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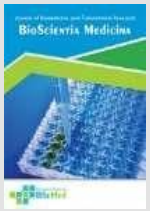

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Molecular Mechanisms of Ozonized *Nigella sativa* Oil in Wound Repair: Albumin as Biomarker in Rat Model

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A B S T R A C T

Background: Wound healing is a complex physiological process that can be impaired in various conditions. *Nigella sativa* oil, rich in bioactive compounds like thymoquinone, has shown promise in promoting wound healing. Ozone therapy, through the generation of reactive oxygen species (ROS), has also been explored for its potential to accelerate wound repair. This study aimed to investigate the molecular mechanisms underlying the effects of ozonized *Nigella sativa* oil on wound healing, with a focus on albumin as a biomarker of tissue regeneration in a rat model. **Methods:** Full-thickness skin wounds were created on the backs of Sprague Dawley rats. The rats were randomly divided into four groups: a control group receiving no treatment, and three treatment groups receiving topical applications of ozonized *Nigella sativa* oil at different ozone concentrations (1400 mg/ml, 1800 mg/ml, and 2200 mg/ml) for 7 days. Wound healing was assessed by measuring wound closure rates and histological analysis. Albumin levels in wound tissue were quantified using immunohistochemistry. Additionally, the expression of key genes involved in wound healing, including growth factors, cytokines, and matrix metalloproteinases, was evaluated using quantitative real-time PCR. **Results:** Ozonized *Nigella sativa* oil significantly accelerated wound closure compared to the control group. Histological analysis revealed improved tissue regeneration and collagen deposition in the treated groups. Albumin levels were significantly elevated in the wound tissue of rats treated with ozonized *Nigella sativa* oil, particularly at the highest ozone concentration. Furthermore, the expression of growth factors (VEGF, TGF-β), pro-inflammatory cytokines (IL-1β, TNF-α), and matrix metalloproteinases (MMP-2, MMP-9) was modulated in a manner consistent with enhanced wound healing. **Conclusion:** Ozonized *Nigella sativa* oil promotes wound healing in a rat model through multiple molecular mechanisms, including the stimulation of albumin synthesis, growth factor expression, and controlled inflammation. These findings suggest that ozonized *Nigella sativa* oil may have therapeutic potential for enhancing wound repair in clinical settings.

1. Introduction

Wound healing, an intricate symphony of biological processes orchestrated to restore tissue integrity, is a fundamental aspect of human physiology. The skin, the body's largest organ, serves as the first line of defense against the external environment, shielding us from pathogens, preventing dehydration, and regulating body temperature. When this protective barrier is breached, a cascade of events is triggered to repair the damage and restore homeostasis. However, this process is not always seamless. In various pathological conditions, such as diabetes, chronic inflammation, and aging, wound healing can be significantly impaired, leading to chronic wounds that pose a substantial burden to patients and healthcare systems. Chronic wounds, characterized by their prolonged duration and resistance to conventional therapies, affect millions of people worldwide. These wounds not only cause physical discomfort and pain but also impair quality of life, increase the risk of

infections, and impose a significant economic burden. The management of chronic wounds remains a major challenge in clinical practice, highlighting the urgent need for innovative therapeutic approaches that can accelerate wound closure and promote tissue regeneration.[1,2]

In the quest for effective wound healing agents, natural products have garnered increasing attention due to their rich repertoire of bioactive compounds and their potential for synergistic effects. Among these, *Nigella sativa* oil, extracted from the seeds of the black cumin plant, has emerged as a promising candidate for wound management. Revered for centuries in traditional medicine for its diverse therapeutic properties, *Nigella sativa* oil has been shown to possess anti-inflammatory, antioxidant, antimicrobial, and immunomodulatory activities, making it an attractive option for promoting wound repair. The oil's therapeutic potential is attributed to its complex chemical composition, which includes thymoquinone, a potent bioactive compound with multifaceted pharmacological properties. Thymoquinone has been shown to modulate various signaling pathways involved in wound healing, including the nuclear factor kappa B (NF-κB) pathway, mitogen-activated protein kinase (MAPK) pathways, and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. These pathways regulate key processes such as inflammation, cell proliferation, migration, and angiogenesis, all of which are critical for successful wound healing. Preclinical studies have demonstrated the efficacy of *Nigella sativa* oil in accelerating wound closure, enhancing tissue regeneration, and reducing scar formation in various animal models. The oil's ability to modulate the inflammatory response, scavenge free radicals, and promote angiogenesis contributes to its wound-healing properties. Moreover, *Nigella sativa* oil has been shown to stimulate the production of collagen, a key structural protein that provides strength and support to the healing tissue.[3,4]

