ginekologia

In vitro photodynamic therapy of cervical cancer

Terapia fotodynamiczna komórek raka szyjki macicy w warunkach *in vitro*

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Abstract

Objectives: *Photodynamic therapy is one of the modern therapeutic techniques, in which cytotoxic effects are induced by light. Currently, investigators show that it can be used to eliminate cervical cancer cells.*

Aim: *In this study we decided to check whether the Low Level Lasers Therapy (LLLT) can induce the phototoxic changes in HeLa cells, after their photosesitization.*

Material and methods: The studies were performed in vitro on HeLa cervical cancer cell line. Protoporphyrin IX (PpIX) in final concentrations: 0.5, 1.0, 5.0 and 10.0μmol/l was used as photosensitizer. The cells were preincubated with specific concentrations for 6 and 18 hours. After these defined periods of time the cultures were illuminated for 8 minutes by laser light (635nm and 30mW/cm^2). The cytotoxic effects were assessed by a colorimetric test XTT, 24 and 48 hours after irradiation.

Results: *Significantly augmented cytotoxic changes were found in HeLa cells 18 hours after preincubation and 48 hours after illumination. Moreover, biostimulating laser exposure preceded by preincubation with protoporphyrin IX caused the cytotoxic changes in cervical cancer cells.*

Conclusion: *The obtained results allow us to assume that photodynamic therapy of cervical cancer using biostimulating laser light should be performed 18 hours after the application of protoporphyrin IX.*

Key words: **cervical cancer** / ***in vitro*** / **photodynamic therapy** / **protoporphyrin IX** / / **Low Level Lasers Therapy** /

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Otrzymano: **30.03.2011**
Zaakceptowano do druku: **20.05.2011**

Streszczenie

Wstęp: *Terapia fotodynamiczna jest jedną z nowych metod leczniczych, w których efekty cytotoksyczne są wywołane przez fotouczulacz po jego aktywacji światłem. Obecnie wskazuje się możliwość zastosowania tej metody w celu eliminacji komórek raka szyjki macicy.*

Cel badań: *Celem niniejszych badań było sprawdzenie czy lasery niskoenergetyczne mogą zostać wykorzystane jako źródło wzbudzenia fotouczulaczy w komórkach raka szyjki macicy.*

Materał i metody: Badania przeprowadzono w warunkach in vitro na linii komórek raka szyjki macicy HeLa. Jako substancję fotouczulającą zastosowano protoporfirynę IX (PpIX) o końcowych stężeniach: 0,5, 1,0, 5,0 i 10,0mmol/l. Komórki preinkubowano z protoporfiryną IX przez okres 6 i 18 godzin. Po tych czasach zawartą w komórkach protoporfirynę IX aktywowano przez 8 minut światłem laserowym (635nm, 30mW/cm2). Komórki inkubowano dalsze 24 i 48 godzin a zmiany cytotoksyczne oceniano kolorymetrycznym testem XTT.

Wyniki: *Zaobserwowano, że najwyższe efekty cytotoksyczne w komórkach HeLa można otrzymać podczas 18 godzinnej preinkubacji oraz 48 godzinnej inkubacji po naświetlaniu. Wyniki wskazują na możliwość zastosowania laserów biostymulacyjnych w terapii fotodynamicznej komórek raka szyjki macicy.*

Wnioski: *Uzyskane wyniki pozwalają przypuszczać, że terapię fotodynamiczną raka szyjki macicy przy pomocy laserów niskoenergetycznych należy wykonać w czasie 18 godzin od momentu aplikacji protoporfiryny IX.*

Słowa kluczowe: **rak szyjki macicy** / **in vitro** / **terapia fotodynamiczna** / / **protoporfiryna IX** / **lasery niskoenergetyczne** /

Introduction

Lasers – the source of coherent light – have been known since 1960. Their action is based on a stimulated emission of electromagnetic radiation. Laser light has specific properties, such as high intensity (radiation rate), monochromatism, coherence and small divergence of laser beam. These properties provide the base for use of lasers in medicine [1].

