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THE EFFECTS OF ELECTRICAL STIMULATION AND LASER RADIATION ON F-ACTIN REORGANIZATION IN A549 AND CHO AA8 CELL LINES

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ABSTRACT

Background: The aim of this report was to determine the effects of electrical stimulation and laser radiation on non-small lung cancer (A549) and Chinese Hamster Ovary cell line (CHO AA8). Furthermore, we also analyzed viability and size of the extracellular spaces in CHO AA8 and A549 cells.
Methods: In order to evaluate the cell viability Tali® Image-Based Cytometer was used. The material (non-small lung cancer cell – A549 and Chinese Hamster Ovary – CHO AA8) was evaluated by the light and confocal fluorescence microscope.
Results: The data demonstrated that exposure to both electrical stimulation and laser radiation for 3 and 5 minutes showed non statistical differences in the percentage of live cells. The morphological abnormalities and microfilaments reorganization indicated induction non apoptotic type of cell death such as mitotic catastrophe. Moreover, CHO AA8 as non-cancerous cells exhibited lower sensitivity for laser and electrical stimulation in comparison to A549 cell line.
Conclusions: Our results confirmed contraindicated the use of these methods, especially due to an increase of the risk of metastasis. Moreover, our study suggests that the laser radiation and electrical stimulation may have limited applications in the cancer patients and that choice of these treatment methods should be used in carefully selected patients.
Keywords: electrical stimulation, laser radiation, F-actin, A549, CHO AA8, metastasis

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INTRODUCTION

Since many decades light and electric current have been used in the treatment of various diseases, including psoriasis, vitiligo, rickets and skin disorders. Light therapy (phototherapy) is a treatment modality that employs different wavelengths of light from many sources, such as sunlight, incandescent lamps, fluorescent lamps, electric arcs, light emitting diodes and lasers. In phototherapy, the use wavelengths include UV (100–400 nm), visible light (400–800 nm) and infrared light (near-infrared 0.8–1.5 µm, middle-infrared 1.5–5.6 µm and far-infrared 5.6–1000 µm). The term "phototherapy" has been widespread by Niels Ryberg Fiensen, who has indicated that the treatment with infrared prevented the rash and blisters caused by smallpox. The cited author has also revealed that the tuberculosis can be treated using ultraviolet radiation.[1,2,3] These discoveries marked the beginning of modern photomedicine and were awarded the Nobel Prize in physiology or medicine in 1903.[4]

The field of phototherapy has seen several major advances in the recent years, one of which being the development of improved laser systems.[5] Laser therapy is widely used in dermatology for the acceleration of wound healing (ulcers, necrosis of the skin, burns and bedsores) and the treatment of aphthae and herpes as well. Moreover, infrared light may increase blood oxygen level, strengthen the immune system and it has also the analgesic and the anti-inflammatory properties.[6] Furthermore, laser therapy has attracted the interest of cancer researchers in recent years since its positive therapeutic effects through a number of research studies. Tudjman and Ostojic (2013) have pointed that laser therapy is a highly effective and safe therapeutic method if it is performed by well-equipped and skilled team of experts, in carefully selected patients.[7] Similarly, Santana-Blank et al., (2003) have suggested that an infrared pulsed laser treatment is safe for a clinical use and may have a potential antitumor activity in patients with advanced neoplasms.[8] Likewise, the *in vitro* studies have revealed the inhibitory properties of low-power 808 nm laser irradiation on the proliferation of human glioblastoma cells.[9] On the other hand, numerous studies have reported on the stimulatory effects of laser light on the tumor growth, invasion and metastasis.[10, 11]

