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Effect of Autologous Serum Derived from Advanced Platelet-rich Fibrin on the Healing of Experimentally-induced Corneal Ulcer in Donkeys (*Equus asinus*)

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ABSTRACT

The present study aimed to investigate the effect of serum derived from advanced platelet-rich fibrin on the healing of experimentally-induced corneal ulceration in donkeys. Nine adult apparently healthy donkeys of both sexes were used after being proofed normal after ophthalmological examination. A 6-mm-diameter centric corneal ulceration was induced chemically by using 1N sodium hydroxide solution. The donkeys were then randomly allocated into two main groups; control group (A), in which the corneal ulcer left for the period of the study without application of medicaments, and group (B), in which serum derived from advanced platelet-rich fibrin (s-PRF-drops) was applied to the eye three times/day for 35 days. Evaluation criteria included; clinical and external ophthalmic examinations, fluorescein staining, ulcer healing by analyzing serial digital photographs and histopathology. Results revealed no significant changes in the evaluation criteria between treatment and control groups. Corneal ulcer healing was associated with corneal opacity, vascularization, melanosis, and other complications that likely negate any potential benefit of administration of s-PRF as a treatment for corneal ulcer in donkeys. Histological results of the s-PRF-drops group were similar to the other group in the degree of re-epithelialization and regularity of the collagen bundles, type and maturity of the collagen. However, treatment by s-PRF drops resulted in no epithelial keratinization and slightly less sub-epithelial stromal inflammatory reaction.

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Introduction

Equine species frequently suffer from corneal ulcers or ulcerative keratitis, which are of major concern to veterinarians and equine industry (Nasisse and Nelms, 1992; Brooks, 1999 and 2002; Andrew and Willis, 2005; Brooks and Matthews, 2007).

Secondary infection combined with infiltrating polymorphonuclear leukocytes stimulates production and activity of proteolytic enzymes resulting in corneal melting (Brooks and Matthews, 2007; Clode and Matthews, 2011; Johns *et al*., 2011). Corneal ulceration involving the deep layers of stroma with establishment of infection is considered complex ulcer (Brown *et al*., 1974; and Monod *et al*., 2002). Therapies aim to prevent secondary bacterial and fungal infection and to facilitate rapid re-epithelialization (Brooks and Matthews. 2007). However, optimal medical therapy remains controversial and not firmly established.

Regenerative medicine aims to restore the damaged tissues in different pathologies in the ophthalmology field (Miron *et al*., 2017). Recently, blood derivatives have been described as a treatment for ophthalmology disorders including persistent corneal epithelial defects (Anitua *et al*., 2015). Platelet concentrates collect the most active components from the blood including platelets, which are rich in growth factors, fibrin and leukocytes (Dohan *et al*., 2009c; Bielecki and Dohan Ehrenfest, 2012). These preparations can be in the form of solutions or gels and can be injected or placed in a surgical site, on a wound or in an injured area, to regenerate the damaged tissues (Bielecki and Dohan Ehrenfest, 2012). Platelet-rich fibrin (PRF) is an autologous fibrin-based membrane, matrix, or scaffold, living biomaterial, derived from patient's blood (Choukroun *et al*., 2001; Dohan *et al*., 2006a; Prakash and Thakur, 2011). The present study aimed to investigate the effect of serum derived from advanced platelet-rich fibrin (A-PRF) on the healing of experimentally-induced corneal ulceration in donkeys (*Equus asinus*).

Materials and methods

Ethical approval

The experiment was carried out as a prospective cohort

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study at the Department of Surgery, Anesthesiology and Radiology, Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt. The National Ethical Committee of the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, has approved all the procedures of this study in accordance with the Egyptian bylaws and OIE animal welfare standards for animal care and use in research and education.

Animals

The present investigation was carried out on nine apparently healthy donkeys (*Equus asinus*) of both sexes (males=3 and non-pregnant non-lactating females=6). Their ages ranged from 7 to 8 years (average=7.4years) and their body weight ranged from 110 to 150 kg (average=135kg). Based on the ophthalmic examination, all donkeys in the study did not show any ocular abnormalities. Donkeys were kept in well-equipped stables during the period of study. They were managed by giving them two times balanced concentrate diets with suitable amount of green food once a day and water was supplied ad libitum.

Clinical Examination

Donkeys were monitored during recovery from anesthesia and examined after recovery of anesthesia for 3-5 day for their activities, return of food and water intake, and expression of pain. Physiological parameters body temperature (BT), heart rate (HR) and respiratory rate (RR) were recorded before surgery and after recovery.

Eyes were examined for their normal configuration, ocular discharges, orbits, globe positions and movements, palpebral and corneal reflexes and menace response. In a dark room, the pupillary light reflex, eyelids, nictitans, bulbar and palpebral conjunctiva, cornea, iris, and lens were examined (Stoppini and Gilger, 2016).

Anesthesia

Donkeys were anesthetized by intravenous (IV) administration of 1.1 mg/kg Xylazine HCl 2% (Xyla-Ject, ADWIA Co., SAE, Egypt) and 2.2 mg/kg ketamine HCl 5% (Ketamine, Sigma-tec Pharmaceutical Industries, SAE, Egypt). In addition, 3 drops of Benoxinate hydrochloride 0.4% (Benox, Sterile Ophthalmic Solution, Egyptian Int. Pharmaceutical Industries Co., Egypt) as surface analgesia were installed on the corneal surface.

Induction of centric corneal ulcer

After anesthesia, the donkey was positioned on lateral recumbency with the operated eye being the uppermost. A 6-mm-diameter centric corneal ulceration was induced chemically by using 1N Sodium Hydroxide (NaOH) solution (Schultz *et al.*, 1992). A 4- mm diameter sterile cotton swab was immersed in 1N NaOH solution for 5 seconds. The eye was opened by an eyelid dilator and the swab was placed on the corneal surface for 1 minute (Levinson *et al.*, 1976). A sterile stainless steel washer of 6 mm internal diameter was used as a template to help in inducing a uniform circular ulcer. The eye was then irrigated with 20 ml physiological saline (Öztürk *et al.*, 2000). The donkeys (n.=9) were then randomly allocated into two main groups: Control group (A) (n=3) in which the corneal ulcer was left for the period of the study without application of medicaments and treated group (B) (n=6) in which 4 drops of serum derived from Platelet-rich fibrin (s-PRF) in the form of eye drops were installed topically 3 times daily (q 8 h) during the study period from day 0 to day 35 (Fig. 1a-c).