Ozone therapy, involving the administration of ozone gas, has also gained recognition for its potential applications in wound management. Ozone, a triatomic form of oxygen, is a potent oxidant that can generate reactive oxygen species (ROS) upon contact with biological tissues. While excessive ROS production can be detrimental, controlled levels of ROS play a crucial role in wound healing by acting as signaling molecules that trigger various cellular responses. In the context of wound healing, ozone therapy has been shown to promote angiogenesis, stimulate cell proliferation and migration, and enhance extracellular matrix remodeling. These effects are mediated through the modulation of various signaling pathways, including the hypoxia-inducible factor-1 alpha (HIF-1α) pathway, the vascular endothelial growth factor (VEGF) pathway, and the transforming growth factor beta (TGF-β) pathway. Ozone therapy has also been shown to possess antimicrobial properties, which can help to prevent infections and promote a clean wound environment conducive to healing.[4,5]

The combination of *Nigella sativa* oil and ozone therapy, in the form of ozonized *Nigella sativa* oil, represents a novel approach to wound management that harnesses the synergistic potential of these two agents. Ozonation, the process of bubbling ozone gas through the oil, enhances the oil's therapeutic properties by increasing its oxidative potential and generating additional bioactive compounds. Ozonized *Nigella sativa* oil has been shown to exhibit superior wound-healing properties compared to non-ozonized oil in preclinical studies. The ozonized oil has been shown to accelerate wound closure, improve tissue regeneration, and reduce scar formation more effectively than the non-ozonized oil. These enhanced effects are likely due to the combined action of thymoquinone and ozone-derived ROS, which can modulate a wider range of signaling pathways and cellular responses involved in wound healing.[5,6]

Albumin, the most abundant protein in blood plasma, plays a vital role in maintaining oncotic pressure and transporting various molecules, including hormones, fatty acids, and drugs. In the context of wound healing, albumin has been

recognized as a key player in tissue regeneration. It provides a source of amino acids for protein synthesis, promotes cell migration and proliferation, and modulates the inflammatory response. Moreover, albumin levels have been shown to correlate with wound healing outcomes. Studies have demonstrated that patients with chronic wounds often exhibit lower serum albumin levels compared to healthy individuals. Furthermore, interventions that promote wound healing, such as nutritional supplementation and growth factor therapy, have been shown to increase albumin levels in wound tissue. These findings suggest that albumin may serve as a valuable biomarker for monitoring the progress of wound repair and evaluating the efficacy of therapeutic interventions.[6,7] The study aimed to investigate the molecular mechanisms underlying the effects of ozonized *Nigella sativa* oil on wound healing, with a particular focus on the role of albumin as a biomarker of tissue regeneration.

2. Methods

The present study employed a rigorous and well-established experimental design, the randomized controlled trial (RCT), to investigate the molecular mechanisms underlying the effects of ozonized *Nigella sativa* oil on wound healing. The RCT design is considered the gold standard for evaluating the efficacy of interventions, as it minimizes bias and allows for causal inferences to be drawn. In this study, the RCT design involved the random allocation of Sprague Dawley rats to four distinct groups: a control group receiving no treatment, and three treatment groups receiving topical applications of ozonized *Nigella sativa* oil at varying ozone concentrations. This approach enabled us to assess the dose-dependent effects of ozonized *Nigella sativa* oil on wound healing and identify the optimal ozone concentration for maximizing therapeutic benefits. The selection of Sprague Dawley rats as the animal model was based on several factors. Firstly, rats are widely used in wound healing research due to their physiological and anatomical similarities to humans. The skin of rats shares many structural and functional characteristics with human skin, making it a suitable model for studying wound repair processes. Secondly, Sprague Dawley rats are a well-characterized strain with a docile temperament, facilitating handling and experimental procedures. Finally, the availability of extensive literature on wound healing in Sprague Dawley rats allows for comparisons and contextualization of our findings. The study duration of 7 days was chosen based on previous research demonstrating significant changes in wound healing parameters within this timeframe. This duration allowed us to capture the early and intermediate phases of wound healing, including inflammation, proliferation, and the onset of remodeling. By focusing on these critical phases, we aimed to gain insights into the molecular mechanisms by which ozonized *Nigella sativa* oil influences wound repair.

The ethical and humane treatment of animals is of paramount importance in scientific research. In this study, all animal procedures were conducted in strict accordance with the guidelines of the Institutional Animal Care and Use Committee. Male Sprague Dawley rats, aged 8-10 weeks and weighing 250-300 grams, were selected for this study. This age range represents young adult rats that are physiologically mature and less susceptible to age-related variations in wound healing. The use of male rats minimized potential confounding factors associated with hormonal fluctuations in females. The rats were housed in standard cages under controlled conditions of temperature (22 ± 2°C) and humidity (55 ± 5%) with a 12-hour light/dark cycle. These conditions ensured the animals' comfort and well-being throughout the study. The rats were provided with ad libitum access to food and water, allowing them to maintain their nutritional status and hydration levels. Prior to any experimental procedures, the rats were acclimatized to the laboratory environment for one week. This acclimatization period allowed the animals to adjust to their new surroundings and minimize stress, which could potentially influence wound healing.

