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Research Paper

Combination effect of low-level laser and orthokine for improving wound healing: In vitro study

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ABSTRACT

Wound healing is a complex biological process that relies heavily on fibroblast viability, oxidative balance, and regulated cell proliferation. Low-level laser therapy (LLLT) and autologous conditioned serum (ACS), under the tradename of Orthokine, have each been reported to modulate inflammation and support tissue repair, yet their combined cellular effects remain insufficiently defined. This study aimed to evaluate the individual and combined effects of these compounds on the human dermal fibroblast (HDF) cell line. The HDF cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS). Following this, the cell line was treated with LLLT doses (2, 5, 7, and 10 J/cm²) and Orthokine concentrations (1, 2, 3, 5, and 10% v/v). Viability and proliferation were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Reactive Oxygen Species (ROS) measurement, apoptosis, and cell cycle phase analysis were performed by flow cytometry. Results showed that cell viability following Orthokine treatment increased in a dose- and time-dependent manner, and the highest effects were observed at 5% and 10%. LLLT treatment also enhanced viability. The combination of 5% Orthokine with 5 J/cm² LLLT produced the most notable effects, improving viability, decreasing apoptosis, and increasing S-phase frequency. LLLT and Orthokine positively influence fibroblast function for wound healing. Their combination improved cell viability, preventing apoptosis and promoting cell cycle progression. This approach may contribute to the development of more effective, non-invasive wound healing therapies.

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1. Introduction

Skin, the largest organ of the human body, serves as a crucial protective barrier and constitutes one of the body's primary lines of defense. As a part of the integumentary system, the skin consists of a complex interplay of cells and structures that protect the body against environmental threats and maintain homeostasis. Structurally, skin consists of three layers: the epidermis, the dermis, and the subcutaneous tissue [1,2]. The skin serves as a vital protective barrier, and any damage can lead to various problems, including increased infection risk, impaired homeostasis, and delayed healing. Severe trauma, such as major burns, results in significant disability and approximately 180,000 global deaths each year. Such injuries trigger local and systemic inflammatory responses that hinder healing and induce hemodynamic changes [3]. Wound healing involves the coordinated actions of cell types, growth factors, and cytokines to re-establish tissue integrity and facilitate wound closure. Due to its role as the body's primary barrier, the skin is particularly vulnerable to injury. Skin wounds represent a major public health issue, often associated with significant morbidity, pain, functional impairment, decreased mobility, and adverse psychological outcomes such as depression, anxiety, and social withdrawal [4].

A wound compromises the integrity of the epithelial tissues and can impact the underlying layers, such as the dermis, fascia, and muscle [5]. Wound healing is a complex and dynamic process that involves the coordinated interactions of cellular, molecular, and humoral mechanisms [6]. Wound healing involves three interrelated phases: hemostasis, or inflammation, proliferation, and remodeling. While the phases are executed in sequence, they overlap significantly. Dysregulation in any of these phases can lead to aberrant wound healing, resulting in issues such as excessive wound healing (hypertrophic scars and keloids) or chronic wounds (ulcers) [7].

The first phase, hemostasis, begins immediately after injury to control bleeding through vascular constriction, platelet aggregation, and fibrin clot formation. Platelets release key growth factors like Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF), and Transforming Growth Factor-beta (TGF-β). Once bleeding is controlled, inflammation begins with immune cell infiltration, guided by cytokines such as IL-1, to clear bacteria and debris. This phase supports the activation of keratinocytes and fibroblasts, as well as angiogenesis.

However, prolonged inflammation can result in chronic wounds due to elevated pro-inflammatory cytokines and matrix metalloproteases that degrade growth factors. In the proliferation phase, epithelial cells migrate and divide, new vessels form, and fibroblasts produce collagen and Extracellular matrix (ECM) components to build granulation tissue. Some fibroblasts differentiate into myofibroblasts, which contract the wound. Finally, during remodeling, granulated tissue is replaced by collagen and elastin fibers, forming scar tissue. TGF-β mediates new collagen synthesis, while PDGF helps break down the old collagen. Vascular density normalizes, and the ECM is reorganized to restore tissue architecture—a process that may continue for years [8,9].

Fibroblasts are crucial in wound healing and play a central role in tissue repair and fibrosis. They are the most common cells in the body's connective tissue, responsible for producing, depositing, and integrating ECM proteins. Beyond their direct functions in producing and maintaining the ECM, fibroblasts are also critical to other processes, such as inflammation. In the skin, fibroblasts meet the high demand for structural resiliency to repair daily wear and tear and significant wounds.

