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Clinical Evaluation of the Efficacy of Bovine Amniotic Fluid on Healing of Experimental Corneal Defects in Rabbits

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Abstract

This study was conducted to investigate the effectiveness of amniotic fluid in defects in the epithelial tissue of the cornea. In the study, 14 New Zealand rabbits, which were experimentally created with a 3 mm diameter full-thickness defect in the corneal epithelium, were used. After the rabbits were randomly divided into two groups, amniotic fluid was applied to the rabbits in the first group (AF) and saline solution was applied to the rabbits in the second group (CG) three times a day for 20 days. Clinical examinations and tests were performed on the 7th, 14th, and 20th days of the study and the condition of the corneal defects was photographed. Clinical examinations revealed that tear break-up time (TBTT), fluorescein staining, rose bengal staining, corneal turbidity, and corneal vascularization were better in the AF group than in the CG group. As a result, it is concluded that amniotic fluid is effective in corneal epithelial defects and it would be beneficial to use it in addition to medical or operative treatment.

Anahtar Kelimeler: Amnion, cornea, defect, epithelial, treatment

Tavşanlarda Deneysel Kornea Defektlerinin Iyileşmesi Üzerinde Sığır Amniyotik Sıvısının Etkinliğinin Klinik Değerlendirmesi

Öz

Bu çalışma, amniyotik sıvının kornea epitel dokusunda oluşan defektlerdeki etkinliğini araştırmak için yapıldı. Çalışmada, deneysel olarak kornea epitelinde 3 mm çapında tam kat defekt oluşturulmuş 14 adet Yeni Zelanda tavşanı kullanıldı. Tavşanlar rastgele iki gruba ayrıldıktan sonra birinci gruptaki tavşanlara (AF) amniyon sıvısı, ikinci gruptaki tavşanlara (CG) fizyolojik tuzlu su 20 gün süreyle günde üç kez uygulandı. Çalışmanın 7., 14. ve 20. günlerinde klinik muayene ve testler yapılarak kornea defektlerinin durumu fotoğraflandı. Klinik incelemeler sonucunda AF grubundaki deneklerde gözyaşı kırılma zamanı (TBTT), floresein boyama, rose bengal boyama, korneal bulanıklık ve korneal vaskülarizasyon durumunun CG grubuna göre daha iyi olduğu ortaya konuldu. Sonuç olarak amniyon sıvısının kornea epitel defektlerinde etkili olduğu, medikal veya operatif tedavilere ek olarak kullanılmasının faydalı olacağı sonucuna varıldı.

Key Words: Amniyon, defekt, epitel, kornea, tedavi

INTRODUCTION

Corneal epithelium originating from limbal stem cells develops in the 8th week of intrauterine life. Corneal epithelium with a thickness of 40-50 microns has a very resistant structure against infections (1, 2). Corneal defects may develop due to systemic inflammatory diseases, eye diseases (KCS, keratopathy), and limbal stem cell deficiencies. Traumatic or chemical damage to corneal cells can also cause corneal defects (1-5). In patients with corneal defects, clinical symptoms such as increased tears, blepharospasm, pain, loss of corneal transparency, and increased corneal vascularization occur (1, 2). Corneal defects may develop according to the depth of the damage: epithelial defect, epithelial and anterior stromal defect, deep stromal defect, and defect of all layers (2, 4-6).

Defects in the corneal epithelium heal when intact epithelial cells (wing cells and basal epithelial cells) around the damaged area migrate towards the lesioned area (4, 7, 8). As the epithelial cells migrate towards the lesioned area, mitosis is formed and the multilayer epithelium tissue is completed (9). The epithelial tissue formed as a result of the healing of the corneal defect is thinner than the normal corneal epithelium. However, ongoing mitotic cell division ensures the normal thickness and morphological structure of this thin epithelium within a few months (7).

While treating defects of the corneal epithelium, is aimed to reduce pain in the patient, prevent a perforation in the eye tissue and minimize sequelae in the eye. For this reason, medical and operative treatments are used. Local antibiotics, anti-collagenase drugs and vitamins are used in medical treatment. Keratotomy, keratoplasty, conjunctival

flaps, cauterization, and soft contact lens applications are used as operative treatment (10-19).

