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Effects of boron compounds and ozonated olive oil on experimental *Microsporum canis* ınfection in rats

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Abstract: This study aimed to investigate the treatment outcomes of topical application of boric acid, boron-doped gel, and ozonated olive oil in cases of *Microsporum canis*-induced dermatophytosis. Furthermore, the outcomes were compared to those of terbinafine administration. We included 39 female Wistar albino rats weighing 200–250 g and created an *M. canis*-infected area on the skin of their backs. The rats were clinically scored on days 0, 7, 14, 21, and 28 and underwent histopathological evaluation. All the treated groups demonstrated significantly lower clinical scores than the control group ($P < 0.05$). Fewer inflammation cells were observed in the samples of groups treated with 3% boric acid and sodium pentaborate pentahydrate gel than in those of the control group. According to the histopathological evaluation, the groups treated with 3% boric acid and sodium pentaborate pentahydrate gel were statistically different from the control and other treatment groups ($P < 0.05$). Our results indicated that treatment with 3% boric acid and sodium pentaborate gel was adequate in resolving *M. canis*-induced infection in rats. Therefore, gels containing 3% boric acid and sodium pentaborate pentahydrate may be alternatives to antifungal agents such as terbinafine by ensuring easy, reliable, inexpensive, and effective treatment modalities.

Keywords: Boron, *Microsporum canis*, ozonated olive oil, terbinafine, sodium pentaborate pentahydrate, rats

1. Introduction

The skin is the largest organ of the body. Considering the fact that it covers the outer surface of the body, it is directly exposed to external factors that may damage it and cause skin diseases [1–3]. The most common fungal infections of the skin are dermatophyte infections [4,5]. Dermatophytosis is noted in both humans and animals globally. Studies have reported that *M. canis* spores were observed in 4%–10% of healthy dogs and 2%–7% of cats [6,7]. Fungal species that are etiologically associated with dermatophytosis may demonstrate certain degrees of regional variations [8]. Animal and human studies have revealed that the most commonly detected dermatophyte species include *M. canis* and *M. nanum*, *T. mentagrophytes*, *M. equinum*, *T. equinum*, and *T. verrucosum*. Approximately 2/3 of dermal diseases in children aged 2–10 years are caused by zoophilic dermatophytes [9–11]. Dermatophyte fungal spores multiply and divide into hyphae within an incubation period of 1–4 weeks and settle in keratinized cells and hair follicles, following which they penetrate the stratum corneum layer with keratolytic enzymes and extend into hair follicles. This entire process takes approximately 7 days; during this period (telogen phase), dermatophytes digest the keratin layer using keratolytic enzymes, which results in hair loss. They may exist by either wrapping around the hair roots in the form of cuffs (ectothrix) or by settling in the hair (endothrix), consequently impeding hair growth. The metabolic products that are generated by the proliferating fungi have been causatively associated with the occurrence of inflammatory reactions in the stratum corneum layer. Inflammatory reactions of fungi on the skin demonstrate healing by the presence of typical erythematous, inflammatory, rounded areas without fungal elements in the center. The severity of the inflammation depends on the status of the organism's immune system, and the amount and type of metabolic residues [7].

Dermatophytosis diagnosis is based on anamnesis, clinical lesions, macroscopic and microscopic examination of skin scrapings and hairs, examination of hairs under ultraviolet light, skin biopsies, and fungal cultures [8,12]. Apart from the histopathologic forms of inflammation, dermatophyte infection has also been associated with the presence of folliculitis and furunculosis lesions along with prominent orthokeratotic and parakeratotic hyperkeratosis

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lesions (specifically when associated with neutrophilic microabscesses). Generally, *Microsporum* infections are characterized by polygonal arthrospores in ectotric form, and *Trichophyton* infections are characterized by round arthrosporic chains in both endotrix and ectotric forms [8].

Drug resistance, toxicity, and drug interactions limit the efficacy of antifungals to treat dermatophytosis. Antifungal drugs are more toxic than antibacterial drugs since fungal cells are eukaryotic and are similar to mammalian cells, contrary to the prokaryotic bacterial cells. Antifungal drugs are therefore less selective between fungal and mammalian cells; furthermore, they have been associated with a wide range of side effects, which are not limited to skin irritation and allergic contact dermatitis [13]. Lesions are ash-shaped with excessive crust and are particularly near the head region (tinea capitis) or the extremities of the entire body (tinea corporis). The disease is transmitted by spores and hyphae, which are attached to hairs and keratin-containing materials [5].

