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Studies on the Effectiveness of Ozone Therapy on the Treatment of Experimentally Induced Keratitis with *Candida albicans* in Rabbits

Kemal Varol[a], Ayşe Nedret Koç[b], Latife Çakır Bayram[c], Hatice Arda[d], İhsan Keleş[e], Metin Ünlü[d], Vehbi Güneş[e], Gencay Ekinci[e], İlknur Karaca Bekdik[e], and Mustafa Altay Atalay[b]

[a]Department of Veterinary, Food, Agriculture and Livestock Vocational College of Burdur, University of Burdur Mehmet Akif Ersoy, Burdur, Turkey; [b]Department of Microbiology, Medical Faculty, Erciyes University, Kayseri, Turkey; [c]Department of Pathology, Faculty of Veterinary Medicine, University of Erciyes, Kayseri, Turkey; [d]Department of Ophthalmology, Medical Faculty, Erciyes University, Kayseri, Turkey; [e]Department of Internal Medicine, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Turkey

ABSTRACT

Purpose: In the present study, antifungal activity of ozonated olive oil (OZO) and ozonated distilled water (ODW) in the treatment of experimentally induced keratitis with *C. albicans* in rabbits were investigated.
Methods: The Groups were composed of as 1, 2, 3, 4, 5, 7 (n = 5 rabbits, 10 eyes/in each group) and Group 6 (n = 10 rabbits, 20 eyes/in the group). Fourty-eight hours after *C. albicans* inoculation; Group 1 received fluconazle (FLU)+OZO drops, Group 2 received FLU drop, Group 3 received OZO drop, Group 4 received FLU+ODW drops, Group 5 received ODW drop, Group 6 (infected control group) and Group 7 received PBS drop (negative control group). Treatment continued in all groups for 22 days for every 8 hours.
Results: Cornea cultures made 24 days post inoculation revealed statistically significant differences ($p < 0,05$) with concern to *C. albicans* amounts between Group 6 and Group 1–5. Statistical comparison of corneal opacity and corneal ulcer and conjunctivitis values among the Group 6 and Group 1–5 were also different significantly ($p < 0,05$) on days 20 and 24 post inoculation.
Conclusion: OZO and ODW were found to be effective in treating *C. albicans* keratitis in the present study. It has also been proven by this study that ODW contain 26 µg/ml was the most effective in the treatment of *C. albicans* keratitis.

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INTRODUCTION

Corneal diseases are one of the most important causes of loss of sight after cataracts. In the world, the most common cause of unilateral blindness is infectious keratitis.[1] Since the clinical features of the disease in corneal ulcers can mimic other infectious keratitis, it may not be initially determined whether the agent is bacterial or fungal. In fungal keratitis, losses of sight results are often worse than other infectious keratitis. Accordingly, misdiagnosis and improper treatment are common problems. The most common causes of fungal keratitis etiologically are Candida and Aspergillus species. There are studies showing that the frequency of the disease has increased during the last 40 years.[2–5]

Many antifungal drugs used in the treatment of superficial mycoses are effective. However, they are less effective in treating deep fungal infiltrates. They are disappointing when treating keratomycosis due to limited tissue penetration and the lack of wide antimicrobial spectrum and toxic effects of existing antifungals.[6] In addition, excessive use of drugs in infectious diseases, short-term treatment has led to the development of resistance to fungal strains and has led to the search for new antimicrobial agents and new treatment methods.[7]

One of these new treatment methods is ozone therapy.[8] Ozone is a chemical compound made up of three oxygen atoms. Ozone is used for disinfection of drinking water and dirty water with its strong oxidant effect. No bacterial resistance, no pollution of the atmosphere has been reported about ozone up to date. Ozone; inactivates bacteria, viruses, fungi, yeast and protozoa.[9] It has also a fungicide effect. In this way, it inhibits the growth of candida.[7] Ozone has analgesic and anti-inflammatory effects too. Different forms of ozone have recently been used in a wide range of medical indications. Especially the application of ozone gas in the form of locally ozonated water and perioxic oil is common. Ozonated water has gained much importance these days. Ozone is present in water as a molecule. Ozonated water is obtained by ozonizing pure water at room temperature for at least 5 to 20 minutes with an ozone concentration of 60–120 µg/ml.[10] During this process, ozone is directly bubbled into water. Saturating water with gaseous ozone depends on the water volume, gas flow and temperature. Ozone has a short half-life in water. Therefore, water should be used immediately after ozonation.[9,10] Peroxic oils are formed by mixing medical oil with ozone gas. Disinfectant properties of ozone are also demonstrated in the use of ozone vegetable oil. Ozonated oil should be stored in a dark opaque container. It can be stored for 4 months at room temperature. In the fridge, this period is 2 years.[11] Different solutions containing ozone have also been used successfully against different infections such as otitis, intraocular infections and vaginitis.[7,12]

CONTACT Kemal Varol kmlvrl@yahoo.com Department of Veterinary, Food, Agriculture and Livestock Vocational College of Burdur, University of Burdur Mehmet Akif Ersoy, Burdur, Turkey

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In the light of above information, new features of the ozone aimed to be obtained by using ozonated olive oil (OZO) and ozonated distilled water (ODW) in the treatment of experimentally induced fungal keratitis in the present study. In addition to the cytology performed in the current study, as a result of clinical and histopathological data, it will inform us about the effects of OZO and ODW on the eyes and will make a great contribution to clinical applications.

MATERIALS AND METHODS

This study was supported by Erciyes University, Scientific Research Projects Coordination Unit with the project number TSA-2016-6849 as a Normal Research Project, made in Erciyes University Experimental Research Application and Research Center and the permit for Animal Experiments dated 13.01.2016 and with 16/014 decision number from the Erciyes University Animal Experiments Local Ethics Committee. Animal materials of the study were 42 New Zealand white male rabbits which were 3–4 months of age, weighing 2 to 2.5 kg. The experimental animals were supplied from private farms licensed by the Ministry of Food, Agriculture and Livestock. The animals were kept in standard rabbit cages (each rabbit in a separate cage) and the animals were fed as adlibitum and the room temperature was approximately 21 ± 1°C, the lighting was set to be 12 hours light (06:00–18:00) 12 hours dark.

In the present study, the *Candida (C.) albicans* strain, as a clinical isolate, injected 10 µl ($2.5x10^5$ cfu/ml)] intrastromally into 35 randomly selected New Zealand white male rabbits out of a total 42 rabbits. Five New Zealand white male rabbits constituted the negative control group (Group 7) and 10 µl sterile PBS were injected intrastromally on their both eyes. The remaining 2 animals at the beginning of the study before fungal inoculation were sedated with xylazine then euthanized by intracardiac pentobarbital injection. After euthanasia, corneas were then removed humanely for histopathologic examination.

After *C. albicans* inoculation, the infected rabbits were randomly divided into six groups. The Groups were composed of as 1, 2, 3, 4, 5, 7 (n = 5 rabbits, 10 eyes/in each group) and Group 6 (n = 10 rabbits, 20 eyes/in the group). Group 1 received fluconazole (FLU) + OZO drops, Group 2 received FLU drop, Group 3 received OZO drop, Group 4 received FLU + ODW drops, Group 5 received ODW drop. Five animals in Group 6 euthanized for quantification of *C. albicans* 48 hours after inoculation. The remaining 5 animals in Group 6 received PBS drop (infected control group) 48 hours after *C. albicans* inoculation, Group 7 also received PBS drop (negative control group) 48 hours after *PBS* injection. Treatment continued in all groups for 22 days for every 8 hours. In this study, from all preparations (FLU, OZO, ODW and PBS), only one drop was then instilled into each eye upon every succesive treatment.

Eye swabs were taken for microbiological analysis before fungal inoculation to determine for other bacterial presence and 48 hours after fungal inoculation swabs were also taken to determine fungal growth. A slit-lamp biomicroscope was used to assess keratitis 48 hours after fungal inoculation and on days 3, 8, 12, 16, 20, 24. Corneal opacity, conjunctivitis, corneal ulcer and neovascularisation were evaluated 48 hours after fungal inoculation and on days 3, 8, 12, 16, 20, 24. Samples were taken from eyes for cytology before fungal inoculation, 48 hours and 24 days after fungal inoculation. At the end of the 24^{th} day post inoculation, corneas of the remaining all animals used in the experiment were removed humanely as described above.

Generation of C. Albicans

The *C. albicans* strain isolated from human blood obtained from the Microbiology laboratory of Erciyes University Medical School. *C. albicans* strain was planted on Sabouroud Dextrose Agar, incubated at 37°C for 24 hours. The colonies formed were passaged again. After the fresh culture obtained, it was suspended in peptone broth, McFarland 0.5 was suspended by spectrophotometric method to be (Densimat Biomérieux spectrophotometer device) 2.5×10^5 cfu/ml (Colony-forming unit/milliliter) in saline and kept at + 4°C until inoculation.

Evaluation of Antimicrobial Effectiveness

Antimicrobial activity of OZO and FLU; It was determined by antifungal sensitivity test according to CLSI M27-A3[13] and CLSI M60-Ed2[14] reference method.