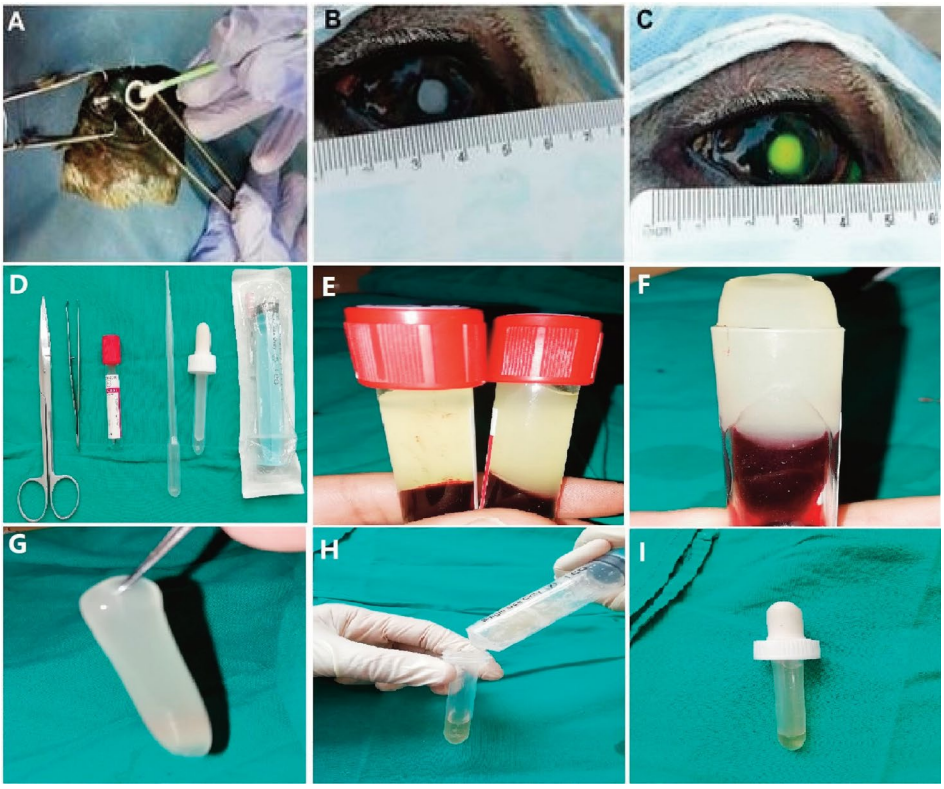


Fig. 1. Corneal ulcer induction procedures and steps for preparation of the serum derived from the A-PRF; a cotton swap immersed in 1N NaOH was applied to the corneal surface after application of a sterile stainless steel washer as a template (A), a central corneal ulcer was now induced and being radiographed with a ruler as a scale (B), and fluorescein staining of the ulcer (C), instruments which used for preparation (D), A-PRF formation after centrifugation (E), extraction the A-PRF gel from the blood collecting plain tube (F), separation the A-PRF gel from the layer of the RBCs (G), collecting the serum after shaking the gel into sterilized dropper (H), serum derived from the PRF ready to use (I).

Preparation of the s-PRF

Autologous blood was used in this study. A 10-ml venous blood was obtained from the jugular vein (using 10 ml size sterile syringe and hypodermic needle size 18G×1.1/2) in sterile plain glass-based vacuum tube. The blood sample was immediately centrifuged at 1500 rpm for 14 minutes (CENTRIFUGE PLC SERIES, model: PLC-05, SN.818555, Gemmy Industrial Corp., Taiwan). After centrifugation, three layers were obtained: platelet poor plasma at the top, red blood cells at the bottom and the A-PRF gel or clot at the middle (Ghanaati *et al*., 2014). The A-PRF gel was exteriorized from the tube by a sterile forceps and the layer of the red blood cells was cut by a sterile scissor to produce a clean A-PRF gel. The A-PRF gel was placed in a 20-ml sterile syringe and was agitated until this gel was turned into a liquid form. The solution was collected inside a sterilized eye dropper (A-PRF drops) to facilitate its usage. A separate sterile dropper was used for each animal. This preparation was done without usage of anticoagulants, thrombin, calcium chloride or any other chemical or biological substances (Fig. 1d-i).

Evaluation Criteria

Animals were examined and evaluated at days 2, 4, 6, 9, 13, 20, 27, and 35 after induction of corneal ulceration. The evaluation criteria included: clinical and external ophthalmic examinations, fluorescein staining, ulcer healing and histopathology. All animals were kept under complete observation after induction of ulcer. For ocular examination and photographing, the animal was sedated by intravenous administration of 1.1 mg/kg Xylazine HCl 2%, in addition to retrobulbar and auriculopalpebral nerve blocks using 20mL and 5 mL of 2% lidocaine hydrochloride respectively.

External ophthalmic examination

The cornea and conjunctiva were examined at days 2, 4, 6, 9, 13, 20, 27, and 35 after induction of corneal ulceration by naked eye for presence of swelling, epiphora, ocular discharges, blepharospasm and by using binocular magnifier and focal light source for presence of corneal opacity, neovascularization, pigmentation, and conjunctivitis. The induced corneal ulcer was inspected for ulcer healing and scar tissue formation.

Fluorescein staining tests

The test was carried out after ophthalmic examination at days 2, 4, 6, 9, 13, 20, 27, and 35 after induction of corneal ulceration. One drop of 2% fluorescein solution was instilled in the eye and left for one min. The eye was then rinsed with sterile normal saline (Petroutsos *et al*., 1983) to remove excess stain and examined for any retained stain using penlight illumination and a binocular magnifier.

Monitoring of ulcer healing

The eye was photographed at days 2, 4, 6, 9, 13, 20, 27, and 35 after induction of corneal ulceration by a digital camera after flushing with normal saline with a ruler as near as possible to the ulcer. The eye was photographed twice; after finishing external ophthalmic examination and after fluorescein staining. The surface areas of the corneal ulcer (in cm^2) was measured and analyzed using the Image J software (ImageJ 4.48v software, National Institutes of Health, USA) to measure and monitor the rate of ulcer healing.

Histopathological examination

Donkeys were euthanized (Knottenbelt and Malalana, 2014) by intravenous injection of xylazine HCl (1.1 mg/kg) followed by rapid intravenous injection of thiopental sodium (Thiopental sodium 1 gm vial, EPICO, Egypt) at a dose of 35 mg/kg. The corneas were excised from eyes and fixed in 10% neutral buffered formalin. The formalin-fixed samples were dehydrated in ascending grades of ethanol, cleared in methyl benzoate, and then embedded in paraffin wax. Paraffin sections at 5 µm in thickness were cut and stained with the following histological stains: Haematoxylin and Eosin for general histological examination (Fischer *et al*., 2008), Periodic acid Schiff (PAS) technique for demonstration of neutral mucopolysaccharides (Aterman and Norkin, 1963), Crossmon's trichrome technique to stain corneal collagen fibers (Crossmon, 1937), and Picro-Sirius red technique to differentiate between mature and immature corneal collagen (Bhutda *et al*., 2017). Paraffin sections were examined by an Olympus BX51 microscope and the photos were taken by an Olympus DP72 camera adapted into the microscope.

Statistical analysis

The data were expressed as mean±SE and were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, 2009). When treatment effects were significant, differences between the least squares means were tested using Duncan's multiple range test and differences were considered significant at the level of $P < 0.05$.

Results

Clinical Examination

On day zero (after induction of ulceration), there was expression of pain as restlessness, dropping of the head, and isolation. The animals showed decrease in the food intake. On the second day, the food intake gradually increased. Animals returned to normal food intake by day four. Dropping of the head disappeared in all donkeys except two animals that showed head dropping and decreased activity up to the fourth day after induction of ulceration. All donkeys of the study returned to their normal activity and behavior by the

Table 1. Values (mean±SE) of physiological parameters of donkeys before induction of corneal ulceration and after recovery from anesthesia.

Groups	Parameters	Preoperative	After recovery
A (Control)	BT (˚C)	38.93±0.3	38.87±0.3
	HR (Beat/min)	102.3±6.1	96.1± 3.6
	RR (Breath/min)	74.3±4.2	69.8± 3.6
B (s-PRF drops)	BT (˚C)	39.37±0.2	38.93±0.3
	HR (Beat/min)	108.3±11	104.1±8.1
	RR (Breath/min)	76±10.5	79.1±3.6

BT: Body Temperature; HR: Heart Rate, RR; Respiratory Rate

fifth day of the study. Physiological parameters (HR, RR, and BT) were within normal range without significant changes between mean values before induction of ulceration and after recovery (Table 1).

