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The Role of Crocetin-Loaded PLGA Nanoparticles as a Pre-Treatment Agent on Indocyanine-Photodynamic Therapy of Breast Cancer Cell

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ABSTRACT

Introduction: Photodynamic therapy (PDT) can be considered as a non-invasive method for cancer treatment. One of the most commonly of a water-soluble dye photosensitizer (PS) used in photothermal therapy (PTT) and PDT is Indocyanine Green (ICG). However, high cytotoxicity in high concentration and instability in aqueous media were limited its application. It was shown that using nanoparticles or plant extracts in combination with PS could improve PDT efficiency. In this study, anti-cancer properties of crocetin (Crt) loaded PLGA (Poly lactic-co-glycolic acid) nanoparticles (NPs) were utilized to increase the PDT efficacy with ICG on the MCF-7 cells.

Material and Methods: Crt was encapsulated into PLGA NPs and its particle size distribution and encapsulation efficiency were evaluated. IC_{10} of Crt, PLGA-Crt NPs and ICG was determined by MTT assay in MCF-7 cancer cells. At these concentrations, the cells were pre-treated with Crt or PLG-Crt, then treated with ICG and finally exposure to near infrared (NIR) laser with 2.5 W powers at different times. The cells viability was evaluated by the MTT assay.

Results: The findings showed no dark cytotoxicity due to ICG (12.9 µM), Crt or PLGA-Crt alone. But NIR laser irradiation in the presence of ICG after cells pre-treatment by the Crt or PLGA-Crt NPs leads to induce cell death to (61.6 ±7) % and (75.5 ±5) %, respectively ($P<0.05$).

Conclusion: The results demonstrated that PLGA-Crt NPs in combination with ICG could improve PDT outcomes more efficiently in comparison with Crt and ICG. Therefore, this method could be effective in breast cancer therapy with low cytotoxicity.

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Introduction

Photodynamic therapy (PDT) has attracted considerable attention as a non-invasive therapeutic technique for cancer because of low cost, short treatment time and low systemic toxicity properties [1-4]. In this method, a light-sensitive agent named photosensitizer (PS) conducts to cancer cells and after stimulation by specific wavelength, singlet oxygen converts into various reactive oxygen species (ROS) that led to the death of tumor cells [5-8]. The effect of PS on cancer cells could be influenced by different factors such as the type or concentration of Ps [9] and the amount of light radiation [10]. On the other hand, the combination of PDT and a secondary treatment can be designed to increase the effectiveness of PDT [7] and reduce toxicity due to the use of a lower dose of PS. recently, more attention has been focused on the using of plant extracts in combination with PDT for cancer treatment [3, 11]. Another approach to enhance PDT is using nanoparticles (NPs) to improve the solubility of hydrophobic PSs, controlled release and increase the concentration of PSs at desired sites [12, 13]. Various types of NPs such as liposomes, quantum dots, gold NPs, polymers, micelles, magnetic NPs, dendrimers, and carbon-based NPs have been developed to improve PSs efficiency [12].

One of the most commonly used PS is Indocyanine green (ICG) or 4, 5-Benzoindotricarbocyanine, a tricarbocyanine dye with amphiphilic molecular structure [14, 15]. This PS is approved by the U.S. Food and Drug Administration (FDA) [10, 16]. Compared to other sensitizers, ICG shows the unique specifications such as strong absorption (between 700 and 800 nm) and emission peaks at the NIR region [10], high accumulation in tumor tissue and water solubility [17, 18]. The problems associated with ICG are high cytotoxicity at high concentration [19] and instability in aqueous media [17].

Recently, the attention to medicinal herbs has been paid, significantly in the treatment of tumors. Saffron, as coloring for foods in plant Crocus sativus L, has been suggested to treat different diseases, especially cancer [20-22]. Crocetin (Crt) is a carboxylic

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carotenoid compound in saffron with different therapeutic effects such as anti-cancer properties with different mechanisms [21, 23, 24]. However, the main limitation for using of Crt in medical applications is the lack of solubility in aqueous solution [25]. One of the ideal approaches to overcome of this obstacle is encapsulation of Crt in appropriate NP to improve its pharmacokinetics and bioavailability [25, 26]. PLGA is one of the most important biodegradable and biocompatible polymers for drug delivery system which approved by FDA and European Medicine Agency (EMA) [13, 27]. In our previous study, it was shown that encapsulation of Crt into PLGA NPs could improve solubility and anticancer effects of Crt against breast cancer cell lines [25, 26]. In this study, it is hypothesized that crocetin-loaded PLGA NPs could also act as enhancer of the PDT efficiency with ICG.

