

Low level laser application reduces the effect of 5 fluorouracil/leucovorin combination on human breast cancer cells

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Abstract

Aim : 5-fluorouracil has been widely used in breast cancer treatment. Low level laser therapy (LLLT) has been shown to modulate biological processes and used in cancer treatment. It has been reported that apoptosis is induced by stimulation of transient receptor potential protein channels in numerous cancer cells, including breast cancer. We aimed to reveal the effects of 5-fluorouracil / Leucovorin (5-FU/LV) and . low level laser on apoptosis via transient receptor protein ancyrin 1 (TRPA1) channels.

Material and Methods: Breast cancer cells (MCF-7) were cultured and cells were divided into seven main groups. Cells were incubated with 5-FU/LV and LLLT exposure separately and together performed on MCF-7 cell cultures. Cell cultures incubated with TRPV1 channel antagonist capsazepin and stimulator capsaicin. The effects of 5-FU and LLLT were investigated on all molecular pathways of apoptosis.

Results: It was found that the levels of apoptosis in 5-FU/LV group were significantly increased in cancer cells compared to control group .TRPA1 channel stimulator administration resulted in significantly increased apoptosis levels compared to the control group, in 5-FU/LV + low level laser group the apoptosis level significantly reduced compared to 5-FU/LV-only group. (p<0.001)

Conclusion: It has been shown that 5-FU/LV significantly increases apoptosis in breast cancer cells, however low level laser administration decreases apoptosis and suppressed apoptotic effect of 5-FU/LV significantly on breast cancer cells.

Keywords: Breast cancer; 5-fluorouracil; leucovorin; low level laser; TRPA1

INTRODUCTION

Breast cancer is the leading causes of cancer-related deaths in women (1), and chemotherapy is widely used in the preoperative and postoperative period. 5-fluorouracil (5-FU) has been widely used in breast cancer since the 1980s. The most important known antitumor effect of 5-FU involves its ability to stop DNA synthesis and to inhibit cell proliferation by binding to DNA and RNA (2). 5-FU is usually used in clinical chemotherapy in combination with leucovorin (LV).

Laser treatment is also commonly used in many medical fields for the treatment of several cancer types. Low-level laser therapy (LLLT), also known as photobiomodulation therapy, has been shown to have secondary in vitro effects on many cell types such as fibroblasts, osteoblasts, stem cells, lymphocytes and smooth muscle cells (3). LLLT is

a form of photon therapy that uses low wavelengths and provides low irradiance for biological modulation and is a safe technique. LLLT is a non-invasive method and low intensity (450-800 nm) light is used in cancer treatment. LLLT has been investigated in many cell and animal model studies where the far and near infrared spectral irradiation ranging between 400 and 1000 nm wavelength modulates different biological processes (4). The effect of LLLT primarily begins after absorption of specific wavelengths of light by the mitochondrial respiratory chain such as cytochromes and flavin dehydrogenase. Absorption of the beam leads to a reduction-oxidation reaction (REDOX) change in the cytoplasm and mitochondria, resulting in an increase in ATP synthesis. This primary reaction triggers intracellular signals at the cellular level, causing secondary reactions such as the production of nitric oxide by stimulation of cytokine reactions (5).

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LLLT is known to have effects on wound healing, nerve regeneration, collagen synthesis, bone remodeling and repair, restoration of nerve function after injury, correction of abnormal hormone secretion, pain relief, stimulation of endorphin secretion, and immune system modulation (6). There are studies examining the efficacy of LLLT, which is used clinically in the treatment of numerous diseases, on many types of cancer such as lung, bone, breast, colon cancer.

There are six subtypes of transient receptor potential (TRP) channels in mammals: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin) and TRPA (anycrin) (7). TRP channels are sensitive to stimuli such as receptor stimulation, heat, plant-derived compounds, environmental irritants, mechanical stress, pH, intra and extracellular voltage, and respond very well to oxidative stress mediators such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and other electrophiles (8,9).

Intracellular calcium (Ca^{2+}) plays an important role in oxidative stress and apoptosis by taking part in the activation of TRP channels by receptor stimulation and through some Ca^{2+} dependent enzymes (10). It has been reported that non-canonical oxidative-stress defense mechanism directly related to TRPA1, and TRPA1 upregulates Ca^{2+} dependent anti-apoptotic pathways in cancer cells (9). In addition, it has been reported that overexpression and apoptosis is induced by stimulation of TRPA1 channels in numerous cancer cells, including breast cancer (9,11).

