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Research Article

Comparison of the Efficacy of Amniotic Membrane Transplantation, Topical Water-Based Propolis Extract, Corticosteroid and Antibiotic Use in Different Combinations on Subacute Corneal Alkali Burns in Rabbits [1][2]

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Abstract

Investigation of the superiority of the usage of propolis extract, amniotic membrane transplantation, corticosteroid and antibiotic both separately and in different combinations was aimed. A total of 40 (20 male, 20 female) New Zealand breed rabbits weighing 2.5-3.0 kg were used in the study. The rabbits were divided into 5 groups on day 7 of alkali burn injury. Amniotic membrane transplantation was applied to 3 groups and each one of these 3 groups had different local medications as 1% water-based propolis extract (AMN+PRP), dexamethasone and tobramycin (AMN+AC) and only tobramycin (AMN+A). The other two groups, which were not performed amniotic membran transplantation, were either treated with propolis (PRP) or left as a control. The defect area results obtained from AMN+PRP group were lower than AMN+A group, and both AMN+PRP and control group at the day 9 and day 14, respectively (P≤0.05). In the measurements made after the second week, AMN+PRP group showed significantly better results in all weeks in terms of defect area (P≤0.05). Corneal thickness was found to be lower in all groups treated with amniotic membrane than in the other groups (P<0.05). This study aimed to find treatment effectiveness of combining the amniotic membrane with propolis, especially as an anti-inflammatory agent comparing to corticosteroids, and it was decided the outcomes can be improved on the further studies..

Keywords: *Rabbit, Cornea, Chemical corneal burn, Amniotic membrane, Propolis*

Tavşanlarda Korneanın Subakut Alkali Yanıklarında Amniyotik Membran Transplantasyonu, Topikal Su Bazlı Propolis Ekstraktı, Kortikosteriod ve Antibiyotiğin Farklı Kombinasyonlarda Kullanımının Etkinliğinin Karşılaştırılması

Öz

Propolis ekstraktı, amniotik membran transplantasyonu, kortikosteroid ve antibiyotiğin ayrı ayrı ve farklı kombinasyonlarda kullanımının üstünlüğünün araştırılması amaçlandı. Çalışmada, 2.5-3.0 kg ağırlıkta toplam 40 Yeni Zelanda ırkı tavşan kullanıldı. Tavşanlar alkali yanık oluşturulmasının 7. gününde 5 gruba ayrıldı. Üç gruba amniyotik membran tranplantasyonu yapıldı ve bu gruplardan birine %1 su bazlı propolis ekstraktı (AMN+PRP), birine deksametazon ile tobramisin (AMN+AK), diğerine de sadece tobramisin (AMN+A) lokal olarak uygulandı. Diğer iki gruba amniyotik memran transplantasyonu yapılmadı, propolis ile tedavi edildi (PRP) veya kontrol olarak bırakıldı. AMN+PRP grubundan elde edilen defekt alanı sonuçları 9. günde AMN+A grubundan 14. günde hem AMN+A hem de kontrol grubundan daha düşüktü (P≤0.05). İkinci haftadan sonraki ölçümlerde AMN+PRP grubu defekt alanı bakımından tüm haftalarda daha iyi sonuç verdi (P≤0.05). Korneal kalınlık sonuçları ise amniotik membran ile tedavi gören tüm gruplarda düşüktü (P<0.05). Bu çalışmada, amniotik membranın propolisle kombine edilmesinin, özellikle kortikosteroidlerle karşılaştırıldığında antienflamatuar bir ajan olarak, tedavi etkinliğinin belirlenmesi amaçlandı ve sonuçların ileriki çalışmalarda geliştirebileceği kanısına varıldı.

Anahtar sözcükler: *Tavşan, Kornea, Kimyasal korneal yanık, Amniotik membran, Propolis*

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INTRODUCTION

Cornea is a transparent and avascular barrier between the eye and environment that allows the light coming into inside. However, corneal respond to the pathologies usually comes out as corneal edema, opacity and neovascularization; and adversely affects vision quality. Protecting the transparency of the cornea following pathologies is one of the most important goals of corneal treatment [1]. Many studies have been done, and many methods have been tried for many years to keep the vision clarity. These methods include medical treatment options for superficial defects and surgical methods such as corneal and conjunctival graft, third eyelid flap application, tissue adhesives, contact lenses, corneal-scleral transposition, autogenous/homologous corneal graft, use of synthetic stitch materials, trans-plantation of amniotic membrane or other organic materials for deeper defects [1-4].

