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Research Article

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The Effect of Combination Therapy of Ultrasound and Aloe Vera gel Containing Zinc Oxide Nanoparticles on Skin Wound Healing in Mice

Zahra Ganjkhani[a], Ali Ebrahiminia[b], Babak Arji Roudsari[a], Saman Nasiri[a], Arash Zaminy[c], Zahra Hesari[d], Rouhollah Gazor[a], Ebrahim Nasiri[e*]

[a] *Department of Anatomical Sciences, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran.*
[b] *Department of medical physics, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran.*
[c] *Burn and regenerative medicine research center, Guilan University of Medical Sciences, Rasht, Iran.*
[d] *Department of Pharmaceutics, School of Pharmacy, Guilan University of Medical Sciences, Rasht, Iran.*
[e] *Neuroscience research center, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran.*

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Abstract

Wound healing is an intricate pathway of cellular and biochemical events that restore the architecture of cutaneous and subcutaneous tissue. This study investigates the effects of ultrasound (US) radiation combined with Aloe vera gel containing zinc oxide nanoparticles (ZnO NPs) on wound healing. The study was conducted on male mice, which were divided into five groups: control (A), US-treated (B), US + Aloe vera gel-treated (C), US + ZnO NPs-treated (D), and US + Aloe vera gel containing ZnO NPs-treated (E). After making a surgical wound of 1.5 cm on the back of the mice, the local use of gel and US radiation started promptly and proceeded for 15 days. After 15 days, the animals were euthanized for histological examination. US with ZnO NPs led to a substantial increase in collagen count compared with the control cohort. US with ZnO NPs, as well as US alone, significantly increased epithelial thickness and notably elevated collagen and elastin levels in the tissue. The study suggests that combining US radiation with ZnO NPs may significantly increase collagen and elastin levels in the skin's connective tissue and enhance epithelial thickness during wound healing in mice. Improved skin structure can help prevent wound infections by reducing the risk of microbial colonization and promoting faster recovery.

Keywords: Wound; Zinc Oxide Nanoparticle; Ultrasound Waves; Aloe Vera.

1. Introduction

Skin wounds result from various factors, including physical, chemical, and biological damage. They are categorized as chronic or acute based on their cause and healing duration. Acute wounds, such as burns and cuts, are typically shallow and heal completely within 8 to 12 weeks[1, 2]. Chronic wounds, such as venous or arterial leg ulcers, diabetic foot ulcers, and pressure ulcers, are associated with physiological disorders that slow or halt healing. These wounds take longer than 2 weeks to heal. The wound-healing process begins with an inflammatory response that starts within the first 24-48 hours [3]. Platelets release growth factors that attract neutrophils and macrophages. In the second stage, fibroblasts

*** Corresponding Author**:
Ebrahim Nasiri, Neuroscience research center, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran. E-mail: ebrahimnasiri@gmail.com.

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proliferate and differentiate, producing collagen that forms the wound structure. Fibroblasts also facilitate angiogenesis and collagen remodeling. In the final stage, collagen synthesis and breakdown reach equilibrium, and fibroblasts coordinate their arrangement [4, 5].

1.1. Combination therapy

There is increasing focus on combined methods for treating wounds. Some researchers have found that combining two treatment mechanisms can improve outcomes for pressure ulcers and diabetic ulcers [6-8]. These methods include using high-pressure oxygen therapy with platelet-enriched plasma simultaneously [9], using US waves and electric field stimulation simultaneously [10, 11], and simultaneously irradiating with laser and US waves [9]. Studies have shown that using US and laser radiation simultaneously for treating pressure ulcers in patients with spinal cord injury shortens the healing process and leads to complete wound healing within 12 weeks [10]. Ultrasound (US) waves have been observed to have various positive effects on wound healing. These effects include influencing cellular protein synthesis and secretion, blood flow and vascular permeability, promoting angiogenesis, and regulating collagen content [11]. For treating skin wounds, frequencies ranging from 3MHz to 5.0MHz have been shown to aid healing in surgical, diabetic, and venous wounds [12]. Ultrasound radiation is beneficial during the inflammatory and proliferative phases of healing because it helps remove dead tissue, increase granulation tissue formation, and reduce wound size. However, it is not effective during the regeneration phase because it affects the continuation of angiogenesis and mononuclear infiltration [13, 14]. In recent years, nanoparticles have gained attention for their use in biomedical and pharmaceutical fields. They are used in wound dressings, drug delivery, and other therapeutic applications. Various nano-scale techniques have been studied to target different phases of wound healing, including nanomaterials [15, 16].

