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Effects of Bovine Amniotic Fluid on Acute Corneal Alkali Burns in the Rat

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Abstract: Bovine Amniotic Fluid (BAF) was topically applied for treatment group, whereas isotonic saline solution was applied for saline group in the study after an alkali burn was created in the right eye of 16 rats with 2.5 N NaOH. Density and area of the corneal opacity, area of the corneal epithelial defect, corneal neovascularization and complications were clinically evaluated and scored using a modified model at post-injury days 0, 3, 7 and 14. At the end of the experiment, all the eyes were histologically examined after enucleation. It was resultantly found that BAF has slightly decreased corneal opacification, increased reepithelization and limited neovascularization, hyphema and total corneal erosions. But these clinical ocular damage scores were not statistically significant. In histological examination, the number of polymorphonuclear leucocytes, corneal thicknesses, edema, keratinization, vascularization and inflammation were significantly reduced by BAF compared to the saline group. Based on these positive effects in severe burns, it was concluded that BAF could be a new topical alternative agent for veterinary ophthalmology thereafter.

Key words: Alkali burn, BAF, bovine amniotic fluid, cornea, rat

INTRODUCTION

The corneal alkali injury is characterized by the massive infiltration of PMN (*Polymorphonuclear leucocytes*) into the stroma and the severe destruction of corneal collagens (Sotozono *et al*., 1999). Alkali burns may also produce denaturation of the anterior layers of the cornea. Further, stromal injuries may induce fibroblastic activity and produce disorganized collagen that can lead to the formation of corneal scarring and neovas-cularization (Gao *et al*., 1994; Karacal *et al*., 2005a; Herretes *et al*., 2006).

A number of therapies have been reported for alkali injuries; including ascorbate (Pfister *et al*., 1982), citrate (Pfister *et al*., 1991), fibronectin (Phan *et al*., 1991), sodium hyaluronan (Chung *et al*., 1996, 1999), metalloproteinase inhibitor (Sotozono *et al*., 1999; Kato *et al*., 2006), Epidermal Growth Factor (EGF) (Gonul *et al*., 1995; Kim *et al*., 2001), amniotic membranes (Kim and Tseng, 1995; Pan *et al*., 2003) and human amniotic fluid (Lee and Kim, 1996; Ozgenel and Filiz, 2003; Ozgenel *et al*., 2004; Karacal *et al*., 2005b; Pan *et al*., 2006).

Human Amniotic Fluid (HAF), which is in contact with the ocular surface during embryonic development modulates wound healing in the fetus by increasing both endogenous and exogenous Hyaluronic Acid (HA) in the application region (Longaker *et al*., 1990). Because HA reduces scar formation during wound healing by inhibiting lymphocyte migration, proliferation and chemotaxis, granulocyte phagocytosis and degranulation and macrophage motility, Human Amniotic Fluid (HAF) may prevent periimplant fibrosis or capsule formation (Gao *et al*., 1994; Karacal *et al*., 2005a). Furthermore, Hyaluronic Acid Stimulating Activator (HASA), which is present at high concentrations, as well as HA, in HAF stimulates the wound to increase the production of endogenous hyaluronic acid (Karacal *et al*., 2005a). In addition, the rich content of trophic and growth factors in HAF may enhance the healing process (Karacal *et al*., 2005a). HAF has been shown to contain certain antiinflammatory cytokines (Interleukins such as IL-1α and 1β, IL-6 and IL-10) and HAF has also special properties that minimize the contraction of the wound and inhibit various processes that ultimately cause scarring (Dudley *et al*., 1997; Fukuda *et al*., 2002; Herretes *et al*., 2006).

The major objectives of this study were to evaluate the efficacy of topical Bovine Amniotic Fluid (BAF) and to investigate if BAF might be used as a new alternative agent to HAF and other topics in future studies especially for veterinary ophthalmology.