Amniotic membrane is the inner layer of placenta that provides hemostasis [5,6]. It has avascular nature including anti-angiogenic factors and inhibiting proteinase activity, and so it reduces neovascularization and fibrosis, and induces epithelization when it is transplanted to the cornea [2,5,7-10].

Propolis has been used in various parts of the world for treatments because of its antibacterial, antifungal, antiviral, anti-inflammatory, antitumoral, immunostimulatory and regenerative effects on tissues in recent years [11-16]. Local application of the propolis extract has been proposed as an effective treatment options for corneal neovascularization because it suppress the angiogenesis [17].

Using the amniotic membrane for repairing corneal defects has long been known. However, its effectiveness is still controversial. This study aimed to compare the clinical efficiency of amniotic membrane combined with topical propolis extract, topical antibiotic and corticosteroid. Also, investigation of anti-inflammatory effect of the propolis was the priority for the study to find an alternative to corticosteroid usage, because of threatening complications.

MATERIAL and METHODS

Ethical Approval

The study design was approved by Adnan Menderes University Animal Experiments Local Ethics Committee (HADYEK) on 9.10.2014 and numbered 64583101/2014/172.

Animal Studied

A total of 40 (20 male, 20 female) New Zealand breed rabbits weighing 2.5-3.0 kg were used in the study. During the test period, animals were housed in transparent polycarbonate cages with a temperature of 22°C, 50-70% humidity and 12/12 h of light/dark conditions and feeded *ad libitum*. Before the trial, 15 days of acclimatization and standard clinical and ophthalmological examinations executed.

Experimental Design

All rabbits were anesthetized with 2.5 mg/kg xylazine HCl and 20 mg/kg ketamine HCl, and topical anesthesia of the eyeball was achieved by using 0.5% proparacaine HCl. The 6 mm wide filter paper which was soaked in 1 N NaOH, was held on the cornea of the right eye for 1 min to form the alkali burn and immediately after the eye was washed with a sterile eye wash solution. Before the trial groups assignation, 7 days was waited for the burn lesion become subacute form. Waiting period to create subacute lesion was decided based on the references [18-20].

To prevent possible effect of gender, 4 males 4 females rabbit randomly assigned to the groups as following *Table 1*;

Amniotic Membrane Preparation and Transplantation

A placenta from healthy dog cleaned flushed with saline solution containing 50 µg penicillin (1000 IU)/mL, 20 µg/mL streptomycin, 100 µg/mL neomycin, and 2.5 mg/mL amphotericin B. Amniotic membrane was then separated and placed on nitrocellulose paper of 5x5 cm pieces with epithelial surface up, and stored in 99% glycerin at room temperature (as described before [3]). Fifteen days after the amniotic membrane obtained, it was transplanted to the rabbits.

Clinical Ophthalmological Examinations

Reflex tests (pupillary, threat, dazzle and palpebral were scored as positive, weak and negative), corneal defect area measurement (using Photoshop software following calibration of the pictures), corneal thickness measurement (POCKET II Pachymeter, Quantel Medical, USA), corneal opacity scoring (scored between 0-3 as indicated by Lee et al.[18]), corneal neovascularization scoring, conjunctival examination, fluorescein staining and Shirmer I tear test were performed on postoperative day 1, 3, 5, 7, 9 and 14 days and then once a week until the end of 12th week.