The creation of full-thickness skin wounds was a critical step in this study, as it allowed us to simulate a clinically relevant injury and evaluate the effects of ozonized *Nigella sativa* oil on wound repair. The rats were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. Ketamine, a dissociative anesthetic, induces a state of catalepsy and analgesia, while xylazine, an alpha-2 adrenergic agonist, provides sedation and muscle relaxation. This combination ensured adequate anesthesia and pain management throughout the wound creation procedure. The dorsal hair was carefully shaved using electric clippers, exposing the skin surface. The skin was then disinfected with povidone-iodine, a broad-spectrum antiseptic that effectively eliminates bacteria, viruses, and fungi. A full-thickness excisional wound, measuring 1 cm in diameter, was created on the back of each rat using a sterile biopsy punch. The biopsy punch, a cylindrical cutting instrument, ensured the creation of a uniform and well-defined wound, minimizing variability between animals. Following wound creation, the rats were randomly assigned to the four experimental groups. The control group received no treatment, while the treatment groups received topical applications of ozonized *Nigella sativa* oil at different ozone concentrations (1400 mg/ml, 1800 mg/ml, and 2200 mg/ml). The ozonized oil was applied directly to the wound surface using a sterile cotton swab once daily for 7 days. This topical application allowed for direct contact of the oil with the wound bed, maximizing its therapeutic potential.

The preparation of ozonized *Nigella sativa* oil involved a carefully controlled process to ensure the generation of a stable and effective therapeutic agent. *Nigella sativa* oil was obtained from a reputable commercial supplier, ensuring its purity and quality. The oil was then ozonized using a medical-grade ozone generator. Ozone gas was bubbled through the oil at different concentrations (1400 mg/ml, 1800 mg/ml, and 2200 mg/ml) for 30 minutes. The ozonation process resulted in the formation of ozone-derived ROS and other bioactive compounds within the oil, enhancing its therapeutic potential. The ozonized oil was stored at 4°C until use, ensuring its stability and preventing degradation of its active components. The use of different ozone concentrations allowed us to investigate the dose-dependent effects of ozonized *Nigella sativa* oil on wound healing and identify the optimal concentration for maximizing therapeutic benefits.

The assessment of wound healing involved a multi-faceted approach that included both macroscopic and microscopic evaluations. Wound closure, a key indicator of healing progress, was assessed by measuring the wound area using digital calipers on days 0, 3, 5, and 7. Digital calipers, with their high precision and accuracy, allowed for precise measurements of wound dimensions, minimizing measurement errors. The percentage of wound closure was calculated based on the initial wound area and the wound area on each measurement day. This approach provided a quantitative measure of wound healing dynamics and enabled comparisons between the different experimental groups. Histological analysis was performed to evaluate the microscopic changes associated with wound healing. On day 7, the rats were euthanized, and the wound tissue was harvested. The tissue samples were fixed in 10% formalin, a widely used fixative that preserves tissue morphology and prevents autolysis. The fixed tissues were then embedded in paraffin, a wax-like substance that provides support during sectioning. The paraffin-embedded tissues were sectioned at 5 µm thickness using a microtome, generating thin slices suitable for microscopic examination. The tissue sections were stained with hematoxylin and eosin (H&E), a classic staining technique that differentiates cellular and extracellular components. H&E staining allowed for the visualization of key histological features, including re-epithelialization, granulation tissue formation, and collagen deposition. Additionally, Masson's trichrome staining was performed to specifically visualize collagen fibers, a major component of the extracellular matrix that provides structural support to the healing tissue. The histological features were evaluated by a

blinded pathologist, ensuring objectivity and minimizing bias in the assessment.

Immunohistochemical staining was employed to quantify albumin levels in the wound tissue. This technique utilizes antibodies to specifically detect and visualize target proteins within tissue sections. In this study, a primary antibody against albumin was used to label albumin molecules in the wound tissue. The primary antibody was then detected using a biotinylated secondary antibody and streptavidin-horseradish peroxidase conjugate. The signal was visualized using diaminobenzidine (DAB) as a chromogen, resulting in a brown precipitate at the sites of albumin localization. The intensity of albumin staining was quantified using image analysis software, providing a quantitative measure of albumin levels in the wound tissue.

Quantitative real-time PCR (qPCR) was performed to measure the expression of key genes involved in wound healing. This technique allows for the precise quantification of mRNA levels, reflecting gene expression. Total RNA was extracted from the wound tissue using a commercial kit, ensuring high-quality RNA isolation. cDNA was synthesized from the RNA using reverse transcriptase, an enzyme that converts RNA into complementary DNA (cDNA). qPCR was then performed using gene-specific primers and a fluorescent dye that binds to double-stranded DNA. The relative expression of each gene was normalized to the expression of GAPDH, a housekeeping gene that serves as an internal control. The genes selected for qPCR analysis included growth factors (VEGF, TGF-β), cytokines (IL-1β, TNF-α), and matrix metalloproteinases (MMP-2, MMP-9). These genes play critical roles in various aspects of wound healing, including angiogenesis, cell proliferation, inflammation, and extracellular matrix remodeling. By measuring the expression of these genes, we aimed to gain insights into the molecular mechanisms by which ozonized *Nigella sativa* oil influences wound repair. The data collected in this study were analyzed using appropriate statistical tests to determine the significance of the observed effects. One-way ANOVA was used to compare the means of multiple groups, followed by Tukey's post hoc test for pairwise comparisons. A p-value of less than 0.05 was considered statistically significant.

