COMBINED THERAPY OF OZONIZED OIL AND PHOTOBIOMODULATION IN THE TREATMENT OF CHRONIC WOUNDS

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ABSTRACT: Hard-to-heal wounds pose significant therapeutic challenges due to pathogenic microorganisms and associated infections, leading to delays in tissue healing. The present study aimed to carry out a clinical trial to identify the microbial agent present in hard-to-heal wounds on the lower limbs, assess the antimicrobial effects of ozonated oil and examine the benefits of photobiomodulation combined with ozonated oil in the treatment of those wounds. Then we examined the antimicrobial kinetics of ozonated oil on microorganisms found in wounds by an in vitro study. For this study, some commercial ozonated sunflower oil was used as well as the LED photobiomodulation therapy, 660 nm, 30 seconds, punctual 2 cm away on the wound bed, 120 mW, 3.6 J per point. The procedure was performed three times a week. The clinical results evidenced a significant healing of injuries treated with ozonated oil and photobiomodulation, evidenced by a

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reduction in traces of infection, presence of granulation tissue, wound area reduction and, in some cases, full wound closure. Among the eight patients monitored, only one had negative microbiology. Among the microorganisms identified are Klebsiella sp, Staphylococcus epidermidis and Escherichia coli, all proved resistant to the antibiotics tested. In vitro results confirmed the antimicrobial efficacy of ozonated oil, demonstrating its potential as a viable therapeutic agent for chronic wounds in all microorganisms tested when subjected to concentrations deemed low, ranging from 1.6% to 12.5%, demonstrating antibacterial activity. In conclusion, ozonated oil associated with photobiomodulation therapy represents a promising resource for the treatment of hard-to-heal wounds. However, new clinical trials are necessary to establish more improved treatment protocols.
KEYWORDS: Chronic wound; Ozonated oil; Photobiomodulation therapy; Antimicrobial activity; Antimicrobial resistance.

TERAPIA COMBINADA DE ÓLEO OZONIZADO E FOTOBIOMODULAÇÃO NO TRATAMENTO DE FERIDAS CRÔNICAS

RESUMO: As feridas de difícil reparo apresentam desafios significativos em seu contexto terapêutico devido à presença de microrganismos patogênicos e infecções associadas, o que resulta em atrasos no processo de reparação tecidual. O presente estudo teve como objetivo realizar um ensaio clínico para identificar o agente microbiano presente em feridas de difícil reparo de membros inferiores, avaliar os efeitos antimicrobianos do óleo ozonizado e investigar os benefícios da fotobiomodulação associada ao óleo ozonizado na terapêutica dessas feridas. Em seguida, em um estudo *in vitro,* investigar a cinética antimicrobiana do óleo ozonizado nos microrganismos encontrados nas feridas. Para o estudo foi utilizado óleo de girassol ozonizado comercial e a terapia por fotobiomodulação a LED, 660 nm, 30 segundos, pontual a 2 cm de distância no leito da ferida, 120 mW, 3,6 J por ponto. O procedimento foi realizado 3 vezes por semana. Os resultados clínicos indicaram uma melhora significativa nas lesões tratadas com óleo ozonizado e fotobiomodulação, evidenciada pela redução nos sinais de infecção, presença de tecido de granulação, diminuição da área da ferida e, em alguns casos, o completo fechamento da ferida. Entre oito pacientes monitorados, apenas um apresentou microbiologia negativa. Entre os microrganismos identificados estão *Klebsiella sp, Staphylococcus epidermidis* e *Escherichia coli*, todos demonstrando resistência aos antibióticos testados. Os resultados *in vitro* confirmaram a eficácia antimicrobiana do óleo ozonizado, demonstrando seu potencial como agente terapêutico viável para feridas crônicas em todos os microrganismos testados quando submetidos a concentrações consideradas baixas, variando de 1,6% a 12,5%, demonstrando atividade antibacteriana. Em conclusão, o óleo ozonizado, associado à terapia por fotobiomodulação, representa um recurso promissor para o tratamento de feridas de difícil reparo. Contudo, são necessários novos ensaios clínicos para estabelecer protocolos de tratamento mais aprimorados.
PALAVRAS-CHAVE: Ferida crônica; Óleo ozonizado; Terapia de fotobiomodulação; Atividade antimicrobiana; Resistência antimicrobiana.

TERAPIA COMBINADA DE ACEITE OZONIZADO Y FOTOBIOMODULACIÓN EN EL TRATAMIENTO DE HERIDAS CRÓNICAS

RESUMEN: Las heridas de difícil cicatrización plantean importantes retos terapéuticos debido a los microorganismos patógenos y las infecciones asociadas, lo que provoca retrasos en la cicatrización de los tejidos. El presente estudio tuvo como objetivo realizar un ensayo clínico para identificar el agente microbiano presente en heridas de difícil cicatrización en los miembros inferiores, evaluar los efectos antimicrobianos del aceite ozonizado y examinar los beneficios de la fotobiomodulación combinada con aceite ozonizado en el tratamiento de dichas heridas. A continuación, examinamos la cinética antimicrobiana del aceite ozonizado sobre los microorganismos encontrados en las heridas mediante un estudio *in vitro*. Para este estudio, se utilizó un poco de aceite de girasol ozonizado comercial, así como la terapia de fotobiomodulación LED, 660 nm, 30 segundos, puntual a 2 cm de distancia sobre el lecho de la herida, 120 mW, 3,6 J por punto. El procedimiento se realizó tres veces por semana. Los resultados clínicos evidenciaron una cicatrización significativa de las heridas tratadas con aceite ozonizado y fotobiomodulación, evidenciada por una reducción de los rastros de infección, presencia de tejido de granulación, reducción del área de la herida y, en algunos casos, cierre completo de la herida. Entre los ocho pacientes monitoreados, solo uno presentó microbiología negativa. Entre los microorganismos identificados se encuentran *Klebsiella sp*, *Staphylococcus epidermidis* y *Escherichia coli*, todos demostraron ser resistentes a los antibióticos probados. Los resultados *in vitro* confirmaron la eficacia antimicrobiana del aceite ozonizado, demostrando su potencial como agente terapéutico viable para heridas crónicas en todos los microorganismos probados cuando se sometieron a concentraciones consideradas bajas, que oscilaron entre 1,6% y 12,5%, demostrando actividad antibacteriana. En conclusión, el aceite ozonizado asociado a la terapia de fotobiomodulación representa un recurso prometedor para el tratamiento de heridas de difícil cicatrización. Sin embargo, son necesarios nuevos ensayos clínicos para establecer protocolos de tratamiento más mejorados.
PALABRAS CLAVE: Herida crónica; Aceite ozonizado; Terapia de fotobiomodulación; Actividad antimicrobiana; Resistencia antimicrobiana.

