Cytological Assessment of Healing Palatal Donor Site Wounds and Grafted Gingival Wounds after Application of Ozonated Oil: An Eighteen-Month Randomized Controlled Clinical Trial

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Therapeutic effect of topical ozonated oil on the epithelial healing of palatal wound sites: a planimetrical and cytological study

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Introduction

Oral wound healing is a complex and dynamic process of restoring cellular structures and tissue layers. The wound-healing response can be divided into several distinct but overlapping phases: inflammation, re-epithelialization, granulation tissue formation, matrix formation, and tissue remodelling.¹ Re-epithelialization or epithelial healing is an important, complex process that involves the interactions between keratinocytes and the extracellular matrix upon which cells migrate, proliferate, and differentiate, thereby restoring tissue structure and function.¹ ²

The oral cavity provides the unique environmental challenge for the epithelial healing of oral wounds produced during various periodontal procedures.³ ⁴ Trauma from mastication, relatively large commensal flora, and elevated levels of dental plaque impair the normal sequence of the healing process.³ ⁴ Therefore, there is concern regarding the delayed healing of oral cavity wounds.⁵

Appropriate antimicrobial treatment and oral wound care can accelerate the epithelial healing process, thereby preventing infection and chronicity of the wound. Thus far, different adjunctive chemotherapeutic agents have been used to achieve enhanced epithelial bridging or shorter healing times. Despite extensive efforts to improve wound healing, the outcomes of existing chemotherapeutic agents are far from optimal.⁶ ⁷

Abstract

Aim: To evaluate the effect of ozonated oil on palatal wounds.

Methods: Eighteen patients were randomized and allocated to either the ozone group (n = 8) or control (n = 10) group. Free gingival graft surgery was performed, and post-harvested palatal wounds were treated with either 2 mL ozonated oil or control oil daily for 1 week. A planimetrical analysis analyzed the digital image for the wound sizes and shape factor at baseline, at 24 h, and days 5, 7, 14, 21, and 28, postoperatively. A cytological analysis used the keratinization and superficial cell indices at baseline, 24 h, and days 3, 7, 14, and 21 and the second and third months, postoperatively.

Results: Planimetrical results showed a significant (P ≤ 0.05) improvement in wound size on days 5, 7, 14, 21, and 28, postoperatively, in the ozone group compared to the control group. Cytological results showed a significant (P ≤ 0.001) improvement in epithelial healing on days 7, 14, and 21, and the second and third months, postoperatively, after the application of ozonated oil compared to control oil.

Conclusion: Our results showed significant improvement in wound size and epithelial healing after topical ozonated oil application compared to control oil on palatal wounds.
have also shown that chemotherapeutic agents, such as chlorhexidine, sodium hypochlorite, povidone iodine, and hydrogen peroxide have cytotoxic effects on human oral epithelial and gingival fibroblast cells.

Currently, ozone gas dissolved in water or in plant oils, such as olive oil (ozonized oil), is being discussed in dentistry for its excellent antimicrobial property, without the development of drug resistance and facilitation of wound healing in the oral cavity. The most cited explanation for ozone’s bactericidal effects centers on the disruption of cell membrane integrity through oxidation of its phospholipids and lipoproteins, penetration of ozone through the cell membrane, reaction with cytoplasmic contents, and conversion of the closed circular plasmid DNA to open circular DNA, which would presumably diminish the efficiency of bacterial procreation.6–10

Moreover, under the influence of ozone, improved rheological properties,11 activated cellular metabolism,12 raised intracellular ATP concentrations,13 and the expression of cytokines relevant to wound healing, especially transforming growth factor-β1 (TGF-β1),14 have been observed.

It has been hypothesized that the wound-healing action mechanism of ozonized oil might be in part connected to its antimicrobial effect, but also with its ability to promote the liberation of growth factors, activate local antioxidant mechanisms, and promote tissue repair.15

However, there is no consensus or direct evidence that ozone plays a central role in the healing process. Thus, this clinical trial was designed to investigate the effect of ozone on the epithelial healing of palatal excisional wounds (wounds generated after free gingival autograft surgery) with large epithelial and connective tissue defects that heal with secondary intention. The rate of re-epithelialization was a criterion to indicate the influence of the ozonated oil on the healing of palatal wound sites.