1.2. Zinc oxide:

Zinc is present both inside cells and in the extracellular matrix (ECM) of the epidermis and dermis, where it is found within protein complexes. In this context, zinc stabilizes cell membranes and acts as an essential cofactor. It plays a vital role in mitosis, cell migration, and maturation. Introducing additional zinc ions can accelerate numerous biochemical and molecular processes involved in wound healing by enhancing the activity of metallothioneins and zinc-dependent enzymes. Additionally, shortcomings in the expression of zinc-finger transcription factors in growth factor-associated mRNA correlate with hindered wound healing [17]. Zinc oxide is an inorganic antibacterial agent that is more stable than natural substances. Zinc, with a long half-life in cells, is crucial for wound healing, especially in the delayed healing of wounds and burns [18]. Topical zinc has been reported to reduce inflammation, promote epithelial regeneration, and reduce bacterial growth in chronic wounds. Zinc is a cofactor for metalloproteinases and also plays an important role in ECM regeneration. Zinc oxide nanoparticles have antibacterial, anti-inflammatory, and cleansing properties and are widely used in cosmetics, skin creams, and ointments [19]. These small-diameter nanoparticles exhibit strong antibacterial properties, which can be enhanced by ultrasound radiation. This study demonstrated that combining low-frequency ultrasound with silver nanoparticles reduces the inflammatory response, eliminates microorganisms, and facilitates new tissue formation with complete epithelialization in the treatment of purulent wounds [20].

1.3. Aloe vera:

Aloe vera is regarded as one of the top healing plants and has been extensively utilized in traditional herbal medicine for many years. Reported plant properties include immune modulation, anti-inflammatory effects, antiprotozoal activity, protection against ultraviolet light, and enhanced wound and burn healing. Research indicates that A. vera gel not only boosts the collagen content in healing wounds but also alters the collagen composition and enhances the degree of collagen cross-linking [21]. Furthermore, there have been reports of increased production of hyaluronic acid and dermatan sulfate in the granulation tissue of healing wounds following either oral or topical use of A. vera. Additionally, the gel promotes angiogenesis and improves oxygen access. [22, 23]. This plant is rich in water, vitamins, minerals, enzymes, lignin, saponins, and

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amino acids. Additionally, its antioxidant, antibacterial, and antifungal properties play a crucial role in promoting wound healing [24]. The biologically active compounds in Aloe vera gel are known to stimulate the movement of fibroblasts, macrophages, and epidermal cells, which in turn promotes the formation of epidermal tissue and enhances collagen synthesis [25].

1.4. Gelatin compounds

Acrylic hydrogels are a fascinating type of hydrogel that possess pH-sensitive properties. This characteristic makes them suitable for various biomedical applications, including drug delivery systems. These unique properties stem from ionizable groups in their backbones and side or end groups. "Smart" polymers can alter their conformation using acidic (e.g., carboxylic and sulfonic acids) or basic (e.g., ammonium salts) groups, which can accept or release protons depending on changes in the pH of the physiological environment [26]. Using gelatin compounds with drugs can increase the durability of the therapeutic agent at the wound site and prevent its premature disappearance. To produce a pharmaceutical gelatin compound, researchers have used the confirmed and safe method of creating polyacrylic acid as a gelatin agent in numerous studies. Polyether sulfone (PES) and polyacrylic acid stand out as key polymer materials. It possesses remarkable resistance to oxidation, heat, and moisture, as well as strong mechanical and film-forming traits. As a result, it has been widely used in membrane separation for a range of applications, including biomedicine, hemodialysis, and plasma separation [27, 28].

In this research, we created and analyzed hybrid acrylic hydrogel materials containing Aloe vera powder. Our goal was to study how ultrasound waves could enhance the antibacterial properties of Aloe vera gel containing ZnO nanoparticles and their combined impact on wound healing in mice.

2. Materials and methods:

2.1. Materials

Zinc oxide nanoparticles (ZnO, 99+%, 10-30nm) were obtained from US Research Nanomaterial Company Inc. (CAS#: 1314-13-2) (**Figure 1**). We purchased aloe vera powder from Tehran-Chem Company (Iran). We acquired all additional reagents from Merck Co. (Darmstadt, Germany).

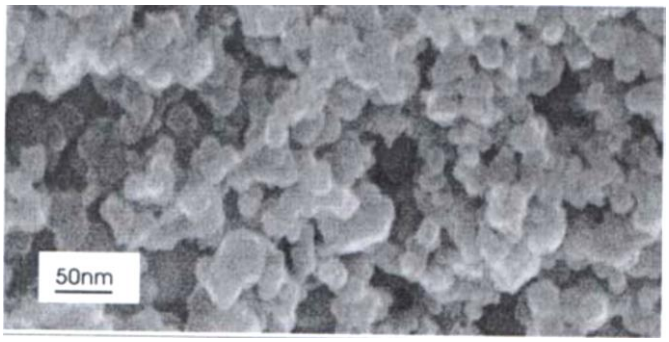


Figure 1. ZnO Characterization. (A) SEM micrographs of ZnO nanoparticles

2.2. Five treatment formulations were used in this study

(A) PAA hydrogel alone (control), (B) ultrasound (US) + PAA hydrogel, (C) US + Aloe vera hydrogel (2% w/v Aloe vera), (D) US + ZnO nanoparticle hydrogel (0.081% w/v ZnO NPs), and (E) US + Aloe vera/ZnO nanoparticle hydrogel containing 2% w/v Aloe vera and 0.081% w/v ZnO NPs. Ultrasound was applied at 1 MHz, 0.5 W/cm², and a 20% duty cycle for 5 minutes daily over 15 consecutive days.