Materials and Methods

Chemicals

Poly (D, L-lactic-co-glycolic acid) (PLGA) (Average M: 7000–17.000; lactic acid: glycolic acid 50:50), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) and Indocyanine Green (ICG, cardiogreen, chemical formula C43H47N2NaO6S2 and MW: 774.69) were purchased from (Sigma Aldrich). Crt was extracted from plant Crocus sativus L. based on the method represented in the Iran patent no. 84459. Fetal Bovine Serum (FBS) and RPMI 1640 with L-glutamine and $NaHCO_3$ and penicillin - streptomycin was obtained from (Gibco, USA). Polyvinyl alcohol (PVA, 87–89% hydrolyzed, and average Mw¼ 88, 000–97,000) and other solvent and chemical reagents were procured from Merck (Darmstadt, Germany).

Cell culture

MCF-7 cell line (Human Mammary Carcinoma, Epithelial-like) was purchased from Pasteur Institute of Iran and maintained in RPMI-1640, enriched with of 10% fetal bovine serum (FBS), 2 mM L-glutamine, antibiotic solution (100 IU/mL penicillin and 100 µg/mL streptomycin) in T75 culture flasks. The cells were grown in the temperature of 37°C, the atmosphere with 5% of CO_2 and humidity 95%.

Light source

The cells were exposed to a NIR (near-infrared) laser light source (MDL-III808–13020113, P.R.China). This device generates a wavelength of 808 nm in the NIR region with a power of 2.5W and beam surface of 40 mm^2.

Preparation of PLGA NPs loaded with Crocetin (PLGA-Crt)

PLGA NPs loaded with Crt were prepared by double emulsification and solvent evaporation technique as described previously [25, 26]. Briefly, 25mg PLGA polymer was dissolvent in dichloromethane (DCM). Then, 1.25 mg Crt powder dissolved in pyridine was added to the polymer and stirred for 30 min. Afterwards, the mixture was added to 4 mL 5% polyvinyl alcohol (PVA) and sonicated in ice bath (pulse on 1 s, pulse off 1 s, amplitude: 90%) for 10 min, using a probe solicitor (Fisons Instruments Ltd, Crawley, U.K). The emulsion was added to 10 ml 0.1% PVA and was kept at room temperature for (18-20) h to remove the organic solvent. Finally, NPs were washed three times with deionized water and lyophilized. PLGA NPs without Crt was also synthetized as control.

Physicochemical properties of PLGA-Crt NPs

Particle size distribution and zeta potential of the prepared NPs were determined by a particle size analyzer (PSA, Zetasizer, Molvern, USA). Encapsulation efficiency (EE) was determined by dissolving of one mg of lyophilized Crt-PLGA NPs in 1 ml dimethyl sulfoxide (DMSO). The concentration of released Crt was determined by a UV spectrophotometer (Shimadzu UV-1700 Pharma Spec, Kyoto, Japan) at 430 nm and using a Crt standard curve. The EE% (Encapsulation Efficiency) of Crt is the percentage of Crt that is successfully entrapped into the nanoparticles. EE% is calculated as the below:

EE% = (total Crt entrapped into the NPs divided by the total Crt added) ×100

Cytotoxicity assay

MCF-7 cells were seeded in 96-well plates at a density of 3×10^3 cells per well and incubated for 24 h. Then, the media was removed and 5 different concentrations of Crt (0-400 µM), PLGA NPs containing the same amount of Crt and ICG (0-250 µM) in RPMI comprising 3% FBS were added to each well. After 48 and 24 h incubation of cells with Crt or PLGA-Crt and ICG, respectively, the media was removed and the cells were washed with PBS. Then the fresh media with 10% FBS was added to each well and incubate for 48 h again. Then 10 µl of MTT (3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide) (5mg/ml) reagent was added to each well and after 4 h incubation at 37℃, the formazan crystals were dissolved in 200 µl DMSO. Optical density of the wells was read at 570 nm against 630 nm by a microplate reader (Stat Fax model 2100, USA). By performing this protocol IC_{10} (The agent concentration to induce 10% cytotoxicity) and IC_{50} (the agent concentration to induce 50% cytotoxicity) were obtained.

Evaluating the effect of Crt or PLGA-Crt on the PDT efficiency of ICG

Crt in IC_{10} concentration (15 µM) or PLGA-Crt containing equal amount of Crt was added to MCF-7 cells seeded in 96-well plates at a density of 3×10^3 cells per well.