Fibroblasts have several important roles throughout the healing process of wounds. They synthesize and remodel ECM proteins and regulate matrix turnover by producing enzymes that cross-link ECM proteins or promote or inhibit their degradation [10]. Fibroblasts release specific proteolytic enzymes, such as collagenase (Matrix Metalloproteinase-1 (MMP-1)), gelatinase (MMP-2 and MMP-9), and stromelysin (MMP-3), which facilitate their movement within the extracellular matrix. Various factors, including PDGF and TGF-β, regulate their migration to the wound core and subsequent activity [11].

Following an injury, fibroblasts are recruited to the wound site around days 5–7. They contribute to wound contracture by differentiating into contractile myofibroblasts under the influence of TGF-β and mechanical tension. In the remodeling phase, fibroblasts further refine the matrix by replacing collagen type III with type I, strengthening the tissue and forming mature scar architecture. While fibroblasts are key contributors to the repair process and formation of new tissue following damage, excessive ECM deposition can lead to scarring and loss of tissue function, resulting in fibrosis [10]. Although more than 5,000 wound care products are currently available (mainly in the form of dressings), there remains a strong demand for more effective therapies. Treatments that can reduce hospitalization time and costs while also supporting patients' psychological well-being are sought after [12]. Innovative methods are being explored to pave the way for more effective healing solutions in patient care. This study aimed to contribute to the development of advanced wound treatment strategies by evaluating two approaches rooted in regenerative medicine and biophysics: ACS and LLLT.

Photobiomodulation, also termed LLLT, is a non-invasive and effective approach for managing a wide array of medical conditions and rehabilitating injuries. It utilizes low fluences (0.04–50.00 J/cm²) and power densities (<100 mW/cm²). The efficacy of LLLT depends on using the correct settings, as improper choices can lead to adverse outcomes [13]. LLLT exerts

its effects by initiating photochemical reactions within mitochondria. It enhances repair by stimulating photochemical reactions in mitochondria through photon absorption by cytochrome c oxidase, an essential enzyme in the mitochondrial respiratory chain. This process enhances electron transport and Adenosine Tri phosphate (ATP) synthesis while simultaneously generating reactive oxygen species (ROS). This process triggers various signaling pathways, activating transcription factors that promote protein synthesis, cell proliferation, and anti-inflammatory responses. Beyond these cellular effects, LLLT can also stimulate stem cell activity, fostering enhanced tissue repair and regeneration processes [14]. This technology has been shown to effectively reduce wrinkles, acne scars, and hypertrophic scars in dermatology [13]. Furthermore, LLLT is employed in the treatment of a wide range of wounds, including, but not limited to, burns, traumatic amputation injuries, surgical skin grafts, infected wounds, and entrapment wounds [15].

ACS, under the Orthokine trademark, is a regenerative treatment made by incubating whole blood with glass spheres. This results in an increase in the concentration of anti-inflammatory cytokines, including IL-1ra, and several growth factors, PDGF and TGF-β1, in the liquid blood phase [16]. This, in turn, can lead to reduced pain and enhanced functional outcomes and has been studied mainly in inflammation and tissue damage in musculoskeletal conditions [17]. Some studies have explored the potential of ACS as a regenerative substance for facial skin revitalization and rejuvenation, significantly increasing the skin's mechanical properties [18].

The high levels of cytokines and growth factors in ACS suggest that it may improve wound healing and enhance the skin's overall appearance and health.

This study investigates the combined effects of LLLT at 685 nm and Orthokine treatment on the HDF cell line to identify new strategies to enhance cellular regeneration and healing. These advancements could improve recovery for patients with skin injuries, surgical wounds, and age-related conditions, leading to optimized treatment protocols and better patient care in clinical settings.

2. Materials and Methods

2.1 *Materials*

The HDF cell line was obtained from the National Center for Genetic and Biological Resources of Iran. Cells were cultured in DMEM (Gibco, Germany) supplemented with fetal bovine serum (10%) and penicillin-streptomycin (1%) in a CO_2 incubator at 37°C. Orthokine was prepared using the EOT Orthokine kit, with whole-blood samples incubated for eight hours and centrifuged to collect serum. Orthokine was applied at concentrations of 1-50% (v/v).