External Ophthalmic Examination

Control group (A) (n=3)

Blepharospasm was recorded from day two after induction of the ulcer and remained till the end of the study, except in one animal where it disappeared by day 20. Epiphora and corneal opacity were recorded at day two after induction and gradually decreased starting from day 20 until day 35. Corneal vascularization was observed to be variable. It was either superficial neovascularization (n=2) or deep neovascularization (n=1). Generally, corneal vascularization gradually decreased from day 20 to day 35 after induction of ulceration. Concomitant conjunctivitis was recorded in two donkeys of the control group, which subsided in one eye at day 13. The eye remained with conjunctivitis until the end of the study (Fig. 2).

Drop treated group (B) (n=6)

Blepharospasm and epiphora were noticed in five animals by day two. These two symptoms disappeared by day 20 in two animals and by day 35 in three animals. Swelling was not recorded in animal of this group. Opacity of the cornea was recorded in five animals from day two and in one donkey from day nine. By the 13th day, intensity of opacity increased and remained till the day 35 (n=5). In one animal, mild opacity persisted and disappeared within 35 days. Neovascularization was variable; it started from day two (n=2) and from day four (n=3). However, one animal did not show any neovascularization except few blood vessels that were observed around the dorsal border of the ulcer in the day 35. Neovascularization started as superficial vascularization and changed into deep vascularization by day six. Conjunctival congestion was observed from the day two and remained till the day 35 (n=2). It disappeared from the day nine then reappeared in the day 27 (n=3). One donkey showed conjunctival congestion in the day two then disappeared till the day 35 (Fig. 2).

Fluorescein Staining Results

In the control group (A), the fluorescein uptake disappeared at day 20 (n=2) and at day 35 (n=1). In s-PRF drops group (B), fluorescein uptake persisted to the day 35 (n=3), day 27 (n=2). One animal showed positive fluorescein uptake

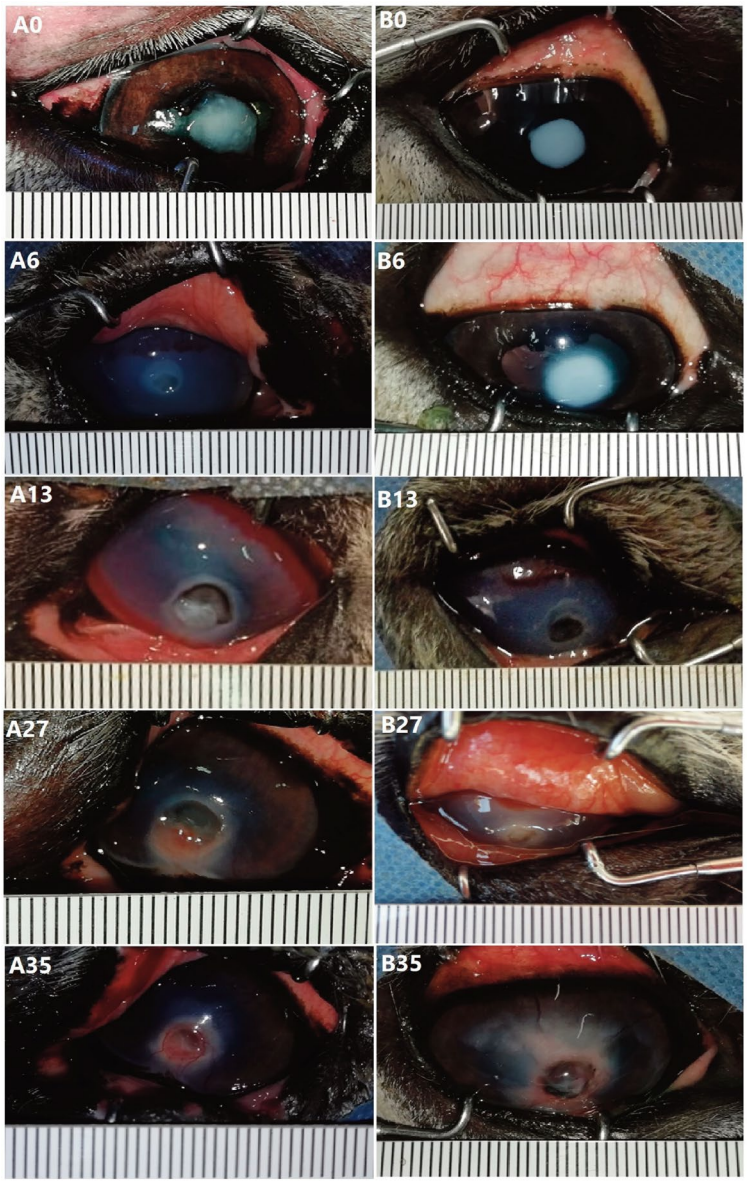


Fig. 2. Serial photographs of the follow-up of the induced corneal ulceration in the 2 groups in day 0, 6, 13, 27 and 35 in donkeys.

test on day two after ulcer induction then negative test until the end of study period (Fig. 3).

Some complications were recorded during this study (Fig. 4). In group (A), pigmentary keratitis was observed in one donkey on day 20 till the end of study. In group (B), anterior synechia was noticed in three donkeys; two of them on day 13 and the other one on day nine. In addition, iris prolapse was observed in one donkey on the day 20 and pigmentary keratitis was observed in two donkeys on day 35 of the study.

Results of Ulcer healing

In group (A), mean value of the surface area (cm^2) of the ulcer at day 35 was decreased significantly ($P < 0.041$) when

Table 2. Values (mean±SE) of the surface area (cm^2) of the corneal ulceration and fluorescein-stained corneal ulceration of the donkeys as measured by the software (Image J).

Day	Non-stained corneal ulceration		Fluorescein-stained corneal ulceration	
	Group (A) (*n*=3)	Group (B) (*n*=6)	Group (A) (*n*=3)	Group(B) (*n*=6)
0	0.43±0.09^{a}	0.46±0.05^{a}	0.93±0.22^{a}	0.71±0.02 ^{a}
2	0.39±0.09 ^{a}	0.39±0.04 ^{a}	0.66±0.19 ^{aA}	0.22±0.05 ^{bB}
4	0.36±0.08 ^{a}	0.38±0.05 ^{a}	0.23±0.07^{b}	0.11±0.03 ^{b}
6	0.32±0.09 ^{a}	0.35±0.06 ^{a}	0.24±0.08^{b}	0.08±0.03 ^{b}
9	0.27±0.02 ^{a}	0.37±0.05 ^{a}	0.13±0.05^{b}	0.17±0.11 ^{b}
13	0.34±0.08 ^{a}	0.31±0.04 ^{a}	0.12±0.07^{b}	0.22±0.11 ^{b}
20	0.49±0.15 ^{aA}	0.27±0.05^{bB}	0.06±0.07^{b}	0.2±0.1 ^{b}
27	0.31±0.08 ^{a}	0.22±0.03 ^{b}	0.04±0.04^{b}	0.17±0.09 ^{b}
35	0.15±0.08^{b}	0.20±0.01 ^{b}	0±0 ^{bA}	0.14±0.09 ^{bB}

Values of different small superscript letters over time (within column) and capital superscript letters between groups (within row) are significantly different at $p<0.05$.