Histopathological Examination

Rabbits were euthanized with high dose isoflurane on the 14th days of post-alkali burn (two animals from each group)

Table 1. *Study groups*

Group	Procedure
Control	Saline
AMN+A	Amniotic membrane transplantation + Topical* antibiotic[1]
AMN+AC	Amniotic membrane transplantation + Topical* antibiotic[1] + Topical* corticosteroid[2]
AMN+PRP	Amniotic membrane transplantation + Topical* water-based propolis extract, 1%
PRP	Topical* water-based propolis extract, 1%[3]

** All topical applications were administered 2 drops 3 times a day until the end of the trial; [1] Tobrased® Eye Drop (tobramycin 0.3%, Bilim Drug Company); [2] Dekort® Eye Drop (dexamethasone 0.5%, Deva Drug Company); [3] Eğriçayır® Water-Based Propolis 33% (Diluted to 1% when using)*

and at the end of experiment (six animals from each group). For histopathological examination, the eyeballs were extirpated and fixed in Bouin's solution for 24-48 h. After fixation, the tissues were embedded in paraffin, sectioned in 4 µm thickness and stained with hematoxylin-eosin (H-E). The tissue sections were examined under light microscopy.

Statistical Analysis

Statistical analysis of the data was performed using the SPSS 22 statistical package program (Inc., Chicago, II, USA). Statistical analysis of STT, defect area, fluorescein and corneal thickness determined by measurement; In order the dimensional differences of the corneal defects would not affect the results, all the data were initially accepted as 100 and the proportional recovery level was determined. The normal distribution of these parameters was investigated by Kolmogorov-Smirnov test and the differences between the group means of these parameters were compared using the one-way ANOVA test. The significance control of the differences between the groups was done by Duncan multiple comparison test. The differences between the mean scores of the parameters determined by scoring (conjunctival examination, corneal opacity, neovascularization) were compared using the Kruskal-Wallis test. Bilateral comparisons were made with Mann-Whitney U test. Reflexes were compared between groups by using Chi-square test.

RESULTS

Since the defects covered a small portion of the cornea and was not sized to precisely prevent vision, reflexes were not disappeared in any rabbit during the trial, and no difference was found associated with recovery or treatment. Also, there was no statistically significant difference in terms of opacity and neovascularization scores.

Fluorescein staining could not be obtained properly due to the placed membrane in groups where amniotic membrane was placed. No statistical difference was found in the analysis of the data obtained from the control group and the PRP group in any stages of the study. Therefore, visible size of corneal defect without staining were evaluated.

For the parameters determined by the measurement, since the other results obtained from the measurements on the intermediate days were not very different, the statistical results of the first two weeks on the 0, 5, 9 and 14 days were mentioned *(Fig. 1)*.

