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Surgery

Compare The Efficacy of Corneal Healing Between Autologous Serum and Eye drops Platelet Rich Plasma Treatment in Dogs

Shaaban M. Gadallah[1], Tarek N. Misk[1], Alaa Ghaith [2], Eslam Reda[1]*, Anis Anis [3]

(1) Department of Surgery, Faculty of Vet. Medicine, University of Sadat City, Egypt.

(2) Department of Ophthalmology, Faculty of Medicine, University of Alexandria, Egypt.

(3) Department of Pathology, Faculty of Vet. Medicine, University of Sadat City, Egypt.

**corresponding author: eslamredaedrees@gmail.com* *Received: 3/12/2020 Accepted: 29/12/2020*

ABSTRACT

Corneal ulcer is considered one of the most common ocular surface diseases, it may lead to blepharospasm, photophobia, lacrimation, conjunctival hyperemia, and corneal oedema. Conventional treatments are sometimes not effective in solving the pathological condition therefore blood derivatives which are rich in growth factors were used to accelerate wound healing as well as its ability to induce regeneration of affected tissues. Blood derivatives include autologous serum (AS), eye drops platelets rich plasma (E-PRP). A 6 mm induced corneal wound were performed at the center of the cornea at one eye at a 240 M depth in 20 mongrel dogs. Dogs divided into three groups, E-PRP group (8 dogs) and AS group (8 dogs) and a control group (4 dogs) received normal saline eye drops. All dogs were received 4-time treatment per day until negative fluorescein stain test is obtained. Histological study was performed for 3 dogs (one from each group) two months from the beginning of the experiment. Clinically the E-PRP group showed faster healing time with a significant difference (3.62±0.23 day) from AS group (4.75±0.25 day) and control group (6.75±0.30 day). Histologically, E-PRP group showed more arrangement of the epithelial layers compared with AS group and control group. Aim of our study: to compare the efficacy of AS and E-PRP in the healing of induced corneal wound in dogs. The aim of our study: to compare the efficacy of AS and E-PRP in the healing of induced corneal wound in dogs. In conclusion, E-PRP and AS showed a significant faster epithelial healing from control group. As well as E-PRP showed also a significant improvement over AS in heling time and quality of regenerated epithelial tissue.

Keywords: *platelets rich plasma (E-PRP), Autologous serum (AS), Corneal wound healing.*

INTRODUCTION

Corneal ulcer is one of the most common ocular surface diseases in dogs. Ulcers which include the outer two layers of the cornea; epithelial layer and stroma, mainly occurs due to eyelid, eye lashes and tear film dysfunctions. This usually leads to pain, blepharospasm, photophobia, lacrimation, conjunctival hyperemia and corneal oedema (Gelatt, 2013, Maggs, 2013). Corneal wound healing is a complex mechanism that involves cell proliferation, migration, reattachment of the epithelium to its extracellular matrix, and cell differentiation. These processes are sustained by glucose, vitamins, and growth factors (Freire, *et al.*, 2014).

During last years, The administration of blood products as a therapeutic option has acquired great prominence, due to its biological composition and its ability to induce the

regeneration of affected tissues (Nugent and Lee 2015). There are a variety of ways for obtaining autologous blood derivatives, with variable amounts of platelets and growth factors (GFs) (Alio, *et al.*, 2007). The GFs, including platelet-derived GF (PDGF), transforming GF-β (TGF-β), vascular endothelial GF (VEGF), fibroblastic GF (FGF), insulin-like GF (IGF) and epidermal GF (EGF), have a main role in wound healing and increase the physiological process at the site of the injury (Di Pietro, 2017).

In human ophthalmology, the use of blood derivatives as autologous serum and platelets-enriched plasma eye drops represents an alternative therapeutic approach in regenerative medicine due to its potential to stimulate and accelerate tissue healing (Anitua, *et al.*, 2015).

