Rabbit corneal wound treatment using small intestinal submucosa (SIS) and platelet rich plasma (PRP) scaffold

Abstract

Traumatic corneal wounds trigger numerous inflammatory reactions. This severe inflammation can lead to fibrosis or scarring on the cornea's surface by inhibiting the growth of the natural epithelium. In this study, the healing effects of two simultaneous treatments of small intestine submucosal graft (SIS) and platelet-rich plasma (PRP) in rabbit corneal wound healing were investigated. Twenty white New Zealand rabbits weighing 2.5 to 3 kg, clinically healthy, and with no history of eye disease were selected and divided into four groups (N = 5) and subjected to a wound induction test by crescent knife. Following wound formation, the studied groups included control (absence of corneal wound covering with only physiological serum), PRP+SIS, SIS, and PRP in the form of 1 cc subconjunctival drops of PRP every 12 hours. In groups with SIS, the dressing was placed on the wound with a circumferential suture. With clinical eye examination and fluorescein staining, the wounds were examined in terms of size, infection, turbidity, and edema. 21 days after the operation, half of the animals from each group were killed, and their corneas were evaluated by histopathology. On the 21st day of the study, the PRP+SIS group had the lowest amount of corneal opacity. In the histopathological evaluation, the calculation of the number of rows of epithelium was not significant. The corneas of the PRP and SIS + PRP groups, as well as the SIS group, exhibited significantly less vascularization compared to the control group. The order of stromal collagens was significant in both the SIS group with SIS + PRP and the control group with SIS + PRP. The amount of edema between the control group and the SIS + PRP and PRP groups was significant. The level of inflammation was significantly lower only between the control and SIS+PRP groups had significantly lower levels of inflammation. SIS and PRP alone cannot have the simultaneous use effect that we saw in the SIS and PRP groups. As a result, using the SIS + PRP method in such corneal wounds may be an effective method with less vascularization and inflammation.

Keywords: corneal ulcers, platelet-rich plasma, small intestinal submucosa, healing

1. Introduction

The cornea is the clear covering layer of the eye that serves as anatomical barriers between the outside world and the eye, as well as having refractive properties. Severe corneal damage can result in loss of clarity and associated visual problems. Corneal injuries commonly cause damage to the epithelium and its basement membrane. Active fibroblasts, keratocytes, and myofibroblasts start healing until they terminate. TNF-α, IL-1, and TGF-β are among the growth factors and cytokines that orchestrate these processes. Myofibroblasts, due to their role in preserving the cornea's integrity, have an impact on corneal ulcer healing in both positive and negative ways. Persisted scarring can also be caused by myofibroblasts. Therefore, after severe corneal injury, correct differentiation and the elimination of myofibroblasts are essential for remodeling (1). Corneal neovascularization is the consequence of excessive wound healing after surgery, trauma, or infection. The development of new vascular structures in previously avascular regions is known as neovascularization. Corneal neovascularization, for example, is typically linked to infectious or inflammatory diseases of the eye surface. Research on cancer angiogenesis has demonstrated that the cornea exhibits a balance between anti-angiogenic chemicals, such as endostatin, angiostatin, or pigment epithelium-derived factor, and angiogenic factors, such as VEGF and FGF. Neovascularization, or the activation of angiogenesis in the cornea, is a result of issues such as inflammation, infection, damage, and injury. Proteolytic enzymes such as metalloproteinases (MMPs) may have an impact on corneal neovascularization (2). Various materials are used for corneal transplantation, including platelet-rich plasma (PRP) and small intestinal submucosa (SIS) methods. Platelet-rich plasma (PRP) is an autologous concentration of platelets in a small volume of plasma obtained by centrifugation of whole blood. The alpha granules of platelets are rich in growth factors that promote angiogenesis, reduce inflammation and collagen deposition, and provide background material outside the cells. In keratectomy rabbits, subconjunctival injection of PRP causes faster corneal re-epithelialization and regeneration, fibroblast migration, and less inflammation. Prescribing PRP alone, without topical antibiotics, gives the best results (3). In 2021, Farghali et al. demonstrated that using PRP in corneal wound healing in dogs and cats accelerates re-epithelialization (2). To date, in canine keratoconjunctivitis sicca, studies on the effects of PRP on metalloproteinases have been limited, and the results are controversial (4, 5). In

2023, Piso et al. demonstrated that the application of platelet-rich plasma eye drops influences the production of matrix metalloproteinases involved in corneal healing (4).

SIS is also a type of biological material that is used in clinical cases. SIS's structure, biological activity, and immune response make it suitable for body repair. This material is useful in tissue engineering and regenerative medicine of organs such as vessels, bladders, gastrointestinal tracts, valves, and tendons (6). SIS consists of various compounds such as collagen, elastin, fibronectin, laminin, glycosaminoglycans, and proteoglycans. It also has fibroblast growth factors (FGF-2), beta-growth factors (TGF-β), and vascular endothelial growth factors (VEGF). Most of the SIS is made up of collagen, and the other aforementioned parts contribute a small amount. Collagen helps to repair wounds by causing them to contract (4). The multifunctional glycoprotein regulates cell attachment to the ECM, and proteoglycans provide cell adhesion sites and inhibit substrate-degrading enzymes (Mulloy et al., 2006). In addition, it causes SIS to release growth factors such as VEGF and TGF-β (7). However, which of the methods is more effective in the treatment of corneal ulcers is still unclear. For this reason, this research aims to identify and introduce the most efficient method for treating corneal ulcers by comparing various biological materials used in wound healing with each other.