LLLT was delivered at 685 nm with fluences of 2, 5, 7, and 10 J/cm². MTT reagent (0.5 mg/mL, Sigma, USA) and 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) were used for viability assays and reactive oxygen species detection, respectively.

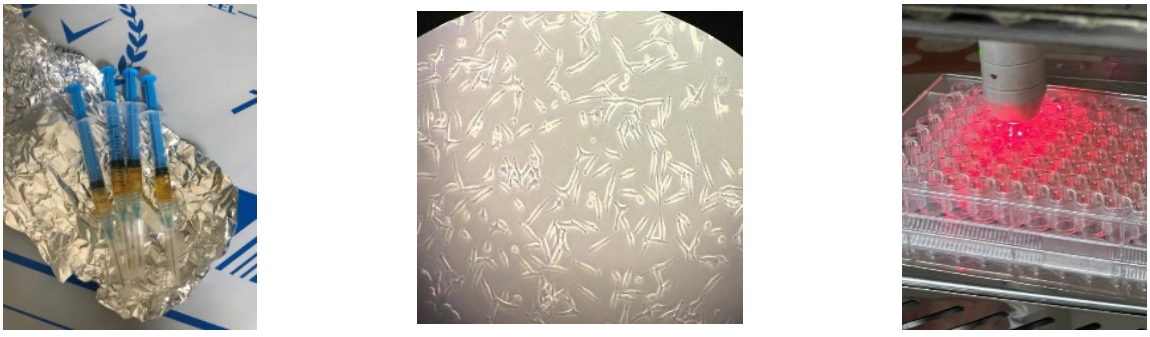
Flow cytometric analysis was performed using standard equipment, and data were analyzed with FlowJo software. Statistical comparisons were conducted with t-tests and ANOVA.

2.2 *Cell culture*

The HDF cell line was cultured in DMEM medium supplemented with 10% FBS and 1% Penicillin-Streptomycin (PEN/STREP) (0.5 mL). The cells were incubated at 37°C with 5% CO_2. The culture medium was replaced every 24 hours. After reaching the third passage, the cells were treated with LLLT and Orthokine. The cells were confirmed to be free of mycoplasma contamination prior to experimentation.

2.3 *Orthokine Treatment*

One blood sample (10 cc of blood was taken from a 30-year-old woman) was transferred into an Orthokine kit with chromium sulfate ($CrSO_4$)-coated glass beads and incubated for 8 hours. The sample was centrifuged at 3000 RPM for 15 minutes, and the Orthokine serum was separated, aliquoted into smaller syringes, and then stored in a freezer at -20°C (Figure 1). For treatment, cells were cultured and incubated for 24 hours at 37°C with 5% CO_2.



(a) (b) (c)

Figure 1. a: The prepared syringes containing ACS derived from a clinical blood sample. b: low-level laser irradiation treatment at 685 nm to the cells in a 96-well plate. c: The cultured HDF cell line was observed to adhere to the bottom of the flask under light microscopy.

Orthokine was added at concentrations of 1, 2, 3, 5, 10, 15, 25, and 50% (v/v) in a 96-well plate (10000 cells per well). After treatment, the cells were incubated for 24 or 48 hours to evaluate the cellular response over time. For treatment, cells were cultured and incubated for 24 hours at 37°C with 5% CO_2. Orthokine was added at concentrations of 1, 2, 3, 5, 10, 15, 25, and 50% (v/v) in a 96-well plate (10000 cells per well). After treatment, the cells were incubated for 24 or 48 hours to evaluate the cellular response over time.

2.4 *Treatment of Low-Level Laser*

Low-level laser irradiation at 685 nm was applied to the HDF cell line with adjusted dose, power, and spot size settings. Cells cultured for 24 hours were moved to a biosafety hood, and laser doses of 2, 5, 7, and 10 J/cm² were applied to specified wells in a 96-well plate, excluding the first row. After exposure, the cells were incubated for 24 or 48 hours to evaluate the cellular response over time.

2.5 *Combined Orthokine and low-level laser irradiation*

After incubating the cells for 24 hours and then washing with Phosphate-Buffered Saline (PBS), various ACS concentrations (1%, 2%, 3%, 5%, 10%, 15%, 25%, and 50% (v/v)) were added, followed by a 24-hour incubation. Subsequently, cells were exposed to low-level laser irradiation at doses of 2, 5, 7, and 10 J/cm². After 24 hours, cell viability was assessed using the MTT assay. Further flow cytometry analyses were conducted, including ROS production, apoptosis, and cell cycle assessment.