1. INTRODUCTION

Chronic wounds are defined as injuries requiring more than three weeks to heal, often stagnating in the inflammatory phase (Wen *et al*., 2022; Martinengo *et al*., 2019). Several factors contribute to this stagnation at the inflammatory phase and consequently to the delay in the wound-healing process of hard-to-heal wounds. It is important to highlight that the presence of microorganisms and local infection is one of the main factors that significantly impair the healing process (Versey *et al*., 2021). In the event of an infection occurs, the organism triggers an extended inflammatory reaction resulting from the migration of leukocytes and high production of pro-inflammatory cytokines. There is also an increase in the activity of metalloproteinases and a decrease in the release

of growth factors, significantly impairing the wound-healing process (Gushiken *et al.*, 2021).

An additional factor related to infection that makes the scenario more complex concerns with the ability of microorganisms to form biofilms in the wound. Biofilms are defined as clusters of microorganisms that are immersed in a self-generated matrix of extracellular polymeric substances, allowing them to adhere to each other and/or to a surface, resulting in the formation of a barrier (Wu; Cheng; Cheng, 2019). This barrier provides protection to microorganisms against the immune system of the host, giving them one hundred to one thousand times greater resistance to antimicrobial agents, approximately. As a consequence, this phenomenon causes a noticiable delay in the wound-healing process, representing a substantial challenge in the treatment of hard-to-heal wounds (Patrulea; Borchard; Jordan, 2020). This delay in the wound-healing process caused by the existence of biofilms is intrinsically related to the continuous inflammatory response induced in the host's immune system, which can lead to the deregulation of the wound extracellular matrix, affecting cell migration and proliferation as well as the appropriate deposition of the connective tissue components. Furthermore, the unbalanced release of inflammatory mediators can cause the disorganization of angiogenesis and tissue repairing processes, thus delaying the formation of a quality extracellular matrix and, therefore, impairing the proper wound-healing process (Tomic-Canic *et al.*, 2020).

At about 60% of the chronic wounds harbor biofilm-forming microorganisms, highlighting Staphylococcus aureus and Pseudomonas aeruginosa as those most frequently associated with this phenomenology (Drago *et al.*, 2019). However, researches reveal the presence of a diversity of other microorganisms in this context, emphasizing the complexity of the microbiota in hard-to-heal wounds, including bacteria, fungi and viruses. In view of the resistance presented by infections related to biofilms, it is imperative to identify the preponderant microbiological species in each wound. To this end, analysis of tissue cultures or swab collections plays a crucial role, providing essential information to guide appropriate therapies, considering the sensitivity of these microorganisms. In this context, the exploitation of innovative approaches becomes vital to face the challenge represented by the resistance of biofilm-related infections (Puca *et al.*, 2021; Kaiser; Wächter; Windbergs, 2021).

Faced with the need of antimicrobial therapies for tissue healing, ozone has been studied and supported as an advanced clinical therapeutic agent for the treatment of hard-

to-heal wounds (Fitzpatrick; Holland; Vanderlelie, 2018). Topical application of ozonated oils emerges as an effective strategy, providing ease of handling, possibility of prolonged storage, prevention of rapid degradation, enabling treatment without hospitalization and mitigating risks inherent to the use of ozone in its gaseous form. The interaction between ozone and the double bonds of fatty acids presents in vegetable oils results in the predominant formation of ozonides, particularly 1,2,4-trioxolanes, and peroxides, which justifies the antimicrobial activity and stimulates the healing and regeneration properties of tissues (Anzolin; Silveira-Kaross; Bertol, 2020; Zeng; Lu, 2018).

In the context of innovative therapies, in addition to the demand for an antimicrobial therapeutic agent such as ozonated oil, the photobiomodulation has been the subject of investigation as a therapeutic approach that influences cell growth. Photobiomodulation (PBM) treatment, which uses non-ionizing light, offers therapeutic benefits that include the improvement of the tissue repairing, modulation of the inflammatory process, pain relief and reduction of the oxidative stress. PBM stimulates mitochondria, resulting in increased production of adenosine triphosphate (ATP) and the release of growth factors. These growth factors bind to receptors on the cell surface and activate signaling responses that promote cell proliferation, viability and migration, contributing to the hard-to-heal wounds repairing process (Leyane; Jere; Houreld, 2021).

In this scenario, the main purpose of the present study is the identification of the microbial agent involved in hard-to-heal wounds on the lower limbs, the analysis of the antimicrobial resistance profile, the investigation of the antimicrobial effects of ozonated oil and the exploration of the benefits of photobiomodulation combined with ozonated oil in the therapeutic process of hard-to-heal wounds on the lower limbs. Then, in an *in vitro* study, we investigated the antimicrobial kinetics of ozonated oil on microorganisms found in wounds.

2. METHOD

This paper is about a clinical trial carried out at the Paulo Sano Basic Health Unit and Dr. Antônio Milton Zambom Basic Health Unit in the municipality of Fernandópolis, which explored the benefits of photobiomodulation combined with ozonated oil in the therapeutic process of hard-to-heal wounds on lower members. Followed by an *in vitro* experimental study that investigated the antimicrobial kinetics of ozonated oil in the

Microbiology Laboratory of Universidade Brasil, located in the city of Fernandópolis-São Paulo.

2.1 Clinical Study

The present study complies with the ethical precepts contained in CONEP Resolution No. 466, of December 12, 2012. The study was approved by the Research Ethics Committee under CAAE number: 37021620.2.0000.5554. The researcher on charge clarified and guided all patients and/or guardians about the objectives and procedures to which they would be subjected, explaining all the risks and benefits of those procedures, in addition to the freedom to withdraw from participating of the research at any time without any penalty or harm. After all clarifications, only patients who signed the Free and Informed Consent Form participated in the present study. Furthermore, the Authorization Term for image use is part of the research instruments, declaring that this material shall be used solely and exclusively for these research purposes.

