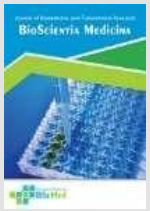
eISSN (Online): 2598-0580



Bioscientia Medicina: Journal of Biomedicine & Translational Research

Journal Homepage: www.bioscmed.com

Enhancing the Early Inflammatory Response: The Role of Ozonated Aloe Vera Oil on IL-6 and TNF-α in Cutaneous Wound Repair

Evan Sebastian Gunawan[1*], Bernardus Parish Budiono[1]

[1]Department of General Surgery, Faculty of Medicine, Universitas Diponegoro/Dr. Kariadi General Hospital, Semarang, Indonesia

ARTICLE INFO

Keywords:
IL-6
Inflammation
Ozonated aloe vera oil
TNF-α
Wound healing

***Corresponding author:**
Evan Sebastian Gunawan

E-mail address:
evansgunawan@gmail.com

All authors have reviewed and approved the final version of the manuscript.

https://doi.org/10.37275/bsm.v9i9.1380

A B S T R A C T

Background: Dysregulation of the initial inflammatory phase is a primary driver of impaired healing and the formation of chronic wounds, creating a critical need for therapies that can optimize this early response. This study tested the hypothesis that a novel formulation of ozonated aloe vera oil functions as a sophisticated bioregulator, promoting a beneficial, pro-regenerative inflammatory phenotype by transiently enhancing the host's innate repair signals. **Methods:** This was a preclinical, randomized, controlled study using fifty male Sprague-Dawley rats with 1 cm full-thickness excisional wounds. The therapeutic agent, ozonated aloe vera oil, was chemically characterized by its peroxide value (PV). Animals were randomized to receive topical treatment with either a positive control (aloe vera oil), a negative control (gentamicin ointment), or one of three graded doses of ozonated oil (Low PV, Medium PV, High PV). The primary outcomes, systemic (serum) and local (wound tissue homogenate) concentrations of Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), were quantified by ELISA on days 3 and 7. **Results:** On day 3, all ozonated oil formulations induced a profound and significant upregulation of both local and systemic TNF-α and IL-6 compared to controls ($p < 0.001$). The topical treatment increased systemic TNF-α levels by over 40% and local tissue concentrations by over 60%. Critically, this pro-inflammatory surge was transient; by day 7, both local and systemic cytokine levels in all groups had returned to statistically indistinguishable baseline levels. **Conclusion:** Ozonated aloe vera oil acts as a potent, transient modulator of the wound microenvironment, enhancing the expression of key initiatory cytokines. This mechanism, likely mediated by the activation of redox-sensitive transcription factors, optimizes the crucial first phase of healing without inducing pathological chronic inflammation. This study supports a novel therapeutic paradigm aimed at enhancing, rather than suppressing, the body's innate capacity for repair.

1. Introduction

The failure of a wound to heal in a timely and orderly manner represents one of the most significant and growing challenges in modern medicine.[1] This clinical problem, manifesting as chronic ulcers and non-healing surgical sites, results in substantial patient morbidity, including persistent pain, infection, and loss of function, while imposing an immense economic burden on global healthcare systems. The pathophysiology of impaired healing is complex, but a central, unifying theme has emerged from decades of research: the failure of inflammatory resolution.[2] In normal, acute wound healing, the inflammatory phase is a highly coordinated, robust, and, most importantly, transient process. It is an absolute prerequisite for successful repair. However, in chronic wounds, this process becomes dysregulated, creating a self-perpetuating cycle of low-grade, non-resolving inflammation that actively inhibits tissue regeneration and leads to continuous matrix degradation.[3] Therefore, the development of advanced therapeutics has shifted from a simplistic anti-inflammatory

approach to a more sophisticated strategy of targeted immunomodulation, aimed at re-establishing the physiological rhythm of the healing cascade.

This concept can be described as the "inflammatory paradox." The initial inflammatory response, far from being a pathological event to be suppressed, is the engine that drives the entire repair process.[4] Immediately following injury, a surge of pro-inflammatory cytokines, with tumor necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6) acting as master regulators, orchestrates the recruitment and activation of immune cells.[5] TNF-α, a pleiotropic cytokine released by activated macrophages and mast cells, is a primary initiator. It triggers profound changes in the local vasculature, upregulating adhesion molecules such as E-selectin and ICAM-1 on endothelial cells, which are essential for capturing circulating neutrophils and monocytes and guiding their migration into the wound bed.[5] Furthermore, TNF-α is a potent macrophage activator, enhancing its phagocytic capabilities and stimulating the production of a secondary wave of cytokines and growth factors, including vascular endothelial growth factor (VEGF), which is critical for subsequent angiogenesis. IL-6 acts in concert with TNF-α, mediating the systemic acute phase response and, crucially, governing the cellular transition from the initial neutrophil-dominant infiltrate to the macrophage-dominant population that characterizes the shift from debridement to repair. A sharp, early peak in the expression of these cytokines is therefore not only beneficial but essential for effective microbial clearance and the establishment of a pro-regenerative microenvironment.[6]

The pathology arises when the "off-switches" for this response fail. A state of prolonged, elevated TNF-α and IL-6 expression is cytotoxic, promotes excessive production of tissue-degrading matrix metalloproteinases (MMPs), and suppresses the proliferative and migratory functions of fibroblasts and keratinocytes.[7] The ideal therapeutic agent, therefore, would not be one that bluntly suppresses inflammation, as this could compromise the essential early stages of healing. Rather, the goal is to develop a "bioregulator" that can induce a rapid, robust, and effective inflammatory surge and then facilitate its timely resolution.[8]