Autologous serum (AS) eye drops was the first hemoderivative product used for the treatment of corneal pathologies (Fox, *et al.*, 1984, Tsubota, *et al.*, 1999). Their use was referred to its content of growth factors provided by destructed blood platelet content due to high speed centrifugation (Nurden, *et al.*, 2008, Blair and Flaumenhaft 2009).

Some studies reported that AS has higher levels of EGF more than PRP. which accelerates corneal wound healing leading to high migrating ability of cells in primary culture corneal epithelium in rats (PRCE) and (human corneal epithelium) HCE cultures. Furthermore, the lowest concentrations of EGF and weakest effect on corneal epithelial cell growth and migration were observed with PRP, in which platelet activation has not occurred (Burmeister, *et al.*, 2009, Freire, *et al.*, 2014)

The major drawbacks of AS was it had no standard protocol for preparation either its percentage of dilution is ranging from 20% to 100% (Geerling and Hartwig 2006). Furthermore, AS contain pro-inflammatory agents such as metalloproteinase and acid hydrolases derived from leukocytes degranulation, which may induce negative effects in ocular tissue regeneration (Schnabel, *et al.*, 2007).

On the other hand, other studies reported superior effect of E-PRP over AS (Alio, Abad *et al.*, 2007, Lee, *et al.*, 2016) . They found that a significant higher concentration of TGF-β1 and EGF, PDGF and Fibronectin in E-PRP than found in AS. Moreover, E-PRP induces a significantly higher epithelization ratio promoting the rearrangement of actin filaments stabilizing the tear film and prevent apoptosis (Kim, *et al.*, 2012, Rodríguez and Alió 2019). Although there isn`t activation of platelets in E-PRP, its efficacy didn`t depend on platelets activation due to plasma contain growth factors, plasma protein, and epitheliotrophic factors that accelerate corneal wound healing, Indicating a positive and significant correlation between PDGF-BB and platelets in E-PRP (Rodriguez, *et al.*, 2020).

This study aims to evaluate autologous blood derivatives eye drops (AS & E-PRP) clinically and histo-pathologically that was previously used in human ophthalmology to be applied in veterinary ophthalmology using a canine animal model with special reference to its storage and preservation techniques.

MATERIALS AND METHODS

This study was conducted at Faculty of Veterinary Medicine Hospital and its Laboratory at Sadat City University after following the ethical guidelines for animal care and use in scientific research at Sadat City University.

Animals

The present study was carried out on 20 apparently healthy dogs for only one eye for each dog. 16 males and 4 females mixed breed (mongrel) dogs were used, aging 1.52±0.31 years and weighing 15.7±2.13 kg. Dogs classified to three groups; group I (E-PRP), group II (AS) each include 8 dogs and group III (Control Group) which received saline eye drops.

The dogs were anaesthetized by intramuscular injection of 1 mg/kg xylazine HCl (Xylaject ® by Amoun pharmaceutical CO. Egypt) as tranquilizer followed by intravenous injection of 10 mg/kg ketamine HCl (ketamine hydrochloride inj. USP ® by Rotexmedica – Trittau Germany) as general anesthesia. Benoxinate hydrochloride eye drops (Benox ® by EIPICO, Egypt.) was used topically as a local anesthesia. The dogs were placed in lateral recumbency. lid retractor was placed for exposure of the cornea. A 6-mm calibrated corneal trephine was placed in the center of the cornea. The trephine depth was 240 µ which include the anterior third of the corneal stroma then a crescent bevel-up blade was used to curette the trephined button from the center of the cornea (Aldavood, *et al.*, 2003). Fluorescein stain 10% eye drops (fluorescein inj. USP ® by

Sunways –India) was applied immediately after removing the incised button to stain the corneal wound as shown in figure (1) to ensure the defect. Fluorescein test was used in a daily basis for all animal groups until showed negative stain indicating intact corneal epithelial layer. All dog groups were received 4 times eye drops per day until showed negative Fluorescein test. Each Dog was kept in a clean separate cage with neck collar. Corneal defect of each dog was photographed every 24 hours immediately after Fluorescein testing until epithelial healing was complete.

