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Evaluation of the Effects of Different Therapeutic Agents on Experimental Dry Eye (DE) for the Purpose of Ocular Surface Impairments in Mice

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Abstract: Dry Eye Syndrome (DES) may impair ocular surface integrity, damaging corneal and conjunctival epithelial cells which play an important role on ocular surface health. The present study intended to evaluate the effects of different therapeutic agents on ocular surface impairments in Experimental Dry Eye Model. In the study, 112 BALB-C breed female mice were allocated equally as well as randomly to two groups, Control (Cont.) and Experimental (Exp.) with 8 subgroups within themselves. Cont. and Exp. subgroups were called as Formal Saline (SF), Sodium Hyaluronate (SH), Diclofenac Sodium (DS), Olopatadine (O), Retinoic Acid (RA), Fluoromethanole (FML), Cyclosporine-A (CsA) an Doxycycline Hyclate (DH) according to agents administrated. Exp. groups were kept in dry eye cabinet, Cont. groups were maintained at room conditions during 6 weeks. While all animals received no agents between 0 (baseline) to 2 weeks, above mentioned agents were administrated topically to their right eyes twice a day, 5 μL per time between 2-6 weeks. The effects of these agents were evaluated at the 0, 2, 4 and 6 weeks in terms of corneal fluorescein staining and clearance as well as impression cytology and additionally on the 6 weeks with regard to corneal fluorescein permeability. The difference between Cont. and Exp. groups as regards corneal fluorescein and clearance including impression cytology were determined as non-significant ($p>0.05$) for 0 week (baseline) however, it was significant ($p<0.05$) for 2, 4 and 6 weeks ($p<0.05$). When the therapeutics were evaluated to all parameters considering variables such as subgroups and measuring times, CsA in terms of corneal fluorescein and permeability and DS in terms of tear clearance and impression cytology were detected to be the most effective agents. In conclusion, it was determined that ocular surface impairment caused by DES may be markedly prevented by immunomodulator efficacious CsA and anti-inflammatory efficacious DS.

Key words: Mice, evaporative dry eye, diagnosis, treatment, fluorescein, Turkey

INTRODUCTION

Dry Eye Syndrome (DES) is a multifactorial disorder characterized by inflammation, tear film hyperosmolarity and instability and vision impairment with having a potential to induce ocular surface damage (Anonymous, 2007). Its development may be due to some risk factors including age (Gelatt, 1991), gender (Foulks, 2008), environment (Kjaergaard *et al*., 2004; Wolkoff and Kjergaard, 2007), hormones (Foulks, 2008), nourishment (Rashid *et al*., 2008), systemic and local effective drugs (Erdem *et al*., 2007; Foulks, 2008) and refractive surgery (Ang *et al*., 2001). DES is more common in women and the elderly amongst humans (McCabe and Narayanan, 2009) and dogs within animal species (Hartley *et al*., 2006).

In DES, ocular integrity is apt to impairment owing to damaging of the corneal and conjunctival epithelial cells which play an important role on ocular surface health (Fahim *et al*., 2006). For determining such this impairment, corneal fluorescein staining (Savini *et al*., 2008), clearance (Macri *et al*., 2000) and permeability (McNamara *et al*., 1997) tests along with impression cytology (Altinors *et al*., 2007) are used.

DE Models are frequently applied for investigating the factors playing a crucial role on its pathogenesis and alternative treatment modalities (Dursun *et al*., 2002; Suwan-apichon *et al*., 2006; Altinors *et al*., 2007). For this purpose, models such as mechanical (Moore *et al*., 2001), hormonal and neural (Altinors *et al*., 2007) inhibitions of lacrimal secretion as well as evaporative DE (Barabino and Dana, 2004; Chen *et al*., 2008) have been tried.

DES treatment is mainly based on either prevention of the clinical signs or removals of the factors primarily inflammations that are believed to play an important role on the disease pathogenesis or both (Foulks, 2008; Gayton, 2009). The symptomatic treatment modalities administered for alleviating the clinical signs of DES usually continue throughout life span which is