In this study, by applying LLLT and 5-FU/LV on breast cancer cell cultures, we aimed to reveal the effects of 5-FU/LV and LLLT on apoptosis via TRPA1 channels, which are commonly found in breast cancer cells and are known to be susceptible to oxidative stress.

MATERIAL and METHODS

Cell culture and Low Level Laser Application

Human Breast Cancer Cell line (MCF-7) was maintained from Culture Collection of Animal Cells, Foot and Mouth Disease (ŞAP) Institute, Ankara, Turkey. MCF-7 cells were cultured in RPMI-1640 medium. Mediums were used for cell cultures with containing 10% fetal bovine serum (FBS) (Fisher Scientific, and 1% pen./strep. Antibiotic combination (Thermo-Fischer) and? MCF-7 cells were evenly distributed as 1×10^6 cells in each of 8-10 flasks (filter cap, sterile, 5 ml, 25 cm^2). A humidified incubator was used to incubate at 37°C at 5% CO_2 . After cells have reached 75-80% confluence, cells were incubated with 5-FU/LV combination as described in groups section. Following the 5-FU/LV incubation, detached (0.25 Trypsin-EDTA) and washed cells were put into sterile 9.6 cm^2 plate wells quickly (2×10^6 cells for per well) and laser applications (Lasport GD-P-1 Pain Laser Therapy Device, U.S.A) were performed at 808 nm wavelengths at 6 Joule/ cm^2 as covering the entire well. Cells were examined daily for evidence of contamination. After laser application, the

cells were split into the sterile falcon tubes for analyses.

Reagents/Dyes

Caspase-3 (AC-DEVD-AMC) and Caspase-9 (AC-LEHD-AMC) substrates were obtained from Enzo (Lausen, Switzerland). APO Percentage assay with releasing buffer were obtained from Biocolor (Belfast, Northern Ireland). A mitochondrial stain 5,50, 6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) and Probenecid were obtained from Santa Cruz (Dallas, Texas, USA). Pluronic® F-127 was obtained from Biovision (San Francisco, USA). Dihydrorhodamine-123 (DHR 123), was obtained from Sigma Aldrich (St. Louis, MO), Fura 2 (AM) calcium florescent dye was obtained from Calbiochem (Darmstadt, Germany) and MTT (Cell proliferation reagent) was obtained from Biovision (Milpitas, CA, USA).

Cell viability (MTT) assay

MCF-7 cell viability was evaluated by the MTT assay based on the ability of viable cells to convert a water-soluble, yellow tetrazolium salt into a water-insoluble, purple formazan product. The enzymatic reduction of the tetrazolium salt happens only in living, metabolically active cells, not in dead ones. Optical density was measured in an automatic multiplate reader (Synergy™ H1, Biotek, USA) at 490 and 650 nm (as reference wavelength) and presented as the fold experimental/control as described elsewhere (12). Effects of 5-FU and Folinic acid (Leucovorin) on cell viability were assayed by MTT. Cytotoxicity was examined in different 5-FU doses and fixed folinic acid dose (3 μM). Cells were treated with different 0, 1, 3, 5 and 10 μM 5-FU doses and fixed 3 μM folinic acid doses in all groups for 24 h (Figure 1).

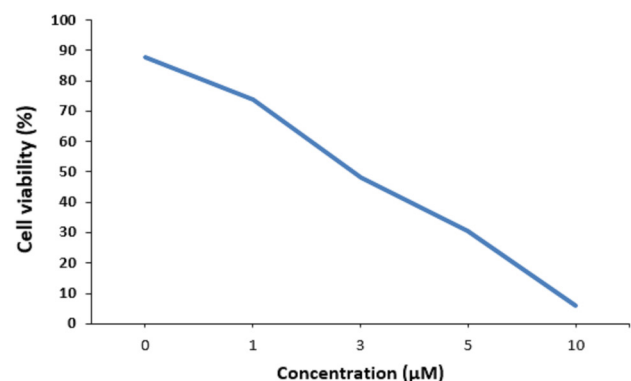


Figure 1. Cytotoxicity was examined in different 5-FU doses (0, 1, 3, 5 and 10 μM) and fixed folinic acid dose (3 μM) in all groups for 24 h

Groups

The study was planned as 7 main groups below,

Group 1 (Control): None of the study drugs were used and breast cancer cells were preserved in a flask with the same cell culture condition.

