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The therapeutic effect of ozone–enriched propolis oil extraction in cats with gingivitis

Efecto terapéutico de la extracción de aceite de propóleo enriquecido con ozono en gatos con gingivitis

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ABSTRACT

In cats, gingivitis treatment typically involves professional dental cleaning under anesthesia, pain management, and antibiotic administration to reduce inflammation. Considering the disadvantages of antibiotic administration, the necessity for alternative acute treatment protocols arises. It is believed that enhancing the antibacterial, anti–inflammatory, and local anesthetic effects of propolis with ozone could shorten the treatment duration, help reduce the risks associated with gingivitis, and also support the overall health of the cat. The study included 20 domestic cats diagnosed with the causative agent of gingivitis presented to private clinics. The cats included in the study were grouped based on bacterial agents (n=10; Female=4, Male=6) and viral agents (n=10; Female=5, Male=5). Propolis extracted with ozone–enriched oil was administered in spray form for 14 days (d). Before the application and on the 7th and 14th d of the treatment, the gingival indices of the cats were recorded, scored, saliva samples were collected, and photographs were taken. The levels of VEGF and TNF–α in saliva were determined using a cat–specific ELISA kit. When the data were evaluated, the application of ozone–enriched propolis demonstrated a statistically significant reducing effect on the levels of TNF–α in saliva in both groups (P<0.01). Salivary VEGF levels showed a significant increase during application, especially in gingivitis caused by bacterial agents (P<0.05). In the viral group, application was found to be more effective in increasing VEGF levels during the first 7 d. In gingivitis caused by bacterial agents, the gingival index (GI) and plaque index (PI) decreased compared to pre–treatment values (P<0.05). In gingivitis associated with viral agents, the decrease in the GI was statistically significant, while the decrease in the PI was found to be non–significant. In conclusion, this study demonstrated that the application of ozone–enriched propolis might be an alternative treatment option for cats with gingivitis.

Key words: Feline gingivitis; ozone–enriched propolis; saliva VEGF; saliva TNF–α

RESUMEN

En los gatos, el tratamiento de la gingivitis suele consistir en una limpieza dental profesional bajo anestesia, tratamiento del dolor y administración de antibióticos para reducir la inflamación. Teniendo en cuenta los inconvenientes de la administración de antibióticos, surge la necesidad de protocolos alternativos de tratamiento agudo. Se cree que potenciar los efectos antibacterianos, antiinflamatorios y anestésicos locales del propóleo con ozono podría acortar la duración del tratamiento, ayudar a reducir los riesgos asociados a la gingivitis y también favorecer la salud general del gato. El estudio incluyó 20 gatos domésticos diagnosticados con el agente causante de la gingivitis presentados en clínicas privadas. Los gatos incluidos en el estudio se agruparon por agentes bacterianos (n=10; Hembra=4, Macho=6) y virales (n=10; Hembra=5, Macho=5). El propóleo extraído con aceite enriquecido con ozono se administró en forma de aerosol durante 14 días (d). Antes de la aplicación y en los d 7 y 14 del tratamiento, se registraron y puntuaron los índices gingivales de los gatos, se recogieron muestras de saliva y se tomaron fotografías. Los niveles de VEGF y TNF–α en saliva se determinaron mediante un kit ELISA específico para gatos. Cuando se evaluaron los datos, la aplicación de propóleos enriquecidos con ozono demostró un efecto reductor estadísticamente significativo sobre los niveles de TNF–α en saliva en ambos grupos (P<0,01). Los niveles de VEGF salival mostraron un aumento significativo durante la aplicación, especialmente en la gingivitis causada por agentes bacterianos (P<0,05). En el grupo vírico, la aplicación resultó más eficaz para aumentar los niveles de VEGF durante los primeros 7 d. En la gingivitis causada por agentes bacterianos, el índice gingival (IG) y el índice de placa (IP) disminuyeron en comparación con los valores previos al tratamiento (P<0,05). En la gingivitis asociada a agentes virales, la disminución del IG fue estadísticamente significativa, mientras que la disminución del IP resultó no significativa. En conclusión, este estudio demostró que la aplicación de propóleo enriquecido con ozono podría ser una opción de tratamiento alternativa para los gatos con gingivitis.