Many clinical and laboratory studies are carried out to obtain successful results by shortening the treatment process in defects in the corneal epithelium. These studies mostly focused on the use of agents that affect cellular activities such as migration, mitosis, and apoptosis in the corneal epithelium. The most important agents used are growth factors, fibrinonectins, and retinoids (1, 20).

In this study, it was aimed to investigate the effectiveness of bovine amniotic fluid in the treatment of epithelial damage induced experimentally in the corneas of rabbits. In this study, the view that growth factors, anti-inflammatory proteins, extracellular matrix precursors, cytokines, and antiangiogenic proteins in bovine amniotic fluid may have limiting effects on ocular damage was influential in the establishment of the hypothesis.

MATERIAL AND METHOD

Animal Material

In this experimental study, 14 female, 4-5 months old New Zealand rabbits obtained from Fırat University Experimental Research Center were used. Before starting the study, the approval of Fırat University Animal Experiments Local Ethics Committee dated 05.05.2017 and numbered 2017/09 was obtained. During the study, rabbits were kept in special laboratory conditions (24 ± 3 ℃ 40-60% humidity, 12 hours dark, 12 hours light) and in special compartments. Its feeding was done using standard pellet feed.

Obtaining and Preparation of Amnion Fluid

Amniotic fluids taken under sterile conditions during the cesarean operation of cows who had a healthy pregnancy period were used in the study. Amniotic fluids were centrifuged at 2000 rpm for 15 minutes after bacteriological examinations. The supernatant was placed in sterile tubes and stored at -20 degrees. Amniotic fluid, which was taken to +4 degrees one day before use, was used in the applications.

Anesthesia Protocol

For the corneal defect formation phase, sedation was provided to rabbits by first administering xylazine hydrochloride (Rompun 23.32 mg / ml, Bayer) at a dose of 4 mg/kg intramuscularly. After 10 minutes, 50 mg / kg dose of ketamine hydrochloride (Ketalar 50 mg / ml, Parke-Davis) was administered intramuscularly and anesthetized.

Creating the Corneal Defect Model

After the rabbits were anesthetized, antisepsis was performed in the left eye to be defected. Local anesthesia was provided by dropping 0.5% proparacaine (Alcain) 10 minutes before the defect was created in the cornea. The boundaries of the standard corneal epithelial defect were determined with a 3 mm diameter punch trepan (Figure 1a). The corneal epithelium was completely scraped from this border and a round defect with smooth edges was created (Figure 1b).

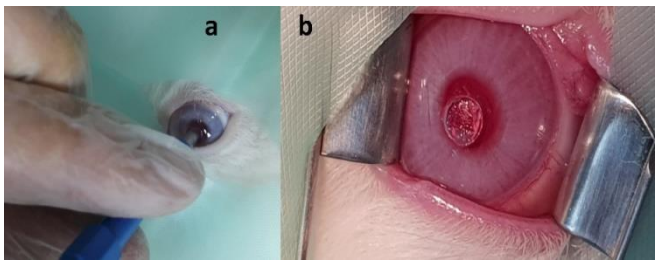

Figure 1. Creating the corneal defect area with a punch trepan (a), Creating a corneal full-thickness epithelial defect (b)

Creating Groups

After the epithelial defect was created, the rabbits were randomly divided into two equal groups. Applications started on the same day. The first group was determined as the amniotic fluid group (AF), and the second group was the control group (CG). Three drops of amniotic fluid were instilled into the left eyes of rabbits in the AF group three times a day. Three drops of physiological saline was instilled into the left eyes of rabbits in the CG group three times a day. Applications were continued for 20 days in both groups.

Postoperative Care

After the groups were formed, the rabbits were placed in special compartments as three and four, and pellet feed and water were continued daily. Postoperative antibiotics were not administered to animals in either group.

Macroscopic Evaluation

The rabbits in AF and CG groups were clinically examined on the 7th, 14th, and 20th days of the study and macroscopic findings were evaluated. In clinical examination, intraocular pressure measurement, Schirmer tear test, tear break-up time measurement (TBTT), rose bengal staining, and fluorescein staining were performed on rabbits. In addition, ocular disorders such as photophobia, tear discharge, redness, conjunctivitis, corneal edema, corneal vascularization that may be seen in rabbits during the study were evaluated as direct (Riester Ophthalmoscope Pen-Scope R2076) and indirect (Heine Omega® 500-German brand indirect binocular ophthalmoscope).