Group 2 (5-FU/LV): Cells were incubated with 5-Flourouracil (5-FU, 3 μM) and Leucovorin (LV, 3 μM) for 24 hrs. simultaneously (12).

Group 3 (5-FU/LV+AP18): Cells were incubated with 5-Flourouracil (5-FU, 3 μ M) and Leucovorin (LV, 3 μ M) for 24 hrs. and then incubated with AP18 (AP18, 0.1 mM, 30 min).

Group 4 (5-FU/LV+LLLT): Cells were incubated with 5-Flourouracil (5-FU, 3 μ M) and Leucovorin (LV, 3 μ M) for 24 hrs. and then exposed to laser beams at 808 nm at 6 Joule/cm².

Group 5 (5-FU/LV+LLLT+AP18): Cells were incubated with 5-Flourouracil (5-FU, 3 μ M) and Leucovorin (LV, 3 μ M) for 24 hrs. and then exposed to laser beams at 808 nm at 6 Joule/cm² and then incubated with AP18 (AP18, 0.1 mM, 30 min).

Group 6 (LLLT): Cells in the group were exposed to laser beams at 808 nm at 6 Joule/cm².

Group 7 (LLLT+AP18): Cells in the group were exposed to laser beams at 808 nm at 6 Joule/cm² and then incubated with AP18 (AP18, 0.1 mM, 30 min).

For all experiments (except for calcium signaling), the cells were further treated with cinnamaldehyde (Cnm, 0.1 mM, 10 min) for activation of TRPA1 channel before related analysis. During calcium signaling analysis (Fura-2/AM), cells were stimulated on 20th cycles with 0.1 mM Cnm in the existence of 1.2 mM calcium and calcium free buffer in extracellular environment.

Measurements of Intracellular Calcium and Fura-2 Loading

Fura 2 AM (acetoxymethyl ester) dye was used for measuring intracellular calcium level in MCF-7 cells. The cells were incubated with HEPES-buffered saline [HBS; 5 mM KCl, 145 mM NaCl, 10 mM D-glucose, 1 mM MgCl₂, 1,2 mM CaCl₂, 10 mM HEPES and 0.1% (w/v) bovine serum albumin (BSA); pH 7.4] containing 5 μ M Fura-2 AM and 0.05% (w/v) Pluronic F-127 for 60 min at 37°C in the dark. The loaded cells were washed twice with HBS and covered with 1ml of HBS supplemented with 2.5 mM probenecid for at least 20 min at 37°C in the dark to allow for Fura-2 AM de-esterification. Fluorescence intensity at 510 nm (emission) was determined in individual wells using a plate reader equipped with an automated injection system (SynergyTM H1, Biotek, USA) at alternating excitation wavelengths of 340 and 380 nm every 3 s for 50 acquisition cycles. During the measurement of intracellular calcium signaling, TRPA1 channels were stimulated by automatic injector with Cnm (0.1 mM) on 20th cycle. Measurement of [Ca²⁺]_i including staining process modification was performed to according to method of Martinez et al (13).

Intracellular ROS production measurement

Non-charged and non-fluorescent dye [Dihydrorhodamine-123 (DHR-123)] go through the cell membrane easily. Inside the MCF-7 cells, DHR-123 is oxidized to cationic rhodamine-123 (Rh-123) which is localized in the mitochondria and demonstrates green fluorescence. The cells (106 cells/ml for per group) were incubated with 20 μ M DHR-123 as florescent oxidant dye at 37 °C for 25 min (13). SynergyTM H1 automatic microplate reader device was used for determining Rh-

123 fluorescent intensities. Analyzes were performed at 488 nm excitation wavelength and 543 nm emission wavelength. We presented the data as fold change over the level before treatment.

Apoptosis assay

The cell apoptosis assay (APOPercentageTM) was used for the detection and quantification of apoptosis. The APOPercentage dye is actively bound to phosphatidylserine lipids and transferred into the cells and apoptotic cells are stained red. The apoptosis analyzes procedure was performed according to the manufacturer instruction and Oz et al (14). The MCF-7 cells were analyzed for apoptotic cells detection by spectrophotometry (multiplate reader) at 550 nm (SynergyTM H1, Biotek, USA), and results were shown as fold change over the level before treatment (experiment/control).