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cumbersome for the patients and animal owners and imposes an important cost on the country economy. Therefore, recent clinical (Avunduk *et al*., 2003; Yang *et al*., 2006) and model works (Dursun *et al*., 2002; Altinors *et al*., 2007; Chen *et al*., 2008) appeared to have been focused commonly on the factors such as inflammation, hyperosmolarity and evaporation effective on the disease processes. The agents including corticosteroids (Yang *et al*., 2006), Nonsteroid Anti-Inflammatory Drugs (NSAIDs) (Avunduk *et al*., 2003), immunosuppressive or immunomodulators (Kunert *et al*., 2002), tetracyclines (Quarterman *et al*., 1997), vitamins, essential fatty acids (Rashid *et al*., 2008), sexual hormones (Mathers *et al*., 1998), authologue serum (Poon *et al*., 2001), mast cell stabilizators (Mah and Kim, 2008) and botulinum toxins (Keegan *et al*., 2002) are administered. The present study was aimed at comparing the therapeutic effects of Sodium Hyaluronate (SH), Diclofenac Sodium (DS), Olopatadine (O), Retinoic Acid (RA), Fluoromethanole (FML), Cyclosporine-A (CsA) and Doxycycline Hyclate (DH) on ocular surface damage in evaporatively induced DE and to determine the most relevant one for this particular purpose.

MATERIALS AND METHODS

The materials of the study consisted of 112 BALB-C breed female mice of 8 weeks old, provided by Experimental Research Center of Firat University. The study was initiated on an official approval for experimental animal use from local ethic board (Protocol num: 2011/09-119). All experiments on animals have been conducted adhering strictly to ethic principles for use and care of experimental animals. The animals had an *ad libitum* access to food and water throughout the experiment.

The animals were allocated equally and randomly to Cont. and Exp. groups which were also divided within themselves into 8 subgroups of 7 animals each. These subgroups included SF (0.9% formal saline, Fizyol, Vilsan), SH (0.3% Sodium Hyaluronate, Artelac Advanced, Abdi Ibrahim), DS (0.1% Diclofenac Sodium, Inflased, Bilim), O (0.1% Olopatadine, Patanol, Alcon), RA (0.01% Retinoic Acid, Sigma and Aldrich), FML (1% Fluoromethanole, FML, Abdi Ibrahim), CsA (0.05% Cyclosporine-A, Restasis, Abdi Ibrahim) and DH (0.025% Doxycycline Hyclate, Multigen).

Exp. group animals were placed in a specially designed Dry Eye Cabinet (DEC) (Temperature 22.5±0.4°C, RH 25.1±0.61%, air flow rate 15 L min^{-1}, air current rate 2.3±0.5 m sec^{-1}) during 6 weeks of the experiment. Air turbulence within the cabinet was further accelerated with a couple of air fans (1200±250 RPM, 50 CFM) installed in the Experiment Animal Division (EAD) (Fig. 1). Cont. group subjects were kept in cages of a room with RH of 50-80% and temperature between 21-23°C during the study.

On the 1st 2 weeks of the study (0-2 weeks), Exp. subgroup subjects were left in EAD just being exposed to evaporative stress but during the same period Cont. subgroups remained in their cages without any applications. Then, the right eyes of Cont. and Exp. subgroups subjects was instilled 5 μL the agents mentioned above twice a day between 2-6 weeks. During this period, the subjects in EAD continued to be exposed to evaporative stress.

Both Cont. and Exp. groups were evaluated in corneal fluorescein staining, tear clearance and impression cytology on 0, 2, 4 and 6 weeks. After completion of the required tests on the 6th week, all subjects were euthanazied with carbon monoxide gas and their treated eyes were removed and the corneal tissue were analyzed in terms of corneal fluorescein permeability test.

For Corneal Fluorescein Staining test, 1 μL 1% flourescein sodium (Bereket Kimya Lab. Istanbul, Turkey) was instilled into the right eyes of the subjects, 10 min latter the stained corneas were examined with a slit-lamp biomicroscope (XL-1, Shin Nippon, Japan) under cobalt blue filter light and lesions determined were photographed with Operation Microscope (OPMI 1-FR, Zeiss, Germany). The corneal surface was graded as no (0), 1/8 or less (1), 1/4 or less (2), 1/2 or less (score 3, Fig. 2) and more than an half or entire (score 4, Fig. 3) corneal surface stained.