Evaluation of the difference in corneal opacity

In the evaluation of opacity changes in the cornea, Yoeruk et al (2008) was made considering the criteria (Table 1). Corneal turbidity was scored from 0 to 4, and was evaluated by direct ophthalmoscope on days 7th, 14th, and 20th.

Table 1. Macroscopic scoring of corneal cloudiness

Score	The severity of corneal cloudiness
0	Clear cornea
1	Transparent cornea with visible anterior chamber details
2	Blur to see the anterior chamber clearly
3	A blur so that the pupil and iris details cannot be seen
4	Turbidity where no structure other than the cornea is seen

Evaluation of corneal neovascularization

Vascularizations in the cornea, sclera, conjunctiva, and third eyelid of the rabbits were evaluated. These changes were examined and evaluated because they gave clinical clues about the degree of conjunctival and scleral damage. Vascularization formed in the cornea was evaluated according to the criteria determined (21) (Table 2).

Table 2. Macroscopically corneal neovascularization scoring

Score	Neovascularization severity
0	No neovascularization
1	Limited neovascularization around the cornea
2	Neovascularization extending to the pupillary border
3	Neovascularization extending beyond the edge of the pupil into the central cornea

Measurement of intraocular pressure

In the measurement of intraocular pressure in rabbits, "Reicher Technologies Tono-Pen" brand veterinary tonometer was used (Figure 2a).

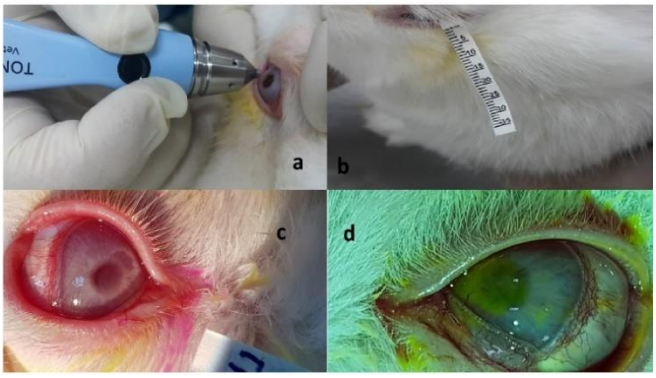

Figure 2. Measurement of intraocular pressure (a), Application of Schirmer test strips (b), Corneal examination with rose bengal staining and white light (c), Fluorescein sodium staining and corneal examination under a blue cobalt filter (d).

Application of the Schirmer tear test

In the application of this test, standard Schirmer test "Schirmer Tear Test, Ophthalmik Strips-Optitech" measurement strips were used without applying any topical anesthesia to the eye (Figure 2b) . For this purpose, measuring strips placed in the conjunctival space (lateral canthus) of the lower eyelid were taken one minute later and the amount of tear was measured (22-27).

Tear Break-up Time Test (TBTT)

In the study, a full blink was performed after 1 drop of 1% fluorescein was instilled into the defected left eyes of the rabbits. Then, using a 20-diopter loop, an indirect ophthalmoscope with a cobalt blue or yellow filter and without allowing a second blink, the time until the first black dry spot was seen, which is considered to be an indicator of tear breakage in the cornea, was determined (2, 22, 24). The TBTT test was determined to be 27 seconds on average in normal individuals and 5 seconds in patients with KCS (23).

Rose Bengal staining test

During the study, 1% rose bengal (Institut Pourquier, Montpellier France) dye was applied to the cornea of rabbits in both groups for this test. Five minutes after the dye was instilled, the cornea was examined under white light using an indirect ophthalmoscope using a 20 dioptric loop (Figure 2c). Changes detected in the cornea were scored between 0 and 3 (Table 3) and recorded (4, 23, 24, 27, 28).