Table 1. Conditions of the experimental groups

Group no.	ICG	PLGA-Crt	Crt	Laser exposure
1(control)	-	-	-	-
2	five concentrations	-	-	-
3	-	five concentrations	-	-
4	-	-	five concentrations	-
5	+	-	+	-
6	+	+	-	-
7	+	+	+	three exposure times
8	-	-	-	three exposure times
9	+	-	-	three exposure times
10	+	-	+	three exposure times

After 48h, the supernatants of cells were removed and ICG with concentration of 12.9 µM (IC_{10}) were added to cells following by incubation overnight. Finally, the cells were exposed with the NIR laser for different exposure time (3, 5, 10 min). Then, the cells were incubated for 48 h at 37˚C. To assess cell survival and comparing the different experimental groups together, MTT test was utilized. The different conditions of the experimental groups have been summarized in Table 1.

Judgment indexes and statistical analysis

In order to compare cytotoxicity of the agents and their photodynamic efficacy IC10, IC50 and ED50 indexes were determined and to compare the effect of the laser in different conditions of treatment, the coefficient of laser effect defined as follows (Table 2):

$$\text{Coefficient of laser effect} = \frac{(\text{Cell survival without laser radiation})\%}{(\text{Cell survival with laser radiation})\%}$$

Table 2. Evaluation of coefficient of laser

coefficient of laser	>1	negative synergistic effect
	=1	Ineffective
	<1	positive synergistic effect

Similarly, the effect of pre-treatment by PLGA-Crt, PLGA or Crt before applying ICG- mediated PDT with different laser exposures was determined as the below:

$$\text{Coefficient of pre-treatment effects} = \frac{\text{Cell survival in the presence of ICG (\%)}}{\text{Cell survival pretreated with each agent in the presence of ICG (\%)}}$$

Results

Characterization of PLGA NPs containing Crt

Size distribution and PdI (polydispersity index) of the PLGA-Crt NPs were measured. The results showed a diameter of 239.8 ± 9 nm (Fig. 1.A) and PDI of 0.3 and its zeta potential was measured at -12.4 mV (Fig. 1.B). Based on the results obtained from the standard curve, the efficiency of encapsulation of Crt was obtained about 80%. The absorption spectrum of the PLGA-Crt NPs is observed at the concentration of 1 mg/ml in a solution of DMSO in the UV-Visible (Fig. 1.C). Two absorption peaks (465, 441 nm) are shown between 200 and 900 nm.

Characterization of ICG

The absorbance spectrum of ICG (10 µg /ml in water) was measured at a wavelength range of 200-900 nm. According to this spectrum is observed two storage peaks at (714 and 779 nm) (Fig. 1.D).

Cytotoxicity assay

MTT test was used to determine the non-toxic concentration of ICG, Crt and PLGA-Crt on the MCF-7 cells (Figure 2). The obtained IC_{10} and IC_{50} of the agents were presented in Table 3. At similar concentrations, PLGA-Crt showed more cytotoxicity effects compared to Crt. PLGA formulation without Crt did not show cytotoxicity (data not shown). For next experiments, all tested compounds were used at IC_{10} concentration.

Table 3. IC50 (a concentration of the agent which leads to 50% cell death) and IC10 (The agent concentration to induce 10% cytotoxicity) obtained for the agents

Agent	IC10 (µM)	IC50 (µM)
Crt	15	200
PLGA-Crt	10	50
ICG	12.9	40

Cells viability after applying PDT mediated by ICG

Figure 3-A represents the percentage cell viability in the presence of ICG after laser irradiating for 3, 5 and 10 min. The results showed no significant difference in cell death between the treated groups.

The findings show that there is no different significant ($P>0.05$) in reduction of cell survival with pre- treatment PLGA-Crt, PLGA, Crt, in the present ICG and without ICG, while the use of ICG as PS is effective on viability of MCF-7 cells of pretreated with PLGA-Crt, PLGA and Crt (Fig. 3-B).