There was no difference between the groups in the

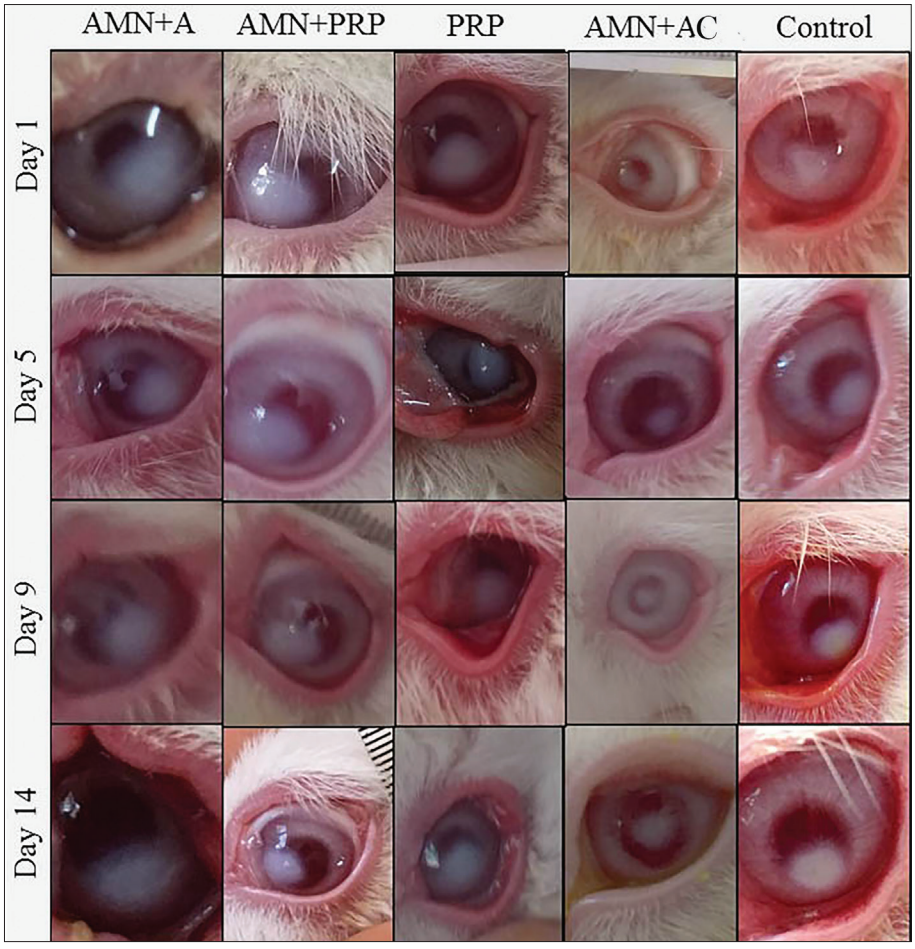

Fig 1. Images of the first 2 weeks of the corneal lesion of rabbits randomly selected from different groups

Table 2. *Shirmer tear test results until the 14th day*

Groups	Day 0		Day 5		Day 9		Day 14	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	100±0	8	160.14±44.22	8	196.27±64.99	8	174.59±46.98
PRP	8	100±0	8	123.26±16.99	8	152.84±19.64	8	118.44±14.04
AMN+PRP	7	100±0	7	106.10±7.13	7	105.20±26.28	7	85.46±12.49
AMN+A	7	100±0	7	104.13±10.48	7	98.49±11.86	7	91.39±7.94
AMN+AC	7	100±0	7	109.03±11.8	7	84.82±16.98	7	98.02±23.43
P	-		-		-		-	

** Differences between the mean indicated by different letters in the same column are significant (P<0.05)*

Table 3. *Defect area measurement results until the 14th day*

Groups	Day 0		Day 5		Day 9		Day 14	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	100±0	8	93.21±2.28	8	89.17±2.45^{ab}	8	87.65±2.93^{a}
PRP	8	100±0	8	95.49±0.97	8	88.82±2.31^{ab}	8	84.08±2.96^{ab}
AMN+PRP	7	100±0	7	91.81±1.60	7	82.06±2.81^{b}	7	77.43±3.20^{b}
AMN+A	7	100±0	7	96.76±1.27	7	92.63±1.62^{a}	7	89.73±1.99^{a}
AMN+AC	7	100±0	7	95.15±1.23	7	87.45±2.01^{ab}	7	82.90±2.89^{ab}
P	*		-		*		*	

** Differences between the mean indicated by different letters in the same column are significant (P≤0.05)*

Table 4. *Corneal thickness measurement results until the 14th day*

Groups	Day 0		Day 5		Day 9		Day 14	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	100±0	8	121.56±5.77^{a}	8	128.02±6.24^{a}	8	124.52±5.89
PRP	8	100±0	8	111.76±7.42^{ab}	8	114.05±10.25^{ab}	8	112.97±10.98
AMN+PRP	7	100±0	7	104.98±2.59^{b}	7	110.80±5.31^{ab}	7	110.27±5.40
AMN+A	7	100±0	7	99.59±1.75^{b}	7	100.19±2.20^{b}	7	99.48±2.14
AMN+AC	7	100±0	7	101.64±1.21^{b}	7	100.47±1.38^{b}	7	99.33±2.09
P	-		*		*		-	

** Differences between the mean indicated by different letters in the same column are significant (P<0.05)*

evaluation of the data obtained from Shirmer tear test until the 14th day *(Table 2)*.

As shown in *Table 3*, the results of AMN + PRP were significantly lower than AMN + A on 9th day, and control and AMN + A group on 14th day (P<0.05).

As shown in *Table 4*, the results of corneal thickness measurement from all of AMN groups at 14th day were found to be significantly better on the 5th and 9th day than the control group (P<0.05).

Histopathologically, at 2nd week post-alkali burn, diffuse corneal edema was observed in all groups. Collagen lamellae were separated into a fine feltwork of pale-staining fibrils. It was noted that the corneal epithelium desquamated and ulcers were formed in the defect area. There was an accumulation of neutrophil leukocytes around the limbal vessels in the control group. In AMN + A, AMN + PRP and PRP groups, there was also seen neovascularization and mild neutrophil leukocyte infiltrations in the superficial 1/3 part of the stroma. In AMN + AC groups, neovascularization accompanied by moderate neutrophil leukocyte infiltrations was observed in the superficial 1/2 part of the stroma.