Table 3. Scoring used in rose bengal staining

Score	Dye intensity
0	No painting
1	Light painting
2	Intermediate staining
3	Common painting

Fluorescein staining test

A drop of 1% fluorescein sodium dye was applied to the injured left eyes of the rabbits in both groups. After the application, the cornea was washed with saline and examined under a cobalt blue filter with a 20-dioptric loop and indirect ophthalmoscope (Figure 2d). The detected changes were made in consideration of the scoring system reported by the researchers (27, 28) (Table 4).

Table 4. Scoring used in fluorescein staining

Score	Dye intensity
0	No painting
1	Staining of 1/8 or less of the corneal surface
2	Painting 1/4 of the corneal surface
3	Staining 1/2 of the corneal surface
4	Staining the corneal surface

Statistical Analysis

SPSS (version 22.0) was used for statistical analysis. The Kruskal-Wallis test was used to compare the mean differences between groups, and the Mann-Whitney U test was used to compare the differences between the two groups. Differences between groups were analyzed using the Paired-Samples T test. Data are presented as mean ± standard error. Statistical significance was accepted when P≤0.05 (29).

RESULTS

The number of rabbits with blepharospasm, photophobia, conjunctivitis, scleral vascularization and vascularization in the third eyelid in AF and CG groups as a result of the clinical examinations performed on the 7th, 14th and, 20th days of the study and the severity of the findings are presented in Table 5 and Table 6 (Figure 3, 4, 5).

Statistical significance of intraocular pressure, corneal turbidity, corneal vascularization, Schirmer tear test, fluorescein staining, rose bengal staining, TBTT findings on the 7th, 14th, and 20th days of the study are presented in Table 7 and Table 8 (Figure 3, 4, 5).

Table 5. Numbers of rabbits with blepharospasm and photophobia in AF and CG groups at examinations on days 7th, 14th, and 20th.

	AF		CG	
	Blepharospasm	Photophobia	Blepharospasm	Photophobia
7th day	3	5	5	7
14th day	0	0	2	0
21th day	0	0	1	0

Table 6. Number of rabbits with conjunctivitis, scleral vascularization and vascularization in the third eyelid in AF and CG groups in the examinations performed on days 7th, 14th and 20th, and the severity of the findings

	AF			CG		
	Conjunctivitis	Scleral Vascularization	Vascularization in the Third Eyelid	Conjunctivitis	Scleral Vascularization	Vascularization in the Third Eyelid
7th day	3 (+++)	3 (+++), 1(++), 3 (+)	2 (+++), 4 (+)	5 (+++)	1 (+)	4 (+++), 2 (++), 1 (+)
14th day	2 (+)	1(++), 3(+)	1(+)	2 (+++)	2 (+++), 1 (++), 2 (+)	2 (++)
21th day	0	3(+)	0	1 (+++)	1 (+++), 3(++), 2(+)	1 (++)

Mild: + Moderate Severe: ++ Severe: +++

Table 7. Statistical analysis of schirmer tear test, fluorescein staining, rose bengal staining, TBTT measurement values

Groups	Schirmer	Fluorescein Staining	Rose Bengal Staining	TBTT
CG	7.04±2.53	3.37±0.70	2.28±0.74	9.99±2.40
AF	9.37±1.95	2.75±1.06	1.42±0.52	6.66±1.88
P value	0.514	0.001	0.000	0.000
Days				
7th	8.92±1.00	3.50±0.20	2.50±0.17	4.42±0.38
14th	7.28±1.10	2.57±0.27	1.71±0.26	8.78±1.30
20th	8.42±1.08	3.14±0.31	1.35±0.28	11.78±1.21
P value				
Between days				
7-14	0.339	0.000	0.001	0.009
7-20	0.687	0.208	0.000	0.000
14-20	0.422	0.040	0.055	0.003

Table 8. Statistical analysis of intraocular pressure, corneal turbidity and corneal vascularization findings

Groups	Intraocular Pressure (Hg)	Corneal Turbidity	Corneal Vascularization
CG	24.85±2.56	1.61±1.02	1.42±0.53
AF	23.63±2.64	0.85±0.81	0.71±0.60
P value	0.596	0.355	0.126
			Days
7th	22.71±1.68	1.08±0.28	0.69±0.18
14th	24.71±2.07	0.73±0.19	0.61±0.16
20th	25.29±1.36	1.12±0.29	0.73±0.19
			P value
			Between days
7-14	0.476	0.165	0.165
7-20	0.318	0.104	0.104
14-20	0.822	0.336	0.336