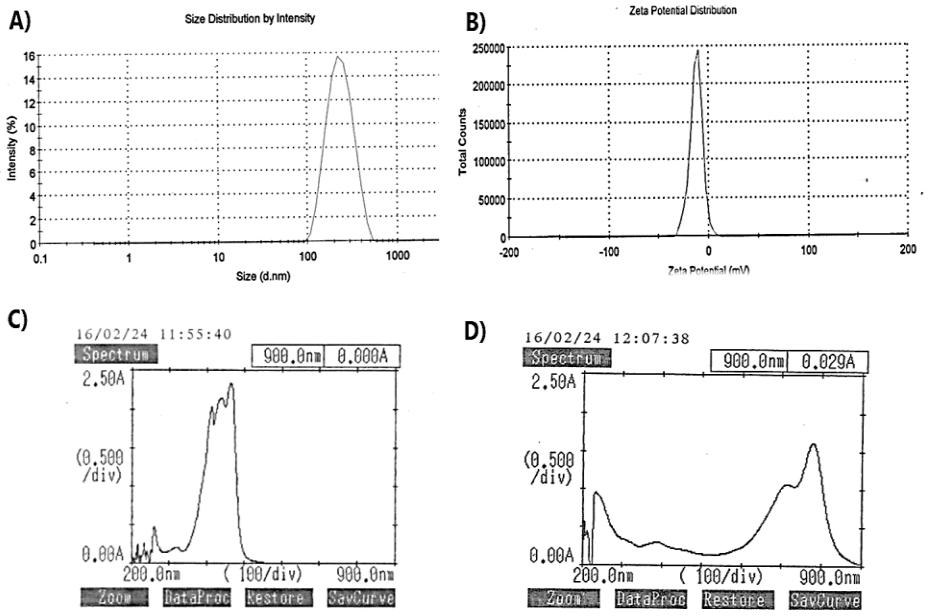

Figure 1. Characterization of agents. A) Size distribution of the PLGA-Crt nanoparticles. B) Zeta potential of the PLGA-Crt nanoparticles. C) The UV-vis spectrum of PLGA-Crt (1 mg /ml in DMSO). D) The UV-vis spectrum of ICG (10 µg /ml in water).

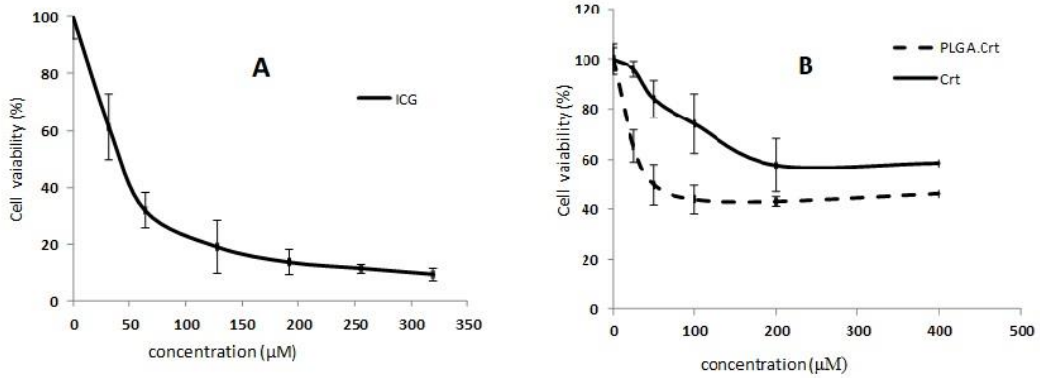

Figure 2. The effect of different concentrations of: A) ICG, B) Crt and PLGA NPs containing the same amount of Crt, on the MCF-7 cells survival. The data present the mean of at least three repetitions ± SD.

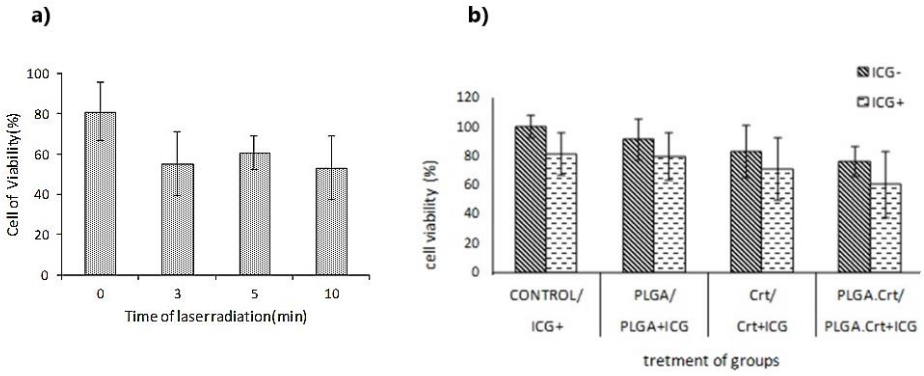

Figure 3. a) The effect of NIR laser radiation on the survival of MCF-7 cells in the presence of ICG (12.9 µM). b) The effect of ICG (12.9 µM) as PS on viability of MCF-7 cells with pre-treatment of PLGA-Crt (10 µM), PLGA and Crt (15 µM). (The data present the mean of at least three repetitions ± SD.)