Reflex examinations were scored normal in almost all rabbits and no difference was found between the treatment groups. In terms of opacity and neovascularization, opacity scores showed statistically significant difference. AMN + PRP were significantly better than the other groups after 8 weeks. In the twelfth week, the results obtained from the

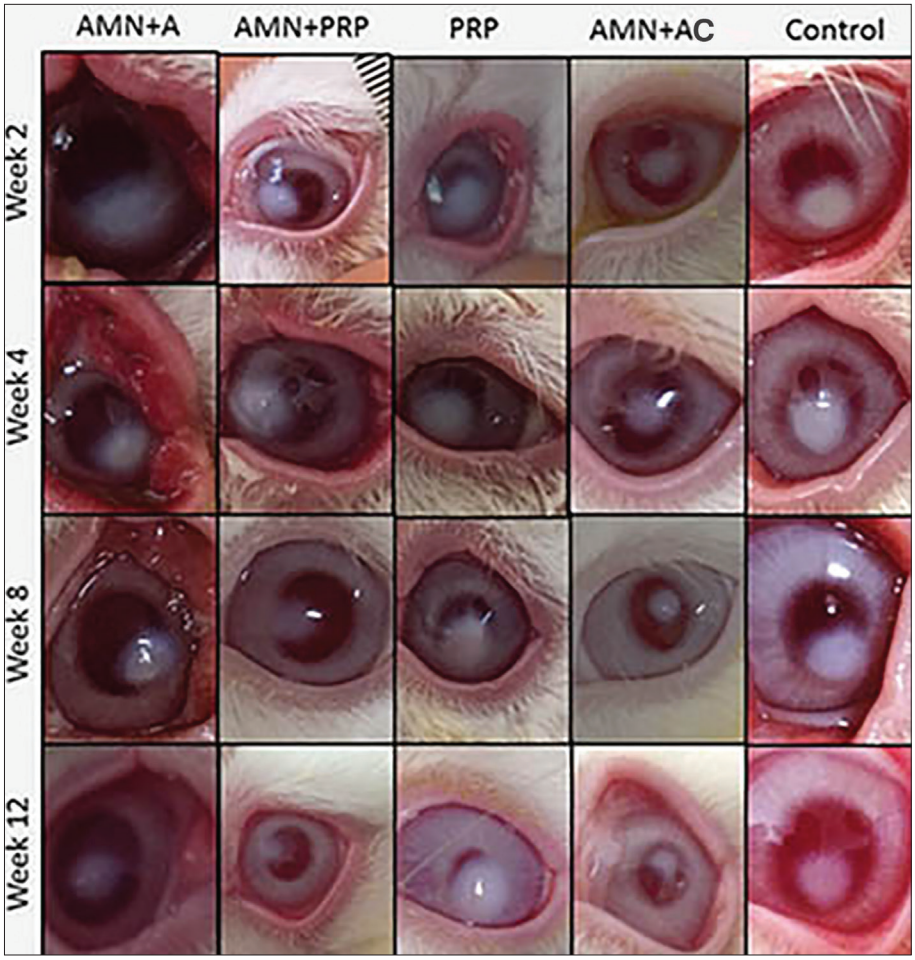

Fig 2. Images of between Week 2 and 12 of the corneal lesion of rabbits randomly selected from different groups

Table 5. *Shirmer tear test results between week 2 and 12*

Groups	Week 2		Week 4		Week 8		Week 12	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	174.59±46.98	6	171.79±37.09[a]	6	122.32±46.13	6	105.83±48.92
PRP	8	118.44±14.04	6	89.80±13.92[b]	6	82.88±12.22	6	52.98±6.69
AMN+PRP	7	85.46±12.49	5	75.76±12.12[b]	5	85.40±23.85	5	60.36±20.53
AMN+A	7	91.39±7.95	5	80.34±6.95[b]	5	65.86±7.95	5	74.38±8.73
AMN+AC	7	98.01±13.43	5	71.42±11.02[b]	5	79.92±12.81	5	82.32±4.18
P	-		*		-		-	

** Differences between the mean indicated by different letters in the same column are significant (P<0.05)*

control and the AMN + AK group were found to be similar to the AMN + PRP *(Fig. 2)*.