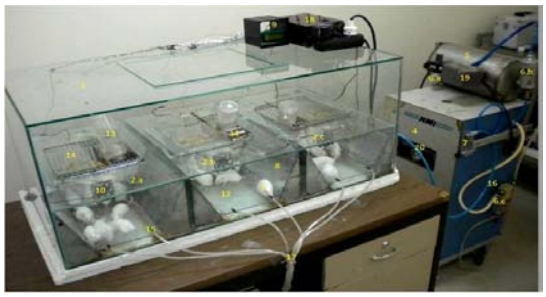

Fig. 1: Appearance of DE Model; 1: DEC (Dry Eye Cabin); 2 (a-c): EAD (Experimental Animal Division); 3: The subjects; 4: AC (Air compressor); 5: AC tube; 6 (a-c): Water separator; 7: Flowmeter; 8: Desiccators' containers, 9: Control board; 10: Fan; 11: Humidity-Temperature monitor; 12: Mobile glass plates; 13: Watering; 14: Feeder; 15: Air inlet holes; 16: Pneumatic pipes; 17: Flexible inhalation hose; 18: Fan switch board; 19: AC pressure monitor; 20: AC pressure gauge

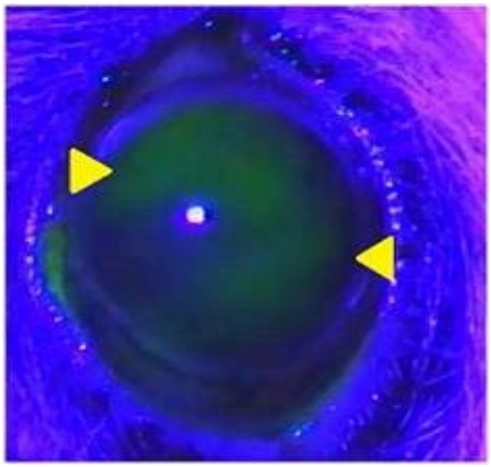

Fig. 2: Appearance of corneal fluorescein staining pattern (score 3) in an Exp.-SF case at the 4th week of experiment

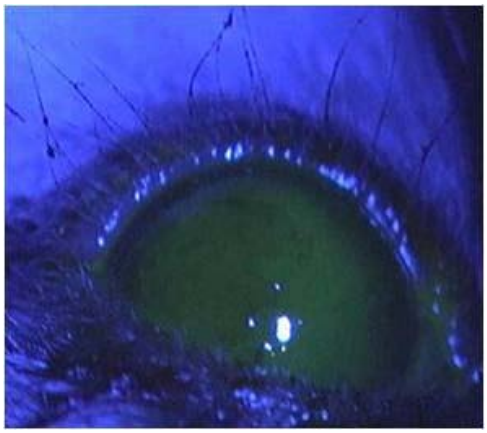

Fig. 3: Appearance of corneal fluorescein staining pattern (score 4) in an Exp.-SF case at the 4th week of experiment

Tear fluorescein clearance was applied as described by Dursun *et al.* (2002). In short, 1 μL 1% fluorescein sodium was instilled into the right eye of each subject and 15 min later an absorbent paper point (Absorbent paper points, Sure Dent Corporation, Korea) was placed gently between the lower lid and the globe near the lateral cantus for 1 min. The tear soaked point was moved to a labeled eppendorf tube that was kept in a dark container until being fluorophotometrically analyzed. For analysis, 100 μL phosphate buffer solution was added to the tube which was centrifuged at 12000 rpm for 5 min and the solution was moved to 384 microplates (QG Hellma, Germany) that was assessed fluorophotometrically between 485-530 nm using a fluorescence multiple reader (FluOSTAR, BMG Lab. Tech. Germany). The data were recorded as FU μL^{-1}. For impression cytology, conjunctival tissue sample from each subject was obtained from the inferior fornix of its right eye with nitrocellulose filter paper strip (Nitrocellulose/Filter Paper Sandwich, Invitrogen, USA) with 0.45 μm porous size. The strip was placed to the inferior fornix through the lateral cantus approach and pressed gently against the globe for 2-3 sec for conjunctival surface epithelial cells to adhere. This strip was processed according to the technique of Nelson *et al.* (1983). The preparations were examined under a digital microscope with x100 magnification. For each preparation, four randomly selected areas were photographed, their goblet cell numbers were counted and the result was recorded as mean.

Corneal fluorescein permeability test was performed at the end of the 6th week. The subjects were euthanized with carbon monoxide gas 10 min after instilling 1 μL 1% fluorescein sodium into their right eyes. Their corneas were harvested and washed twice with balanced salt solution and placed in 1.5 mL eppendorf tubes Cont.aining 200 μL form the same solution which were then protected from light until being analyzed. Prior to analysis these samples were left in an ultrasonic shaker (Elmasonic S30 H, Elma, Germany) during 90 min and the solution extracted was centrifuged at 12000 rpm for 5 min and analyzed fluorophotometrically as it was in Tear Fluorescein Clearance test.