We aimed to compare the effects of topical application of boric acid, boron-doped gel, and ozonated olive oil in cases of *M. canis*-associated experimentally induced dermatophytosis to those of terbinafine administration in rats.

2. Materials and methods

2.1. Animal material

We included 39 Wistar albino breed female rats aged 6–8 weeks and weighing 200–250 g. Animals were obtained from the Celal Bayar University Experimental Animal Application and Research Center (Manisa Celal Bayar University Animal Experiments Local Ethics Committee Permission No. 77.637.435) and housing, maintenance, and experimental procedures were carried out at this location. Room temperature was adjusted to approximately 21 ± 1 °C, and lighting was fixed at 12 h of light and 12 h of dark. Food and water were given ad libitum.

2.2. Creation of skin infections

2.2.1. Cultivation of *M. canis*

This stage of the study was performed in the İstanbul University Faculty of Veterinary Medicine's Department of Microbiology. Reference *M. canis* (ATCC 36299) was cultured in commercial Sabouraud dextrose agar (SDA, Merck, Darmstadt, Germany) and incubated at 25 °C for 7–14 days. During the incubation period, the morphologies of the colonies formed in SDA were examined once every 3 days and lactophenol cotton-blue slides of the same were prepared with the help of a microscope. Colony purity was checked by judging the microscopic appearance of the fungi.

Pure *M. canis* colonies were collected from the 14-day culture of SDA using physiological saline (0.85% FTS), transferred into sterile tubes, and subsequently homogenized. The suspension density was adjusted spectrophotometrically to a concentration of 0.5 McFarland (1.0×10^6 cfu/mL) and made ready for use, and 1 mL of the same was reserved for each test animal (1.0×10^6 cfu/mL).

2.2.2. Creation of *M. canis* infection

We determined the center of the dorsal region of the rats that was spotted under general anesthesia (xylazine 10 mg/kg, Alfazyne 2%, Atafen; ketamine, 50 mg/kg, Alfamine 10%, Atafen) with a 3 × 3 cm square that was subsequently used to create a hairless area with a machine pet clipper. The hairless area was rubbed with sandpaper for 10 s, following which we applied 1 mL of *M. canis* suspension (1.0×10^6 cfu/mL) to the same. Lesions were visible on the hairless area within 3 weeks and treatment was initiated.

2.3. Experimental design

Rats were randomly categorized into 4 treatment groups, and the lesions in each case were directly subjected to their respective treatment modalities. Accordingly, Group A (n = 8) underwent topical treatment with 0.03 g of 1% terbinafine fungus cream once daily; Group B (n = 8) was topically treated with ozonated olive oil once daily; Group C (n = 8) underwent topical treatment with 0.5 mL of 3% boric acid once daily; and Group D (n = 8) was treated with a topical gel that contained 0.8 g of 3% sodium pentaborate pentahydrate once daily. Seven rats did not receive any treatment and constituted a negative control group (Group E). Treatment continued until day 29.

On days 0, 7, 14, 21, and 28 of the experiment, we subjected members of each group to clinical scoring according to the data presented in Table 1, following which their photographs were taken [14]. On day 29 of the experiment, we stopped treatment, the animals were euthanized under general anesthesia, and skin samples were acquired using the punch biopsy method.

2.4. Histopathological examination

The biopsy samples were fixed in 10% buffered formaldehyde solution for histopathological examination. The skin samples were embedded in paraffin blocks after fixation and stained according to hematoxylin and eosin (H&E) and Mallory's trichrome protocols [15]. We used two Grocott fungi staining kits (Grocott, Bio-Optica Cat. No. 04-043823 and Grocott Methenamine Silver, BesLab Cat. No. 0059) for *M. canis*. The samples were stained and the sections were examined under light microscope. We then evaluated the amount of granulation and inflammation cell infiltration in the dermis, collagen strands organization, collagen pattern, young and mature collagen amount parameters, and histopathological healing status of lesions. Cases demonstrating weak repair were scored as 1, moderate repair as 2, and good repair as 3 for the purposes of statistical evaluation.