Materials and methods

Initially, a total of 26 patients (10 males and 16 females, mean age: 30.19 ± 8.57 years) diagnosed with localized gingival recession were considered eligible for the study. All the patients were recruited between February 2010 and June 2010, and were randomly selected from the outpatient department at Jagadguru Sri Shivarathreeshwara Dental College and Hospital, Karnataka, India. Four patients were excluded, as they did not meet the inclusion criteria. The remaining 22 (nine males and 13 females) were randomized and allocated to either the ozone group (n = 11) or the control group (n = 11). During the study, one patient from the control group and three patients from the ozone group failed to appear for follow up or discontinued the study. Finally, a total of 18 patients (eight males and 10 females, mean age: 28.13 ± 6.38 years) were considered, and they completed the study.

The study was a single-centered (Jagadguru Sri Shivarathreeshwara [JSS Dental College and Hospital, India], longitudinal, triple-blinded (patients, clinical investigator/cytologist, and statistician) randomized, placebo-controlled, parallel-armed, clinical trial. The duration of the study was 3 months, during which planimetric parameters were recorded at baseline, 24 h, and days 5, 7, 14, 21, and 28, postoperatively; cytological parameters were recorded at baseline, 24 h, days 3, 7, 14, and 21, the second and third months, postoperatively.

Prior written, informed consent was obtained from each patient, based on the recommendations of the Institutional Review Board (IRB) of JSS University. The IRB approved the study. All procedures in this clinical trial were performed according to the ethical principles established by the Declaration of Helsinki.16

Inclusion criteria were age of 20–40 years, male/female, systemically healthy, single tooth labial recession in lower anterior sextant that required soft tissue graft involving donor tissue from the palate, and all patients should have read, understood, and signed an IRB-approved informed consent form, and should be able and willing to follow study procedures and instructions. Exclusion criteria were patients who had used any tobacco product within the past 3 months; systemic conditions (i.e. uncontrolled diabetes mellitus, cancer, HIV, bone metabolic diseases) that could compromise wound healing, and systemic corticosteroids, immunosuppressive agents, radiation therapy, and/or chemotherapy prescribed or received within 2 months prior to study entry, which could compromise wound healing and preclude periodontal surgery.

The patients were randomly allocated to either the ozone group (where the patients received cold-pressed olive oil treated with ozone at a concentration of 14 μg/mL) or the control group (where the patients received olive oil that had not been treated with ozone). One of the authors (SKG) generated the random allocation sequence. The random allocation sequence was concealed from the main investigator (PVP) and cytological investigator (VGD) until the study was completed.

Clinical procedure

At the initial study visit, all of the included patients underwent free gingival graft surgery involving donor tissue removal from the palate. The masked main investigator (PVP) performed all the surgical procedures. In order to obtain a uniform palatal wound, a standardized tinfoil template (9 × 10 mm) was used to mark the donor area (Figure 1a). Subsequently, a standardized
A cuboid-shaped palatal graft measuring 9 × 10 mm was harvested from premolars and the first molar region of the hard palate, leaving behind a 2 mm-deep, standard-sized (9 × 10 mm), post-harvested wound area in the donor site (Figure 1b). Finally, the harvested graft underwent a routine free gingival graft procedure to cover the denuded root surface, and the generated standard sized palatal wound (9 × 10 mm) was considered and evaluated in the study.

After the surgical procedure, routine postoperative instructions were given to the each patient. Oral hygiene status was monitored by measuring the plaque index at various time intervals. All of the treated patients were scheduled for recall appointments. During the first-week visit, all of the patients were asked to report to the clinic on a daily basis and have their allocated therapeutic medication applied by one of the masked investigators (PVP) to their respective palatal donor site wounds (Figure 2). The wounds of the ozone group patients were treated with 2 mL of ozonated oil per day, with a concentration of 14 µg of ozone per mL of olive oil. The control group patients’ palatal wounds were treated with 2 mL of non-ozonated oil per day. A soft stent was used to protect the palatal donor wound site in both groups. Patients were instructed not to disturb the stent for 1 week. The stent was removed and reseated by one of the investigators (PVP), only during the application of medications, collection of smears, and capturing of digital photographs for the analysis. The protective stent also facilitated the retention of test oils in the post-harvested palatal wound sites. Postoperatively, protective stents were discontinued after 7 days.