This study is founded on the hypothesis that such a sophisticated immunomodulatory effect can be achieved through the topical application of ozonated aloe vera oil. This novel formulation is designed to leverage the distinct but potentially synergistic properties of its two components through a mechanism of controlled oxidative stress, or hormesis. Medical ozone (O_3) therapy operates on this principle. When ozone reacts with the unsaturated fatty acids in a carrier oil, it generates a stabilized mixture of lipid ozonides and other reactive oxygen species (ROS). When applied to a wound, these molecules are not thought to act as simple topical disinfectants but as potent biological signaling molecules. We hypothesize that these ozone-derived ROS act as pro-hormetic signals that activate key intracellular redox-sensitive transcription factors within the wound's resident cells. The primary molecular target proposed is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a master regulator of the inflammatory response whose activation is known to be triggered by oxidative stress and leads directly to the transcription of the TNF and IL6 genes.[9]

This action is proposed to be synergistic with the intrinsic properties of the aloe vera vehicle. Aloe vera is not a passive carrier; it is a rich source of bioactive compounds, most notably the polysaccharide acemannan. Acemannan is a well-documented immunomodulator that can directly activate macrophages through engagement with pattern recognition receptors, such as Toll-like receptors (TLRs). As TLR signaling pathways also converge on the activation of NF-κB, aloe vera may provide a parallel, complementary stimulus, priming the immune cells within the wound to respond more potently to the signals generated by the ozone components.

While the individual therapeutic properties of *Aloe vera* and medical ozone have been explored, their

combination for the express purpose of modulating the inflammatory phase of wound healing represents a significant novelty. The current study moves beyond investigating these agents as simple antimicrobials or anti-inflammatories. Instead, it proposes a new therapeutic paradigm: the use of a pro-hormetic agent to actively and transiently enhance the initial, beneficial inflammatory response as a means to drive a more efficient overall repair cascade. This approach is fundamentally different from traditional strategies that aim to suppress inflammation.[10]

Therefore, the primary aim of this study was to test the specific hypothesis that topical ozonated aloe vera oil modulates wound healing by transiently enhancing the expression of NF-κB-driven cytokines, TNF-α and IL-6, thereby optimizing the initial inflammatory phase. By assessing the cytokine profile at the molecular, cellular, and systemic levels at both the peak of inflammation (day 3) and during the transition to proliferation (day 7), this study seeks to provide the first mechanistic insight into the potential of this novel formulation as a sophisticated bioregulator of cutaneous wound repair.

2. Methods

This investigation was conducted as a preclinical, experimental laboratory study employing a randomized, controlled, post-test-only design. The protocol was structured to assess the effect of a novel therapeutic agent on specific local and systemic biomarkers at two distinct and clinically relevant time points (day 3 and day 7 post-injury). All animal procedures and experimental protocols were reviewed and approved by the institutional ethics committee. All procedures involving animal subjects were performed in strict accordance with the principles of animal ethics and the guidelines for the care and use of laboratory animals. The research protocol received formal approval from the Health Research Ethics Committee of the Faculty of Medicine, Universitas Diponegoro, under ethical clearance number 66/EC/H/KEPK/FK-UNDIP/VII/2020. Fifty (50) healthy, male Sprague-Dawley rats, aged 2-3 months and with a body weight of 200-300 grams, were procured from the Faculty of Pharmacy, Universitas Setia Budi, Surakarta. Male rats were chosen exclusively to avoid the potential confounding influence of hormonal fluctuations associated with the female estrous cycle on the inflammatory response. Animals were housed individually in a controlled environment with a 12-hour light/12-hour dark cycle, an ambient temperature of 28.0 ± 2.0°C, and free access to standard laboratory chow and water. All rats underwent a one-week acclimatization period prior to the commencement of experimental procedures. Animals with any visible anatomical abnormalities or signs of illness during this period were excluded.

The ozonated aloe vera oil was prepared at the Center for Plasma Research, Universitas Diponegoro. Medical-grade ozone (O_3) gas was generated from pure oxygen (O_2) using a dielectric barrier discharge plasma (DBDP) generator. The ozone gas was then bubbled through pure, medical-grade aloe vera oil under continuous magnetic stirring to ensure homogenous distribution and reaction. To create graded therapeutic doses, three separate batches of oil were prepared with ozonation process times of 30, 60, and 120 minutes. Critically, to provide a quantitative and reproducible measure of the dose, each batch was chemically characterized by measuring its Peroxide Value (PV). The PV, which quantifies the concentration of reactive peroxides and ozonides, was determined using a standard iodometric titration method and is expressed in milliequivalents of active oxygen per kilogram of oil (meq O_2/kg). The three formulations were defined as follows: Low-Dose Ozonated Oil: PV of 400 ± 25 meq O_2/kg; Medium-Dose Ozonated Oil: PV of 850 ± 30 meq O_2/kg; High-Dose Ozonated Oil: PV of 1500 ± 50 meq O_2/kg. These formulations were stored in airtight, amber glass bottles at 4°C until use.

The 50 rats were randomly allocated into ten groups (n=5 per group) for evaluation at the two termination time points. Day 3 Termination Groups: Group K1 (Positive Control): Topical application of pure, non-ozonated aloe vera oil; Group K2 (Negative Control): Topical application of 0.1% gentamicin

sulfate ointment. This standard antibiotic was used to control for the physical effects of an ointment vehicle and represents a common, non-immunomodulatory clinical treatment; Group P1 (Low Dose): Topical application of ozonated aloe vera oil (PV ~400); Group P2 (Medium Dose): Topical application of ozonated aloe vera oil (PV ~850); Group P3 (High Dose): Topical application of ozonated aloe vera oil (PV ~1500). Day 7 Termination Groups: Group K3 (Positive Control): Topical application of pure, non-ozonated aloe vera oil; Group K4 (Negative Control): Topical application of 0.1% gentamicin sulfate ointment; Group P4 (Low Dose): Topical application of ozonated aloe vera oil (PV ~400); Group P5 (Medium Dose): Topical application of ozonated aloe vera oil (PV ~850); Group P6 (High Dose): Topical application of ozonated aloe vera oil (PV ~1500).