As shown in the following table *(Table 5)*, the statistical difference of the data obtained from Schirmer tear test on the 4th week was interpreted as unrelated to treatment (P<0.5).

Defect area results *(Table 6)* was significantly better in AMN + PRP the group than other groups for all weeks (P<0.5).

As shown in corneal thickness measurements *(Table 7)*, higher results were obtained in the PRP group from the 4th week onwards. The results from all of AMN groups was found that was lower than the other groups.

To evaluate the effects of treatments on wound healing, the eye sections were examined histopathologically at 12th-week post-alkali burn. In the control group, corneal ulcer extending into the outer third of the stroma was observed in the defect area. The ulcer area was filled with fibroblast proliferation and fine collagen fibers *(Fig. 3)*. Scattered polymorphonuclear leukocytes infiltration between connective tissue cells was seen. At the base of the ulcer, there were new vessel formation and moderate

Table 6. *Defect area measurement results between week 2 and 12*

Groups	Week 2		Week 4		Week 8		Week 12	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	87.64±2.93^{a}	6	75.48±4.05^{a}	6	63.75±4.42^{bc}	6	55.43±4.62^{bc}
PRP	8	84.08±2.97^{ab}	6	78.68±3.64^{ab}	6	68.20±4.80^{ab}	6	59.84±4.82^{ab}
AMN+PRP	7	77.43±3.20^{b}	5	67.06±4.65^{b}	5	51.20±7.14^{c}	5	43.58±7.53^{c}
AMN+A	7	89.73±1.99^{a}	5	87.72±3.13^{ab}	5	79.89±2.93^{a}	5	72.43±2.84^{a}
AMN+AC	7	82.90±2.89^{ab}	5	76.55±4.03^{ab}	5	62.29±3.19^{bc}	5	53.00±2.45^{bc}
P	*		*		**		**	

** Differences between the mean indicated by different letters in the same column are significant (P≤0.05)*

Table 7. *Corneal thickness measurement results between week 2 and 12*

Groups	Week 2		Week 4		Week 8		Week 12	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Control	8	124.52±5.89	6	121.69±7.51^{ab}	6	102.30±5.17^{ab}	6	91.49±3.99^{ab}
PRP	8	112.97±10.98	6	139.19±13.59^{a}	6	112.19±12.93^{a}	6	101.52±10.99^{a}
AMN+PRP	7	110.27±5.40	5	100.61±5.31^{bc}	5	85.84±5.53^{b}	5	72.00±3.17^{bc}
AMN+A	7	99.48±2.14	5	93.44±3.729^{c}	5	86.75±1.02^{b}	5	76.28±1.71^{bc}
AMN+AC	7	99.33±2.09	5	93.87±3.15^{c}	5	81.08±3.86^{b}	5	70.52±1.34^{c}
P	-		**		*		**	