Similarly as in the case of phototherapy, the use of therapeutic electric current in medicine is not a new approach and it has expanded in recent years to include the treatment of muscular pain, muscular spasms, hair loss, infections, arthritis, rheumatic diseases as well as many types of cancer. Despite the fact that the antitumor effectiveness of electrical stimulation has been reported in numerous cancer cell lines, several experimental tumor models and clinical trials, the use of electrotherapy in clinical practice of cancer treatment is limited, *inter alia*: by the incomplete knowledge and controversy regarding the effect of electric current on cancer cells. For these reasons, the manufacturers of the multiple electro-medical devices (including the Duoter Plus which was used in the presented study) contraindicate the use of electrotherapy in the cancer patients. Thus, further studies are needed to clarify the controversy concerning the impact of electric current and laser radiation on cancer cells. Therefore, the aim of this study was to examine the effect of electric current and laser radiation on the viability and morphological changes of two different cell lines – A549, non-small cell lung cancer cell line and as a model of non-cancerous cell line, the CHO AA8 Chinese hamster ovary fibroblasts. The influence of electric current and laser light on the organization of actin cytoskeleton was also evaluated.

METHODS

Cell culture

The human non-small cell lung cancer cell line A549 and Chinese hamster ovary cell line were cultured at 37^{0}C in 5% CO_2 atmosphere in DMEM (Dulbecco's Modified Eagle's Medium) and MEM (Modified Eagle's Medium) respectively, supplemented with 10% FBS (Fetal Bovine Serum) and gentamycin. After 24h of culture, the cells were exposed to: (i) electrostimulation for 3 and 5 min or (ii) laser radiation at wave length 808 nm for 3 and 5 min using Duoter Plus (Astar; basic frequency - 100 Hz and spectrum 50 Hz). This device is widely used to treatment with average frequency of bipolar currents (bidirectional) and low frequency of unipolar currents (unidirectional). The control cells were grown under identical conditions but without exposure to electric current or laser light treatment.

The survival measurement

The effect of electrical stimulation and laser light on the number of viable and death A549 and CHO AA8 cells was determined using Tali Viability Kit – Dead Cell Green (Invitrogen, Life Technologies) according to the manufacturer's instructions. The survival of CHO AA8 and A549 cells was analyzed using the Tali Image-Based Cytometer (Invitrogen, Life Technologies). For statistical analysis GraphPad Prism (Ver5.00; GraphPad Software) was used (the non-parametric Mann-Whitney U test; $P \leq 0.05$).

The extracellular areas analysis

To evaluate the extracellular spaces, the areas between the cells demarcated by cellular membrane in CHO AA8 and A549 cells were measured. The size of fields was analyzed using ImageJ software (Ver1.45s; NIH). For statistical analysis GraphPad Prism (Ver5.00; GraphPad Software) was used (the non-parametric Mann-Whitney U test; $P \leq 0.05$).

The Hematoxylin staining

In order to examine the alterations in cellular morphology, the CHO AA8 and A549 cells were fixed with 4% paraformaldehyde (20 min, RT). After washing with PBS (3 x 5min, RT), the cells were stained by Mayer's hematoxylin (5 min, RT) and rinsed under running tap water (5 min, RT) and with PBS (10min, RT). Then, the preparations were mounted using Aqua-Poly/Mount and analyzed using an Eclipse E800 microscope (Nikon) equipped with a CCD camera (DS-5Mc-U1; Nikon) and NIS-Elements image analysis system (Ver3.30, Nikon).

The fluorescence staining of the F-actin

For the F-actin analysis, the A549 and CHO AA8 cell line were fixed with 4% paraformaldehyde (20min, RT). Then, Triton X-100 was used for cell permabilization (5min, RT) and the cells were washed with PBS (3x5 min, RT). In order to visualize actin filaments, cells were incubated with phalloidin conjugated to Alexa 488 (diluted 1:40 in PBS, 20 min, dark, RT). The nuclei were labeled by DAPI (diluted 1:20 000 in PBS; 10 min, dark, RT). Slides were mounted in Aqua-Poly/Mount and analyzed using an Eclipse E800 microscope with a Y-FL fluorescence attachment (Nikon), NIS-Elements image analysis system (Ver3.30; Nikon) and a CCD camera (DS-5Mc-U1; Nikon).