The research participants were selected through an active search in all the Health Units of the mentioned municipality. In addition, the project was publicized to the community through informative posters made available in the health services and with the support of the nurses responsible for primary care in the municipality. After screening the candidates participating in the research, an evaluation was carried out following the inclusion criteria: patients aged 30 to 80 years, with hard-to-heal wounds of vascular, diabetic or traumatic etiology. The exclusion criteria were adopted: bedridden, people with neoplasms, people with Leprosy, people with neurological impairment, pregnant women, people living with HIV/AIDS.

An initial assessment of the injury was carried out on each participant, the following instruments used for data collection: application of the patient data collection instrument questionnaire in which factors related to demographic aspects were evaluated, such as age, comorbidities, family income, main complaint, history of family illnesses, main comorbidities, clinical appearance of the lesions such as wound location, tissue characteristics, amount of exudate, characteristics of the edge and injured skin.

The analysis for the presence of microorganisms was carried out by collecting swabs from the lesion(s) on the first day before starting treatment. The material was collected by a professional properly trained for this procedure, respecting aseptic techniques and biosafety standards.

After cleaning with 0.9% saline solution, the swab was moved across the wound bed in a zig-zag way, reaching at least ten points, taking care not to touch the wound edges aiming to avoid the sample contamination. After obtaining the wound material by swab, the samples were identified properly with the patient number, date, time, anatomical site of the wound and wound number where necessary. Then the samples were sent to the Institute of Hematology and Clinical Analysis of Fernandópolis at once.

The culture medium used to identify the bacteria was Mac Conkey Agar. It is a method used to isolate gram-negative bacteria. In this medium, lactose-fermenting bacteria can be differentiated through the formation of pink colonies, and non-lactose-fermenting bactéria can be identified with the formation of colorless colonies. Blood agar medium was also used. It acts as a selective and differential medium used to isolate and identify pathogenic microorganisms. The sheep blood agar culture medium provides the growth of the vast majority of gram-positive and gram-negative bacteria as well as fungi, from a rich and supplemented base, offering excellent conditions of development for non-fastidious microorganisms.

An antibiogram was performed after identifying the microorganisms. The antibiogram procedure consisted of preparing a sterile saline suspension with the bacteria that were isolated, using the standard on the McFarland scale whose turbidity standard was 0.5 (1/2).

That sterile saline suspension is inoculated on the surface of a Mueller-Hinton agar plate, using a sterile swab and then paper discs containing antibiotics impregnated at a standardized concentration are applied. Antibiotics of 12 different types were used in each antibiogram plate. Each disc has its code (abbreviation for the antibiotic) and the numerical value of its concentration printed on one side. After incubation in a microbiological oven for 24 hours at a temperature ranging between 36 and 37°C, the pattern of growth or inhibition around each disc is analyzed, being sensitive (S) when the antibiotic was effective and there was no growth bacterial, so the infection can be treated with the recommended dosage of antimicrobial; resistant (R) when the antibiotic was not effective and there was bacterial growth, thus usual systemic concentrations of the antimicrobial do not inhibit the microorganism, generating clinical ineffectiveness.

Before applying the dressing, first of all the wound was cleaned with 0.9% saline solution and then the LED was applied, using the wavelength 660 nm, 30 seconds, punctual 2 cm away on the wound bed, 120 mW, 3.6 J per point. After applying

photobiomodulation therapy, ozonated sunflower oil from the Blustratum® brand was applied to the entire wound bed. It was covered with a dry sterile gauze, wrapped in a bandage and fixed with a masking tape.

All patients received the same procedure three times a week for a maximum of 12 weeks. Treatment would be stopped in the event of the wound closed before 12 weeks or at the patient's discretion.

Aiming to follow the wounds, some photographic records were taken to observe the evolution of them weekly, using the iPhone 11 cell phone camera, with 12MP; a standard distance was maintained by using a support to verify the progress of tissue healing.

2.2 *In Vitro* Study

An *in vitro* study was carried out to better understand and determine the best dosage and administration time of ozonated oil for the treatment of bacterial infections. The following strains were used: *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10031, all of them belonging to the Microbiology Laboratory collection of Universidade Brasil, in Fernandópolis campus.

The tests for verifying the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (CBM) were carried out according to the procedure recommended by CLSI, 2018.

The strains of microorganisms were suspended in an appropriate media.

The minimum inhibitory concentration essay is a technique used to determine the lowest concentration of an antimicrobial agent able to inhibit the visible growth of a particular microorganism. The MIC was performed in a 96-well plate. A serial dilution was used and the bacterial cell density of the suspension was 106 /mL for testing.

Columns 1, 2 and 3 were designated as controls, with column 1 receiving only culture medium; column 2 receiving ozonated oil; column 3 receiving only microorganism suspension. The serial dilution started from the 4th column, being represented by the concentration of 100% ozonated oil. The procedure was repeated up to the last column, representing the dilutions 50%, 25%, 12.50%, 6.25%, 3.12%, 1.56%, 0.78% and 0.39%. At last, the suspension with 106 / mL microorganisms was added to all wells, excepting the negative controls (columns 1 and 2).