Palabras clave: Gingivitis felina; propóleo enriquecido con ozono; VEGF salival; TNF–α salival

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INTRODUCTION

Problems with a pet's oral and dental health can affect its overall health and quality of life [1]. Although the prevalence of oral cavity and periodontal diseases in cats (*Felis catus*) varies in the literature, ranging from 70-85% [2] to 85-95% [3] depending on the age groups of the studied population, all reports agree that these conditions are frequently observed in cats older than two years, with an incidence rate of up to 95% [4]. Gingivitis, the earliest stage of periodontal disease, is an inflammation of the gums caused by subgingival plaque bacteria or microbial by–products such as cytotoxins and endotoxins in response to the host's immune response [5, 6, 7]. The presence of anaerobic species of gram–negative bacteria is typically found in these bacterial plaques [8].

In cats with compromised immune systems, opportunistic bacteria have been reported to cause gingivitis, with samples from these cats primarily isolating gram–positive bacteria such as *Staphylococcus, Corynebacterium*, and *Streptococcus*, as well as gram–negative bacteria such as *Pseudomonas* spp., *Proteus mirabilis*, and *Klebsiella pneumoniae* [9].

Gingivitis in cats can occur in all breeds and at any age with varying severity; however, it is diagnosed more frequently in adult or older cats. Its occurrence is not attributed to a single cause [10, 11]. Gingivitis is more frequently observed in cats during the periods of secondary teeth emergence (3-5 months), sexual maturity (6-9 months), and later in life due to the accumulation of tartar and dental calculus on the tooth surfaces [12]. Predisposing factors include systemic diseases, particularly immune–suppressing conditions such as feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV), as well as diet. Additionally, local dental diseases such as excessive tooth crowding (commonly seen in many Persian cats), tooth morphology, feline odontoclastic resorptive lesions (FORL), and fractured teeth are present [13].

The primary goal of treatment is to control gingivitis by cleaning both supra – and subgingival dental plaque and tartar, as well as addressing the predisposing factors [14, 15]. Broad–spectrum antibiotics are utilized in the treatment of gingivitis. The most commonly used antibiotics are amoxicillin–clavulanate acid, cephadroxil, and clindamycin [16]. Although gingivitis is a reversible inflammation, if treatment is not performed in a timely manner or if resistant bacterial strains are involved, the ongoing infection can lead to the loss of supporting dental tissues and progress to periodontitis. In the advanced stages of periodontitis, it can lead to tooth loss, bone infection, and allow pathogenic bacteria to enter the bloodstream, potentially causing organ damage. Oral diseases can also be indicative of other systemic conditions [7, 17]. It has even been suggested that dental disease is a risk factor for the development of chronic kidney disease in cats [18]. Therefore, easy–to–apply treatment options that prevent the formation of antibiotic–resistant bacteria should be developed for the treatment of gingivitis.

Propolis, a natural product whose use has become widespread in various forms due to its pharmacological effects and beneficial properties in recent years, was chosen. Propolis has gained popularity in recent years due to its therapeutic properties, containing numerous natural compounds such as polyphenols, phenolic aldehydes, sesquiterpene quinones, coumarins, amino acids, and steroids [19]. Many studies have shown that propolis has a wide range of biological and pharmacological effects, including antimicrobial, antioxidant, anti–inflammatory, immunomodulatory, antitumor, anticancer, anti–ulcer, hepatoprotective, cardioprotective, and neuroprotective actions. These properties have been studied for their potential use in veterinary medicine and have shown promise in various formulations [20].

Most ozonated oils have been reported to be used in the treatment of infections and skin diseases without causing side effects [21, 22, 23]. It has been reported that ozone stimulates the immune system by inducing leukocytosis and phagocytosis at low doses [24], can promote tissue healing, and exhibits anti–inflammatory effects [21]. Especially due to its biocompatibility with epithelial and periodontal mucosal cells, positive results have been achieved in the prevention, control, and treatment of oral infections [22, 23]. Ozone therapy is used in veterinary medicine for the local treatment of various lesions and neuromuscular diseases, including mastitis, metritis, endometritis, fetal membrane retention, vaginitis, urovagina, enteritis, and laminitis [24, 25, 26].