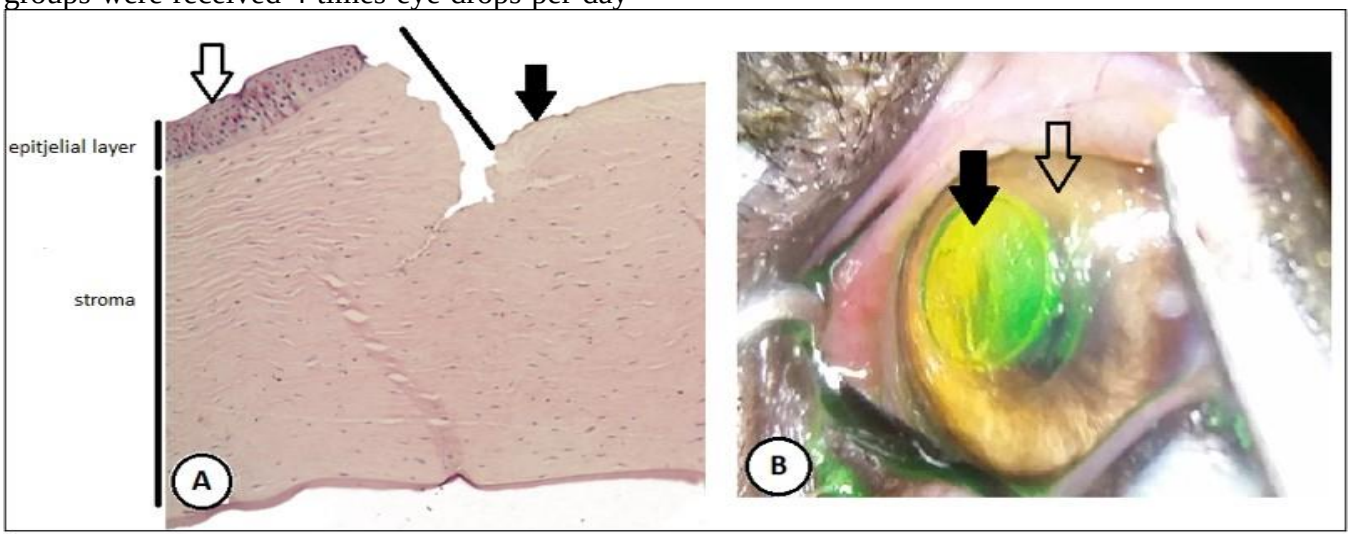

Fig. (1): Cornea, Dog cornea after incision: A) showing incision include epithelium and 1/3 of stroma represent around 240µ (solid arrow) by H&E stainX4. B) showing total incision button at center of cornea around 6 mm width stained by fluorescein stain surrounded by normal corneal layers (empty arrow).

Eye drops preparation

E-PRP preparation was carried out under strict sterile conditions using sterile and disposable materials at the laboratory of faculty of veterinary medicine Sadat City University. Blood sample collection from jugular vein 10 ml, then E-PRP preparation method as prepared previously in 2019 for human (Rodríguez and Alió 2019). technique and methods of preservation as performed as described by in human (Kim, *et al.*, 2012)

Autologous serum was prepared by spontaneous coagulation of the blood extracted without anticoagulant in a test tube. Incubation for 2 h at room temperature. Centrifugate at 3000 rpm for 15 min, Followed by collection of supernatant fraction then diluted to 20 % by using 0.9% physiological saline as TGF-β concentrations are five times higher in serum than in tears (Pan, *et al.*, 2017).

