

# Effects of Combined Sonodynamic and Photodynamic Therapies on a Colon Carcinoma Tumor Model

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## Abstract

#### **Objective**(s)

Although photodynamic therapy is considered as a noninvasive method, most photosensitizers are susceptible to ultrasound. Therefore, it is expected that the combination of two activation methods might have a synergistic effect. This probable effect has been investigated in this study.

#### **Materials and Methods**

This study was conducted on colon carcinoma tumor in Balb/c mice. The tumors were induced by subcutaneous injection of CT26 cells. Ultrasound and light irradiations were performed on tumors 24 hr after injection of liposomal Zn (II)-phthalocyanine. The treatment efficacy was evaluated using daily measurement of the tumor dimensions.

#### Results

Ten days post treatment, relative tumor volumes of all groups were significantly reduced in comparison with the main control group. The best response was observed when one of the two treatment methods had been applied. The longest doubling time of tumor was related to the treatment group namely photodynamic, sonodynamic and combination technique, while the shortest belonged to the control group.

#### Conclusion

This study showed that liposomal Zn phthalocyanine is both photosensitizer and sonosensitizer. Photodynamic and sonodynamic therapies can be efficient in retarding tumor growth rate. In this study, the combination of two methods didn't show any improvement in therapeutic outcomes. It is predicted that latest results are related to the treatments sequence and could be optimized in the future.

Keywords: Carcinoma, Colon, Liposome, Photodynamic therapy, Sonodynamic therapy, Zn (II)-phthalocyanine

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## Introduction

Surgery, radiotherapy and chemotherapy are three appropriate methods used for the treatment of large invasive tumors (1). However, development of new techniques and/or improvement of detection techniques of malignant cells are crucial. In that case, there would be potent treatment methods for smaller tumors with less invasiveness. Photodynamic therapy (PDT) is a treatment modality that combines a photosensitizing agent with a proper wavelength of light in order to selectively destroy cells. The interaction of photons with photosensitizers in the presence of oxygen molecules results in the formation of oxygen radicals and singlet oxygen that causes damage to the irradiated tissue; thus, the outcome will be necrosis and apoptosis (2).

Currently, PDT is routinely used in a number of countries such as Russia, England and Italy (3). However, only some studies have been reported on *in vivo* and *in vitro* application sonodynamic therapy in combination with the second or third generations of sensitizers (4-8).

There are two important limitations on PDT; the first one is the side effects of the photosensitizers, and the second is the lack of penetration of visible light in tissues (9). Therefore, it seems logical to look for new modalities in order to decrease undesired side effects, and at the same time to increase the depth of treatment. On the other hand, there have also been reports confirming the activation of certain photosensitizers by ultrasound (10). Some studies on cell death mechanisms after ultrasonic activation of photosensitizers have been carried out. Based to these studies, cavitation is mainly responsible for producing free radicals and consequently cell death. This procedure is called sonodynamic therapy (SDT) and the dyes being used are known as sonosensitizer (11)

Jin *et al* (2000) evaluated the effect of combined sonodynamic and photodynamic therapies on squamous cell carcinoma of C3H/He mice in the presence of ATX-70 and PH-1126 as sensitizers. Based on their report, single treatment stopped tumor growth by 27-77 percent, whereas combined treatment inhibited

the tumor growth to 92-98 percent, and increased the animals' survival from 77 to 95 days. Pathological examinations have indicated 2-3 fold increase in the depth of tumor necrosis (12)

Hachimine *et al* (2007) applied sonodynamic therapy after the administration of DCPH-P-Na (I) to Balb/c athymic nude mice. MKN-45 cells were subcutaneously injected into the back of nude mice and led to tumors with a diameter of approximately 5 mm. After 24 hr, ultrasound irradiation has been done for 10 min at 1 MHz, and two different intensities (1.0 or 2.0 W/cm<sup>2</sup>) and a 50% duty cycle. The growth of the MKN-45 tumors was significantly inhibited within 15 days after the treatment in comparison with the control group (13).