Statistical analysis was carried out using SPSS 13.0 (Statistical Package for the Social Sciences for Windows, SPSS Inc., Chicago, IL, USA) Version. The differences within subgroups in terms of measurement times were assessed using Friedman test applied commonly for non-parametric and repeated measures. When a significant difference between subgroups was found, Wilcoxon test was applied further to determine the difference between which measurement times to exist. The differences between the same subgroups of Cont. and Exp. or those within their own subgroups for each measurement time were analyzed with Tukey test from ANOVA. It was considered as significant at $p<0.05$ or $p>0.05$ with 95 confident interval.

RESULTS AND DISCUSSION

The differences between Cont. and Exp. groups in terms of corneal fluorescein staining and clearance as well as impression cytology were significant at all measurement times except for the baseline values (0 week) ($p<0.05$). According to corneal fluorescein permeability test, the difference was also found to be significant ($p<0.05$).

When the values of Corneal Fluorescein test for the same agent was tested between the subgroups of Cont. and Exp. groups the presence of significant differences between DSs, Os, RAs CsAs and DHs in 2 weeks; SFs and SHs in 4 and 6 weeks ($p<0.05$) were determined.

According to Impression Cytology test, no significant difference ($p>0.05$) was determined between Cont. and Exp. groups when the data of the same agent obtained at the first measurement period were evaluated. In this respect, other three measurement periods presented a significant difference at $p<0.05$. From these results, it was determined that mean goblet cell counts of Cont. subgroups were higher than those of Exp. subgroups. When each subgroup data of Cont. group were evaluated taking various measurement periods into account the difference was found to be non-significant ($p>0.05$) but that was significant for Exp. groups ($p<0.05$) when the data were analyzed for the same content.

Tear Fluorescein Clearance test data demonstrated the presence of significant differences between all subgroups of the same agent of Cont. and Exp. in 2 weeks ($p<0.05$) and all ($p<0.05$) except CsA ($p>0.05$) for 4 and 6 weeks. Also the mean data of all subgroups belonging to all agents were greater in Cont. than in Exp. group. When Cont. subgroup data were evaluated in alterations within themselves regarding measurement times, the differences in Cont. group were significant for O and FML ($p<0.05$). This difference for O was present between weeks 0-2, 0-4, 2-4 and 2-6 and weeks 0-4 and 0-6 for FML. In case of Exp. group however, this difference ($p<0.05$) existed in all subgroups.

It was detected that in the sense of corneal Fluorescein Permeability test there was statistically significant difference ($p<0.05$) between the total mean values of both Cont. and Exp. groups. The data of subgroups of these two were compared within themselves Exp.-SF was seen to be the highest (359.29±108.42) in contrast to the lowest value (67.86±15.68) of Cont.-DS. When the data of an agent of Cont. groups was compared to those of the Exp. group, the marked difference ($p<0.05$) was observed to exist between SFs and RAs. In other words, these two agent values were higher in Exp. group than Cont. one.

Corneal Fluorescein Staining is a test to have been used commonly in many clinical (Avunduk *et al*., 2003; Yang *et al*., 2006) and model (Dursun *et al*., 2002; Lekhanont *et al*., 2007a, b) studies for specification of ocular surface damage occurring during DES process. According to the result of this test in the present study, it was seen an increase in mean corneal staining score (2.05±1.17) in 2 weeks in reference to baseline value (0 week) (0.35±0.46) in all Exp. subgroups. The data of Exp. subgroups in the mentioned period compared to those of the Cont. group indicates the development of ocular surface damage in the first subgroups being exposed to evaporative stress. Similar results have been reported by other model works (Barabino *et al*., 2005; Chen *et al*., 2008).

A persisting increase in corneal fluorescein score mean in Exp.-SF subgroup in 4 weeks, a first measurement time after 2 weeks of administration of therapeutic agents indicates that evaporative stress condition became more aggressive in due course. Apart from Exp.-SF, Exp.-SH subgroup also demonstrated an increase in corneal staining areas. These findings imply that SF and SH may have no effect on corneal epithelial healing during 2 weeks in the subjects received them. A similar result related to the effect of SH has also been reported by Aragona *et al*. (2002) who have stated that SH produce no positive effect on corneal fluorescein in short period.