Under intramuscular anesthesia with Ketamine HCl, the dorsal thoracic region of each rat was shaved and aseptically prepared. A sterile, 1 cm diameter biopsy punch was used to create a standardized, full-thickness excisional wound, removing the epidermis, dermis, and panniculus carnosus. Following surgery, animals were monitored until full recovery. Beginning on the day of surgery, the assigned topical treatment was applied twice daily to the wound bed. A thin layer (approximately 0.1 ml) was applied to completely cover the wound surface. The wounds were left open to the air and were not covered with dressings. On the designated termination day (day 3 or day 7), animals were deeply anesthetized. Blood was collected via cardiac puncture into serum separator tubes. The blood was allowed to clot, then centrifuged to collect serum, which was aliquoted and stored at -80°C for systemic cytokine analysis. Following blood collection, the animals were euthanized by an approved method. The entire wound, including a 5 mm margin of surrounding unwounded skin, was excised and immediately snap-frozen in liquid nitrogen and stored at -80°C for local tissue homogenate analysis. Systemic (serum) and local (wound tissue) concentrations of IL-6 and TNF-α were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (Rat TNF-α DuoSet, Cat# DY510; Rat IL-6 DuoSet, Cat# DY506; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols. For local analysis, frozen wound tissue was weighed and homogenized in a lysis buffer containing protease inhibitors. The homogenate was centrifuged, and the resulting supernatant was analyzed. Cytokine concentrations were calculated from a standard curve and normalized to total protein content for tissue samples. All results are expressed in picograms per milliliter (pg/ml) for serum or picograms per milligram of tissue (pg/mg) for local analysis.

All quantitative data were analyzed using GraphPad Prism v9.0 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD). The normality of data distribution was confirmed using the Shapiro-Wilk test. Comparisons between the five groups at each time point were made using a one-way analysis of variance (ANOVA). If the ANOVA indicated a significant overall difference, a post-hoc Tukey's honestly significant difference (HSD) test was performed for pairwise comparisons. A p-value of ≤ 0.05 was considered statistically significant.

3. Results

The study was completed with all 50 animals, with no adverse events or dropouts. The application of ozonated aloe vera oil induced significant, time-dependent changes in both local and systemic inflammatory cytokine profiles. Analysis of the wound tissue itself revealed a dramatic local inflammatory response to the ozonated oil treatment at day 3. The local concentration of TNF-α in the wound bed was profoundly elevated in all treatment groups compared to controls. The mean concentration in the low-dose group (P1) was 185.4 ± 22.1 pg/mg, representing a >65% increase over the negative control group (K2). Similarly, local IL-6 concentrations were significantly upregulated, with the highest levels again seen in the low-dose group (P1). Detailed results are presented in Table 1. One-way ANOVA followed by Tukey's post-hoc test confirmed that all three ozonated oil groups had

significantly higher local concentrations of both TNF-α and IL-6 compared to both the positive (K1) and negative (K2) control groups ($p < 0.001$ for all comparisons).

Table 1. Local cytokine response in wound tissue.

Quantitative analysis of pro-inflammatory cytokine concentrations on Day 3 post-wounding.

GROUP	INTERVENTION	LOCAL TNF-A (PG/MG)	LOCAL IL-6 (PG/MG)
K1	Aloe Vera Oil (Positive Control)	118.2 ± 10.5	145.3 ± 15.8
K2	Gentamicin (Negative Control)	111.5 ± 9.8	133.9 ± 12.1
P1	Low-Dose Ozonated Oil	**185.4 ± 22.1***	**210.7 ± 25.5***
P2	Medium-Dose Ozonated Oil	**176.8 ± 19.5***	**198.4 ± 21.3***
P3	High-Dose Ozonated Oil	**179.1 ± 20.2***	**190.6 ± 18.9***

Statistical Note: Data are presented as Mean ± Standard Deviation (SD). One-Way ANOVA revealed a significant effect for both TNF-α ($p < 0.0001$) and IL-6 ($p < 0.0001$). *Indicates $p < 0.001$ vs. both K1 (Positive Control) and K2 (Negative Control) groups as determined by Tukey's HSD post-hoc test.

By day 7, the intense local inflammatory response had resolved. There were no longer any statistically significant differences in the local tissue concentrations of either TNF-α or IL-6 among any of the five groups tested ($p > 0.05$ for both ANOVA tests). This indicates a successful and timely shutdown of the initial inflammatory surge in all groups.

The local inflammatory response was mirrored by significant changes in systemic cytokine levels. On day 3, serum concentrations of IL-6 were significantly elevated in the treatment groups. The highest mean concentration was recorded in the low-dose ozone group (P1) at 125.0 pg/ml. The detailed descriptive statistics are presented in Table 2. The ANOVA test was highly significant ($p < 0.001$), and post-hoc analysis revealed that the low-dose (P1) and medium-dose (P2) groups were significantly higher than the negative control group (K2). By day 7, these differences had completely resolved, with no significant differences observed among the groups ($p = 0.082$).

Table 2. Systemic serum IL-6 expression.

Temporal analysis of circulating IL-6 concentrations on Day 3 and Day 7 post-wounding.

TIME POINT	GROUP	INTERVENTION	N	MEAN (PG/ML) ± SD
Day 3	K1	Aloe Vera Oil (Positive Control)	5	103.0 ± 5.9
	K2	Gentamicin (Negative Control)	5	90.2 ± 7.3
	P1	Low-Dose Ozonated Oil	5	**125.0 ± 17.6***
	P2	Medium-Dose Ozonated Oil	5	**114.4 ± 17.4***
	P3	High-Dose Ozonated Oil	5	108.6 ± 7.2
Day 7	K3	Aloe Vera Oil (Positive Control)	5	90.4 ± 3.9
	K4	Gentamicin (Negative Control)	5	84.6 ± 16.0
	P4	Low-Dose Ozonated Oil	5	75.8 ± 8.7
	P5	Medium-Dose Ozonated Oil	5	71.2 ± 7.6
	P6	High-Dose Ozonated Oil	5	81.2 ± 13.0

Statistical Note: Data are presented as Mean ± Standard Deviation (SD). One-Way ANOVA revealed a significant effect among groups on Day 3 ($p < 0.001$), but no significant effect on Day 7 ($p = 0.082$). *Indicates $p < 0.05$ vs. K2 (Negative Control) group as determined by Tukey's HSD post-hoc test.