Digital photographs of the palatal wounds were taken at baseline, 24 h, and days 5, 7, 14, 21, and 28, postoperatively, by using a digital camera (Kodak C713; Eastman Kodak Company, Rochester, NY, USA). The digital camera was placed at a 20-cm distance from an intraoral mirror placed at the level of the occlusal plane of the lower teeth. The image was captured only when the palatal wound margin was clearly visible on the LCD screen of the digital camera. A small acrylic plate with a standard diameter of 3 mm was also placed adjacent to the wound as a reference, as suggested by Filippi,18 for the planimetric measurement of human palatal wounds. Thus, any difference (and thus, possible mistake in measurement) in camera–wound distance or angle could be adjusted and/or corrected. A set of 50 images was captured, and the 20 best reproducible images of each interval were analyzed with 10 analyses per image, and an average was considered for each time interval. The photographic images were analyzed by image analysis computer software (UTHSCSA Image Tool program, University of Texas Health Science Center, San Antonio, TX, USA) for the size and shape of the wounds19 at the indicated time intervals. For the measurement, the software was
calibrated, and the scale setting was changed from pixels to millimeters (mm). The instructions were followed, as per the software manual, for measuring the wound sizes. The wound outline was traced using a cursor on the screen, and the software automatically calculated the wound size (area) in square millimeters (mm²), and the perimeter of the wound in millimeters (mm). The shape factor\(^{19}\) was calculated using the obtained data of wound size (area in mm²) and perimeter (in mm) using the following formula: \(SF = \frac{4\pi S}{P^2}\), where \(SF\) is the shape factor, and \(S\) and \(P\) are the surface area and perimeter of the wound, respectively. All of the wound dimension measurements were performed double blinded so that the operator could not identify anyone in the groups during the analysis.

Cytological procedures

Cytological technique was used in the study to evaluate epithelial keratinization, regeneration, and degeneration, as this technique facilitated the repeated scraping of epithelial cells, which can be evaluated over a period of time. A sterilized, disposable, interproximal brush (STIM interprox brushes; DENT–AIDS, New Delhi, India) was used to scrape the palatal donor wound site. The flat surface of the interproximal brush was placed against the margin of the wound with firm pressure, and rotated four to five times to collect cellular material (Figure 3).\(^{20}\) The collected material was smeared onto the coded glass slide in parallel fashion, and rapidly fixed with spray fixative (RAPID PAP spray fixative; Biolab Diagnostics, Mumbai, India) to avoid the smears from air drying. Smear slides were sent to the histopathological laboratory for cytological analysis. Smears from donor wound sites were collected at baseline (before the surgical procedure), 24 h, days 3, 7, 14, and 21, and the second and third months, postoperatively.

Staining and examination of sample smear

The smeared slides were stained by rapid Papanicolaou technique (RAPID PAP stain; Biolab Diagnostics, India). The masked cytological investigator (VGD) performed all of the staining and examinations of the selected smear slides. The stained smeared slides were examined under a microscope at 40× resolution, and the cell count was systematically performed via a meandering movement of the stage. For each smear, a minimum of 200 cells were examined for cellular and nuclear changes. The cellular and nuclear characteristics were evaluated according to the cell-type classification of Lange et al.\(^{21}\) Cells were classified into superficial cells (ST2 and ST1), intermediate cells (IT2 and IT1), parabasal cells (OBT), and basal cells (BT) (Figure 4).