Mitochondrial membrane potential (JC-1) analyses

Mitochondrial membrane potential fluorescence dye [JC1 (1 μ M)] intensity was evaluated by 485 nm (green) excitation wavelength and the emission wavelength of 535 nm, the red signal at the 540 nm (excitation) and 590 nm (emission) wavelengths (SynergyTM H1, Biotek, USA) (14). Data are presented as emission ratios (590/535). Mitochondrial membrane potential changes were quantified as the integral of the decrease in JC1 fluorescence ratio of experimental/control.

Caspase 3 - 9 activity assays

Caspase 9 (AC-LEHD-AMC) and Caspase 3 (AC-DEVD-AMC) substrates cleavages were measured with SynergyTM H1 microplate reader (Biotek, USA) with 360 nm and 460 nm wavelengths (excitation/emission). Caspase 9 and Caspase 3 activity evaluation methods were based on previously reported (15). The values were evaluated as fluorescent units/mg protein and shown as fold change from the level before treatment (experimental/control).

Statistical analyses

All results were presented as means \pm standard deviation (SD). Significant values in the groups were assessed with one-way ANOVA. Statistical analyses were calculated using GraphPad and ?Prism version 7.04 for windows (GraphPad Software, San Diego California, the USA). P<0.05 was considered significant.

RESULTS

Effects of 5-FU/LV and LLLT treatments on Cnm-induced [Ca²⁺]_i concentration, intracellular ROS production, mitochondrial membrane depolarization (JC-1), Caspase 3 and Caspase 9 values through TRPA1 channels activation in the MCF-7 cells.

As a result of 5-FU/LV and LLLT application on MCF-7 cell cultures separately and together, it was found that intracellular Ca²⁺ levels, intracellular ROS, mitochondrial depolarization, caspase 3 and caspase 9 levels were increased significantly in both cancer cells in 5-FU/LV treated group compared to the control group. (p<0.001) Intracellular Ca²⁺, intracellular ROS, mitochondrial depolarization, caspase 3 and caspase 9 levels were

significantly decreased by the application of TRPA1 channel inhibitor AP18 on cancer cells ($p < 0.05$ for intracellular Ca^{2+} and intracellular ROS levels, $p < 0.001$ for mitochondrial depolarization, caspase 3 and caspase 9 levels). In the 808 nm-LLLT group, intracellular Ca^{2+} levels, intracellular ROS, mitochondrial depolarization, caspase 3 and caspase 9 levels were significantly decreased in the cancer cell compared to the control group. ($p < 0.05$ for intracellular Ca^{2+} and ROS levels, $p < 0.001$ for the others)

Intracellular Ca^{2+} levels, intracellular ROS, mitochondrial depolarization, caspase 3 and caspase 9 levels in 5-FU/LV +808 nm-LLLT groups were found to be statistically significantly decreased compared to the 5-FU/LV-only group in breast cancer cell. ($p < 0.001$) In these groups, the effects of TRPA1 channel stimulator Cnm in cancer cells were significantly diminished compared to the channel inhibitor AP18 ($p < 0.001$) (Figure 2).