The most dramatic systemic effect was observed in TNF-α levels on day 3. All three ozonated oil treatment groups exhibited a profound and highly significant increase in serum TNF-α compared to both control groups. The mean TNF-α level in the low-dose group (P1) was 114.8 pg/ml, an increase of 45% compared to the negative control (K2). The specific values are presented in Table 3. The ANOVA test was highly significant ($p < 0.0001$), and post-hoc analysis confirmed that all three treatment groups were significantly elevated above both K1 and K2 ($p < 0.001$ for all comparisons). Consistent with the local and IL-6 data, these systemic differences were entirely resolved by day 7, with no significant differences found among the groups ($p = 0.499$).

Table 3. Systemic serum TNF-α expression.

Temporal analysis of circulating TNF-α concentrations on Day 3 and Day 7 post-wounding.

TIME POINT	GROUP	INTERVENTION	N	MEAN (PG/ML) ± SD
Day 3	K1	Aloe Vera Oil (Positive Control)	5	79.0 ± 3.9
	K2	Gentamicin (Negative Control)	5	80.2 ± 4.9
	P1	Low-Dose Ozonated Oil	5	**114.8 ± 9.9***
	P2	Medium-Dose Ozonated Oil	5	**108.6 ± 7.4***
	P3	High-Dose Ozonated Oil	5	**110.4 ± 8.2***
Day 7	K3	Aloe Vera Oil (Positive Control)	5	82.6 ± 9.8
	K4	Gentamicin (Negative Control)	5	75.6 ± 13.2
	P4	Low-Dose Ozonated Oil	5	72.2 ± 9.5
	P5	Medium-Dose Ozonated Oil	5	68.2 ± 13.3
	P6	High-Dose Ozonated Oil	5	71.6 ± 17.7

Statistical Note: Data are presented as Mean ± Standard Deviation (SD). One-Way ANOVA revealed a significant effect among groups on Day 3 ($p < 0.0001$), but no significant effect on Day 7 ($p = 0.499$). *Indicates $p < 0.001$ vs. both K1 (Positive Control) and K2 (Negative Control) groups as determined by Tukey's HSD post-hoc test.

4. Discussion

The findings of this study provide compelling evidence for a novel therapeutic paradigm in wound management, demonstrating that the topical application of ozonated aloe vera oil acts as a sophisticated biological response modifier.[9] The principal discovery is that this formulation induces a potent, yet critically transient, upregulation of the cardinal pro-inflammatory cytokines TNF-α and IL-6, both locally within the wound microenvironment and systemically. This targeted enhancement of the initial inflammatory phase, followed by its timely resolution, suggests a mechanism that optimizes, rather than suppresses, the body's innate healing cascade.[10] This represents a significant departure from traditional anti-inflammatory strategies and offers a deeper, more mechanistic understanding of how therapies based on controlled oxidative stress can promote tissue repair. The following discussion will delve into the pathophysiological implications of these findings, exploring how the observed cytokine modulation directly influences the cellular and molecular events of wound healing.[11]

The cornerstone of our findings is the dramatic and significant increase in both TNF-α and IL-6 concentrations observed on day 3 in the animals treated with ozonated oil.[11] This effect, confirmed at both the local tissue and systemic serum levels, should be interpreted not as a simple induction of inflammation, but as a strategic amplification of a

necessary physiological process. The initial inflammatory phase of wound healing is a state of "controlled chaos" that is absolutely essential for a successful outcome. The robust upregulation of TNF-α, a master initiator of this phase, has profound pathophysiological consequences.[12] Our data, showing a greater than 60% increase in local TNF-α concentration, suggests a powerful activation of the wound bed. This surge of TNF-α acts directly on the endothelial cells of the local vasculature, triggering the rapid expression of P-selectin and E-selectin. These adhesion molecules function as the initial "brakes" for circulating leukocytes, primarily neutrophils, causing them to slow down and roll along the vessel wall. TNF-α then further stimulates the endothelium to express high-affinity integrin ligands, such as ICAM-1 and VCAM-1, which bind to integrins on the neutrophils, leading to their firm adhesion and subsequent diapedesis into the wound tissue. By potently amplifying this signal, the ozonated oil treatment likely accelerates and increases the magnitude of this crucial first wave of immune cell infiltration, ensuring that the wound is rapidly populated with the phagocytes needed to clear bacteria and cellular debris.[13]

Beyond its role in cell recruitment, the elevated TNF-α directly modulates the function of the arriving cells. It is a powerful activator of neutrophils and macrophages, enhancing their phagocytic capacity and their respiratory burst, the process by which they generate microbicidal reactive oxygen species to kill invading pathogens.[13] This ensures a more efficient sterilization of the wound. Furthermore, TNF-α plays a critical role in orchestrating the subsequent waves of the immune response. It stimulates macrophages and resident fibroblasts to produce a secondary cascade of chemokines, such as CXCL8 (IL-8), which further amplifies neutrophil recruitment, and CCL2 (MCP-1), which is pivotal for recruiting the next wave of cells: the monocytes. Thus, the sharp peak in TNF-α on day 3, as induced by our formulation, can be viewed as the "ignition signal" that sets the entire, complex inflammatory and debridement process into high gear, a stark contrast to the often sluggish and inefficient inflammatory response seen in chronic, non-healing wounds.