RESULTS

The survival of CHO AA8 cells following laser radiation and electrical stimulation

The analysis of cell viability was performed using a Tali Image-Based Cytometer and Tali® Viability Kit – Dead Cell Green, which contains the SYTOX® Blue, a dye which is a live cell-impermeant fluorogenic DNA-binding dye that has been extensively used to identify necrotic cells. The CHO AA8 cells exhibited minimal susceptibility to laser and current treatment. No statistical differences in the cell viability were found in the CHO AA8 cells following their exposure for 3 and 5 min, as compared to the control. As it has been showed in Figure 1A, the mean percentage of live cells was 97.6% in control, 95.3% after 3 min and 93.2% following 5 min of laser treatment (Fig.1A). In turn, the exposure of CHO AA8 on electrical stimulation revealed 96% of viable cells in untreated population, 94.1% and 91.5% of cells after 3 and 5 min of treatment, respectively (Fig.1B).

The survival of A549 cells exposed to laser light and electrical stimulation

The staining with SYTOX® Blue dye revealed that there was no statistically significant differences in the percentage of live cells after the treatment with laser light and electric current. After laser radiation treatment, the average percentage of A549 live cells was 92.6%, 87.1% after 3 and 5 min exposure, respectively, and 97.5% in control cells (Fig.1C). In turn, as it has been showed in Figure 1D, 86.7% of cells remained viable following 5 min exposure to electrical stimulation, 92.7% after 3 min of physical stimuli treatment, and 95.1% in the control (Fig.1D).

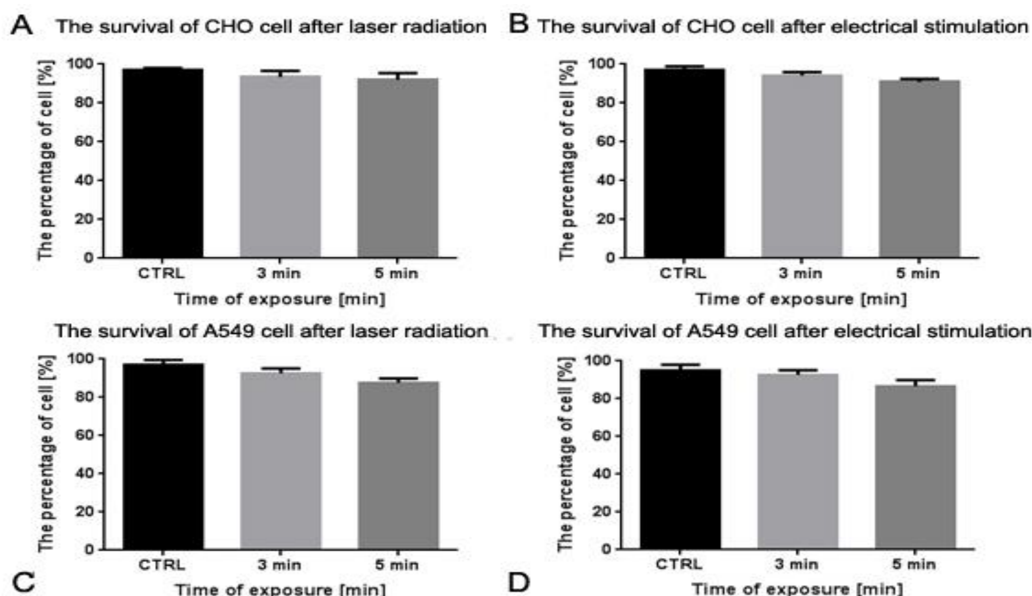


FIGURE 1: The effects of laser and electrotherapy on survival in A549 and CHO AA8 cell line. A – the percentage of live CHO AA8 cell after laser radiation. B – the percentage of live CHO AA8 cell following electrical stimulation. C – the percentage of live A549 cell after exposure to infrared radiation. D – the percentage of live A549 cell after electrical stimulation. The data showed non statistical differences in non-small lung cancer cell line and Chinese Hamster Ovary cell line survival as compared to control. The average values of the percentage of live cell were described in text.