2.5. Statistical analysis

The groups were evaluated statistically considering the clinical scoring outcomes using SPSS 20.0 (IBM Corp., Armonk, NY, USA). We used the median of the clinical scoring results; furthermore, we compared the intra- and intergroup data using Friedman and Kruskal–Wallis tests. In the Kruskal–Wallis and Friedman tests, we used the post hoc Dunn test (multiple comparison tests) to identify the groups that created the difference. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical scoring findings

Among the 8 rats in Group A, 4 demonstrated complete clinical improvement, while 1 in Group B, 6 in Group C, and 3 in Group D showed complete recovery. All rats showed clinical improvement and decreased ulcerated areas, improved dandruff, improved areas of hair loss, and reduced inflammation. Rats in Group C demonstrated the highest degree of clinical progress. None of the patients in Group E (no treatment) showed clinical improvement. At the end of day 28, the most significant clinical improvement was observed in Group C (treated with 3% boric acid) and the least improvement was noted in Group E (no treatment) (Figure 1).

The improvements observed on days 0, 7, 14, 21, and 28 are summarized in Table 2. Improvement findings categorized according to days are given in Table 3. Considering these outcomes, we noted that the most significant improvement was observed in Group C,

Table 1. Clinical scoring parameters [14].

0: Normal
1: Mild erythematous areas
2: Well-defined redness, hair loss, less dandruff
3: Inflammation, redness that protrudes outside the lesioned area, also dandruff
4: Fully shed bristles, severe dandruff, less ulcer formation, serious clinical signs
5: Large ulcerative areas

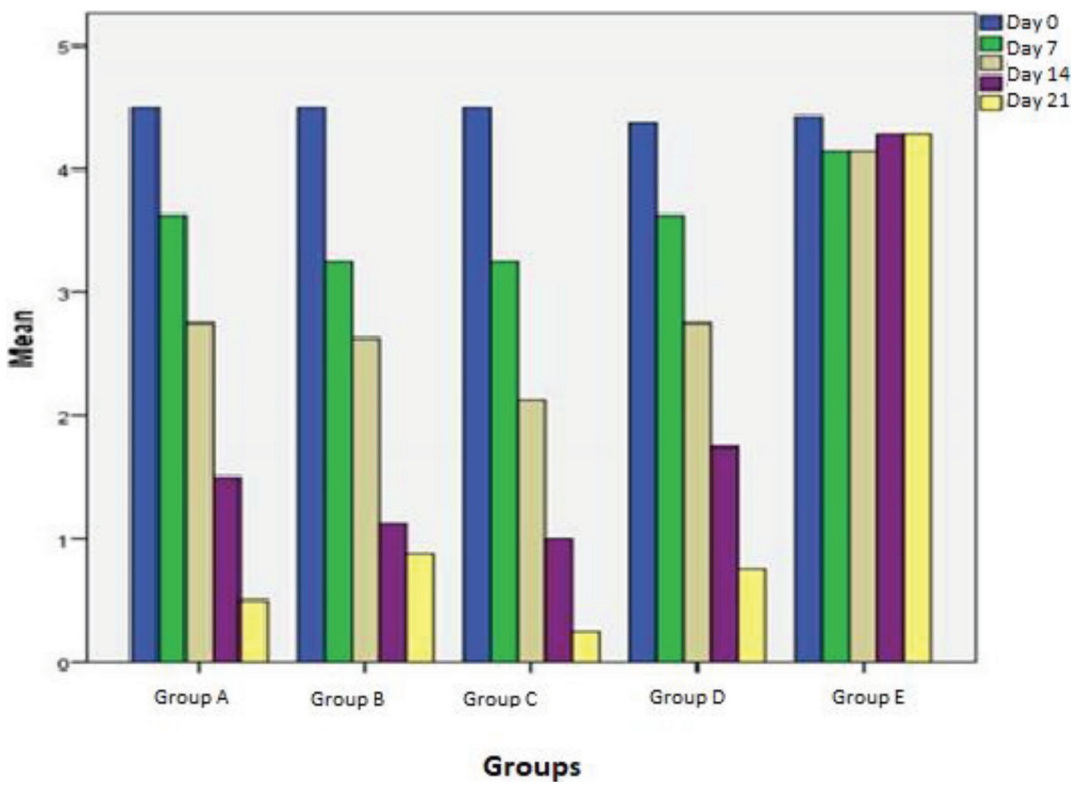

Figure 1. Comparison of average recovery status of groups by days.