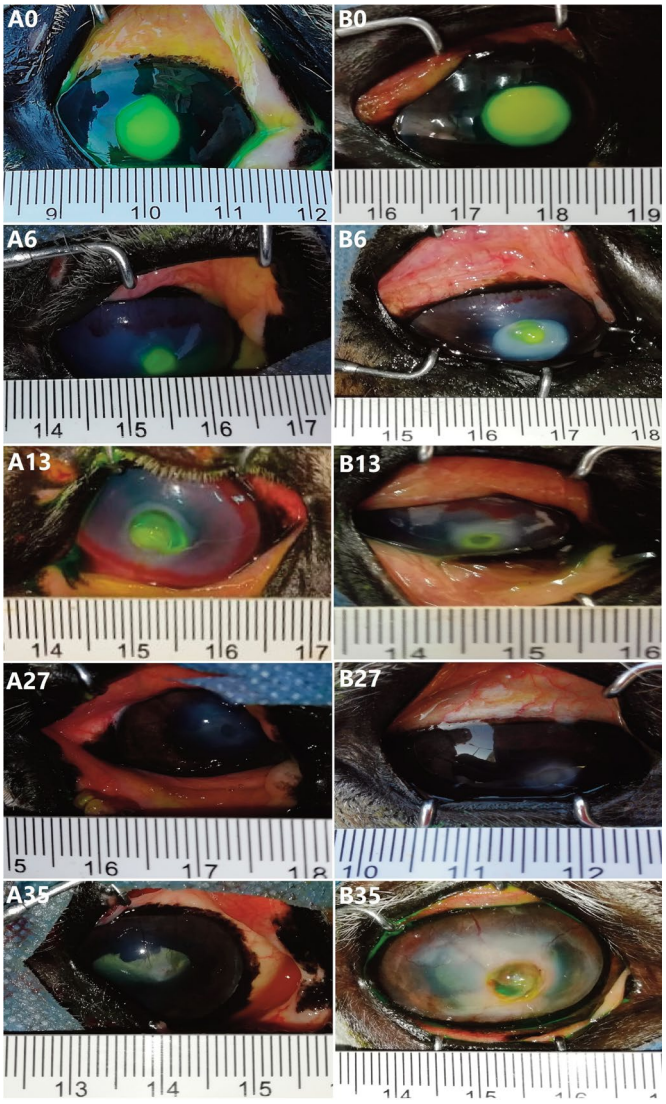


Fig. 3. Serial photographs of the follow-up of the induced corneal ulceration after fluorescein staining in the 2 groups in day 0, 6, 13, 27 and 35 in donkeys.

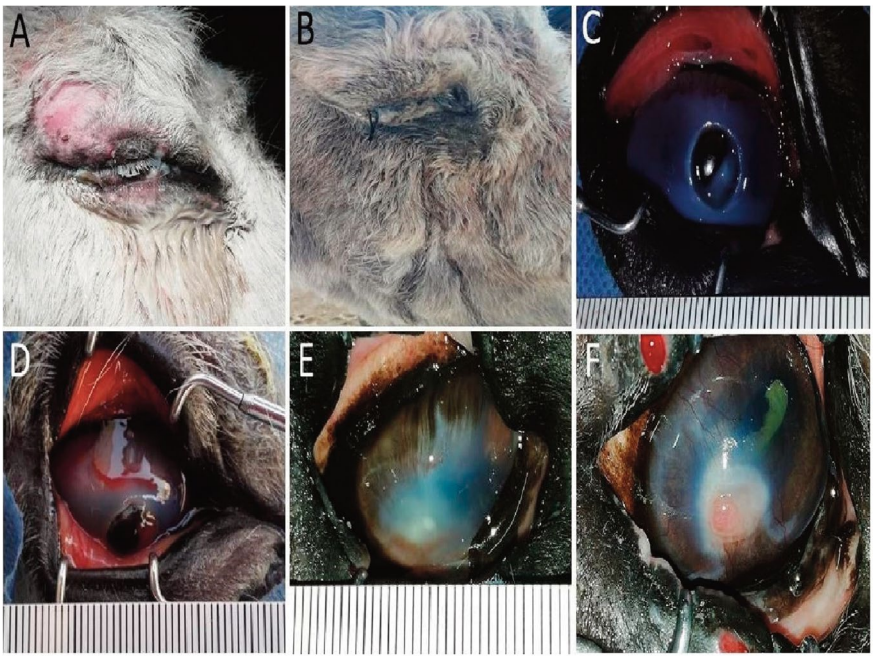


Fig. 4. Complications recorded during the follow-up period of the induced corneal ulceration in donkeys; eyelid swelling and blepharospasm (A), epiphora (B), anterior synechia (C), iris prolapse (D), pigmentary keratitis (E), pigmentary keratitis with scar formation (F).

compared to day zero value (Table 2, Fig. 5). In group (B), mean values of the surface area (cm^2) of the ulcer at days 20, 27 and 35 were decreased significantly ($P < 0.005$) when compared to day zero value (Table 2, Fig. 5). On day 20, mean value of the surface area (cm^2) of the ulcer for group (B) was decreased significantly ($P < 0.04$) when compared to group (A).

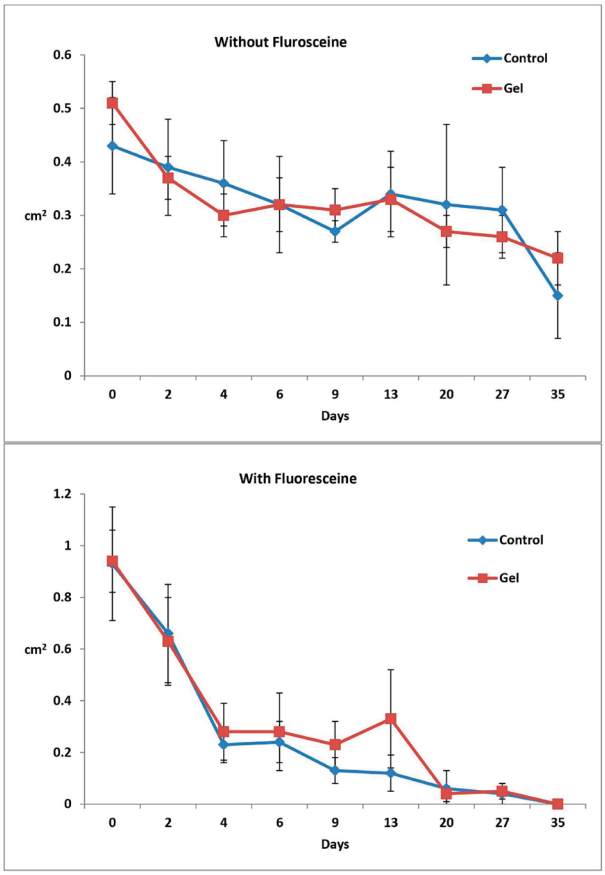


Fig. 5. Histogram of the mean (±SE) values of the surface area (cm^2) of the corneal ulceration before fluorescein-staining (upper histogram) and of the fluorescein-stained corneal ulceration (lower histogram) of the donkeys as measured by the software (ImageJ).

Results of image analysis after fluorescein staining revealed that in group (A), mean values of the surface area (cm^2) of the ulcer at days 4, 6, 9, 13, 20, 27 and 35 showed significant ($P < 0.0001$) decreases when compared to day zero value (Table 2, Fig. 5). In group (B), mean values of the surface area (cm^2) of the ulcer at days 2, 4, 6, 9, 13, 20, 27 and 35 were decreased significantly ($P < 0.0001$) when compared to day zero value.