2.3. Animal model

Thirty male mice (20-25 grams and 8-10 weeks old) were obtained from the laboratory animal house of Guilan University of Medical Sciences. The mice were housed in the animal-keeping room of the Faculty of Medicine under standard conditions of 22±2ºC and a 12-hour light/12-hour dark cycle. They were kept in groups of three in cages and had access to water and food throughout the two-week study period. The mice were randomly divided into experimental groups, with 6 animals in each group. Group A (control) was treated with PAA gel; Group B received ultrasonic (US) waves and PAA gel; Group C received US waves and PAA gel containing Aloe vera; Group D received US waves and PAA gel containing ZnO Nps and Group E received US waves and PAA gel containing ZnO Nps and Aloe vera. The treatment lasted for 5 minutes per day. The animals were euthanized 15 days after treatment, and skin samples were collected for further testing.

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2.4. Ethical statement

All experimental procedures were approved by GUMS.AEC.1401.004 registration code and followed the guidelines for working with laboratory animals provided in the research ethics guidelines of Guilan University of Medical Sciences.

2.5. Skin surgical incision

The animals were anesthetized using an intraperitoneal injection of a ketamine-xylazine combination at a dose of 100 to 10 mg/kg [29]. After sterilizing and shaving the hair on the back of the mice, they were placed in a supine position on the surgical table under aseptic conditions. Subsequently, a 1.5 cm incision was made to full thickness, including both the dermis and epidermis on the animal's back [30].

2.6. Preparation of polyacrylic acid (PAA) gel

For the preparation of the gel, 0.5g of polyacrylic acid (PAA) was accurately weighed and dissolved in 100cc of deionized water. The PAA was stirred with a magnetic stirrer until completely dissolved, forming a gel-like solution. Next, 4 drops of triethanolamine were carefully added to adjust the pH. The pH of the resulting gel was measured using a calibrated pH meter and found to be approximately 7, which corresponds to the normal pH of human skin [31, 32].

2.7. Preparation of Aloe Vera gel

0.5 grams of polyacrylic acid (PAA) were dissolved in 80 milliliters of distilled water. Separately, 2 grams of Aloe vera powder were dispersed into 20 milliliters of water and mixed thoroughly to obtain a homogeneous solution. Subsequently, this solution was incorporated into the PAA gel, and the pH of the resulting mixture was adjusted following the aforementioned procedure [28].

2.8. Preparation of gel containing ZnO Nps

A solution was prepared by dissolving 0.5g of polyacrylic acid (PAA) in 80cc of distilled water. Separately, 0.0814g of zinc oxide nanoparticles (ZnO NPs) was dispersed in 20cc of water until a homogeneous solution was achieved. The resulting ZnO NPs solution was then combined with the PAA gel. Subsequently, the pH of the solution was adjusted [33].

2.9. Preparation of Aloe vera gel containing ZnO Nps

0.5 grams of poly acrylic acid (PAA) were accurately weighed and dissolved in 80 milliliters of distilled water. Following this, 2 grams of Aloe vera powder were dispersed in 10 milliliters of water, and 0.0814 grams of zinc oxide nanoparticles (ZnO NPs) were dispersed in 10 milliliters of water. Next, the Aloe vera and ZnO NP solutions were thoroughly mixed until homogeneous. The resulting solutions were then incorporated into the PAA gel, and the pH of the overall solution was adjusted to the desired level by carefully adding 4 to 5 drops of triethanolamine, as specified. The effective concentration of ZnO nanoparticles used in the hydrogel formulation was 0.081% w/v (0.814 mg/mL). Aloe vera powder was incorporated at a final concentration of 2% w/v (20 mg/mL). These concentrations were selected based on previous studies showing wound-healing and antibacterial efficacy of ZnO nanoparticles and Aloe vera formulations without inducing tissue toxicity. [28, 33].

2.10. Ultrasonic parameters

The ultrasonic treatment involved a duty cycle of 20% and a frequency of 1 MHz, with an intensity of 0.5 W/cm2, using an effective irradiation area (ERA) of 1.0 cm2. The ultrasonic waves were applied for 5 minutes, utilizing the direct coupling method and oscillatory rubbing movements. Before and after treatment, the ultrasonic equipment was meticulously calibrated to ensure no loss of intensity during the animal treatment, maintaining the procedure's efficacy [34, 35].

2.11. Histomorphometry

Skin tissue samples were collected on day 15. They were fixed in 10% formalin for 7 days. The samples were then processed, paraffin-embedded, and prepared for sectioning. Hematoxylin-eosin staining was used to evaluate epithelium thickness, Masson's trichrome staining was used to assess collagen deposition, and van Gieson-Verhoeff staining was used to evaluate tissue elastin.