Preparation of Antifungal Materials

Ozone olive oil material

Olive oil (OL) and OZO were provided from a certified (Aktifoks, Işık Kozmetik/İzmir/Turkey) commercial company. According to the information given by the manufacturer, 10 liters of OL was ozonized for 10 days at 18°C - 20°C with Hansler ozone device, which produces 25 grams of ozone per hour. Acidity, iodine number, peroxite number, pH, viscosity and *P*-Anisidin values of OL and OZO were determined in our previous study.[15]

Ozonated water

The ozone concentration was adjusted to 80 µg/ml in the ozone generator (Evozone Basic Plus GMBH Germany), fed with pure oxygen that flow rate of 500 ml/min. The sterile distilled water (PF Sterile Distilled Water/Polifarma) was ozonized ten minutes at 20°C and maximum ozone saturation was obtained. Ozone concentrations of ozonated distilled water was detected spectrophotometrically, according to Rakness et al.[16] method in a medical ozone device (Medozone Eser Medikal/Turkey) at 20°C at 253.7 nm wavelength.

Fluconazole and 0.3% drop of fluconazole

FLU and 0.3% drop of FLU (Fluzamed 0.3% eye drops, World Medicine) were obtained from the commercial company.

Formation of Experimental Keratitis

The rabbits were treated humanely according to the guidelines of the Vision and Ophthalmology Research Association. Anesthesia was done by intramuscularly injected ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (3.5 mg/kg). Anesthetized rabbits were fixed under a stereoscopic

microscope. For eye applications, the eyelid was opened with a wire speculum. A drop of 0.5% proparacain (Alcain/Alcon) was dropped in order to create a better anesthesia on the eye. Using a micro-injector having a 10-gauge needle containing 10 µl *C. albicans* ($2.5x10^5$ cfu/ml) suspension injected into each cornea inrastromally. Correct, stroma was not penetrated in the anterior eye chamber.[17–19]

Clinical Evaluation of Keratitis

Evaluation of corneal opacity; Corneal opacity was evaluated according to the scale: 0; clear cornea, 1; minor edema, 2; corneal opacity in more than two-quarter circles, 3; total corneal opacity.[17,18]

Evaluation of conjunctivitis; Degree of redness based on grading conjunctivitis: (1; low, 2; medium and 3; high).[17,18]

Evaluation of corneal ulcer; It was evaluated according to the cornea lesion: (1; low, 2; medium and 3; high).[17,18]

Evaluation of Neovascularisation; Neovascularisation was determined according to the intensity of vascularisation (1; low, 2; medium and 3; high) by modifying the method of Schreiber et al.[18]

Quantification of *C. albicans* per Cornea

Following euthanasia; corneas; the limbus was removed from the eyes, to make an extended coronal incision approximately to the middle eye. The corneas were removed with sterile micro dissecting scissors after completing the cut of the reverse limbus. The corneas were homogenized in a sterile 1 ml protease peptone (1%). Each sample was directly diluted 1/10, 1/100, 1/1000. The diluted sample was inoculated to 0.1 ml on Sabouraud Dextrose Agar plates. It was incubated at 37°C for 24 hours and the colonies were counted. All identified cultures were duplicated. The average of the results was determined for each eye. The results are expressed as the number of organisms per millimeter of the corneal sample as $\log_{10}$.

Histopathological Examination

Application of the cytospin technique

For this purpose, corneal cells were collected with a brush used in humans (Cytobrush, Gynobrush Plus, Germany). After anesthetic application to each eye, samples were taken by rotating the brushes from the ocular surface. After the handle of the brushes was cut, the head of the brushes was placed in the fixative solution (Cytospin collection fluid, Thermo Shandon 6768001, USA) in a tube. The fixatives taken to the megafunnels were cytocentrifuged for 15 minutes at 1500 cycles (Cytospin; Thermo Shandon Southern Ltd, Cheshire, England). The collected cells were taken into glasses with poly L-lysin. Wet glasses that were cytocentrifuged were dried, stained with Hematoxylin-eosin (HE) and Papanicolau (PAP) and closed with mounting medium (Merck). Sections were examined under a light microscope and photographed. Pictures were taken using an Olympus DP71 microscope-compatible digital camera (Olympus DP71) and a digital program (DP Controller and the DP Manager).[20]

Histopathological evaluation of keratitis

To make histopathological examinations of the cornea, they were placed in 10% formol solution. After the samples taken, they had been passed through routine follow-up procedures, and then taken into paraffin blocks and then 5 µm thick sections were prepared. HE, Periodic Acit Schifft (PAS) staining was applied. Each preparation was evaluated at least on 4 microscope fields at x10, x40, and x100 magnifications. Sections were examined under a light microscope and photographed. The pictures were taken with a microscope (Olympus BX-51) compatible digital camera and digital program (DP Controller and the DP Manager).

Statistical Analysis

The findings were evaluated using the IBM SPSS 22.0 for Windows package program. Shapiro–Wilk test was used for the compatibility of the data to the normal distribution. Since the data was normally distributed, it was determined by making two-way analysis of variance in microbiological findings in repeated and intra-group comparisons. Benferoni corrected multiple-comparison tests were used. Inter-group and intra-group comparisons of clinical findings were analyzed with the Kruskal–Wallis test. p value was considered significant ($p < .05$).

RESULTS

Ozone Oil Analysis Findings

Due to the result of the OZO analysis; the peroxide number was determined as 1352 mmol-mEq/kg, Acid number was determined as 8.9951 unit, Iodine number was determined as 2.2040 unit, *P*-Anisidine value was determined as 0.516 mmol/g. Furthermore, viscosity was determined as 1000 centipoise at 34°C and pH was determined as 2.1 at 24°C.

Ozonated Distilled Water Analysis Findings

Ozone concentration of ozonated distilled water was 26 µg/ml at 20°C.

Microdilution Findings

Microdilution findings are given in Table 1. In summary, microdilution findings for *C. albicans* strain, a clinical isolate at 24th hour, MICs values of OZO and FLU were 1.56% and 0.25 µg/mL, respectively. At 48th hour, MICs values of OZO

Table 1. Minimum inhibitory concentrations of ozonated olive oil and fluconazole.

MIC values						
			Incubation time (24 hours)		Incubation time (48 hours)	
			%(v/v)	In µg/ml	%(v/v)	In µg/ml
Number	Strain	Material	OZO	FLU	OZO	FLU
1	*C. albicans*	Blood	1,56	0,25	3,125	0,25
2	*C. albicans ATCC 90028*	Standart	0,1	0,25	0,1	0,25
Total			1,57	0,50	3.126	0.50

and FLU were 3.125% (v/v) and 0.25 µg/mL, respectively. Since the half-life of ozone is very short in ozonated water, microdilution was not performed for ozonated water (Table 1).

Microbiological Findings of Corneal Infection

No bacterial growth was observed in the analysis of swabs from all animals before fungal inoculation. Microbiological findings of corneal infection are given in Table 2. With concern to this table; When the amount of yeast growth on the 24th day between the groups were compared; It was determined that there was a statistical ($p < .05$) difference in the amount of yeast grown between Group 6 and Group 1, 2, 3, 4, 5, and 7. When the amount of yeast grown within the group is compared; A statistical ($p < .05$) difference was determined between 48th hour and 24th day in Groups 1, 2, 3, 4 and 5 (Table 2).

Clinical Findings of Keratitis

Clinical findings (corneal opacity, corneal ulcer, conjunctivitis and neovascularisation) are given in Tables 3, 4, 5 and Figures 5, 6, 7.

Table 2. The amount of *C. albicans* growing in the eye before and after treatment.

Groups	48th hour before treatment (CFU/ml) $\bar{x} \pm sd$	24th day after treatment (CFU/ml) $\bar{x} \pm sd$
G.1. *(n = 10)*	6288306,76 ± 10328053,02[a]	28044,72 ± 65932,37[a]*
G.2. *(n = 10)*	6288306,76 ± 10328053,02[a]	67178,55 ± 113716,07[a]*
G.3. *(n = 10)*	6288306,76 ± 10328053,02[a]	31101,83 ± 82930,96[a]*
G.4. *(n = 10)*	6288306,76 ± 10328053,02[a]	59471,68 ± 94132,90[a]*
G.5. *(n = 10)*	6288306,76 ± 10328053,02[a]	14168,09 ± 24914,97[a]*
G.6. *(n = 10)*	6288306,76 ± 10328053,02[a]	4568366,95 ± 8403236,35[b]
G.7. *(n = 10)*	0,0 ± 0,0[a]	0,0 ± 0,0[a]

Groups; G.1. FLU and OZO drops applied group, **G.2**. FLU drop applied group, **G.3**. OZO drop applied group, **G.4**. FLU and ODW drops applied group, **G.5**. ODW drop applied group, **G.6**. PBS drop applied group (Infected control group), **G.7**. PBS drop applied group (Negative control group). *Indicates that there is a statistical difference (*$p < 0.05$, **$p < 0.001$) between the same columns in comparisons between groups. There is a statistical difference ($p < 0.05$) between different lowercase (a-c) (superscript) lines in comparison of measurements within the group.