2.6 *MTT Assay*

The MTT assay is a colorimetric test used to assess cell viability and proliferation. In this study, MTT assays were performed to determine the viability and proliferation of the HDF cell line following treatment. The culture medium was removed, and 200 µL of fresh medium containing 0.5 mg/mL MTT reagent was added to each well. Plates were incubated at 37°C for 2–4 hours. The insoluble formazan crystals were dissolved in Dimethyl Sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate Enzyme-Linked Immunosorbent Assay (ELISA) reader with a reference wavelength at 630. The number of technical and biological replicates was 5.

2.7 *Measurement of the Intracellular ROS*

Cells were washed with PBS and centrifuged at 1500 rpm for 5 minutes. The pellet was resuspended in 400 µL PBS. To assess ROS levels and compensate for spectral overlap between DCFH-DA and PI, four sample groups were prepared: unstained, DCFH-DA only, PI only, and dual-stained. Cells were incubated with 5 µL DCFH-DA at 37°C for 45 minutes. After washing with PBS and centrifugation, pellets were resuspended in 500 µL PBS, and 3 µL PI was added to the relevant tubes before flow cytometric analysis. The model and manufacturer of the flow cytometer was the BD FACS Calibur (Figure 4).

2.8 *Quantification of Apoptosis*

Cells were washed and resuspended in 500 µL of 1X Binding Buffer. For Annexin V/PI staining, four tubes were prepared (unstained, Annexin V-FITC, PI only, and dual-stained). Cells were incubated with 5 µL Annexin V-FITC at 4°C in the dark for 15 minutes, followed by washing and addition of 3 µL PI. Samples were analyzed by flow cytometry(Figure 5).

2.9 *Cell Cycle S-phase Analysis*

Cells were fixed in 70% cold ethanol (dropwise into 50 µL PBS) and stored at 4°C for 2 hours with gentle shaking to prevent clumping. After fixation, cells were washed with PBS and stained with PI Master Mix (40 µL PI, 10 µL RNase A, 950 µL PBS; freshly prepared). Samples (5×10^5 cells/mL) were incubated at room temperature for 30 minutes, then analyzed by flow cytometry. Data were processed using FlowJo v. 7.6.1 software (Figure 6).

2.10 *Statistical Analysis*

Data analysis was performed using Excel (version 16.0). All data were shown as the mean ± standard deviation. Analysis of variance (ANOVA) and t-tests were used to compare the treated and control groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 *Cell Culture*

The HDF cell line was cultured in DMEM medium with 10% FBS (Figure 1). After the third passage, the cells were treated individually and in combination with low-level laser irradiation and Orthokine.

3.2 *Orthokine treatment*

Cell viability following Orthokine treatment at various concentrations (1, 2, 3, 5, 10, 15, 25, and 50% v/v) was assessed at 24 and 48 hours.

The highest viability after 24 hours was observed with Orthokine 5% v/v treatment, resulting in approximately a 62.1% increase in cell viability. The 10% v/v Orthokine treatment showed the most significant increase in viability at 48 hours, with a 118.7% increase compared to the control group. All experiments were performed in triplicate, and statistical significance was considered at $P < 0.05$.

3.3 *low-level laser irradiation treatment*

Cell viability after treating the HDF cell line with low-level laser irradiation at energy densities ranging from 1

to 20 J/cm² was assessed over 24- and 48-hour intervals. Based on the viability results, the doses of 2, 5, 7, and 10 J/cm² were selected for subsequent combination experiments with Orthokine. The maximum increase in viability was observed at 10 J/cm² in both 48 hours and 24 hours after the treatment, with 113.3% and 16% increases, respectively (Figure 2). All experiments were performed in triplicate, and statistical significance was considered at $P < 0.05$.

3.4 *Combination treatment*

Effective low-level laser irradiation (2, 5, 7, and 10 J/cm²) and optimal Orthokine concentrations (1, 2, 3, 5, and 10% v/v) were selected for combination therapy to assess potential new protocols in treatment.

Among all combinations, 5% Orthokine with 5 J/cm² low-level laser irradiation showed the highest viability (115.3%) compared to the control group(Figure 3).

This combination also reduced ROS generation(Figure 4) by 1.8% (not statistically significant) and significantly decreased apoptosis by approximately 55% (Figure 5).