The F-actin organization and morphological changes in CHO AA8 cells after electrical stimulation and laser radiation

The control CHO AA8 cells were characterized by typical for fibroblast spindle shape and oval nuclei (Fig. 2A). Following electrical stimulation at both time points, the small population of morphologically altered cells was noticed. These changes included mostly the enlargement of cell size and the fragmentation of cell nuclei (Fig. 2B, C).

The fluorescence studies revealed stress fibers, especially at the cell cortex, in control CHO AA8 cells (Fig. 2A). In turn, the treatment of cells with electric current resulted in the extension of F-actin network in the enlarged multinucleated cells (Fig.2B'). Furthermore, in some cells, the thickening of the actin filaments was seen (Fig. 2C').

The morphology of CHO AA8 cells treated with laser light was similar to that observed in cells exposed to electrical stimulation. Low level of morphological changes, associated mostly with the formation of enlarged multinucleated cells, was found in the CHO AA8 cells exposed to electric current (Fig. 3B, C). In these cells, F-actin was present in the form of strongly expanded networks (Fig. 3B',C'). There was lack of evidence of apoptosis features in the cells exposed to both electric current and laser light.

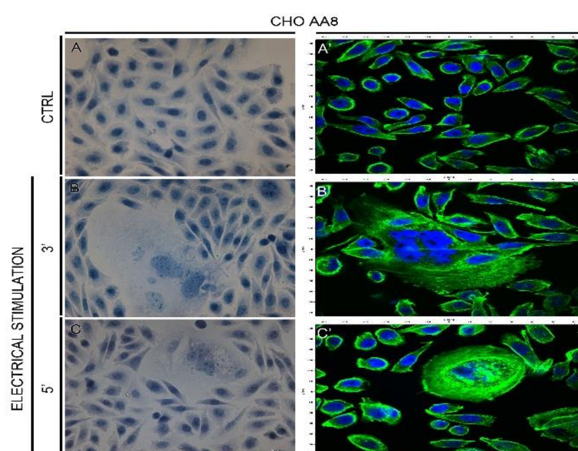


FIGURE 2: The F-actin organization and morphological changes in Chinese Hamster Ovary Cell Line (CHO AA8) after electrical stimulation. A-C – the Hematoxylin's staining. We observed giant cells with multinucleated after 3 min interference current radiation (B). The CHO AA8 cells with mitotic catastrophe-like phenotype were noticed (C); A'-C' – the fluorescence labeling of the F-actin. The actin filaments thick bundles and multinucleated were seen (B',C'). Bar = 50 µm.

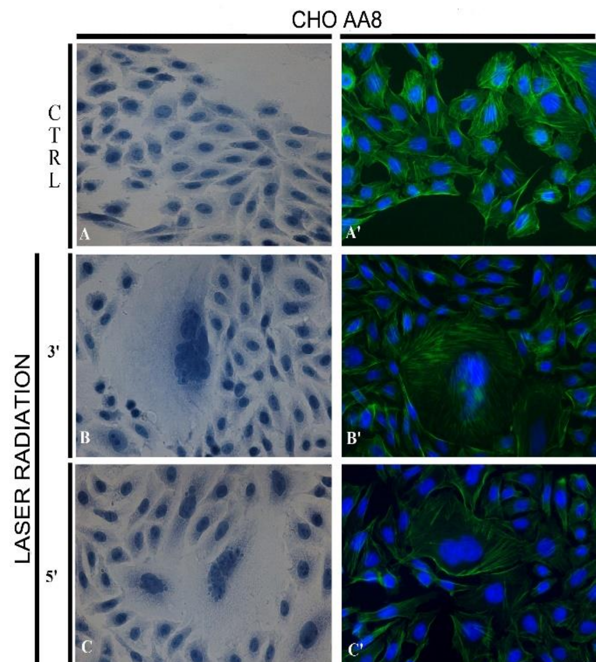


FIGURE 3: The F-actin reorganization and morphological changes in Chinese Hamster Ovary Cell Line (CHO AA8) after laser radiation. A-C – the Hematoxylin's staining. The cells with large size and multinucleated were observed (B,C). A'-C' – F-actin labeling with Alexa488 conjugate with phalloidin. The presence of giant cells, where F-actin were noticed on the edge of cell and a lot of stress fibers the entire surface of the cells were observed (B',C'). Bar = 50 µm.