Saliva is an important fluid from a periodontal perspective. It washes the inside of the mouth and protects the oral mucosa by coating it against external factors. Pro–inflammatory cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor–alpha (TNF–α) play a key role in the pathogenesis of periodontal diseases, and the inhibition of these cytokines reduces bone loss associated with periodontitis [27, 28]. TNF–α is a cytokine that mediates some of the events that occur during periodontal disease. VEGF stimulates the proliferation of vascular endothelial cells necessary for angiogenesis, activates the release of proteolytic enzymes, and intensifies chemotaxis and migration [29]. Additionally, it plays a significant role in angiogenesis, bone formation, wound healing, and the regeneration of oral epithelium.

As a result, the undesirable side effects of modern medications have led to a preference for pharmaceuticals derived from natural sources in recent years. Due to these negative aspects worldwide, there has been a shift toward new explorations, and herbal therapy research has gained importance. Literature studies have reported that propolis is effective in the treatment of gingivitis in humans and dogs; however, no such study has been found in cats. Therefore, the aim of the study was to present an alternative treatment option for gingivitis in cats.

MATERIALS AND METHODS

Research design

The domestic cats brought to private clinics located in the Efeler district of Aydin province underwent careful oral and dental examinations. Cats with naturally occurring varying degrees of gingival inflammation, who had undergone a general examination and had the causative factor of gingivitis identified, were included in this study.

In the preliminary assessment for the oral and dental examination, factors such as the number of teeth, presence of dental caries, color and volume of the gingiva, halitosis, presence of deciduous teeth, symmetry of the mouth, presence of lesions in the oral mucosa, enamel defects, enamel hypoplasia, fractures,

gingival hyperplasia and retraction, oral masses, oro–nasal fistula, and exposure of the pulp were taken into consideration.

The study material consisted of a total of 20 cats, with 10 having a bacterial causative factor (female=4, male=6) and 10 having a viral causative factor (female=5, male=5) in TABLE I. Although the causative factors differed, all cats were diagnosed with chronic gingivitis. Among the cats with viral–induced gingivitis, previuosly 2 were diagnosed with Feline Herpes Virus and Feline Calicivirus, while 8 were diagnosed with Feline Immunodeficiency Virus (FIV).

TABLE I
Distribution of cases according to race, age, gender and the causative agent

Case	Rase	Age (Year)	Gender	Diagnosis	Causative Agent
1	Tabby	4	Male	Gingivitis	Bacterial
2	British	3	Female	Gingivitis	Bacterial
3	British	5	Male	Calculus+Gingivitis	Bacterial
4	Tabby	8	Female	Calculus+Gingivitis	Bacterial
5	British	2	Male	Calculus+Gingivitis	Bacterial
6	Tabby	6	Male	Gingivitis	Bacterial
7	Tabby	7	Female	Calculus+Gingivitis	Bacterial
8	Tabby	3	Male	Calculus+Gingivitis	Bacterial
9	Tabby	5	Female	Gingivitis	Bacterial
10	Tabby	4	Male	Calculus+Gingivitis	Bacterial
11	Tabby	1	Female	Calculus+Gingivitis	Viral
12	Tabby	6	Female	Calculus+Gingivitis	Viral
13	Tabby	7	Male	Calculus+ Gingivitis	Viral
14	Tabby	1	Male	Gingivitis	Viral
15	Tabby	1	Male	Calculus+Gingivitis	Viral
16	Tabby	1	Female	Calculus+Gingivitis	Viral
17	British	2	Female	Gingivitis	Viral
18	Tabby	4	Male	Calculus+Gingivitis	Viral
19	Tabby	4	Male	Calculus+ Gingivitis	Viral
20	Tabby	5	Male	Calculus+Gingivitis	Viral

Ozonation and application of propolis

Ozone (O_2/O_3 mixture) was generated from medical–grade oxygen using ozonator equipment (Sorande; Model: SAIR-2,Türkiye). The amount of ozone in the oily propolis was measured using a UV spectrophotometer (Shımadzu,Model: UV-1601, Australia) at 254 nm. Propolis extracted in ozone–enriched (5%) olive oil was placed in a spray bottle and applied once a day (d) for 14 d, delivering 2-3 puffs (0.3 mL) each time.