3. Results

Table 1 displays the percentage of wound closure over time (Days 0, 3, 5, and 7) for four different groups: a control group and three treatment groups receiving ozonized *Nigella sativa* oil at varying ozone concentrations (1400 mg/ml, 1800 mg/ml, and 2200 mg/ml). All groups exhibit an increase in the percentage of wound closure over time, indicating the natural healing process. The treatment groups consistently demonstrated higher wound closure percentages compared to the control group at all time points, $p<0,05$. This suggests that ozonized *Nigella sativa* oil accelerates wound healing. The treatment group receiving the highest ozone concentration (2200 mg/ml) exhibits the most rapid wound closure, followed by the 1800 mg/ml and then the 1400 mg/ml groups. This suggests a dose-dependent relationship between ozone concentration and wound healing efficacy.

Table 1. Percentage of wound closure (%).

Group	Day 0	Day 3	Day 5	Day 7
Control	0 ± 0	20 ± 3	35 ± 5	50 ± 7
1400 mg/ml Ozone	0 ± 0	30 ± 4*	45 ± 6*	65 ± 8*
1800 mg/ml Ozone	0 ± 0	35 ± 5*	55 ± 7*	75 ± 9*
2200 mg/ml Ozone	0 ± 0	40 ± 5*	60 ± 7*	80 ± 10*

*p<0,05 VS control group.

Table 2 presents the histological scores for various parameters of wound healing on day 7, comparing the control group to three groups treated with ozonized *Nigella sativa* oil at different ozone concentrations. The parameters evaluated include re-epithelialization, granulation tissue formation, and collagen deposition, each scored on a scale of 0 to 3, with 3 representing the most advanced or mature state. Table 2 shows a clear trend of higher scores for all three histological parameters in the groups treated with ozonized *Nigella sativa* oil compared to the control group. This indicates that the treatment promoted better tissue regeneration. The scores generally increase with increasing ozone concentration, suggesting a dose-dependent effect of the treatment. The group treated with the highest ozone concentration (2200 mg/ml) consistently shows the highest scores, indicating the most advanced tissue regeneration. The treated groups show improved re-epithelialization (covering of the wound with new epithelial cells) compared to the control, with the 2200 mg/ml group exhibiting near-complete re-epithelialization. The formation of granulation tissue, which is crucial for wound healing, is also enhanced in the treated groups, particularly in the 2200 mg/ml group, suggesting better wound bed preparation for subsequent healing phases. Collagen is a key structural protein that provides strength and support to the healing tissue. The treated groups, especially the 2200 mg/ml group, show increased collagen deposition, indicating improved tissue remodeling and scar formation. Overall, table 2 supports the conclusion that ozonized *Nigella sativa* oil enhances various aspects of tissue regeneration in a dose-dependent manner, $p<0,05$, with the highest ozone concentration (2200 mg/ml) showing the most pronounced effects.

Table 2. Histological assessment of wound tissue on day 7.

Group	**Re-epithelialization (score 0-3)**	**Granulation tissue formation (score 0-3)**	**Collagen deposition (score 0-3)**
Control	1 ± 0.5	1 ± 0.3	1 ± 0.4
1400 mg/ml Ozone	2 ± 0.6*	2 ± 0.5*	2 ± 0.5*
1800 mg/ml Ozone	2.5 ± 0.4*	2.5 ± 0.3*	2.5 ± 0.4*
2200 mg/ml Ozone	3 ± 0.2*	3 ± 0.1*	3 ± 0.2*

*p value <0,05 VS Control; Scoring Criteria: 0: Absent or minimal, 1: Mild, 2: Moderate, 3: Extensive/Mature.

Table 3 presents the albumin levels in the wound tissue on day 7, comparing the control group to the three groups treated with ozonized *Nigella sativa* oil at different ozone concentrations. Table 3 clearly shows that the groups treated with ozonized *Nigella sativa* oil have significantly higher albumin levels compared to the control group. This suggests that the treatment stimulates albumin synthesis or accumulation in the wound tissue. The albumin levels increase with the increasing concentration of ozone in the treatment. The group treated with the highest ozone concentration (2200 mg/ml) exhibits the highest albumin levels, suggesting a dose-dependent relationship between ozone concentration and albumin synthesis or accumulation. Overall, table 3 supports the conclusion that ozonized *Nigella sativa* oil stimulates albumin synthesis or accumulation in the wound tissue in a dose-dependent manner.