Regenerative changes that consisted of new intermediate cells (IT2 and IT1), OBT and BT, and degenerative changes consisting of cytolysis, karyolysis, perinuclear halo formation, karyorrhexis, nuclear wall hyperchromasia, and pyknotic nucleus formation were analyzed. Noted pyknotic changes were considered when their nuclei were spherical and shrunk with the loss of nucleoli. The analysis was followed by calculation of the keratinization and superficial cell indices.\(^{21}\)

![Figure 3. Collection of cytological smear from margin of wound using an interprox brush.](image3)

![Figure 4. Cellular and nuclear type classification. BT, basal cells; IT, intermediate cells; OBT, parabasal cells; ST, superficial cells.](image4)
The parameters recorded for the exfoliative cytological analysis were:

1. Keratinization index:  \[ \text{Keratinization index} = \frac{\text{Cells without nuclei}}{\text{Total no. examined cells (n = 200)}} \times 100. \] (1)

2. Superficial cell index:  \[ \text{Superficial cell index} = \frac{\text{Cells with pyknotic nuclei and with no nuclei}}{\text{Total no. examined cells (n = 200)}} \times 100. \] (2)

Statistical analysis

Recorded planimetrical and cytological data were analyzed using SPSS software (version 11; SPSS, Chicago, IL, USA). All parametric variables were analyzed by two-factor ANOVA with repeated measures and Student’s t-test (independent samples). Two-factor ANOVA with repeated measures was conducted to determine whether there was a statistical significance between two different treatment groups for improvement in healing over the period of time. Student’s t-test (independent samples) was used for the comparison of mean differences between treatment groups at specific time intervals. An alpha level of 0.05 was utilized for both statistical analyses.

Results

There were no unwanted side-effects observed in the ozone or control group after the application of the respective oils. A clinical view of the typical healing sequence of palatal wounds treated by ozonated oil and placebo oil at various time intervals is shown in Figure 5. The mean plaque score of both the groups at various time intervals decreased over time. The result of the Student’s t-test showed that there was a significant decrease in the mean plaque score on day 7 in the ozone group (0.16 ± 0.033) compared to the control group (0.8 ± 0.191; \( t(16) = -9.32, P < 0.001 \)). However, by day 14 and 21, and the second and third months, the differences in the mean plaque scores of both groups were not statistically significant \( (P > 0.05) \).

Planimetrical analysis

Comparison of wound size and shape factor

The mean wound size and mean shape factor over time for the ozone and control groups are shown in Table 1. The mean wound size and shape factor changed over time in both groups, with a significant change in the ozone-treatment group when compared to the control group. The result of the two-factor, repeated-measures ANOVA for differences in wound size and shape factor over time showed that there was a statistically significant interaction between the treatment groups and time intervals for wound size \( (F = 22.119 \ [d.f. = 6.96], P < 0.001) \) and shape factor \( (F = 42.669 \ [d.f. = 6.96], P < 0.001) \). The result of the main effect of time interval was also significant for wound size \( (F = 2625.609 \ [d.f. = 6.96], P < 0.001) \) and shape factor \( (F = 70.332 \ [d.f. = 6.96], P < 0.001) \). Further, there was a significant main effect in the treatment groups for wound size \( (F = 63.311 \ [d.f. = 1.16], P < 0.001) \), but the main effect in the treatment groups did not reach significance for the shape factor \( (F = 3.542 \ [d.f. = 1.16], P < 0.078) \).

The results of the Student’s t-test (independent samples) for differences in the mean wound size and mean shape factor between the ozone and control groups at specific time intervals are shown in Table 1. No significant difference was found in the mean wound size at 24 h between the groups. On days 5, 7, 14, 21, and 28, there was a significant decrease \( (P \leq 0.05) \) in both the mean wound size, and a significant differential change \( (P \leq 0.05) \) in the mean shape factor in the ozone group as compared to the control group. On day 28, the mean wound size and mean shape factor were reduced to zero in the ozone group, indicating complete epithelization and no residual wound areas in the donor site wounds. The results suggest that the topical application of ozone on palatal wound sites has an enhancing effect on epithelial healing.

Cytological analysis

The stained epithelial cells revealed no sign of morphological alterations or pathological condition. The epithelial cells observed during the initial phase of wound healing and after complete epithelization are shown in Figure 6.