The F-actin organization and morphological changes in A549 cells after electrical stimulation and laser radiation

The A549 control cells were characterized by the presence of polygonal, epithelial shape and lobar cell nuclei (Fig. 4A) their actin cytoskeleton was found in the form of short fibers in the cytoplasm as well as stress fibers traversing the cells. The most intense fluorescence for F-actin occurred at the cell periphery. The control cells maintained the tight cell-cell contacts that were enriched in cortical F-actin.

Following the 3 min exposure of A549 cells to electric current, the giant multinucleated cells were observed, in which a well-developed F-actin was present mainly in the form of short fibers that

were seen as bright fluorescent puncta (Fig. 4B'). In turn, after the 5 min exposure of A549 cells to above physical stimuli, the characteristic organization of actin filaments for migrating cells was seen. The appearance of cells with F-actin-rich lamelliopodia as well as the accumulation of actin filaments at the leading edge of cells was noted (Fig. 4'C).

The A549 cells exposed to laser radiation were characterized by an increase in size and the fragmentation of cell nuclei. After the exposure of cells to 3 min laser radiation, the F-actin was seen as short fibers accumulated around the nucleus , in the enlarged cells (Fig. 5B'). Moreover, in the cells exposed to laser radiation for 5 min, the actin filaments became concentrated in the front of the cells (Fig. 5 C').

Following the treatment with electrical stimulation and laser radiation, the A549 cells gradually lost their cell-cell contact which was accompanied by the extension of actin-rich protrusions towards adjacent cells (Fig. 5). Similar to CHO AA8 cells, the A549 cells exposed to electrical stimulation or laser radiation did not exhibited the morphological features associated with apoptosis.

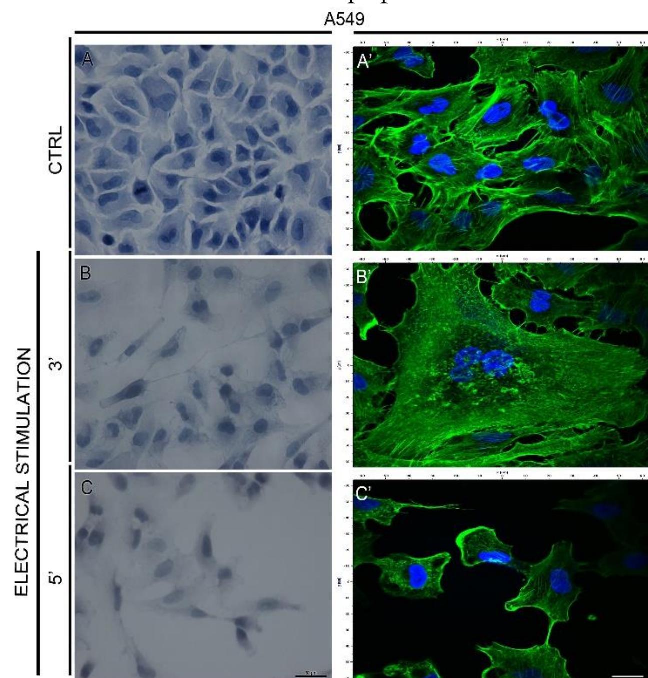


FIGURE 4: The F-actin organization and morphological changes in non-small lung cancer cell line (A549) after electrical stimulation. A,B,C - the Hematoxylin's stainig. A'-C' – the labeling cytoskeletal F-actin with phalloidin conjugate with Alexa 488. The changes in size and shape were noticed (B,C). The giant cell with small aggregates of F-actin were noticed (B'). Furthermore, lammelopodium with strongly F-actin fluorescence on the edge of cells were also observed (C'). Bar = 50 µm.