** Differences between the mean indicated by different letters in the same column are significant (P<0.05)*

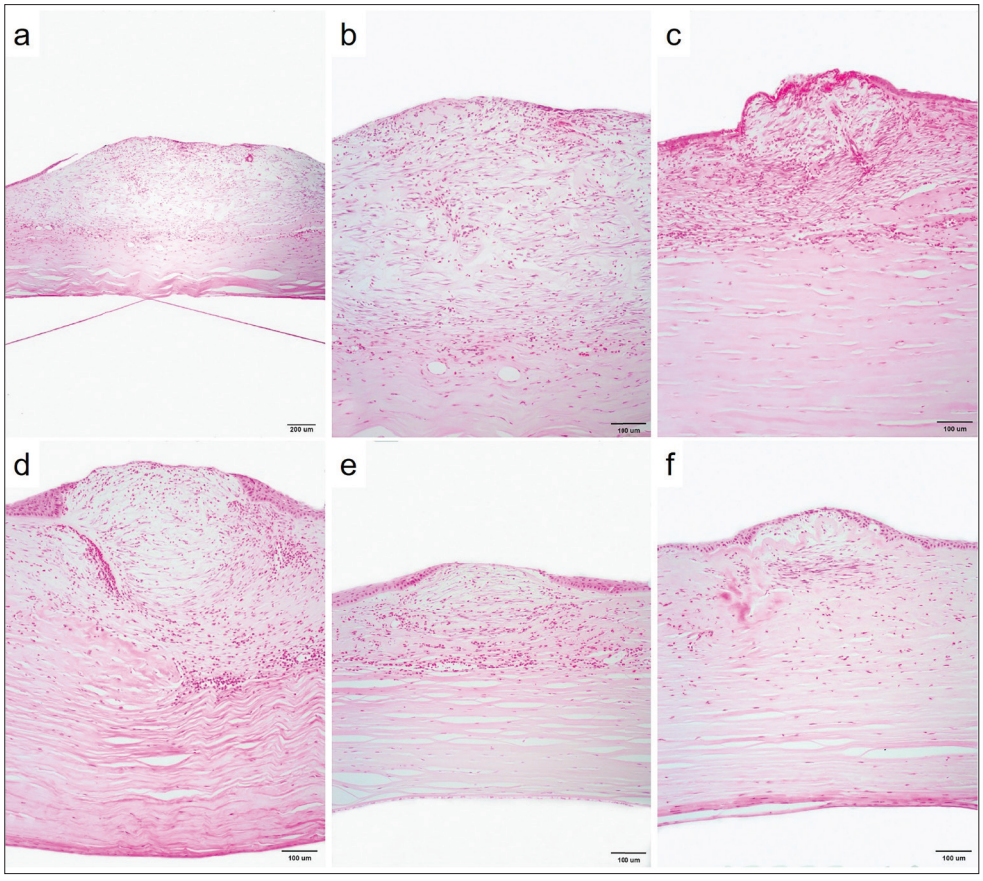

Fig 3. The effects of treatments on alkali burn-induced histopathological changes. Representative photomicrographs of eye sections stained with H-E from each treatment group (12th week post-alkali burn). **A-B.** Control group, **B.** PRP group, **C.** AMN + A group, **D.** AMN + PRP group, **E.** AMN + AC group

polymorphonuclear leukocytes infiltration extending from limbus toward to the defect area.

Treatments with AMN + A, AMN + PRP, AMN + AC or PRP was determined to accelerate the wound healing process. The majority of the defect area was filled with mature granulation tissue. In the defect area, immature connective tissue cell (fibroblast) proliferation and new vessel formations were also seen. The corneal epithelium at the edges of the defect was hyperplastic and the ulcer surface was covered with a single layer of squamous epithelium. There was no difference in the severity of inflammatory changes between AMN + A, AMN + PRP, PRP, and control group. In AMN + AC group, mild polymorphonuclear leukocyte infiltrations were observed in the defect area and the severity of inflammatory changes was lower than that in the control group. It was detected that treatments with AMN + AC and AMN + PRP were diminished the size of the defect area compared to the control group. There was no difference in the size of the defect area between AMN+A, PRP, and the control group.

DISCUSSION

Effectiveness of corneal graft surgery has been widely proven [19-24]. According to literature information, the amniotic membrane promotes epithelialization, reduces inflammation, neovascularization, fibrosis and pain, acts as a substrate for cell growth and biological bandage and, shows antimicrobial effects [5,9-10,25]. Considering all of these, to compare anti-inflammatory and anti-microbial effect of topical propolis treatment following amniotic membrane transplantation with topical antibiotic and corticosteroids usage without any debridement in the subacute alkali burns of the cornea was aimed in this study.

Some clinical data suggests that the amniotic membrane shows anti-angiogenic effects on the cornea [20], and neovascularization [26,27] and an effective treatment option of corneal lesions of the horse [2,28], dog [29], cat [3], rabbit [7] and humans [10,30-32]. In the present study, no statistically significant difference was found between the first two weeks in terms of opacity and neovascularization scores, but after the second week opacity significantly decreased from 8th to 12th weeks in AMN + PRP group. On the 12th week, opacification in the control group and AMN + AC group was similar to the AMN + PRP group. In PRP group and AMN + A group, opacity scoring was high in all weeks compared to the other groups. These findings were assessed as that usage of AMN + A or PRP would not provide efficient contribution to the regression of opacity. And when looked dissenting investigations, some researchers stated that the amniotic membrane transplantation is ineffective, even exacerbating, in reducing inflammation, opacity and neovascularization especially in the early phases of the corneal alkali burn lesion [33]. Our results might be interpreted as that combining the amniotic membrane with propolis might accelerate the reducing of the opacity but might not affect the final outcome.