Histopathological analysis

Histopathological analysis was performed after two months from starting the experiment. Corneas were harvested and processed for hematoxylin and eosin staining. Cotneal tissues were removed curefully and fixed in 10% formalin for 72 h. For histopathological investigation samples were trimmed, washed, dehydrated, embedded in paraffin wax, serially sectioned with a microtome at 3 µm thickness and stained with hematoxylin and eosin (H&E) stain. Additionally, histological photos were taken by using Lieca DMLB microscopes and Leica EC3 digital camera

Statistical Analysis

Mean, and standard deviation of the duration of corneal epithelial healing after topical application of eye props calculated using ANOVA using SPSS statistical software (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, version 26.0. Armonk, NY: IBM Corp) and a *P* value less than 0.05 was considered statistically significant.

RESULT

Group (I) showed progression of epithelial healing rapidly from the first day till complete healing which occur after the third day of treatment with mean healing time 3.62±0.23 day (Table (1) and figure (2)). Group (II) showed complete healing after fourth day with mean healing time 4.75±0.25 day (Table (1) and figure (2)). Group (III) which received normal saline as control group showed mean healing time 6.75±0.30 day (Table (1) and figure (2)).

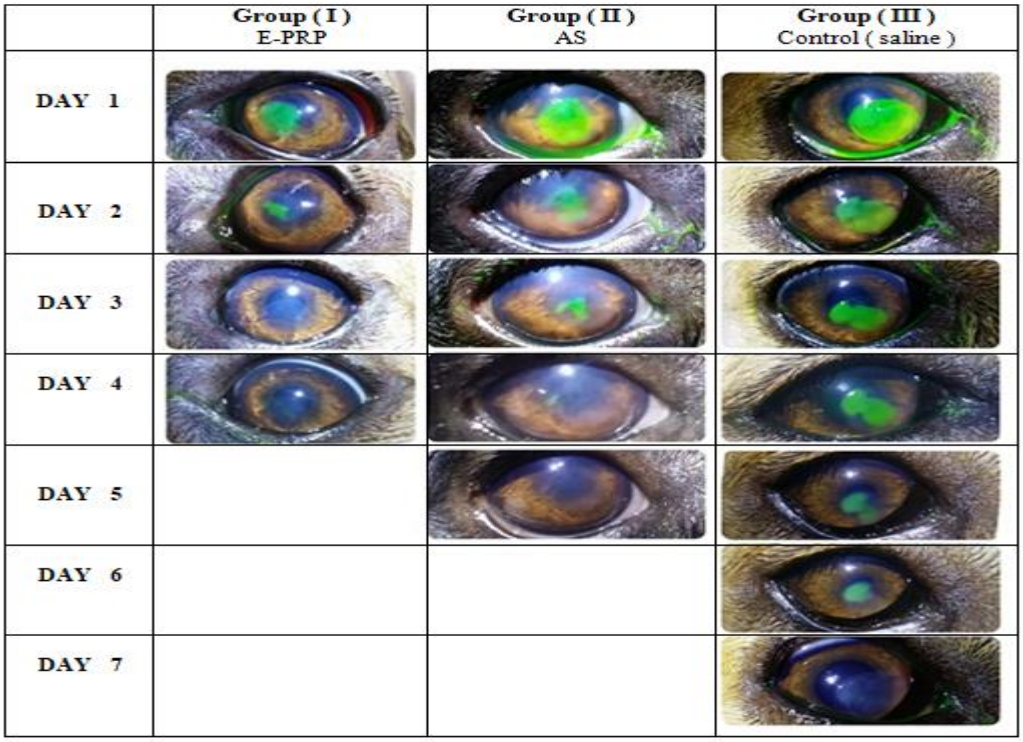

Fig. (2): Showing fluorescein stain each day for group (I) E-PRP, (II) AS and (III) control group received saline till complete healing in dog cornea.