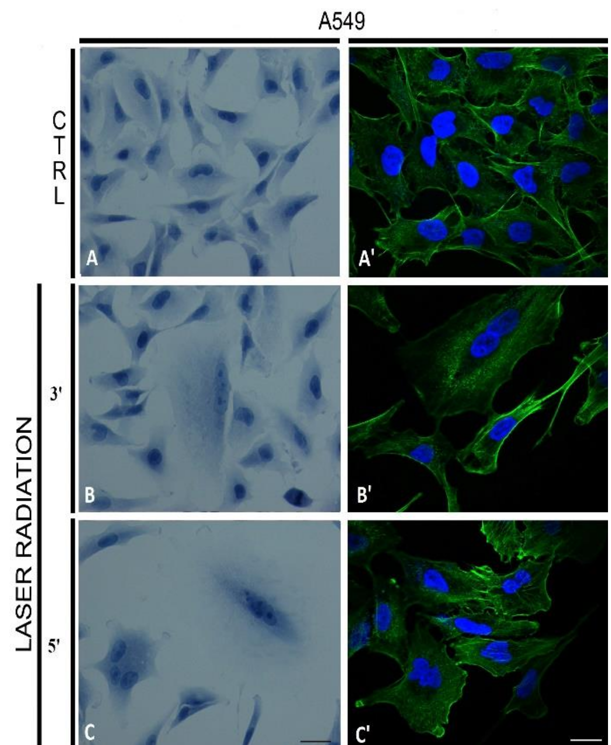
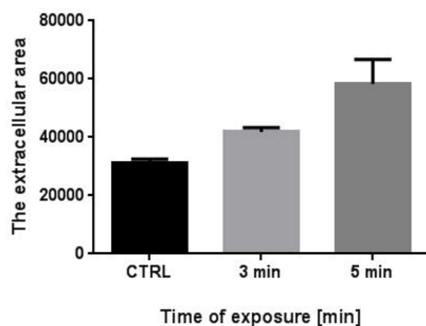


FIGURE 5: The F-actin organization and morphological changes in non-small lung cancer cell line (A549) after laser radiation. A-C – the Hematoxylin's staining. The large size cells were seen. A'-C' – the fluorescence labeling of F-actin. After exposure to laser therapy cells were characterized by the presence of short actin polymers on the cytoplasm and the strongly fluorescence of F-actin on the edge cells (B',C'). Bar = 50 µm.

The measurement of extracellular areas in CHO AA8 and A549 cells after electrical stimulation

The extracellular spaces were measured at fluorescence images using ImageJ software. The mean value of size of extracellular spaces after exposure to electrotherapy was presented in Figure 6. The obtained data revealed that exposure to electrical stimulation for 3 and 5 min resulted in an increase of areas between cells, as compared to the control. The extracellular spaces in CHO AA8 control was 31357.5, whereas after cell exposure to electric current 41856.3 and 58347.8, respectively after 3 and 5 min treatment (Fig. 6A). The data showed no statistically differences in extracellular area in 3 and 5 exposure time as compared to control. On the other hand, as shown in Figure 6, A549 were characterized by larger spaces and their increase in comparison to non-cancerous cell line (CHO AA8). In A549 control cells, the average size of extracellular spaces was 21490.4, while after current radiation for 3 and 5 min were 45877.8 and 216002, respectively (Fig. 6B). The exposure to interference current resulted in statistically

significant increase of the size of extracellular spaces in A549 cell line, as compared to control.