Sampling

In this study, the effectiveness of the treatment was monitored through photography on d 0, 1, 3, 5, 7, 10, and 14. Additionally, plaque index (PI) and gingival index (GI) data were recorded. Saliva samples were collected at the beginning of the treatment, as well as on the 7th and 14th d. To collect the saliva sample, the patients' mouths were rinsed thoroughly with water. then cats were placed on their side with their mouths closed and the saliva accumulated in the oral cavity was collected approximately 0.5 mL of saliva was obtained using a dropper into Eppendorf tubes. The samples were then centrifuged (Nüve, Model:NF800R, Türkiye) at 3500 g for 10 min to remove cellular components and plaque. The samples were stored at -80 °C (Nuaıre, Model: NU6617W35, USA) until they were evaluated.

Measurement of plaque index (PI) and gingival index (GI)

Using a periodontal probe, the depth (periodontal pocket) was measured by inserting it into at least three points on each tooth, and the average value was recorded. To assess the severity of gingivitis, the Plaque Index (PI) by Silness and Löe [30] and the Gingival Index (GI) by Löe and Silness [31] were used.

Determination of TNF–α and VEGF levels based on ELISA method

In the study, the levels of TNF–α and VEGF in saliva samples were measured using a cat–specific commercial VEGF ELISA kit (BT LAB, E0139 Cat, China) and a TNF–α ELISA kit (BT LAB, E0031 Cat, China). The saliva samples were analyzed in accordance with the procedures outlined in the kit, with two replicates performed for each analysis. The results of the ELISA analyses were calculated in ng/mL using CurveExpert Professional v. 2.7.3 software.

Exclusion criteria

Pregnant cats, cats that had recently undergone surgery, and cats that had used antibiotics within the last 15 d were excluded from the study, as their physiological conditions could lead to different results. Additionally, cats that had received long–term antibiotic and corticosteroid treatment were excluded from the study due to the potential for differing levels of VEGF and TNF–α.

Statistical analyses

All statistical analyses were performed using the SPSS for Windows Version 29.0 software package (SPSS Inc., Chicago, IL, USA). A *P*–value of less than 0.05 was considered an indicator of a statistically significant difference in the decisions. The normal distribution of the continuous variables was assessed using the Shapiro–Wilks test. It was determined that all data, except for GI and PI values, followed a normal distribution. To compare means between the groups, all ELISA results were evaluated by using repeated measures ANOVA. The Bonferroni correction was used to adjust *P*–values in multiple comparison tests. The Friedman test was applied to evaluate the PI and GI data. The Wilcoxon signed–rank test was applied to assess differences between the groups.

RESULTS AND DISCUSSIONS

According to the data from a small survey conducted with pet owners during our study, pet owners preferred the use of oral spray over procedures that would distress and increase the anxiety of their cats, such as administering medication and taking blood samples. They were also more positively inclined towards providing saliva samples instead of taking blood during the treatment process. This study has presented a more easily

applicable alternative treatment method compared to other treatment options. Prior to applying ozone–enriched oily propolis extraction to the cats meeting our criteria, PI and GI measurements were taken and photographed (FIG 1).

FIGURE 1. Images taken before and on the 14th day of application of ozone–enriched oily propolis extract

The comparison of saliva TNF–α levels due to the application of ozone–enriched propolis in gingivitis caused by both viral and bacterial agents revealed a statistically significant difference between TNF–α levels on days 7 and 14 compared to the beginning of the application process ($P<0.01$). (TABLE II, FIG 2).