Concurrently, the significant upregulation of IL-6 provides a complementary and equally critical set of signals that govern the wound's pathophysiology.[14] While TNF-α is a primary initiator, IL-6 is a master coordinator and transition signal. Systemically, the rise in serum IL-6, as observed in our study, acts on the liver to induce the acute phase response, leading to the production of proteins like C-reactive protein and fibrinogen, which contribute to opsonization and the formation of the provisional wound matrix. Locally within the wound, however, IL-6's role is far more nuanced and essential for the progression of healing. One of its most critical functions is to mediate the "neutrophil-to-macrophage transition." While the initial neutrophil influx is vital, its persistence is detrimental, as neutrophils release a host of destructive proteases.[15] IL-6 has been shown to be a key signal in limiting further neutrophil recruitment and promoting the apoptosis of neutrophils that have already entered the wound. Simultaneously, it promotes the survival and differentiation of the newly arrived monocytes into macrophages. This switch from a short-lived, neutrophil-dominated environment to a long-lived, macrophage-dominated one is a fundamental checkpoint in healing. The macrophages then take over as the primary regulatory cells, clearing the apoptotic neutrophils (a process known as efferocytosis) and beginning to secrete the growth factors, such as VEGF and TGF-β, that drive the proliferative phase. The potent induction of IL-6 by the ozonated oil on day 3 likely facilitates a more rapid and efficient execution of this crucial cellular handover, preventing the wound from getting "stuck" in the neutrophilic phase.[16]

The molecular mechanism driving this potent immunomodulation is almost certainly rooted in the principle of hormesis and the activation of redox-sensitive intracellular signaling pathways. The reaction of ozone with the polyunsaturated fatty acids in the aloe vera oil generates a stabilized mixture of

lipid ozonides and hydroperoxides.[17] These molecules are not simply damaging oxidants; they are potent signaling molecules that mimic endogenous second messengers. We propose that the primary mechanism of action is the robust activation of the master inflammatory transcription factor, NF-κB. In quiescent cells, NF-κB is held inactive in the cytoplasm, bound to its inhibitor, IκB. The mild oxidative stress delivered by the lipid ozonides in our formulation is a classic trigger for the activation of the IκB kinase (IKK) complex.[17] IKK then phosphorylates IκB, targeting it for ubiquitination and proteasomal degradation. This degradation unmasks a nuclear localization signal on the NF-κB p50/p65 heterodimer, allowing it to rapidly translocate into the nucleus. Once inside, it binds to specific κB consensus sequences in the promoter and enhancer regions of hundreds of pro-inflammatory genes, most notably those encoding TNF-α and IL-6. This provides a direct, causal link from the chemical properties of the ozonated oil to the observed surge in cytokine gene transcription and protein expression.

This pro-inflammatory stimulus is likely amplified by a synergistic effect with the aloe vera vehicle. The primary bioactive polysaccharide in aloe, acemannan, is structurally similar to microbial surface molecules and is recognized as a pathogen-associated molecular pattern (PAMP) by the innate immune system. Acemannan is known to engage Toll-like receptor 4 (TLR4) on the surface of macrophages.[18] TLR4 signaling, via its downstream adaptors MyD88 and TRIF, also culminates in the potent activation of the NF-κB pathway. Therefore, the formulation delivers a "two-hit" signal to the wound's immune cells: an oxidative stress signal from the ozonides and a receptor-mediated signal from the acemannan. This dual activation of the same master inflammatory pathway likely explains the profound magnitude of the cytokine response, which was significantly greater than that induced by aloe vera oil alone.

Of paramount importance to the therapeutic potential of this formulation is the transient nature of the inflammatory surge. Our data unequivocally show that by day 7, the cytokine levels, both locally and systemically, had returned to baseline and were statistically indistinguishable from controls. This demonstrates that the treatment does not create a state of pathological, non-resolving inflammation. This timely resolution is as biologically significant as the initial activation. The key to this resolution likely lies in a second, parallel signaling pathway activated by the same oxidative stimulus: the Nrf2 pathway. Nrf2 is the master regulator of the cellular antioxidant and cytoprotective response.[18] Under basal conditions, it is sequestered in the cytoplasm by its inhibitor, Keap1. Oxidative stress, such as that provided by our formulation, causes Keap1 to release Nrf2, allowing it to translocate to the nucleus. There, it binds to the Antioxidant Response Element (ARE) in the promoter regions of a host of protective genes, including *HMOX1* (Heme Oxygenase-1) and genes for glutathione synthesis. The products of these genes are potently anti-inflammatory. For instance, HO-1 and its products, carbon monoxide and bilirubin, are known to directly inhibit the NF-κB pathway. Therefore, we propose a "two-phase" signaling model: the ozonated oil first triggers a rapid, NF-κB-driven pro-inflammatory phase (the day 3 peak), which is then actively terminated by a slightly delayed but more sustained Nrf2-driven anti-inflammatory and resolving phase. This elegant dual activation of both "on" and "off" signals explains the ideal kinetic profile observed in our results and is the hallmark of a sophisticated bioregulatory agent.

The lack of a clear dose-response relationship among the three different Peroxide Values tested is another intriguing finding. It suggests that the biological effect, in terms of activating the NF-κB and Nrf2 pathways, reaches a saturation point at or below the PV of our low-dose formulation.[19] From a clinical standpoint, this is highly advantageous, as it implies that a lower, and therefore potentially safer, dose with less overall oxidative load may be sufficient to achieve the maximal desired therapeutic effect. Finally, the inclusion of local tissue analysis in our study was critical. It definitely demonstrates that the observed

systemic effects are a direct consequence of a powerful local event within the wound's pathophysiology, confirming that the topical agent is acting precisely at the intended site of injury.