Additionally, the S-phase frequency increased by 31.7% (Figure 6).

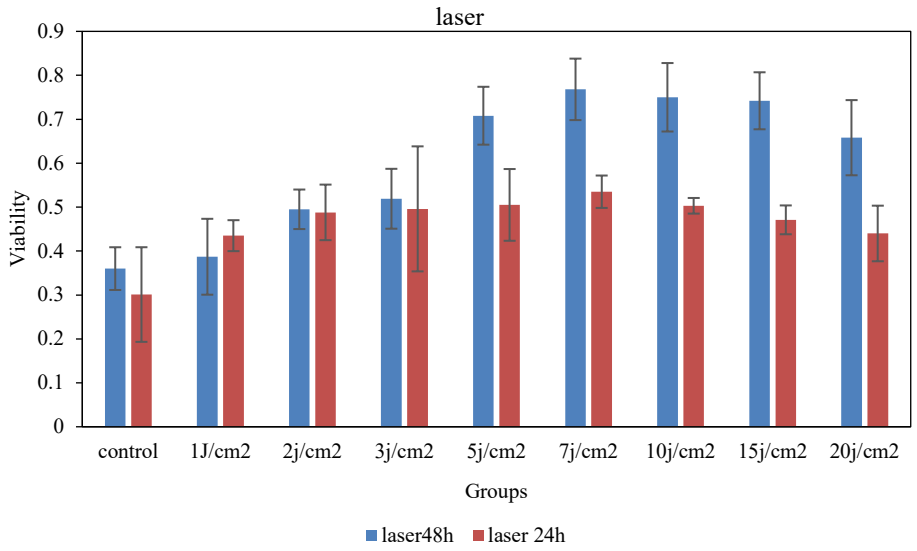


Figure 2. Effects of low-level laser irradiation at different energy densities (1, 2, 3, 5, 7, 10, 15, and 20 J/cm²) on HDF cell viability after 24 and 48 h. Based on the viability findings, selected doses of 2, 5, 7, and 10 J/cm² were used for subsequent combination experiments with Orthokine. Statistical significance was considered at $P < 0.05$.

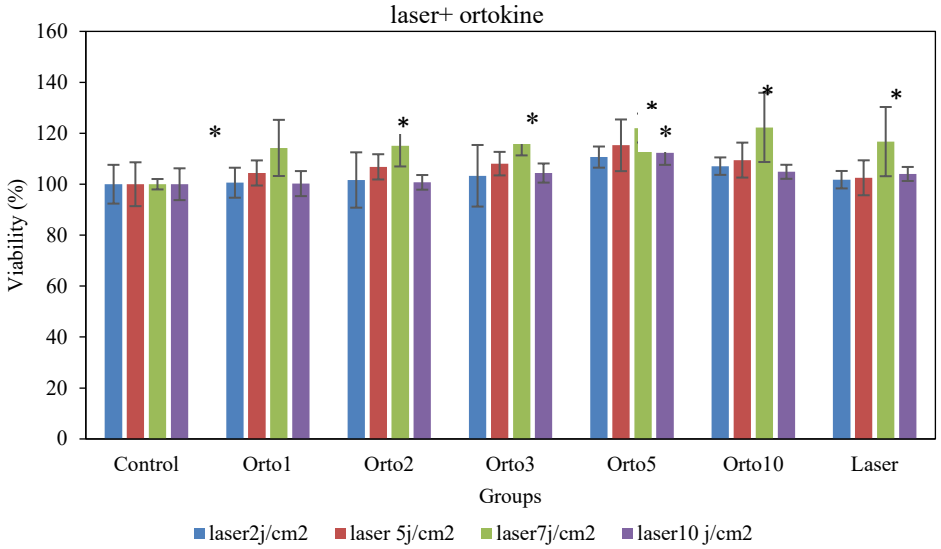


Figure 3. Combination therapy of various dosages of Orthokine (1, 2, 3, 5, 10)% v/v and laser exposure with fluences of 2, 5, 7, and 10 J/cm², P value < 0.05.