Results of Histopathological Examination

The cornea of the control negative group showed the normal histological structure of the cornea in donkey. It was formed of the following layers: corneal epithelium, Bowman's membrane, corneal stroma, Descemet's membrane and corneal endothelium. The corneal epithelium was a stratified squamous non-keratinized epithelium. It was formed of a basal cuboidal layer, a second polyhedral layer and four to five gradually flattening layers. The Bowman's membrane was the basement membrane for the stratified epithelium. It was a collagenous acellular zone (mainly type I collagen fibrils and laminin). The corneal stroma (substantia propria) was the thickest transparent avascular layer of the cornea. It was formed of regular parallel bundles of collagen fibers (evenly spaced) along with sparsely distributed interconnected keratocytes, which are the cells for general repair and maintenance. The Descemet's membrane was a modified basement membrane of corneal endothelium. Corneal endothelium was a layer of simple squamous epithelium (Figs. 6 & 7a-12a).

Microscopical examination of the cornea after induction of ulcer (0-day) showed a circumscribed area of epithelial loss and sub-epithelial stromal reaction in the form of oedema and dispersed and disorganized collagen fibers (Figs. 7b-12b). The histopathological examination of the cornea of the control positive group (after 35 day) showed re-epithelialization of the epithelial defect and sub-epithelial stromal inflammatory reaction in the form of oedema, blood clotting, new vascularization, congestion, leucocytic infiltration, dispersed, disorganized collagen bundles and thickening of the stroma (Figs. 7c-9c).

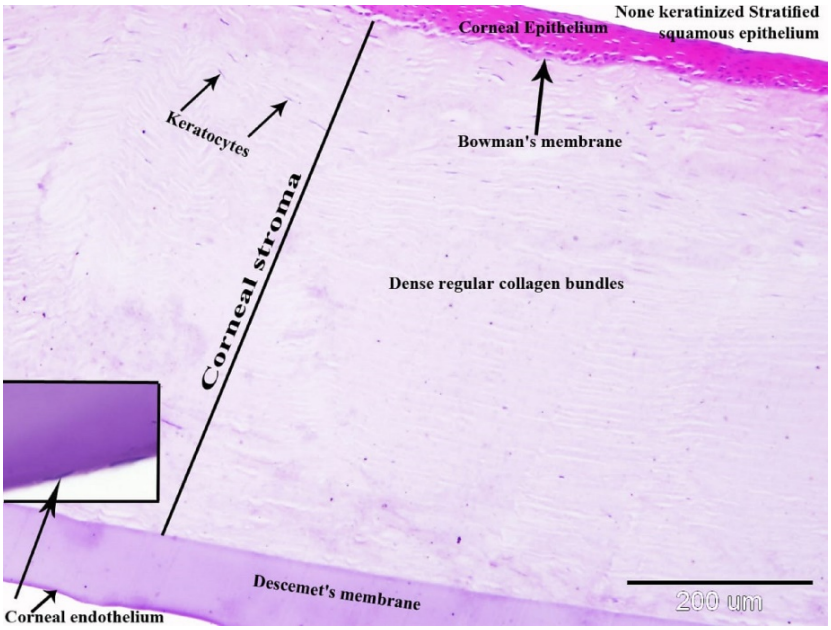
The histopathological examination of the cornea of the

PRF drop group (after 35 day) showed epithelial defect and in some cases re-epithelialization of the epithelial defect. It also showed sub-epithelial stromal inflammatory reaction in the form of edema, new vascularization, leucocytic infiltration, dispersed, disorganized collagen bundles and slight thickening of the stroma (Figs. 7d-9d).

The present study showed activation and proliferation of fibroblasts in control positive and PRF drop groups compared to control (Figs. 9a-d).

By using Crossmon's trichrome technique to examine the regularity and density of the collagen bundles, 0-day induced corneal ulcer group showed dispersed irregular collagen bundles. While the control positive group showed dense irregular collagen bundles and the PRF drop group showed dispersed irregular collagen bundles. (Figs.10a-d). By using Picro-Sirius red stain technique to examine the type of the collagen fibers, 0-day induced corneal ulcer group showed dispersed collagen type I. while the control positive group and the PRF drop group showed abnormal collagen types (Figs. 11a-d). The PRF drop group also showed regenerated keratinized corneal epithelium (Figs. 11d).

Paraffin sections stained with PAS technique revealed that 0-day induced corneal ulcer group showed dispersed weak PAS positive collagen bundles of the corneal stroma. However, the control positive group showed regenerated corneal epithelium, ill clear and interrupted PAS positive Bowman's membrane and PAS positive collagen bundles of the corneal stroma. While the PRF drop group showed regenerated



Fi. 6. Photomicrograph of paraffin sections in the cornea of the donkey of the control negative group showing the normal histological structure of the cornea. Original magnification; X100, Scale bars = 200 µm, Hematoxylin and Eosin stain.

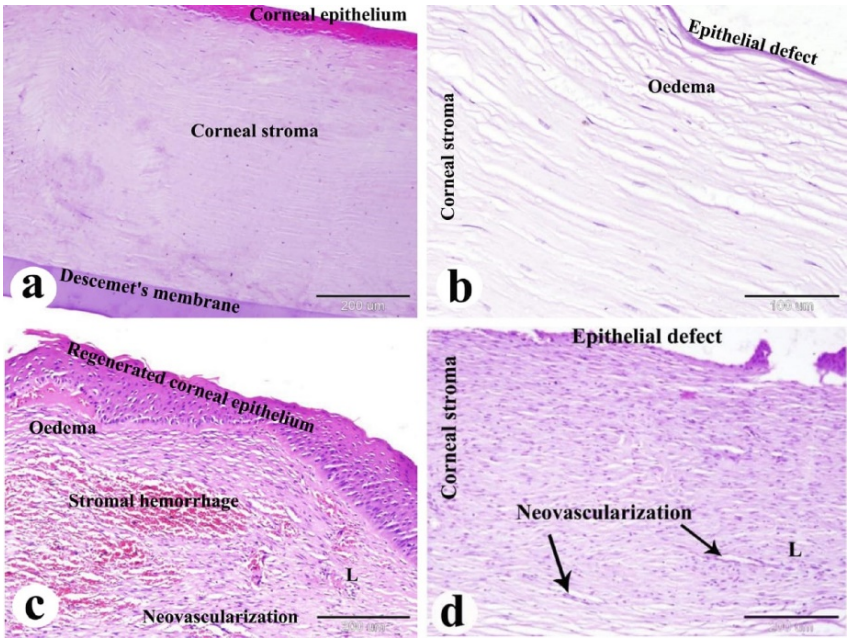


Fig. 7. Photomicrograph of paraffin sections in the cornea of the donkey. a: The control negative group showing the normal architecture of the cornea. b: 0-day induced corneal ulcer group showing epithelial defect and oedema in the corneal stroma. c: The control positive group showing regenerated corneal epithelium, stromal hemorrhage, oedema, leucocytic infiltration (L), and neovascularization. d: The PRF drop group showing epithelial defect, leucocytic infiltration (L) and neovascularization. Original magnification; a, c & d: X100, Scale bars = 200 µm, b: X200, Scale bars = 100 µm, Hematoxylin and Eosin stain.

corneal epithelium, ill clear and interrupted PAS positive Bowman's membrane and weak PAS positive collagen bundles of the corneal stroma (Fig. 12a-d). Table 3 Showed the histopathological changes and healing score in the present study.

Discussion

Subjective and objective signs of keratitis were recorded with variable degrees and intensities in all animals of this study. Fluorescein staining test was positive in all eyes after ulcer induction and disappeared at different times hereafter. The rate of the ulcer healing, which was indicated by measuring the surface area of the ulcer, showed significant decrease in s-PRF-drops and control groups. A significant decrease in the fluorescein-stained ulcer surface area in all groups was recorded over time. Histopathological examination of the cornea showed re-epithelialization of the corneal defect and slightly less sub-epithelial stromal inflammatory reaction in the treated group. Moreover, activation and proliferation of fibroblasts were noticed in control positive and s-PRF-drops groups compared to control negative group.