Table 2. Improvement levels observed between 0, 7, 14, 21, and 28 days of treatment.

Groups	Day 0	Day 7	Day 14	Day 21	Day 28	P
Group A	4.5	4	3	1.5	0.5	0.000*
Group B	4.5	3	3	1	1	0.000*
Group C	4.5	3	2	1	0	0.000*
Group D	4	4	3	2	1	0.000*
Group E	4	4	4	5	4	0.837

*: Statistical difference was significant ($P < 0.05$).

Table 3. Findings of improvement between treatment groups by days.

Groups	Day 0	Day 7	Day 14	Day 21	Day 28
Group A	4.5	4	3	1.5	0.5
Group B	4.5	3	3	1	1
Group C	4.5	3	2	1	0
Group D	4	4	3	2	1
Group E	4	4	4	5	4
P	0.982	0.027*	0.000*	0.000*	0.000*

*: Statistical difference was significant ($P < 0.05$).

followed by Group A, Group D, and Group B. The most significant degree of improvement by day 28 was observed in Group C and the least in Group E (Figure 1). Despite the lack of statistically significant differences between the groups on day 0 ($P > 0.05$), significant difference between the groups could be observed from day 7 ($P < 0.05$). Furthermore, the difference between the groups at days 14, 21, and 28 demonstrated considerable statistically significant ($P < 0.05$). Therefore, Groups A, B, C, and D showed statistically significant clinical improvement compared to Group E during the experimental period between 0 and 28 days, according to our expectations. Differences in clinical improvement between Groups A, B, C, and D and the control, Group E, were statistically significant after 14 days ($P < 0.05$) (Tables 2 and 3).

3.2. Histopathological findings

We noted varying degrees of orthokeratotic hyperkeratosis, edema in the dermis layer, coarseness and hyalinization areas in collagen, significant organization at the dermal epidermal border, and areas of young connective tissue in each group.

The collagen fibers were generally organized vertically and horizontally (Figure 2), contrary to that in Group D, which was relatively mild and horizontal (Figures 2 and 3). Mallory trichrome staining indicated that the lesion areas in Group E samples were wider than those of the other groups (Figure 3). Furthermore, we observed the development of reticular connective tissue in the samples of Group E. We also noted the presence of mild and moderate mononuclear inflammatory cell infiltrations in a number of cases, which were concentrated in the dermal epidermal border. Fewer inflammatory cells were observed in the samples of Groups C and D (Figure 2), contrary to those in Group E. The rats demonstrated different densities of atrophy in hair follicles.

Considering the histopathology data, the groups treated with 3% boric acid (Group C) and sodium pentaborate pentahydrate gel (Group D) showed statistically significant improvement compared to the control group (Group E) ($P < 0.05$). We also noted differences in the amount of repair between Groups C and D, despite the lack of statistical significance ($P > 0.05$). According to the histopathological evaluation, groups treated with the two boron-based products showed the most significant improvement. In contrast, based on the histopathological repair scores, improvement in Groups A and B was higher than in the control, i.e. Group E. However, we observed that this difference and the differences in histopathological repair between Groups A and B were not statistically significant ($P > 0.05$) (Figure 4).

4. Discussion

Dermatophytosis is a superficial fungal skin disease that is typically caused by species belonging to the genus

