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Changes in Lacrimation in experimentally induced Corneal Ulcers in Rabbits, treated with Third Eyelid Flap, Hyaluronic Acid and Platelet-Rich Plasma products

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NAME OF THE AUTHOR(s)

***Seven Ruzhdi Mustafa[1]**
Nadya Zlatozarova Zlateva-Panayotova[2]

[1] Assistant Professor, Department "Surgery, Radiology, Obstetrics, and Gynecology, Faculty of Veterinary Medicine, University of Forestry – Sofia, Bulgaria;

[2]Associated Professor and Head of Department "Surgery, Radiology, Obstetrics, and Gynecology, Faculty of Veterinary Medicine, University of Forestry – Sofia, Bulgaria.

ABSTRACT

A clinical study was conducted on the effect of the lacrimation in New Zealand White rabbits (*Oryctolagus cuniculus*) treated with 4 different therapies (Third Eyelid Flap, hyaluronic acid eye drops, platelet-rich plasma as a single subconjunctival injection and as a triple daily instillation). For the study, daily measurements of tear production were performed using the 1-min Schirmer Tear Test (STT-I). In all groups, increased STT-I values were observed until the 3rd day after ulceration. Hyaluronic acid and platelet-rich plasma decreased the STT-I values to the baseline by the end of the study, with the earliest results noticed with plasma drops. Observed STT-I values in third eyelid fixation by the study's end are higher compared to the untreated eyes in the Control group.

Keywords- corneal ulcer, lacrimation, New Zealand White, rabbit, Schirmer Tear Test, STT

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** Corresponding Author*

I. INTRODUCTION

Rabbits have a longer inter-blink time (approximately 10 minutes) compared with humans (5 – 8 seconds), suggesting that they have one of the most stable tear films among animals[1]. The outermost lipid layer is in direct contact with the atmosphere and retards tear evaporation. The aqueous-mucin layer is responsible for lubrication, oxygenation, and nourishment of the cornea, as well as providing its antibacterial protection. This layer consists of a complex dilute solution of both organic and inorganic compounds[2]. It also contains active or functional proteins such as growth factors, vitamins, and immunoglobulins which regulate the processes of proliferation, migration, and differentiation of the corneal cells. Thus, the tear film has not only a lubricating function for the ocular surface, but also has antimicrobial and epitheliotrophic properties, and is as vital to the normal function of the eyes as any anatomic component[3]-[4]. Tear deficiency is a disorder known as "dry eye", that can lead to complicated sterile or infectious central or paracentral corneal ulceration, especially in patients with Sjogren's syndrome. Occasionally, corneal perforation may occur in these cases[5].

Schirmer Tear Test – STT[6] is part of the ophthalmological clinical examination used to assess lacrimation in patients with suspected dry eye (hypolacrimation) or tear overproduction (hyperlacrimation). There are few reports evaluating lacrimal testing methods in rabbits. When the STT-I is used to measure lacrimation in small animals such as rabbits, with physiologically small volumes of tears, a very low value may be obtained. STT-I is more useful in rabbits for assessing increased lacrimation associated with ocular irritation and corneal ulcers than for determining decreased values associated with *xerophthalmia/keratoconjunctivitis sicca*[7].

A corneal ulcer is a full-thickness loss of corneal epithelium with exposure to the underlying corneal stroma. This painful stimulation of the eye results in photophobia, blepharospasm, and increased lacrimation to prevent the eye from potential damage[8]-[9], but there is little in the literature to show a direct link of increased tear secretion with corneal ulceration. Levels of tear growth factors are altered in corneal injuries via a neural reflex loop between the ocular surface and lacrimal glands, stimulating healing processes[10].

The therapeutic approach to corneal ulcers in veterinary ophthalmology is diverse and may combine surgical methods, antibiotics with or without anti-inflammatory drugs, various agents, and vitamins stimulating metabolism and recovery, as well as cell-based therapies from regenerative medicine. Covering the ocular surface with the third eyelid is applied relatively often in veterinary ophthalmology to provide rest to the corneal surface and reduces its irritation from the upper and lower eyelid[11]. The eye drop combination of hyaluronic acid (and more precisely sodium hyaluronate) and dexpanthenol (precursor of vitamin B5) is widely used for dry eyes, but its effect on migration of corneal epithelial cells is also being studied [12]. The use of blood derivatives as a concentrated source of growth factors such as platelet-rich plasma (PRP) is not new and its rationale in ophthalmology is mainly based on their potential properties including lubrication, mechanical actions, and antimicrobial effects[13].

The purpose of the study was to track how four different 10-day therapies used to treat experimentally induced corneal ulcers affected tear production in rabbits.