Table 3. Albumin levels in wound tissue on day 7.

Group	Albumin levels (arbitrary units)
Control	10 ± 2
1400 mg/ml Ozone	15 ± 3*
1800 mg/ml Ozone	18 ± 4*
2200 mg/ml Ozone	22 ± 5*

*p value < 0,05 VS control

Table 4 illustrates the changes in gene expression levels in wound tissue after 7 days of treatment with ozonized *Nigella sativa* oil at different ozone concentrations. The data is presented as fold change relative to the control group, which received no treatment. The expression of VEGF and TGF-β, key growth factors involved in angiogenesis (new blood vessel formation) and cell proliferation, is significantly increased in the treated groups compared to the control. This upregulation is dose-dependent, with the highest ozone concentration (2200 mg/ml) showing the most pronounced effect. This suggests that ozonized *Nigella sativa* oil promotes tissue regeneration by stimulating the production of these growth factors. The expression of pro-inflammatory cytokines IL-1β and TNF-α shows an interesting pattern. This suggests that the treatment helps in resolving inflammation, which is crucial for the transition from the inflammatory phase to the proliferative phase of wound healing. Matrix metalloproteinases (MMPs) like MMP-2 and MMP-9 play a vital role in extracellular matrix remodeling, which is essential for cell migration and tissue reconstruction during wound healing. Table 4 shows a moderate upregulation of these MMPs in the treated groups, suggesting that the treatment facilitates proper tissue remodeling. Overall, Table 4 supports the idea that ozonized *Nigella sativa* oil modulates the expression of key genes involved in wound healing.

Table 4. Gene expression in wound tissue on day 7.

Gene	Control	1400 mg/ml ozone	1800 mg/ml ozone	2200 mg/ml ozone
VEGF	1.0 ± 0.2	1.5 ± 0.3*	1.8 ± 0.4*	2.0 ± 0.5*
TGF-β	1.0 ± 0.1	1.3 ± 0.2*	1.6 ± 0.3*	1.8 ± 0.4*
IL-1β	1.0 ± 0.3	1.2 ± 0.4*	1.0 ± 0.3	0.8 ± 0.2*
TNF-α	1.0 ± 0.2	1.3 ± 0.3*	1.1 ± 0.2	0.9 ± 0.2*
MMP-2	1.0 ± 0.2	1.4 ± 0.3*	1.6 ± 0.3*	1.7 ± 0.4*
MMP-9	1.0 ± 0.3	1.2 ± 0.4*	1.4 ± 0.3*	1.5 ± 0.4*

*p value <0,05 VS control.

4. Discussion

The role of albumin in wound healing is multifaceted and crucial, extending beyond its primary function of maintaining oncotic pressure in the circulatory system. The observed increase in albumin levels within the wound tissue of rats treated with ozonized *Nigella sativa* oil underscores its significance in the context of tissue regeneration and repair. The mechanisms by which albumin contributes to wound healing can be broadly categorized into its function as a nutrient source, its role in cellular processes, and its modulation of the inflammatory response. Albumin, being the most abundant protein in blood plasma, serves as a reservoir of amino acids, the fundamental building blocks of proteins. During wound healing, there is a heightened demand for protein synthesis to facilitate the formation of new cells and extracellular matrix

components. The increased albumin levels in the wound tissue provide a readily available source of amino acids, fueling the protein synthesis machinery and supporting the construction of new tissue. The availability of these amino acids is particularly crucial in the proliferative phase of wound healing, where rapid cell division and matrix deposition occur. Furthermore, albumin also binds and transports various nutrients, such as vitamins, minerals, and fatty acids, to the wound site. These nutrients are essential for cellular metabolism and energy production, further supporting the anabolic processes involved in tissue regeneration. The increased albumin levels in the treated groups may thus enhance the delivery of these vital nutrients to the wound, creating a favorable microenvironment for healing.[7,8]

Beyond its function as a nutrient source, albumin also actively participates in various cellular processes that are critical for wound healing. Albumin has been shown to promote cell migration and proliferation, two key events in the proliferative phase of wound healing. The mechanisms by which albumin exerts these effects are complex and involve interactions with various cell surface receptors and signaling pathways. For instance, albumin can bind to the transforming growth factor-beta (TGF-β) receptor, leading to the activation of downstream signaling cascades that promote cell proliferation and differentiation. Additionally, albumin can interact with integrins, a family of cell adhesion molecules that mediate cell-matrix interactions. These interactions can trigger intracellular signaling events that promote cell migration and adhesion, facilitating the movement of cells into the wound bed and their subsequent integration into the newly formed tissue. The increased albumin levels in the wound tissue of the treated rats may thus create a more conducive environment for cell migration and proliferation, accelerating the repopulation of the wound bed with new cells and promoting tissue regeneration.[9,10]