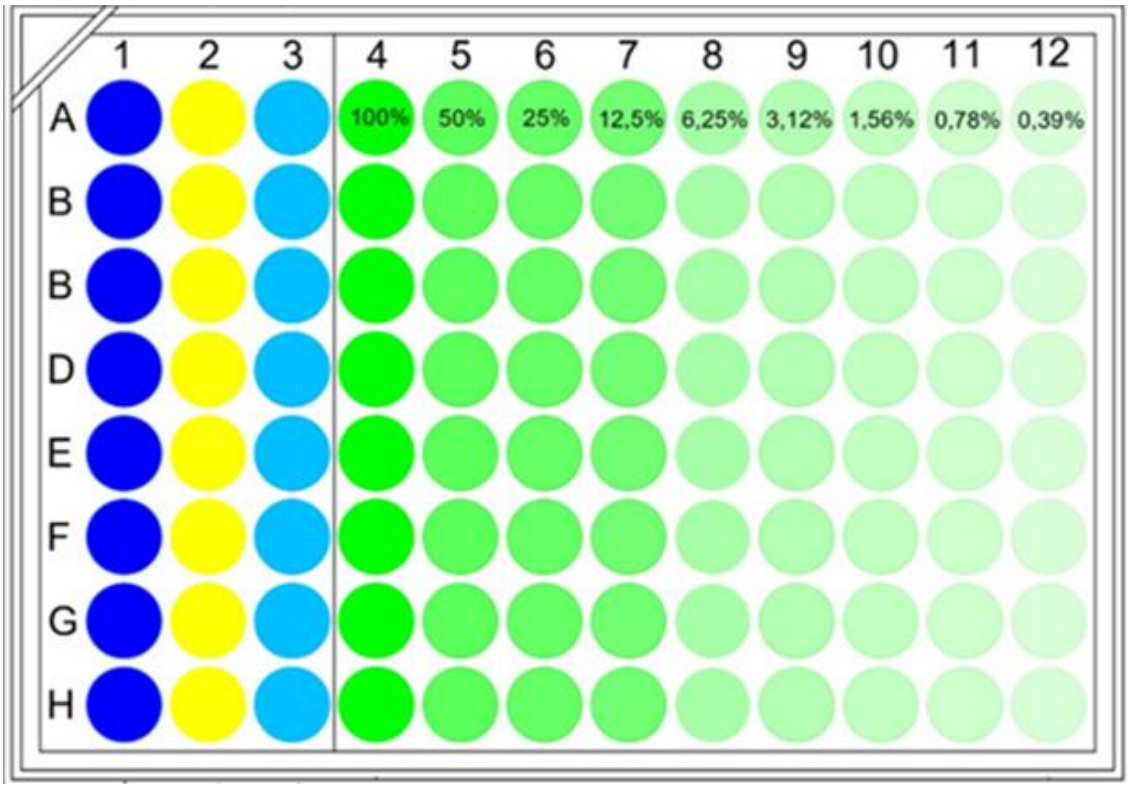


Image 1: Representative scheme of the microdilution methodology.
Source: prepared by the authors (2024).

The plates were incubated in a microbiological oven at a 37ºC temperature for a 24 hours period. After the incubation time, 50 µL of the dye 2,3,5-triphenyl tetrazolium chloride (TTC) that is an indicator of bacterial cell viability was added to each well. Colorimetric evaluation is carried out in this method. When metabolically active bacteria come into contact with the TTC, they reduce the compound to1,3,5- triphenylformazan in reddish color and then can be visually observed. They remain colorless in wells where bacteria are not growing. In this way, the MIC was determined for each microorganism as the lowest concentration of the antibacterial agent (ozonated oil), expressed in mg/L (µg/ml), which prevents bacterial growth completely under strictly controlled in vitro conditions (Kowalska-Krochmal; Dudek-Wicher, 2021).

Aiming to determine the minimum bacterial concentration, an aliquot (20 µl) of the wells, in which no bacterial growth was observed, was seeded in Petri dishes containing Tryptone Soy Agar (TSA) for the bacteria *Escherichia coli, Klebsiella sp* and *Staphylococcus epidermidis*. The plates were incubated at a 28°C temperature for 72 hours. After this period of time the spots where microbial growth occurred were observed, thus determining the CBM, which is defined as the lowest concentration tested where no

microbial growth occurred after performing the subculture (Aiemsaard; Punareewattana, 2017).

For determining how the concentration of ozonated oil changes over time and how this affects bacterial viability, the "time-kill" or bactericidal kinetics assay was performed (Allahghadri *et al*., 2010).

In order to that determination, the microorganisms were cultivated in an appropriate médium. Then 950 µL of each microorganism were used, at a concentration of 106 / mL, and 50 µL of ozonated oil were added at the dilution determined by CBM. Then the samples were incubated at 28 ºC temperature and collected 100 µL, at pre-determined time intervals and plated on Tryptone Soy Agar (TSA) medium for Escherichia coli, Klebsiella sp and Staphylococcus epidermidis with the aid of a Drigalski loop. Plating occurred at times of 0', 5', 10', 20', 40', 80', 160' and 320' and incubated 24/48 at 37ºC temperature. All assessments were performed in triplicate. After incubation the colonies were counted manually.

The same methodology was used to identify the interference of LED in the bactericidal and fungicidal kinetics of ozonated oil, however the samples of *Escherichia coli*, *Klebsiella sp* and *Staphylococcus epidermidis* were irradiated with LED before being spread in culture. All assessments were performed in triplicate.

2.3 Data Analysis

Data were analyzed using descriptive statistics. The microbial count data was approached using a graph in order to observe the evolution of the microbial count variation over time.

To evaluate the regression of the wound area, the initial area and final area values measured by Image J®, version 1.3.1 software (US National Institutes of Health, Bethesda, MD).

3. RESULTS

3.1 Clinical Study

3.1.1 Sociodemographic Characterization of Patients

Ten patients with hard-to-heal wounds on the lower limbs underwent clinical evaluation when they spontaneously sought care at Basic Health Units and met the

inclusion criteria established. Of the total ten patients, two did not show up to start treatment, resulting in eight patients who were followed throughout the study. During the development of the research three patients chose to discontinue treatment, while two of them had complete tissue healing before the 12-week period and three patients completed treatment submitting to the established protocol over 12 weeks.