2. Material and Methods

2.1. Ethical approval

This research involves animals, and its ethical management was approved by the Ethics Committee. The Ethics Committee considered details and information about the ethical management of animals, including an ophthalmic assessment format for each member who participated in the study and who analyzed the severity of the lessons. In optimal conditions, animal welfare was observed, and nose contact between patients was avoided. An animal observation assessment was conducted, consisting of three separate daily checks. Two of the above checks included an examination of each individual. In addition, a daily intramuscular injection of 2.2 mg/kg flunixin meglumine was administered for five days to decrease post-surgical swelling and pain. It is significant to note that in order to treat corneal ulcers, which are known to be the most painful for patients, pre-anesthetic medicine containing xylazine is used. In these circumstances, xylazine is essential for reducing pain. Additionally, deep general anesthesia is used throughout the surgical procedure.

Throughout the investigation, the patients did not exhibit any notable indications of severe pain, such as discomfort or other symptoms. In fact, they maintained their food intake and physical activity at optimal levels without any noteworthy changes that would warrant special attention or exclusion from the experiment.

2.2. Platelet-rich plasma preparation

To maintain the product's autologous principle, platelet-rich plasma (PRP) was taken from the same animal the day before the ulceration on which it is intended to be administered. Using a two-fold centrifuged procedure of 10 ml of whole blood with an anticoagulant citrate dextrose solution, 0.7 ml of non-activated PRP was obtained, regardless of the application form.

2.3. Preparation of the sheep decellularized small intestinal submucosa (SIS)

The decellularization protocol consisted of mechanical separation of intestinal layers from each other, detergent treatment, and washes with 0.9% saline solution in between. SIS was prepared by mechanical removal of the tunica serosa and tunica muscularis from the small intestines and cleaned by repeated washes with saline solution. The SIS was further treated with 1% sodium dodecyl sulfate (SDS) under continuous shaking for two days, thoroughly rinsed with saline solution, and treated with 1% triton X-100 afterward. The detergent was removed by thoroughly rinsing with a saline solution containing 1% pen/strep. At the end of the decellularization protocol, all SIS membranes were collected and lyophilized overnight using SCANVACVR Coolsafe.

2.4. Animals

Twenty New Zealand white rabbits from the Experimental Animal Center, weighing 2.5 to 3.0 kg, were used for cell animal transplantation. Clinical observations were made for animal health. The rabbit small intestine was obtained from the donor group in the laboratory in compliance with the ethical principles of laboratory animals.

2.4.1. Studied groups

Following ulceration, the studied groups included control (3 drops of only physiological serum a day for 14 days), PRP+SIS, PRP groups as 8 drops of PRP equally four times a day for 14 days, and SIS group consist of SIS graft only.

2.4.2. Corneal ulceration

In order to create anesthesia, ketamine (10% Rotex Pharmaceutical Company, Germany) in the amount of 20 mg/kg and xylazine (2% Bremerpharma, Pharmaceutical Company, Germany) in the amount of 1 mg/kg were used intramuscularly. After complete anesthesia, the left eye of each

rabbit was smeared with 2 drops of 0.5% tetracaine, and after 5 minutes, the cornea was ulcerated in the 1 mm dimension using a crescent knife. The eye was washed with 2 ml of sterile normal saline, and immediately after the ulceration, the eye was stained with fluorescein to ensure the sameness of the wounds. To prevent post-operative pain, a single dose of flunoxine meglumine (Royan Daru Pharmaceutical Company) at 1 mg/kg was injected subcutaneously into all groups (8).

2.4.3. Fixation of SIS

The resulting SIS in the sterile saline solution was trimmed to cover the corneal ulcer. The SIS was fixed with simple sutures of 8-0 (Vicryl resorbable suture, Ethicon, United Kingdom), placed in four circular positions around it.

2.4.4. Care after surgery

To prevent infection, enrofloxacin (30 mg/kg/d) was injected intramuscularly for 5 days, and tramadol (20 mg/kg) was injected daily for 3 days for analgesic effect. Additionally, we checked the area for swelling or inflammation, the presence of secretions, and any potential local infections.

2.4.5. Measurement of Corneal Opacity

Using the slit lamp, the degree of corneal opacity was assessed in accordance with a previously reported technique (9) and scored as follows: The following were the scores: There are four types of areas: 1. diffuse or scattered with visible iris details; 2. easily observable translucent with somewhat veiled iris details; 3. opalescent with barely perceptible pupil size and no visible iris details; and 4. opaque with no apparent iris details.

2.5. Microscopy

2.5.1. Histopathology

At the end of the third week, the animals were anesthetized, and the eyeballs were removed. After separating the eyeballs, the samples were placed in 10% formalin and sent to the pathology laboratory. In the laboratory, the cornea disk was separated from the eyeball, and after the preparation of the paraffin block, 5µ sections were prepared and stained with hematoxylin-eosin and finally scored according to the evaluation criteria of the indicators.

