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Therapeutic effect of bovine amniotic fluid in murine dry eye model

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Abstract

This study investigated the therapeutic effect of bovine amniotic fluid (BAF) in dry eye (DE) model in 60 female BALB/c mice divided equally into 6 groups. Control group (CG) received 5 μL formal saline and experimental groups 0.2% benzalkonium chloride in both eyes twice a day during 14 days. From 15 to 30 days while CG and DE group only was administered saline, other groups called BAF20, BAF35, BAF50 and BAF100 received 5 μL 20, 35, 50 and 100% BAF three times a day, respectively. On day 15, in all experimental groups tear production decreased, tear break-up time shortened, corneal fluorescein staining score increased compared to baseline; on day 30, for these parameters the most effective BAF concentrations were 35%, 50% and 100%. According to western blot analysis the lowest levels were obtained in CG and BAF35 groups for tumor necrosis factor α (TNF-α), vascular endothelial growth factor (VEGF) and cytokeratin 10 (K10); CG for adiponectin receptor-1 (AdipoR1); BAF35 and CG for adiponectin receptor-2 (AdipoR2) ($P < 0.05$). In the immunohistochemical analysis the lowest levels were in CG, BAF35, BAF50 and BAF100 groups for TNF-α, VEGF and K10; CG, BAF35 and BAF50 groups for AdipoR1; CG, BAF20, BAF35 and BAF50 groups for AdipoR2 ($P < 0.05$). TUNEL method revealed a lower apoptotic cell score in all BAF groups ($P > 0.05$). In conclusion; moderate to high concentrations of BAF have the more beneficial effects on DE at molecular and clinical signs and it can be used for the treatment.

Key Words: bovine amniotic fluid, dry eye, inflammation, mice, treatment

Introduction

Dry eye (DE) is a chronic multifactorial disorder induced primarily by high rate of inflammatory cytokine release as a result of tear hyperosmolarity, increased precorneal tear film (PTF) instability and hyperactivity of immune components in ocular surface cells[17,57]. DE is characterized by ocular surface impairment, inflammation, precorneal tear film instability and visual disturbances[49,54,57]. The current DE treatment modalities are based on increasing PTF stability, maintaining normal tear osmolarity, preventing apoptosis and inflammation and providing ocular surface homeostasis[52,57]. The main purpose of DE management is to remove the underlying causes of the disease playing roles in its pathogenesis[14,15] with resultants of relieved clinical symptoms and improved the patient of life quality[9,14,27]. Recently, the therapeutic benefits of

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some biological agents, similar to natural tears including autologous serum, umbilical cord serum and human amniotic membrane and fluid, on DE widely have been investigated[44,45,53]. In earlier studies indicated that human amniotic membrane and fluid contain high amounts of growth factors vital for fetal development and revealed to induce corneal sensitivity, promote nerve regeneration and decrease epithelial damage as well as scar tissue formation in alkali burn cornea[18,26]. It has also been reported that these agents contain hyaluronic acid which is an anti-inflammatory polysaccharide[11,43,48]. In a mouse DE model study[44] in which the therapeutic effects of some body fluids and tear preparations were compared, it was found that human amniotic fluid yielded better results in terms of corneal fluorescein staining and goblet cell density than artificial tear preparations and human serum. Another study reported[45] that higher concentration of human amniotic fluid has better therapeutic effects on DE than its lower concentrations. Bovine has a well-developed allantoic cavity and a large amount of amniotic fluid. Bovine amniotic fluid (BAF), which is rich in proteins, minerals and cells, has the advantages of being inexpensive and easy to obtain[12]. In a study on acute corneal alkali burns of rat, BAF was found to reduce corneal erosions, edema and keratinization. These results indicate that BAF may be an alternative therapeutic agent in veterinary ophthalmology[16]. To our knowledge, up to now no study has investigated the therapeutic benefits of BAF on DE.

DE models can be established utilizing aquaternary ammonium compound, benzalkonium chloride (BEC), an active ingredient of ophthalmic preparations with antimicrobial action[36,56].BEC rapidly induces DE via disrupting the lipid phase of PTF and disintegrating corneal epithelial cell membrane[36].

The aim of this study was to investigate the therapeutic effects of 4 different concentrations of BAF, a biological fluid, in murine DE model induced with BEC.

Materials and Methods

Experimental procedure: This study was conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and it was approved by the Firat University Animal Experiments Local Ethics Committee (2016/4/34). The material in the study consisted of 60 BALB/c female mice with average weights of 18–20 g and average ages of 6–8 weeks. During the study, the subjects were kept in a room with an ambient temperature of 24ºC ± 1ºC, relative humidity of 60% ± 10%, and a light-dark cycle of 12 hours. No food and water restrictions were applied during the study. Following clinical and ophthalmological examinations, the subjects that were determined to be healthy were included the study. One week prior to the experiment, all the subjects were transferred to an independent room, where they were kept in conventional mouse cages. Experimental procedures were performed using a funnel shaped hard plastic restraint[22], the narrower end of which had a hole large enough for a mouse to pass its head through. To obtain the measurements, the animals were encouraged to pass through the restraint several times. Then, they were randomly divided into 6 groups (n=10 x 6). Group I served as the control group (CG); the other 5 groups (1 experimental group, the dry eye [DE] group and 4 BAF treatment groups) were topically administered 5 μL of 0.2% BEC in both eyes twice a day (09:00, 21:00) for 14 days to induce the DE model. During this time, the CG subjects received the same amount of saline and the same time points[55]. The subjects in the CG and DE groups (Group II) received 0.9% saline 3 times a day (09:00, 15:00, and 21:00) for a 15-day therapeutic period; the subjects in the other 4 groups (BAF treatment groups) were administered 20% BAF (Group III, BAF20), 35% BAF (Group IV, BAF35), 50% BAF (Group V, BAF50), and 100% BAF (Group VI, BAF100) at the same intervals and the same time points. At the end of the study, the mice were

decapitated, and the eye tissues were carefully collected and stored at -80°C until the Western blot (WBlot), immunohistochemical (IHC), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analyses were conducted.

Procurement and Preparation of BAF:BAF was obtained from a 270±2 day pregnant cow that had a prolapsed vagina and a closed cervix uterus. The fluid was collected during the caesarean section procedure under sterile conditions, centrifuged at 3,000 rpm for 15 min and stored at -20ºC until used[12]. During use, it was kept at 4ºC to prevent bacterial contamination[12,16]. The BAF in Group III, Group IV, and Group V was diluted at concentrations of 20% (BAF20), 35% (BAF35), and 50% (BAF50), respectively, with isotonic saline; the BAF in Group VI (BAF100) was pure or used without dilution solution.