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MATERIALS AND METHODS

Animals: Sixteen adult female rats aged between 7 and 8 months were used in this study. All rats were free from ocular diseases and were fed with a standard caloric diet for their ages. The animals were monitored before procedures and until fully recovered.

BAF: Bovine Amniotic Fluid was obtained under aseptic conditions from a healthy pregnant cow brought to a slaughterhouse postmortem at 7.5 month of gestational age. The fluid was centrifuged at 2000 rpm for 10 min. The supernatant was preserved at -20°C approximately 2-3 days. The samples were kept frozen until immediately before application and then stored at 4°C during the study in an effort to minimize potential bacterial proliferation in the samples.

Corneal alkali burns: An alkali burn was created in the right eye of each rat with 2.5 N NaOH (Sodium hydroxide). Rats were anesthetized with 4 mg kg^{-1} xylazine (®Rompun, Bayer) and 50 mg kg^{-1} ketamine (®Alfamine, Alfasan) intramuscularly. After stabilization of the right eye globe, the alkali agent was applied on the ocular surface for 40 sec using a 4 mm in diameter filter paper ring immersed with 2.5 N NaOH. Following the standardized exposure to the alkali agent, the eyes were washed thoroughly with 10 mL of isotonic saline solution (0.9% NaCl) to remove the residual chemical agent and protein coagulum.

Experimental design: The right eyes of the animals were assigned to 2 groups as active control (saline, n = 8) and treatment (BAF, n = 8) groups. All left eyes (n = 16) of the same animals were used as passive control. Approximately 10 μL of BAF was topically applied 4 times a day (40 μL day^{-1}) during the 2 weeks for BAF group, whereas, same amount of isotonic saline solution was applied for saline group. In addition, topical lomefloxacine (®Okacin, Novartis) eye drops were also administered 3 times a day for a week to these both groups. At the injury day and post injury days 3, 7 and 14; the eyes were photographed with a machine (Olympus DP12, Japan) attached to a stereomicroscope (Olympus SZ2-ILST, Japan) at magnification making the eye clearly seen (approximately 10 times) after each eye was centered in the screen.

Clinical assessment of the ocular burn: Closure of the epithelial defect was monitored at the injury day and post injury days 3, 7 and 14 by the instillation of sodium fluorescein in the affected eye. Excess fluorescein was rinsed away with 1 mL of isotonic saline solution and then photographs were taken. Density and area of the corneal opacity, area of the corneal epithelial defect, neovascularization and presence of any complications, such as, hyphema, corneal erosion and collapse of ocular surfaces were evaluated and scored as described in Table 1 using a model modified from that Herretes *et al.* (2006) had applied.

Table 1: Clinical assessment and scores of ocular damage for treatment (BAF) and active positive (saline) groups using a modified model (Herretes *et al.*, 2006)

Score	Assessment
Area of the epithelial defect	
0	Non: No defect or no green area
1	Mild: Changes <1/3 of burning day
2	Moderate: Green area >1/3 and <2/3 of burning day
3	Severe: Not changed or >2/3 of burning day
Neovascularization	
0	No neovascularization and hyphema
1	Only dilate episcleral vasodilatation or hyperemia
2	Peripheral (1/3 of the cornea) corneal neovascularization
3	Paraxial (peripheral 2/3 of the cornea), not central, corneal neovascularization
4	Axial (peripheral + central) corneal neovascularization
Complications	
0	No any complications
1	Partial hyphema involving up to one half of corneal surface
2	Red eye, close to total hyphema
3	Total corneal erosion
4	Perforative corneal collapse