To the authors' knowledge, this is the first report for an in vivo experiment to evaluate the effect of s-PRF-drops on the healing of corneal ulceration in equines. PRF as a gel has been

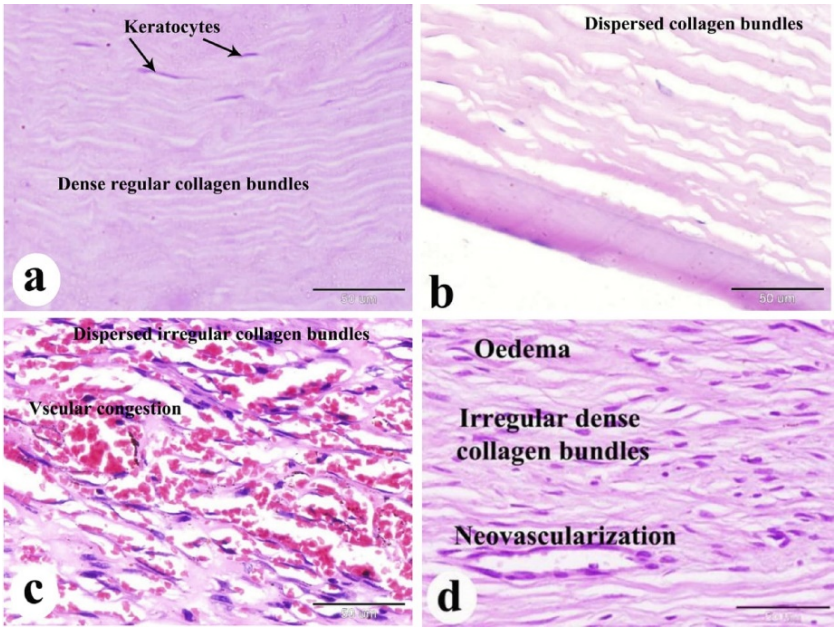


Fig. 8. Photomicrograph of paraffin sections in the cornea of the donkey. a: The control negative group showing the normal structure of the corneal stroma. b: 0-day induced corneal ulcer group showing dispersed collagen bundles. c: The control positive group showing dispersed collagen bundles and vascular congestion. d: The PRF drop group showing irregular dense collagen bundles, oedema and neovascularization. Original magnification; X400, Scale bars = 50 µm, Hematoxylin and Eosin stain.

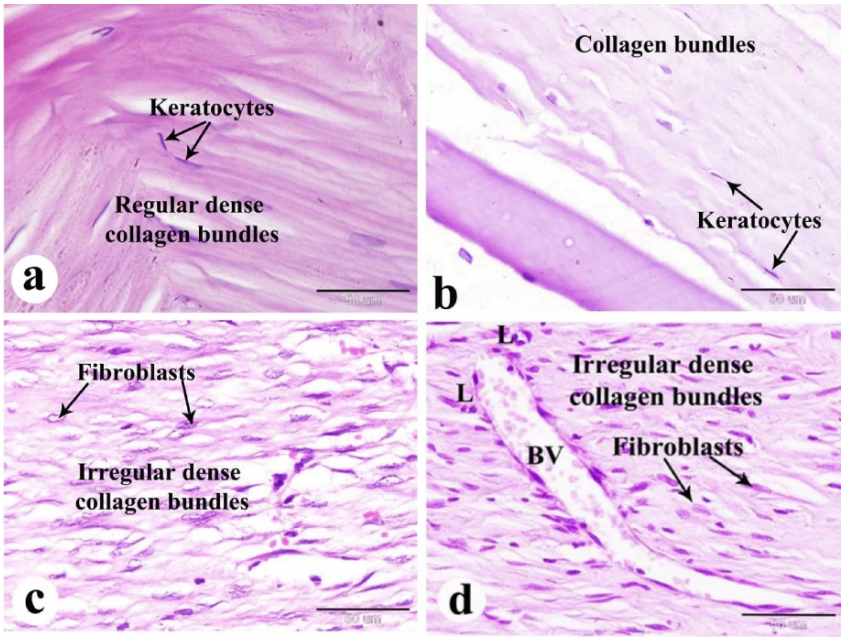


Fig. 9. Photomicrograph of paraffin sections in the cornea of the donkey. a: The control negative group showing numerous keratocytes between the dense regular collagen bundles. b: 0-day induced corneal ulcer group showing numerous keratocytes between the collagen bundles c: The control positive group showing numerous fibroblasts between the irregular dense collagen bundles. d: The PRF drop group showing numerous fibroblasts between the irregular dense collagen bundles, neovascularization (BV), and leucocytic infiltration (L). Original magnification; X400, Scale bars = 50 µm, Hematoxylin and Eosin stain.

used previously for treatment of the corneal ulcer in horses (Burling *et al*., 2000), in dogs (Mishra *et al*., 2021) and in human (Can *et al*., 2016; Emma *et al*., 2020). PRF has been used directly as a clot or after compression, as a membrane or plug. The supernatant can be aspirated from the vacuumed tube and used in injectable form (He *et al*., 2009). Serum, derived from advanced PRF (A-PRF) by shaking the gel resulted in separation of the fluid content from the fibrin mesh, was used in the present study as s-PRF drops. The resultant s-PRF drops; however, contained lower concentrations of growth factors, matrix proteins, leukocytes and stem cells compared to the original gel. The reason is that platelets have been reported to be trapped massively in the fibrin meshes of the PRF-gel, which is also loaded or seeded with leukocytes (B- and T-lymphocytes), monocytes, and neutrophilic granulocytes and mesenchymal stem cells (Perut *et al*., 2013; and Choukroun, 2014; Saluja *et al*., 2011)

Animals in the present study tolerated the experiment and expressed signs of distress only during the first four days after induction of the corneal ulceration. Nearly similar ophthalmic procedure has been tolerated in donkeys without major complications (Ibrahim and Ahmed, 2021). No significant clinical changes were recorded in animals of the current study similar to what reported in previous studies during the whole study periods (Gunay *et al*., 2015; Can *et al*., 2016; Ibrahim and Ahmed, 2021).

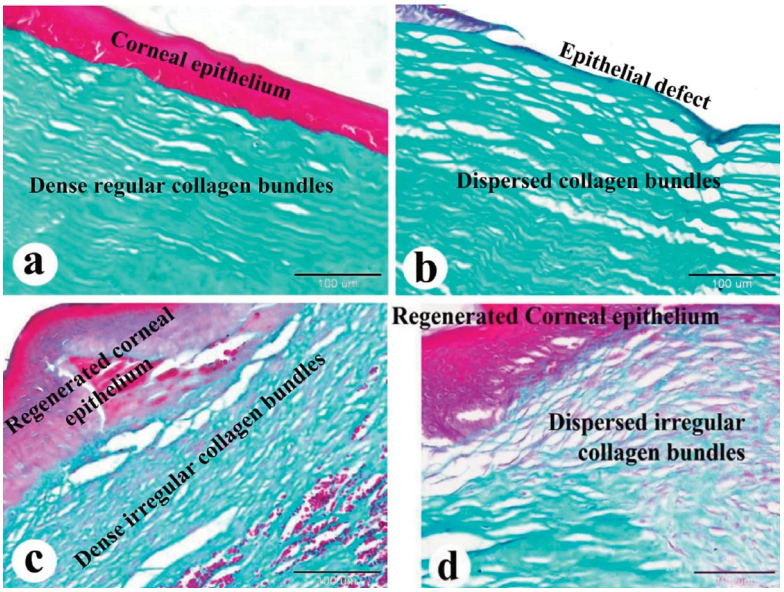


Fig. 10. Photomicrograph of paraffin sections in the cornea of the donkey. a: The control negative group showing the corneal epithelium and dense regular collagen bundles. b: 0-day induced corneal ulcer group showing dispersed collagen bundles and epithelial defect. c: The control positive group showing regenerated corneal epithelium and dense irregular collagen bundles. d: The PRF drop group showing regenerated corneal epithelium and dispersed irregular collagen bundles. Original magnification; X200, Scale bars = 100 µm, Crossmon's trichrome stain.