Oral diseases in cats are an important issue in veterinary medicine. Oral health is a critical factor that affects the overall health of cats [7, 32]. Gingivitis is primarily caused by bacterial plaques that colonize the gingival sulcus. These plaques produce various toxins and by–products that lead to inflammation of the gums. The immune system secretes cytokines in response to these bacteria and by–products, including IL-1β, interleukin-8 (IL-8), prostaglandins, and TNF–α [7]. These cytokines lead to the

TABLE II
TNF–α levels (ng·L^{-1}) in saliva samples collected at the beginning of treatment, as well as on days 7 and 14, in gingivitis caused by viral and bacterial agents following the application of ozone–enriched propolis

Application of ozone–enriched propolis	TNF–α levels in gingivitis caused by bacterial agents. Mean ± SD (n=10)	TNF–α levels in gingivitis caused by viral agents. Mean ± SD (n=10)
Before application	126.26 ± 8.80	162.30 ± 18.65
7th day of application	66.07 ± 9.72***	128.25 ± 13.80**
14th day of application	37.09 ± 6.54***	106.64 ± 11.37**

**: $P<0.01$, ***: $P<0.001$ indicates significance when comparing the beginning of the treatment with the beginning of the treatment as well as on days 7 and 14 according to repeated measures ANOVA

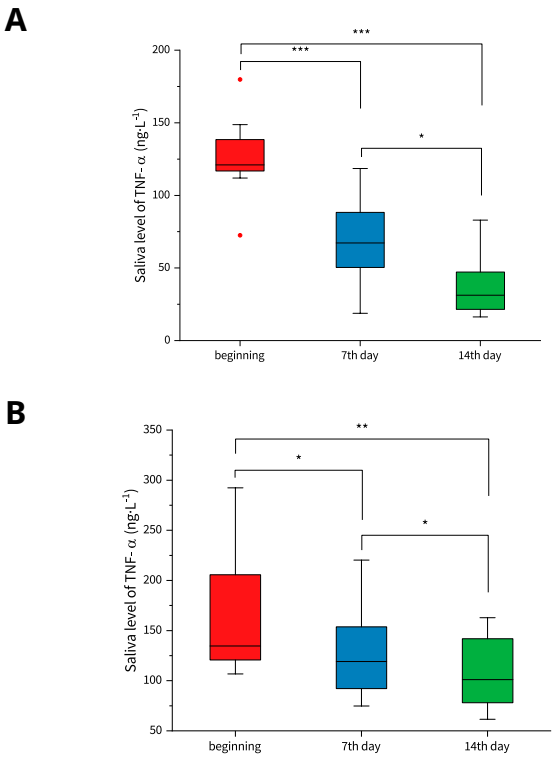

FIGURE 2. The changes in salivary TNF–α levels in gingivitis caused by bacterial agents (A) and viral agents (B) following the application of ozone–enriched olive oil–extracted propolis (*$P<0.05$; **$P<0.01$; ***$P<0.001$)

accumulation of inflammatory cells and initiate the inflammatory process [1, 13]. There have been many studies in humans on the relationship between some inflammatory mediators in saliva and the pathogenesis of periodontitis. They reported that salivary TNF–α levels were higher in individuals with periodontal disease compared to healthy individuals [33, 34, 35]. Geng [36] and Rai [37] have proposed that TNF–α levels in saliva may serve as a potential biomarker for periodontal disease. In a study conducted in dogs, TNF–α levels in saliva samples were found to be higher in healthy dogs with gingivitis than in healthy dogs without gingivitis [38]. In addition, TNF–α has been closely associated with tissue destruction and immune response, and thus has been reported to play an important role in periodontitis–induced bone loss [39]. When all these data are evaluated, it can be considered that the decrease in TNF–α levels with ozone–enriched olive oil propolis reflects the positive effects of the treatment.

When examining the VEGF levels in saliva, it was observed that the levels increased during the application in gingivitis caused by bacterial and viral agents, and this increase fluctuated throughout the process. However, when the values obtained before and after the final application were examined, a significant increase was detected. Our results are summarized in FIG. 3 and Table III.