Histological evaluation: The eyes of all animals were enucleated bilaterally at the end of the treatment for histopathological evaluation, corneal thickness measurements and morphometric analyses. All the eyes were fixed in 10% neutral buffered formalin solution for 24 h and prepared for paraffin embedding. Sections (6 μ) of corneas were stained with heamtoxylin and eosin (H and E) and evaluated by light microscopy (Olympus B×50, Japan) for cell deaths, as well as for the structure of the basement membrane, epithelium and stroma. Corneal thicknesses and measurements were determined to evaluate directly from paraffin sections using an ocular micrometer. The distance between the epithelial surface and inner endothelial layer of the cornea was measured. For each cornea 2 peripheral (left and right) and 2 central regions were measured. For each region, 2 measurements were made. Thus, 8 measurements were obtained for each cornea. To quantify histologically, the infiltrating polymorphonuclear cells were counted by an observer unaware of the passive control, saline and treatment groups. The number of inflammatory cells of the cornea represented the cells counted in the stromal layers. The left eyes of both groups were used as passive control histologically.

Statistical analysis: For statistical analysis of the present study, SPSS for windows version 13 was used.

The average burn score among the three readers was used in all analyses. The ocular damage scores were evaluated using nonparametric chi-square (χ^2) test. Histologic results were analyzed statistically using the Kruskal-Wallis one-way analysis of variance and intergroup comparisons were made by Mann-Whitney U-test. All results were expressed as mean±SD. Differences were considered significant when $p<0.05$.

RESULTS

Every rat was followed up during 14 days after alkali burns. The clinical reports were done at the day 0 and 3, 7 and 14th days after the injury. Assessment categories including corneal opacification, reepithelization, neovascularization and complications were evaluated and scored by the investigators independently using a modified and semiquantitative method as described in Table 1. In general consideration, BAF was found as superior to saline, however, there were not any significant differences between BAF and saline groups, except for the neovascularization at 7th day ($p<0.05$), statistically (Table 2).

Clinical evaluation

Corneal opacification: Density of the corneal opacity did not change throughout the whole follow up period in both groups. Therefore, seeing of the iris and pupil in all burned eyes could not be achieved and they were obscured and hazy throughout the study. Central area of the corneal opacity did not also change during the study. But, the borderline of corneal opacification has slightly lost at 7th day and this situation has continued at 14th day after the injury in both groups. As extensive neovascularization and hyphema camouflaged the corneal opacity, corneal density could not be clinically evaluated sufficiently for scoral assessment of corneal opacification (Fig. 1 and 2).

Corneal reepithelization: The epithelial damage was almost full of the cornea immediately after creation of alkali burns (Fig. 1e). This defect was nearly approximated down one-half at 3rd days, ⅓ at 7th days and <⅓ of the corneal surface to nearly complete epithelial healing at 14th days of examinations in both positive control (saline) and treatment (BAF) groups. But reepithelization was more prominent in BAF group (Fig. 1). However, epithelial defects have contrastly been deteriorated and have increased in subsequent examinations of resultant complicated animals with hyphema and total corneal erosions (Fig. 2).

Table 2: Corneal damage scores in treatment (BAF) and active control (saline) groups

Groups	3rd day	7th day	14th day
Area of the epithelial defect			
BAF	1.87±0.64	2.37±0.51	1.87±0.99
Saline	1.75±0.46	2.12±0.64	1.87±0.83
Neovascularization			
BAF	1.14±0.37	1.50±0.83*	2.50±0.54
Saline	1.37±0.51	1.85±0.37	2.83±0.40
Complications			
BAF	0.50±1.41	1.12±1.64	1.25±1.58
Saline	0.00±0.00	0.75±1.38	1.50±1.51

Data were presented as mean± SD; *$p<0.05$ versus saline; *There were not any significant differences between BAF and saline groups, except for the neovascularization at seventh day ($p<0.05$)