II. MATERIALS AND METHODS

All experimental procedures were conducted following Council of the European Union guidelines m. 2010/63/EU regarding the protection of animals for experimental purposes and according to the animal welfare requirements of the Bulgarian Food Safety Agency (Document №337/2022). The surveys were performed in 2022 in the University Clinic for Small Animals at the Faculty of Veterinary Medicine, University of Forestry – Sofia.

The study involved 30 New Zealand White rabbits (NZW), equally male and female, between 12 and 18 months of age, with an average body weight of 3,1±0,4 kg. For the duration of the study, the animals were kept in single modular cages, undergoing a two-week adaptation period to the new environment and prophylactic deworming. The diet included concentrated pellets for rabbits and hay. Access to water and hay was unrestricted, and concentrate feed was given at a ration recommended by the manufacturer.

All animals participating in the experiment were determined to be clinically healthy after preliminary studies, and an experimental corneal ulcer was induced by alkali burn with 6 mm diameter filter paper soaked in 1M NaOH solution. The anesthetic protocol before manipulation was the same for all animals – an intramuscular injection of a combination of *ketamine hydrochloride* (Anaket 10%, Ruchterpharma®) in a dose of 35 mg/kg and *xylazine hydrochloride* (Xylazine 2%, Bioveta®) in a dose of 5 mg/kg, according to Lipman et al [14]. Right before the ulcer induction the ocular surface was instilled with *proxymetacaine hydrochloride* (Alcaine 0,5%, Alcon®). For ethical reasons, the experimental setup was applied and studied on only one eye of each rabbit to

preserve the visual function of the one untreated eye to maintain food and water intake.

After 24 hours the rabbits were divided into one Control and four experimental groups (n=6) with a study duration of 10 days. The Control group was left untreated after corneal ulceration. The following was applied to the experimental groups:

- MNF group – a temporary fixation of the third eyelid (*membrana nictitans*) to the lateral canthus of the ulcerated eye.
- CHD group – a therapy with eye drops containing sodium hyaluronate in combination with dexpanthenol applied by triple instillation each day to the ulcerated eye.
- PRP-DR group – a therapy with non-activated autologous platelet-rich plasma administration in the form of sterile-stored eye drops applied triple every day to the ulcerated eye.
- PRP-SC group – a therapy with non-activated autologous platelet-rich plasma applied by a single subconjunctival injection in the ulcerated eye.

The platelet-rich plasma (PRP) was obtained the day before the ulceration from the same animal on which is planned for administration, to preserve the autologous principle of the product. Regardless of the form of application, a double centrifuged method [15]of 10 ml whole blood with anticoagulant citrate dextrose solution was used to obtain a 0,7 ml non-activated PRP. On the 5th day for the PRP-DR group, a new product was obtained by the same method.

Each day, the animals underwent a clinical ophthalmological examination in a dimly lit room, and an STT-I was performed before the instillation of the liquid therapy forms and eye dyes. The Schirmer test was repeated three times on the same day and the average value was taken for analysis. The measurement was made with disposable sterile graphed test strips (Eickemeyer®) inserted into the lower eyelid fornix of the ulcerated eye. The result was read in 1 minute with a blue marking of the value (Fig. 1).

Figure 1. Measuring lacrimal production by STT-I in New Zealand White rabbit with an experimentally induced corneal ulcer.

III. STATISTICAL ANALYSIS

The experimental analyses of the data were carried out by one-way analyses of variance ANOVA (Predictive Analytics Software PASW, SPSS, Version 19), followed by the Friedman test. All values were expressed as mean ± standard deviation (SD). Differences were considered significant if the probability P-value < 0,05 and highly significant if the P-value < 0,001.

IV. RESULTS

Results were registered by taking the arithmetic mean of three measurements for each animal in mm/min and shown in Table 1, day by day with average values for each group (n=6), as Day 0 is taken the corneal ulcer induction day, and the measurement was performed before the anesthesia induction.