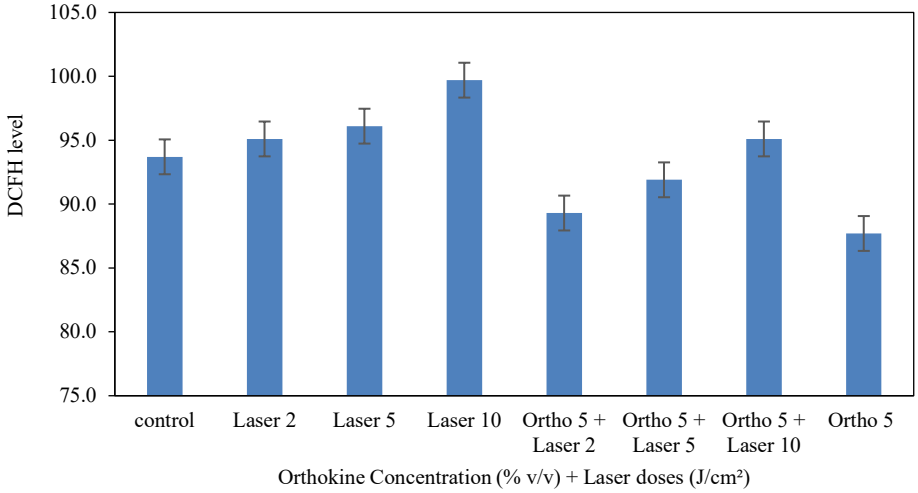


Figure 4. ROS production induced by Orthokine 5% v/v and laser exposure with fluences of 2, 5, and 10 J/cm² (P value < 0.05)

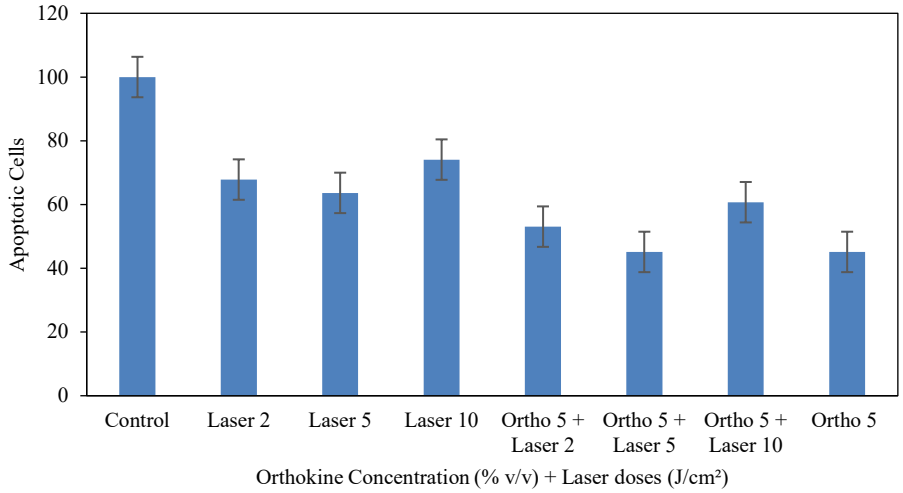


Figure 5. Percentage of apoptotic cells in the HDF cell line treated with Orthokine at a 5% (v/v) concentration and laser exposure with fluences of 2, 5, and 10 J/cm² doses, P value < 0.05.

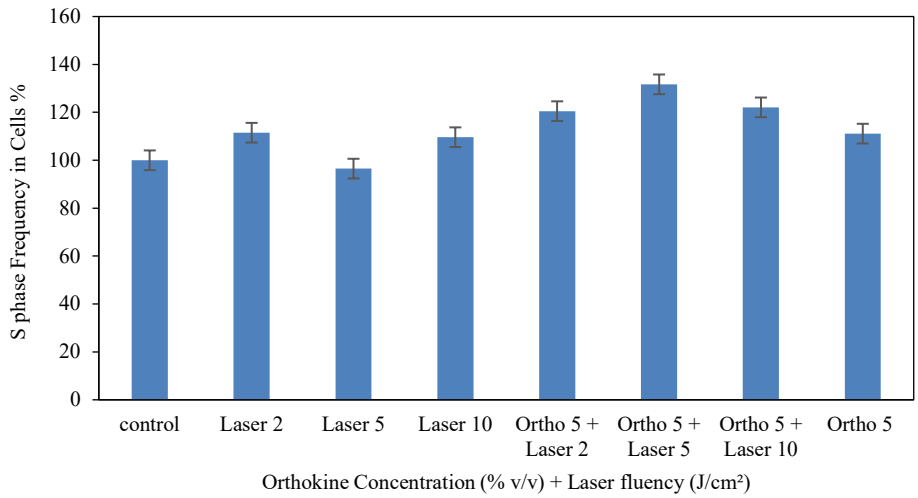


Figure 6. Comparative S-phase frequency in the HDF cell line with different treatments of Orthokine (5% v/v) and laser exposure with fluences of 2, 5, and 10 J/cm², P value < 0.05

4. Discussion

This study aimed to investigate the effects of low-level laser irradiation at 685 nm and Orthokine on the HDF cell line. The results indicate that low-level laser irradiation and Orthokine modulate cellular behavior, with potentially therapeutic implications in tissue repair and anti-inflammatory applications.