Table 1. showing mean complete healing time for all three groups

Group	Healing time (by Days)
PRP treated	**3.62±0.23^{c}**
AS treated	**4.75±0.25^{b}**
Saline treated	**6.75±0.30^{a}**

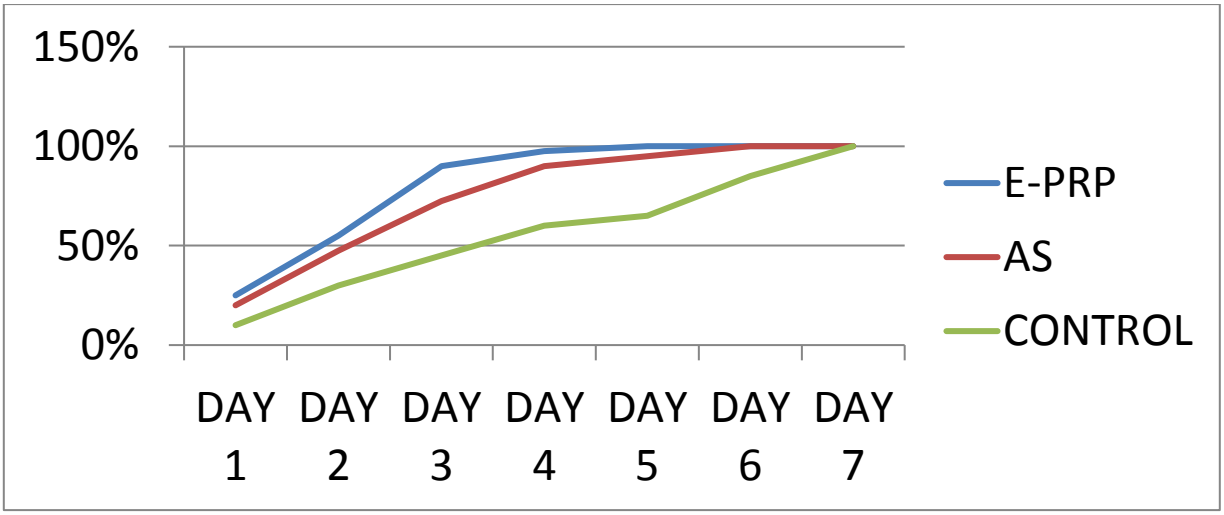

Fig. (3). Showing the percent of corneal healing over time for all three groups

Clinically using fluorescein test, the first group E-PRP showed that the mean healing rate at first day was 25.2% ±3.3 and at the second day was 65.4% ±5.2 while at third day 90.3 % ±3.8 and finally at fourth day mean healing rate was 97.6 ±2.5. in the group (II) (AS group) the mean healing rate at first day was 21.3% ±0.8 while in second day was 47.6 ±5.3 and it found at the third day was 72.7 ±5.2 while in fourth day was 90.4% ±3.7 and finally fifth day 97.1 % ±2.3 till complete healing. Finally, in Group (III) (control group), the mean healing rate was 9.2%±5.3, 30.1%±5.2, 44.7%±5.4, 59.5%±6.3, 70.6%±5.1, 86.3%±4.7 and finally 100% at 1,2,3,4,5,6 and 7 days respectively as shown in figure (3).