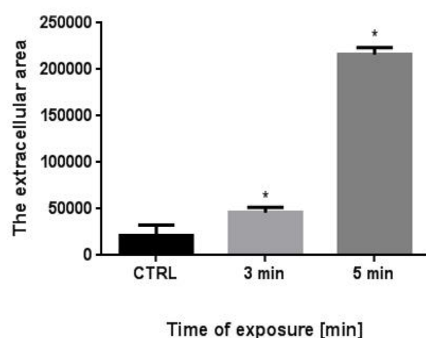
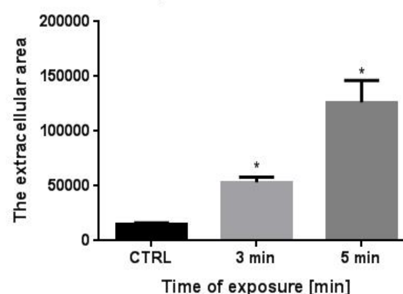


FIGURE 6: The measurement of extracellular spaces in CHO AA8 and A549 cells after electrical stimulation. A–the average value of size extracellular spaces in CHO AA8 cell line following interference current. B–the average value of size extracellular spaces in A549 cells after electrotherapy. The data showed statistically differences in 3 and 5 time of exposure as compared to control (*).

The measurement of extracellular areas in A549 and CHO AA8 cells after laser radiation

Similar to the previously described results (The measurement of extracellular spaces in A549 and CHO AA8 after electrical radiation), the changes in size of extracellular spaces after laser radiation were also evaluated. The measurement showed an increase in extent of extracellular areas in CHO AA8 and A549 cell lines. As it has been showed in Figure 7A, in control A549 cells the area of extracellular spaces was 15444, whereas 53448.8 and 126213 following infrared radiation for 3 and 5 min, respectively (Fig. 7A). The exposure to laser therapy resulted in statistically significant increase of the size of extracellular spaces in A549 cells, as compared to the control. In turn, exposure to laser radiation, CHO AA8 exhibited extracellular spaces in size 10903 in untreated cells, 16077 after 3 min and 25074.3 after 5 min (Fig. 7B). The data showed no statistically differences in extracellular area in 3 and 5 exposure time as compared to control.



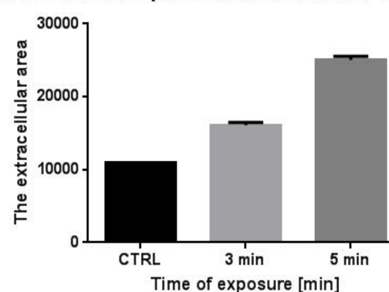


FIGURE 7: The measurement of extracellular spaces in CHO AA8 and A549 cells after laser radiation. A–the average value of size extracellular spaces in CHO AA8 cell line following infrared radiation. B–the average value of size extracellular spaces in A549 cells after laser therapy. The data showed statistically differences in extracellular area in 3 and 5 exposure time as compared to control (*).

DISSCUSION

In present study, we have revealed the effect of electrical stimulation and laser radiation on two cell lines: non-small lung cancer cell line (A549) and Chinese hamster ovary cell line (CHO AA8). CHO AA8 cells were used as a model of non-cancerous cells. The first aim of present investigation was to evaluate the influence of both factors on the survival of above mentioned cell types. The image-based cytometery analysis of SYTOX ® Blue staining revealed non-statistical differences in the percentage of live cells in both cell lines. Therefore, these results suggest that electrical stimulation and laser radiation itself does not induce cell death in A549 and CHO AA8 cells. Likewise, Fernandez et al., (2012) have reported that laser irradiation did not resulted in decrease in the viability of glioblastoma cells (1321N1 cell line).[12] Similar, Morino et al., (2008) have shown that the exposure of A549 cells to mild electrical stimulation (MES) did not cause cell death.[13] Our light microscopy observations of cells exposed to laser radiation or electric current stimulation demonstrated that the cells with hallmarks of apoptosis (cell shrinkage, nuclear condensation and membrane blebbing) were not present in both

cell lines. Muaryama et al., (2012) have shown that even if laser radiation (wavelength 808 nm) induced an decrease in the proliferation of A-172 cells, the morphological abnormalities typical for apoptosis or necrosis have not been observed.[9]