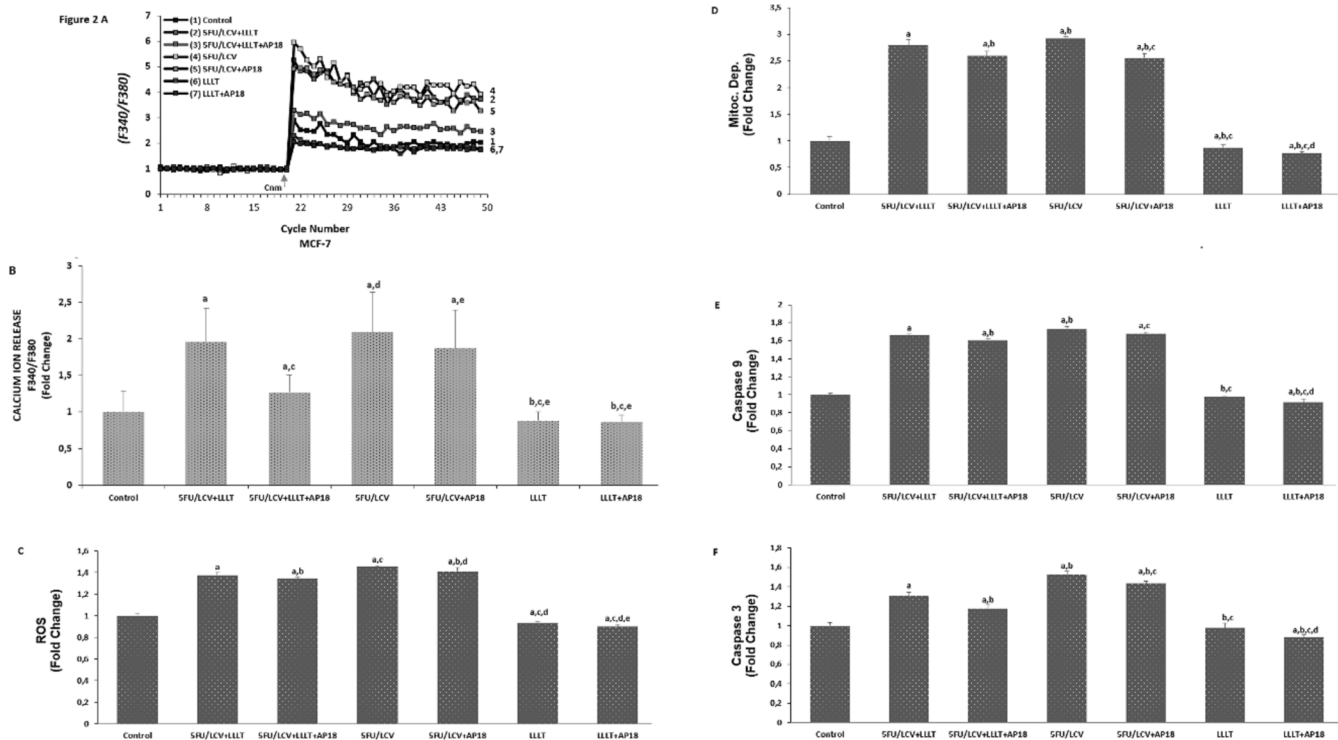


Figure 2. A,B. The effect of 5-FU/LV and LLLT on cytosolic calcium levels in MCF-7 cells. Cells are stimulated by Cinnamaldehyde (Cnm, 0.1 mM and on 20th cycle) but they were inhibited by AP18 (AP18, 0.1 mM for 30 min) (mean \pm SD and $n=3$). ^a $p < 0.001$ and ^b $p < 0.05$ vs control, ^c $p < 0.001$ and ^d $p < 0.05$ vs 5-FU/LV+LLLT group, ^e $p < 0.001$ vs 5-FU/LV group and ^f $p < 0.05$ vs LLLT group. **C.** The effect of 5-FU/LV and LLLT on Reactive Oxygen Species levels in the MCF-7 cells. Cells are stimulated by Cinnamaldehyde (Cnm, 0.1 mM for 10 min) but they were inhibited by AP18 (AP18, 0.1 mM, 30 min) (mean \pm SD and $n=10$). ^a $p < 0.001$ vs control, ^b $p < 0.05$ and ^c $p < 0.001$ vs 5-FU/LV+LLLT group, ^d $p < 0.001$ vs 5-FU/LV group and ^e $p < 0.05$ vs LLLT group. **D.** The effect of 5-FU/LV and LLLT on mitochondrial depolarization levels in the MCF-7 cells. Cells are stimulated by Cinnamaldehyde (Cnm, 0.1 mM for 10 min) but they were inhibited by AP18 (AP18, 0.1 mM, 30 min) (mean \pm SD and $n=10$). ^a $p < 0.001$ vs control, ^b $p < 0.001$ vs 5-FU/LV+LLLT group, ^c $p < 0.001$ vs 5-FU/LV group and ^d $p < 0.001$ vs LLLT group. **E,F.** The effect of 5-FU/LV and LLLT on caspase 9 (A) caspase 3 (B) levels in the MCF-7 cells. Cells are stimulated by Cinnamaldehyde (Cnm, 0.1 mM for 10 min) but they were inhibited by AP18 (AP18, 0.1 mM, 30 min) (mean \pm SD and $n=10$). ^a $p < 0.001$ vs control, ^b $p < 0.001$ vs 5-FU/LV+LLLT group, ^c $p < 0.001$ vs 5-FU/LV group and ^d $p < 0.001$ vs LLLT group

Results of apoptosis values in MCF-7 Cells

As a result of administration of 5-FU/LV and LLLT separately and together on breast cancer (MCF-7) cell culture, apoptosis levels increased significantly in 5-FU/LV treated group compared to the control group, ($p < 0.001$) while these levels were decreased significantly with the use of TRPA1 channel inhibitor AP18. In the 808 nm LLLT groups however, apoptosis levels in breast cancer cells were diminished significantly ($p < 0.001$) whereas these levels increased significantly with the use of