Procedure of the clinical parametric tests: In all the groups, the tear production rate, tear break-up time (TBUT), and corneal fluorescein staining tests were measured 7 times; baseline (before the experiment) and after inducing the DE model on days 15, 18, 22, 25, 28, and 30 at the same time points (13:00–15:00). During each measurement, any ocular abnormalities (photophobia, ocular discharge and redness, conjunctivitis, keratitis) were also noted.

Measurement of tear production rate with Endodontic Absorbent Paper Point Test (EAPPTT): As test material Roeko Color (Color size 30, Langenau, Germany) brand, a gold standard for this particular measurement[29,30,40] was used. After ensuring the physical restraint, the test strips were placed in the lower conjunctival fornix near the lateral canthus of each eye and left in place for 1 min. Measurement was performed in both eyes simultaneously. After the test strips were removed, the length of wetness on the strips was read on amillimeter scale and average data of both eyes were recorded.

TBUT: With a micro-injector (901 N, Hamilton, USA) 1 µL 0.1% liquid sodium fluorescein (Fertility Chemical Laboratory, Istanbul, Turkey) was instilled into the left and right eyes of each subject. After 3 blinks, the eye lids were withheld with fingers and under the slit-lamp biomicroscope (XL-1, Shin-Nippon, Osaka, Japan) the moment the tear break up occurred the first was noted and the average value of the both eyes was recorded[21,36].

Corneal fluorescein staining test: In all the experimental subjects, 90 sec after the TBUT, the corneal staining rate was assessed using the slit-lamp biomicroscope with a cobalt blue filter; and the detected corneal lesions were photographed under an operation microscope[25,36]. The lesions were scored as 0: no staining on the corneal surface; 1: a staining area equal to or less than 1/8; 2: a staining area equal to or less than 1/4; 3: a staining area equal to or less than 1/2; and 4: a staining area more than 1/2 or covering the entire corneal surface[25].

Total protein isolation:Corneoconjunctival tissue samples (Half of each group; 5 samples of both eyes) were washed with cold phosphate buffered saline (PBS) and protein isolation was performed with UPX Universal Protein Extraction Kit (Expedeon, UK), after protein isolation, the protein samples were kept at -20 °C until analysis[2].

Analysis of VEGF, TNF-α, K10, adiponectin receptor 1 (AdipoR1), and adiponectin receptor 2 (AdipoR2) protein expression levels using the WBlot technique: First, the protein concentrations of corneoconjunctival tissue were measured with a Lowry kit. About 30 µg of the protein samples was loaded into each wells of Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), later, the protein samples of the corneoconjunctival tissue were run on 12% gel via the SDS-PAGE and carried to a nitrocellulose membrane. The samples were then incubated overnight at 4 °C with primary antibodies against VEGF (sc-7269, Santa Cruz-Germany), TNF-α (ab8348, Abcam-UK), K10 (NBP1-97795, Novus Bio-USA), AdipoR1 (ab126611, Abcam-U.K.), AdipoR2 (ab77612, Abcam-U.K.), and beta-actin (sc-47778, Santa Cruz-Germany) (dilution: 1/500 for all antibodies).

After, the protein samples were incubated by the HRP-conjugated secondary antibody (1/1000) for 60 min at room temperature, the nitrocellulose membrane was colored with DAB and the protein levels calculated with an image program (Image J; National Institutes of Health, Bethesda, MD, USA)[3,2,28].

Tissue fixation and processing: After the mice were decapitated, their left and right eyes were removed for WBlot and IHC, fixed in 10% neutral buffered formaldehyde, sectioned in the vertical plane adjacent to the optic nerve, and embedded in paraffin. Serial sections in 5 µm thickness including the cornea, retina and optic nerve were cut.

IHC analysis: The paraffin embedded sections were deparaffinized in xylene and dehydrated using a series of graded alcohol. UltraVision™ ONE Detection System: HRP Polymer/ AEC Chromogen (ThermoFisher Scientific, Rockford, IL, USA) was used according to the manufacturer's protocol. Briefly; antigen retrieval was accomplished by microwaving the sections for 15 min in Citrate Buffer at pH6, then allowed to cool for 20 min. The sections were washed in PBS and primary antibodies were applied after Hydrogen Peroxide Block (5 min) and Ultra-V Block (5 min). The sections were incubated in primary antibodies,including VEGF (1/100, sc-7269), TNF-α (1/100, ab8348), K10 (1/100, NBP1-97795), AdipoR1 (1:20, ab126611), AdipoR2 (1:250, ab77612). Then incubation with primary antibodies against immunodetection was performed for 60 min at 37 ˚C with biotinylated goat anti–polyvalent, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with a 3- Amino-9-Ethylcarbazol(AEC) (ThermoFisher Scientific, Rockford, IL, USA), as the chromogen substrate. The sections were counterstained with Gill's hematoxylin, and coverslips were attached using aqueous mounting media. The intensity and prevalence of the IHC staining was scored on a scale of 0 to +3 (0: absent, +1: weak, +2: medium, +3: strong). Scoring was done for each mouse,individually.

The TUNEL method: Sections were stained using In-Situ Cell Death Detection POD apoptosis kit (Boehringer Mannheim, Mannheim, Germany) following recommended standard procedures. After the tissue sections were taken on positively charged slides at a thickness of 5 µm, they were left to dry overnight in a 60 °C oven, deparaffinized in xylol, and dehydrated in serial alcohols. After the contours of the sections were drawn with a bounding pencil, they were kept with a 1:500 dilution of Protinase K solution for 7 min at room temperature. To stop endogenous peroxidase activity, 5 min of a blockage in 3% H_2O_2 prepared in methanol was applied. Then, each section was washed with 13 µL/cm^2 of equilibration buffer solution for 6 min at room temperature and covered with a plastic coverslip, then left in 100 µL terminal deoxynucleotidyl transferase (TdT) enzyme solution at 37 °C for 1 hour before being transferred to protein blocking serum for 10 min to prevent non-antigenic binding. All the tissue sections were incubated with anti-digoxigenin conjugate for 30 min at room temperature. They were washed 3 times with PBS for 5 min after each procedure, except for the incubation step with the protein blocking serum. DAB was used as the chromogen. For contrast staining, Gill's hematoxylin was used. Finally, the slides were covered with water-based adhesive (Bio-Optica, Milan, Italy). TUNEL staining was scored using the same scoring that was used in the IHC method described earlier.