When comparing corneal opacity, corneal ulcer and conjunctivitis values in intergroup comparisons; between Group 1 and Group 6 on the 20th and 24th days, between Group 2 and Group 6 on the 20th and 24th days, between Group 3 and Group 6 on the 20th and 24th days, between Group 4 and Group 6 on the 20th and 24th days, between Group 5 and Group 6 on the 12th, 16th, 20th and 24th days, and between Group 7 and Group 6 on the 48th hour, 4th, 8th, 12th, 16th, 20th and 24th days were found significantly ($p < .05$) different (Tables 3, 4, 5 and Figures 5, 6, 7).

In intragroup comparisons, it was observed that corneal opacity, corneal ulcer and conjunctivitis values decreased statistically ($p < .05$) in Group 1, Group 2, Group 3, Group 4 and Group 5 on the 20th and 24th days. In addition to these findings, it was observed that corneal opacity decreased statistically in Group 5, Group 1 and Group 3, starting from the 16th day. It was observed that the corneal ulcer decreased significantly from the 16th day in Group 5 and Group 1. In addition, it was observed that conjunctivitis decreased significantly starting from the 12th day in Group 5 (Tables 3, 4, 5).

There was no statistically significant difference in neovascularisation findings between the infected control group and the treatment groups. However, signs of neovascularisation decreased in the treatment groups. Recovery findings of groups 1, 2, 3, 4, 5, 6 and 7. are given in Figure 1.

Among the clinical findings, the best improvement was seen in Group 5 (in the ODW applied group) according to the results and averages of the intergroup comparison of corneal opacity, corneal ulcer, and conjunctivitis values. In the second place, the group with the best improvement was Group 1 (the group in which FLU and OZO were applied together). The group in which improvement is seen in the third place was Group 3 (the group in which OZO was applied). The group in which improvement is seen in the fourth place was Group 4 (the group in which FLU and ODW were applied together). The group with improvement in the fifth rank was Group 2 (FLU group).

Table 3. Corneal opacity findings in the eye.

Groups	**G.1.** *(n = 10)* $\bar{x} \pm sd$ *m(min-max)*	**G.2.** *(n = 10)* $\bar{x} \pm sd$ *m(min-max)*	**G.3.** *(n = 10)* $\bar{x} \pm sd$ *m(min-max)*	**G.4.** *(n = 10)* $\bar{x} \pm sd$ *m(min-max)*	**G.5.** *(n = 10)* $\bar{x} \pm sd$ *m(min-max)*	**G.6.** *(n = 10)* $\bar{x} \pm sd$ *m(min-max)*	**G.7.** *(n = 10)* $\bar{x} \pm sd$ *m(min-max)*
48th hour	2,70 ± 0,48[a] 3,00 (2,00–3,00)	2,70 ± 0,48[a] 3,00 (2,00–3,00)	2,80 ± 0,42[a] 3,00 (2,00–3,00)	3,10 ± 0,31[a] 3,00 (3,00–4,00)	3,00 ± 0,47[a] 3,00 (2,00–4,00)	2,80 ± 0,78 3,00 (2,00–4,00)	0,00 ± 0,00** 0,00 (0,00–0,00)
4th day	2,90 ± 0,56[a] 3,00 (2,00–4,00)	2,70 ± 0,48[a] 3,00 (2,00–3,00)	2,80 ± 0,42[a] 3,00 (2,00–4,00)	2,90 ± 0,31[a] 3,00 (2,00–3,00)	3,10 ± 0,31[a] 3,00 (3,00–4,00)	3,00 ± 0,00 3,00 (3,00–3,00)	0,00 ± 0,00** 0,00 (0,00–0,00)
8th day	2,80 ± 0,63[a] 3,00 (2,00–4,00)	2,80 ± 0,42[a] 3,00 (2,00–3,00)	3,10 ± 0,31[a] 3,00 (3,00–4,00)	3,20 ± 0,42[a] 3,00 (3,00–4,00)	3,00 ± ±0,47[a] 3,00 (2,00–4,00)	3,30 ± 0,48 3,00 (3,00–4,00)	0,00 ± 0,00** 0,00 (0,00–0,00)
12nd day	2,80 ± 0,42[a] 3,00 (2,00–4,00)	2,90 ± 0,3 [a] 3,00 (2,00–3,00)	2,90 ± 0,56[a] 3,00 (2,00–4,00)	3,00 ± 0,66[a] 3,00 (2,00–4,00)	2,60RHPE_A_19944430,51[ac] 3,00 (2,00–3,00)	3,50 ± 0,52 3,50 (3,00–4,00)	0,00 ± 0,00** 0,00 (0,00–0,00)
16th day	2,10 ± 0,31[ab*] 2,00 (2,00–3,00)	2,30 ± 0,48[ab] 2,00 (2,00–3,00)	2,10 ± 0,31[ab*] 2,00 (2,00–3,00)	2,30 ± 0,48[ab] 2,00 (2,00–3,00)	2,00 ± 0,00[ab*] 2,00 (2,00–2,00)	3,30 ± 0,48 3,00 (3,00–4,00)	0,00 ± 0,00** 0,00 (0,00–0,00)
20th day	0,90 ± 0,31[b**] 1,00 (0,00–1,00)	1,30 ± 0,48[b*] 1,00 (1,00–2,00)	1,10 ± 0,31[b*] 1,00 (1,00–2,00)	1,20 ± 0,42[b*] 1,00 (1,00–2,00)	1,000,47 [bc**] 1,00 (0,00–2,00)	3,50 ± 0,52 3,50 (3,00–4,00)	0,00 ± 0,00** 0,00 (0,00–0,00)
24th day	0,50 ± 0,52[b**] 0,50 (0,00–1,00)	0,80 ± 0,42 [b*] 1,00 (0,00–1,00)	0,60 ± 0,51[b*] 1,00 (0,00–1,00)	0,70 ± 0,42[b*] 1,00 (0,00–1,00)	0,40 ± 0,51[b**] 0,00 (0,00–1,00)	3,50 ± 0,52 3,50 (3,00–4,00)	0,00 ± 0,00** 0,00 (0,00–0,00)

Groups; G.1. FLU and OZO drops applied group, **G.2**. FLU drop applied group, **G.3**. OZO drop applied group, **G.4**. FLU and ODW drops applied group, **G.5**. ODW drop applied group, **G.6**. PBS drop applied group (Infected control group), **G.7**. PBS drop applied group (Negative control group). *Indicates that there is a statistical difference (*$p < 0.05$, **$p < 0.001$) between the same columns in comparisons between groups. There is a statistical difference ($p < 0.05$) between different lowercase (a-c) (superscript) lines in comparison of measurements within the group.

Table 4. Findings of corneal ulcer in the eye.

Groups	G.1. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.2. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.3. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.4. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.5. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.6. (n = 10)$\bar{x} \pm sdm$ (min-max)	G.7. (n = 10)$\bar{x} \pm sd$ m(min-max)
48th hour	$2{,}60 \pm 00{,}51^{ac}$	$2{,}50 \pm 00{,}52^{ac}$	$2{,}70 \pm 00{,}48^{ac}$	$2{,}80 \pm 00{,}42^{a}$	$2{,}90 \pm 00{,}31^{a}$	$2{,}60 \pm 00{,}51^{a}$	$0{,}00 \pm 00{,}00^{*}$
	3,00 (2,00–3,00)	2,50 (2,00–3,00)	3,00 (2,00–3,00)	3,00 (2,00–3,00)	3,00 (2,00–3,00)	3,00 (2,00–3,00)	0,00 (0,00–0,00)
4th day	$2{,}80 \pm 00{,}42^{a}$	$2{,}70 \pm 00{,}48^{ac}$	$2{,}80 \pm 00{,}63^{a}$	$3{,}10 \pm 00{,}56^{a}$	$3{,}30 \pm 00{,}48^{a}$	$3{,}10 \pm 00{,}0{,}31^{ab}$	$0{,}00 \pm 00{,}00^{*}$
	3,00 (2,00–3,00)	3,00 (2,00–3,00)	3,00 (2,00–4,00)	3,00 (2,00–4,00)	3,00 (3,00–4,00)	3,00 (3,00–4,00)	0,00 (0,00–0,00)
8th day	$3{,}30 \pm 00{,}48^{a}$	$3{,}60 \pm 00{,}51^{a}$	$3{,}50 \pm 00{,}52^{a}$	$3{,}40 \pm 00{,}51^{a}$	$3{,}40 \pm 00{,}51^{a}$	$3{,}40 \pm 00{,}51^{ab}$	$0{,}00 \pm 00{,}00^{*}$
	3,00 (3,00–4,00)	4,00 (3,00–4,00)	3,50 (3,00–4,00)	3,00 (3,00–4,00)	3,00 (3,00–4,00)	3,00 (3,00–4,00)	0,00 (0,00–0,00)
12nd day	$3{,}30 \pm 00{,}48^{a}$	$3{,}20 \pm 00{,}63^{a}$	$3{,}10 \pm 00{,}56^{a}$	$2{,}70 \pm 00{,}67^{ac}$	$2{,}60 \pm 00{,}51^{ab}$	$3{,}60 \pm 00{,}51^{ab}$	$0{,}00 \pm 00{,}00^{*}$
	3,00 (3,00–4,00)	3,00 (2,00–4,00)	3,00 (2,00–4,00)	3,00 (2,00–4,00)	3,00 (2,00–3,00)	4,00 (3,00–4,00)	0,00 (0,00–0,00)
16th day	$2{,}50 \pm 00{,}52^{ab*}$	$2{,}60 \pm 00{,}51^{ab}$	$2{,}60 \pm 00{,}51^{ab}$	$2{,}70 \pm 00{,}4^{ac}$	$2{,}50 \pm 00{,}70^{ab*}$	$3{,}70 \pm 00{,}48^{b}$	$0{,}00 \pm 00{,}00^{*}$
	2,50 (2,00–3,00)	3,00 (2,00–3,00)	3,00 (2,00–3,00)	3,00 (2,00–3,00)	2,00 (2,00–4,00)	4,00 (3,00–4,00)	0,00 (0,00–0,00)
20th day	$1{,}00 \pm 00{,}00^{bc**}$	$1{,}30 \pm 00{,}48^{bc*}$	$1{,}10 \pm 00{,}31^{bc*}$	$1{,}20 \pm 00{,}42^{bc*}$	$1{,}00 \pm 00{,}47^{b**+}$	$3{,}60 \pm 00{,}51^{ab}$	$0{,}00 \pm 00{,}00^{**}$
	1,00 (1,00–1,00)	1,00 (1,00–2,00)	1,00 (1,00–2,00)	1,00 (1,00–2,00)	1,00 (0,00–2,00)	4,00 (3,00–4,00)	0,00 (0,00–0,00)
24th day	$0{,}60 \pm 00{,}51^{b**}$	$0{,}90 \pm 00{,}31^{b*}$	$0{,}70 \pm 00{,}48^{b*}$	$0{,}80 \pm 00{,}42^{b*}$	$0{,}50 \pm 00{,}52^{b**}$	$3{,}60 \pm 00{,}51^{ab}$	$0{,}00 \pm 00{,}00^{**}$
	1,00 (0,00–1,00)	1,00 (0,00–1,00)	1,00 (0,00–1,00)	1,00 (0,00–1,00)	0,50 (0,00–1,00)	4,00 (3,00–4,00)	0,00 (0,00–0,00)