Inflammation is an integral part of the wound healing process, serving to eliminate pathogens and debris from the wound site. However, excessive or prolonged inflammation can be detrimental, hindering wound closure and leading to chronic wounds. Albumin plays a crucial role in modulating the inflammatory response, ensuring that it is both effective and timely. Albumin possesses antioxidant properties that can scavenge free radicals and reactive oxygen species (ROS), which are generated during the inflammatory response and can cause tissue damage. By neutralizing these harmful molecules, albumin helps to protect the wound tissue and limit the extent of inflammation. Furthermore, albumin can bind to various pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), thereby reducing their bioavailability and mitigating their inflammatory effects. This cytokine-binding capacity of albumin helps to prevent excessive inflammation and promote a balanced inflammatory response that is conducive to healing. The increased albumin levels in the treated groups may thus contribute to the controlled inflammatory response observed in these animals. By scavenging free radicals and binding pro-inflammatory cytokines, albumin may help to create a more favorable wound environment, facilitating the transition from the inflammatory phase to the proliferative phase of wound healing.[10,11]

The findings of this study highlight the multifaceted role of albumin in wound healing and its potential as a biomarker for monitoring tissue regeneration. The increased albumin levels in the wound tissue of rats treated with ozonized *Nigella sativa* oil suggest that the oil stimulates albumin synthesis, contributing to the enhanced wound healing observed in these groups. The mechanisms by which albumin promotes wound healing are complex and involve its function as a nutrient source, its role in cellular processes, and its modulation of the inflammatory response. The observed increase in albumin levels, coupled with the improved histological features and accelerated wound closure in the treated groups, provides compelling evidence for the therapeutic potential of ozonized *Nigella sativa* oil in

wound management. Further studies are warranted to explore the safety and efficacy of this approach in human subjects and to elucidate the precise molecular mechanisms underlying its effects. The identification of albumin as a biomarker of tissue regeneration may also facilitate the development of novel diagnostic and therapeutic strategies for wound management.[11,12]

The manuscript highlights the critical role of growth factors, particularly vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β), in the intricate process of wound healing. The upregulation of these growth factors in the groups treated with ozonized *Nigella sativa* oil underscores the potential of this intervention to stimulate and enhance the natural healing cascade, leading to accelerated wound closure and superior tissue regeneration. VEGF, a potent signaling protein, is a key orchestrator of angiogenesis, the process of new blood vessel formation. In the context of wound healing, angiogenesis is vital for supplying oxygen and nutrients to the injured tissue, facilitating the removal of waste products, and supporting the migration and proliferation of cells involved in repair. The upregulation of VEGF expression in the treated groups suggests that ozonized *Nigella sativa* oil actively promotes angiogenesis in the wound bed. This enhanced vascularization creates a conducive microenvironment for tissue regeneration, enabling the delivery of essential factors and cells required for repair. The molecular mechanisms by which ozonized *Nigella sativa* oil stimulates VEGF expression are likely multifaceted. Thymoquinone, a major bioactive component of the oil, has been shown to activate the hypoxia-inducible factor-1 alpha (HIF-1α) pathway, a key regulator of VEGF expression. Under hypoxic conditions, HIF-1α induces the transcription of VEGF, leading to increased angiogenesis. The oxidative stress generated by ozone may also contribute to VEGF upregulation by mimicking hypoxic conditions and activating the HIF-1α pathway. Furthermore, ozone may directly stimulate VEGF expression through the modulation of other signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway. The enhanced angiogenesis resulting from VEGF upregulation has several beneficial effects on wound healing. The increased blood flow to the wound bed improves oxygen and nutrient delivery, facilitating cellular metabolism and energy production. This, in turn, supports the proliferation and migration of fibroblasts, keratinocytes, and endothelial cells, which are essential for wound closure and tissue regeneration. Moreover, the newly formed blood vessels provide a conduit for the infiltration of immune cells, such as macrophages and neutrophils, which play crucial roles in clearing debris and fighting infection.[12,13]

TGF-β, a multifunctional cytokine, plays a pivotal role in various stages of wound healing, including inflammation, proliferation, and remodeling. In the early stages of wound healing, TGF-β promotes the recruitment and activation of inflammatory cells, such as neutrophils and macrophages, which are essential for clearing debris and preventing infection. Subsequently, TGF-β stimulates the proliferation and migration of fibroblasts, the key cells responsible for synthesizing collagen and other extracellular matrix components. In the later stages of wound healing, TGF-β orchestrates the remodeling of the extracellular matrix, ensuring the proper organization and maturation of the scar tissue. The upregulation of TGF-β expression in the treated groups suggests that ozonized *Nigella sativa* oil modulates the inflammatory response and promotes fibroblast activity, leading to enhanced tissue regeneration. Thymoquinone, the main bioactive component of the oil, has been shown to regulate TGF-β signaling by inhibiting the phosphorylation of Smad2/3, key downstream mediators of TGF-β signaling. This inhibition may lead to a controlled inflammatory response and prevent excessive scar formation. The increased fibroblast activity resulting from TGF-β upregulation has several beneficial effects on wound healing. Fibroblasts synthesize collagen, elastin, and other extracellular matrix components that provide structural support and tensile strength to the healing tissue. The

enhanced collagen deposition observed in the treated groups, particularly in the group receiving the highest ozone concentration, suggests that ozonized *Nigella sativa* oil promotes the formation of a strong and resilient scar.[13,14]