Current studies demonstrating the effects of propolis on oral and dental health show that the flavonoids found in propolis possess antimicrobial, anti–inflammatory, and immunomodulatory properties, which are highly beneficial in the treatment of aphthous ulcers, candidiasis, gingivitis, and periodontitis [40, 41]. Thus, it is believed that propolis will reduce oxidative stress and prevent the formation of damage through its antioxidant effects.

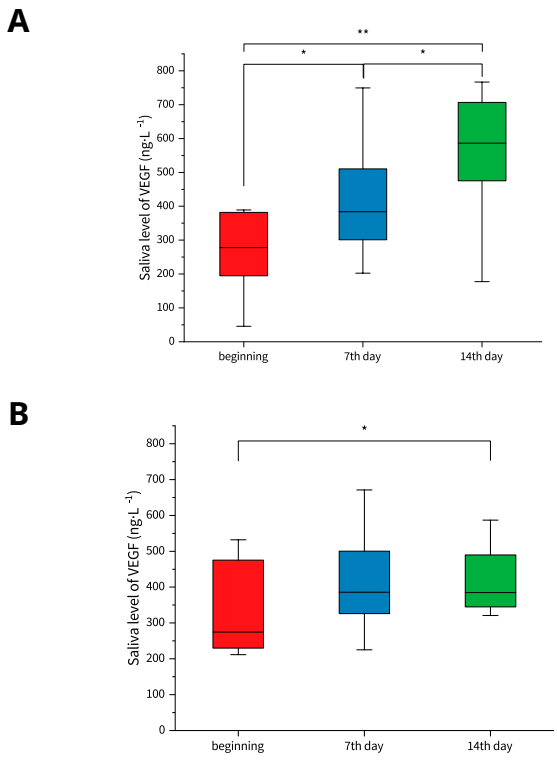

FIGURE 3. The changes in salivary VEGF levels in gingivitis caused by bacterial agents (A) and viral agents (B) following the application of ozone–enriched olive oil–extracted propolis (*P<0.05; **P<0.01)

TABLE III
VEGF levels (ng·L^{-1}) in saliva samples collected at the beginning of treatment, as well as on days 7 and 14, following the application of ozone–enriched propolis in relation to viral and bacterial agents

Application of ozone–enriched propolis	VEGF levels in gingivitis caused by bacterial agents. Mean ± SD (n=10)	VEGF levels in gingivitis caused by viral agents. Mean ± SD (n=10)
Before application	267.54 ± 37.82	325.72 ± 39.87
7th day of application	411.48 ± 53.14*	415.98 ± 41.13
14th day of application	511.56 ± 65.19**	418.62 ± 29.71*

*: P<0.05, **: P<0.01 indicates significance when comparing the beginning of the treatment with the beginning of the treatment as well as on days 7 and 14 according to repeated measures ANOVA

In veterinary medicine, propolis is used as an ointment to control mastitis in dairy cows. In pig herds, 5% propolis is added to the milk as a prophylactic agent for respiratory and gastrointestinal diseases. It is also added to the diets of rams, pigs, and calves to stimulate growth. It is also used for wound healing and as a local anesthetic for surgery [42]. The effects of propolis have been studied in dogs for dermatophytosis, sarcoptic mange [43], and fungal otitis [44]. Recently, it has also started to be used as a treatment option for disinfection in regenerative endodontics [45]. No studies have been found regarding the use of propolis in cats.

In a study conducted in the early stages of gingivitis, it was determined that VEGF levels decreased and returned to the level before the onset of normal gingivitis with oral health practices [46]. Another study reported a decrease in salivary VEGF levels at the onset of experimental gingivitis [47]. When our results are analyzed, it is thought that VEGF levels increase especially in gingivitis caused by bacterial agents, and VEGF levels, which decrease due to gingivitis, increase due to the elimination of the agent during treatment.

Measurements taken with a periodontal probe during the study were recorded according to the Löe and Silness gingival index scoring system [31]. In cats with gingivitis caused by bacterial agents, the GI was measured as 1.90 ± 0.82 at the beginning of the application, 1.40 ± 0.69 on d 7, and 1.30 ± 0.48 on d 14. When statistically evaluating the application, the difference between the beginning and the end of the treatment was found to be statistically significant (P<0.05). When the pre–application and d 7 application were statistically evaluated, the difference between them was found to be statistically insignificant.