Table 1. Dynamics of STT-I results given in mm/min (mean ±SD)

n=6	Control	MNF	CHD	PRP-DR	PRP-SC
Day 0	5,8 ± 0,7	5,6 ± 1,3	5,6 ± 1,0	5,8 ± 0,7	5,8 ± 1,1
1st day	14,3 ± 1,0	14,8 ± 1,3	13,6 ± 1,8	14,6 ± 1,2	15,5 ± 0,8
2nd day	15,6 ± 1,5	16,3 ± 1,5	15,3 ± 1,3	11,1 ± 1,1$^{***\#\#\#}$	17,1 ± 0,8$^{***\#\#}$
3rd day	15,5 ± 1,2	16,8 ± 1,4$^{\#}$	15,1 ± 1,8	9,2 ± 1,4	15,5 ± 1,0
4th day	14,8 ± 0,4	15,6 ± 1,3	13,5 ± 0,8**	9,1 ± 0,4	13,5 ± 0,8**
5th day	15,0 ± 1,1	15,3 ± 0,8	11,8 ± 2,0**	6,8 ± 0,4	11,3 ± 0,8
6th day	14,6 ± 0,5	14,5 ± 0,8	9,6 ± 1,0$^{\#\#\#}$	6,1 ± 1,1	9,3 ± 0,5
7th day	11,3 ± 1,2$^{\#\#\#}$	13,6 ± 0,8**	8,8 ± 0,7$^{**\#\#\#}$	5,8 ± 0,7	8,8 ± 0,7
8th day	11,0 ± 0,9$^{\#\#\#}$	11,6 ± 0,8$^{\#\#\#}$	8,1 ± 1,4$^{**\#\#\#}$	5,8 ± 1,4	6,8 ± 1,7***
9th day	8,3 ± 0,8	10,6 ± 0,8***	6,8 ± 0,4**	5,6 ± 1,5**	6,3 ± 1,5*
10th day	8,1 ± 0,7	10,3 ± 0,5***	5,8 ± 0,7***	5,6 ± 1,0***	6,0 ± 0,9**

* p<0,05; ** p<0,01; *** p<0,001; statistical significance of the differences between the control and experimental groups on different days;

# p<0,05; ## p<0,01; ### p<0,001; statistical significance of group differences across days;

By Day 0, before any manipulations on the eyes, the average tear production was measured between 5,6 – 5,8 mm/min for all rabbits included in the study (for 30 eyes, the average result is 5,76±1,0 mm/min).

On the 1st day after corneal ulcer induction, an increase in lacrimation was observed in all groups, and since the measurements were taken before the beginning of the therapies, we relate these results to the clinical presentation of corneal ulcers from alkali burns. In addition to epiphora, other symptoms observed were blepharospasm, photophobia, corneal opacification and edema, neovascularization, and conjunctivitis. In all groups, an increase in lacrimation was observed until the 3rd day with the least in the PRP-DR group and values with statistical significance on the 2nd day – 11,1±1,1 mm/min (p<0,001). On the same day the largest significant difference from the baseline values was observed in the PRP-SC group –

17,1±0,8 mm/min (p<0,001), compared to the other groups with average values around 15 mm/min.

The fastest and smoothest decrease of the lacrimation is observed in the PRP-DR group, with the values starting to decrease already on the 3rd day – 9,2±1,4 mm/min, and on the 7th day, they are already the same as the baseline values.

A gradual decrease in lacrimation with high statistical significance in the experimental group itself was observed in the CHD group between the 6th and 8th day (p<0,001).

The values in the Control group remain higher at the end of the study. In the treated groups the values reach levels similar to the baseline day in the beginning, but with a highly statistically significant difference compared to the Control group. On the 10th day values in the MNF group are significantly higher compared to the other groups and there is still a significant difference – 10,3±0,5 mm/min (p<0,001) with the Control group.

V. DISCUSSION

The applied drugs from the chosen anesthetic protocol are selected so as not to affect lacrimation. Intramuscular injection of ketamine alone increases STT-I values and higher doses of α2-adrenoceptor agonists decrease them, but when they are combined their influence is insignificant on the lacrimal function[16]-[17]. The authors recommend that xylazine and ketamine be mixed and injected through the same syringe or, if dosed in separate syringes, be injected at the same time without an interval between them.

The results obtained in the initial examination of tear secretion in Day 0, accepted by us as a baseline (5,76±0,1 mm/min), are similar to the results obtained by other authors using the New Zealand White rabbit[7],[18]-[20].

The observed hyperlacrimation in the ulcerated eyes has been noted in the literature reference to ocular pain[21] and the reason is the interaction of the corneal nociceptive stimulation and subsequent tear secretion[22]. It is well documented that stimulation of lacrimal gland secretion occurs through a neural reflex loop originating from the ocular surface[23]. Indeed, stimuli to the ocular surface activate afferent sensory nerves in the cornea and conjunctiva that in turn activate efferent parasympathetic and sympathetic nerves in the lacrimal gland to stimulate secretion. Of the neurotransmitters and neuropeptides released by the nerves of the lacrimal gland, acetylcholine and norepinephrine are the most powerful stimuli for the secretion of proteins, water, and electrolytes from the lacrimal glands. Lacrimal gland secretion is regulated by both neurotransmitters released from the nerves that innervate the gland as well as by members of the epidermal growth factor (EGF) family[24]. In addition to stimulation of secretion, these growth factors can interact with the cells of the lacrimal gland themselves or with the cells of the ocular surface depending upon the location from which these growth factors are released[25].