Statistical analysis: SPSS 13.0 (SPSS Inc., Chicago, IL, USA) version was used for the statistical analysis of clinical parametric tests. An independent statistical analysis was applied to each test. Friedman test, used for non-parametric and repeated measurements, was applied to determine the difference between the different measurement times of a group for a specific test. In a group with significant differences, the Wilcoxon test was used to determine if there was a difference between the measurement times. The difference between the groups at each measurement time was assessed using Tukey's

test in one-way analysis of variance (ANOVA). Statistical results, set to $P < 0.05$ or $P < 0.001$, were accepted as significant.

The IBM SPSS Statistics 20.0 package program was used for statistical analysis of the WBlot, IHC, and TUNEL techniques. Group variability was tested with one-way ANOVA; post-hoc, Duncan, and Games-Howell tests were used for WBlot analysis; Tukey's test was used for the IHC and TUNEL analyses. Statistical results were accepted as significant at $P < 0.05$. For the reliability of the statistics, the measurements were repeated at least 3 times in the WBlot analysis.

Results

EAPPTT: For the EAPPTT parameter, the difference between all the groups was significant at all the measurement times ($P < 0.001$; Table 1) except at baseline ($P > 0.05$; Table 1). On the 15th day, the first measurement after BEC application, the highest aqueous tear production rate was recorded in CG, and the difference between this group and all the other groups was significant ($P < 0.05$; Table 1); however, the differences in the EAPPTT results between all the groups, except the CG, were insignificant ($P > 0.05$; Table 1). At the last measurement time of the study (30th day), the mean tear production rate in the treatment groups, ranging from highest to lowest, were: BAF35, BAF50, BAF100, and BAF20, although the difference was not statistically significant ($P > 0.05$; Table 1).

TBUT: For all measurement times, except baseline the difference between groups was statistically significant ($P < 0.001$; Table 2). On the 15th day, the highest mean TBUT mean (6.99±0.48 sec) was recorded in the CG subjects and the lowest TBUT was recorded in the DE group. The differences between all the groups on the 30th day, the last measurement time of the study, were found to be statistically significant ($P < 0.05$; Table 2). Among the treatment groups, it was determined that the BAF50 group had longest TBUT values and the BAF20 group had the shortest TBUT values.

Corneal fluorescein staining test: The baseline score for all the groups was 0 ($P > 0.05$; Table 3). For the other measurement times, a significant difference ($P < 0.001$; Table 3) was observed between the groups. On the 15th day of measurements, the difference between all the groups, except CG, was statistically insignificant ($P > 0.05$; Table 3). In the last measurement time (the 30th day), the lowest mean scores among the treatment groups were recorded in BAF35, BAF50, and BAF100 groups, with no statistically significant difference between them($P > 0.05$; Table 3). On the 30th day, the highest mean score among these groups was recorded in the BAF20 group.

WBlot analysis: VEGF and TNF-α inflammatory cytokine expression levels were measured in the highest DE group and the lowest in the CG and BAF35 groups, and these groups showed the significant differences with the other groups ($P < 0.05$; Fig. 1A,B,C). For K10, the highest levels were found in the DE, BAF20 and BAF100 groups ($P < 0.05$), and the lowest levels in the CG and BAF35 groups ($P < 0.05$; 1A,D). AdipoR1 and AdipoR2 protein expression levels determined higher in DE group in comparison to all the groups ($P < 0.05$; Fig. 1A,E,F). Compared to all other groups, the lowest AdipoR1 expression levels are in the CG group; the lowest AdipoR2 levels were measured in CG and BAF35 groups ($P < 0.05$; Fig. 1A,E,F).

IHC analysis and TUNEL method: For VEGF, it was found that immunoreactivity was evident in the corneal epithelium of the DE group (Fig. 2Aa). It was found that immunoreactivity was low in BAF50 and BAF100 groups along with BAF35 (Fig. 2Ab) in treatment groups, but not in CG (Fig. 2Ac). Immunoreactivity for TNF-a was found to be evident in the corneal epithelium and subepithelial corneal stromal cells of the DE group (Fig. 2Ba). In the treatment groups, immunoreactivity was lower in BAF35 (Fig.

Table 1. Statistical analysis of EAPPTT data according to measurement times and group variables (Mean ± standard deviation). *Baseline values of the study, a-e: There is a significant difference between groups containing different letters in the same column. ($P < 0.05$), ANOVA-Posthoc, Tukey test were used. A-F: The differences between groups that contain different letters on the same line are significant. ($P < 0.05$). Friedman was used for non-parametric tests and Wilcoxon test for intra-group comparisons. (n=10x 6 group)

Groups	Measurement Times							---*P*---
	Baseline* (mm/min)	15th day (mm/min)	18th day (mm/min)	22nd day (mm/min)	25th day (mm/min)	28th day (mm/min)	30th day (mm/min)	
CG	4.59±0.32	$4.74±0.37^{a}$	$4.79±0.15^{a}$	$4.67±0.26^{a}$	$4.61±0.40^{a}$	$4.39±0.25^{a}$	$4.55±0.41^{a}$	0.066
DE	$4.79±0.41^{A}$	$2.47±0.23^{Bb}$	$2.45±0.34^{Bd}$	$2.41±0.20^{Bd}$	$2.37±0.30^{Be}$	$2.42±0.32^{Be}$	$2.54±0.33^{Bd}$	0.001
BAF20	$4.50±0.36^{A}$	$2.39±0.27^{Cb}$	$2.94±0.20^{Bc}$	$2.97±0.20^{Bc}$	$3.04±0.17^{Bd}$	$3.07±0.19^{Bd}$	$3.07±0.16^{Bc}$	0.001
BAF35	$4.60±0.29^{A}$	$2.47±0.26^{Eb}$	$3.30±0.19^{Db}$	$3.34±0.21^{Db}$	$3.68±0.22^{Cb}$	$4.05±0.15^{Bb}$	$4.08±0.13^{Bb}$	0.001
BAF50	$4.59±0.31^{A}$	$2.42±0.28^{Fb}$	$2.95±0.19^{Ec}$	$3.15±0.13^{Dbc}$	$3.14±0.09^{DEcd}$	$3.52±0.19^{Cc}$	$3.95±0.18^{Bb}$	0.001
BAF100	$4.44±0.29^{A}$	$2.43±0.19^{Fb}$	$2.79±0.19^{Ec}$	$3.18±0.15^{Dbc}$	$3.39±0.12^{Cbc}$	$3.46±0.13^{Cc}$	$3.94±0.15^{Bb}$	0.001
---*P*---	0.292	0.001	0.001	0.001	0.001	0.001	0.001	