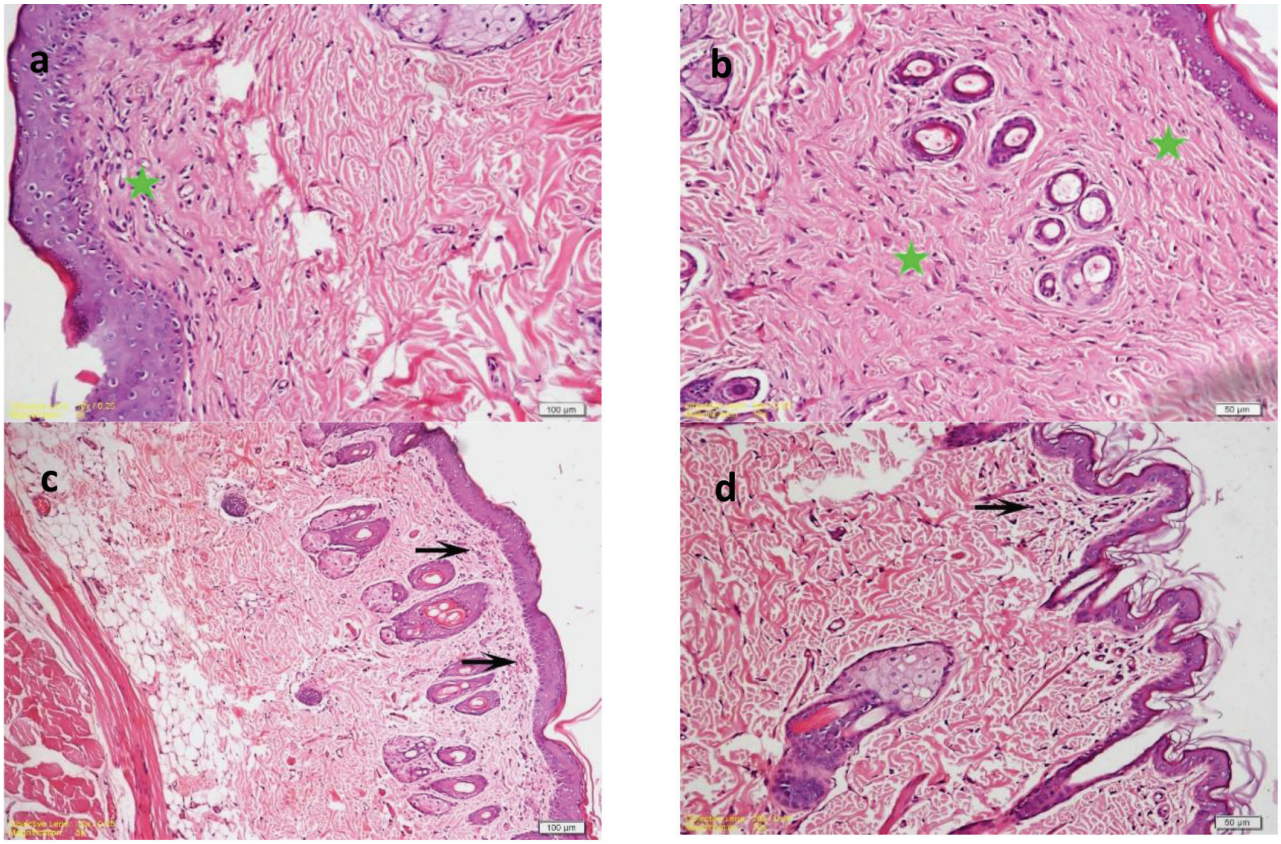

Figure 2. a) Young connective tissue in the dermis, vertical and horizontally located collagen fibers (star), bar = 100 µm. b) Young connective tissue in the dermis, horizontally located collagen strands (star), bar = 100 µm. c) Superficial inflammation in subepidermal area, few mononuclear inflammation cells (arrows), bar = 100 µm. d) Superficial inflammation in the subepidermal area, few mononuclear inflammation cells (arrow), bar = 50 µm, H&E.

Microsporum. Due to its zoonotic and highly infectious nature, topical antifungals are applied to minimize the infectious, contagious, and zoonotic risks of transmission [5]. Petranyi et al. [16] reported that terbinafine, an allylamine derivative, demonstrated a primary fungicidal effect against dermatophytes, other filamentous fungi, and *Sporothrix schenckii* in vitro. Slim and Karelson [17] highlighted the fact that oral and topical terbinafine treatment could be used to treat children infected with *M. canis;* however, the side effects include vomiting, pruritus, and local erythema. Studies associated with the treatment of *M. canis* infections with terbinafine have reported successful outcomes in both human and animal cases [14,18]. Here, we applied a terbinafine topical cream as a positive control group and compared its efficacy to alternative treatment modalities.