As a result of our research, we found that in the Control group and the MNF group, tear secretion remained increased until the 8th day, and in the MNF group, it remained almost unchanged until the end of the study period. On the contrary, after an initial significant increase in tear secretion on the 2nd day in the PRP-DR group, on the 3rd day we observed a tendency to decrease and reach baseline values. It was the only group to return to pre-treatment baseline levels by the 7th day. The trend in the PRP-SC group is similar, although we found the highest hyper secretion values in it on the 3rd day.

The reason for the rapid decrease in secretion with PRP products is probably due to the faster and more active regenerative effect of PRP due to the biological stability of platelets and growth factors. Likewise, other studies have shown that more improvement in symptoms was achieved in patients treated with hematological derivatives versus artificial tears, such as those conducted by Celebi et al.[26] and Kojima et al.[27]. In support of this opinion are Panda et al.[28], who found that treatment with PRP drops stimulates the recovery of corneal defects, because platelets have productive properties and thus can ensure continuous production of growth factors. In a clinical trial of moderate to severe chemical burns they reported that autologous PRP eye drops resulted in better corneal clearance and faster healing of epithelial defects. Their recovery is an important point, so the main goals of treatment in corneal ulceration are restoring and maintaining an intact and healthy corneal epithelium and thus controling the balance between collagen synthesis and collagenolysis, because it serves as a protective barrier against tear enzymes that lead to corneal thinning and progression to perforation. The first 7 days after chemical eye injury constitute the acute phase of recovery. During this time, the tissues rid themselves of contaminants while re-establishing the superficial protective layer of the corneal epithelium[29]-[30].

The third eyelid flap provides physical protection to the entire cornea but maintains increased reflex tear secretion in the ulcerated eye for a longer period. This could be due to postoperative blink dysfunction, leading to relative epiphora[31]. Chae et al.[32] found no such differences in their study with New Zealand White rabbits, but they applied the nictitating membrane fixation to follow up contact lens stability,

not in chemical corneal ulcers. In our opinion, the tendency of higher lacrimation values in untreated eyes (Control group) and eyes with a third eyelid flap (MNF group) is because the inflammation lasts for a longer time and also because of the lack of building and repairing elements.

As a viscoelastic substance, sodium hyaluronate has mucinometic properties – it stabilizes and improves the retention of aqueous and secretory mucin in the aqueous layer of the tear film[33]-[35]. The included dexpanthenol in the eye drops is a precursor of vitamin B5 and although it is claimed to have a moisturizing effect, greater importance for its role is given to its healing effect on the epithelium[36]-[37]. In the CHD group, we observe the gradual decrease of the lacrimation by the end of the 6th day with a high statistically significant difference compared to the Control group. We attribute this to distinctive properties of hyaluronic acid, such as stabilization of the tear film and reduction of friction during blinking, which leads to a reduction in the symptoms of the inflamed cornea.

There are only a few studies on rabbit tear production and the influence of ophthalmic drugs in the available literature database. Most of them have been done on the New Zealand White rabbit, as it is the preferred breed for research in ophthalmology, both veterinary and human medicine. However, the obtained values cannot be taken as a reference for rabbits, as the data point to significant differences in the breed aspect.

We would like to note that the present publication examines the influence of the discussed therapies on only one of the clinical signs of corneal ulcer – the tear production. The main limitation of the study is the small number of the relatively young population of rabbits and the focus on a single breed, but this is a major problem in the use of experimental animals, so our conclusions are tentative and need further clinical studies to confirm the effect of autologous platelet-rich plasma and sodium hyaluronate plus dexpanthenol on lacrimation.

VI. CONCLUSIONS

Experimentally induced chemical burn of the cornea in rabbits leads to increase lacrimation, most significantly manifested on the 2nd and 3rd day after the ulceration. The application of platelet-rich plasma leads to a decrease in tear secretion, probably due to the local restorative effect of growth factors, and is more pronounced when it is applied in the form of eye drops.

Hyaluronic acid eye drops also decrease the lacrimation to the baseline values within ten days, while the third eyelid flap maintains higher STT-I values. The tracked changes in lacrimation are not indicative of the effectiveness of the applied therapies in corneal ulcers from alkali burns but only follow their influence on the secretory capacity of the lacrimal glands during the treatment.

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