Table 2. Statistical analysis of TBUT data according to measurement times and group variables (Mean ± standard deviation). *Baseline values of the study, a-f: There is a significant difference between groups containing different letters in the same column. ($P < 0.05$), ANOVA-Posthoc, Tukey test were used. A-F: The differences between groups that contain different letters on the same line are significant. ($P < 0.05$). Friedman was used for non-parametric tests and Wilcoxon test for intra-group comparisons. (n=10x 6 group)

Groups	Measurement Times							---*P*---
	Baseline* (sec)	15th day (sec)	18th day (sec)	22nd day (sec)	25th day (sec)	28th day (sec)	30th day (sec)	
CG	7.25±0.44	$6.99±0.48^{a}$	$7.21±0.30^{a}$	$6.93±0.22^{a}$	$6.81±0.30^{a}$	$6.89±0.38^{a}$	$6.96±0.29^{a}$	0.161
DE	$6.99±0.33^{A}$	$2.15±0.32^{Be}$	$2.04±0.17^{Be}$	$2.08±0.29^{Be}$	$2.02±0.17^{Bd}$	$2.13±0.20^{Be}$	$2.18±0.26^{Bf}$	0.001
BAF20	$7.37±0.25^{A}$	$2.49±0.30^{Dbc}$	$3.53±0.22^{Cd}$	$4.39±0.24^{Bd}$	$4.59±0.24^{Bc}$	$4.48±0.29^{Bd}$	$4.49±0.18^{Be}$	0.001
BAF35	$7.19±0.21^{A}$	$2.69±0.21^{Eb}$	$3.92±0.20^{Dc}$	$4.98±0.25^{Cbc}$	$5.27±0.22^{Cb}$	$5.40±0.23^{BCc}$	$5.58±0.34^{Bc}$	0.001
BAF50	$7.27±0.41^{A}$	$2.17±0.29^{Fc}$	$4.29±0.28^{Eb}$	$5.02±0.29^{Db}$	$5.25±0.29^{Cb}$	$6.42±0.31^{Bb}$	$6.54±0.17^{Bb}$	0.001
BAF100	$7.30±0.38^{A}$	$2.62±0.27^{Eb}$	$4.29±0.32^{Db}$	$4.65±0.30^{Ccd}$	$4.55±0.40^{CDc}$	$4.69±0.51^{Cd}$	$4.99±0.33^{Bd}$	0.001
---*P*---	0.231	0.001	0.001	0.001	0.001	0.001	0.001	

Table 3. Statistical analysis of corneal fluorescein staining test scores according to measurement times and group variables (Mean ± standard deviation). *Baseline values of the study, a-d: There is a significant difference between groups containing different letters in the same column. ($P < 0.05$), ANOVA-Posthoc, Tukey test were used. A-G: The differences between groups that contain different letters on the same line are significant. ($P < 0.05$). Friedman was used for non-parametric tests and Wilcoxon test for intra-group comparisons. (n=10x 6 group)

Groups	Measurement Times							---*P*---
	Baseline* (score)	15th day (score)	18th day (score)	22nd day (score)	25th day (score)	28th day (score)	30th day (score)	
CG	0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	------
DE	0^{A}	$3.55±0.37^{Cb}$	$3.45±0.37^{Cc}$	$3.45±0.37^{Cc}$	$3.45±0.37^{Cc}$	$3.25±0.26^{Bd}$	$3.10±0.21^{Bd}$	0.001
BAF20	0^{A}	$3.65±0.34^{Fb}$	$3.10±0.21^{Ebc}$	$2.80±0.26^{Db}$	$2.30±0.26^{Cb}$	$2.15±0.24^{Cc}$	$1.90±0.21^{Bc}$	0.001
BAF35	0^{A}	$3.60±0.39^{Gb}$	$3.10±0.32^{Fbc}$	$2.40±0.39^{Eb}$	$2.15±0.34^{Db}$	$1.50±0.41^{Cb}$	$0.75±0.59^{Bb}$	0.001
BAF50	0^{A}	$3.61±0.32^{Gb}$	$2.78±0.48^{Fb}$	$2.39±0.39^{Eb}$	$1.83±0.41^{Db}$	$1.22±0.48^{Cb}$	$0.50±0.41^{Bb}$	0.001
BAF100	0^{A}	$3.40±0.39^{Gb}$	$2.90±0.21^{Fb}$	$2.55±0.28^{Eb}$	$1.95±0.44^{Db}$	$1.60±0.52^{Cb}$	$0.55±0.37^{Bb}$	0.001
---*P*---	------	0.001	0.001	0.001	0.001	0.001	0.001	

2Bb) and BAF50 and BAF100. In CG, there was no immunoreactivity (Fig. 2Bc). For K10, immunoreactivity was most prominently detected in the corneal epithelium of the DE group (Fig. 2Ca). Immunoreactivity was found to be less pronounced in BAF50 (Fig. 2Cb) and BAF35 and BAF100. In CG, there was no immunoreactivity (Fig. 2Cc). Immunoreactivity for AdipoR1 was evident detected in the corneal epithelium of the DE group (Fig. 2Da). Immunoreactivity was less pronounced in the BAF50 group with BAF35 (Fig. 2Db). There was no AdipoR1 immunoreactivity in CG (Fig. 2Dc). Immunoreactivity for AdipoR2 was evident in the corneal epithelium of the DE group (Fig. 2Ea). It was found less in BAF20 and BAF35 groups with BAF50 (Fig. 2Eb). There was no AdipoR2 immunoreactivity in CG (Fig. 2Ec). In TUNEL staining, the number of apoptotic cells in the corneal epithelium of the DE group was found to be high (Fig. 2Fa). While the number of apoptotic cells was less common in BAF50 (Fig. 2Fb) and other treatment groups, it was not detected in CG (Fig. 2Fc).When all groups were evaluated according to the severity scores of the immunoreactive cells; for VEGF, the immunoreactive cell score was measured at the highest DE ($P < 0.05$) and the lowest at CG($P < 0.05$). In the treatment groups, the severity score of all the groups except for BAF20 was low (Fig. 3A). For TNF-α ve K10, the immunoreactive cell score was highest in the DE group($P < 0.05$) and the lowest in all other groups except BAF20 (Fig. 3B,C). For AdipoR1, the immunoreactive cell score was highest in the DE group ($P < 0.05$) and lowest in the CG, BAF35 and BAF50 groups (Fig. 3D). For AdipoR2, the immunoreactive cell score was highest in the DE group($P < 0.05$)and lowest in all other groups except BAF100 (Fig. 3E). For TUNEL, the immunoreactive cell score was highest in the DE group($P < 0.05$), lowest in the CG group($P < 0.05$), and low in all treatment groups with no statistically significant difference ($P > 0.05$) between them (Fig. 3F).