Local SDT combined with whole body PDT via photoflora as sensitizer is now being used at Opal clinic in Australia to treat breast and prostate carcinomas (14). Pharmacokinetic characteristics of zinc phthalocyanine (ZnPc) make this molecule a promising second generation phototherapeutic agent. Its quantum efficiency is relatively high to produce singlet oxygen, and its toxicity in the absence of light is low. Photoactivation of this dye in wavelength of 670 nm provides an extra feasibility in the treatment of relatively thick and deep tumors (15). Furthermore, Miloska et al (2005) have shown that ZnPc has sonosensitizing property (4), while preparing pharmaceutical formulations that enables ZnPc's systemic administration is highly difficult. Due to their low water solubility, hydrophobic photosensitizers cannot directly be injected intravenously. As a result, several different strategies have been employed to prepare stable formulations of hydrophobic photosensitizers such as conjugation to watersoluble polymers or encapsulation liposomes, gold and polymer nanoparticles (16). The current study has investigated photo and sono sensitizing properties of a liposomal ZnPc form. Because ultrasound would penetrate in soft tissues more than visible light (14), it was predicted that the combination of these treatments modalities could improve PDT efficacy in a certain dye dose.

In this research, the combined effects of SDT and PDT on a colon carcinoma tumor model

have been evaluated using liposomal ZnPc.

# **Materials and Methods**

#### Cell line and culture conditions

CT26 cell line derived from a tumor colon carcinoma of a Balb/c mouse was grown in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 50 units/ml penicillin and 50  $\mu$ g/ml streptomycin. Cell culture was performed at 37 °C in a 5% CO<sub>2</sub> humidified incubator. They covered bottom of the flask as a monolayer after 2-3 days of the growth and proliferation of the cells. Exponentially growing cells were trypsinized using 0.05% trypsin-EDTA. The cell survival rate and their number were determined by a hemocytometer using trypan blue.

## Tumor models

Female and male Balb/c mice, aged 6 - 8 weeks weighing 20-22 g were purchased from Iranian Pasteur Institute. The mice were housed in an animal facility in Medical Physics Research Center at  $23\pm2$  °C, 65% moisture, and 12 hr darkness and brightness, alternatively. In order to create a tumor model, CT26 tumor cells (5×10<sup>5</sup> cells per mouse) were implanted subcutaneously in the right dorsum of animals. When the tumor volume reached 100±20 mm<sup>3</sup>, the mice underwent the study. After tumor induction (nearly 30 days post injection), the tumor tissue was subjected to the pathological examinations and affirmed to be a tumor.

## Animals' anesthesia

The mice were anesthetized before the illumination or the exposure to ultrasound, via intraperitoneal injection of ketamine hydrochloride (100 mg/kg), and chanazine (5 mg/kg) (17).

## Chemicals and preparation of liposomal ZnPc

ZnPc was obtained from Sigma-Aldrich (97% dye content). In order to formulate the photosensitizer in a liposomal form, 300 mg of egg lecithin, 100 mg of cholesterol, 400 mg of glucose and the required amount of ZnPc powder were dissolved in 10 ml of pyridine. The solution was frozen through particular processes

using dry ice and subsequently dried via freeze dryer (Labco Co-USA) during two consecutive stages of -40 °C and -25 °C temperature designed for a period of 24 hr (18). Eventually, to prepare the final stable solution, 2 ml of distilled water was added and mixed completely by a vortex. Based on the spectroscopic results from the liposomal suspension supernatant, the encapsulation rate of ZnPc was determined to be more than 85% by this technique. Distribution and average of liposoms size were estimated as 1- 6.5 µm and 1.6 µm, respectively (19).

## Light source

An incoherent light source, as LUMACARE, equipped with a piece of fibers optic bundle and a band pass filter of  $670\pm20$  nm was utilized for illumining the tumors. Light homogeneity of the source was  $\pm5\%$ . Illumination intensity was 160 mW/cm<sup>2</sup> and total exposed light was 300 J/cm<sup>2</sup> (20). Illumination parameters were assessed by a photometer (CON-TROL-CURE IL1400; UVPROCESS, USA).

## Ultrasound generator system

Irradiation of ultrasound was conducted with a 215A ultrasound generator in continuous mode and frequency of 1.1 MHz with maximum intensity of 1 W/cm<sup>2</sup> for 10 min (21). Ultrasound probe was planar and surface area of the piezoelectric crystal was 7.0 cm<sup>2</sup>.