Histological grading of the corneal ulcer was done om 0 till 3 for Vascularization, Epithelialization, Inflammation, Edema and Collagen regularity. The vascularization phenomena were 0: ansent, 1: mild, 2: moderate and 3: high. The vascularization phenomena were 0: ansent, 1: mild, 2: moderate and 3: high. The vascularization phenomena were 0: no, 1: mild, 2: moderate

and 3: high. The epithelialization phenomena were 0: ansent, 1: 1-2 layers, 2: 3-4 layers and 3: ≥5 layers. The Inflammation phenomena were 0: no, 1: mild and scatered, 2: moderate and 3: High and diffuse. The edema phenomena were 0: no, 1: mild and focal, 2: moderate and focal and 3: High and diffuse. The Collagen regularity phenomena were 0: no, 1: mild, 2: moderate and 3: Normal.

2.5.2. Immunohistochemistry (IHC)

The CD31 and αSMA were evaluated using the IHC method to define the rate of the vascularization and myofibroblast population, respectively. The IHC test gives a score of 0 to 3, indicating the amount of special receptor protein on the surface of cells in a related tissue sample. If the score is 0 to 1, it is called negative. If the score is 2, it's called borderline. A score of 3 or higher is called positive (10).

2.6. Statistical analysis

For statistical analysis and pathological results analysis, all qualitative data were converted into quantitative data by Graphpad Prism software version 9 and graded on an incremental scale from 0 to 3. The data were evaluated using the non-parametric method, Kruskal-Wallis post hoc test for comparison between different groups, and Mann-Whitney U for comparison between two groups, and the differences were considered significant at the level of $p<0.05$.

3. Results

3.1. Corneal Opacity (Edema)

Corneal opacity on day 21 was higher in the control group than in the PRP, SIS, and PRP+SIS groups. Also, a significant difference was observed between the treatment groups, indicating that the PRP+SIS group has the least opacity compared to all groups (Figures 1 and 2).

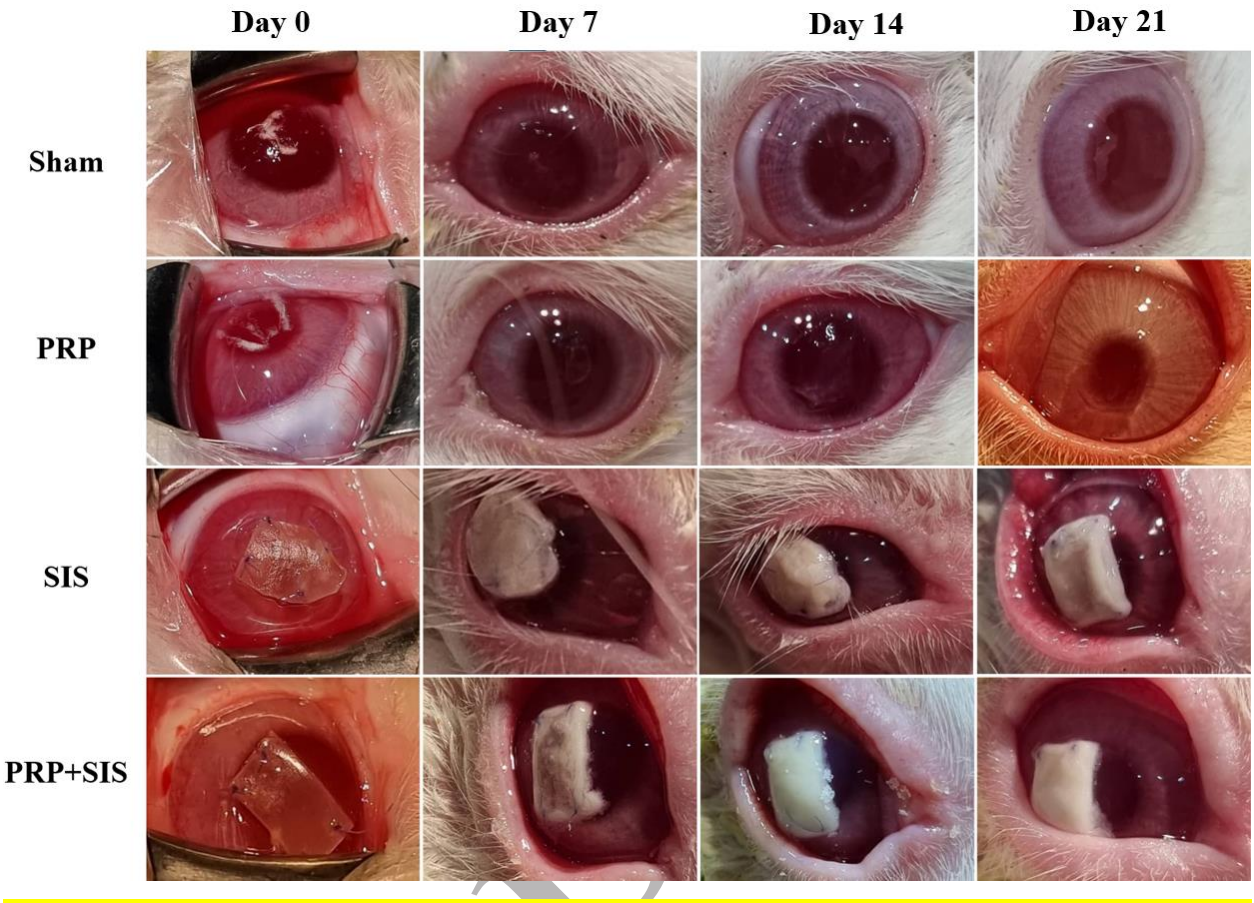

Figure 1. In vivo observations of corneal opacity in ulcerated rabbit. Photographs of corneas from treated rabbit for 0, 7, 14 and 21 days.