Geweely [19] used ozonated olive oil to successfully treat infections of *Aspergillus fumigatus*, *Candida albicans*, *Epidermophyton floccosum*, *M. canis*, and *Trichopyton rubrum*. Daud et al. [20] reported considerable improvements in rabbits that were experimentally infected with *M. canis* and subsequently treated with 1% terbinafine cream and ozonized sunflower oil. In this study, the topical ozonated olive oil treatment group showed positive outcomes in comparison with the untreated control group; however, these results were not as good as those of groups treated with boron-based creams.

Topical antifungal agents containing boron in adults are characteristically used to treat onychomycosis caused by the agents of *T. rubrum* and *T. mentagrophytes*, including the causative agents associated with *M. canis* [21]. Demirci et al. [22] reported the antimicrobial effects of both boric acid and sodium pentaborate pentahydrate in their in vitro study against tested pathogenic agents (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Aspergillus niger*, and *Candida albicans*). In their study, boric acid and sodium pentaborate demonstrated moderate levels of antibacterial activity and superior anticandidal and antifungal activities against a variety of pathogenic species. Demirci et al. [23] reported that certain active biological polymers combined with sodium pentaborate pentahydrate to

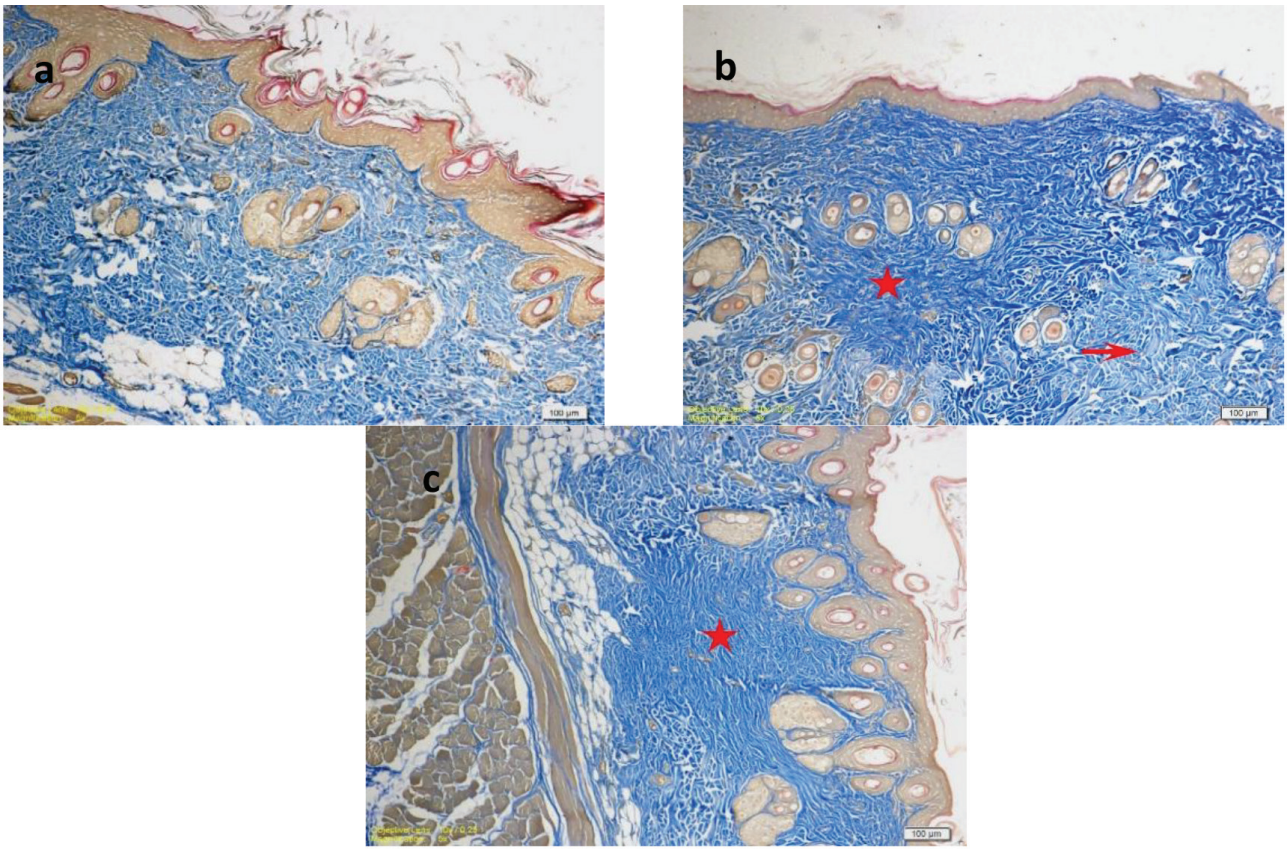

Figure 3. a) The usual collagen structure in the dermis, bar = 100 µm. b) Young connective tissue in the dermis (star), original collagen tissue (arrow), bar = 100 µm. c) Young connective tissue development in the dermis (star), bar = 100 µm, Mallory's trichrome.