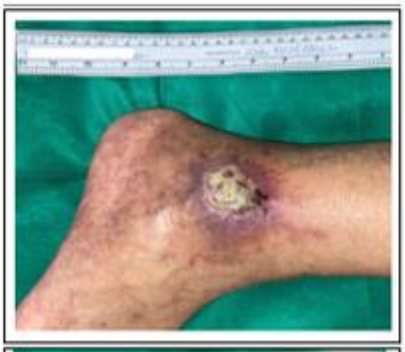
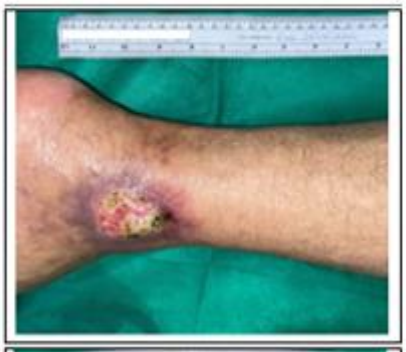
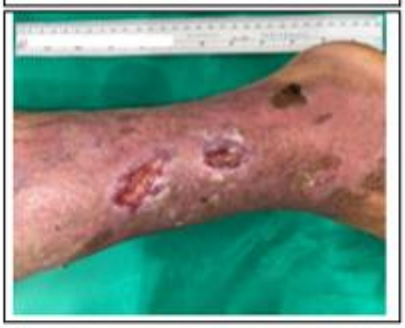
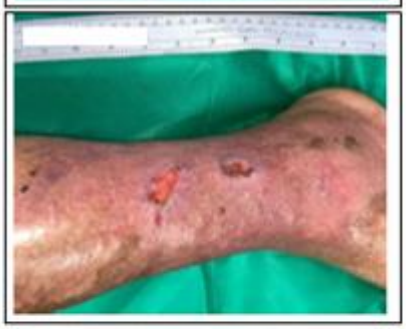
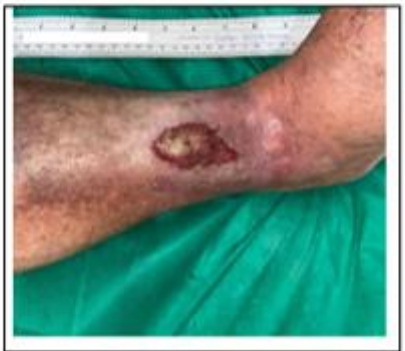
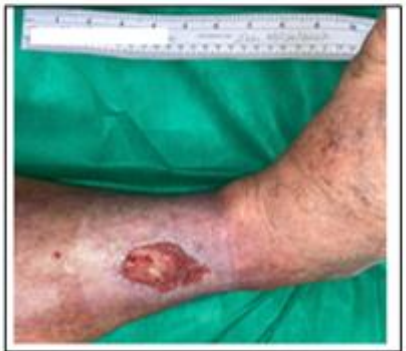
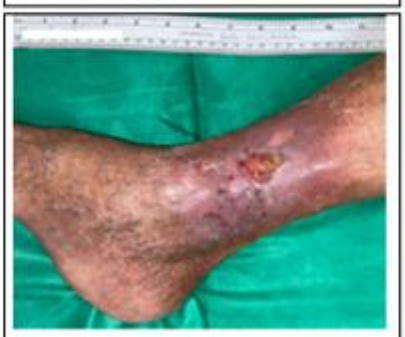
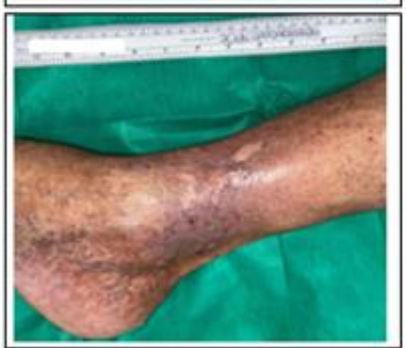
The sample was characterized by six male individuals (75%) and two female individuals (25%), with ages ranging from 41 to 79 years old. As for ethnic-racial self-identification, five participants declared themselves as white (62.5%), two as black (25%) and one as mixed race (12.5%). Regarding education, five interviewees had incomplete primary education (62.5%) and three had completed secondary education (37.5%). As for occupational status, three were retired (37.5%), two were on leave (25%), two were employed (25%) and one was unemployed (12.5%). It was observed that three (37.5%) participants had income equal to or less than one minimum wage while three (37.5%) had income between one and two minimum wages and two (25%) had income between two and three minimum wages.

3.1.2 Wound Characterization

Regarding the temporality of the wound, the variation was from 3 months to 10 years. Regarding the type of wound, four (50%) were mixed ulcers and four (50%) were venous ulcers. Regarding the location of the wounds, six (75%) participants had injuries in the lower third of the leg and two (25%) in the malleolus. Five (62.5%) participants had one single wound, while three (37.5%) participants had double wounds.

3.1.3 Macroscopic Assessment

After the period of following-up, a significant improvement in the lesions was observed, such as a reduction in traces of infection, the presence of granulation tissue, a reduction in the wound area and, in some cases, closure of the wound fully.