This study provides a deep mechanistic insight into the therapeutic action of ozonated aloe vera oil. It reframes the agent not as a simple antimicrobial, but as a pro-hormetic bioregulator that strategically manipulates the wound's own pathophysiological processes. It effectively "hijacks" and amplifies the initial, necessary inflammatory signals through the NF-κB pathway to ensure a rapid and efficient start to healing. It then facilitates the resolution of this inflammation, likely through the activation of the Nrf2 protective pathway, preventing the transition to a chronic state. This mechanism of enhancing and then resolving the body's innate repair capacity represents a more physiologically attuned and highly promising paradigm for the future of wound care.[18,19]

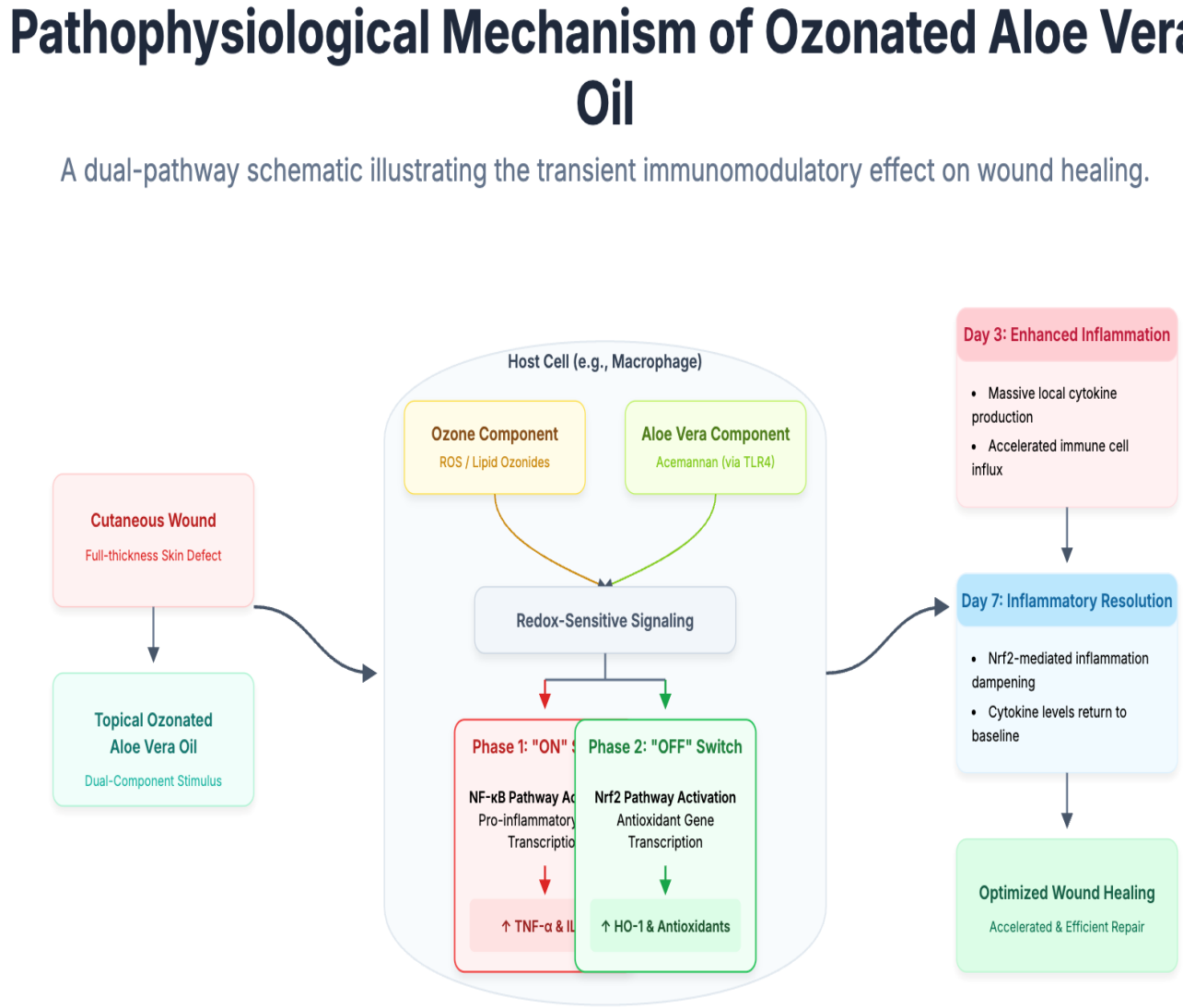


Figure 1. Pathophysiological mechanism of ozonated aloe vera oil.

Figure 1 showed a detailed schematic that elegantly illustrates the proposed pathophysiological mechanism of action for topically applied ozonated aloe vera oil in the context of cutaneous wound healing. The figure presents a conceptual model that moves far beyond a simple interpretation of the therapeutic agent as merely an antimicrobial or a generic anti-inflammatory substance. Instead, it posits a sophisticated, dual-pathway biological modulation, a carefully orchestrated sequence of events that first enhances and then resolves the initial inflammatory phase, ultimately leading to an optimized and more efficient repair process. This narrative interpretation will deconstruct the figure, step-by-step, to explain the complex interplay between the therapeutic stimulus, the host cellular response, and the time-dependent physiological outcomes that culminate in successful wound healing. The entire cascade, as depicted on the far left of the diagram, begins with an initial insult: the creation of a cutaneous wound, specifically described as a full-thickness skin defect. This represents a significant breach of the body's primary protective barrier, an event that immediately triggers a complex danger response. Into this environment of cellular damage and exposure, the therapeutic intervention is introduced through the topical application of ozonated aloe vera oil. The figure designates this as a "Dual-Component Stimulus," a crucial distinction that forms the foundation of the proposed mechanism. This label implies that the oil is not a monolithic entity but rather delivers two distinct sets of bioactive signals to the wound microenvironment, each contributing to the subsequent cellular response. An arrow logically connects the initial wound state to the application of the oil, indicating that the treatment is a direct response to the injury. The diagram then follows the stimulus to its primary target, a representative host cell, which is identified as a macrophage. The macrophage is an excellent choice for this model, as it is widely recognized as the master regulator of the entire wound healing process, orchestrating the transition from inflammation to proliferation. The figure illustrates that this host cell receives and processes the dual signals emanating from the ozonated aloe vera oil.