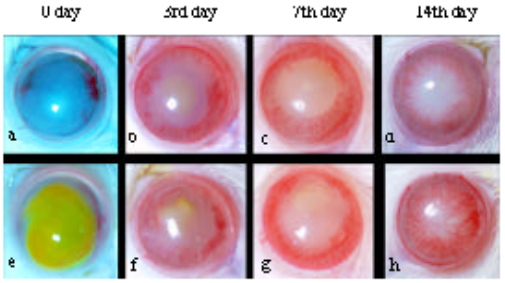

Fig. 1: A representation of the corneal damage for BAF (a-d) and saline (e-h) groups of all the assessment days. It was appeared that extent of neovascularization and reepithelization completion were more favourable in BAF group than in the other, but iris and pupil were obscured in all photographs of both groups because of corneal opacification

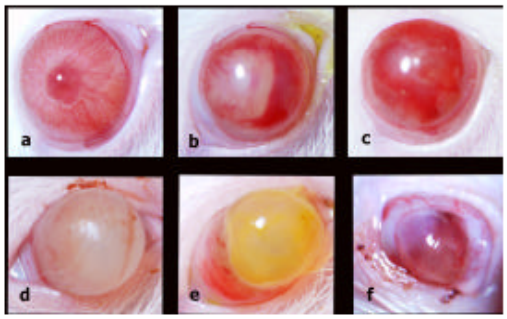

Fig. 2: Viewing of mild to highly severe complications, including various types of hyphema (a-c), total corneal erosions with and without flourescein staining (d-e) and ocular collapse (f) encountered in both groups

Corneal neovascularization: Neovascularization did not improve on the day after burning except for the mild episcleral-limbal neovascularization and hyperemia. But other assessment days, it has increasingly and gradually persisted until the end of the experiment in both groups

(Fig. 1). At 3rd day examination, neovascularization has occupied the cornea adjacent the limbus and usually extended from the limbus toward the peripheral one-third to one-half of corneas circumferentially. In addition, there were opacific areas between these uncountable vessels. Further, it has increased, densened and has also continued to extend toward the corneal center (approximately ⅔ of the cornea) at 7th day. Results of this examination have only been found significant ($p<0.05$) between BAF and saline groups (Table 2). Then these vessels have thickened, darkened and extended to the center almost completely at the last examination. However, this situation was less prominent in BAF than the other group (Fig. 1). In spite of above findings, neovascularization remained limited with the limbal cornea at former assessments has resulted in either total erosions or collapses at latter examinations.

Corneal complications: Of 10 complications, 4 in BAF and 6 in saline groups were seen, namely, hyphema, total corneal erosion and perforative collapse of the cornea (Fig. 2). These disorders have generally performed during latter part of the treatment, which one (in BAF) is at 3rd day, 4 (2 in each group) are at 7th day and 5 (1 in BAF and 4 in saline groups) are at 14th day; including, 2 partial hyphema (1 in each group), 3 total hyphema (1 in BAF and 2 in saline), 3 corneal erosion (1 in BAF and 2 in saline) and 2 corneal collapse (1 in each group). Briefly, it has been stated that partial to total hyphema (red eye) and corneal erosions in saline group were more prominent than the other and BAF has slightly limited hyphema severity and the erosion.

Histological evaluation: The parameters such as corneal thickneses and polymorphonuclear leucocytes and other histologic findings were observed on microscopy (Table 3).

Passive control group: No differences in the histology of the corneal stroma and endothelial layers were observed in rats of passive control group. These control corneas showed 4-5 layers of cells with normal orientation and appearances of basal, wing and squamous cells. Keratocytes populated the stroma lying between tightly packed collagen bundles. There were no infiltrating cells in this group. Histological examination showed no indication of keratinization. There also had no blood vessels and edema in the stroma (Fig. 3a).

Active control (saline) group: The epithelium showed slight enlargement with the irregularity of basal cells. The basal cells had lost their columnar appearances. The stroma was edematous with enlarged pericellular spaces. Some endothelial cells were swollen. In this group, many inflammatory cells had infiltrated the anterior stroma ($15.25\pm4.33/1\ mm^3$) and were marginating the corneal endothelium in the anterior chamber. The corneas of rats with saline showed severe keratinization and many abnormal vessels were also seen in the anterior stroma (Fig. 3b and c).