TRPA1 channel inhibitor AP18 compared to the control group ($p < 0.001$). It was found that apoptosis levels were decreased significantly in the groups which received 5-FU/LV +808 nm LLLT on the breast cancer cell compared to the 5-FU/LV-only group. ($p < 0.001$) In these groups, the effects of TRPA1 channel stimulator Cnm in both cancer cells were found to be significantly decreased compared to administration of the channel inhibitor AP18. ($p < 0.001$ for stimulation, $p < 0.05$ for inhibition) (Figure 3).

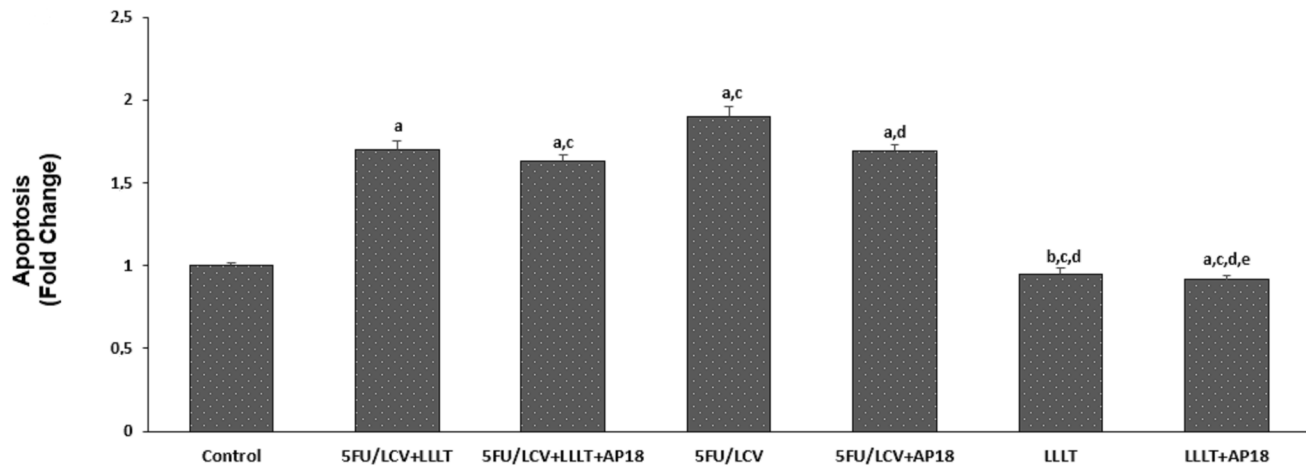


Figure 3. The effect of 5-FU/LV and LLLT on apoptosis levels in the MCF-7 cells. Cells are stimulated by Cinnamaldehyde (Cnm, 0.1 mM for 10 min) but they were inhibited by AP18 (AP18, 0.1 mM, 30 min) (mean \pm SD and n=10). ^ap<0.001 and ^bp<0.05 vs control, ^cp<0.001 vs 5-FU/LV+LLLT group, ^dp<0.001 vs 5-FU/LV group and ^ep<0.001 vs LLLT group

DISCUSSION

It is known that TRP channels and play important roles in intracellular oxidative stress and apoptosis and TRP channels change intracellular calcium concentrations and have effects on regulation of Ca^{2+} secretion in many cell and TRP channels change the Ca^{2+} concentration through hyperpolarization (16). The intracellular Ca^{2+} concentration is play crucial roles in cancer cells and it has been shown that intracellular Ca^{2+} regulate cellular survival, growth, differentiation, metabolism, and apoptosis in cells (17). 5-FU, which is used in the treatment of many cancer types including breast cancer, is an antimetabolite chemotherapeutic agent and inhibitor of thymidylate synthase. 5-FU induced apoptosis through to increase ROS levels and mitochondrial dysfunction in cancer cells.. Oxidative stress is triggered by the increase in the amount of ROS and as a result, irreversible changes occurred in components in cancer cells (18). ROS regulates critical cellular events such as protein phosphorylation, gene expression, transcription factor activation, proliferation and apoptosis (19). It has been shown that inhibition of caspase-dependent pathway of apoptosis death has important role of chemotherapeutic drug resistance (20) and 5-FU induced apoptosis through caspase-dependent pathway in colon cancer cells (21).