The MTT assay revealed that the combined treatment, particularly with a laser dose of 5 J/cm² and an Orthokine concentration of 5% v/v, resulted in a notable increase in cell viability compared to control groups and, in some instances, individual treatments. This observation implies that both fibroblast cell line survival and proliferation are notably improved, which are critical processes in tissue repair and regeneration. Fibroblasts are integral to wound-healing processes, engaging in extracellular matrix synthesis, wound contraction, and interactions with immune cells, depending on their specific subtype and anatomical localization [19]. Huiling Ma et al. found that LLLT at 60 J/cm², delivered with either an 830 nm or a 635 nm/830 nm laser, increases fibroblast proliferation and collagen synthesis [20]. Similarly, Gholian *et al.* demonstrated that ACS substantially reduces wound surface area and improves healing scores in patients with chronic wounds compared with traditional saline dressings [21]. The 5 J/cm² dose, effective in the primary study's fibroblast cell line, is corroborated by studies on hair follicle stem cells (HFSC), which are essential for tissue regeneration and hair growth [22]. Naderi *et al.* demonstrated that LLLT at 685 nm optimally increased rat HFSC viability to 5 J/cm² after 48 hours of irradiation. Similar results were observed in human HFSC, where MTT assays revealed maximal cell viability at 5 J/cm² across doses from 1–20 J/cm² [23]. These findings collectively establish 5 J/cm² as a consistently optimal dose for cellular biostimulation and proliferation relevant to regenerative medicine and cell-based therapies.

Furthermore, the analysis of ROS levels shows a notable increase in intracellular ROS at higher laser dosages (10 J/cm²). This suggests a dose-dependent oxidative stress response. Moderate ROS levels serve as secondary messengers to facilitate healing and angiogenesis, but excessive ROS can compromise cell integrity [24]. The highest ROS production (99.7%) was observed at 10 J/cm². By contrast, Orthokine administration alone, or in combination with lower laser doses (2–5 J/cm²), effectively mitigated ROS accumulation. The combined treatment with a laser dose of 5 J/cm² and 5% v/v Orthokine reduced ROS levels to 91.9%. These findings may suggest a potential redox-modulating role for Orthokine, although some ROS-related changes were not statistically significant. Similarly, in an evaluation of human skin fibroblast cells (Hu02), Naderi *et al.* reported that low-level laser irradiation at 660 nm significantly increased intracellular ROS levels in a dose-dependent manner at 1, 5, and 10 J/cm² [25]. Mohamed et al. found that LLLT effectively reduced ROS and enhanced antioxidant defenses in rats with carbon tetrachloride-induced liver fibrosis [26]. This is consistent with the redox-modulating effect of Orthokine observed in our study. Shirokova et al. reported that ACS significantly reduced conjugated dienes (ROS footprints) in synovial fluid, with a 35.3% decrease after one month and a 53.5% decrease after three months [27].

The apoptosis assay further supported the regenerative potential of the combined therapy. The lowest levels of apoptotic cells were observed in the group treated with 5 J/cm² low-level laser irradiation and 5% v/v Orthokine, with only 32.4% apoptotic cells. This indicates a protective effect of the combined treatment against cell death. The laser's biphasic dose response was again evident, as higher doses (10 J/cm²) reversed this protective effect and elevated apoptosis rates. The biphasic dose response of LLLT may be explained by mitochondrial photobiomodulation mechanisms. Moderate laser doses can stimulate cytochrome c oxidase activity, enhance ATP production, and promote controlled ROS signaling involved in proliferation and tissue repair. In contrast, excessive laser doses may induce oxidative stress and mitochondrial dysfunction, leading to increased apoptosis and reduced cellular viability. Chu et al. concluded that LLLT protects endothelial cells from TNF-α- and cycloheximide-induced apoptosis. This effect results from LLLT's ability to reduce caspase activation, suppress the p38 Mitogen-Activated Protein Kinase (MAPK) pathway, inhibit Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, and suppress inducible nitric oxide synthase expression [28]. Spinello et al. discovered that IL-1Ra reduces apoptosis by directly interacting with and non-competitively inhibiting initiator caspases-8 and -9, preventing the formation of the active dimeric structures and blocking the apoptotic cascade [29].