Lasers can influence tissues in different ways which allows to categorize lasers into high-powered ones (power above 500 W/cm2), used for tissue destruction and removal (cutting, vaporization and coagulation), and low-powered, biostimulating ones (power below 500W/cm^2), used in physiotherapy, sports medicine, dermatology, rheumatology and dentistry [2].

Biostimulating lasers emit red and infrared light of wavelengths from 632 to about 900nm. They cause no thermal effects in tissue, unlike during laser surgery. This effect is called pre-thermal (specific) [3]. In physiotherapy, biostimulating lasers are used for treatment of inflammatory conditions, such as rheumatic diseases, neuropathies, tendinitis, fascitis, tenosynovitis, bursitis, entezopathies, etc. The widely known analgesic effect of laser light is used for treatment of lesions from overloading or degenerative lesions in musculoskeletal system. Laser light improves regeneration of tissue, is used for treatment of wounds, ulcerations and, in cases of improper healing, of bones [2].

Biostimulating lasers are of special interest to scientists who treat cancer by photodynamic therapy (PDT). This method involves a kind of selective detection and destruction of tissue sensitized by special dye, thanks to the photochemical reactions [4]. Three factors are used to achieve it: the substance with photosensitizing features, the photosensitizer, the light of appropriate wavelength and the oxygen [5]. Used individually, each of the factors should be harmless for tissue. Their joined action causes toxic effect on tissues, based mainly on action of free radicals, created due to induction of photosensitizer the light [6]. PDT is used for the treatment of cancers, especially melanoma.

Currently, there have appeared some scientific studies concerning the use of this method for therapy of cervical cancer, as an alternative to available treatment methods of this cancer [7]. Search for new treatment methods of cervical cancer seems to be appropriate since it is a very common tumor, burdened with a high degree of mortality [8].

The aim of this work was to investigate the potential of using biostimulating lasers in the treatment of cervical cancer cells after photosensitization.

Materials and methods

Chemicals

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO). The stock solution of protoporphyrin IX (2.5mmol/l) was obtained by dissolution in dimethyl sulfoxide. The final concentrations (0,5; 1,0; 5,0 and 10,0μmol/l) were obtained directly in the culture medium at the time of incubation.

Cell culture

The studies were performed on tumor HeLa cells, obtained from human epithelial cervical cancer of a 31-year-old African American woman, Henrietta Lacks, who died in 1951 [9]. The cells were cultured in RPMI 1640 supplemented with 5% FCS, 2 mmol/l L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin in a 5% CO2-humidified atmosphere at 37oC. After 24–48 hours incubation the cells were transferred to 96-well plates. The cells were rinsed off using 96% solution of PBS (phosphate-buffered saline) and digested with 0.05% trypsin (3 minutes at 37oC). Then, trypsin was removed and the cell suspension was prepared in 2 ml of medium. The cells were spun down and the sediment was suspended in 4 ml of fresh medium. The suspension was divided into 4 test-tubes and the medium was added to each test-tube in 1:8 proportion. The average density of cells in 1 ml of suspension was 885 thousand/ml. Hundred μl of cell suspension was placed in each well of a 96-well plate. In the tests we used 48-hours cell cultures.

Photodynamic therapy treatment

HeLa cells were preincubated for 6 or 18 hours with protoporphyrin IX final concentrations as above. After this period PpIX was photoactivated by laser light (diode laser), at the wavelength of 635 nm and power of 30mW/cm^2.

What is important, the laser head was placed directly on the cover of 96-well plate. Irradiation continued for 8 minutes and was the same in all experiments. Then, the cells were incubated for 24 or 48 hours. After this period the analysis of PDT toxicity was carried out. Each experiment was repeated 14 times and PDT results were compared with a control group of cells, which were incubated with non-activated PpIX.