	Home	Final	Weeks of treatment
1			3
2			6
3			6
4			8

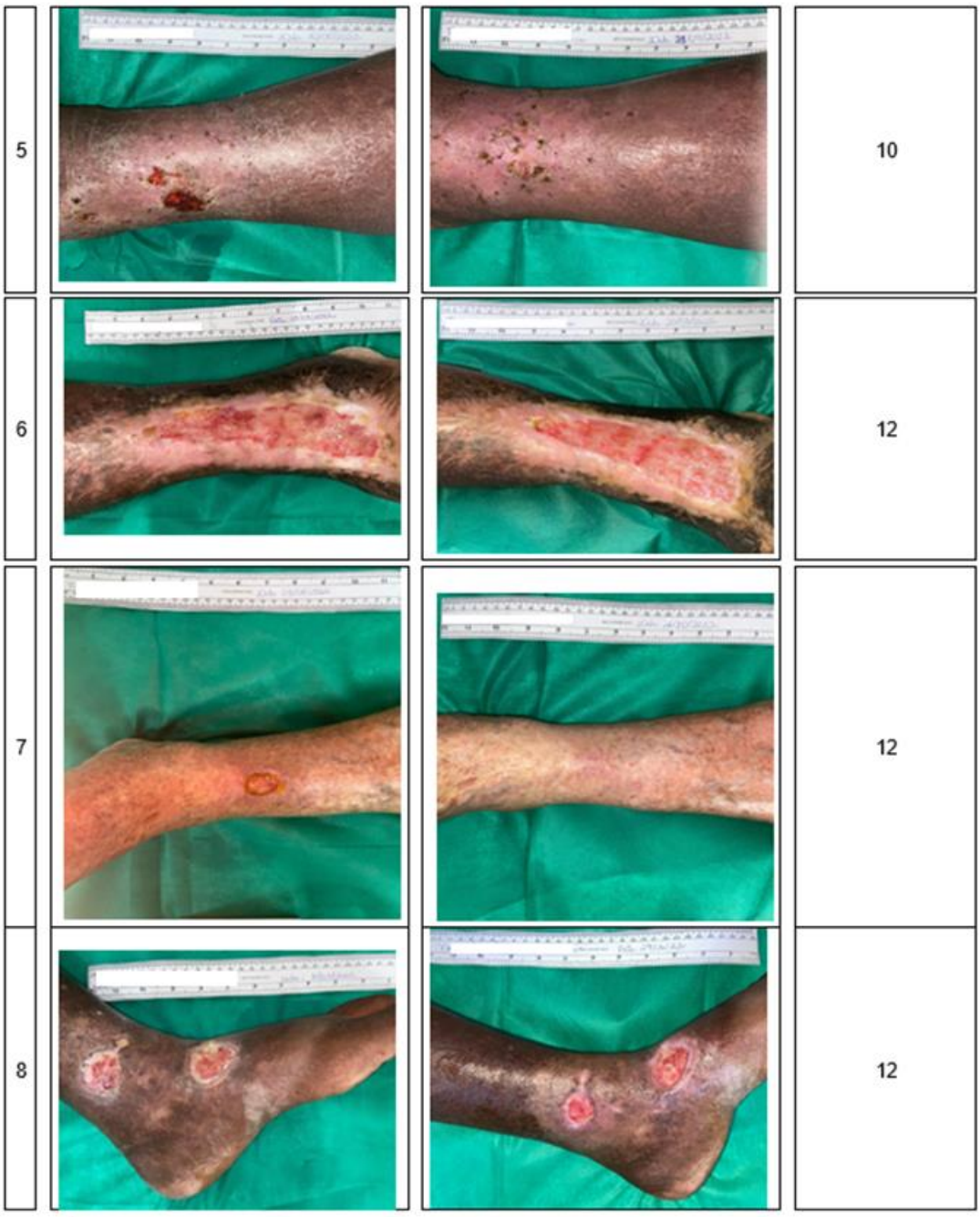


Image 2. Macroscopic findings of the evaluated wounds.
Source: prepared by the authors (2024).

3.1.4 Wound Healing Index

Among the eight patients monitored (Table 3), five individuals fully completed the therapeutic regimen, with patients 6, 7 and 8 undergoing 12 weeks of treatment and patients 4 and 5 having full wound healing before the stipulated period of 12 weeks. Three participants (Patients 1, 2 and 3) interrupted treatment, basing their decisions upon the lack of time available or upon the difficulties associated with traveling to the Health Unit

to have their dressings carried out three times a week. Even receiving partial treatment, the three patients got a reduction in the initial wound area.

Table 1. Healing index analysis.

Patient	Wounded existence time	Follow-up time (weeks)	Initial area (cm²)	Final area (cm²)	Reduction area (cm2)	Reduction Rate (%)
1	4 months	3	14.7	13.9	0.8	5.4
2	2 years	6	9.3	2.4	6.9	74.2
	2 years	6	5.3	1.3	4.0	75.5
3	6 months	6	18.2	17.7	0.5	2.7
4	10 years	8	6.3	0.0	6.3	100.0
5	1 year	10	1.2	0.0	1.2	100.0
	1 year	10	4.2	0.0	4.2	100.0
6	3 years	12	71.2	66.1	5.1	7.2
7	5 years	12	6.6	0.0	6.6	100.0
8	2 years	12	12.8	5.1	7.7	60.2
	2 years	12	10.7	10.5	0.2	1.9

Source: prepared by the authors (2024).

3.1.5 Microbiological Identification

Among the 8 patients monitored, only 1 patient showed negative microbiology while 6 patients showed the presence of only 1 microorganism in the wound and 1 patient showed the presence of 2 microorganisms in a single wound. In six cultures performed, gram-negative bacteria were identified with 5 cases of *Escherichia coli* and 1 case of *Klebsiella sp.* In one culture, the presence of a gram-positive bacterium identified as *Staphylococcus epidermidis* was observed while yeasts were identified in another culture.

3.1.6 Antibiogram

The identified bacteria were evaluated based on the antibiogram being divided into resistant, sensitive and low sensitive. The *Klebisiella sp.* found in only one patient proved resistant to ampicillin, nitrofurantoin, oxofloxacin, amoxicillin, tetracycline, norfloxacin, gentamicin, nadixylic acid, phosphomycin, sulfazotrim, clavulanic acid+amoxicillin. The *Staphylococcus epidermidis*, also found in a single patient, proved resistant to tetracycline. The *Escherichia coli* was found in five patients and proved resistant to ampicillin, amoxicillin, tetracycline, gentamicin and sulfazotrim.

Table 2. Antibiotics tested and frequency of results

Antibiotics tested	Resistant-R	S-sensitive	Little Sensitive-PS
ampicillin	6	0	1
cefazolin	2	3	2
levofloxacin	3	4	0
nitrofurantoin	4	1	2
cefeepime	1	3	3
amoxicillin	6	1	0
ciprofloxacin	3	4	0
tetracycline	7	0	0
norfloxacin	4	3	0
gentamicin	6	1	0
nadoxylic acid	5	2	0
fosfomycin	4	2	1
meropenem	0	7	0
sulfazotrim	6	0	1
clavulanic acid+amoxicillin	5	2	0
ceftriaxone	1	4	2

Source: prepared by the authors (2024).

3.2 *In Vitro* Study

3.2.1 Minimum Inhibitory Concentration

In the minimum inhibitory concentration test, we evaluate the effectiveness of ozonated oil against various bacterial and fungal strains. We observed that, for the bacteria *Escherichia coli* and *Klebsiella sp*., a minimum concentration of 6.25% of ozonated oil was necessary to visibly inhibit the growth of these microorganisms. On the other hand, for the bacterium *Staphylococcus epidermidis*, inhibition occurred at a lower concentration, 3.12% of ozonated oil.