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2.12. Evaluation of the thickness of the epithelium

The pre-prepared samples in all groups were stained with hematoxylin and eosin, Masson's trichrome, and Van Giesen-Verhoef. Subsequently, the entire field of view of each section was examined using an optical microscope to observe the thickness of the epithelium. Five fields were randomly selected and photographed in each section. In each field, 15 epithelial thickness measurements were randomly taken using Digimizer software (version 11). We collected the average values, analyzed them in SPSS, and compared them across groups [36].

2.13. Evaluation of the amount of collagen

In order to measure the amount of collagen, we utilized Masson's trichrome staining, which specifically targets collagen strands. After staining, we observed the samples using a microscope at 400X magnification. We then evaluated 10 microscopic fields, each 1500 square micrometers, in each animal. We categorized the degree of collagenization into four grades based on histopathological criteria: 0 for absence, 1 for thick fiber and high dyeing reaction, 2 for thin fiber and poor dyeing reaction, and 3 for thin fiber and very weak dyeing reaction. Finally, we compared the averages across groups.

2.14. Evaluation of the amount of elastin

To assess the amount of elastin, we used Van Gieson-Verhoeff staining, which specifically targets elastin fibers. After staining, we observed the samples under a microscope at 400X magnification. We evaluated 10 microscopic fields, each covering 1500 square micrometers, in each animal. We categorized elastin production into four grades based on histopathological criteria: 0 for absence, 1 for thick fiber and high dyeing reaction, 2 for thin fiber and poor dyeing reaction, and 3 for thin fiber and very weak dyeing reaction. We then compared the averages between groups.

2.15. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 19. Quantitative data were expressed as mean ± standard deviation (Mean ± SD). Comparisons among the five experimental groups were carried out using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. A p-value less than 0.05 was considered statistically significant.

3. Results:

3.1. Histological findings

Histological findings showed that the thickness of the epithelium in the control group was 0.23 ± 2.49 micrometers. Wound treatments using ultrasound (US), US + Aloe vera, US + ZnO Nps, and the combination of US + Aloe vera + ZnO Nps all led to an increase in the thickness of the epithelium. However, this increase was not statistically significant, and there were no significant differences in epithelial thickness among the treatment groups (**Figures 2** and **3**).

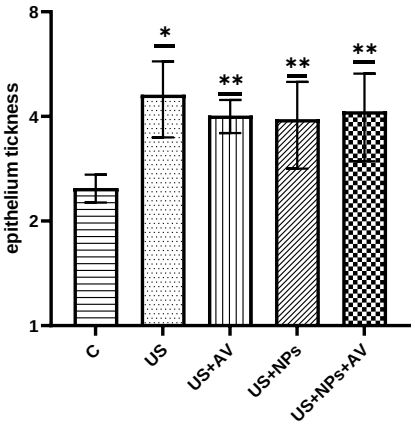


Figure 2. Quantitative analysis of epithelial thickness in different experimental groups on day 15 after wound induction. Data are presented as Mean ± SD. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. P < 0.05 and *P < 0.01** versus control group.

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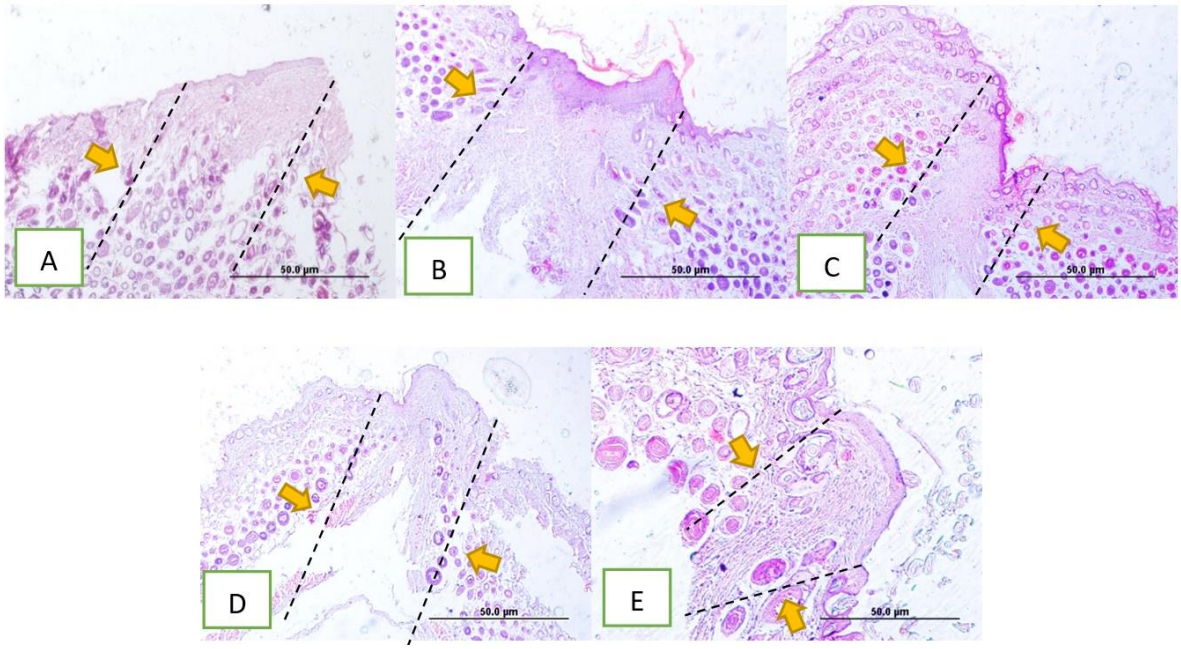


