activation occurs shortly after contact with the centrifugation tube wall, forming a dense fibrin network and a usable PRF clot. As a result, the time from when the blood sample is drawn until it is placed in the centrifuge tube must be as short as feasible, ideally 2 minutes and 30 seconds. If this time is extended, diffuse fibrin polymerization may develop, making PRF ineffective.29

Among the growth factors present in PRF are TGF-β, platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF). In addition, PRF contains a number of cytokines, including IL-1, IL-6, IL-4, and TNF-α, in addition to the growth factors generated by platelets. According to studies, PRF is abundant with leukocytes and other kinds of cytokines in addition to having high quantities of platelets and growth factors. These results have significant scientific significance since lymphogenic substances released by leukocytes, which are crucial for cellular crosstalk during tissue regeneration, are the primary regulators of bone and soft tissue regeneration. Following the findings of these studies, more recent research has revealed that a reduction in centrifugation force in PRF preparations is connected to an increase in the total levels of platelets, leukocytes, and growth factors, suggesting that the idea of low-speed centrifugation increases the PRF’s capacity for regeneration. The recruitment and activation of neutrophils as well as the release of protease enzymes by neutrophils are induced by fibrin and fibrin breakdown products. These neutrophils eliminate bacterial contaminants through the formation of oxidative radicals. Additionally, fibrin’s interaction with monocytes and macrophages affects phagocytosis, demonstrating the significance of macrophages in the transition from inflammation to tissue healing. Tissue regeneration requires precise cell-to-cell communication, which is only feasible with leukocytes, proving that leukocytes play a crucial role in tissue regeneration in addition to platelets.29

PRF membranes have been used in several medical specialties, including ophthalmology, orthopedic surgery, plastic surgery, and surgery of the mouth, ear, nose, and throat. In three patients with severe corneal stromal melting and central desemetocoele brought on by neurotrophic keratopathy and infective keratitis, Can et al. reported using PRF membrane grafts. In patients with infectious keratitis, a single-layer PRF membrane graft was applied with a therapeutic bandage contact lens (TBCL) in addition to topical medications, including moxifloxacin, fluorometholone, sodium hyaluronate, and tetracycline. At one day postoperative follow-up, the PRF membrane successfully filled the corneal ulcer and blended with the corneal tissue. Administration of PRF membranes has also been shown to reduce pain intensity and conjunctival injection. In the three months postoperative follow-up period, the cornea was found to be thickened without transparency. There was no recurrence or decrease in corneal function in the one-year postoperative follow-up period.11

Thrombospondin-1, fibronectin, vitronectin, autologous platelet concentrates, and leukocytes make up the three-dimensional fibrin architecture of
PRF membranes, making them suitable for use as a scaffold in tissue engineering. Slowly released growth factors like PDGF, TGF-β, FGF, IGF-1, IGF-2, VEGF, and EGF regulate cellular connections and speed up matrix creation to enhance tissue repair as a result of platelet degradation and matrix disintegration. PRF membranes can be employed as an alternative biomaterial to cure the cornea, according to research by Can et al. (2016). Another study described the use of autologous fibrin membrane grafts in conjunction with PRP clots as a novel alternative treatment for cases of corneal perforation. In that investigation, the perforated area of the cornea was filled with a PRP clot as a stopper to keep the intraocular tissue from communicating with the outside world. After that, the surrounding conjunctival tissue was covered with an autologous fibrin membrane to cover the entire ocular surface and the PRP clot. The complicated process of creating autologous fibrin membranes, which takes 1.5 to 3 hours, involves platelet-poor plasma, autologous thrombin, calcium chloride, certain conditions, and a laminar flow hood. Contrarily, PRF membranes can be made more rapidly and easily utilizing a straightforward approach that needs a blood tube devoid of anticoagulants, centrifugation equipment, and 15 minutes before the surgical surgery. Additionally, PRF membrane preparations don’t contain any chemicals that can have negative local or systemic adverse effects.

Although leukocytes are present in the PRF membrane in relatively small amounts, there is concern that they could cause an exaggerated inflammatory response due to pre-inflammatory mediators, lysosomes, and other collagen or lytic derivatives present in leukocytes. However, Choukroun et al. showed that PRF membrane functions as an anti-inflammatory adjunct during surgical procedures because of its capacity to retroactively regulate inflammation. Choukroun et al. provided the first description of the process for manufacturing platelet-rich fibrin, in which no extra anticoagulant was needed throughout the production process. Up to 5 mL of blood was drawn from the rabbit’s auricular vein and stored in a glass tube. In addition, the blood sample was centrifuged at 2700 rpm for 12 minutes, resulting in the appearance of three distinct layers in the blood sample. The tube’s bottom layer comprises erythrocytes, the middle layer contains a fibrin clot, and the top layer is platelet-poor plasma (PPP). The PPP layer is removed first using a pipette, followed by the separation of the fibrin clot from the erythrocyte layer using scissors, the removal of the fibrin layer with forceps, and compression of the fibrin layer using a PRF box (PRF box), a membrane. The PRF membrane is subsequently trimmed to fit the application’s requirements for the cornea. Then placed in an overlay, covering the entire corneal surface.

CONCLUSION
According to the studies listed above, PRF has been shown to hasten tissue regeneration, heal wounds faster, and stop fibrosis from forming. The most recent state-of-the-art biomechanical membrane, PRF, is particularly useful in rebuilding the ocular surface. More research is still needed to properly understand how the PRF membrane impacts the expression of TGF-β and -SMA.

CONFLICT OF INTEREST
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