Discussion

DE is an complex ocular disorder characterized by excessive evaporation of the aqueous and alteration of the mucin components of PTF due to increased tear osmolarity, corneal and conjunctival epithelial damage, squamous metaplasia, and dysfunction or inflammation of the ocular surface and the tear glands[19,52,55,57]. To date, many experimental DE models have been established to investigate its complex pathogenesis and realize novel and effective treatment options[1,4,5,8,10,21,37,50]. Xiong *et al*[56] suggested that, although these models provide important data about the disease, each of them has its own characteristics and limitations; therefore, they do not fully reflect the complex etiopathogenesis and chronic form of DE. In their DE rabbit model, these investigators used BEC, a preservative constituent of ophthalmic preparations, as the DE inductive agent, which is well-known to rapidly and progressively destabilize PTF via altering its lipid and mucin layers. They found that BEC can reduce the amount of aqueous tear production and conjunctival goblet cells and increase corneal fluorescein and Rose Bengal staining areas in subjects. Many in vivo studies performed using mice[24,36], rats[41], cats[6] and rabbits[56] have shown that BEC also induces corneal and conjunctival inflammatory cell infiltration, excessive release of pre-inflammatory mediators, PGF instability, conjunctival goblet cell loss, corneal epithelial desquamation, erosions, ulceration, and corneal neovascularization. Considering its negative effects on the ocular surface, many researchers[19,36,55,56,59] have recently used BEC to establish a successful DE model in different animal species. The present study established murine DE model by topically applying 5 μL 0.2% BEC to both eyes of the subjects for 14 days. At the end of this period, the average tear production rate decreased, the TBUT shortened and corneal fluorescein staining scores increased significantly in comparison to the baseline values

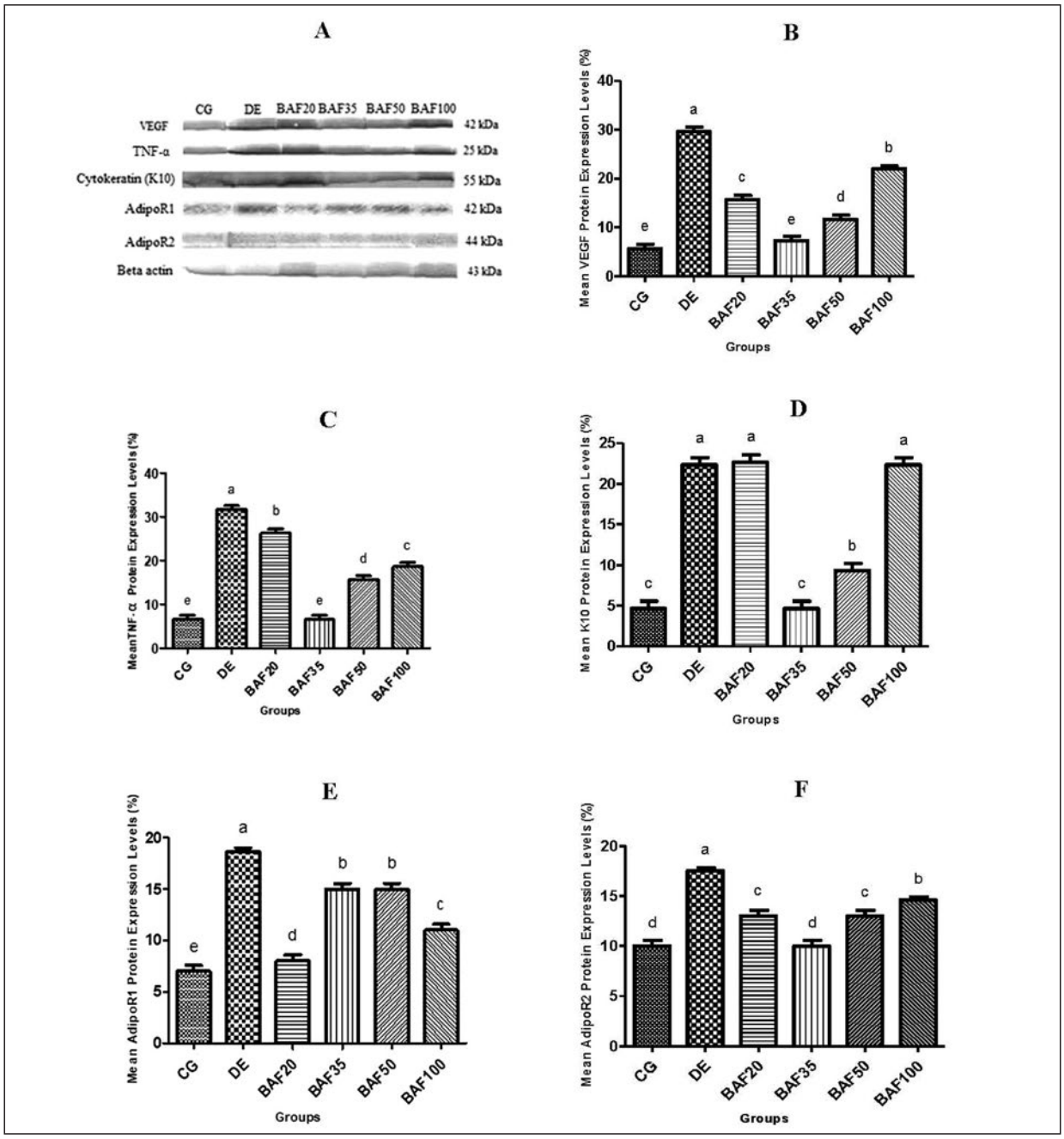

Fig. 1. Western blotting mean protein expression results (CG: Control group, DE: Dry Eye group). **A.** VEGF, **B.** TNF-α, **C.** K10, **D.** AdipoR1, **E.** AdipoR2, **F.** Western blotting protein bands. *a-e: Differences between groups with different letters are statistically significant ($P < 0.05$). One-Way ANOVA Post Hoc Duncan Test. (n = 3 x 6 groups, mean ± standard deviation). The protein expression levels in y-axis defined as percent of control.

in all subjects except for those in the CG having no BEC. It was also observed that all these abnormalities continued in the DE group until the end of the study. According to WBlot and IHC analyses of the corneal and conjunctival tissues obtained from all the group subjects at the end of the study, the levels of inflammatory cytokines, such as TNF-α and VEGF, and K10, markers of squamous metaplasia, and levels of AdipoR1 and AdipoR2, indicators of inflammatory