Figure 3. Representative histological sections of wound tissues on day 15 after wound induction stained with hematoxylin and eosin (H&E). (A) Control group treated with PAA gel, (B) ultrasound-treated group, (C) ultrasound + Aloe vera-treated group, (D) ultrasound + ZnO nanoparticle-treated group, and (E) ultrasound + Aloe vera/ZnO nanoparticle-treated group. Images were captured at ×40 magnification.

The experimental group that received US + ZnO NPs (p=0.00) and the group treated with US alone (p=0.020) showed a statistically significant increase in collagen quantity relative to the control group. This increase in the amount of collagen in the US waves-treated group (P = 0.02), the US waves + ZnO Nps- treated group (P = 0.00), and the US waves + Aloe vera + ZnO Nps- treated group (P = 0.011) was statistically significant compared to the group treated with US + Aloe vera. The study employed histology with Masson's trichrome staining to evaluate tissue collagen levels. The results revealed that the control group displayed weak collagen production, measured at 2.75 ± 0.129. In contrast, treatment with US waves + ZnO NPs, US waves alone, and US waves + ZnO NPs + Aloe vera showed a notable increase in collagen production (**Figures 4**, **5** and **6**).

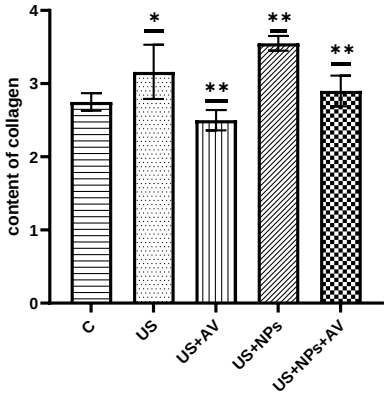


Figure 4. Quantitative evaluation of collagen deposition in wound tissues of different experimental groups on day 15. Data are presented as Mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's multiple comparison test. P < 0.05* and P < 0.01** versus control group.

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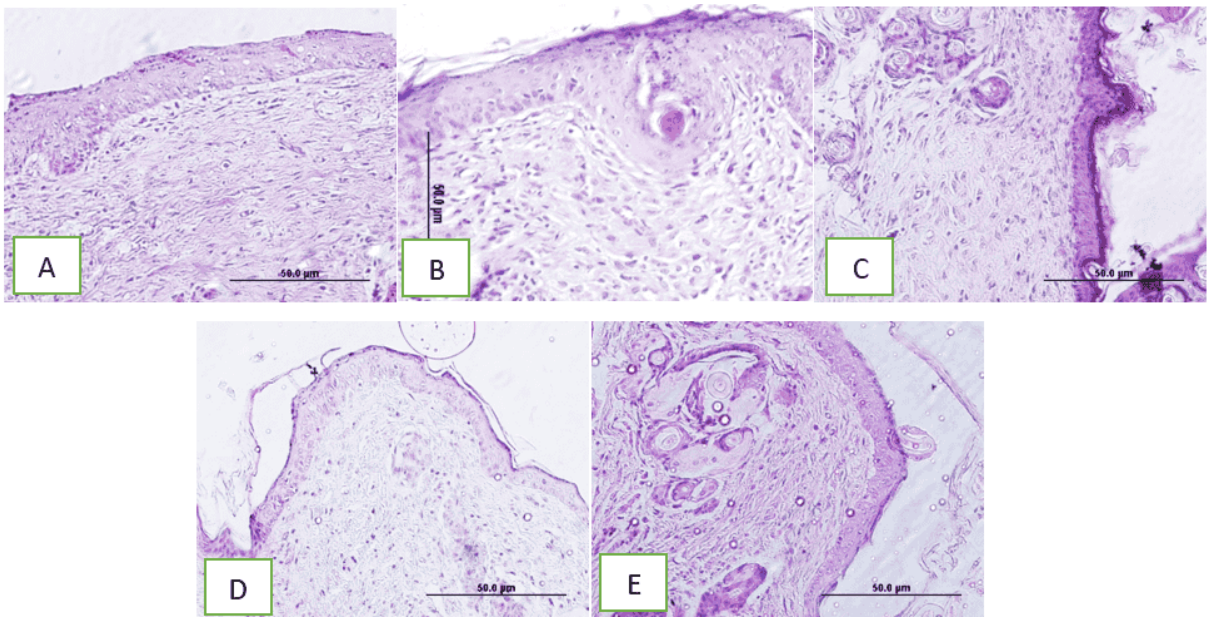


Figure 5. Histological evaluation of collagen fibers in wound tissues using Masson's trichrome staining on day 15 after wound induction. (A) Control group, (B) ultrasound-treated group, (C) ultrasound + Aloe vera-treated group, (D) ultrasound + ZnO nanoparticle-treated group, and (E) ultrasound + Aloe vera/ZnO nanoparticle-treated group. Images were obtained at ×400 magnification.