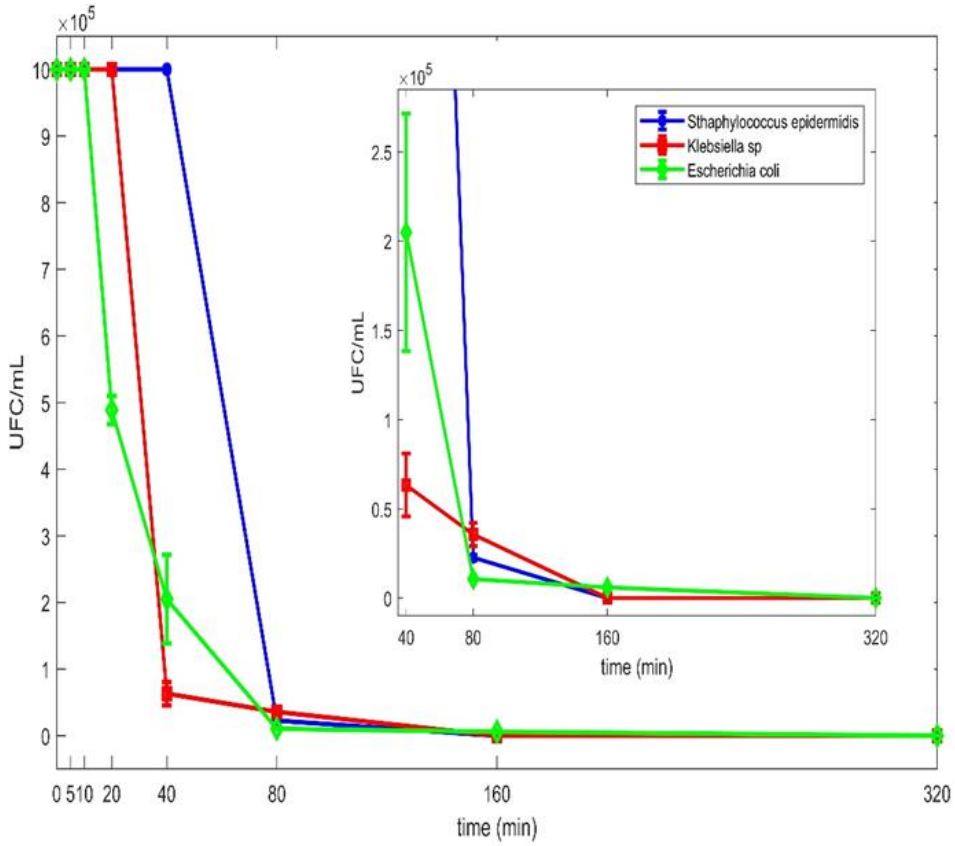
3.2.2 Bactericidal Concentration

Based on the results of the test that determined the minimum concentration of ozonated oil able of inhibiting the growth of each microorganism, we carried out tests on the minimum concentration necessary to promote the bactericidal effect. For the bacteria *Escherichia coli* and *Klebsiella sp*., it was necessary to reach a concentration of 12.5% of ozonated oil to obtain the bactericidal effect. In the case of the bacterium *Staphylococcus epidermidis*, a concentration of 6.25% of ozonated oil was sufficient to achieve the bactericidal effect.

3.2.3 Antimicrobial Potential

The antimicrobial effect of the ozonated oil was observed on all the microorganisms tested with variations only in the exposure time required to achieve the bactericidal/bacteriostatic effect.

In the case of the bacterium *Escherichia coli*, it was observed that the bacteriostatic effect was evident after 20 minutes of contact with the ozonated oil. However, 320 minutes of exposure were required to achieve the bactericidal effect. For *Klebsiella sp*., the bacteriostatic effect was observed after 80 minutes of exposure, while the bactericidal effect was achieved after 160 minutes. As for the bacterium *Staphylococcus epidermidis*, both bacteriostatic and bactericidal effects were observed after 80 minutes of exposure.



Graph 1. Growth kinetics of microorganisms found.
Source: prepared by the authors (2024).

3.2.4 LED interference in the bactericidal and fungicidal kinetics of ozonated oil

It was found that LED irradiation did not interfere with the antimicrobial potential of the ozonated oil.