Groups; G.1. FLU and OZO drops applied group, **G.2**. FLU drop applied group, **G.3**. OZO drop applied group, **G.4**. FLU and ODW drops applied group, **G.5**. ODW drop applied group, **G.6**. PBS drop applied group (Infected control group), **G.7**. PBS drop applied group (Negative control group). *Indicates that there is a statistical difference (*$p < 0.05$, **$p < 0.001$) between the same columns in comparisons between groups. There is a statistical difference ($p < 0.05$) between different lowercase (a-c) (superscript) lines in comparison of measurements within the group.

Table 5. Conjunctivitis findings in the eye.

Groups	G.1. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.2. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.3. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.4. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.5. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.6. (n = 10)$\bar{x} \pm sd$ m(min-max)	G.7. (n = 10)$\bar{x} \pm sd$ m(min-max)
48th hour	$2{,}50 \pm 00{,}52^{ac}$	$2{,}40 \pm 00{,}51^{ab}$	$2{,}50 \pm 00{,}52^{ab}$	$2{,}60 \pm 00{,}51^{ac}$	$3{,}00 \pm 00{,}00^{a}$	$2{,}40 \pm 00{,}51^{a}$	$0{,}00 \pm 00{,}00^{**}$
	2,50 (2,00–3,00)	2,00 (2,00–3,00)	2,50 (2,00–3,00)	3,00 (2,00–3,00)	3,00 (3,00–3,00)	2,00 (2,00–3,00)	0,00 (0,00–0,00)
4th day	$2{,}80 \pm 00{,}42^{a}$	$2{,}90 \pm 00{,}56^{a}$	$2{,}90 \pm 00{,}56^{a}$	$3{,}20 \pm 00{,}63^{a}$	$3{,}20 \pm 00{,}42^{a}$	$3{,}40 \pm 00{,}51^{ab}$	$0{,}00 \pm 00{,}00^{**}$
	3,00 (2,00–3,00)	3,00 (2,00–4,00)	3,00 (2,00–4,00)	3,00 (2,00–4,00)	3,00 (3,00–4,00)	3,00 (3,00–4,00)	0,00 (0,00–0,00)
8th day	$3{,}10 \pm 00{,}56^{a}$	$3{,}10 \pm 00{,}56^{a}$	$3{,}10 \pm 00{,}56^{a}$	$3{,}30 \pm 00{,}48^{a}$	$3{,}10 \pm 00{,}56^{a}$	$3{,}50 \pm 00{,}52^{ab}$	$0{,}00 \pm 00{,}00^{**}$
	3,00 (2,00–4,00)	3,00 (2,00–4,00)	3,00 (2,00–4,00)	3,00 (3,00–4,00)	3,00 (2,00–4,00)	3,50 (3,00–4,00)	0,00 (0,00–0,00)
12nd day	$3{,}30 \pm 00{,}48^{a}$	$3{,}40 \pm 00{,}51^{a}$	$3{,}20 \pm 00{,}63^{a}$	$3{,}00 \pm 00{,}81^{a}$	$2{,}90 \pm 00{,}87^{a*}$	$3{,}70 \pm 00{,}48^{b}$	$0{,}00 \pm 00{,}00^{**}$
	3,00 (3,00–4,00)	3,00 (3,00–4,00)	3,00 (2,00–4,00)	3,00 (2,00–4,00)	3,00 (2,00–4,00)	4,00 (3,00–4,00)	0,00 (0,00–0,00)
16th day	$2{,}30 \pm 00{,}48^{ab}$	$2{,}60 \pm 00{,}51^{ac}$	$2{,}90 \pm 00{,}56^{a}$	$2{,}60 \pm 00{,}51^{ac}$	$2{,}20 \pm 00{,}42^{ab*}$	$3{,}30 \pm 00{,}48^{ab}$	$0{,}00 \pm 00{,}00^{**}$
	2,00 (2,00–3,00)	3,00 (2,00–3,00)	3,00 (2,00–4,00)	3,00 (2,00–3,00)	2,00 (2,00–3,00)	3,00 (3,00–4,00)	0,00 (0,00–0,00)
20th day	$1{,}00 \pm 00{,}00^{bc*}$	$1{,}10 \pm 00{,}31^{bc*}$	$1{,}10 \pm 00{,}31^{b*}$	$1{,}10 \pm 00{,}31^{bc*}$	$0{,}80 \pm 0{,}42^{b*}$	$3{,}40 \pm 00{,}51^{ab**}$	$0{,}00 \pm 00{,}00^{**}$
	1,00 (1,00–1,00)	1,00 (1,00–2,00)	1,00 (1,00–2,00)	1,00 (1,00–2,00)	1,00 (0,00–1,00)	3,00 (3,00–4,00)	0,00 (0,00–0,00)
24th day	$0{,}60 \pm 00{,}51^{b*}$	$0{,}80 \pm 00{,}42^{b*}$	$0{,}60 \pm 00{,}51^{b*}$	$0{,}80 \pm 00{,}42^{b*}$	$0{,}40 \pm 00{,}51^{b*}$	$3{,}50 \pm 00{,}52^{ab**}$	$0{,}00 \pm 00{,}00^{**}$
	1,00 (0,00–1,00)	1,00 (0,00–1,00)	1,00 (0,00–1,00)	1,00 (0,00–1,00)	0,00 (0,00–1,00)	3,50 (3,00–4,00)	0,00 (0,00–0,00)

Groups; G.1. FLU and OZO drops applied group, **G.2**. FLU drop applied group, **G.3**. OZO drop applied group, **G.4**. FLU and ODW drops applied group, **G.5**. ODW drop applied group, **G.6**. PBS drop applied group (Infected control group), **G.7**. PBS drop applied group (Negative control group). *Indicates that there is a statistical difference (*$p < 0.05$, **$p < 0.001$) between the same columns in comparisons between groups. There is a statistical difference ($p < 0.05$) between different lowercase (a-c) (superscript) lines in comparison of measurements within the group.

Histopathological Findings

Histopathological findings in the negative control group (Group 7)

On light microscopic examination, the cornea was observed to consist of five layers. Basal columnar cells, intermediate polygon cells, and superficial squamous cells were present in the multi-layer squamous nonkeratinized layer. Under the corneal epithelium (Bowman's layer). Secondary propria consisted of collagen fibers and scattered spindle-shaped stromal cells arranged at regular intervals. The Descemet membrane was present just below the stroma and was covered by the endothelium of Descemet. Normal corneal morphology was observed in the negative control group. In this group, the lamellar structure of the stroma was regular. There was no change in the size and shape of the corneal epithelial cells. Keratocytes are neatly sorted. Keratin is not available (Figure 2. A.1).

Histopathological findings in treatment groups

Corneal keratin amount was very low in treatment groups (Groups 1–5). Among these groups, Groups 1, 3 and 5; cell infiltration was almost completely eliminated compared to other treatment groups. Stromal cell infiltration was prominent in Groups 2 and 4 compared to the control group. Especially in the group that was only applied to FLU (Group 2), the cell infiltration and edema in the corneal stroma was more severe than other treatment groups. Cellular infiltration in the corneal stroma was significantly reduced when compared with Group 1 and Group 5 among the treatment groups. These groups (Groups 1–5) were very similar to the control group (Figure 2. B.1-F.1, B.2-F.2).