Treatment (BAF) group: Histologic sections of the cornea from rats that received BAF showed differences compared to corneas of rats received saline solution. The infiltration of PMN and edema in the stroma were showed

Table 3: Measurements of corneal thicknesses and numbers of PMN in eyes of passive control, active control (saline) and treatment (BAF) groups

Groups	Corneal thicknesses	No. PMN/1 mm³
Passive control	151.97±1.06	0.00±0.00
Active control (saline)	232.72±7.47$	15.25±4.33$
Treatment (BAF)	178.23±11.07+$	3.25±1.28+$

Data are presented as mean±SD; +p<0.001 versus saline, $: p<0.001 versus passive control

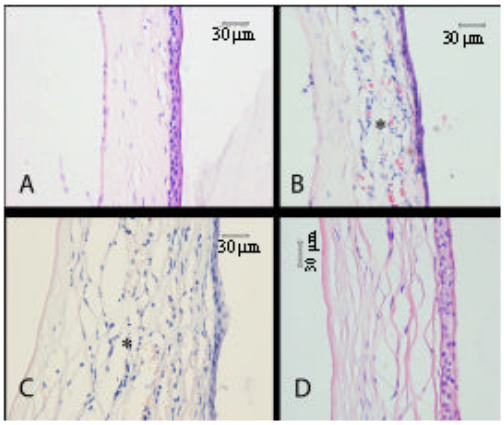

Fig. 3: The normal appearance of the cornea possessed regular corneal epithelial layers and stromal fibers in the passive control eye of rats. The normal corneas had the columnar features of basal epithelial cells and there was no keratinization (a). The corneas of saline group showed severe keratinization. Some abnormal vessels were seen in the anterior stroma and the basal cells had lost their columnar appearances. Many inflammatory cells had infiltrated the stroma (asterisk) (b and c). The corneas of BAF treated rats looked almost normal and had the columnar features of basal epithelial cells. Epithelium layers showed thinner keratinization (early stages) than saline group. Photomicrographs of histologic sections were stained with Haemtoxylin and Eosin (d)

to be significantly reduced in the group treated by BAF. The blood vessel in the stroma was decreased in BAF group compared to saline group. Additionally, histological examination showed no indication of extreme keratinization, but the early stages of keratinization (desquamation and cellular enucleation) were evident. The mean number of infiltrating cells in the cornea of rats treated by BAF was 3.25±1.28 cells section^{-1}, whereas, it was 15.25±4.33 cells section^{-1} in saline group (Fig. 3d). The cell infiltration was significantly reduced by BAF treatment compared to the other ($p<0.001$).

Corneal thicknesses: Mean corneal thickness of passive control group was 151.97±97 μ. In the group that received BAF treatment, the mean corneal thickness was significantly decreased (178.23±11.07 μ) compared to saline (232.72±7.47 μ) group ($p<0.001$). Thus, the treated group with BAF had effects on the corneal thickness compared to rats that received only saline solution (Table 3).

