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# In Vitro Synergistic Effects of Ciprofloxacin, Vitamin E, And Low Power Laser on Human Dermal Fibroblasts

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## ABSTRACT

**Background and objectives:** Human Dermal Fibroblasts (HDF) are involved in the production of the extracellular matrix, formation of the connective tissue, and wound healing. Considering the role of ciprofloxacin in the treatment of skin infections and the subsequent oxidative stress as well as the protective effects of vitamin E and low power laser against inflammation and oxidative stress, we evaluated combined effects of low power laser and vitamin E on inflammation and oxidative stress in HDF cells treated with ciprofloxacin.

**Methods:** Morphology of the cells was studied using an inverted microscope. Viability of the cells was assessed using the MTT assay, and the concentration of reactive oxygen species was determined after exposure of the cells to ciprofloxacin (5, 25, 50, 75, and 100 µg/ml), vitamin E (1 mg/ml), and low power laser (660 nm; power density: 30 $mW.cm^{-2}$).

**Results:** The survival rate of the cells increased significantly after the treatment with ciprofloxacin, vitamin E, and low power laser compared with the cells treated with ciprofloxacin and vitamin E ($p$<0.001). The amount of reactive oxygen species increased in the treated cells when compared with those only treated with ciprofloxacin and vitamin E.

**Conclusion:** The low power laser treatment has favorable effects on the growth of HDF cells, which can be beneficial for wound healing, even in the presence of ciprofloxacin.

**Keywords:** Ciprofloxacin, Vitamin E, Lasers, Reactive Oxygen Species.



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## INTRODUCTION

The skin is the largest tissue in the vertebrate body and has many functions, including heat regulation, directing the senses, and acting as a mechanical barrier to protect the body against the invasion of microorganisms and harmful environmental factors such as radiation, mechanical damage, and burns _(1). Ciprofloxacin (CFX) is a second-generation fluoroquinolone antibiotic that was introduced to the clinical treatment system in 1987. This antibiotic was produced by substituting the N-1 ethyl group of norfloxacin with the cyclopropyl group, which creates a wide range of antibacterial properties (2). It has been widely used for treatment of various human infections (3). In addition to the antimicrobial properties, fluoroquinolones, especially CFX, have a variety of effects on eukaryotic cells in vitro (4). This drug can affect mammalian topoisomerase II, degrade mitochondrial DNA replication proteins, and thus induce a gradual decrease in mitochondrial DNA content in CFX-treated cells (5). Research has shown that CFX induces apoptosis by inhibiting cell cycle and cell proliferation (6). Some studies have also shown that CFX induces apoptotic, cytotoxic, and oxidative effects in tumors at different levels (7).

Low power laser therapy was first proposed as a treatment method by Mester et al., who showed the positive effects of low energy ruby laser (1 J/cm$^2$) on wound healing. This finding has attracted the attention of scientists for understanding this technology and its applications (8). In the study of Mester et al., the power of the device was between $3^{-10}$ to $1^{-10}$ W, and the doses used in the wound healing process were between $10^{-2}$ and $10^2$ J/cm$^2$ (8). In low power lasers, the thermal changes are small and imperceptible. For this reason, low power lasers are also referred to as low-intensity lasers or cold lasers. These lasers cause biological changes without generating heat. Recent light-based skin therapies are more focused on low power lasers (9). Moreover, the effects of laser radiation at the cellular and molecular levels have been demonstrated by various studies. To improve wound healing, non-surgical lasers have been used as tissue stimulators (10). Lasers with different wavelengths have different effects on fibroblasts as well as anti-inflammatory and analgesic effects (11).

Considering the role of fluoroquinolones in oxidative stress and the role of low power lasers as well as vitamin E in reducing inflammation and oxidative stress, in this study, for the first time, combined effects of CFX, vitamin E, and low power laser on human skin fibroblast cells was studied.