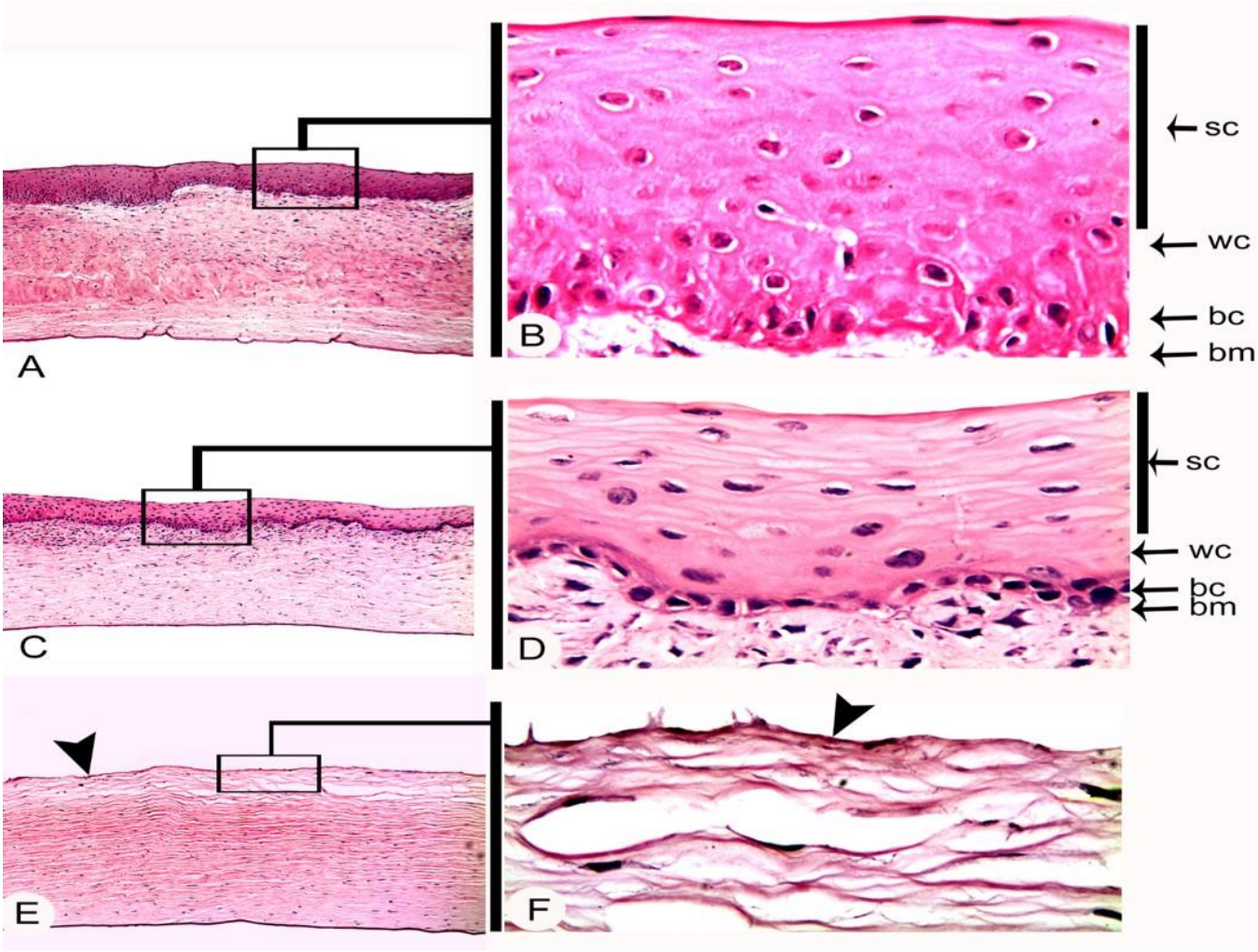

Fig. (4): Cornea, Dog. A & B) Group I, eye drops platelets rich plasma treated group: A) showing healing of corneal wound with regular epithelial layer. B) Higher magnification of Fig. A; showing complete corneal epithelium structure which consists of superficial cells (sc), complete wing cells layer (wc), columnar basal cells layer (bc) and basement membrane (bm). C & D) Group II, autologous serum treated group: C) showing healing of corneal wound with tortuous epithelial layer. D) Higher magnification of Fig. C, showing incomplete corneal epithelium structure which consists of superficial cells (sc), incomplete wing cells layer (wc), cuboidal basal cells layer (bc) and basement membrane (bm). E & F) Group III, saline treated group: E) showing absence of corneal epithelium (arrowhead). F) Higher magnification of Fig. E, showing complete absence of corneal epithelium (arrowhead) and presence of thin layer basement membrane.