Histopathological findings in the infected control group

In the infected control group (Group 6), intense neutrophil leuklocyte and histiocyte infiltration was noted in the stroma. It was observed that stromal thickness increased markedly with stromal edema. The epithelial layer consisted of 3-row cells with a single infection, and severe keratin was present. Especially the cell forming, the collagen layer close to the anterior stroma was irregular. Inflammatory cells, polymorphonuclear leukocytes, which were observed mostly in corneal material with fungal keratitis, also had varying degrees of lymphocyte and plasma cells. Stroma coagulation necrosis was evident. Filamentous fungi were observed between the stromal lamellae in the deep (Figure 3. A.1-F.1, A.2-F.2).

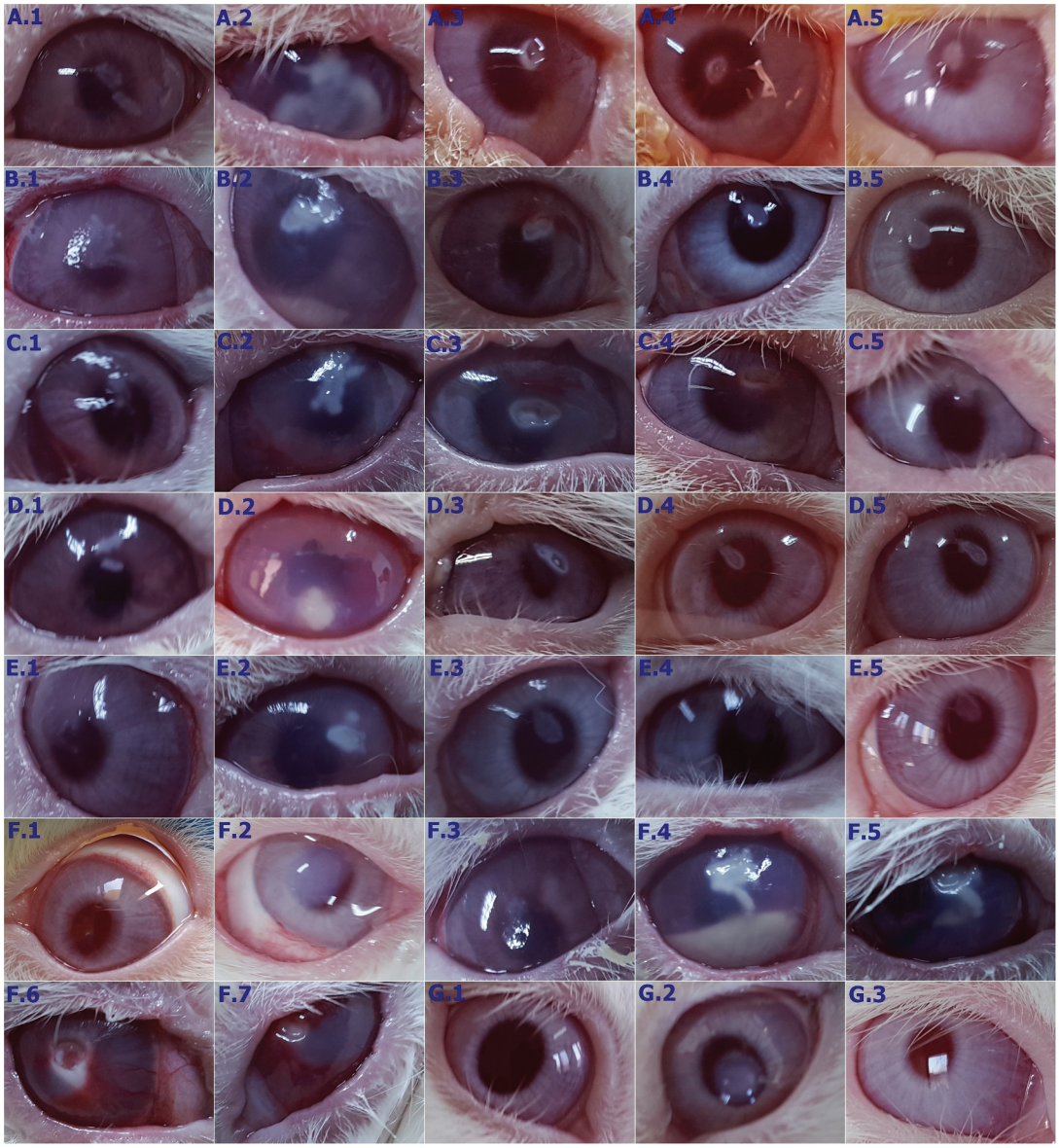


Figure 1. Before and after treatment photos of Group 1, 2, 3, 4, 5, 6, 7. **(A.1-A.5)** FLU and OZO drops applied group; **A.1**: 48th hour after application of *C. albicans* strain and before treatment; **A.2**: 8th day after treatment; **A.3**: 16th day after treatment; **A.4**: 20th day after treatment; **A.5**: 24th day after treatment. **(B.1-B.5)** FLU drop applied group; **B.1**: 48th hour after application of *C. albicans* strain and before treatment; **B.2**: 8th day after treatment; **B.3**: 16th day after treatment; **B.4**: 20th day after treatment; **B.5**: 24th day after treatment. **(C.1-C.5)** OZO drop applied group; **C.1**: 48th hour after application of *C. albicans* strain and before treatment; **C.2**: 8th day after treatment; **C.3**: 16th day after treatment; **C.4**: 20th day after treatment; **C.5**: 24th day after treatment. (**D.1-D.5**) FLU and ODW drop applied group; **D.1**: 48th hour after application of *C. albicans* strain and before treatment; **D.2**: 8th day after treatment; **D.3**: 16th day after treatment; **D.4**: 20th day after treatment; **D.5**: 24th day after treatment. **(E.1-E.5)** ODW drops applied group; **E.1**: 48th hour after application of *C. albicans* strain and before treatment; E.2: 8th day after treatment; **E.3**: 16th day after treatment; **E.4**: 20th day after treatment; **E.5**: 24th day after treatment. **(F.1-F.7)** PBS drop applied group (Infected control group)**; F.1**: 0th hour *C. albicans* strain before application; **F.2**: 0th hour after *C. albicans* strain application; **F.3**: 48th hour after *C. albicans* strain application and before treatment; **F.4**: 8th day after treatment; **F.5**: 16th day after treatment; **F.6**: 20th day after treatment; **F.7**: 24th day after treatment. **(G.1-G.3)** PBS drop applied group (Negative control group); **G.1**: 0th hour before PBS injection **G.2**: 48th hour after PBS application and before treatment **G.3**: 24th day after treatment.

It was remarkable that the significant pathological findings observed in all treatment groups disappeared (Groups 1–5). Among these symptoms, keratoconjunctivitis, diffuse, severe, acute, neutrophilic (suppurative) and focal corneal ulcer, superficial stromal ulcer, severe neutrophil leukocyte and histiocyte infiltration, degenerative areas in the deep stroma, as well as purulent abscess and stromal necrosis were observed to disappear. In addition, it was observed that the stromal structure of fungal hyphae and spore-like conidia disappeared completely with the intense degeneration that was evident. In addition to these, in Group 1, cell infiltration in the superficial stroma, neovascularization, hyperplasia in the epithelium; In Group 2, mild cell infiltration in the stroma, neovascularization; In Group 3, epithelial hyperplasic stromal edema; In Group 4, very mild cell infiltration in the superficial stroma, hyperplasia in the epithelium and In Group 5, mild neovascularization was evident in the stroma.