In cats with gingivitis caused by viral agents, the gingival index values measured at the beginning of the application were 1.88 ± 0.33, remained at 1.88 ± 0.33 on d 7, and were recorded as 1.44 ± 0.73 on d 14. When comparing the GI values between the beginning of the application and d 7, no statistically significant difference was found between the groups (P>0.05). However, the difference between the GI on d 7 and 14, was found to be statistically significant. When the GI values at the beginning and the end of the study were evaluated, the difference between them was found to be statistically significant (P<0.05).

Throughout the study, measurements taken with a periodontal probe were statistically evaluated based on the Silness and Löe plaque index (PI) [30]. In cats with gingivitis caused by bacterial agents, the PI was measured as 1.70 ± 0.82 at the beginning of the application, 1.30 ± 0.49 on d 7, and 1.30 ± 0.48 on d 14. When statistically evaluating the application, the results from before the application and on d 7 and 14 were found to be statistically significant ($P<0.05$). However, the difference between the results on d 7 and d 14 was found to be statistically insignificant ($P>0.05$).

In cats with gingivitis caused by viral agents, the PI values were measured as 1.89 ± 0.61 before the application, 1.88 ± 0.60 on d 7, and 1.78 ± 0.67 on d 14. When the PI was statistically evaluated during the application period, the difference between the groups was found to be statistically insignificant ($P>0.05$).

Since ozone therapy stimulates the wound healing process, it has been reported to be effective in the treatment of various skin disorders [48, 49]. It was reported to have benefits such as faster tolerance of hyperemia in the gums, quicker healing of gingival bleeding, and rapid reduction of edema at the gingival margin. Researchers applying ozone therapy concluded that it was highly reliable and potentially effective due to the absence of observed side effects and its natural origins. There are numerous studies demonstrating the therapeutic effects of ozone application in the treatment of gingivitis in humans. While there are studies [23] proving that ozone application reduces plaque formation in dogs, no studies have been found examining the effects of ozone application on oral and dental health in cats. In light of this information, our study is unique in investigating the therapeutic efficacy of both propolis and ozone in cats.

The alcoholic extraction of propolis is not suitable for medical use. The aqueous extraction has lower biological activity due to the reduced content of phenolic and volatile oils. Studies have shown that ozone itself does not penetrate cells and quickly reacts with polyunsaturated fatty acids, leading to an increase in the formation of oxidant products. However, when applied with oil, the entry of ozone into the cells occurs in a slow and controlled manner. For this purpose, the oily extract of propolis enriched with ozone was chosen for application in our study.

CONCLUSION

This study will provide a new perspective on scientific research related to herbal products used in veterinary medicine. The use of herbal products is being conducted on animals; however, these applications are not based on scientific data. Studies conducted in this area are considerably insufficient compared to those in human medicine. In this study, the data obtained from the oral application of ozonated oily extract of propolis in cats diagnosed with gingivitis will serve as an important resource for future research on the topic. In our study, saliva samples were also collected to determine the levels of TNF–α and VEGF present in the saliva. This results also enhance its uniqueness as a study demonstrating that the monitoring of periodontal diseases in animals can be conducted through saliva samples. In summary, our study found that the propolis extracted in olive oil enriched with ozone had a local effect and reduced the inflammatory response.

Conflict of interest

There are no conflicts of interest, according to the authors, regarding this article.

Credit author statement

G.S.E.A. was involved in the study design, animal handling, laboratory experiments, and statistical analyses and interpretation of the results. U.K. was involved in the literature review, the application to animals and the evaluation of the results. S. B. was involved in the literature review, the application to animals and the evaluation of the results. F. K. was involved in the literature review, interpretation of results and drafting of the manuscript.

Ethics approval and consent to participate

The study was approved by the Animal Experiments Local Ethics Committee of Adnan Menderes University with the date January 19, 2023 and number 64583101/2023/20 and which were performed in strict accordance with the guidelines of the Experimental Animal Ethics Committee.

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