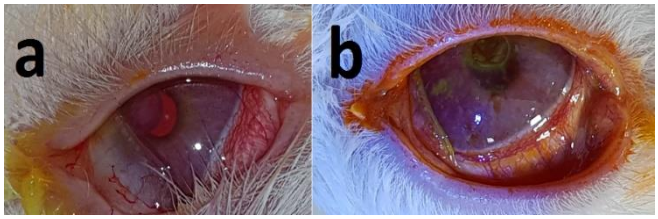

Figure 3. Clinical appearance of a rabbit in the AF group on day 7th (a), Clinical appearance of a rabbit in the CG group on day 7th (b)

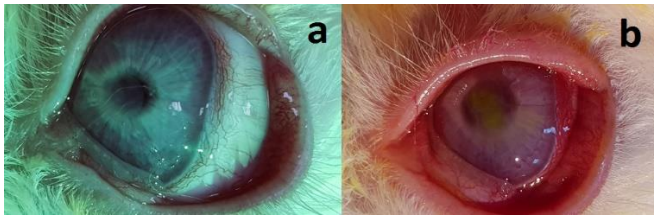

Figure 4. Clinical appearance of a rabbit in the AF group on day 14th (a), Clinical appearance of a rabbit in the CG group on day 14th (b)

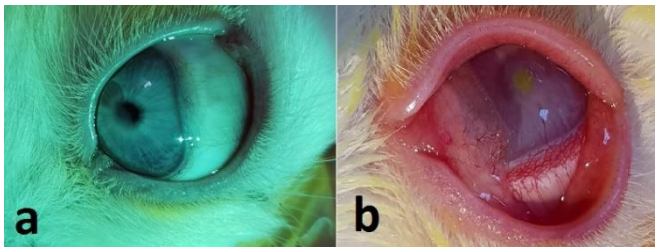

Figure 5. Clinical appearance of a rabbit in the AF group on day 20 (a), Clinical appearance of a rabbit in the CG group on day 20 (b)

DISCUSSION AND CONCLUSION

Corneal diseases such as ulcus cornea, descemetocele, mycotic keratitis, corneal abscess, bullous keratopathy, calcium-deposited senile keratopathy, corneal trauma, limbal melanoma, neurotrophic keratitis, feline corneal necrosis, and corneal dermoid are common in veterinary medicine (30, 31). Diseases that cause corneal surface disorders cause the loss of transparent, non-pigmented, vascular, and cellular features of the cornea and consequently, ophthalmologists have difficulties in treatment. In cases where vision is partially or completely blocked as a result of corneal diseases, various surgical techniques are used to ensure the transparency of the cornea. Penetrating corneal transplants are preferred in the treatment of full-thickness corneal defects. Corneal conjunctival transpositions, corneal scleral transpositions, third eyelid graft applications, and amniotic membrane applications are preferred in the treatment of incomplete corneal defects such as corneal ulcers, corneal neoplasm, corneal dermoid, feline corneal necrosis, desamatocele, infectious, and pigmentary keratitis. Corneal tissue adhesives and limbal stem cell transplantations are other treatment options (7, 31-33). In this study, the efficacy of bovine amniotic fluid in the treatment of corneal epithelial defects created experimentally in rabbits was evaluated clinically. In this study, the efficacy of bovine amniotic fluid in the treatment of corneal epithelial defects created experimentally in rabbits was evaluated clinically. For this purpose, amniotic fluid was instilled in 3 drops 3 times a day to the group of AF in rabbits.

Corneal diseases are easily recognized with symptoms such as epiphora, blepharospasm, photophobia, itching, and increased opacity (31). Since corneal diseases progress rapidly, medical or surgical intervention is required immediately after diagnosis (4, 7, 31). In this study, clinical findings such as epiphora, blepharospasm, photophobia, increased corneal opacity, scleral vascularization, conjunctivitis, and vascularization in the third eyelid were evaluated in the clinical examinations of rabbits in both the AF group and the CG group. After the corneal defect was formed, severe conjunctivitis, scleral vascularization, and vascularization in the third eyelid were found in all rabbits in both groups, together with blepharospasm and photophobia. In the clinical examinations performed at the end of the study, it was observed that only three of the rabbits in the AF group had mild scleral vascularization, and the clinical findings of varying severity continued in the CG group.