DISCUSSION

Of various therapeutics (Pfister *et al*., 1982, 1991; Phan *et al*., 1991; Gonul *et al*., 1995; Chung *et al*., 1996, 1999; Sotozono *et al*., 1999; Kim *et al*., 2001; Herretes *et al*., 2006; Kato *et al*., 2006) used for corneal wound healing, HAF has been tried in different areas and tissues; including hand surgery (Al-Qattan *et al*., 1995), tendons (Ozgenel *et al*., 2001), nerves (Lee and Kim, 1996; Ozgenel and Filiz, 2003; Pan *et al*., 2006), fetal wounds (Ledbetter *et al*., 1991; Gao *et al*., 1994), bones (Karacal *et al*., 2005b) and cartilages (Ozgenel, 2002; Ozgenel *et al*., 2004). In several of these studies, HAF was proposed to minimize fibrosis associated with hand surgery (Al-Qattan *et al*., 1995), to reduce scar formation when applied intraluminally (Karacal *et al*., 2005a) and to appear to be a useful adjunct in the treatment of bone healing because of its ability to be stored in deep freeze if made cell-free (Karacal *et al*., 2005b). Ozgenel (2002) presented in her studies that human amniotic fluid enhanced neochondrogenesis from free perichondrial grafts (Ozgenel *et al*., 2004) and topical application of HAF immediately after tenorrhaphy was significantly effective in preventing peritendinous adhesion formation without impairment of tendon healing in a rabbit model (Ozgenel *et al*., 2001). HAF and amniotic fluid mesenchymal stem cells were also proposed to enhance peripheral nerve regeneration (Ozgenel and Filiz, 2003) and to augment growth of injured sciatic nerve across a nerve gap (Pan *et al*., 2006).

In ophthalmology, amniotic fluid has started to receive great attention in recent years and it has been used in the eye (Lee and Kim, 1996; Herretes *et al*., 2006). It was observed in these reports that HAF promoted faster corneal nerve regeneration and recovery of corneal sensitivity (Lee and Kim, 1996) and it was effective in the reduction of corneal opacity, scarring and promotion of reepithelization following the ocular chemical burn (Herretes *et al*., 2006). Furthermore, the concentrations of HA, HASA and other factors in HAF is well known and studied (Arvidson *et al*., 1972; Das *et al*., 1975; Longaker *et al*., 1990; Gao *et al*., 1994; Zhang *et al*., 2001), but studies on BAF are too insufficient and need further investigations. On the basis of all these observations, we used bovine amniotic fluid for the 1st time to treat ocular alkali burns in a murine model in this study.

Corneal neovascularization characterized by corneal ingrowth of new vessels originating from the limbus is a major sight-threatening complication of corneal infections, chemical injury and keratoplasty (Kwon and Kim, 2006). Mild to moderate corneal alkali burns do not induce consistent neovascularization (Herretes *et al*., 2006). But, the burn in this study was highly dense, deep and severe and it has considerably created epithelial damages, opacities, neovascularization and hyphema, which are the major factors of this study, corneal erosions, collapses and keratinization. So, the eyes of animals treated with HAF in the previous study (Herretes *et al*., 2006) have mild haze and also iris detail and pupil were visible, whereas, the eyes in this study have reasonably severe affected corneas and also iris and pupil obscured in both groups. But, neovascularization, partial and total hyphema were less prominent in BAF group than the other which is more effective, thereby observing limitation of neovascularization and hyphema production following the BAF treatment.

The present study indicated that BAF treatment might ameliorate the infiltration of PMN and edema in the stroma, the mean number of infiltrating cells in the cornea and the mean corneal thickness. It appears from these histologic findings that the number of PMN, corneal thicknesses, edema, keratinization, vascularization, hyphema and inflammation are reduced by BAF after alkali burns compared to saline application and these results were in good accordance with the previous report (Herretes *et al*., 2006) used an alkali injuried mice model.

With these encouraging results, studies must be ongoing to determine the concentration, acquisition, preservation and biologic activity of the amniotic fluid. While maintaining therapeutic approaches, well-studied protocols for amniotic fluid sterilization, acquisition, preservation and transmission to avoid fluid-borne

diseases should be standardized in the next step. Overall, these further investigations should be performed for various animal amniotic fluids as alternative agents to mimic for several keratopathies more important for companion animals with these inflammatory lesions typically seen in veterinary ophthalmology.

CONCLUSION

Clinical and histological observations have certainly shown that topical instillation of BAF has restrictive effects on neovascularization, hyphema production and corneal erosions in severe alkali burns and it has reduced corneal edema, keratinization and inflammation. Hence, it has been also stated that BAF may be a new topical alternative agent for veterinary ophthalmology thereafter.

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