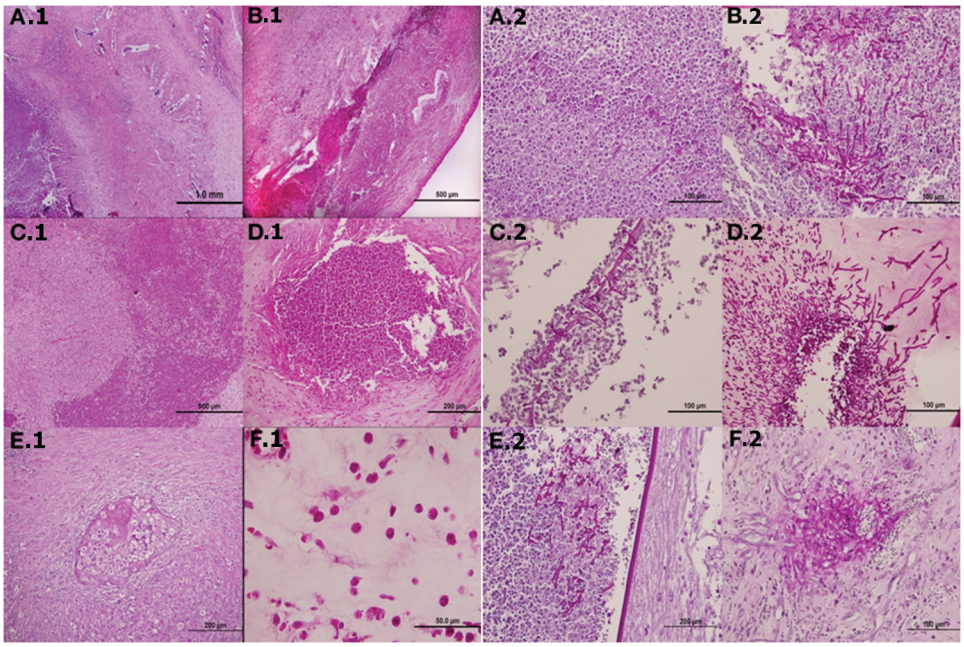


Figure 2. Hematoxylin-eosin and Periodic acid Schiff stained preparations of the infected control group (Group 6). **(A.1-F.1)** Group 6 (Infected control group) Hematoxylin-eosin stained preparations. Keratoconjunctivitis, diffuse, severe, acute, neutrophilic (suppurative) and focal corneal ulcer, eye-striking peripheral corneal vascularization and Candida albicans -like fungal agents in these areas. **(A.1)** intense neovascular vessels, corneal structures have disappeared throughout the stroma, **(B.1)** inflammatory cell infiltration in the superficial stroma, ulcer and bleeding. **(C.1-D.1)** degenerative areas in deep stroma, as well as purulent abscess and stromal necrosis. Bar: 1 mm (A.1), 500 µm (B.1, C.1), 200 µm (D.1, E.1), 50 µm (F.1). **(A.2 – F.2)** Group 6 (Infected control group) preparations painted with rust (Periodic acid Schiff). **(A.2-D.2)** fungal hifa and spor-like conidia stromal with intensive degenerate neutrophil leukocyte and histiocyte infiltration. **(E.2, F.2)** hifa invasion in deep stomatitis. Descemet's membrane and fungal hifa and spores invaded into the anterior chamber. Bar: 100 µm (A.2.-D.2, F.2), 200 µm (E.2).

Cytological Findings

Morphological appearances of corneal epithelial cells were evaluated. Adequate cellularity was present in cytological samples. Before Candida inoculation (0th hour), in the cytological examination of the ocular surface, normal columnar epithelial cells of the conjunctiva, round small cells, non-cornea squamous epithelium were observed in the eyes of the rabbits in the control (Group 7) and treatment groups (Group 1–5). These cells were similar in shape and size with eosinophilic cytoplasm (Figure 4. A, D, H, I).

At 48th hour and 24th day after inoculation, dense neutrophil leukocytes, squamous epithelial cells showing keratoconjunctivitis and corneal ulcer, degenerative neutrophil leukocytes and fungal cells were prominent in Group 6. Numerous mononuclear (macrophage, lymphocyte, plasma cells) cells were also dense, including reactive corneal epithelial cells and conjunctival epithelial cells.

Conjunctivitis and keratitis were observed in rabbits in the infected groups, which attracted attention with intense polymorphonuclear neutrophils. Dysplasia changes were observed in epithelial cells, especially in cases with corneal ulcers. In some cases, squamous metaplasia, characterized by cells with abundant cytoplasm picosic nuclei and nucleus/cytoplasm ratio was considerably reduced. Inflammatory cells observed predominantly in corneal material with fungal keratitis were polymorphonuclear leukocytes, varying degrees of lymphocyte and plasma cells (Figure 4. B, C, E- G).

At the end of the 24th day, dysplasia cells with hyperchromasia and nuclear pleomorphism, clear cytoplasm and increased nucleocytoplasm ratio were evident in Groups 2 and 4. However, normal corneal and conjunctival cells were found in Group 5, similar to Group 7. In Group 3 and 1, although similar to Group 5, inflammatory cells were found in small numbers.

DISCUSSION

Corneal infections are one of the reasons that cause serious and permanent damage to the visual system. Today, although there are various groups of antibiotics used in the treatment of different types of corneal infections, new treatment methods are needed to protect eye health due to the increasing resistance to antibiotics.[21] Especially in case of illness, vascularisation and fast cicatricization are required in the anterior part of the eye, as well as antibacterial and antiviral action.[22] Theoretically, in this case, ozone therapy can be considered, since these pathologies can be difficult and take a long time to be treated with conventional treatments. Ozone has a remarkable germicidal effect. Antimicrobial effects of ozone against viruses, bacteria and fungi have been proven in vitro and in vivo.[7,15] It is known that the OZO has a fungicidal effect against *C. albicans*, many major fungal pathogens, and also for non-albicans Candida and Aspergillus species.[8,15] In this study MIC values of OZO against *C. albicans* supports this (Table 1). Furtermore in the present study, when the amount of 24th day *C. albicans* growth in the cornea compared between groups: there were statistically significant difference ($p < 0,05$) between Group 6 (infected untreated group) and Group 3 (treated with OZO). When the *C. albicans* growth in the cornea

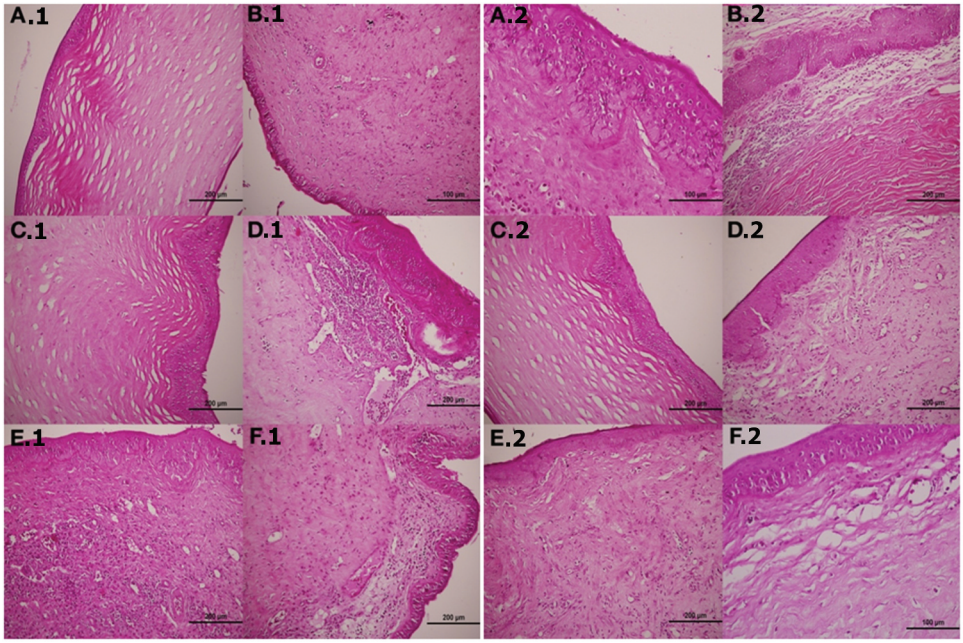


Figure 3. Hematoxylin-eosin stained preparations of treatment groups (Group 1, 2, 3, 4, 5, 7). **(A.1-F.1)** Group 6 (Infected control group) Hematoxylin-eosin stained preparations. Keratoconjunctivitis, diffuse, severe, acute, neutrophilic (suppurative) and focal corneal ulcer, eye-striking peripheral corneal vascularization and *Candida albicans* -like fungal agents in these areas. **(A.1)** intense neovascular vessels, corneal structures have disappeared throughout the stroma **(B.1)** inflammatory cell infiltration in the superficial stroma, ulcer and bleeding. **(C.1-D.1)** degenerative areas in deep stroma; as well as purulent abscess and stromal necrosis. Bar: 1 mm (A.1), 500 µm (B.1, C.1), 200 µm (D.1, E.1), 50 µm (F.1). **(A.2 – F.2)** Group 6 (Infected control group) preparations painted with rust (Periodic acid Schiff). **(A.2-D.2)** fungal hifa and spor-like conidia stromal with intensive degenerate neutrophil leukocyte and histiocyte infiltration. **(E.2, F.2)** hifa invasion in deep stomatitis. Descemet's membrane and fungal hifa and spores invaded into the anterior chamber. Bar: 100 µm (A.2.-D.2, F.2), 200 µm (E.2). **(A.1-C.1)** Group 7 (Negative control group) **(A.1)** control eye, normal rabbit cornea, **(B.1, C.1)** normal corneal structure, combination therapy group; **(D.1)** Group 1 (FLU and OZO applied group) cell infiltration in superficial stroma, neovascularization, hyperplasia in epithelium; **(E.1, F.1)** Group 2. (FLU applied group) mild cell infiltration in stroma, neovascularization, Bar: 100 µm (B.1), 200 µm (A.1, C.1, D.1-F.1). **(A.2)** Group 3 (OZO applied group) epithelial hyperplasic stromal edema; **(B.2)** Group 4. (FLU and ODW applied group) very mild cell infiltration in the superficial stroma, hyperplasia in the epithelium; **(C.2-F.2)** Group 5 (ODW applied group) mild neovascularization in the stroma. Bar: 100 µm (B.2), 200 µm (A.2, C.2, D.2-F.2).

compared within groups; there were statistically significant difference ($p < .05$) between 48th hour and 24th day in Group 3 (Table 2).