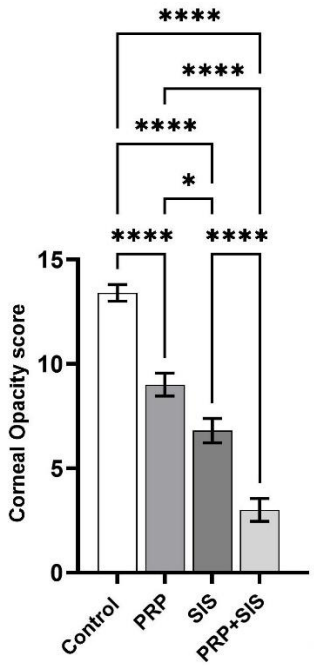

Figure 2. The corneal opacity scoring. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

3.2. Histopathological findings

3.2.1. Vascularization

The groups were very different from each other. There was a significant difference between the control and the other groups. Additionally, the PRP and PRP+SIS groups didn't show any significant differences. On the other hand, there was a significant weekly difference between the control and SIS groups. This suggests that PRP and scaffolding complement each other well to improve corneal lesions and create ideal conditions for recovery (Figures 3 and 4).

3.2.2. Epithelialization

The treated and control groups' epithelialization did not differ from one another. Significant differences were also not observed in the PRP, SIS, and PRP+SIS groups. This finding indicates that PRP and scaffold alone or in combination may not provide greater comfort for corneal ulcer healing compared to the control (Figures 3 and 4).

3.2.3. Edema

The incidence of edema varied between the treatment and control groups. The data demonstrated that the edema distribution in the PRP and PRP+SIS groups was significantly lower than that of the control group (Figures 3 and 4).

3.2.4. Inflammation

There were no differences in inflammation across all groups, except between the control and PRP+SIS groups. Given that neither PRP nor SIS displayed any irritating behavior on the corneal ulcer's surface, this indicates that the inflammatory response was not overblown in the therapy groups, and the combined therapy of them could reduce the inflammatory process (Figures 3 and 4).

3.2.5. Collagen regularity

Wound healing is directly related to the regularity of collagen bundles and fibroblasts. In this study, the collagen regularity of the control group was weaker than the treatments. On the other hand, the PRP+SIS group showed a greater improvement in collagen deposition with more regularity than the control and SIS groups. This shows that the PRP+SIS and PRP groups, which have a higher ability to produce collagen, can improve the corneal wound more (Figures 3 and 4).

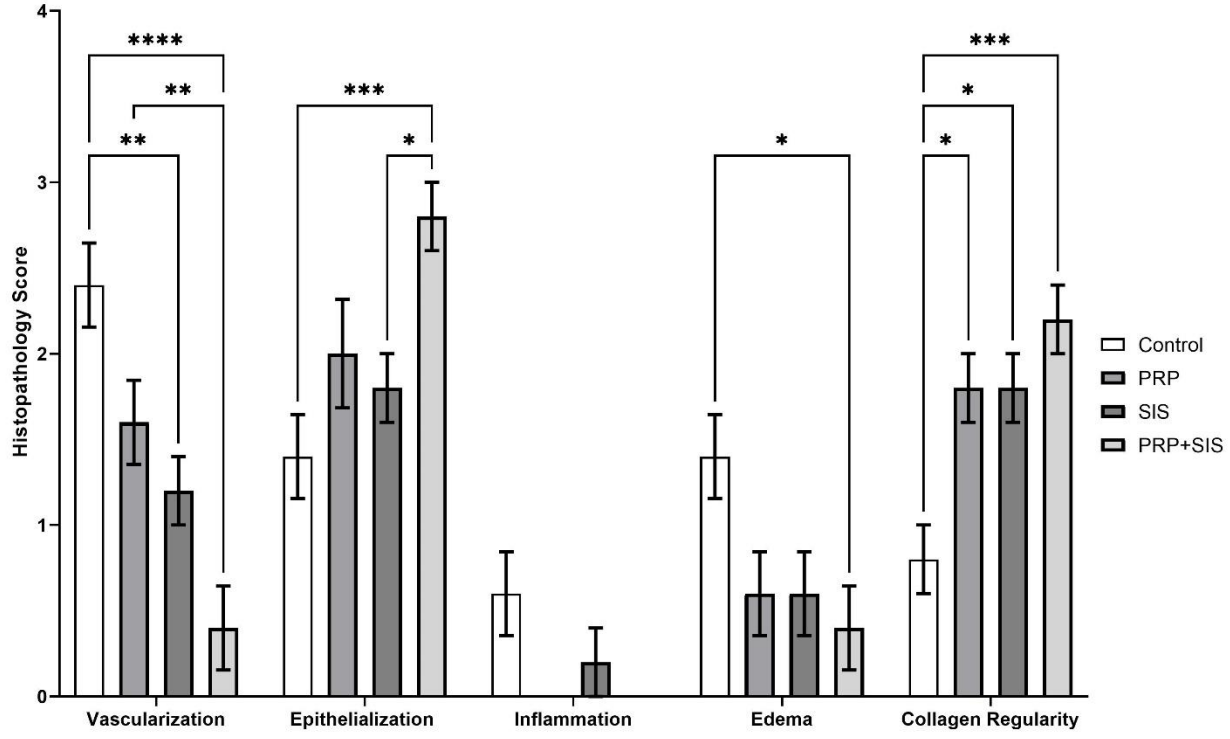

Figure 3. The corneal ulcer histopathology scoring. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