XTT viability test

The viability of cells was analyzed using the XTT colorimetric test, based on dynamics of reduction of XTT pigment (tetrazoline-2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-5-carboxyanilide) by the living cells and production of the colorful product. Intensity of the fluorescence was measured at the wavelength of 450nm [15].

The percentage of mitochondrial activity inhibition was calculated according to the following equation: 100−(OD of drug treated cells−OD of medium alone/OD of untreated cells−OD medium alone×100); where OD is optical density, untreated cells are control cells [10].

Statistical Analysis

Statistical analysis of the results was done with nonparametric Kruskal-Wallis test ANOVA (post-test Dunn's) using the STATISTICA ver.5 software (Statsoft, Krakow, Poland). P value <0.05 was considered statistically significant.

Results

Influence of protoporphyrin IX concentration and preincubation periods on cytotoxic effects in HeLa cells/ 24-hour incubation

In order to assess influence of protoporphyrin IX concentration and preincubation periods on cytotoxic effects in HeLa cells, the cells were preincubated for 6 or 18 hours with different concentrations of PpIX. After these periods of time, the photosensitizer was photoactivated for 8 minutes by laser light wavelength of 635 nm. Then, cells were incubated for 24 hours and, after this period, we assessed the percentage inhibition in mitochondrial activity using the colorimetric XTT test.

Results of cytotoxicity were joined and are graphically shown in Figures 1 and 2.

Figures show the importance of preincubation time of cervical cancer cells with protoporphyrin IX. In the case of a 6-hour exposure of cells to photosensitizer, the inhibition in mitochondrial activity was low as compared to the control group of cells, reaching the value of below 20%.

Elongation of preincubation time to 18 hours caused a significantly higher cytotoxic effect as compared to the 6-hour preincubation ($p<0.01$). In the highest concentration of PpIX used in the study, 10μmol/l, the inhibition reached 34%.

Use of still higher concentration of protoporphyrin IX did not result in a significant increase in the cytotoxic effect ($p>0.05$).

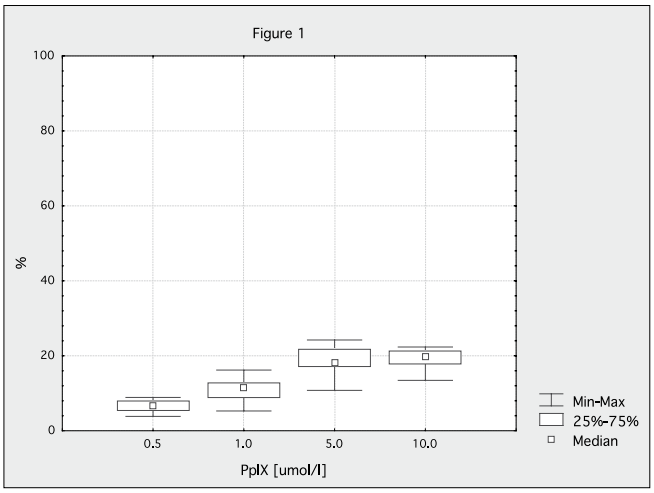

Figure 1. Preincubation time: 6 hours.

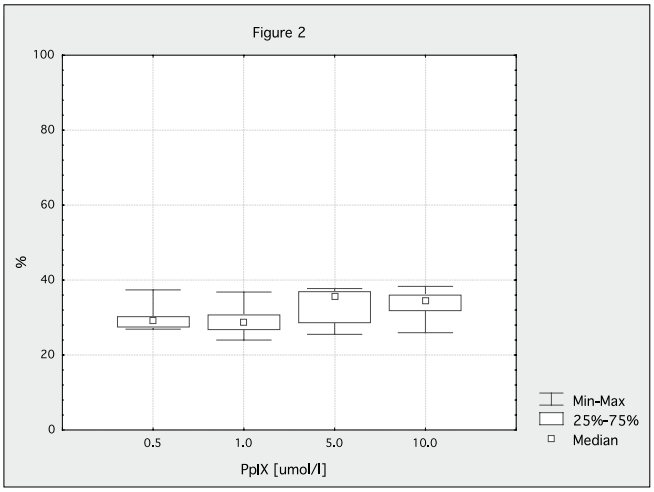

Figure 2. Preincubation time: 18 hours.