Corneal fluorescein score mean was found to reduce in all other subgroups beyond Exp.-SF and SH ones. In parallel to the results of this study FML (Yang *et al*., 2006; Lekhanont *et al*., 2007a), RA (Kim *et al*., 2009), CsA (Kunert *et al*., 2002) and DH (Lekhanont *et al*., 2007b) have been reported to induce a positive whereas DS (Lekhanont *et al*., 2007b) no responses on the parameters mentioned in short period. However, to date no report exists on the effect of O in short or long period.

The present study determined that corneal fluorescein score mean in Exp.-SF subgroup continued to increase along the experiment with being reached at its highest level in 6 weeks, the last measurement time (Table 1). However, during this period in the remaining subgroups this score decreased. These findings as in those of Barabino *et al*. (2005) and Chen *et al*. (2008) show that the evaporative stress in DEC became more effective as the time advanced.

When the effects of different therapeutic agents with regard to corneal fluorescein staining score were evaluated considering variables such as subgroups and measurement time CsA was observed to present the most positive outcome. CsA, an immunomodulator, immunosuppressive and anti-inflammatory agent may produce this action preventing apoptosis (Tatlipinar and Akpek, 2005) and cytoplasmic transcription factors (Fukushima *et al*., 2006) required for inflammatory cytokines and T cell activation. In their DE Model work, Lekhanont *et al*. (2007a) found marked improvement in corneal fluorescein staining areas in the subjects treated with CsA. In addition to the findings of the former study Kim *et al*. (2009) in a clinical study have found that this positive action related to this agent occurred in very short period. The same authors including Park *et al*. (2007) and

Table 1: Statistical evaluation of Corneal Fluorescein Staining test in control and experimental subgroups at different measuring periods (p-tests used)

	Measuring times				
Groups	Week 0	Week 2	Week 4	Week 6	p (Friedman-Wilcoxon)
Control					
SF	0	0.43 ± 0.53^{de}	0.14 ± 0.37^{de}	0.29 ± 0.48^{e}	>0.05
SH	0^{B}	0.71 ± 0.48^{bcdeA}	0^{eB}	0^{cB}	<0.05
DS	0	0.29 ± 0.48^{e}	0.57 ± 0.53^{bcde}	0.29 ± 0.48^{e}	>0.05
O	0	0.14 ± 0.37^{e}	0.29 ± 0.48^{cde}	0.14 ± 0.37^{c}	>0.05
RA	0	0.29 ± 0.48^{e}	0.43 ± 0.53^{cde}	0^{c}	>0.05
FML	0	0.57 ± 0.53^{cde}	0.29 ± 0.48^{cde}	0.57 ± 0.53^{c}	>0.05
CSA	0	0.14 ± 0.37^{e}	0.29 ± 0.48^{cde}	0.29 ± 0.48^{e}	>0.05
DH	0	0.29 ± 0.48^{e}	0.57 ± 0.53^{bcde}	0.57 ± 0.53^{c}	>0.05
Experimental					
SF	0^{C}	1.43 ± 1.13^{abcdeAB}	1.86 ± 0.69^{abAB}	2.29 ± 0.75^{aA}	<0.05
SH	0^{B}	1.43 ± 0.53^{abcdeA}	2.14 ± 1.21^{aA}	1.71 ± 0.75^{abA}	<0.05
DS	0^{C}	2.00 ± 1.27^{aA}	1.43 ± 0.97^{abcdAB}	0.71 ± 0.75^{bcBC}	<0.05
O	0^{C}	2.00 ± 1.00^{abcdA}	1.29 ± 1.38^{abcdeAB}	0.29 ± 0.48^{BC}	<0.05
RA	0^{C}	3.00 ± 1.81^{aA}	1.14 ± 0.69^{abcdeB}	0.86 ± 0.69^{bcB}	<0.05
FML	0^{C}	2.14 ± 1.46^{abcA}	0.57 ± 0.53^{bcdeB}	0.43 ± 0.53^{cB}	<0.05
CSA	0^{B}	2.14 ± 1.06^{abcA}	0.43 ± 0.53^{cdeB}	0.29 ± 0.48^{cB}	<0.05
DH	0^{C}	2.29 ± 1.11^{abA}	1.57 ± 0.78^{abcAB}	1.00 ± 1.00^{bcBC}	<0.05
P Tukey	>0.05	<0.05	<0.05	<0.05	

Table 2: Statistical evaluation of impression cytology test in control and experimental subgroups at different measuring periods (p-tests used)