The focal light source, ophthalmoscope and / or slit-lamp biomicroscope are used in the examination of the cornea (4, 33, 34). Edema, keratitis, tissue loss, thickening, destructive events, and neovascularization in the cornea can be easily recognized in the examination with an ophthalmoscope and slit lamp biomicroscope. In addition to ophthalmoscopy, rose bengal staining and fluorescein staining techniques provide important data in the diagnosis of corneal pathologies. Dead and degenerated epithelial cells in the cornea and conjunctiva are detected with Rose Bengal staining. Fluorescein staining techniques are used in the diagnosis of epithelial defects in the cornea and conjunctiva, ulcus, abrasions, and opening of the nasolacrimal canal (4, 13, 31, 35). In the study, intraocular pressures, corneal turbidity, corneal vascularization, Schirmer tear test, fluorescein staining, rose bengal staining, and TBTT findings were evaluated on the 7th, 14th, and 20th days. When the data were evaluated, there were no statistically significant differences between the AF and CG groups in terms of intraocular pressure and schirmer tests. However, there were statistically significant differences between AF and CG groups in terms of fluorescein staining, rose bengal staining, TBTT, corneal turbidity, and vascularization criteria ($P \leq 0.05$).

The importance of tear function in the healing of corneal epithelial defects has led ophthalmologists to use similar biological products in the treatment of corneal defects. Biological fluids have been used in ocular surface diseases since 1984 (36-41). Among these products, breast milk, blood products, and amniotic fluid with similar protein content with the amniotic membrane, which has a wide range of use in ocular surface diseases. Although the concentration and diversity of biologically active molecules differ among these biological fluids, it seems possible to be used as a tear substitute for ocular surface care, as they contain most of the tear essential components. In a study, corneal epithelial defects were created in ex vivo organ culture in mice and the efficacy of fetal bovine serum, human amniotic fluid and horse amniotic fluid on epithelial defect were compared (42). While there was no difference in the rate of defect closure in the cornea between the control group and fetal cow serum, a faster re-epithelialization was observed in the groups using human amniotic fluid and horse amniotic fluid compared to the control group. Reepithelization of the two groups was faster than the fetal cow serum group, but it was not statistically significant.

There is no standardization in terms of preparation, concentration, and frequency of use of amniotic fluid in various studies. One study investigated the effectiveness of different concentrations of human amniotic fluid in the dry eye model in mice (43). It was determined that amniotic fluid at concentrations of 20-50-100% was superior to the control group in tear production, corneal staining and goblet cell counts compared to the control group that was applied saline. Among amniotic fluids of different concentrations, they reported that mice treated with 20% amnion improved less tear production than other concentrations. In this study, bovine amniotic fluid (concentration of 100%) obtained under sterile conditions was applied to rabbits with a full-thickness

corneal defect of 3 mm in diameter with the help of punch trepan in their left eyes. The amniotic fluid group (AF) was compared with the saline-administered control group (CG) and evaluated according to various clinical criteria. On the first day of the study, blepharospasm and photophobia, severe conjunctivitis, scleral vascularization, and vascularization in the third eyelid were detected in the defected eyes of all rabbits. During the clinical examinations performed on days 7, 14, and 21, there was a rapid improvement in the clinical findings of the rabbits in the AF group, while the rabbits in the CG group showed a slower course of improvement. When the data were evaluated, there were no statistically significant differences between the AF and CG groups in terms of intraocular pressure and Schirmer tests. However, there were statistically significant differences between AF and CG groups in terms of fluorescein staining, rose bengal staining, TBTT, corneal turbidity, and vascularization criteria ($P \leq 0.05$).

As a result, considering the 20-day clinical observations of the subjects, the efficacy of the AF group compared to the control group was revealed. At the same time, it was determined that besides a faster recovery, a more transparent corneal recovery that would not obstruct the vision was provided. Therefore, it was concluded that amniotic fluid is applicable in the treatment of corneal defects. However, it is thought that more comprehensive and long-term studies are needed.

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