Comparison of keratinization index and superficial cell index

The mean keratinization index and superficial cell index over time for the ozone and control groups are shown in Table 2. The mean keratinization index and superficial cell index increased over time in both groups, with a significant differential increase in the ozone group compared to the control group. The results of the two-factor ANOVA with repeated measures showed that the main effect of the treatment groups was significant for the keratinization index \( (F = 359.752 \ [d.f. = 1.16], P < 0.001) \) and the superficial cell index \( (F = 599.066 \ [d.f. = 1.16], P < 0.001) \),
The main effect of time was also significant for the keratinization index \((F = 7005.493 \text{ [d.f. = 7.112], } P < 0.001)\) and the superficial cell index \((F = 19355.209 \text{ [d.f. = 7.112], } P < 0.0001)\). Moreover, the interaction of these two factors was also significant for the keratinization index \((F = 53.754 \text{ [d.f. = 7.112], } P < 0.001)\) and the superficial cell index \((F = 205.145 \text{ [d.f. = 7.112], } P < 0.001)\).

**Figure 5.** Clinical view of typical healing sequence of palatal wounds treated by ozonated oil (a) (same patient) and placebo oil (b) (same patient) at baseline (i), at day 7 (ii), at day 14 (iii), at day 21 (iv), and at day 28 (v).
The results of the Student’s *t*-test (independent samples) for differences in the mean keratinization and superficial cell indices between the two groups at specific time intervals are shown in Table 2. The mean keratinization and superficial cell indices of the ozone group showed rapid decreases, similar to the control group, from baseline to 24 h. On day 3, the ozone group showed a significant (*P* ≤ 0.001) decrease in the keratinization and superficial cell indices compared to the control group. From day 7 to 21, the ozone group showed rapid and significant (*P* ≤ 0.001) increases in the keratinization and superficial cell indices compared to the control group. On the second and third months, the mean keratinization index was significantly (*P* ≤ 0.001) higher in the ozone group compared to the control group. These results suggest that the topical application of ozone on palatal wound sites has an enhancing effect on the re-epithelialization rate of healing epithelial tissue.

### Discussion

The present study was undertaken to evaluate the therapeutic effects of topical ozonated olive oil on the healing of post-harvested palatal donor wound sites, with large epithelial and connective tissue deficiencies that heal by secondary intention. Wound healing is a complex biological process commonly divided into overlapping phases: inflammation, re-epithelialization, granulation tissue formation, matrix formation, and tissue remodelling. The use of antimicrobial agents, such as ozonated oil, can affect the nature and quality of the inflammatory infiltrate...
and that of the granulation tissue component.\textsuperscript{4,5,15} In the present study, the rate of re-epithelialization was evaluated to indicate the influence of the ozonated oil on the healing of palatal tissue. The rate of epithelial healing was assessed by planimetrical and cytological analyses. The result revealed that ozonated oil significantly enhanced re-epithelialization of the palatal donor site wounds.

Re-epithelialization is a major component of the wound-healing process, which is achieved through a complex interplay of diverse growth factors, cytokines, and cell-cycle regulators.\textsuperscript{1,2,22} Currently, ozone or its products are being investigated for its influence on growth factors, cytokines, and cell-cycle regulators in biological systems. Bocci \textit{et al.} conducted a series of studies\textsuperscript{14,23–25} and showed that the contact of ozone with human blood led to an increased release of TGF-\(\beta\)\textsubscript{1}; interferons-\(\alpha\), -\(\beta\), and -\(\gamma\); interleukins-1, -2, -6, and -8), and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), which are important for human wound healing.

Epithelial cell migration is an essential step of wound closure, where epithelial cells migrate from the periphery of the wound towards the central part of the defect. The migration and proliferation of keratinocytes and the close relationship between the migratory cells and the collagen of the newly-formed connective tissue determine the rate of re-epithelialization of the wound.\textsuperscript{2} In our study, these epithelial changes were evident on cytological smears and were confirmed by the appearance of newly-generated OBT and BT in the cytological smears. Moreover, epithelial migration was also confirmed by progressive increase in the pyknotic and keratinized cells (ST1 and ST2) in the cytological smears, ultimately resulting in the gradual increase in the keratinization and superficial cell indices with advancing time intervals.

Our cytological analysis results confirm the findings of Lange \textit{et al.},\textsuperscript{21} where the keratinization index increased gradually from 0\% to 50\% in a 3-month period, demonstrating the long-term cytological changes occurring in healing human palatal mucosa.