## MATERIALS AND METHODS

*Cell culture:* The Human Dermal Fibroblasts (HDF) cell line was provided by the Cell Bank of the Iranian Biological Resource Center (Tehran, Iran). The cells were propagated in 25 ml plastic flasks containing Dulbecco's Modified Eagle Medium (Sigma-Aldrich, USA) supplemented with 10% heat-inactivated fetal bovine serum, and 10 µg/ml penicillin-streptomycin. The cells were incubated at 37 °C with 5% $CO_2$ and 80% humidity. The culture medium was replaced with fresh medium three times a week. Cell counting was performed using a haemocytometer (HGB, Germany) (12).

*In vitro laser irradiation*

Irradiation was performed with a red light source (660 nm; power density: 30 mW.cm$^{-2}$) using a low power laser device (Thor LLLT, HILARIS® laser FL, Austria). The plates were divided into a control group (no irradiation) and a test group that was irradiated with low-level laser (2 J/cm$^2$ for 30 seconds). Irradiation was carefully timed and carried out in a dark laminar flow hood (NU-140 Vertical, AireGard, Malaysia) (13).

*Morphological studies of HDF cells*

The cells were studied using an inverted microscope (ZEISS, Germany), and the morphological characteristics of these cells were analyzed before and after treatment with CFX, vitamin E, and low power laser.

*MTT assay*

The MTT assay was performed to measure cytotoxic effects on the HDF cells. For this purpose, 3× $10^4$ cells were seeded in a 96-well plate, and the plate was incubated overnight. Culture medium was replaced with fresh medium, and the cells were treated with different concentrations of CFX (5, 25, 50, 75, and 100 µg/ml), and vitamin E (1 mg/ml) for24, 48, and 72 hours. The test group was irradiated with low power laser (2 J/cm$^2$) for 30 seconds. Then, 5 µl of MTT stock solution (5 mg/ml) were added to each well. After 1 hour of incubation at 37 °C and observation of



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the purple color precipitates under the microscope, the medium was removed carefully. Then, 100 µl of dimethyl sulfoxide (Merck, Germany) were added to each well to dissolve the formazan crystals, and the plate was incubated at room temperature for 2 hours. Finally, absorbance was read at 570 nm using a microplate reader (Bio-Rad, USA). The assay was performed in triplicate for each concentration, and the cell viability was determined using the following formula (14):

$$\text{Viability}=\frac{\text{OD Sample}-\text{OD Blank}}{\text{OD Negative control}-\text{OD Blank}}$$

*Measurement of reactive oxygen species (ROS) in HDF cells using flow cytometry*

The intracellular accumulation of ROS following the treatments was measured by the 7.2-dichlorofluorescein diacetate (DCFH2-DA) assay. For this purpose, $1\times10^6$ HDF cells were seeded in 6-well plates.

Then, the cells were treated with CFX (5, 25, 50, 75, and 100 µg/ml) and vitamin E (1 mg/l) for 24, 48, and 72 hours, and then were irradiated with a low power laser at 660 nm for 30 seconds. The cells were detached and then incubated with 2 mM DCFH2-DA for 45 minutes in the dark. Then, the cells were washed with phosphate buffer saline and transferred to a flow cytometer (BD Facscalibur, Becton Dickinson, USA) for measuring the level of ROS. Data were analyzed with FlowJo 7.6.1 software (15).

*Statistical analysis*

The tests were repeated three times, and the results were reported as mean ± standard deviation. The obtained data were statistically analyzed using GraphPad InStat software (version 8, La Jolla, USA). One-way analysis of variance (ANOVA) was used for statistical analysis. A *p*-value of less than 0.05 was considered statistically significant.