## Experimental protocol

As the tumor diameter reached about 5 mm, the animals were randomly divided into 8 groups each containing 10 mice (5 males and 5 females) (22). In the beginning of treatments,  $1.46 \,\mu M/kg$ liposomal ZnPc was injected to four animal groups intraperitoneally (18, 23). After 24 hr (6), the tumors of one group were only irradiated by light. In the second group ultrasound was applied to the tumor as shown in Figure 1 (24), and the third group was treated by light followed by ultrasound. No irradiation was applied to the fourth group. In the other four groups of animals, saline injected normal was instead of photosensitizer and the same treatment regimes first four groups were applied. of the



Figure1. The experimental set-up for ultrasound exposure to the animal tumor models.

The treatments was followed up via daily measurement of tumor diameters- including small diameter (a), large diameter (b) and tumor thickness (c)- using a digital caliper with 0.01mm precision, and estimation of tumor volume (V) as  $V = \pi/6$  (a. b. c) (21). The measurements were continued until 120 days after treatment, which is the maximum survival time of animals.

#### **Treatments Efficacy Evaluation**

For each tumor, the first day of treatment was considered as day zero and relative tumor volumes in later days were accordingly normalized. On the basis of daily variations of the relative tumor volume, doubling time of each tumor was determined and the doubling time of the tumors was estimated in each group.

The cumulative survival fraction was also assessed in various groups using Kaplan-Meier method.

#### Statistical analysis

All data were analyzed using SPSS 12 after performing normality test and selection of proper comparative tests. According to the normality test of Kolmogorov-Smirnov, the data distribution was not normal. Consequently, the Mann-Whitney U test was used to compare relative tumor volume with a confidence level of 95%. Moreover, after calculating the cumulative survival fraction of animals via Kaplan-Meier method, log rank test was applied to compare between groups. Doubling time of tumors was also compared in different groups using T-Test.

## Results

Treatment results of different groups were

evaluated from different viewpoints including comparison of relative tumor volume in the post treatment first day, function of relative tumor volume versus day and doubling time of the tumor volume. Relative tumor volume variations are presented in Figure 2. In all groups, the growth of the CT26 tumor was significantly inhibited within 10 days after the treatment in comparison with the control group (P < 0.001). The groups received PDT or SDT showed significant differences in comparison with the groups receiving only dye, light, or ultrasound (P < 0.012). Furthermore, significant difference in the relative tumor volume was observed between the groups receiving combined treatment and PDT or SDT (P < 0.003).

Considering variations of the relative tumor volume and correlation of various mathematical functions fitted into the data obtained from different groups, the most suitable equation with the best regression corresponding to experimental data was evaluated for each group. These functions and their regressions (R<sup>2</sup>) are shown in Table 2.

As it can be seen, mean of tumor volume changes in all groups followed an exponential function except for groups of photodynamic, sonodynamic and combined treatments which were fitted into a third power function.

Tumors doubling time of different groups is shown in Figure 3. The longest doubling times were observed in photodynamic, sonodynamic and combined treatment groups, respectively. The shortest doubling time of tumors was observed in the control group (3.36 days).

Cumulative survival fractions for different groups are recorded in Figure 4.



Figure 2. Relative variations of tumor volume in different groups for the first 10 days post treatment. The data show the mean of 10 tumor volume measurements in each group.

Table 2. Correlation of various mathematical functions with relative tumor volume variations in different groups and the best fitted mathematical function with the  $R^2$ -value corresponding to empirical data for each group (V<sub>0</sub> indicates the relative tumor volume and t shows the days after treatment).

$R^2$	The best fitted mathematical function	Group
0.881	$v_r = e^{(0.0787t)}$	control
0.904	$v_r = e^{(0.0926 t)}$	Receiving light
0.921	$v_r = e^{(0.0793 t)}$	Receiving ultrasound
0.909	$v_r = e^{(0.0919 t)}$	Receiving liposomal ZnPc
0.857	$v_r = e^{(0.0716 t)}$	Receiving light and ultrasound
0.910	$v_r$ = - 0.377 t+ 0.43 t <sup>2</sup> + (0.25×10 <sup>-4</sup> ) t <sup>3</sup>	Photodynamic therapy
0.873	$v_r$ = - 0.367 t+ 0.0388 t <sup>2</sup> + (4×10 <sup>-4</sup> ) t <sup>3</sup>	Sonodynamic therapy
0.845	$v_r$ = - 0.0856 t+ 0.0655 t <sup>2</sup> - (3×10 <sup>-4</sup> ) t <sup>3</sup>	Combined treatment





Iran J Basic Med Sci, Vol. 14, No. 3, May-June 2011 209



Figure 4. Variations of cumulative survival fraction in different treatment groups versus day based on Kaplan-Meier calculations (each group includes 10 mice).