Eye-drop plasma rich platelets treated group showed a complete and perfect healing of the induced corneal ulcer (Figure 4 A & B). In the autologous serum treated group, the healing of the corneal ulcer was completed at the end of the experiment, but the epithelial layers are tortuous with beginning of epithelial layers formation without differentiations of these cells (Figure 4 C & D). In the other hand, in saline treated group there was a complete absence of corneal epithelium and presence of thin layer basement membrane. (Figure 4 E & F).

DISCUSSION

Blood derivatives eye drops contain several types of growth factors which play important role in migration and proliferation of corneal epithelium accelerating the regeneration process. AS and E-PRP are the most common forms used. In dogs, there are a lot of pathological conditions of the cornea and sclera as corneal ulcers and keratoconjunctivitis kicca (KCS) that can be managed using AS and E-PRP (Anitua, *et al.*, 2015, Vatnikov, *et al.*, 2020).

The effect of these blood derivatives is variable and controversial due to way of preparation of these derivatives and the amounts of growth factors produced (reference here). This experimental study may help in clarifying the differences between AS and E-PRP in healing of a 6 mm induced corneal wound.

Clinically, AS group proved significant faster corneal healing than control group. Negative fluorescein test was achieved at 4.75±0.25 days to 6.75±0.30 days in control group. AS have a pH, osmolarity and biomechanical properties

which resemble natural tears and when used topically it supply essential nutrients such as growth factors, vitamins and bacteriostatic products as IgG lysozyme which regulates proliferation processes and migration of the cells of the corneal epithelium (Alio, *et al.,* 2012).

Histologically, AS group showed epithelial layer formation without differentiation of wing cells and cuboidal cells. irregular epithelial layer formation was noticed however it was better than found in control group which had only basement membrane above stromal layer. This result was matching with other authors (Tsubota, *et al.,* 1999, Matsumoto, *et al.,* 2004, Geerling and Hartwig 2006).

Clinically, E-PRP group showed not only significant improvement over control group but also a significant improvement over AS group. Complete corneal healing was achieved at 3.62±0.23 days. This results matches with other authors claiming that E-PRP contains higher GFs than obtained from AS (more reference needed) (Rodríguez and Alió 2019).

Histologically in E-PRP group, regular epithelial layers along with stroma and formation of this layers seem to be as shown in normal cornea was documented. Also, epithelial layers differentiation to basal cuboidal cells which had long axis perpendicular to corneal surface was noticed that was not seen in AS group. This result agrees with authors who found that E-PRP had more advantages than AS referred to a higher concentration of growth factors, which might stimulate the growth of epithelial cells and thus lead to faster healing (Alio, *et al.,* 2012). These result was supported by results indicating superior effect of E-PRP over AS in Patients with persistent epithelial defect PED he claimed these results due to superior epitheliotrophic capacity of E-PRP over AS (Kim, *et al.,* 2012).

On the other hand, some others claim that in vitro use of AS induce faster wound healing than E-PRP in wound healing assay using rabbit primary cultures and human culture line. Moreover, in a vivo study on induced corneal wound in a rabbit showed faster complete corneal reepithelization in AS than in E-PRP. They revealed superior effect of AS over E-PRP due to the last one had platelets didn`t activated yet, but coagulation during AS preparation allow activation of the platelets (Feire, *et al.,* 2014).

However, the concentration of GFs in the E-PRP is not necessarily correlate to the number of platelets, and fibronectin is a plasma protein and its presence does not depend on the activation of platelets (Rodriguez, *et al.,* 2020). Moreover, recent studies found that freezing of E-PRP at -20 ^{0}c for 3 months, increases the concentration of important protein such as PDGF-BB and EGF and maintain the level of other GFs (Rodriguez, *et al.,* 2020). Further studies on blood derivatives byproducts still needed to end these controversial results proven if E-PRP is superior over AS for treating different ophthalmological disorders in canine.

CONCLUSION

Blood derivatives eye drops as AS and E-PRP facilitates re-epithelialization and promotes faster cornel wound healing. E-PRP as new therapeutic approach was superior over AS for treating canine corneal wounds.

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