The rate of re-epithelialization, when analyzed by the cytological method, demonstrated that the ozone group had a rapid and accelerated migration of epithelial cells compared to the control group, as measured by the keratinization and superficial cell indices. The planimetrical analysis also revealed the significantly decreased wound area after the application of ozonated oil when compared to control oil on days 5, 7, and 14 of the analysis. These changes clearly suggest that after the application of ozonated oil, there is an accelerated migration of epithelial cells as part of the healing process in post-harvested palatal wounds.

Our planimetrical analysis results confirm the findings of Filippi,\textsuperscript{18} who concluded that the application of ozonated water accelerates wound healing in the oral cavity within the first 48 h compared to placebo water, resulting in earlier epithelial wound closure after 7 days. Our study

<table>
<thead>
<tr>
<th>Time intervals</th>
<th>Treatment group</th>
<th>Keratinization index</th>
<th>Superficial cell index</th>
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<tr>
<td></td>
<td></td>
<td>Mean SD</td>
<td>t P</td>
</tr>
<tr>
<td>Baseline</td>
<td>Ozone</td>
<td>97.08 1.940</td>
<td>0.292 0.774</td>
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<td></td>
<td>Control</td>
<td>96.73 2.910</td>
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<tr>
<td>24 h</td>
<td>Ozone</td>
<td>0.19 0.072</td>
<td>–1.735 0.102</td>
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<tr>
<td></td>
<td>Control</td>
<td>0.24 0.043</td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>Ozone</td>
<td>2.45 0.330</td>
<td>12.447 0.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.94 0.170</td>
<td></td>
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<tr>
<td>Day 7</td>
<td>Ozone</td>
<td>5.71 0.340</td>
<td>24.531 0.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2.00 0.290</td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>Ozone</td>
<td>21.65 1.920</td>
<td>8.927 0.000</td>
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<tr>
<td></td>
<td>Control</td>
<td>11.39 2.750</td>
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<tr>
<td>Day 21</td>
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<td>38.81 2.490</td>
<td>11.325 0.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>25.02 2.620</td>
<td></td>
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<tr>
<td>Month 2</td>
<td>Ozone</td>
<td>52.25 1.850</td>
<td>11.653 0.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>42.49 1.720</td>
<td></td>
</tr>
<tr>
<td>Month 3</td>
<td>Ozone</td>
<td>65.70 2.220</td>
<td>17.782 0.000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>51.61 1.05</td>
<td></td>
</tr>
</tbody>
</table>

\(\ddagger\) could be computed because the SD of both groups was 0.
is also in favor of the results of two similar studies published by Noritaka et al. and Kim et al., where they found similar wound closure in a rat model after the application of topical ozonated oil.

In the present study, we found that epithelial healing was complete, with minimal residual wounds clinically detectable after 21 days, postoperatively, in both groups. These findings are in agreement with results of those Rossmann and Rees, Del Pizzo et al., and Silva et al., where the authors concluded that the complete epithelialization of palatal wounds occurs in 3–4-week intervals.

The antimicrobial property of ozone is another important mechanism that can explain the enhanced epithelial healing of palatal wounds. Increased bacterial colonization of the wound surface results in an increased area of inflammation and granulation tissue that might prolong the repair. As oral wounds are constantly subjected to a relatively large commensal flora, it is clear that bacteria affect wound healing in the oral cavity. Elevated levels of bacteria (over 10⁵) decrease epithelialization; bacterial metabolites inhibit epithelial cell migration, digest dermal proteins and polysaccharides, and increase the production of neutrophil proteases, cytotoxic enzymes that damage vulnerable epithelium. Thus, the control of bacteria by ozonated oil might act directly or indirectly on epithelial and connective tissue cells and can accelerate oral wound healing.

Our results are in agreement with a recent experimental study by Dmitrieva et al. in 30 Wistar rats. In their study, the rats were divided into three groups, with 10 rats in each group. Skin defects were created and subsequently contaminated with the microorganisms. The wounds were irrigated with 10–15 mL commercial perftorane solution, ozonized perftorane, and physiological solution, depending on their respective groups. The results indicated that in the group with ozonized perftorane irrigations, the mean number of microorganisms in the wound were reduced; granulation tissue growth and epithelization were enhanced when compared with the groups of rats irrigated with physiological solution and non-ozonized perftorane.