Animals' survival didn't show significant difference between group receiving SDT and the groups receiving only ultrasound or dye. The difference of animals' survival between PDT and the groups receiving only light or dye was not significant either. While in combined treatment, there is a significant difference in comparison with the group subjected to SDT (P= 0.03) was observed.

## Discussion

PDT and SDT have been frequently applied to treat small tumors using different dyes as a photosensitizer such as protoporphyrin IX or sonosensitizer, e.g. ATX-70. Recently, metal phthalocyanines have been utilized by many researchers second generation as photosensitizers. The study of Miloska et al (2005) indicated ultrasonic sensitization of ZnPc (4). Considering photo and sono sensitivities and hydrophobic property of ZnPc, the proposing of a proper formulation without canceling its optical and sonic specifications is useful. There are several approaches to prepare stable formulations of hydrophobic photosensitizers such as conjugation to water-soluble polymers, polymer nanoparticles, gold and and encapsulation liposomes (16). In current research photo and sono sensitizing properties of a liposomal ZnPc form have been examined.

Furthermore, PDT and SDT modalities were combined to improve PDT efficacy via a single dose of liposomal dye. Thus far, there has not been reported a similar research considering all parameters of our study to evaluate photo and sono sensitivities of ZnPc.

Based on our results, the best response to the treatment was observed in groups subjected to photodynamic or sonodynamic therapy. Also under these circumstances, relative decrease in tumor volume was more than combined Regarding increased animals' treatment. cumulative survival fraction in combined treatment, it can be introduced as an effective mechanism to prolong survival of the animals in comparison with groups receiving photodynamic or sonodynamic therapies alone, and it can be inferred that attention to the tumor size variations is not sufficient to judge the efficacy of photodynamic and sonodynamic therapies.

Therefore, if the data concerning two parameters of tumor volume variations and animals' cumulative survival fraction are compared with reference to daily variations of the tumor volume after treatment, efficacy in combined treatment would be lower than PDT or SDT, while animals' survival in combined treatment has been recorded higher than individual treatments.

On the other hand, soft tissue can encounter at

least one of these events after ultrasound exposure: permeability in cell membrane, and sonodynamic activation (25). In the present study, utilization of the second procedure was our goal. However, induced permeability in cell membrane is more feasible at lower frequencies of ultrasound (26), nevertheless, after interacting of mechanical strokes with cell membrane, enhancement in cell permeability and subsequent leakage of ZnPc molecules is not impossible. Because SDT was administered before PDT, an assumption is therefore reinforced in relation to increased permeability of cell membrane and excretion of some ZnPc molecules from the tumor cells.

Considering significant loss of animals' survival in the group receiving liposomal ZnPc in comparison with control group, drug toxicity in intraperitoneal injection can be proposed; however, regarding significant increase in animals' survival in the group subjected to combined treatment in comparison with the groups receiving only one treatment, it seems that effective combined treatment was due to drug toxicity. In other words, if the group receiving drug is considered as a sham group, after combined treatment and activation of ZnPc by light and ultrasound, enhancement in cumulative survival would be greater than its loss resulting from drug toxicity.

## Conclusion

Treatment response in groups subjected to photodynamic or sonodynamic therapy confirmed photo and sono activations of liposomal ZnPc. It can be inferred that photodynamic and sonodynamic therapies could be effective in slowing down the tumor growth process, but it is evident that the tumor growth would not stop while just one treatment session is applied.

Since in the combinational treatment, 24 hr after injecting of liposomal ZnPc, the tumors had been subjected to the irradiation of ultrasound and light respectively and leakage of sensitizer molecules from tumor cells after ultrasound exposure is predicted, it is expected that reverse treatment sequence can provide better response.

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Iran J Basic Med Sci, Vol. 14, No. 3, May-June 2011 211

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