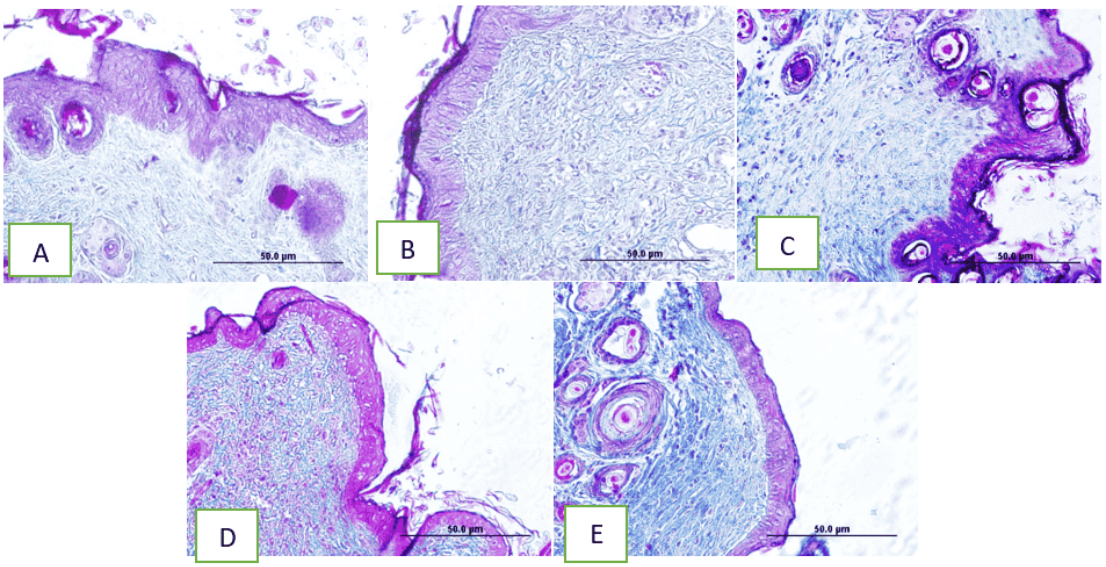


Figure 6. Representative microscopic images showing collagen deposition in wound tissues stained with Masson's trichrome on day 15 after treatment in different experimental groups. Increased collagen density was observed predominantly in the ultrasound + ZnO nanoparticle-treated group.

The tissue analysis showed that only the group treated with ultrasound waves and ZnO NPs had a significantly different elastin level compared with the control group and the other experimental groups. The other experimental groups did not differ significantly in elastin levels among themselves (**Figure 7** and **8**).

Examining the histological parameters revealed the levels of collagen and elastin.

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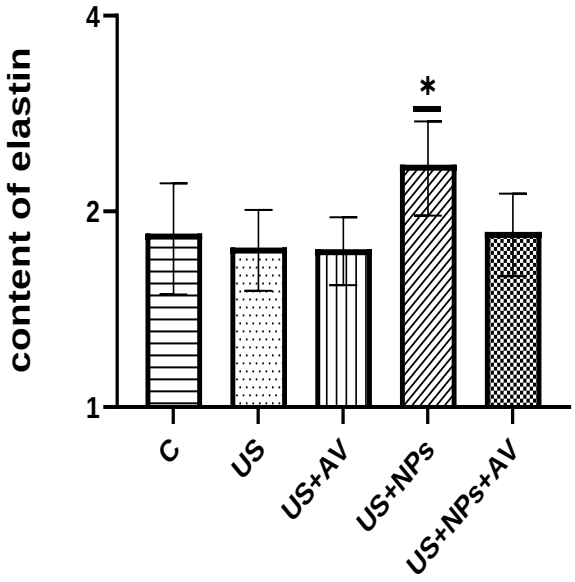


Figure 7. Quantitative analysis of elastin content in wound tissues of experimental groups on day 15. Data are expressed as Mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's multiple comparison test. *P < 0.05 versus control group.