Because of the controversial usage of the amniotic membrane [32,34-36], most researchers have investigated an adjuvant therapy which can be used with it. Corticosteroids are powerful tools to prevent scarring and to preserve transparency in some forms of keratitis, uveitis, conjunctivitis, scleritis/episcleritis [37]. There are many studies using topical or systemic steroids after amniotic membrane transplantation [30,31,38,39]. However, there are some researchers reporting that corneal ulcers could be treated only with adjuvant systemic immunosuppressive therapy [30].

Propolis is a substance that has been suggested which has regenerative, antibacterial, antifungal, antiviral, anti-inflammatory, antitumoral, immunostimulatory effects on tissues [11-16]. In the present study, almost no signs of severe inflammation and chemosis were observed in the eye and surrounding tissues. The results of Shirmer tear test obtained from the control group at the 4th week were found to be significantly higher however; this difference was not found determinative in terms of healing process. Also, since the defect area did not have enough space to block the view, the reflexes were not completely lost in any rabbit during the study. In addition, the results of the reflex examinations of all groups were almost identical. In the initial measurements, the reflexes scored as week improved in a short time by regressing of schemosis; and were not associated with anti-inflammatory treatment or healing.

Some researchers have suggested that propolis extract would be effective when applied locally for the treatment of corneal neovascularization due to its angiogenesis suppressive effect [17]. Hepşen et al.[40] confirmed this hypothesis and reported that topical application of water-based extract of propolis in the rabbits with corneal defect of the-silver nitrate had an inhibitory effect on corneal neovascularization. Also some researchers stated that in rabbits with chemical corneal injuries, topical application of propolis [14] or, caffeic acid phenethyl ester which is the active ingredient of propolis [41], has equal effect with topical dexamethasone usage in terms of suppressing corneal neovascularization.

In this study, the results of defect area from AMN + PRP group were significantly lower than AMN + A on 9th day, and control and AMN + A group on 14th day. In the following weeks, the AMN PRP group showed significantly better results. The control group showed slow improvement than AMN + PRP group until 4 weeks; in the following weeks the recovery in the AMN + A group was found to be significantly weak. However, some studies suggested that amniotic membrane has little effect on wound healing, neovascularization and inflammation following acute ocular surface burns, as well as on improvement of vision [34,42-45]. There are some investigators suggesting

that the efficiency of the amniotic membrane equal to umbilical cord serum [36]. Another study stated that chondrocyte derived extracellular matrix in corneal alkaline burns gave better results than human amnionic membrane on corneal neo-vascularization, opacity and stromal changes [46]. However, when the present study findings were evaluated, it was observed that co-administration of the amnion membrane with corticosteroids or propolis yielded positive results in terms of clinical improvement in the defect area. In the study performed by Zheng et al.[47], conjunctival coverage combined with amnion liquid supernatant eyedrop showed the better therapeutic effect than conjunctival coverage individually. Pessolato et al.[48] evaluated the amniotic membrane and propolis separately in the reepithelization of second degree dermal burns in rats and reported that both provide beneficial results. In addition, they suggested that they can be used together considering reepithelization and anti-inflammatory effect of amniotic membrane and, debridement and collagen tissue production stimulation and scar formation suppression effect of propolis. Although there was no significant difference between the groups in terms of neovascularization in the present study, it was determined that PRP application was less effective compared to the other treatment methods in terms of corneal thickness and all of AMN groups yielded more successful results.

In conclusion, the amniotic membrane when applied with other treatment methods was successful in the treatment of subacute alkaline burns of the cornea; especially when combined with propolis, faster regression of the defect area was determined. Local application of only propolis were not found very effective. When these data are evaluated, it is thought that the amniotic membrane application in combination with propolis can be improved by planning further studies.

Declaration of Conflicting Interest

The authors declare no financial or other conflicts related to this report.

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