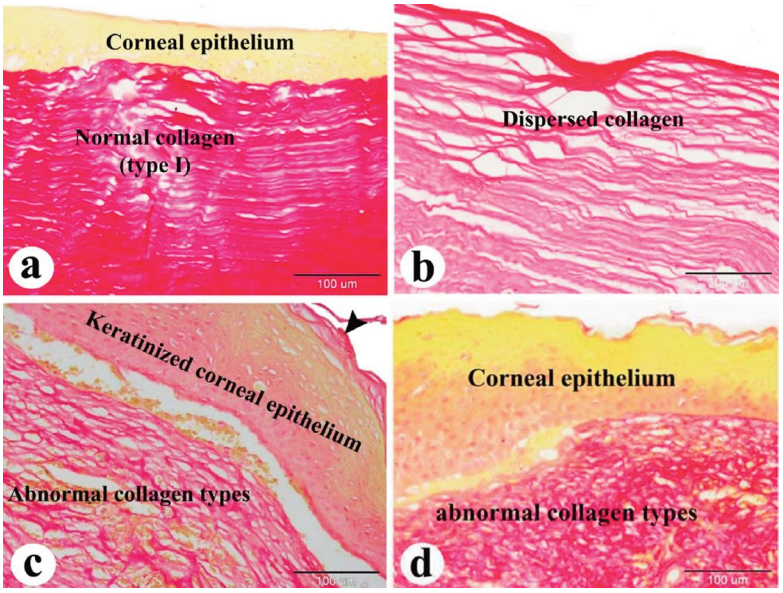


Fig. 11. Photomicrograph of paraffin sections in the cornea of the donkey. a: The control negative group showing the corneal epithelium and normal regular collagen type I. b: 0-day induced corneal ulcer group showing dispersed collagen type I. c: The control positive group showing keratinized corneal epithelium and abnormal collagen types. Note the keratin layer (arrowhead) of the regenerated corneal epithelium. d: The PRF drop group showing regenerated corneal epithelium and abnormal collagen types. Note the new normal mature collagen stained red with Picro-Sirius red stain technique. Original magnification; X200, Scale bars = 100 µm, Picro-Sirius red stain technique.

Table 3. Histopathological changes and healing score of corneal ulcers in donkeys

Histopathological changes and repair events	Control	PRF drop
Edema	+++	+
Stromal disorganization	+++	+++
Leucocytic infiltration	+++	++
Vascular congestion	+++	+
Pigmentary keratitis	+	+
Epithelial and stromal thickening	+++	+
Neovascularization	+++	++
Regular mature collagen replacement	+	+
Irregular fibrous tissue replacement	++	++
Fibroblasts activity	+++	+++
Keratocytes activity	+	+
Epithelial regeneration	+++	+

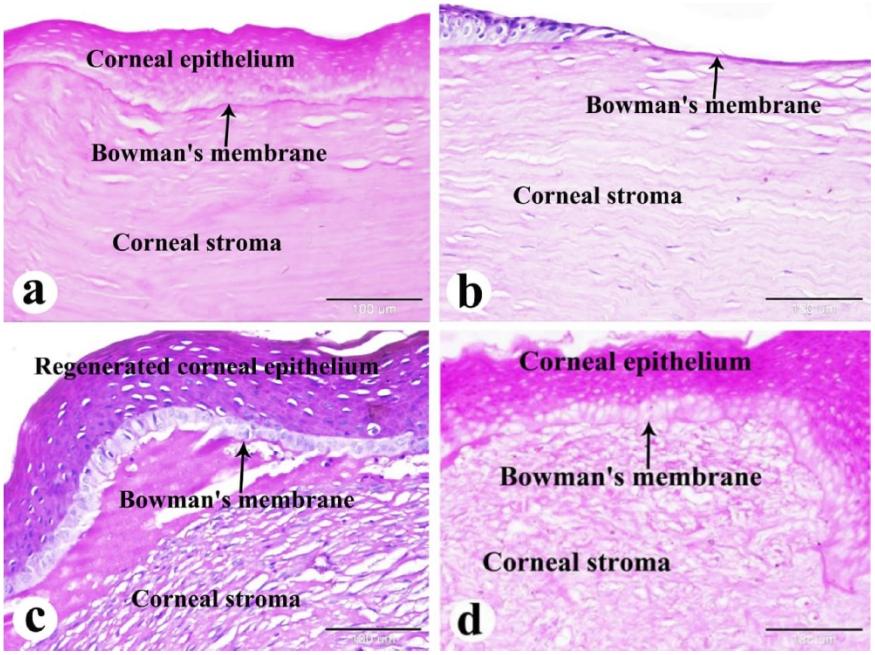


Fig. 12. Photomicrograph of paraffin sections in the cornea of the donkey. a: The control negative group showing the corneal epithelium, clear PAS positive Bowman's membrane and PAS positive collagen bundles of the corneal stroma. b: 0-day induced corneal ulcer group showing PAS positive Bowman's membrane and dispersed weak PAS positive collagen bundles of corneal stroma. c: The control positive group showing regenerated corneal epithelium, ill clear and interrupted PAS positive Bowman's membrane and PAS positive collagen bundles of the corneal stroma. d: The PRF drop group showing regenerated corneal epithelium, ill clear and interrupted PAS positive Bowman's membrane and weak PAS positive collagen bundles of the corneal stroma. Original magnification; X200, Scale bars = 100 µm, PAS stain technique.

Results of the present study revealed that there were ophthalmic signs of inflammation that were recorded in all donkeys after induction of corneal ulceration and during the experiment. These ophthalmic signs could be attributed to the irritating effect of sodium hydroxide that was used for chemical induction of the corneal ulcer in donkeys of the present study. Corneal alkali burns have been reported to induce a strong inflammatory reaction characterized by cell infiltration and production of proteolytic enzymes and cytokines (Levinson *et al*., 1976; Christmas, 1991; Imanishi *et al*., 2000). Similar signs of inflammation including corneal edema, opacity, neovascularization were recorded in another study on donkeys after chemical induction of corneal ulceration (Sobhy, 2016). In another study on rabbits (Gunay *et al*., 2015), corneal edema and vascularization started immediately after burning. The corneal stroma became very opaque within the 24 h following the corneal injury. Within three days following alkali burn, the rabbits showed marked blepharospasm with corneal epithelial defect and ulcer formation. Ophthalmic signs were also evident during s-PRF treatment in the present study. Although PRF is an autogenous product, it has been reported to cause an inflammatory response (Ceyhun *et al*., 2017). Similarly, topical administration of low and high doses of epidermal growth factor (EGF) in horses with experimentally induced corneal ulcers was associated with increased corneal edema, vascularization, melanosis, and scarring, side effects that likely negate any potential benefit of administration of EGF as performed in that study (Burling *et al*., 2000). It has been suggested that prolonged episodes of corneal de-epithelialization may lead to increased production of collagenases in the corneal stroma, and in turn to corneal perforation (De Aracena Del Cid and De Espinosa Escoriaza, 2009).