	Measuring times				
Groups	Week 0	Week 2	Week 4	Week 6	p (Friedman-Wilcoxon)
Control					
SF	15.57 ± 2.82	15.86 ± 4.33^{a}	16.86 ± 3.33^{a}	16.29 ± 2.81^{a}	>0.05
SH	16.43 ± 2.57	15.71 ± 3.54^{a}	16.43 ± 1.71^{a}	17.29 ± 3.54^{a}	>0.05
DS	17.43 ± 4.07	18.29 ± 2.28^{a}	16.71 ± 3.25^{a}	16.43 ± 1.51^{a}	>0.05
O	18.29 ± 2.98	17.43 ± 2.82^{a}	16.00 ± 1.63^{a}	16.29 ± 3.45^{a}	>0.05
RA	17.43 ± 1.98	16.71 ± 3.03^{a}	17.71 ± 2.56^{a}	16.86 ± 2.67^{a}	>0.05
FML	17.14 ± 2.41	14.86 ± 3.02^{a}	15.86 ± 3.02^{a}	16.29 ± 1.79^{a}	>0.05
CSA	17.14 ± 2.67	15.14 ± 2.61^{a}	17.71 ± 3.30^{a}	14.86 ± 3.23^{a}	>0.05
DH	16.00 ± 1.63	15.57 ± 3.40^{a}	16.71 ± 2.36^{a}	16.71 ± 3.25^{a}	>0.05
Experimental					
SF	17.86 ± 3.02^{A}	5.57 ± 2.14^{bB}	5.29 ± 1.38^{cB}	5.14 ± 1.46^{cB}	<0.05
SH	17.57 ± 1.98^{A}	6.43 ± 1.98^{bC}	6.86 ± 1.57^{bcC}	9.29 ± 2.36^{bcB}	<0.05
DS	17.29 ± 2.56^{A}	6.43 ± 1.98^{bC}	9.71 ± 1.79^{bB}	10.43 ± 1.71^{bB}	<0.05
O	16.14 ± 1.34^{A}	6.86 ± 1.46^{bB}	6.57 ± 1.27^{bcB}	7.86 ± 1.06^{bcB}	<0.05
RA	17.29 ± 3.14^{A}	6.43 ± 1.90^{bC}	8.43 ± 1.27^{bcB}	8.71 ± 1.49^{bcBC}	<0.05
FML	17.43 ± 3.40^{A}	5.43 ± 1.81^{bC}	8.14 ± 1.34^{bcB}	8.86 ± 1.21^{bcB}	<0.05
CSA	18.00 ± 3.51^{A}	6.86 ± 2.41^{bC}	8.86 ± 1.46^{bcB}	9.86 ± 0.90^{bB}	<0.05
DH	16.71 ± 2.69^{A}	6.86 ± 2.11^{bC}	8.43 ± 1.51^{bcBC}	9.29 ± 1.11^{bcB}	<0.05
P Tukey	>0.05	<0.05	<0.05	<0.05	

Different letters in the different measurement times and groups in the same line and the same column indicate significance between them

Kunert *et al.* (2002) have claimed that this agent may generate this therapeutic effect by preventing or reducing ocular surface inflammation. From these it can be concludes that CsA have favorable effect on corneal epithelial impairments, possible due to its preventive action on ocular surface inflammation (Lekhanont *et al.*, 2007a; Kim *et al.*, 2009).

The mucin layer, innermost layer of the tear film prevents ocular surface to dry via protecting the tear film integrity (Perry, 2008). This layer is laid down mainly by conjunctival goblet cells (Davidson and Kuonen, 2004; Perry, 2008). A reduction in conjunctival goblet cells may cause corneal epithelial damage because of tear film instability resulting from the mucin layer disintegration (Davidson and Kuonen, 2004; Altinors *et al.*, 2007; Perry, 2008). Many experimental and clinical studies in human (Sahli *et al.*, 2010) and animals (Altinors *et al.*, 2007) as well as the present one have utilized impression cytology technique to assess goblet cell density.

Barabino *et al.* (2005) reported that conjunctival goblet cell density reduces in a DE Model induced via evaporative stress factors, i.e., high air velocity, low RH and temperature. In the study where the same factors were used, similar results were obtained in all Exp. subgroups in 2 weeks and in just Exp.-SF in 4 and 6 weeks in cytological analysis (Table 2). These findings are supported by the results of another model study (Dursun *et al.*, 2002) induced with high air frequency and anticholinergic agent. The study determined that goblet cell density increased in all agents except O in 4 weeks

and all agents including O in 6 weeks which indicate that O has positive response on goblet cell density in prolonged period (Corum *et al*., 2005). In parallel to the result of the current study, SH (Aragona *et al*., 2002), FML (Avunduk *et al*., 2003; Yang *et al*., 2006), RA (Kobayashi *et al*., 1997) and CsA (Kunert *et al*., 2002) have reported to increase goblet cell number in short period.