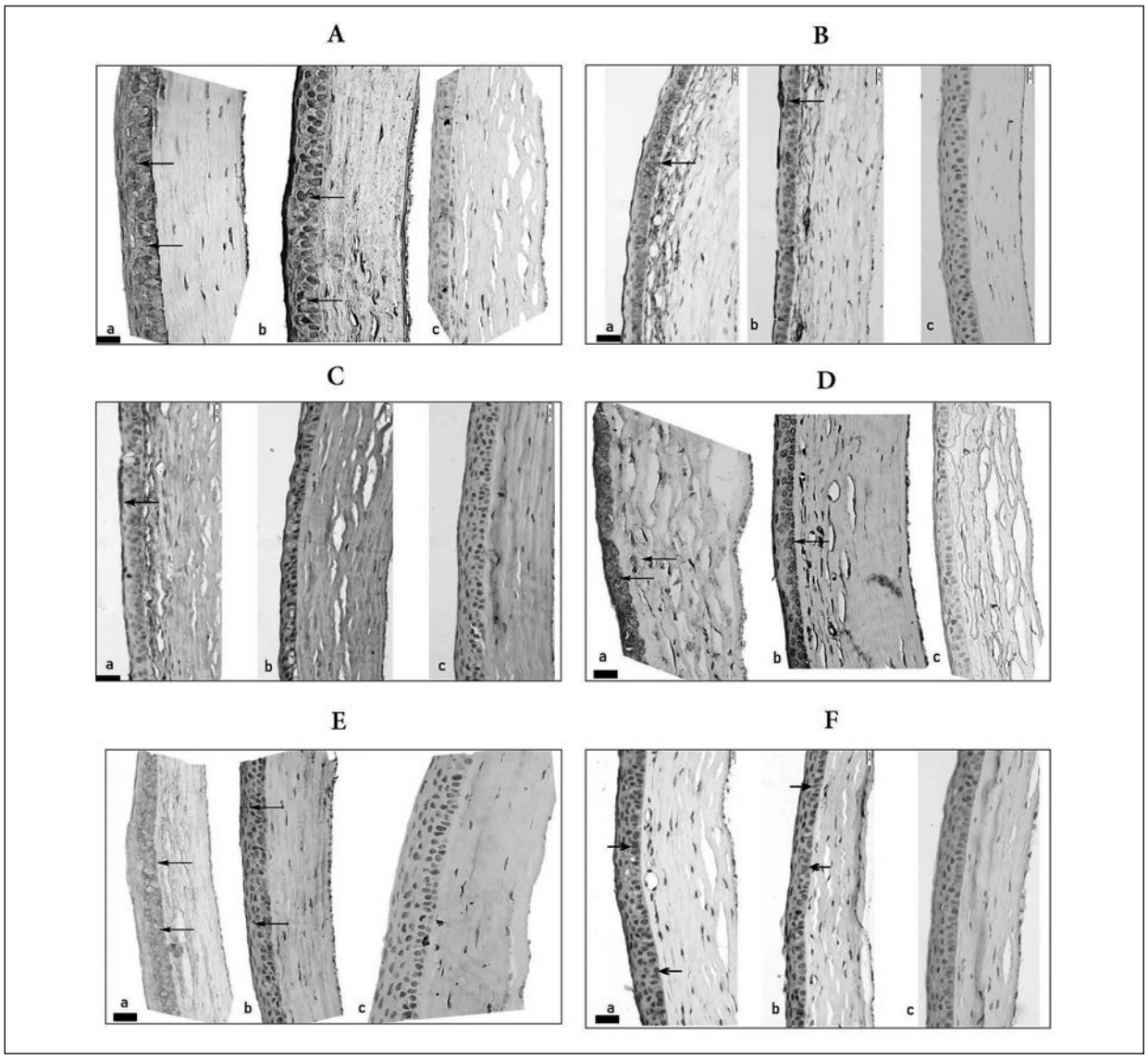

Fig. 2. IHC for immunoreactive cells, TUNEL method. A. VEGF (a) Increased immunoreactivity in corneal epithelium (arrows, mouse no: 1) in DE group, (b) Decreased immunoreactivity of corneal epithelium (arrows, mouse no: 3) in BAF35 group, (c) no immunreactivity in CG (mouse no: 4). **B. TNF-α**(a) Increased immunoreactivity in corneal epithelium (arrow, mouse no: 2) in DE group, (b) Decreased immunoreactivity of corneal epithelium (arrow, mouse no: 4) in BAF35 group, (c) No immunoreactivity in CG (mouse no: 3). **C. K10** (a) Increased immunoreactivity in corneal epithelium (arrow, mouse no: 3) in DE group, (b) Decreased immunoreactivity of corneal epithelium (mouse no: 4) in BAF50 group, (c) No immunoreactivity in CG (mouse no: 2). **D. AdipoR1** (a) Increased immunoreactivity (arrows, mouse no: 1) in DE group, (b) Decreased immunoreactivity (arrow, mouse no: 2) in BAF35 group, (c) No immunoreactivity in CG (mouse no: 5). **E. AdipoR2** (a) Increased immunoreactivity (arrows, mouse no: 5) in DE group (b) Decreased immunoreactive cells (arrows, mouse no: 5) in BAF50 group (c) No immunoreactivity in CG group (mouse no: 3). Immunoperoxidase staining, 400X, Bar:20 μm. **F. TUNEL method** (a) Increased apoptotic cells (arrows, mouse no: 4) in DE group, (b) Decreased apoptotic cells (arrows, mouse no: 3) in BAF50 group, (c) No apoptotic cells (mouse no: 4) CG group. TUNEL staining, 400X, Bars: 20 μm.

reaction, increased in the DE group. When all these parameters are evaluated together, it was concluded that BEC produced an effect similar to DE in both the molecular and clinical levels.