It has been reported that PRF was used for treatment of the corneal ulcer and explored that the PRF has potential effects during treatment of corneal ulcer (Burling *et al*., 2000; Mishra *et al*., 2021); however other studies recorded some adverse effects of PRF on ulcer healing (Can *et al*., 2016; Emma *et al*., 2020).

Results of the present study revealed time-related decreases in the surface area of the induced corneal ulcer in all animals. The inflammatory response secondary to the alkali burn has been quantified through area measurements of re-epithelialization and neovascularization (Conners *et al*., 1997). Healing of the corneal wounds; however, is an exceptionally

complex process involving the combined actions of multiple proteinases, growth factors, cytokines produced by epithelial cells, stromal keratocytes, inflammatory cells and lacrimal glands (Cameron, 1997; Nishida, 1997; Schultz, 1997; Sack *et al*., 2000; Sivak and Fini, 2002).

It has been cited that a decrease in the size of epithelial defects is usually associated with reduction in the risk of infection, as well as in pain and discomfort (De Aracena Del Cid and De Espinosa Escoriaza, 2009). Burling *et al*., (2000) noticed that, after creation of the corneal defect in horses; re-epithelialization was initiated rapidly and progressed in a linear fashion for the first 5 to 7 days after surgery in all horses. The healing rate in his study slowed down after that period as measured by analyzing photographs at different intervals by software. A time-linked decrease in the size of induced corneal ulceration was recorded in another study on donkeys to evaluate the effects of honey, cod liver oil and Aloe Vera on ulcer healing (Sobhy, 2016).

All donkeys in the control group of the present study exhibited negative fluorescein uptake at the end of the study, which indicated occurrence of re-epithelialization. While, 5/6 donkeys in the s-PRF-drops group showed positive staining on the day 35 of the study. Fluorescein dye has been used in diagnosis of corneal ulcer (Gilger and Davidson, 2002). The treatment of ulcers remains until the fluorescein uptake is negative (Williams and Chantale, 2013). Indolent and simple ulcers have been reported to be stained by fluorescein; however, staining can be observed underneath the periphery of the corneal epithelium, indicating detachment from the underlying stroma (Cutler, 2004). In some stages of the present study, fluorescein staining of the corneal stroma underneath the epithelium was observed in donkeys resulting in larger surface area values than concomitant values without staining. In cases of corneal injury, however, topical treatment with blood derivatives is used to compensate for the lack of physiological angiogenesis of this avascular tissue. Blood-derived preparations contain growth factors, cytokines, and other signaling molecules, that are essential for cell turnover in epithelial and stromal tissue in corneal wound healing (Klenkler *et al*., 2007). Furthermore, these molecules may suppress inflammation in the case of impairment of epithelialization and also have antimicrobial effects (Alio *et al*., 2012).

Some key and active components in the PRF help in tissue regeneration such as platelets that are rich in growth factors (Dohan *et al*., 2009b) and cells (mostly the various populations of leukocytes and stem cells) for their antibacterial, neo-vascularization and regenerative properties (Dohan *et al*., 2010). In addition, PRF contains many growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor-alpha (TGF-α), and transforming growth factor-beta (TGF-β) (Dohan *et al*., 2006b; Dohan *et al*., 2009a; Giannini *et al*., 2015).

Shen *et al*. (2011) have reported that concentrations and frequency of administration of blood derivatives may influence the wound-healing process. Undiluted s-PRF-drops were applied three times per day in our study. Several studies have reported the effectiveness of undiluted concentrations of blood derivatives in the epithelial healing process of mechanical corneal wounds (Akyol-Salman, 2006; Akyol-Salman and Gündogdu, 2010), whereas other studies have shown non-significant effects when using 20% dilutions (Shahriari *et al*., 2008).

It has been cited that the mechanism of action of autologous serum relies on its content in proteins, such as fibronectin, and albumin as epidermal growth factors necessary for epithelial regeneration after alkali injury of the cornea (Young *et al*., 2004; Yoon *et al*., 2007; Akyol-Salman and Gündogdu, 2010; Kaya and Akova 2015). Epidermal growth factors have also been shown to be beneficial in promoting epithelial migration in experimental as well as human studies of corneal alkali injury (Campos *et al*., 2003; Ziakas *et al*., 2010). Some complications of corneal ulceration such as pigmentation, anterior synechia and iris prolapse were recorded in the present study in the s-PRF-drops group. Such complications have been recorded in other studies on human corneal ulcerations and descemetocele (Can *et al*., 2016; Emma *et al*., 2020).

Histopathologically, corneal epithelium regeneration was currently recorded in all donkeys of the control group. However, corneal defect was still present in the donkeys of the s-PRF-drops group except one donkey showed corneal re-epithelialization. Signs of inflammation were significantly noticed in both control and s-PRF-drops groups including; sub-epithelial blood clotting, edema, leukocytic infiltration, vascular congestion and neovascularization in the corneal stroma.

In the two groups, numerous fibroblasts were observed among the corneal stromal cells. The rate of corneal regeneration was higher than the control group due to the content of the growth factors in the drops, which extracted from the fibrin gel during the preparation of the drops (Kokdere *et al*., 2015; Can *et al*., 2016). Results of this study revealed thickening of the corneal stroma in donkeys of the control group and slight thickening of the stroma in the s-PRF-drops group.

By Grossmon's trichrome stain, irregular, dispersed and dense collagen bundles were illustrated in both control and s-PRF-drops groups. After the use of Picro-sirius red stain in the present study, corneas of both control and s-PRF-drops groups showed abnormal formation of collagen type I. In this study, PAS stained sections of s-PRF-drops group showed clear and interrupted PAS positive Bowman's membrane and weak PAS positive collagen bundles. While sections of control group showed ill clear and interrupted PAS positive Bowman's membrane. Keratinization was illustrated clear in the regenerated corneal epithelium in the control group and was not observed in the s-PRF treated groups. In another study, epithelial regeneration was normal with a multi-stratified, non-keratinized, and well organized epithelium (Freire *et al*., 2014). Nevertheless, histological sections revealed focal hyperplasia of one or two layers of epithelial cells in all re-epithelialized corneas, regardless of the treatment used compared with the histological sections of a healthy cornea (Freire *et al*., 2014). Serum from plasma rich in growth factors (s-PRGF) has been shown to regenerate rabbit corneas by promoting cell proliferation, migration, differentiation, and adhesion. Treated corneas showed a multistratified non-keratinized and well-organized epithelium (Etxebarria *et al*., 2017). Focal hyperplasia of one to two layers of epithelial cells was observed in all re-epithelialized corneas. No alterations were observed in the endothelium. Stromal depth was maintained across the whole cornea, with healthy collagen fibres. Eyes treated with 100% s-PRGF had keratocytes through the full depth of the stroma (Etxebarria *et al*., 2017). Moreover, treatment with s-PRGF or other blood derivatives has been found to promote proliferation of human corneal epithelial cells (Liu *et al*., 2006; Freire *et al*., 2012; Anitua *et al*., 2013).

Conclusion

Corneal ulcer healing is associated with corneal opacity, vascularization, melanosis, and other complications that likely negate any potential benefit of administration of s-PRF as a treatment for corneal ulcer in donkeys. Histological results of the s-PRF-drops group are similar to the other group in the degree of re-epithelialization and regularity of the collagen bundles, type and maturity of the collagen. Treatment by s-PRF drops results in no epithelial keratinization and slightly less sub-epithelial stromal inflammatory reaction.

Conflict of interest

Authors declared that they have no conflict of interest to disclose.

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