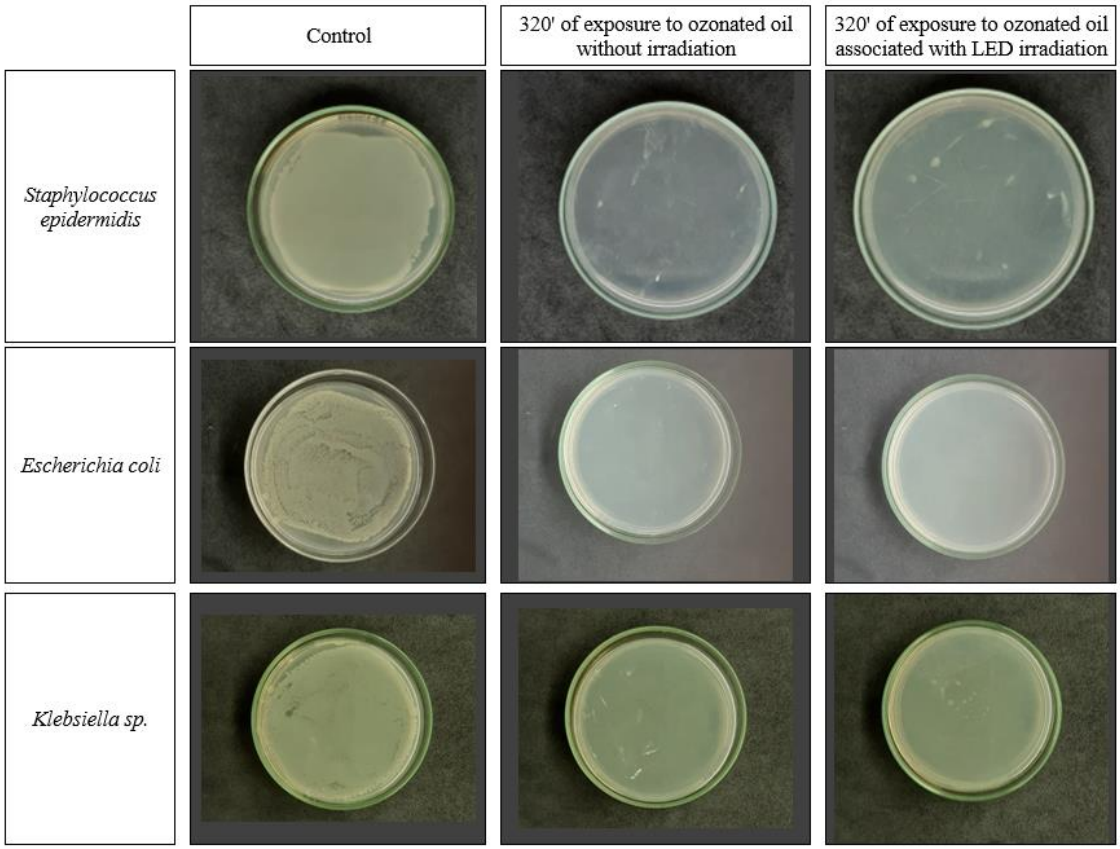


Image 3. Comparison between microorganisms grown in culture medium after 320 minutes of ozonated oil action and microorganisms grown in culture medium after 320 minutes of ozonated oil action and irradiated with LED.
Source: prepared by the authors (2024).

4. DISCUSSION

Choosing the ideal treatment for hard-to-heal wounds aiming to achieve a complete repair of the affected tissue continues to be a significant challenge for healthcare professionals. In this context, ozonated oil and photobiomodulation therapy emerge as promising therapeutic resources, designed to stimulate the healing process of hard-to-heal wounds (Fitzpatrick; Holland; Vanderlelie, 2018; Pavlov *et al*., 2020).

The present study showed that LED photobiomodulation combined with the use of ozonated oil demonstrated effectiveness in promoting tissue repair and, in some cases, enabled closure of the lesion fully. Congruently, a systematic review that analyzed the potential benefits and risks of ozone therapy in advanced wound care revealed consistent results in favor of ozonated oil application as a viable therapy for hard-to-heal wounds, suggesting its potential for conventional clinical practice (Fitzpatrick; Holland; Vanderlelie, 2018). Furthermore, another study covering 28 articles related to the use of ozonated oil in the treatment of wounds also indicated that the effectiveness of ozonated

oil may represent a therapeutic approach in the treatment of tissue injuries due to its antimicrobial, immunological, antioxidants and oxygenating properties (Anzolin; Silveira-Kaross; Bertol, 2020).

In the context of photobiomodulation, histological investigations conducted revealed that the implementation of this therapy accelerated the wound reparative process during the initial phases of a tissue healing, facilitating the reduction of the inflammatory response and promoting faster regeneration of injured tissues (Pavlov *et al*., 2020). Such therapeutic benefits associated with therapy are related to the stimulation of mitochondria, leading to increased production of adenosine triphosphate (ATP) and the release of growth factors. The binding of growth factors to cell surface receptors induces the activation of signaling responses that promote cell proliferation, viability and migration (Leyane; Jere; Houreld, 2021).

Sales, Dantas and Medrado (2022) emphasize that photobiomodulation has the ability to promote cell growth and accelerate the healing process, thus contributing to the patient's clinical recovery and, indirectly, to improving their quality of life.

The results regarding tissue repair evidenced by the use of ozonated oil in the treatment of hard-to-heal wounds in this study may be related to its antimicrobial effect. Of the patients monitored, 88% of them had microorganisms such as *Escherichia coli*, *Klebsiella sp*, *Staphylococcus epidermidis* in the wounds before starting treatment. It should be noted that the microorganisms mentioned revealed resistance to specific conventional antibiotics as identified in the antibiogram. This resistance probably originates from mutations in bacteria and yeast, which induce changes in cell membrane proteins, giving them a configuration that is no longer susceptible to recognition by pharmacological agents. Due to this phenomenon, ozonated oils have experienced an increase in scientific interest and clinical applications (Ugazio *et al*., 2020).

In vitro results confirmed the antimicrobial efficacy of ozonated oil, demonstrating its potential as a viable therapeutic agent for chronic wounds. This finding has significant relevance for clinical practice, especially in contexts in which wounds exhibit exudates susceptible to dilution of ozonated oil after application, whilst maintain their antimicrobial effectiveness. Furthermore, it showed the bactericidal effect in all strains studied.

These results provide valuable parameters for the development of clinical protocols, guiding the application of ozonated oil in wounds that are hard to repair, taking

into account factors such as oil concentration, duration of exposure and frequency of dressing changes to ensure the desired antimicrobial effect. This effect is mainly attributed to the predominant formation of ozonides, aldehydes and peroxides, which react with the bacterial membrane and cytoplasm, inducing an increase in cellular permeability and cytoplasmic changes that culminate in the interruption of bacterial growth (Zeng; Lu, 2018; Silva *et al*., 2021; Varol *et al*., 2017).

It should be emphasized that the presence of microorganisms in wounds can significantly interfere with the tissue reparative process (Martinengo *et al*., 2018). When infection occurs, the organism triggers a prolonged inflammatory response characterized by the migration of leukocytes and a significant production of pro-inflammatory cytokines, exerting harmful effects on the wound-reparative process (Gushiken *et al*., 2021). Therefore, the early identification of microorganisms in hard-to-heal wounds is crucial to determine appropriate treatment. In searching for appropriate therapeutic approaches, the findings of the present study indicate that the combination of antimicrobial therapy with ozonated oil and photobiomodulation may prove to be effective in the wound-reparative process, especially when we take into account that the light emitted by the LED did not stimulate the proliferation of microorganisms. This scenario suggests that the combined antimicrobial and healing effects of ozonated oil and photobiomodulation present a synergistic approach to chronic wound management, mitigating potential challenges associated with microbial proliferation in hard-to-heal wounds.

5. CONCLUSION

This study highlights the antimicrobial and reparative benefits of ozonated oil combined with photobiomodulation for chronic wound management.

Despite encouraging results, it is important to highlight the need to conduct new randomized, controlled and blind clinical trials. These additional studies are essential to improve treatment protocols, optimize therapeutic efficacy and validate the safety of using ozonated oil in specific clinical contexts.

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