Cell cycle analysis revealed a significant increase in the proportion of cells in the S phase in the group treated with 5 J/cm² low-level laser irradiation and 5% v/v Orthokine, reaching 131.6%. This suggests an enhanced rate of DNA synthesis and cellular proliferation.

The use of Orthokine in this study is supported by its known anti-inflammatory properties, primarily attributed to the Interleukin-1 Receptor Antagonist (IL-1Ra). The Interleukin-1 (IL-1) is a key pro-inflammatory cytokine implicated in cartilage degradation in osteoarthritis. By blocking IL-1 effects, Orthokine can modulate the inflammatory response in various conditions, including wound healing. LLLT has also shown anti-inflammatory effects and can influence cellular metabolism. The enhanced effects observed with the combined treatment may be related to the complementary actions of LLLT, which stimulates cellular activity, and Orthokine, which modulates inflammation, creating an environment conducive to fibroblast cell line proliferation and survival. By

exploring these innovative methods, this study aimed to unlock new potential for effective wound management and demonstrate their transformative impact on patient care. The primary limitation of this study is its reliance on an in vitro model using the HDF cell line. While this model allows precise assessment of dose-dependent effects on cell viability, oxidative stress, apoptosis, and cell cycle dynamics, it does not reflect the full biological complexity of clinical wound environments. The controlled laboratory conditions exclude key systemic and tissue-level factors in wound healing, such as immune cell migration, vascular interactions, and the full process of long-term tissue remodeling seen in vivo. The analysis is limited to measurements of viability, ROS production, apoptosis, and S-phase frequency. Further investigation into the molecular mechanisms and signaling pathways modulated by combined laser and Orthokine therapy is warranted to establish evidence-based therapeutic protocols and optimize clinical translation. The clinical relevance and broader efficacy of this combination therapy need further investigation. This study aimed to evaluate the individual and combined effects of LLLT and Orthokine on the HDF cell line. LLLT, a noninvasive treatment that uses light to promote cellular regeneration, stimulates mitochondrial activity, enhancing ATP production and promoting tissue repair. ACS, derived from autologous blood, contains elevated levels of anti-inflammatory cytokines and growth factors that facilitate healing and reduce inflammation. The combination of Orthokine with LLLT produced the most significant effects, improving cell viability, reducing oxidative stress, and decreasing apoptosis. Additionally, the combination promoted cell cycle progression, indicating enhanced cell proliferation. This combination treatment may represent a promising experimental approach for improving wound healing responses in vitro. By improving fibroblast cell line function, reducing inflammation, and enhancing tissue regeneration, further in vivo and clinical studies are required before potential clinical application. These findings highlight the therapeutic potential of combining biophysics and regenerative medicine to enhance tissue repair and optimize healing outcomes, with further research needed to refine treatment parameters and evaluate clinical applicability.

Declaration of artificial intelligence (AI) in the writing process

The authors declare whether AI or AI-assisted technologies were used during the preparation of this manuscript. If used, AI tools were employed solely to improve language quality, grammar, readability, and organizational structure. The authors carefully reviewed and edited all AI-generated content and take full responsibility for the accuracy, integrity, and originality of the final manuscript. No AI tool was used to generate, analyze, or interpret scientific data or images or to draw scientific conclusions. The use of AI-assisted technologies complies with current publication ethics recommendations and journal policies.

Authors' contributions

MSN: Study conception, design, and supervision. DKJ and MH: Data collection and investigation. NA: Writing and original draft preparation. MSN and BK and HM: Critical revision. All authors read and approved the final version of the manuscript.

Conflict of interest

No potential conflict of interest was reported by the authors.

Ethical declarations

Patient confidentiality was strictly maintained throughout the study. Due to the retrospective design and the use of de-identified data from medical records, informed consent was waived. The study protocol was approved by the Institutional Ethics Committee of Guilan University of Medical Sciences (approved code: IR.GUMS.REC.1400.464).

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