Similar to ozone, various topical antimicrobial agents have also been tested for their healing efficacy in different post-surgical situations. Kozlovsky et al. reported that 1% chlorhexidine digluconate (CHX) gel and Listerine solution increased the rate of epithelization in palatal wounds of a rat model. Similarly, Bassetti et al. also reported that the topical application of 0.1% CHX and 0.2% CHX resulted in undisturbed healing on day 7 following injury. Our study also reports similar results, where the antimicrobial property of ozonated oil could be attributed to epithelial changes in healing wounds. However, considering that results from animal experiments cannot necessarily be applied to oral mucosa, it can only be theoretically suggested that the effects of ozonated oil mentioned in the literature are responsible for the influence on the wound healing of oral mucosa.

Ozone gas dissolved in an oil base (olive oil) was used to treat post-harvested palatal wound sites. Studies have revealed that the direct application of gaseous ozone has a cytotoxic effect on human gingival fibroblast and epithelial cells, but the direct application of aqueous ozone is safe on oral tissue. Previous studies have concluded that ozone in an oil base is non-toxic and provides the safest medical therapy devised. There is no evidence of free radical damage. On the contrary, ozone gas liberated from ozonated oil has been proven to stimulate the production of superoxide dismutase, catalase, glutathione peroxidase, and reductase, enzymes that protect the cell from free radical damage. Moreover, studies have also reported that the O₃ molecule can be stabilized as an ozonide between the double bonds of a monounsaturated fatty acid, such as oleic acid; therefore, ozonated olive oil can be an ideal preparation for the topical form of O₃, and it remains stable for 2 years when stored at 4°C.

One millimeter of ozonated oil (estimated at 14 μg O₃/mL) was applied daily for 1 week on post-harvested palatal donor sites. Previous studies have revealed that ozone concentrations in the range of 80–100 μg/mL produce "wound cleansing", that is, disinfection, whereas low-concentration ozonated solutions, ranging from 10 to 40 μg/mL, produce "wound healing", that is, epithelialization and granulation. Ozone concentrations greater than 80 μg/mL were believed to have a cytotoxic effect. In principle, these empirically determined values have been confirmed by studies to impact the effect of ozone on immunocompetent cells in whole blood. The exact stimulation mechanism of the low-dose ozonated oil to the tissue (e.g. granulation and epithelization) is still unclear.

In the present study, palatal wound-healing rates were assessed by the change in wound area and shape factor of the measured wounds. Several investigators have suggested that the use of surface area to its perimeter (S/P ratio) is a useful method to characterize wound-healing rates. Changes in this parameter are a measure of the change in a wound's effective radius, which is an index of movement of a wound's margin towards the center for healing. The S/P ratio has been used to assess healing rates in venous ulcers, and has been reported to be a suitable indicator of linear healing per day. The S/P ratio has also been used to predict time for wound closure based on a non-linear delayed exponential model of healing, which seems to offer some predictive features.
We used an exfoliative cytological technique to assess the epithelial healing of palatal wounds. Selection of the cytological technique was based on the fact that exfoliative cytology, in contrast to histological studies, permits repeated, non-destructive observations. Moreover, cytological techniques have also been widely used for testing therapeutic effects.46 However, if errors of diagnostic interpretation are avoided, and well-established cell indices are used, sufficiently accurate results can be achieved. Experimental long-term observations have also confirmed the consistency of smear results in healing tissues.

Our results showed significant improvement in epithelial healing after topical ozone application on palatal wound sites. At present, there are few published data and anecdotal reports suggesting enhancement of wound healing after the application of ozonated oil. The exact mechanism behind it is still unclear. Therefore, for future prospects, longer-term studies with histopathological and immunohistochemical evaluations should be undertaken to ascertain the efficacy of ozone on the healing of oral wounds.

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References

22 Werner S., Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev 2003; 83: 835–70.
23 Bocci V, Valacchi G, Corradeschi F et al. Studies on the biological effects...