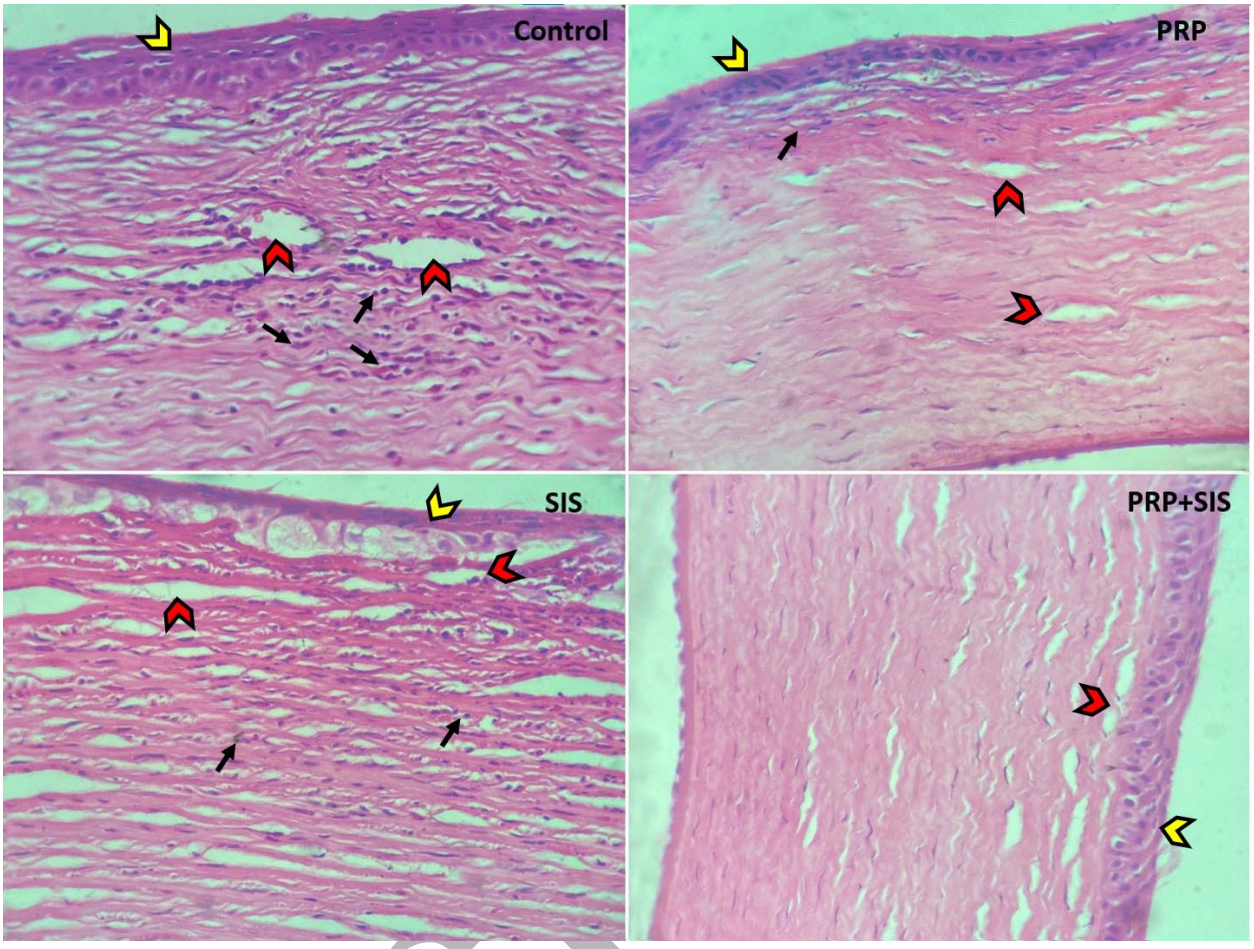

Figure 4. Histological evaluation of corneal ulcers from the 21st day. Corneal sections show healing of the corneal epithelial layer, neovascularization, and inflammation in the peripheral and central cornea. The collagen production and regularity and improvement of ulcers were observed in the following order: PRP+SIS > PRP > SIS > control. All pictures are at the same magnification (HE × 100).

3.2.6. Immunohistochemistry

The CD31 and αSMA were evaluated using IHC method. Three weeks after surgery, immunohistochemistry was performed to estimate the number of blood vessels (CD31) and the differentiation of myofibroblasts (α-SMA). Figures 5 and 6 show that in the PRP+SIS group, α-SMA detection was only observed focally under the corneal basement membrane. In contrast, α-SMA was widely detected in the corneal stroma in the control and SIS groups. In the PRP+SIS group, the detection of -SMA was very rare. Overall, PRP with or without SIS significantly

accelerated myofibroblast reduction and keratocyte dedifferentiation (11). All groups had lower CD31 levels than the control, indicating that clearing and transparency improved these groups.