A decrease in the aqueous tear production rate, the most prominent clinical symptom of DE, triggers many inflammatory eye diseases, including DE, by activating the inflammatory process[15,35,38,46]. In recent years, EAPPTT has been used to measure the amount of aqueous tear

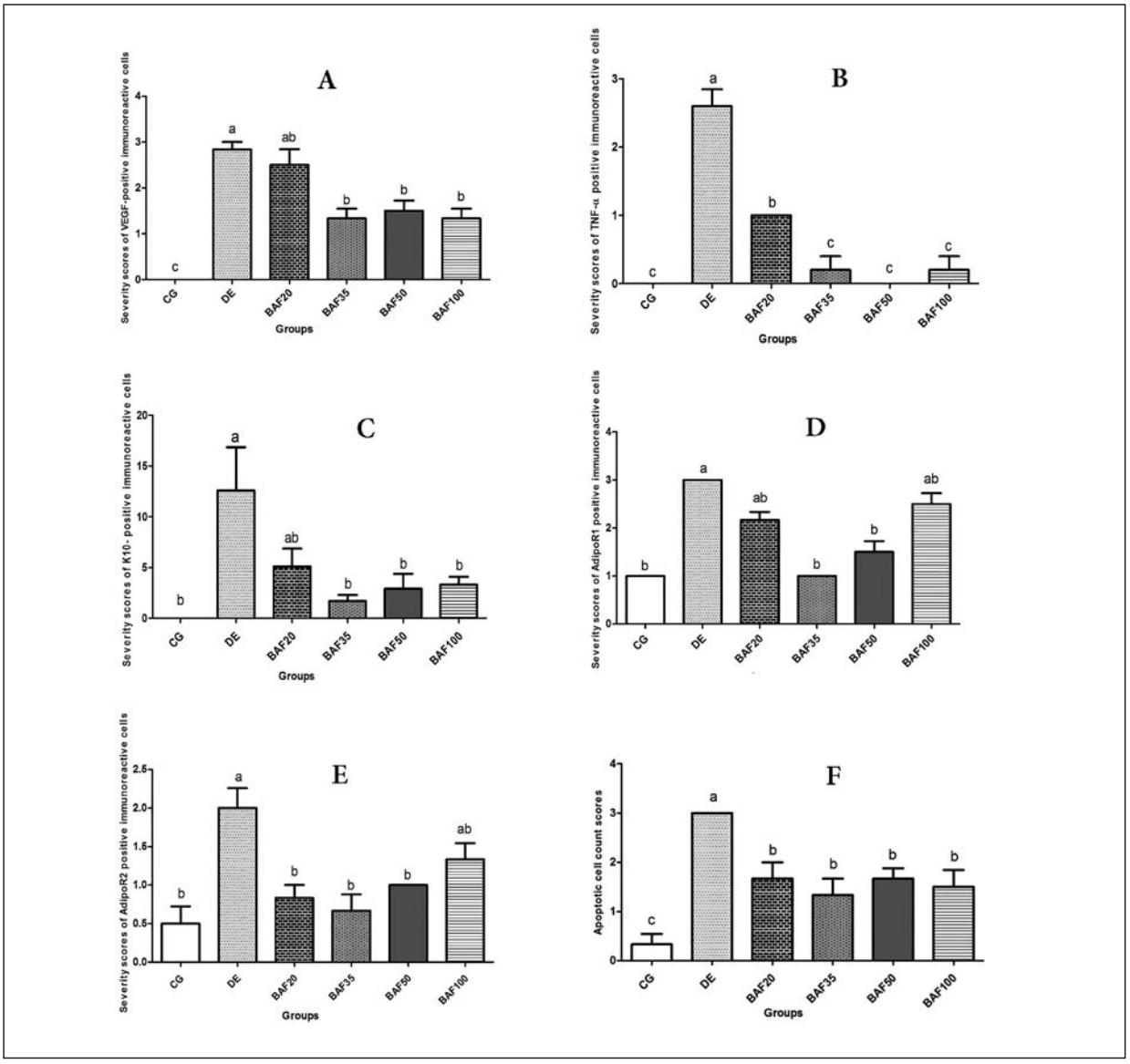

Fig. 3. Statistical bar graph of severity scores of immunoreactive cells in all groups (CG: Control group, DE: Dry eye group). **A.** VEGF, **B.** TNF-α, **C.** K10, **D.** AdipoR1, **E.** AdipoR2, **F.** TUNEL. *a-c: The difference between the groups with the different letters in the columns is significant ($P < 0.05$). (n=3 x 6 groups, mean ± standard deviation).

production in experimental studies using small laboratory and exotic animals[29,30]. This test was also used in the present study. Mean EAPPTT recorded in the initial measurements (baseline) of all subjects showed a statistically significant decrease tear rate in all the experimental groups in comparison to the tear rate at the time (14 days) of the first measurement after BEC administration. These data show that BEC causes a significant reduction in aqueous tear production. This reducing trend, which continued until the end of the study, especially in DE group, indicates that BEC suppresses the amount of aqueous tear production as long as it is applied. In murine DE models, where DE was induced twice daily with 0.2% BEC for 14 days[59] and with 0.1% BEC for 10 days[55], and where phenol red cotton thread test (PRTT) was used to measure aqueous tear production, this agent was found to significantly reduce aqueous tear production. Similar results

have been obtained in other DE model studies using BEC[23,36,54]. In the present study, when the treatment groups were evaluated according to the aqueous tear production parameter, it was found that the mean EAPPTT increased continuously for all the groups, except for the BAF20 group, at all measurement times after 15 days, and the difference between these groups at the last measurement (30th day) was not statistically significant. In a DE model investigating the therapeutic effects of 3 different concentrations of human amniotic fluid (20%, 50% and 100%), it was reported that all concentrations significantly increased aqueous tear production within 2 weeks; however the best result was obtained in amniotic fluid concentrations of 50% and 100%[45]. A similar result was obtained in another study using pure HAF; that study reported that pure HAF significantly decreased the corneal fluorescein score[44]. In the present study, as in the HAF study, the moderate and high concentrations of BAF (35%, 50%, and 100%) were found to have the most beneficial effect on the amount of aqueous tear production.

Evaluation of PTF stability is important, especially in revealing mucin and lipid tear deficiencies and determining dysfunction of meibomian gland[47,52]. In many DE model studies[11,19,32,36,51,54,55,59] tear film stability has been evaluated using TBUT. In a DE model in which 0.2% BEC was administered topically for 7 days in mouse eyes, it was found that TBUT gradually decreased in the experimental group in comparison to the CG, and the difference between these two groups was statistically significant[36]. Similar results were obtained in other DE model studies where 0.1% BEC was applied topically for 10 days[55] and 0.2% BEC for 14 days[59]. In the present study, mean TBUT was recorded as 7.00–7.37 sec in the baseline measurement in all the subjects, which decreased to 2.15–2.69 sec at the first measurement time after 14 days of BEC application. These data show that BEC impairs PTF stability in subjects. Mean TBUT measured on day 15 in all treatment groups was observed to be almost twice as long on day 30, the last measurement time of the study. Xiao *et al.*[55] found that the human amniotic membrane prolongs TBUT in aBEC induced DE model. Our study found beneficial effect on TBUT in all groups, with the longest TBUT value in the BAF50 group.