## RESULTS

*Morphological characteristics of HDF cells*

Figure 1 shows the morphology of HDF cells after the treatment with CFX (5 and 25 µg/ml) and vitamin E (1 mg/l) without laser irradiation for 48 hours. The results showed that the rate of cell growth and survival was reduced compared with the control group. The treatment of the HDF cells with CFX (100 µg/ml), vitamin E (1 mg/l), and low power laser (1 J/cm$^2$, 660 nm for 30 seconds) led to cell proliferation. However, the highest rate of survival was observed after the treatment of the cells with CFX (100 µg/ml), vitamin E (1 mg/l), and low power laser (1 J/cm$^2$, 660 nm for 30 seconds) for 48 hours.

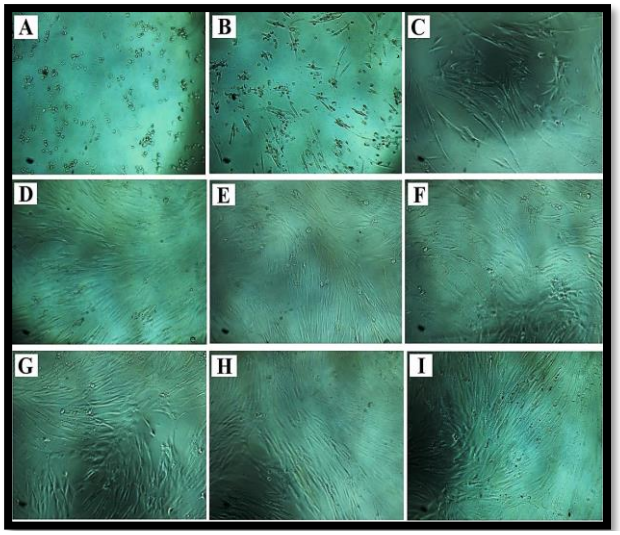


**Figure 1- Morphology of the HDF cells after the treatment with CFX, vitamin E, and low power laser**



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A, B, and C show the morphology of the cells after the treatment with CFX (5 and 25 µg/ml) and vitamin E (1 mg/l) without laser irradiation for 48 hours, respectively. D, E, and F show the morphology of the cells after the treatment with CFX (100 µg/ml), vitamin E (1 mg/l), and low power laser (1 J/cm$^2$, 660 nm for 30 seconds) for 24 hours, respectively. G, H, and I show the morphology of the cells after treatment with CFX (100 µg/ml), vitamin E (1 mg/l), and low power laser (1 J/cm$^2$, 660 nm for 30 seconds) for 48 hours, respectively.

*Results of the MTT assay*

The survival rate of HDF cells changed significantly after the treatment with CFX, vitamin E, and low power laser compared with the cells treated only with CFX and vitamin E ($p$<0.001). As shown in figure 2, after 24 hours of treatment, the survival rate of the cells treated with 100 µg/ml CFX and low power laser was higher than that of the negative control group ($p$<0.01). After 48 hours of treatment, the survival rate of the cells treated with 50 and 75 µg/ml of CFX and low power laser was higher than that of the negative control group ($p$<0.01). However, the survival rate decreased after treatment with 100 µg/ml of CFX and low power laser. After 72 hours of treatment, the survival rate of the cells treated with 75 µg/ml of CFX and low power laser was higher than that of the negative control group ($p$<0.01).

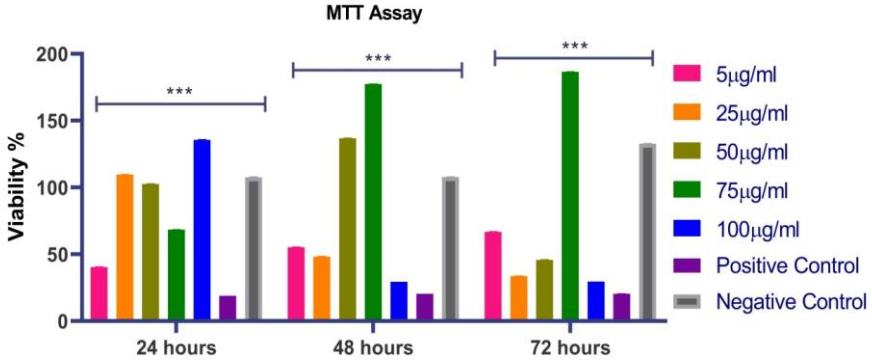


**Figure 2-Survival (%) of HDF cells after the treatment with CFX, vitamin E, and low power laser for 24, 48, and 72 hours (***$p$< 0.01).**