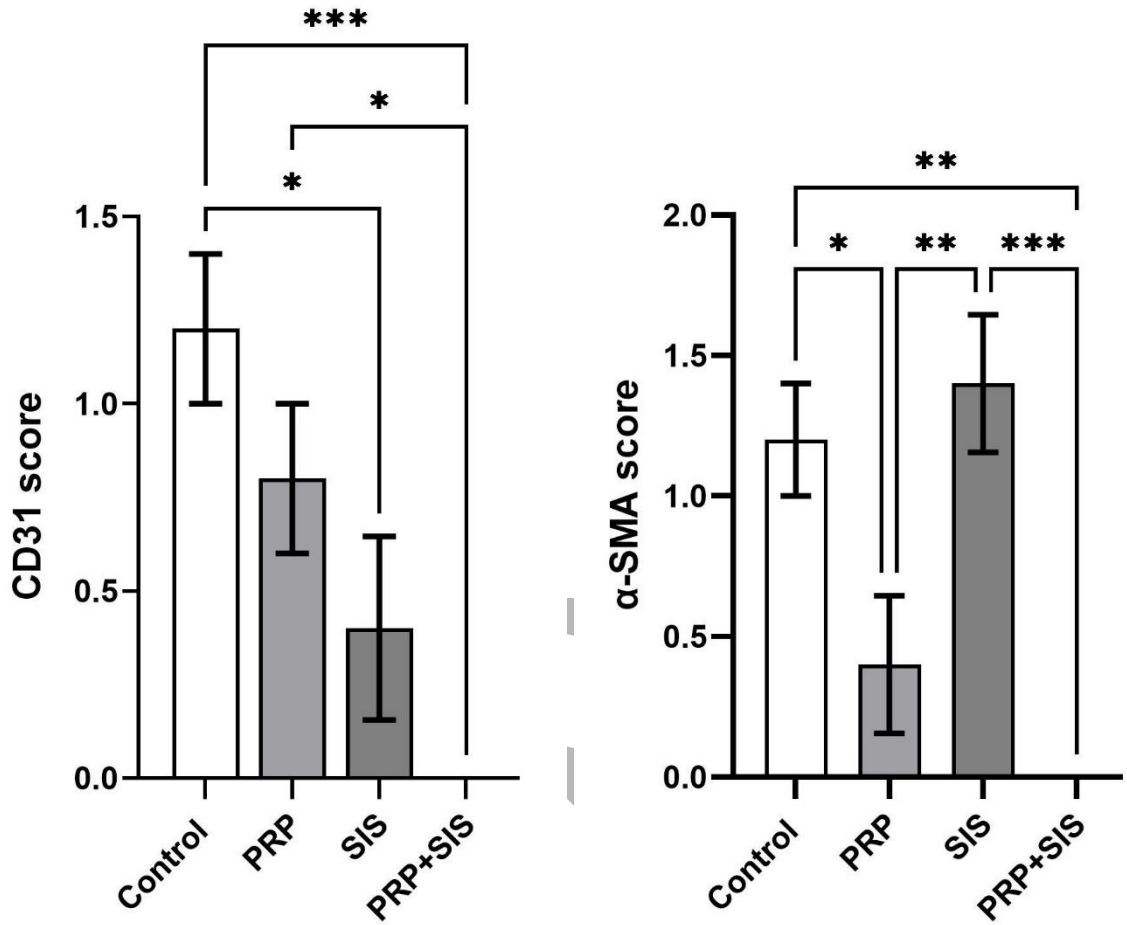

Figure 5. The corneal ulcer IHC scoring. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