DS, a NSAID agent is mainly used for the treatment of ocular inflammation (Lekhanont *et al*., 2007b) and observed to be the most efficient medicine among those used here according to goblet cell density. It may cause this action (Schalnus, 2003) reducing the synthesis of endogenous prostaglandins that initiate inflammatory processes via mediating the inhibition of cyclooxigenase enzyme. It can be deduced from these results that the positive outcome of DS on goblet cell number in mice induced by evaporative stress in DEC can be related to its preventive action on ocular surface inflammatory reaction (Dursun *et al*., 2002; Lekhanont *et al*., 2007b). This assumption appears in the position to support the ideas (De Paiva and Pflugfelder, 2008) that inflammatory processes may have an important role on DES pathogenesis.

The tear film, the most dynamic structure of the functional unit of ocular surface, ensures the clearance, nourishment and lubrication of the ocular surface (Rolando and Zierhut, 2001; McCabe and Narayanan, 2009) and meantime acts as physical and immunological barrier to protect the eye from external influences (Rolando and Zierhut, 2001).

Tear clearance, one of the most important functional units of this structure, plays an effective role on ocular surface health (De Paiva and Pflugfelder, 2004). A stabile PTF (Precorneal tear film) in a healthy eye cleans ocular surface foreign bodies, toxic agents and inflammatory cytokines (Afonso *et al*., 1999). Tear clearance deficiency may be associated with ocular surface inflammation and irritation, epithelial damage and decrease in aqueous tear production (Afonso *et al*., 1999; Dursun *et al*., 2002; De Paiva and Pflugfelder 2004) and this deficiency prompts the cascade of inflammation processes owing to accumulation of cytotoxic factors, proteolytic enzymes and proinflammatory cytokines in tear (De Paiva and Pflugfelder, 2004). Tear Fluorescein Clearance test is utilized for evaluating lacrimal functional unit and PTF quality as well as inflammatory processes in the ocular surface (Afonso *et al*., 1999; De Paiva and Pflugfelder, 2004).

Evaporative stress factors reduce tear quantity which in turn results in PTF instability and reduction in tear clearance (De Paiva and Pflugfelder, 2004). Here, the amount of tear fluorescein in Exp. group was determined to be significantly higher in all subgroups in 2 weeks and SF in 4 and 6 weeks as compared to control (Table 3) indicate that tear amount has reduced which as mentioned (Afonso *et al*., 1999; Dursun *et al*., 2002; De Paiva and Pflugfelder, 2004) lead to decrease in tear clearance. However, in 4 and 6 weeks, apart from Exp.-SF, other agents belonging to the remaining subgroups are seen to have decreased tear fluorescein amount, a condition showing the presence of an inductive action of these agents on tear production rates.

In this study, DS was seen to act most favorably on tear clearance, possible be due to its anti-inflammatory characteristic (Schalnus, 2003). This result confirms the idea (De Paiva and Pflugfelder, 2008) that inflammation plays an important role on DES pathogenesis and also reduction in tear clearance induced inflammation (De Paiva and Pflugfelder, 2004).

Table 3: Statistical evaluation of tear clerance test in control and experimental subgroups at different measuring periods (p-tests used)