*Measurement of ROS in HDF cells using flow cytometry*

Results of flow cytometry showed that in HDF cells treated with CFX, vitamin E, and low power laser (Figure 3B), the ROS level increased significantly compared with the cells treated with CFX and vitamin E without low power laser irradiation ($p$<0.001) (Figure 4A).

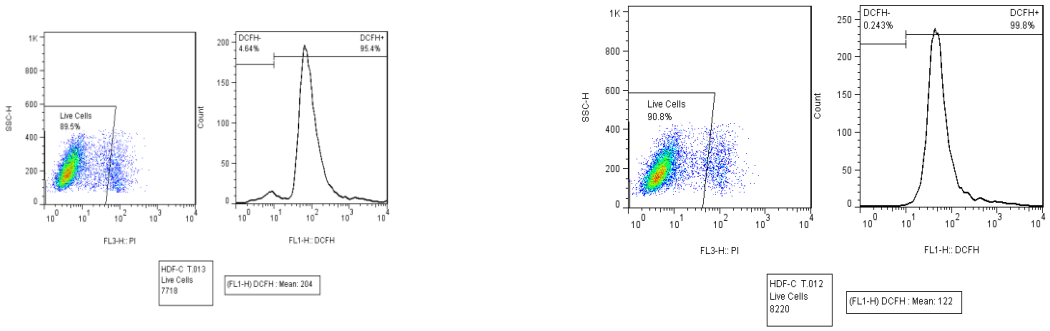


**Figure 3- The level of ROS in HDF cells after the treatment with CFX, vitamin E, and low power laser. A) ROS level after the treatment of HDF cells with CFX and vitamin E without laser irradiation; B) ROS level after the treatment of HDF cells with CFX, vitamin E, and low power laser**



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## DISCUSSION

In this study, we evaluated the effects of CFX, vitamin E, and low power laser on HDF cells' morphology, survival rate, and ROS level. Human dermal fibroblasts are involved in production of extracellular matrix, formation of the connective tissue, and wound healing. These cells are perfect for studying skin biology and wound healing (16). The cells are easily obtained from skin biopsies and can be easily propagated for a long time without genetic manipulation or external growth factors. These cells are genetically stable for 15-20 divisions and are useful for studying various skin factors (17). As fibroblasts, HDF cells can attach to the bottom of culture flask and create a specific morphology. In this study, the morphology of HDF cells was evaluated using an inverted microscope. In general, HDF cells have an elongated morphology and spread to the bottom of the flask. In this study, treatment of the HDF cells with CFX (5 and 25 µg/ml) and vitamin E (1 mg/l) without laser irradiation for 48 hours decreased cell growth and survival compared with other groups. However, the cell growth and survival increased after the treatment of the HDF cells with 100 µg/ml CFX, 1 mg/l vitamin E, and low power laser (1 J/cm$^2$, 660 nm for 30 seconds) for 24 and 48 hours. This indicates the role of low power laser in the growth enhancement of HDF cells.