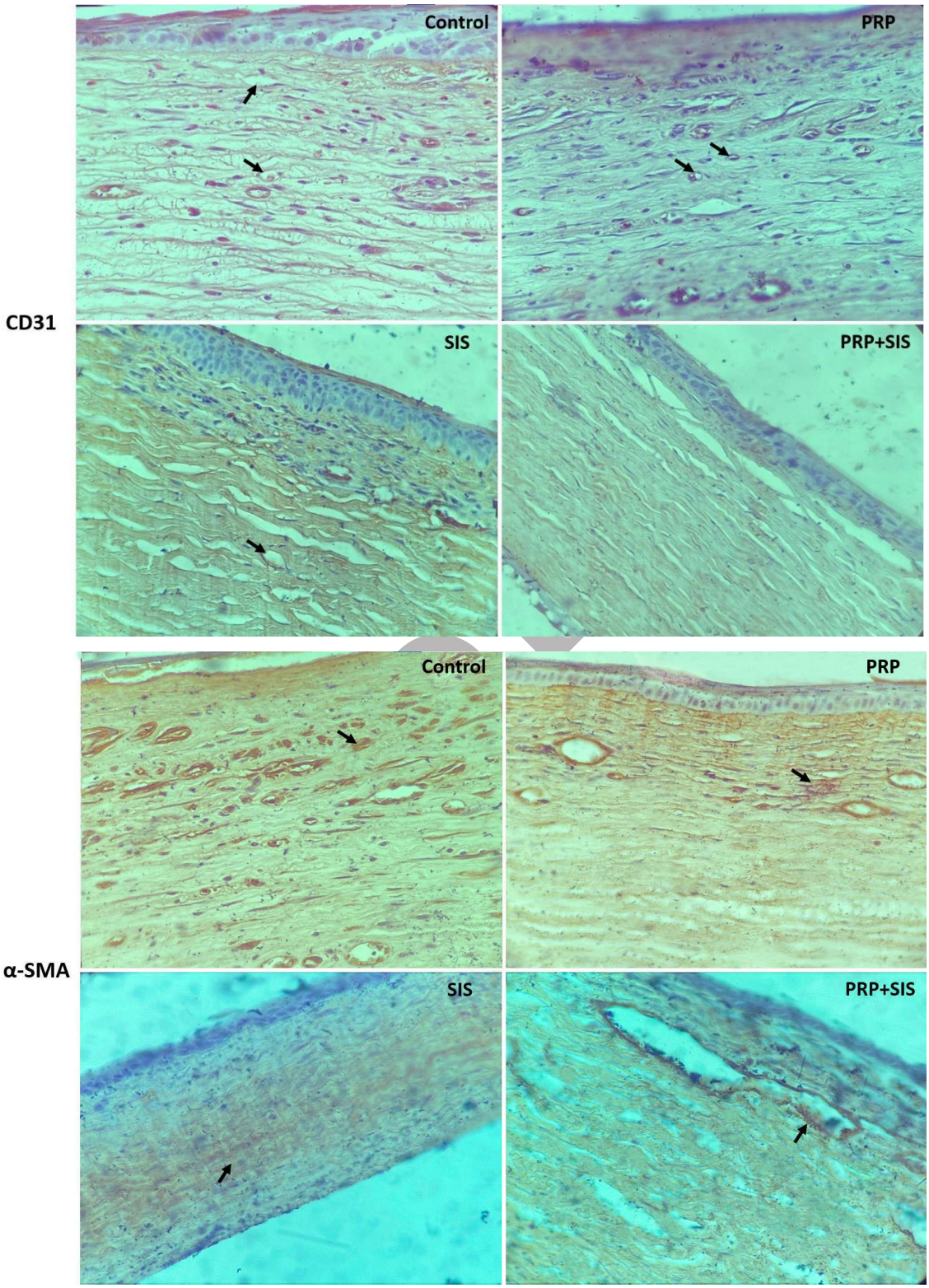

Figure 6. The corneal IHC against CD31 and αSMA molecules (IHC × 100).

4. Discusion

Corneal ulcer is considered one of the eye's emergency diseases, and to prevent the spread of its complications, immediate and appropriate treatment based on clinical and microbiological investigations is necessary. Most ophthalmologists treat patients with corneal ulcers with broad-spectrum antibiotics without laboratory examination, and such treatment is effective for a large number of patients (12). However, this type of treatment is not effective for other patients. For this reason, in all cases of corneal ulcers, sampling and culture are recommended, which, in addition to the diagnostic aspect, improves antibiotic penetration and necrotic tissue delay (13). Many diseases can damage the cornea, disrupting its shape and transparency. One of those diseases is a corneal ulcer. A corneal ulcer develops when its layers are damaged. Various factors contribute to corneal ulcers, including bacterial, fungal, viral, parasitic, Bell's palsy, dry syndrome, and corneal damage and wear (14). If such treatments are not effective, surgery must be performed and a corneal transplant must be performed.

Various biomaterials are used for corneal transplantation, including platelet-rich plasma (PRP) and small intestinal submucosa (SIS) methods. Platelet-rich plasma is a volume of autologous blood plasma that has a high concentration of platelets (15). Inside platelet alpha granules, there are several factors, including tissue growth factor beta (TGFβ), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), which cause corneal regeneration and increase blood supply to the area (16). In this method, TGFβ remains active during inflammation and helps regulate cell migration and proliferation, but VEGF acts as an angiogenesis stimulator after the inflammatory phase (17).

In a separate study conducted in 2021, Farghali et al. treated dogs and cats with corneal ulcers of various origins with PRP. The researchers found that the levels of the matrix genes MMP-2 and MMP-9 were dramatically reduced compared to the control group. Furthermore, in the field of zymography, the animals exhibited full recuperation, regrowth of epithelial tissue in wounds, and restoration of transparency in their corneas within a span of 14 days (2). Sakimoto et al. in 2007 and Pifer et al. in 2014, on the other hand, showed that the administration of PRP in cases of frequent corneal erosion problems depends on the quantity of leukocytes by increasing MMP levels (18, 19). More corneal ulcers result in more corneal edema, according to clinical symptoms. Because the corneal epithelium contains more hydromolecules, conjunctiva ulcers are more susceptible to corneal edema. Some research has shown that corneal edema is linked to leukocyte

infiltrations, which may be caused by PRP-containing leukocytes. Leukocytes in the PRP, on the other hand, could decrease the expression of vascular endothelial growth factor (VEGF) and promote the expression of pro-inflammatory lL12 and IL-16 cytokines.

In 2012, Kim et al. conducted a study titled " Effect of autologous platelet-rich plasma on persistent corneal epithelial defect after infectious keratitis". For this purpose, they extracted platelet-rich plasma from the blood. Then, different PRP factors, including TGFβ, EGF, vitamin A, and fibrinogen, were evaluated with their amounts in the autologous serum. The results of that study indicated that there is no statistically significant difference between the mentioned factors except for EGF, and EGF is significantly higher in PRP than in the autologous serum. Also, the results of their study showed that the degree of healing in the PRP group is significantly higher than that of the animal's own serum (20). In 2014, Acosta et al. investigated the effects of PRP in the treatment of corneal ulcers. The study's results showed that corneal wound healing in the group receiving PRP was statistically significant compared to the control group (8).