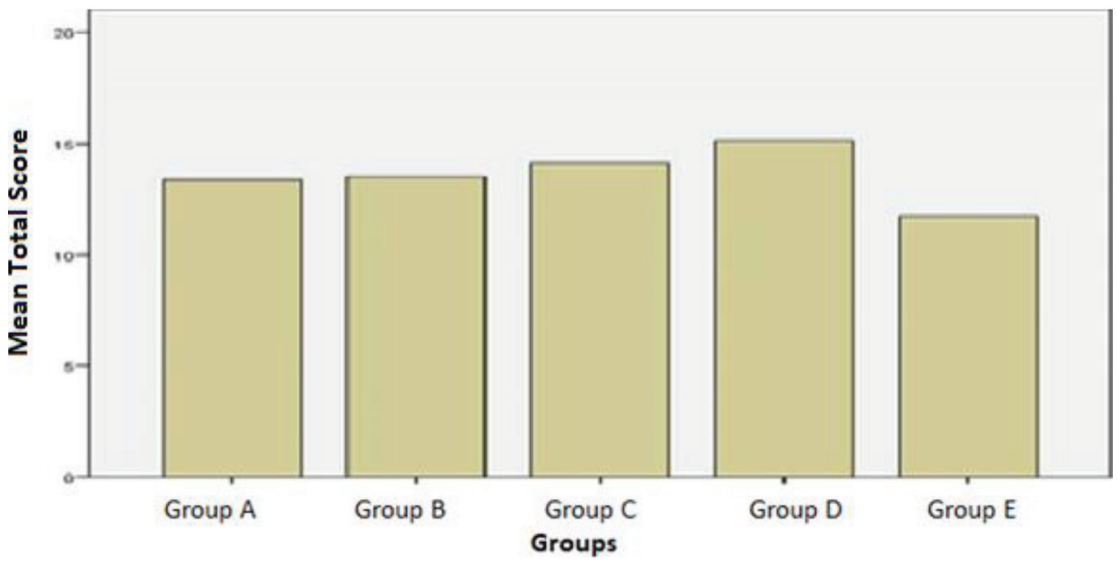

Figure 4. Comparison of experimental groups' histopathological recovery scores.

form a gel healed second-degree burn wounds in rats and showed antimicrobial activity against bacteria, yeast, and a number of fungi. Furthermore, highly diluted solutions of boric acid are used as washing solutions for the eyes (www.etimaden.gov.tr). A hospital study by Blech et al. [24] used 3% boric acid in the deep wounds of patients in

the intensive care unit and highlighted the fact that this treatment could gradually heal deep wounds. In this study, 3% boric acid and 3% sodium pentaborate pentahydrate gel treatment groups demonstrated better macroscopic and histopathological outcomes than terbinafine and ozonated olive oil treatment groups. Considering the clinical score evaluation analyses, we observed that the clinical efficiency levels of ozonated olive oil, 3% boric acid, sodium pentaborate pentahydrate-added gel, and 1% terbinafine used in this study were similar in the improvement of *M. canis* infection. There were several side effects of both oral and topical antifungal drugs used to treat *M. canis*. Considering this, we aimed to find a reliable, low-cost, easy alternative for the treatment of *M. canis* infection. Furthermore, this possibly may be the first study to investigate the effects of topical 3% boric acid and topical sodium pentaborate pentahydrate gel in the treatment of *M. canis* infection in vivo. Although there is a need for a significant amount of further research, our study provides a vital insight regarding *M. canis* infections.

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