The first signal, derived from the ozone component, is described as the delivery of reactive oxygen species and lipid ozonides. These are the chemical products formed when ozone gas reacts with the unsaturated fatty acids within the aloe vera oil. This component is framed as inducing a state of mild oxidative stress, a concept central to the principle of hormesis, where a low dose of a potentially harmful agent can trigger a beneficial, adaptive response. The second signal is derived from the aloe vera component, specifically identifying acemannan as a key molecule that acts via Toll-like receptor 4. Acemannan is a complex polysaccharide found in aloe vera, and its interaction with Toll-like receptor 4 on the surface of macrophages is a well-established mechanism of innate immune activation. This receptor engagement mimics the way the immune system recognizes certain bacterial components, effectively "alerting" the macrophage and priming it for a robust response.[19] The visual representation of these two distinct inputs converging on a central process within the cell, labeled redox-sensitive signaling, is a pivotal element of the schematic. It suggests that the cell integrates the mild oxidative stress from the ozone component with the receptor-mediated signal from the aloe component. This integration leads to the activation of downstream pathways that are sensitive to the cell's internal oxidation-reduction state, setting the stage for the bifurcation of the cellular response into two distinct, time-dependent phases.

Following the integration of the initial stimuli, the cell activates the first of two major pathways, which the figure appropriately labels as Phase 1: The "ON" Switch. This phase is governed by the activation of the Nuclear Factor-kappa B, or NF-κB, pathway. In its narrative of cellular events, the diagram explains that this activation leads to pro-inflammatory gene transcription. NF-κB is a master transcription factor that, in its resting state, is held inactive in the cytoplasm. The convergence of redox signals and Toll-

like receptor activation triggers a signaling cascade that leads to the degradation of NF-κB's inhibitor, allowing the active complex to translocate into the nucleus. Once there, it binds to the promoter regions of hundreds of genes, acting as a powerful switch to turn on the machinery of inflammation. The immediate and primary consequence of this genetic activation, as shown in the schematic, is a significant increase in the production of the pro-inflammatory cytokines tumor necrosis factor-alpha and Interleukin-6.[20] An arrow directly connects this molecular event to the first major physiological outcome, which occurs at Day 3 and is termed Enhanced Inflammation. This section of the figure details the tangible results of the cytokine surge. The "massive local cytokine production" creates a powerful chemical gradient that leads to "accelerated immune cell influx." This refers to the rapid recruitment of neutrophils and, subsequently, more macrophages to the wound site. This amplified cellular army then carries out the crucial task of "efficient wound debridement," which involves clearing away dead tissue, cellular debris, and any invading pathogens. The figure 1 thus narrates a story where the therapeutic agent does not suppress inflammation but strategically and potently enhances it. This initial, controlled inflammatory burst is portrayed as a beneficial and necessary step, ensuring the wound is thoroughly cleaned and prepared for the subsequent phase of tissue reconstruction. It is a process of controlled demolition that is required before new construction can begin.

Crucially, the schematic does not end with the inflammatory surge. It illustrates an elegant, self-regulating mechanism by depicting a second, parallel pathway, labeled Phase 2: The "OFF" Switch. This phase is governed by the activation of the nuclear factor erythroid 2-related factor 2, or Nrf2, pathway. The figure implies that this pathway is also triggered by the initial redox-sensitive signaling, but its effects are functionally opposed to those of NF-κB. The narrative explains that Nrf2 activation leads to antioxidant gene transcription. Similar to NF-κB, Nrf2 is a transcription factor that, upon activation by oxidative stress, translocates to the nucleus. However, instead of turning on inflammatory genes, it activates a suite of powerful protective genes. The outcome of this pathway activation is shown as an increase in Heme Oxygenase-1 and other antioxidants. Heme Oxygenase-1 is a potent enzyme whose byproducts have strong anti-inflammatory and cytoprotective effects.[20] By upregulating the body's own endogenous antioxidant and anti-inflammatory systems, the Nrf2 pathway actively counter-regulates and dampens the initial pro-inflammatory signal driven by NF-κB. This molecular "off" switch leads directly to the second major physiological outcome, which occurs at Day 7 and is termed Inflammatory Resolution. This phase is characterized by "Nrf2-mediated inflammation dampening" and the resulting "return of cytokine levels to baseline." This resolution is not a passive decay of the initial signal but an active, programmed shutdown of the inflammatory process. This is the most critical checkpoint in preventing the wound from transitioning into a chronic, non-healing state. By ensuring the inflammation is resolved in a timely manner, the treatment facilitates the transition to the proliferative phase, where fibroblasts begin to deposit new collagen, and new blood vessels are formed.[20]

The final portion of the diagram shows the logical conclusion of this well-orchestrated sequence of events. The arrows from both the "Enhanced Inflammation" phase and the "Inflammatory Resolution" phase converge on the ultimate goal: Optimized Wound Healing. This final state is described as "Accelerated and Efficient Repair." The narrative presented by the figure is that by first providing a robust "on" signal to kick-start the healing process and then providing a timely and effective "off" signal to resolve it, the therapeutic formulation guides the wound through the natural healing cascade in a more efficient and potentially accelerated manner. It avoids the pitfalls of both an insufficient initial response, which can lead to infection, and an unresolved response, which leads to chronic inflammation. Figure 1 narrates a sophisticated biological story. It shows

how a single therapeutic application can initiate a self-regulating cascade of events within the body's own cells. It leverages the principles of hormesis and synergistic dual-component signaling to first amplify and then resolve inflammation, creating the ideal conditions for tissue regeneration. The schematic beautifully captures the transformation of ozonated aloe vera oil from a simple topical agent into a complex bioregulator that intelligently modulates the intricate pathophysiology of wound repair.

5. Conclusion

This study provides compelling evidence that topical ozonated aloe vera oil functions as a sophisticated biological response modifier, promoting a beneficial, pro-regenerative inflammatory phenotype. The formulation was shown to significantly, yet transiently, upregulate the cardinal pro-inflammatory cytokines TNF-α and IL-6, both locally within the wound and systemically, during the critical initiation phase of repair. This mechanism, likely mediated by the activation of redox-sensitive transcription factors like NF-κB and subsequently resolved by cytoprotective pathways like Nrf2, optimizes the crucial first phase of healing without inducing pathological chronic inflammation. These findings support a novel therapeutic strategy aimed at enhancing, rather than suppressing, the body's innate capacity for repair and establish ozonated aloe vera oil as a promising agent deserving of further investigation for the management of acute cutaneous wounds.