In 2019, Alizade et al. conducted a study with the aim of investigating the effects of PRP eye drops on corneal wound healing after keratoplasty. To achieve this, they chose 34 eyes after keratoplasty for their study. Then, PRP was poured on the eyes of similar wounds every 3 hours. According to their observations, they admitted that the treatment with PRP was completely successful, and the average recovery in the eye with full PRP was significantly lower than the recovery without PRP. They also found no statistical difference between age, gender, or the technique of making changes in the cornea (21). In 2021, Kamiya et al. investigated the effect of platelet-rich plasma on corneal repair after keratectomy. To achieve this, they examined the eyes of 10 patients. Then, at random, PRP was administered topically to the patients' eyes four times a day for two weeks. Afterwards, they quantitatively measured the repair position one, two, and one week after the keratectomy. The results of their study showed that in the group receiving PRP, one day and two days after administration, the wound site was significantly smaller. But a statistically significant difference was not seen until the seventh day. Also, on the first and second days, the healing of epithelial cells in the PRP group was significantly greater than the control group, but on the 7th day, this difference was not statistically significant. Pain and tears were not statistically different between groups.According to their study's findings, they admitted that PRP treatment is useful in healing corneal wounds (22).

The small intestine submucosa is also a type of biological material that is used in clinical cases. SIS's structure, biological activity, and immune response make it suitable for body repair. This substance has been approved in tissue engineering and medicine as a regenerative agent for different organs. SIS consists of various compounds such as collagen, elastin, fibronectin, laminin, glycosaminoglycans, and proteoglycans. Collagen helps to repair wounds by causing them to contract (23). Collagen bundles can be placed in a line or in close proximity to one another, and this phenomenon is associated with regular and beneficial healing. For improved corneal wound healing, fibroblasts must produce collagen and then introduce collagen bundles into the matrix.

In 2005, Yoon and colleagues investigated the regenerative effects of serum extracted from the umbilical cord on repairing corneal epithelial defects. With umbilical cord serum, they treated 14 eyes out of 14 patients with corneal defects for two weeks. Then they evaluated the restoration process with a biomicroscope. The study's results showed that the serum extracted from the umbilical cord had a complete healing effect in six eyes and an incomplete effect in the other six (24). A similar experiment was evaluated of the umbilical cord serum in the corneal ulcrs in the diabetic rabbits that described the effectiveness in corneal healing of diabetic ulcers (25).

In this study, the combined use of PRP drops and SIS resulted in synergistic healing results. Included were the rates of collagen regularity, corneal vascularization, and corneal transparency restoration.

We concentrated on PRP and SIS's early healing benefits. While myofibroblasts (identified by IHC for aSMA) suggest a healing phenotype, corneal opacity can also be brought on by an overabundance of residual myofibroblasts secreting aberrant ECM proteins. In order to clean the cornea, myofibroblast disappearance is necessary following appropriate wound area healing. At day 21, corneal opacity was significantly improved in comparison across all groups in this trial, with scores of roughly 14 in control, 9 in SIS, 6 in PRP, and 4 in PRP+SIS. However, it takes several weeks to months for the cornea to fully rebuild. More research is needed to determine the long-term effects of SIS and PRP on corneal clarity and full reconstruction.

However, while using PRP or SIS alone has therapeutic effects, it is not as effective as using both of them at the same time.

This study sought to investigate the benefits of PRP and improved SIS in accelerating corneal wound healing. At day 21, comparison of corneal opacity showed significant improvement for all groups in the study, with approximate scores of 14 in control, 9 in PRP, 6 in SIS, and 4 in

PRP+SIS. However, it takes several weeks to months for the cornea to completely regenerate. Some studies have shown that the first weeks of inflammation, edema, and angiogenesis are high, and collagen concentration and regularity are low. It was the same in the present study, but different treatment groups showed significant differences in terms of histological components. The presence of myofibroblasts (IHC for α-SMA) is an unpleasant phenotype that causes corneal opacification through oversecretion of atypical ECM proteins. For the cornea to become clear, myofibroblasts must disappear after proper wound healing. In this study, it was observed that the expression of α-SMA in the control and SIS groups was higher than in the PRP and PRP+SIS groups, which suggests that the latter two groups had fewer myofibroblasts and, as a result, improved more. However, the expression of CD31 was different, showing that the control group had the most surviving blood vessels, while it was significantly less in the other groups, indicating the healing effects of both PRP and SIS. Therefore, as we have seen, the PRP and SIS groups had a faster recovery than the control group, and the amount of inflammation and edema was controlled and provided with more regular collagen. The same groups, but with simultaneous administration of both, showed brighter results in terms of pathology grading in the PRP+SIS group.

The result is that simultaneous administration of SIS membrane and PRP drops has beneficial and synergistic effects on the healing of deep corneal wounds. Both the SIS membrane and the PRP alone demonstrated significant healing effects. However, neovascularization and its timely resolution, following an increase in corneal transparency, were observed in the group treated with a combination of PRP and SIS. Its autologous properties, safety, low cost, and therapeutic effects have made PRP a promising therapeutic agent, and its combination with cis also provides additional healing effects.

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Authors' Contribution

MHB: data collection, drafting the manuscript, and supervising the study process; AJ: study design and conducting the study; HF: supervised the surgery process, PM: supervised the histopathological slides.

Conflict of Interests

The authors declare that they have no conflict of interest.

Ethical Issues

The research project was approved by the Ethics Committee of Science and Research Branch Islamic Azad University (code: IR.IAU.SRB.REC.1402.017). as followed this link: https://ethics.research.ac.ir/ProposalCertificateEn.php?id=350655&Print=true&NoPrintHeader=true&NoPrintFooter=true&NoPrintPageBorder=true&LetterPrint=true

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Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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