The upregulation of VEGF and TGF-β in the treated groups highlights the potential of ozonized *Nigella sativa* oil to promote wound healing through multiple molecular mechanisms. The synergistic effects of thymoquinone and ozone-derived ROS may lead to a more comprehensive and effective modulation of the wound healing process compared to either agent alone. The enhanced angiogenesis, cell proliferation, and extracellular matrix deposition observed in the treated groups suggest that ozonized *Nigella sativa* oil may have therapeutic potential for accelerating wound closure and improving tissue regeneration in clinical settings. The findings of this study have several clinical implications. Firstly, ozonized *Nigella sativa* oil may be a valuable adjunct to conventional wound care therapies, particularly in the management of chronic wounds that are resistant to healing. Secondly, the identification of VEGF and TGF-β as key mediators of the oil's effects provides insights into the molecular mechanisms of wound healing and may facilitate the development of novel therapeutic targets. Finally, the use of albumin as a biomarker of tissue regeneration may enable clinicians to monitor the progress of wound healing and tailor treatment strategies accordingly.[14,15]

The inflammatory phase, while crucial for initiating the wound healing cascade, needs to be tightly regulated. An overactive or prolonged inflammatory response can lead to excessive tissue damage, impaired cell function, and delayed wound healing. The delicate balance between pro-inflammatory and anti-inflammatory signals is essential for creating an optimal wound-healing environment. The study's findings suggest that ozonized *Nigella sativa* oil plays a role in modulating this inflammatory response, contributing to its wound-healing properties. Inflammation is the body's initial response to injury, serving to eliminate pathogens, clear debris, and recruit cells necessary for tissue repair. The process is characterized by the release of various pro-inflammatory mediators, such as cytokines and chemokines, which attract immune cells like neutrophils and macrophages to the wound site. These cells phagocytose bacteria and damaged tissue, while also releasing growth factors and other signaling molecules that stimulate angiogenesis, cell proliferation, and extracellular matrix deposition. However, an excessive or prolonged inflammatory response can be detrimental to wound healing. Chronic inflammation can lead to the persistent release of reactive oxygen species (ROS) and proteolytic enzymes, which can damage healthy tissue and impair cell function. Additionally, chronic inflammation can create a pro-fibrotic environment, leading to excessive scar formation and impaired tissue function. Therefore, the ability to modulate the inflammatory response is crucial for achieving optimal wound healing outcomes.[15,16]

Pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), play pivotal roles in the inflammatory phase of wound healing. IL-1β stimulates the production of other pro-inflammatory mediators, promotes angiogenesis, and activates fibroblasts, which are responsible for collagen synthesis. TNF-α, on the other hand, induces apoptosis (programmed cell death) of damaged cells, stimulates angiogenesis, and recruits immune cells to the wound site. While these cytokines are essential for initiating the wound-healing process, their overexpression can lead to chronic inflammation and delayed healing. Therefore, the ability to regulate their expression is crucial for achieving a balanced inflammatory response. The study's findings suggest that ozonized *Nigella sativa* oil modulates the expression of pro-inflammatory cytokines in a manner that promotes optimal wound healing. The initial increase in IL-1β and TNF-α expression observed in the early stages of wound healing is likely a necessary response to injury, serving to initiate the inflammatory cascade and recruit immune cells to the wound site. However, the subsequent decrease in their expression

suggests that the treatment helps to resolve inflammation and prevent its chronicity. This controlled inflammatory response may be attributed to the combined action of thymoquinone, a major bioactive compound in *Nigella sativa* oil, and ozone-derived ROS. Thymoquinone has been shown to possess anti-inflammatory properties by inhibiting the activation of NF-κB, a key transcription factor that regulates the expression of pro-inflammatory genes. Ozone, through the generation of ROS, can also modulate various signaling pathways involved in inflammation. The synergistic effects of these two agents may contribute to the observed regulation of cytokine expression. The controlled inflammatory response observed in the groups treated with ozonized *Nigella sativa* oil likely contributes to the optimal wound healing environment. By preventing excessive or prolonged inflammation, the treatment minimizes tissue damage and promotes the timely transition from the inflammatory phase to the proliferative phase of wound healing. This allows for efficient cell proliferation, migration, and extracellular matrix deposition, leading to accelerated wound closure and improved tissue regeneration. Furthermore, the controlled inflammatory response may also help to reduce scar formation. Chronic inflammation can lead to the excessive production of collagen and other extracellular matrix components, resulting in hypertrophic scars or keloids. By modulating the inflammatory response, ozonized *Nigella sativa* oil may help to prevent excessive scar formation and promote aesthetically pleasing wound healing.[16,17]