6. References

1. Naik K, Singh P, Yadav M, Srivastava SK, Tripathi S, Ranjan R, et al. 3D printable, injectable amyloid-based composite hydrogel of bovine serum albumin and aloe vera for rapid diabetic wound healing. J Mater Chem B Mater Biol Med. 2023; 11(34): 8142–58.
2. Roney M, Issahaku AR, Govinden U, Gazali AM, Aluwi MFFM, Zamri NB. Diabetic wound healing of aloe vera major phytoconstituents through TGF-β1 suppression via in-silico docking, molecular dynamic simulation and pharmacokinetic studies. J Biomol Struct Dyn. 2024; 42(24): 13939–52.
3. Hidayat AT, Budiono P. Ozonated aloe vera: a novel topical agent for improved wound healing and reduced scar formation on TGF-β modulation in vivo study. BioSci Med J Biomed Transl Res. 2024; 9(1): 5854–66.
4. Raj B, Ingole N, Ambad R. Evaluation of the wound healing potential of aloe vera leaf extract containing carbopol 934 as a gelling agent and comparing with sodium CMC and HPMC on surgically induced in wound Wister rats: a preclinical study. J Pharm Bioallied Sci. 2024; 16(Suppl 4): S3699–702.
5. Rizwan M, Usman Abid HM, Akhlaq M, Khan S, Manzoor A, Khiljee B, et al. Comparative efficacy of aloe vera (alobarbadensis) and turmeric (*Curcuma longa*) on burn wound healing in rabbit model. J Popul Ther Clin Pharmacol. 2024; 526–34.
6. Zahra S, Qayyum S, Mansoor M, Essa M, Chohan MA, Ishaq S, et al. Comparative efficacy of aloe vera gel versus normal saline in accelerating episiotomy wound healing: a randomized controlled trial. J Health Rehabil Res. 2024; 4(2): 949–56.
7. Reddy KS, Chittoria RK, Kerakada N, Thomas N, Srinath R, Indushekar TC, et al. Role of topical application of aloe vera for accelerated wound healing of cutaneous burns wound: a case report. J Cutan Aesthet Surg. 2024; 17(3): 246–8.
8. Malhotra P, Kataria N, Mandal A, Kalyani VC, Kumari K. Efficacy of aloe vera gel vs. 1% silver sulfadiazine in first- and second-degree burn wound healing: a systematic review and meta-analysis. Amrita J Med. 2024; 20(3): 94–102.
9. Lee ZM, Goh BH, Khaw KY. Aloe vera and the proliferative phase of cutaneous wound healing: Status quo report on active principles, mechanisms, and applications.

Planta Med. 2025; 91(1–02): 4–18.
10. Arafa NMS, Hummadi HMA, Badr GM. The potential of aloe vera gel utilization for skin wound healing in rats based on GC–MS and HPLC chemical profile. J Basic Appl Zool. 2025; 86(1).
11. Khan A, Ullah S, Ramzan F, Rehman AU, Rehman S, Kholik K, et al. Combined effects of aloe vera leaf extract and vitamin E on wound healing in *Oryctolagus cuniculus* (Rabbits). Open Vet J. 2025; 15(2): 700–8.
12. Patitucci F, Motta MF, Mileti O, Dattilo M, Malivindi R, Pezzi G, et al. Enhancing burn wound care with pre-crosslinked 3D-printed patches: Bromelain delivery and aloe vera bioactives integration for improved healing outcomes. Int J Pharm. 2025; 672(125304): 125304.
13. Panares Arcaya PJ, Claro Capilitan LMD, Decano ZR, Guyo Dugaduga CD, Lamasan Hernane AG, Calo JRD, et al. Phytochemical analysis and wound healing potential of aloe vera (*Aloe massawana*) peel extract. Asian J Biol Life Sci. 2025; 14(1): 147–52.
14. Cao-Luu N-H, Nguyen T-V, Luong H-V-T, Dang H-G, Pham H-G. Engineered polyvinyl alcohol/chitosan/carrageenan nanofibrous membrane loaded with aloe vera for accelerating third-degree burn wound healing. Int J Biol Macromol. 2025; 311(Pt 3): 143880.
15. Hazarika K, Mahato PK, Das P, Mondal S, Ghosh SB, Nandi SK, et al. MAXene-enabled drug free aloe vera hydrogel expedites wound healing in New Zealand white rabbits. J Reinf Plast Compos. 2025.
16. Farshi Azhar F, Mohammadzadeh M, Namvar Aghdash S. Development of sprayable wound dressings based on polyvinyl alcohol-alginate incorporating zinc oxide and aloe vera for enhanced healing: physicochemical evaluation and in-vivo studies in rat models. Polym Bull (Berl). 2025; 82(11): 5637–64.
17. León-Campos MI, Claudio-Rizo JA, Becerra-Rodriguez JJ, Espindola-Serna L, Cano-Salazar LF, Rodríguez-Fuentes N, et al. Aloe vera-enriched collagen-polyurethane hydrogel: supporting tissue regeneration, antibacterial action and drug release for effective wound healing. Biomed Mater. 2025; 20(4).
18. Sari Y, Purnawan I, Kurniawan DW, Sutrisna E. A comparative study of the effects of *Nigella sativa* oil gel and aloe vera gel on wound healing in diabetic rats. J Evid Based Integr Med. 2018; 23: 2515690X18772804.
19. Istek O, Tanrisever M, Kucukler S, Karabulut B, Cevik A. Comparison of the effects of aloe vera gel and coconut oil on the healing of open wounds in rats. Vet Med (Praha). 2023; 68(1): 17–26.
20. Vahlepi R, Arifin MT, Nur M, Muniroh M, Susilaningsih N. Ozonated aloe vera oil improve wound healing by decreasing neutrophil count and wound diameter in Sprague Dawley rats with full-thickness skin defect. Indones J Biomed Sci. 2020; 14(2): 48–54.