Influence of protoporphyrin IX concentation and preincubation periods on cytotoxic effects in HeLa cells/ 48-hours length of incubation

In order to assess influence of protoporphyrin IX concentration and preincubation periods on cytotoxic effects in HeLa cells, the cells were preincubated for 6 or 18 hours with different concentrations of PpIX. After these periods the photosensitizer was photoactivated for 8 minutes using laser light of the wavelength of 635nm. Then, the cells were incubated for 48 hours and, after this period, we assessed the percentage of mitochondrial inhibition by colorimetric test, XTT.

Results of cytotoxicity were joined and are shown graphically in Figures 3 and 4.

Similarly to the case of 24-hour incubation, the figures demonstrate how important the time of preincubation cervical cancer cells with protoporphyrin IX was. In the case of the 6-hour exposure of cells to photosensitizer, the percentage of mitochondrial activity inhibition, as compared to the control

group of cells, varied between 20% and 33%. Elongation of preincubation time to 18 hours caused a significantly higher cytotoxic effect as compared to the 6-hour preincubation ($p<0.001$). The effect reached 63% when the highest PpIX concentration used in the studies (10μmol/l) was applied.

Using still higher concentrations of protoporphyrin IX did not bring any significant increase in the cytotoxic effect ($p>0.05$).

Discussion

Biostimulating lasers (LLLT) cause mainly analgetic, anti-inflammatory and regenerative effects on body cells. As a result of the exposure to low and medium power laser radiation, both molecular and cellular changes can be observed in the cell. The cellular metabolism increases, as demonstrated by an increase in adenosine-5′-triphosphate (ATP), DNA and RNA contents, increase in the membrane enzyme activity and improvement in electrolyte exchange between the cell and external environment. At the level of tissue, an improvement of blood and lymph circulation, hyperpolarization of nerve endings and stimulation of the immune system can be noted. In exposed tissues collagen synthesis manifests an increase. The range of activities allows to use lasers in physical therapy of pain and inflammations [11].

At this point it ought to be mentioned the cancer poses a complete contraindication to laser biostimulation [1]. However, the present study indicates that biostimulating lasers can be used for cancer therapy, on condition that cancer cells had been earlier photosensitized by a special substance, the photosensitizer. An increase in the use of LLLT for treatment of cancer has been noted in recent years. Several reports point to the fact that radiation of tissue gives good results in this type of therapy due to specific features of laser light which allows to direct the light beam precisely at the lesion, without destroying the surrounding tissue. The light induces the photosensitizer in the tissue selectively because it is monochromatic and has high ability of tissue penetration. Low level power of the laser allows to avoid thermal destruction in tissue, which decreases the risk of healthy tissue damage. Laser light can be transferred through an optic fiber, which provides an additional advantage. This light source seems to be the best solution for that sort of therapy [12].

Results of the present study have shown that photodynamic method with laser light is useful for destruction of cervical cancer cells. It is reflected by results of colorimetric test with tetrazolium salts, XTT. This test serves to determine metabolic activity of live cells. Tetrazolium salts are reduced to formazan dye by mitochondrial succinate dehydrogenase. This enzyme is active only in the cells which have an undisturbed metabolism and electron transport chain. The quantity of formazan is calculated photometrically and it correlates with the number of live cells [13].

The examined HeLa cells which were exposed to photodynamic therapy show inhibition of mitochondrial activity in photosensitizer concentration of 0.5 μmol/l and upon 6-hour period of preincubation. This effect has been poor, though. The influence of PpIX on cytotoxicity is well shown in 18-hour preincubation and 48-hour incubation. On the basis of XTT test results, we can conclude that the cytotoxic effect was dependent on photosensitizer concentration and periods of preincubation and incubation.