However, in the presented study, we have noticed the cells with mitotic catastrophe-like phenotype in both cell lines exposed to laser radiation or electric current. Mitotic catastrophe (mitotic cell death) is a relatively new known mechanism of cell death, which is caused by altered mitoses and/or irreparable chromosome damage and characterized by the formation of large multinucleated cells.[14] Several studies have reported on the presence of characteristic morphologic features of mitotic cell death in the normal and tumor cells exposed to ultraviolet or infrared radiation. Chang et al., (2013) showed that the middle-infrared radiation (MIR) promoted an increase in the size of A549 cells.[15] Furthermore, Grzanka et al., (2006) revealed that UV irradiated CHO AA8 cells displayed typical features of mitotic death, including the enlarged cell size, the segmentation of nuclei and the formation of bodies resembling micronuclei.[16] Likewise, Bråthen et al., (2000) indicated the presence of multinucleated cells in UV-exposed populations of 3T3 fibroblasts.[17] In our previous studies, we have observed the formation of giant multinucleated cells in the A549 cells populations after UV irradiation.[18] Thus, the induction of mitotic cell death seems to be a common indicator of ultraviolet and infrared radiation damage. Additionally, in our knowledge, there is no reports on the induction of mitotic cell death after electrical stimulation.

The F-actin is one of the main cytoskeletal protein, which is responsible for cell shape maintenance, cell death, adhesion and cell migration.[19] In the present study, the changes in cell morphology correlated with alterations in microfilaments organization. Depending on the time of exposure to laser light or electric current, in the large multinucleated cells, the actin filaments rearranged to the form of well-developed actin cables or relatively short actin fibers and dot-like aggregates, which were evenly scattered through the cytoplasm or were accumulated near the cell nucleus. These results suggest that laser radiation at 808 nm as well as electrical stimulation may influence the actin filament dynamics to establish optimal cell morphology. Similar results have been presented by Chang et al., (2013) who have revealed that middle-infrared radiation induced morphological changes in A549 cells, by altering the cellular distribution of cytoskeletal components (microtubules and microfilaments).[15] Furthermore, Zhao et al., (2004) have observed that electric fields induced F-actin reorganization was accompanied by cell elongation (in endothelial cells).[20] Moreover, Li and Kolega (2002) have also observed the reorganization of actin cytoskeleton in the conjunction with morphological changes that were observed in bovine aortic endothelial cells (BAECs) following electrical stimulation.[21]

An important observation in our study was that both physical stimuli seem to promote the migration of A549 cells, but not CHO AA8 cells. Cell migration is an early prerequisite for tumor metastasis which, being the most frequent cause of cancer-related death, has a negative impact on patient prognosis.[22, 23] It is well-established that an initial step in cell migration is the accumulation of F-actin at the leading edge of cell and the formation of F-actin-rich cell protrusions such as lamelliopodia or invadopodia/podosomes.[24] Here, after the treatment of A549 cells with electric current, we have observed the cells with F-actin-rich lamelliopodia as well as the accumulation of actin filaments at the leading edge of cells. Likewise, the exposition of A549 cells to laser light resulted in the relocalization of F-actin to one edge of the cell. In addition, the increased motility of A549 cells was manifested by the increase in intercellular space size, what suggest the physical stimuli-induced weakening of cell-cell contact. We have also demonstrated that in contrast to A549 cells, there were no statistical differences in the size of extracellular spaces in non-cancerous cell line (CHO AA8). Therefore, we suggest that electrical stimulation and laser radiation may be dangerous factors which promote tumor cell motility and in consequence metastasis.

CONCLUSIONS

In conclusion, the present investigation showed that treatment with electrical stimulation and 808 nm laser radiation induced migration of cancerous non-small lung cancer A549 cells without affecting the non-cancerous Chinese Hamster Ovary cell line. Furthermore, our results confirmed contraindicated the use of these methods, especially due to an increase of the risk of metastasis. Moreover, our study suggests that the laser radiation and electrical stimulation may have limited applications in the cancer patients and that choice of these treatment methods should be used in carefully selected patients.

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Citation

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