Ozone dissolves in pure water as O_3 and maintains its identity. So ozonated water is used in many medical fields.[23–25] Ozonated physiological saline solution with a concentration of 5 mg/L is effective in treating rabbit fungal keratitis.[26] In this study at the end of the 24th day; when the amount of *C. albicans* growth in the ODW groups compared with the infected control group, statistical difference was also observed (Figure 1, Table 2) When the *C. albicans* growth in the cornea compared within groups; there were statistically significant difference ($p < .05$) between 48th hour and 24th day in Group 5. These results have shown us that ODW with a concentration of 26 µg/ml is perfectly effective in treating rabbit *C. albicans* keratitis (Figure 1, Table 2).

At room temperature, the half-life of ozone in water is very short.[10,25] Therefore, in our in vitro study, MIC values were not determined for ODW. Since at room temperature, the half-life ozone in water is very short, applications were made immediately after ozonating the water in the present study, and even the water was ozonated several times during the application against the possibility of a decrease in the ozone concentration in the water. Contrary to these possibilities; although ozone was more stable in oil than water, it was observed that the ODW group was more effective compared to the other groups. It is thought that this may be due to the good penetration of ODW into ocular tissues.

Antifungal agents currently used in the treatment of keratomycosis are pyrimidines, polyenes, imidazoles. Miconazole and FLU are frequently used in fungal keratitis, as imidazoles have the widest spectrum among antifungal agents, low toxicity, and low irritating properties. FLU shows high solubility in water, weak protein binding and lower molecular gravity. Therefore, it penetrates well into the ocular tissues. However, FLU has only fungicidal activity against most major fungal pathogens. It also has less effect on almost all Aspergillus and non-albicans Candida species.[6,17] Behrens-Baumann et al.[6] reported that FLU was effective in the treatment of keratitis experimentally induced with *C. albicans* in rabbits which was used 48 hours after infection, 1 drop 10 times a day for 24 days. In the present study, FLU was administered 48 hours after infection, 1 drop 3 times a day. At the end of the 24th day; when the amount of *C. albicans* growth in the cornea in the Group 2 (FLU group) and Group 1, Group 4 (ODW and OZO were used together with FLU) compared with the Grup 6 (infected control group), statistical difference was also observed. When the *C. albicans* growth in the cornea compared

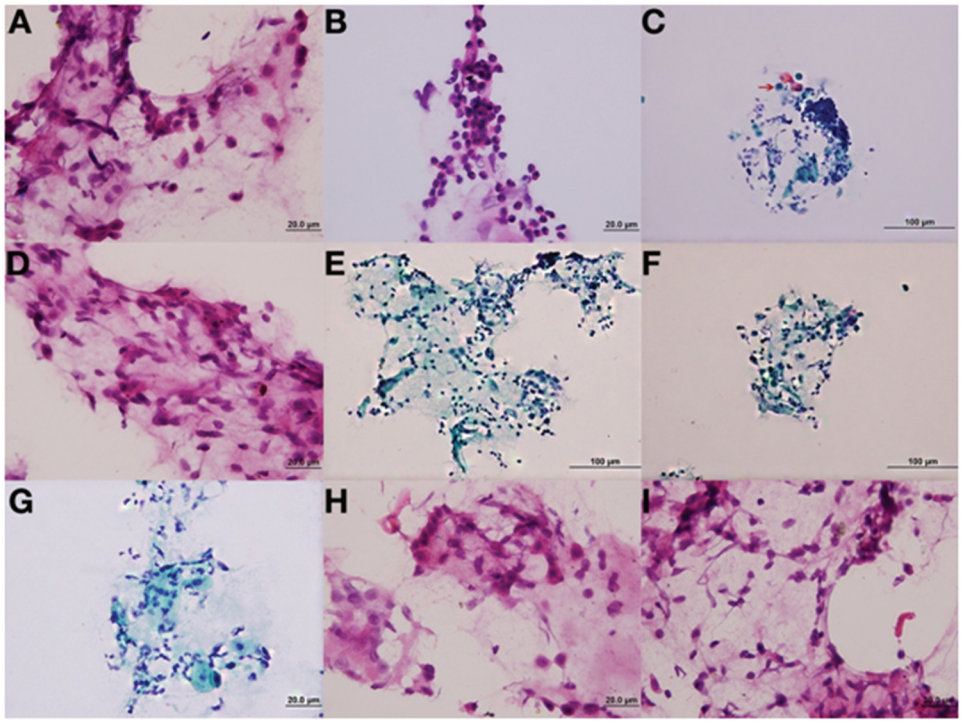


Figure 4. Hematoxylin-eosin **(A, B, D, H)** and Papanicolaou **(C, E, F, G)** dyed preparations of the ocular surface prepared by the cytospin technique. **(A)** Group 7 (Negative control group) control eye, normal rabbit cornea. **(B, C)** Group 6 (Infected control group) dense neutrophil leukocytes and squamous epithelial cells showing keratoconjunctivitis and corneal ulcer. Degenerative neutrophil leukocytes and fungal cells (arrow). Brush sample showing a large number of mononuclear cells (macrophage, lymphocyte, plasma cells), including reactive corneal epithelial cells, conjunctival epithelial cells. **(E, F, G)** treatment groups, Group 2 (FLU applied group) and Group 4 (FLU and ODW applied group) dysplasia cells showing hyperchromamia and nuclear pleomorphism, with clear cytoplasm and increased nucleo-cytoplasm ratio. Cell population similar to Group 7 (Negative control group) in Group 1 (FLU and OZO applied group), Group 3 (OZO applied group) and Group 5 (ODW applied group) from **(D, H, I)** treatment groups. Normal corneal, conjunctival cells. Inflammatory cells in few bears. Bar: 100 µm (E, F), 20 µm (A, B, D, G, H, I).

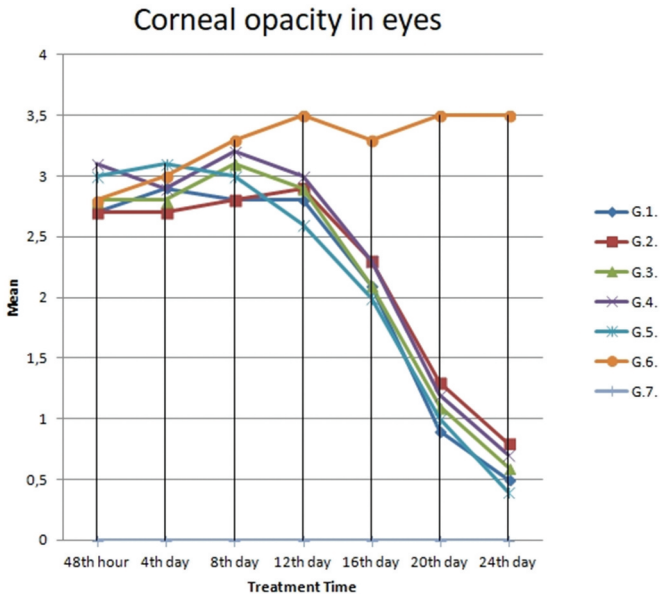


Figure 5. Change of corneal opacity findings during treatment. **Groups; G.1**. FLU and OZO drops applied group, **G.2**. FLU drop applied group, **G.3**. OZO drops applied group, **G.4**. FLU and ODW drop applied group, **G.5**. ODW drops applied group, **G.6**. PBS drop applied group (Infected control group), **G.7**. PBS drop applied group (Negative control group)

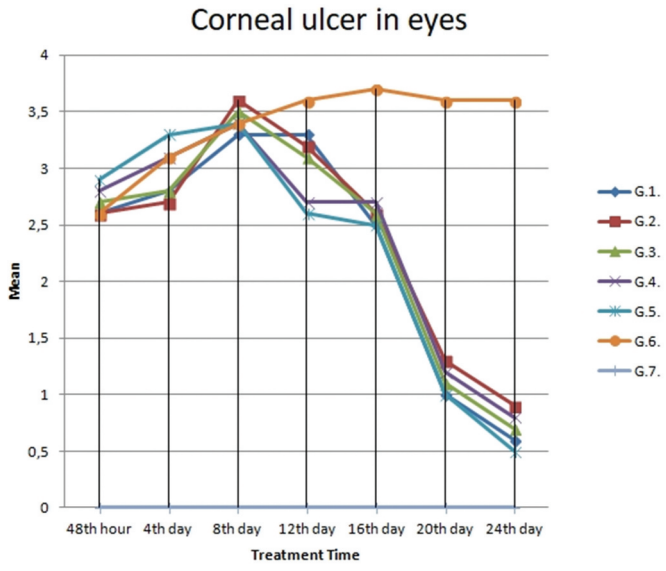


Figure 6. Change of corneal ulcer findings during treatment. **Groups; G.1**. FLU and OZO drops applied group, **G.2**. FLU drop applied group, **G.3**. OZO drop applied group, **G.4**. FLU and ODW drop applied group, **G.5**. ODW drops applied group, **G.6**. PBS drop applied group (Infected control group), **G.7**. PBS drop applied group (Negative control group)

within groups; there were statistically significant difference ($p < .05$) between 48th hour and 24th day in Group 1, Group 2 and Group 4 (Figure 1, Table 2). These results showed us that FLU, OZO and ODW have a synergistic effect when used together.