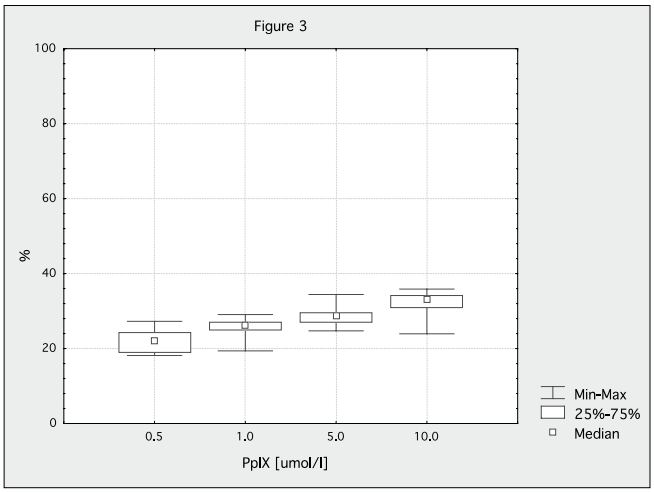

Figure 3. Preincubation time: 6 hours.

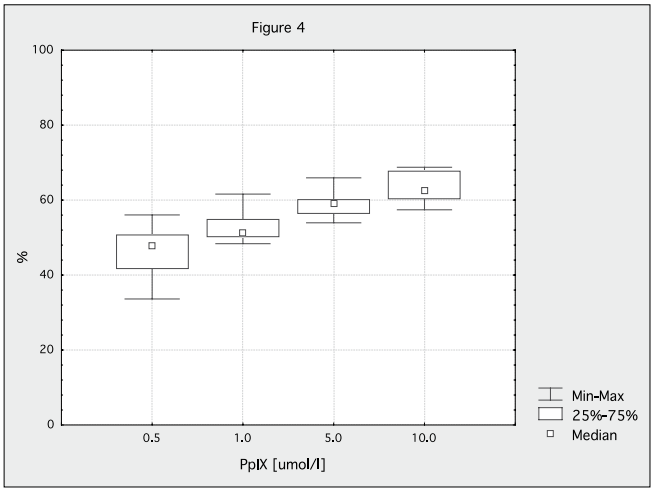

Figure 4. Preincubation time: 18 hours.

What proved important was that each time the period of exposure to laser light was quite short (8 minutes), which manifests the high capacity of laser to excite photosensitizer molecules.

These results allow us to state that photodynamic therapy is a good way to destroy cervical cancer cells. The pre-condition for therapy effectiveness involves application of the proper PpIX concentration and an appropriate period of time between application of the photosensitizer and radiation. It must be remembered, however, that these experiments were conducted in vitro. Acceptance of the hypothesis on efficacy of photodynamic therapy in cervical cancer treatment requires similar studies to be performed in vivo. The references and the fact that photodynamic therapy is accepted as a form of therapy of cervical cancer in Japan, allow us to accept this hypothesis [14].

This work proves the effectiveness of photodynamic therapy on cancer cells in in vitro conditions. These experiments can be treated as an introduction to the clinical use of photodynamic elimination of cancer cells. An important problem will be to determine whether effective elimination of cells takes place also in in vivo conditions. It is necessary to encourage independent investigators to examine photodynamic therapy in order to improve its efficacy.

Conclusion

1. Photodynamic therapy is an effective method of destroying cervical cancer cells.
2. Biostimulating lasers are a proper source of light to be used to destroy cervical cancer cells, providing that cells were earlier photosensitized by protoporphyrin IX.
3. The most important condition to ensure high efficacy of the method is to match appropriate periods of time of preincubation and incubation with protoporphyrin IX.

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