	Measuring times				
Groups	Week 0	Week 2	Week 4	Week 6	p (Friedman-Wilcoxon)
Control					
SF	300.57±41.84	258.14±78.64^{c}	323.00±114.15^{ef}	376.43±119.13^{g}	>0.05
SH	300.00±63.20	360.14±100.85^{c}	284.57±57.32^{f}	326.29±141.64^{g}	>0.05
DS	286.00±85.90	322.43±86.45^{c}	403.14±119.85^{ef}	374.00±148.98^{g}	>0.05
O	261.14±44.46^{B}	326.43±83.49^{cB}	513.14±154.96^{efA}	520.57±226.79^{efgA}	<0.05
RA	316.43±59.97	293.00±52.04^{c}	385.86±112.40^{ef}	376.43±109.79^{g}	>0.05
FML	295.29±50.13^{B}	352.00±86.31^{cAB}	395.43±66.98^{efA}	426.71±98.94^{fgA}	<0.05
CSA	354.00±56.55	360.86±87.07^{c}	333.57±155.14^{ef}	463.57±154.66^{fg}	>0.05
DH	308.43±68.62	313.71±109.03^{c}	387.71±127.28^{ef}	291.14±47.26^{g}	>0.05
Experimental					
SF	296.29±90.02^{C}	2934.43±559.16^{aB}	4083.00±1105.32^{aA}	4415.00±799.21^{aA}	<0.05
SH	291.43±78.96^{D}	2787.57±729.59^{abA}	2286.86±568.97^{bB}	1494.43±432.81^{bC}	<0.05
DS	256.71±57.44^{D}	2575.43±684.17^{abA}	1510.43±464.99^{cB}	884.71±216.43^{defC}	<0.05
O	294.43±68.14^{D}	2502.00±406.28^{abA}	2045.00±235.68^{bcB}	1467.86±233.81^{bcC}	<0.05
RA	234.86±83.99^{D}	2548.43±417.80^{abA}	1891.14±279.83^{bcB}	1230.57±133.33^{bcdC}	<0.05
FML	277.86±54.07^{D}	2444.00±325.19^{abA}	1561.57±368.23^{cdB}	984.86±113.54^{cdeC}	<0.05
CSA	247.43±80.10^{D}	2209.86±324.65^{bA}	1012.29±115.49^{deB}	630.43±82.28^{efgC}	<0.05
DH	293.14±59.54^{D}	2444.71±329.18^{abA}	1640.29±320.55^{bcdB}	1144.00±176.48^{bcdC}	<0.05
P Tukey	>0.05	<0.05	<0.05	<0.05	-

Different letters in the different measurement times and groups in the same line and the same column indicate significance between them

Table 4: Statistical evaluation of Impression Cytology test in control and experimental subgroups at different measuring periods (p-tests used)

Groups	Week 6
Control	
SF	74.14±14.31^{de}
SH	104.86±18.50^{bcde}
DS	67.86±15.68^{e}
O	100.00±23.38^{bcde}
RA	72.00±18.62^{e}
FML	81.86±17.39^{de}
CSA	72.57±9.46^{e}
DH	77.71±16.84^{de}
Experimental	
SF	359.29±108.42^{a}
SH	217.29±85.29^{b}
DS	142.43±40.79^{bcde}
O	171.29±39.36^{bc}
RA	194.86±49.45^{b}
FML	150.00±41.52^{bcde}
CSA	105.29±21.22^{bcde}
DH	158.29±52.93^{bcd}
P Tukey	<0.05

Different letters in the different measurement times and groups in the same line and the same column indicate significance between them

Corneal epithelial cells maintains ocular surface health establishing a barrier to restrict the passage of macromolecules, pathogens and hydrophilic substances (Fahim *et al*., 2006). In case of damage of these cells, corneal epithelial integrity breaks up, resulting in an increase in corneal permeability that allows an easy excess of foreign bodies to ocular surface (McNamara *et al*., 1997). For determining the degrees of clinical and subclinical changes in corneal epithelium, fluorophotometric analyses are usually utilized to test corneal barrier function (Watsky *et al*., 1989). Corneal permeability increases in DES cases (McNamara *et al*., 1997) and is tested measuring amount of fluorescein absorbed by impaired cornea in this present study as established by Dursun *et al*. (2002).

The presence of a positive correlation between corneal permeability rate and the degree of corneal damage is reported (Mc Namara *et al*., 1997; Fahim *et al*., 2006). According to Corneal Permeability test CsA was the most effective agent (Table 4). Lekhanont *et al*. (2007a) and Park *et al*. (2007) noted that CsA may have a preventive effect on corneal surface impairment, a idea also supported by the corneal staining test score of the present study. CsA may generate this effect increasing tear production rate (Lekhanont *et al*., 2007a) or reducing epithelial cell apoptosis (Fukushima *et al*., 2006).

CONCLUSION

When the therapeutic agents were evaluated as a whole considering variables such a subgroups and measurement times, the most effective agent according to Corneal Fluorescein and Permeability test was detected to be CsA and regarding tear clearance and impression cytology tests to be DS. Familiar and prevalent effects of these two agents show that inflammation plays a key role on the pathogenesis of DES and thus it was suggested that these types of anti-inflammatory drugs should be considered when its treatment is the case.

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