Hiraoka et al.[17] compared the subconjuntival efficacy of micafungin and FLU with the *C. albicans* 900028 strains in the treatment of experimental kerattis and continued treatment for 21 days. At the end of the 21st day, in the histopathological

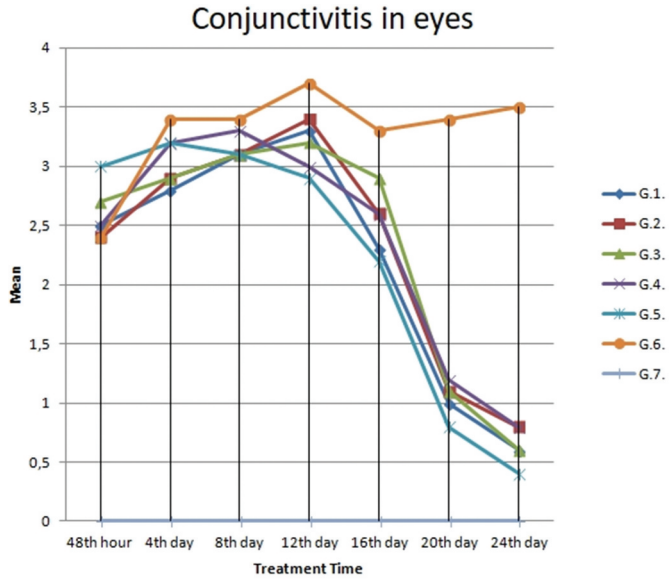


Figure 7. Change of conjunctivitis findings during treatment. **Groups; G.1**. FLU and OZO drop applied group, **G.2**. FLU drops applied group, **G.3**. OZO drops applied group, **G.4**. FLU and ODW drop applied group, **G.5**. ODW drop applied group, **G.6**. PBS drop applied group (Infected control group), **G.7**. PBS drop applied group (Negative control group)

findings of the control group corneas, they detected excessive infiltration of inflammatory cells, large amount of neovascular vessels and destruction of corneal structures over the entire stroma. They saw that the Descemet's membrane and corneal endothelium also disappeared and recorded intense inflammation in the anterior chamber. These findings are very similar to our infected control group (Figure 2, Figure 4). In the FLU applied group, they found that intense infiltration of inflammatory cells, corneal structure and significant destruction of neovascular vessels were noticeable and these findings were relatively localized in the midstroma. They also reported that corneal epithelium, Descemet's membrane and corneal endothelium were intact. Although these findings are similar to the histopathological cytology findings of our FLU and all of our other treatment groups, the findings were milder in our study (Figure 3, Figure 4). In addition, they reported that, on the 18th day of clinical findings, in the infected control group; large corneal lesion, diffuse and severe corneal push and prominent neovascular vessels in the corneoscleral limbus, in the FLU group; central corneal lesion approximately 2 mm in size, middle corneal push and neovascular vessels in the lower limbus, in the micafungin group; there was a small lesion approximately 1 mm in size localized in the middle cornea. In parallel with these findings, it was observed in our study that there were large corneal lesions, diffuse and severe corneal push and prominent neovascular vessels from the corneoscleral limbus in the infected control group. The findings in our study were milder than these findings; although the treatment was subconjuntival, it was thought to be due to the treatment once daily and lasted for 18 days. This is supported by the findings of our treatment groups for 16 and 20 days. Furthermore, the change in the amount of *C. albicans* growth in the eye after treatment in the infected control group and FLU group was similar compared to our infected control group and FLU group and other treatment groups (Figure 1, Table 2).

In our pilot study, the lesions in keratitis created by the *C. albicans* ATCC 900028 strain was milder than the lesions formed by the *C. albicans* strain isolated from blood. In addition, *C. albicans* ATCC 900028 strain proved to be non-resistive to OZO and FLU in our previous study[15] and pilot study (Table 1). But, we did not use this strain (ATCC 900028) due to its mild effect. The fact is that the *C. albicans* strain isolated from existing human blood forms a more stubborn lesion. So, we used this human blood form in the present study. MIC values of the used strain were higher than the standard strain against OZO, and the same against FLU.

However, the amount of C. albicans grown in the eye after treatment was lower in the OZO and ODW groups compared to the FLU group. In addition, the fact that the MIC value was lower in the groups in which OZO and ODW and FLU were used together, compared to the group in which FLU was applied alone, is due to the fungicidal effect of ozone. Moreover, although the MIC findings of FLU are better than OZO against clinical isolate in vitro, ozone is more effective in vivo and clinical isolate produces a more aggressive keratitis, making this study comparable to real cases.

Ozone provides physiological wound healing. Therefore, it minimizes the risk of keloidal scars. In addition, it minimizes the risk of push in the cornea.[27–29] In the treatment groups, a light central corneal lesion approximately 1–2 mm in size was observed, and no neovascular vessels were observed from the middle corneal push and lower limbus. In our study the reason for the absence of push was that ozone reducing the risk of keloidal scar and the risk of push in the cornea (Figure 1).

Ozone has anti-inflammatory effect like prednisolone. It has double bonds containing compounds, arachidonic acid (20:4) and its derivatives, in particular by its ability to oxidize prostaglandins.[7,24,30] Adequate anti-inflammatory therapy is required for inflammatory diseases in the anterior segment. Corticosteroids are one of these anti-inflammatory agents, but the combination of antimycotic agents and corticosteroids in keratomycosis is still controversial. When corticosteroids are used together with antibiotics, it reduces intraocular irritation and neovascularisation and corneal edema. It also reduces non-specific inflammatory processes. However, in many studies, combining corticosteroids with antimycotic agents shows negative effects in fungal infections. Especially in studies, while the healing rates decreased steadily in normal control corneas, they remained stable for days in corneas treated with corticosteroids.[18] O'Day et al.[31,32]reported that although inflammation was similar or considerably less until day 10, on day 15, inflammation in the corneas treated with corticosteroids was more than animals without corticosteroid therapy. Schreiber et al.[18] reported that most of the groups treated with prednisolone until the 24th day showed less corneal clouding and neovascularisation. In our study, decreases in corneal opacity and neovascularisation findings support this. Unlike ozone, when used alone, it shows fungicidal and bactericidal effect and reduces inflammation in the cornea. Bacteriological, clinical, histological, cytological, findings in our study support this (Table 2–6, Figures 1, 2, 4–7).

A special formulation has been developed for the well-tolerated and biocompatible ozonated liposomal sunflower oil plus hypromellose (Ozodrop®) based ophthalmic use and the sensitive tissue of the ocular surface.[29] Spadea et al.[29] reported that Ozodrop® is effective in the treatment of conjunctivitis in the horse, dog, cat, and humans. They also reported that it limited corneal involvement in corneal ulcers in humans. Vigna and Mendenez-Cepero[22] have reported that OLEOZON® colonium three drops twice daily are effective in the treatment of keratitis, conjunctivitis and keratoconjunctivitis cases caused by factors such as herpes virus, chlamydia, mycoplasma, *Chlamidiophila felis*. In parallel with above information's, in the present study, statistical comparison of corneal opacity and corneal ulcer and conjunctivitis values among the Group 6 and Group 1, 2, 3, 4, 5, 7 were also different significantly ($p < 0,05$) on days 20 and 24 post inoculation. When corneal opacity and corneal ulcer and conjunctivitis values within the group compared; statistical difference ($p < .05$) between 48th hour and 20th and, 24th day in Groups 1, 2, 3, 4 and 5 were observed (Table 3–5).

Ozone's analgesic effect is achieved by oxidation of algopeptidites called albuminolysis products, which determine the severity of movement, pain and response on nerve endings in damaged tissue[1,24,29] Although it is seen as a disadvantage that the ozonated oil causes burning cornea tissue, it should be remembered that ozone has an analgesic effect. For this reason, it should be remembered that even though it burns slightly at first, it will reduce the pain later.

CONCLUSION

In conclusion, OZO and ODW were found to be effective in treating fungal keratitis in the present study. OZO and ODW act together with FLU. The growth of the amount of *C. albicans* on the 24th day in the cornea was observed at least in the ODW group. It has also been proven by this study that ODW contain 26 µg/ml was the most effective in the treatment of *C. albicans* keratitis. Especially the effective results of ODW obtained in this study will be used in the treatment of invasive fungal keratitis and ocular infections, and will even be an example for future studies.

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ORCID

Kemal Varol http://orcid.org/0000-0002-3057-2865
Ayşe Nedret Koç http://orcid.org/0000-0002-1736-9707
Latife Çakır Bayram http://orcid.org/0000-0001-9357-0755
Hatice Arda http://orcid.org/0000-0003-1682-2490
İhsan Keleş http://orcid.org/0000-0001-5751-9450
Metin Ünlü http://orcid.org/0000-0003-2505-9853
Vehbi Güneş http://orcid.org/0000-0002-4047-4409
Gencay Ekinci http://orcid.org/0000-0002-4551-8749
İlknur Karaca Bekdik http://orcid.org/0000-0003-3897-5164
Mustafa Altay Atalay http://orcid.org/0000-0003-4169-0637

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