Based on the results of the MTT assay, the survival rate of HDF cells changed significantly after the treatment with CFX, vitamin E, and low power laser when compared with the negative control group (treated only with CFX and vitamin E). After 24 hours of treatment, the survival rate of the cells treated with 100 µg/ml CFX and low power laser was higher than that of the negative control group. After 48 hours of treatment, the survival rate of the cells treated with 50 and 75 µg/ml CFX and low power laser was higher than that of the negative control group; however, treatment with 100 µg/ml CFX decreased the survival rate. After 72 hours of treatment, the survival rate of the cells treated with 75 µg/ml CFX and low power laser was higher than that of the negative control group. Hincal et al. (2003) showed that treatment with CFX at concentrations of 50 and 75 mg/l for 48-72 hours induced cytotoxicity in human fibroblasts (18). In the mentioned study, lipid peroxidation and glutathione, as well as redox enzymes such as catalase, superoxide dismutase, and glutathione peroxidase were studied. The results showed that CFX increased oxidant factors in the cells and decreased the amount of antioxidant compounds. Moreover, pre-treatment of fibroblasts with vitamin E reduced the oxidative activity of CFX so that the level of lipid peroxidation decreased and the amount of total glutathione increased (18). Thus, it can be concluded that vitamin E can prevent the cytotoxic effect of CFX. In 2002, Gürbay et al. studied the cytotoxic effects of CFX on human fibroblast cells and the supportive effect of vitamin E in preventing cytotoxicity. In their study, it was observed that CFX causes cytotoxicity in HDF cells, and pretreatment with vitamin E prevents cytotoxicity (19). According to research, CFX has cytotoxic effects and increases free radicals in cells, which in turn leads to apoptosis or necrosis. Treatment with vitamin E prevents CFX-induced cell death due to its proven antioxidant properties. In this regard, we observed that vitamin E has a protective effect against the CFX-related cytotoxicity in HDF cells.

In 2017, Shingyochi et al. showed that low power laser increases proliferation of fibroblasts by activating protein kinase B-, extracellular signal-regulated kinase-, and c-Jun N-terminal kinase-dependent signaling pathways. According to the authors, this effect is involved in the wound healing effects of low power laser (20). In 2002, Pereira et al. investigated effects of low power laser on fibroblasts' growth and procollagen synthesis. They reported that irradiation with low power laser (3-4 J/cm$^2$) increases the number of fibroblasts by 3 to 6 fold compared with control group. They also demonstrated that increasing the laser energy would halt cell growth, and the optimal growth could be achieved at 3 J/cm$^2$ (21). In 2018, Ma et al. reported that laser radiation increased the proliferation and collagen synthesis in HDF cells (22). In the present study, we also observed that the survival rate of cells increased after the treatment of the HDF cells with 50 and 75 µg/ml of CFX and low power laser for 48 hours; however, at concentration of 100 µg/ml, cell survival decreased. After 72 hours of treatment, the survival rate of the



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cells treated with 75 µg/ml CFX and low power laser was higher than that of the negative control group. In general, the results of the MTT assay showed that the low power laser was able to prevent the cytotoxic effects of CFX, which is consistent with the results of previous studies.

According to the results of flow cytometry analysis, ROS level increased in the HDF cells after the treatment with CFX, vitamin E, and low power laser compared with the cells treated with only CFX and vitamin E. In 2013, Luo et al. reported that low power laser irridation could increase superoxide dismutase activity, which subsequently reduces ROS level in skeletal muscle cells (23). In 2016, a study showed that low power lasers increased collagen synthesis and decreased ROS production in the skin of diabetic rats (24). Fujimaki et al. (2004) also showed that low power laser radiation delays the production of ROS in human neutrophils. They suggested that delayed ROS production in neutrophils might reduce inflammation (25). Inconsistent with this finding, we observed that ROS production increased slightly after the treatment of the cells with low power laser. This might be related to the amount of energy emitted by the low power laser or the duration of irradiation. Moreover, the research mentioned above was performed in vivo, while our study was performed in vitro.

## CONCLUSION

Based on the results, it can be concluded that the treatment with CFX, vitamin E, and low power laser can significantly increase survival of HDF cells. In addition, low power laser can increase the growth of HDF cells, which may be beneficial for wound healing, even in the presence of CFX.

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**Ethics approvals and consent to participate**

Not applicable.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding publication of this article.

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