The extracellular matrix (ECM) is a complex network of proteins, glycoproteins, and proteoglycans that provides structural support and biochemical cues to cells. In wound healing, the ECM undergoes dynamic remodeling, involving both degradation and deposition of its components. This remodeling process is essential for various stages of wound repair, including cell migration, angiogenesis, granulation tissue formation, and scar maturation. MMPs, a family of zinc-dependent endopeptidases, play a pivotal role in ECM degradation. These enzymes cleave various ECM components, such as collagen, elastin, fibronectin, and laminin, creating space for cell migration and facilitating tissue remodeling. The activity of MMPs is tightly regulated during wound healing, as both excessive and insufficient MMP activity can be detrimental. In the early stages of wound healing, MMPs are upregulated to facilitate the removal of damaged tissue and debris, creating a clean wound bed for subsequent repair processes. The inflammatory cells recruited to the wound site, such as neutrophils and macrophages, release MMPs that degrade the provisional matrix formed during the initial stages of healing. This degradation allows for the migration of fibroblasts and endothelial cells into the wound bed, where they initiate the formation of new tissue. During the proliferative phase of wound healing, MMPs continue to play a crucial role in ECM remodeling. Fibroblasts, the key cells responsible for collagen synthesis, also secrete MMPs that degrade the existing collagen matrix, allowing for its replacement with new, organized collagen fibers. This process is essential for the formation of a strong and functional scar tissue. In the final stages of wound healing, MMP activity decreases as the wound matures and the scar tissue contracts. The balance between MMPs and their inhibitors, known as tissue inhibitors of metalloproteinases (TIMPs), is critical for proper scar formation. Excessive MMP activity can lead to excessive ECM degradation and impaired wound healing, while insufficient MMP activity can result in excessive scar tissue formation and fibrosis.[17,18]

The finding that ozonized *Nigella sativa* oil regulates the expression of MMPs in the treated groups suggests that the oil promotes balanced ECM remodeling, which is essential for proper wound repair. The precise mechanisms by which the oil exerts its effects on MMP expression remain to be fully elucidated, but several potential pathways can be hypothesized. Thymoquinone, a major bioactive compound in *Nigella sativa* oil, has been shown to possess anti-inflammatory properties. Inflammation plays a crucial role in wound healing, but excessive or

prolonged inflammation can be detrimental. By modulating the inflammatory response, thymoquinone may indirectly influence MMP expression. Inflammatory cells, such as macrophages and neutrophils, are major sources of MMPs. Therefore, by reducing inflammation, thymoquinone may decrease the infiltration of these cells into the wound site, leading to a decrease in MMP expression. Ozone, through the generation of ROS, can also modulate various signaling pathways involved in wound healing. ROS can act as signaling molecules that activate transcription factors, such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), which regulate the expression of MMPs. The controlled levels of ROS generated by ozonized *Nigella sativa* oil may stimulate these signaling pathways, leading to a balanced upregulation of MMPs that facilitates ECM remodeling without causing excessive tissue degradation. Furthermore, ozonized *Nigella sativa* oil may also influence the expression of TIMPs, the endogenous inhibitors of MMPs. The balance between MMPs and TIMPs is critical for proper ECM remodeling. By modulating the expression of both MMPs and TIMPs, ozonized *Nigella sativa* oil may promote a harmonious interplay between ECM degradation and deposition, leading to optimal wound healing.[18,19]

Balanced ECM remodeling is essential for proper wound repair. In the early stages of wound healing, ECM degradation is necessary to remove damaged tissue and debris, create space for cell migration, and facilitate angiogenesis. However, excessive ECM degradation can lead to impaired wound healing, as it can compromise the structural integrity of the tissue and delay the formation of new tissue. In the later stages of wound healing, ECM deposition is crucial for the formation of new tissue and scar maturation. Collagen, the main structural protein of the ECM, provides strength and support to the healing tissue. However, excessive collagen deposition can lead to fibrosis and scar contracture, which can impair tissue function and aesthetics. The regulated expression of MMPs in the treated groups suggests that ozonized *Nigella sativa* oil promotes a balanced ECM remodeling process, ensuring adequate ECM degradation in the early stages of wound healing and controlled ECM deposition in the later stages. This balanced remodeling contributes to the enhanced wound healing observed in the treated groups, characterized by accelerated wound closure, improved tissue regeneration, and reduced scar formation.[19,20]

5. Conclusion

Ozonized *Nigella sativa* oil promotes wound healing in a rat model through multiple molecular mechanisms, including the stimulation of albumin synthesis, growth factor expression, and controlled inflammation. Albumin serves as a valuable biomarker for monitoring tissue regeneration.

6. References

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