The corneal fluorescein staining test is routinely used to determine epithelial damage, PGF quality and ocular surface abnormalities[39]. Many DE studies on animals have reported that DE causes tear film instability, ocular inflammation, conjunctival goblet cell loss, and corneal neovascularization as well as corneal epithelial cell shedding and corneal erosions and ulceration[19,36,56,59]. Xiao *et al.*[54] determined that the corneal fluorescein staining scores increased in the first measurements of the subjects received 0.2% BEC twice a day for 14 days, the scores gradually started to decrease after BEC administration was discontinued. Using the same model, our study found that the corneal fluorescein staining score significantly increased in all the experimental groups at the first measurement time after administering BEC for 14 days in comparison to the baseline measurement. After stopping the BEC application and starting the BAF treatment, the corneal fluorescein staining scores decreased continuously until the end of the study in all treatment groups except for the BAF20 group. In the terms of the last measurement, the difference in the corneal fluorescein staining scores between the BAF35, BAF50, and BAF100 groups was not significant. Similar to our results, Quinto *et al.*[45] found that moderate and high density human amniotic fluid reduced the corneal fluorescein staining scores more effectively than a20% of BAF.

Ocular surface inflammation plays a key role both as a cause and a result of the pathogenesis of the DE cycle[7,36,59]. Experimental studies have revealed inflammatory cell infiltrations and increased levels of inflammatory cytokine and chemokine (IL-1α-β, TNF-α, VEGF) in the lacrimal tissues and ocular surface epithelium of DE

patients[7,13,59,60]. Recently, many researchers who recognized the importance of the inflammatory process on DE pathogenesis have focused on measuring the levels of inflammatory cytokines and chemokine in the ocular tissues of the subjects using different techniques[19,31,58]. In the present study, the WBlot and IHC analyses showed that the DE group had the highest VEGF and TNF-α levels, suggesting that BEC induces an inflammatory reaction in corneoconjunctival tissues. As a matter of fact, the lowest VEGF and TNF-α levels in the treatment groups were recorded in the BAF35 group in the WBlot analysis and in the BAF35, BAF50, and BAF100 groups in the IHC analysis. These data from the treatment groups indicate that moderate and high concentrations of BAF have a positive effect on the inflammatory process of DE. It has also been suggested[19] that this increase in the concentrations of the cytokines may accelerate the development of squamous metaplasia via inflammatory reaction. Squamous metaplasia, conversion of corneal epithelial cells to keratinizing cells or non-keratinized epithelium into squamous epithelium, is considered to be one of the important symptoms of chronic inflammatory diseases, such as DE[19,31,36]. The presence of squamous metaplasia is demonstrated by increases in the level of K10, an epidermis-specific cytokeratin[19,55]. Recent DE model studies using different measurement techniques[19,55,59] showed that the K10 levels were significantly higher in the corneal epithelium of the DE groups comparison to the controls. In the present study, the highest K10 levels in the corneoconjunctival tissue were recorded in DE, BAF20, and BAF100 groups using the WBlot technique and in the DE and BAF20 groups using IHC staining. As reported in previous studies[36,55,59] the results indicate that BEC-induced DE causes squamous metaplasia in the corneal epithelium of the subjects. In the present study, the lowest K10 levels were measured in the CG and the BAF35 groups according to WBlot analysis and in the BAF35, BAF50, and BAF100 groups according to IHC analysis. When the results of both analyses was evaluated together, the K10 levels were found to decrease significantly in all the treatment groups, except the BAF20 group.

Adiponectin, a protein that secrets from adipose tissues and is abundant in plasma, has anti-inflammatory, anti-diabetic, and anti-angiogenic properties in different tissues[33,34]. From this protein, three different receptors have been identified: AdipoR1, AdipoR2, and T-cadherin[20,34]. Katsiougiannis *et al.*[20] reported that inflammation accelerates adipose and fibrotic tissue formation in the lacrimal glands and ocular tissue; and this suggests that adipocytes may be an important marker in the defense or repair repertoire of these tissues. In the present study, the WBlot and IHC analyses showed that the DE group had the highest AdipoR1 and AdipoR2 levels, which supports the claims of Katsiougiannis *et al.*[20] that adipocyte density increased in cases of inflammation. When the WBlot and IHC analyses were evaluated together, the AdipoR1 and AdipoR2 levels were lower in the BAF treatment groups than the DE group. These results indicate that BEC has an inflammatory effect on the ocular surface and that different concentrations of BAF suppress this inflammatory process to varying degrees.

It is suggested that the inflammatory process that plays a key role in DE pathogenesis and the subsequently development of squamous metaplasia induces epithelial apoptosis[19,36]. Apoptosis positive cells have been reported in the cornea, the conjunctiva, and the lacrimal glands using the TUNEL method[19,31,55]. Some studies[36,42] have reported that a high concentration of preservative agents, such as BEC, cause necrosis while low concentrations cause apoptosis. In the present study, in all groups, apositive staining response was obtained in the epithelial and stromal layers of the cornea with the highest apoptotic cell score in the DE group and the lowest score in CG; no significant difference in the apoptotic cell score was found between the treatment groups. Moreover, the apoptotic cell

score was lower in the BAF treatment groups than the DE group. Similar results have also been reported in previous studies[19,36,55], which found that low BEC concentrations may lead to epithelial apoptosis on the ocular surface. Furthermore, the efficacy of all the BAF concentrations against epithelial apoptosis in which we compared the therapeutic effects supports the view expressed Lin *et al.*[36] that suppression of apoptosis is important in the treatment of ocular surface disorders.

In the present study, all the parameters, including the clinical tests and observations as well as the WBlot, IHC, and TUNEL analyses, demonstrate that moderate to high concentrations of BAF had positive effects on both the molecular and clinical signs of BEC- induced DE. The diagnostic methods used in this study were designed by considering the relationship between the clinical forms of DE and pathogenesis this condition. In two previous DE models[44,45] in which the therapeutic effect of HAF was investigated, the diagnosis was largely established based on the results of clinical tests; however, in addition to these tests, the present study used specific complementary molecular tests, such as IHC, WBlot, and TUNEL, to implement an inclusive diagnostic strategy to investigate the therapeutic effect of BAF, expecting it to be an alternative biological agent to HAF. This approach allowed the results obtained from each measurement technique to be interrelated and supportive, thus enabling a broader evaluation of the study.

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