Many recent studies have shown abnormal TRP channel expression in different cancer types and it has been reported the effects of TRP channels on invasion, proliferation, differentiation and tumor vascularization in cancer cells. In addition, 5-FU has been shown to play an important role in the TRPC5 channel in chemoresistant colon cancer cells (22). Furthermore it has been concluded that TRPA1, TRPC5, and TRPV1–TRPV4 channels are activated via oxidants and TRPA1 inhibition induced chemosensitivity of chemotherapeutic agents which increase ROS production especially (9). In another study, it was reported that similar

to our study, TRPA1 channel activation and subsequently increasing intracellular Ca^{2+} levels on prostate cancer cells (23).

In our study, the effects of administration of 5-FU/LV on breast cancer cell culture (MCF-7) and impact of LLLT on TRPA1 channels and the effects of these channels on apoptosis were investigated. Intracellular Ca^{2+} levels, mitochondrial depolarization, intracellular ROS levels and subsequent apoptosis levels, which are important steps in killing cancer cells, were compared with the control group by administering stimulator (Cnm) and inhibitor (AP18) for TRPA1 channels. As a result of the investigations, it was observed that intracellular Ca^{2+} levels and related mitochondrial depolarization levels and intracellular ROS levels increased significantly in TRPA1 channel activation after 5-FU/LV administration and there was a significant increase in apoptosis levels in cancer cells due to oxidative stress compared to the control group. As a result of TRPA1 channel inhibition however, these effects were significantly reduced and apoptosis levels were significantly lower than the control group.

Studies on the effects of LLLT on cancer cells have been reported that proliferation process is influenced by application time and wavelength (24). It has been reported that LLLT can lead to apoptosis by altering cell membrane permeability, and also cause changes in levels of microRNA expression in cancer cells due to these effects, such as alteration of DNA synthesis, gene expression and inhibition of cellular proliferation (25). Despite the effect of LLLT leading to apoptosis in cancer cells, it has been reported that LLLT increases ATP production in the cell owing to stimulation of the respiratory chain in the mitochondria, causing an increase in the DNA, RNA and protein synthesis, which may lead to an increase in cell proliferation. It has also been reported that this condition may develop in cancer cells and that LLLT may also have

an effect on enhancing cancer cell proliferation, in contrast to its apoptotic effect (26).

There are many studies investigating the efficacy of LLLT on the treatment of various types of cancer and LLLT is recommended in cancer treatment, however there are also numerous studies indicating the need to avoid cancer treatment. Similar to the results of our study, in a study conducted on osteoblast and lung cancer cells about the effects of LLLT on cancer cells (27) and in another study on head and neck cancer cells (26), it was emphasized that LLLT should be avoided in the treatment of cancer because it enhances proliferation in a wavelength-dependent manner. In contrast to these studies and our study, in animal models (4) and cell studies where the efficacy of LLLT in breast cancer was examined (28), it was reported that LLLT was effective in the treatment of breast cancer and could be an alternative treatment.

In our study, the effects of LLLT on MCF-7 through TRPA1 channels regarding apoptosis and 5-FU/LV treatment in these cancer cells were investigated. Stimulator (Cnm) and inhibitor (AP18) agents were applied to TRPA1 channels and apoptosis levels were examined and compared with the control group. Result of the analysis indicated that after the application of LLLT at 808 nm wavelengths, the cell death in MCF-7 diminished significantly compared to the control group. In addition, it was found that the application of LLLT along with 5-FU/LV reduced the apoptotic efficacy of 5-FU/LV.

CONCLUSION

In conclusion, our study has shown that the apoptotic effects of 5-FU/LV on tumor cells are directly associated with TRPA1 channels following administration of 5-FU/LV and LLLT treatment in breast cancer separately and together. It has been found that TRPA1 channels play an important role in the entire molecular pathway of apoptosis by increasing intracellular Ca²⁺ level and enhancing mitochondrial depolarization. It has been also found that 5-FU/LV significantly increases apoptosis in breast cancer cells; however administration of LLLT at 808 nm wavelength reduces apoptosis in breast cancer cells, and also significantly suppresses the apoptotic effect of 5-FU/LV on these cells.

Competing interests: The authors declare that they have no competing interest.

Financial Disclosure: There are no financial supports.

Ethical approval: Not applicable (cell culture study).

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