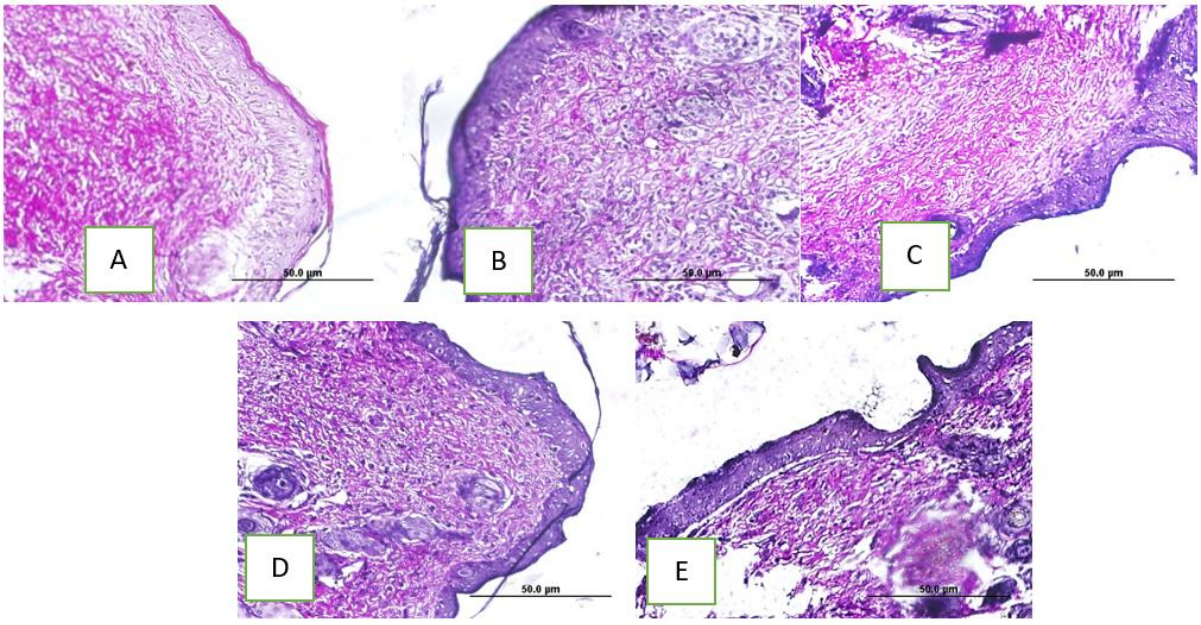


Figure 8. Histological assessment of elastin fibers in wound tissues using Verhoeff–Van Gieson staining on day 15 after wound induction. (A) Control group, (B) ultrasound-treated group, (C) ultrasound + Aloe vera-treated group, (D) ultrasound + ZnO nanoparticle-treated group, and (E) ultrasound + Aloe vera/ZnO nanoparticle-treated group. Images were captured at ×400 magnification.

On the 15th day of the survey, it was observed that hair growth improved similarly in all groups after shaving the back hair. The incisional wound, especially in group D (treated with US waves + ZnO Nps), was hardly visible. Although there is no significant difference between the groups as seen in the picture (**Figure 9**).

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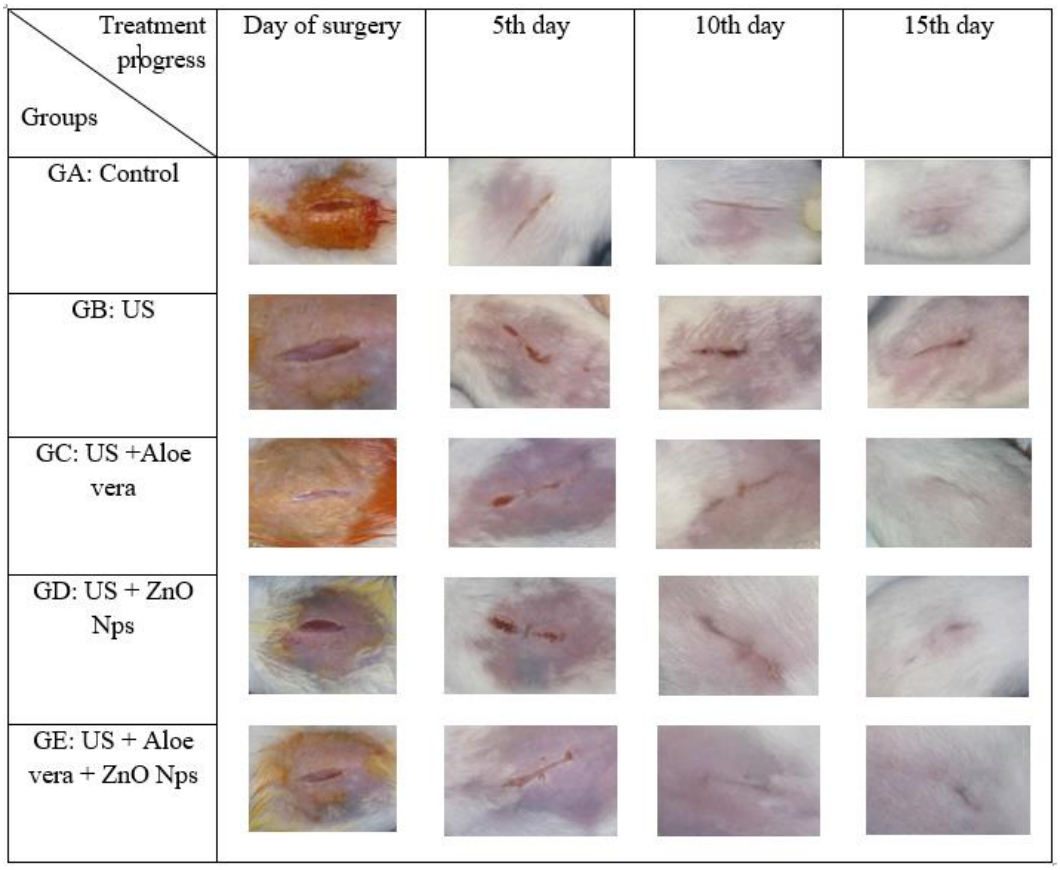


Figure 9. Macroscopic appearance of wound healing progression in different experimental groups on days 0, 5, 10, and 15 after wound induction. Improved wound closure and reduced scar formation were observed in the ultrasound + ZnO nanoparticle-treated group compared with the control group.

3.2. Collagen

In the groups treated with US waves + ZnO Nps, the US wave group, the combination group of US waves + ZnO Nps + Aloe vera, and the Aloe vera group showed a significant difference compared to the control group. The US waves + ZnO NPs group and the US waves group showed significant differences compared with the US waves + ZnO NPs + Aloe vera group and the US waves + Aloe vera group.

3.3. Elastin

Only in the group treated with US waves + ZnO Nps was there a significant difference between the other groups and the control group; the other groups did not have any significant differences in terms of elastin.

3.4. Discussion

The research aimed to investigate how a treatment combining US waves, Aloe vera, and ZnO Nps affected the concentration of collagen and elastin fibers in mice skin after surgery. The study found that collagen fiber levels in treated mice were significantly higher than in the control group. Additionally, elastin fiber evaluation showed a statistically significant increase in elastin quantity. The examination of epithelium thickness among different groups revealed that the thickness of the epithelium in all groups increased compared to the wound group. However, our findings indicate no substantial difference in epithelium thickness among the groups. Although ultrasound (US) waves may not directly reduce inflammation in wound healing, studies

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suggest that exposure to these waves during the inflammatory phase can accelerate it [37-39]. In the proliferative phase, US radiation may boost collagen production by fibroblasts and facilitate epithelial repair, as shown in previous research [40]. Additionally, during the regeneration phase, treating scar tissue with US has been found to result in scar tissue that is stronger and more elastic than conventional scar tissue [41].

The combined therapy of ZnO Nps with US accelerates wound healing and reduces scarring. ZnO NPs have antimicrobial properties and support tissue regeneration, making them effective in promoting wound healing [42]. This combined treatment can speed wound healing by promoting factors such as collagen and elastin fiber production in incisional wounds. Studies have shown that combining US therapy with ZnO NPs significantly increases tissue collagen. Additionally, ZnO NPs assist in skin repair and reduce scars caused by wounds [43, 44]. Their lipophilic properties prevent water loss from the skin, promote hair growth, increase melanin, and prevent wrinkling of wounded skin during repair. The group receiving treatment with US waves and ZnO Nps had reduced scarring compared to other groups.

Furthermore, ZnO NPs accelerate burn wound healing due to their antimicrobial properties. Studies have also shown that ZnO NPs support cellular proliferation and attachment and promote tissue regeneration [42]. In a 2017 study, the combined use of US radiation and Aloe vera gel was less effective than the combination of US radiation and silver nanoparticles in reducing burn wound surface area and expediting wound healing. The difference in results may be due to the type of nanoparticles used in the studies (ZnO Nps instead of silver Nps) [45]. Aloe vera gel has been found to enhance the expression of the TGF gene in the skin of injured mice. It also triggers the release of additional growth factors such as Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF), which in turn aid the wound-healing process [46, 47]. Aloe vera gel primarily reduces inflammation by inhibiting the cyclooxygenase pathway and decreasing the Synthesis of prostaglandin E2 derived from arachidonic acid[48]. In addition, Aloe vera can increase collagen concentration in granular tissue and promote the synthesis of glycosaminoglycans and proteoglycans in the injured area [48]. In a study investigating the use of Aloe vera gel and US waves to treat collagenase-induced tendinitis, researchers found that applying US waves improved gel penetration into the affected tissue, enhancing its effectiveness. As a result, edema and inflammatory cells decreased, and collagen fibers reorganized, restoring the tensile strength of the affected tissue [48]. Subramanian et al. also demonstrated the impact of Aloe vera on promoting wound contraction and collagen synthesis, attributing it to the presence of mannose 6-phosphate (M6P) in Aloe vera leaf gel. Products enriched with mannose have been shown to enhance macrophage activation, thereby stimulating fibroblast activity and collagen synthesis [49]. In our study, Aloe vera-treated skin underwent epidermal regeneration with cell-layer formation, and after two weeks, the wound was nearly healed, although healing in this group was slower than in the other groups.

4. Conclusions:

The results of this study show that using both ultrasound waves (US) and zinc oxide nanoparticles (ZnO Nps) can help improve skin damage in male mice. Previous research did not clearly demonstrate the positive effects of these factors on skin damage, but combining US radiation and ZnO NPs has been shown to improve wound healing in mice. This approach increases collagen and elastin in the skin's connective tissue and also positively affects epithelial thickness.

Conflict of interest

The authors declare no conflicts of interest regarding this study. All authors have contributed to the research and writing processes with complete integrity and transparency.

Using artificial intelligence chatbots

There was no use of artificial intelligence in the making of this article.

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