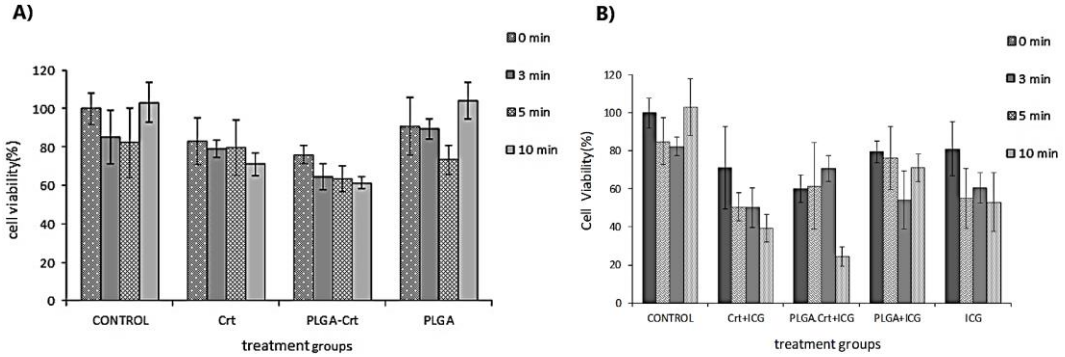

Figure 4. A) The effect of NIR laser radiation at various times on treatment groups PLGA-Crt (10 µM), PLGA and Crt (15 µM) without ICG. B) The effect of NIR laser radiation at various times on different treatment groups PLGA-Crt NPs (10 µM), PLGA and Crt (15 µM) in the presence of ICG (12.9 µM). The data present the mean of at least three repetitions ± SD.

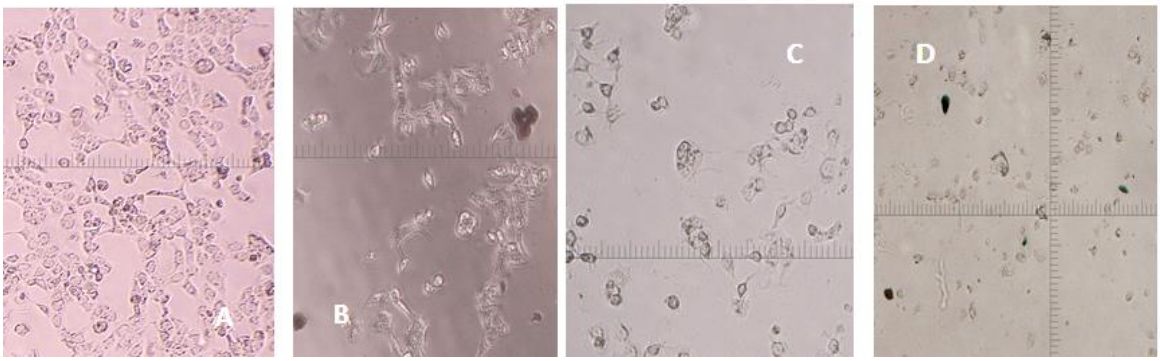

Figure 5. Microscopy images of the MCF-7. (A) Cells Morphology of MCF-7 cells before and after treatments. (B) Control group. (C) Treated cells with PLGA-Crt NPs (10µM) (C). Cells treated by ICG (15µM). (D) Treated cells with PLGA-Crt NPs, ICG and laser 808 nm for

Cytotoxicity induced by NIR laser with pre-treatment agents

Figure 4-A shows the effect NIR laser radiation treated with PLGA, PLGA-Crt and Crt on the MCF-7 cell viability. The following exposing NIR laser, PLGA-Crt showed more cytotoxicity effect compared to the other groups. On the other hand, there is no difference significant in different times of exposure groups ($p<0.001$).

Pre-treatment effect of PLGA, PLGA-Crt and Crt on PDT efficacy with ICG

The effect of exposing NIR laser associated with ICG on viability of the MCF-7 cells pretreated by PLGA, PLGA-Crt or Crt is shown in Figs. 9, 10. NIR laser irradiation in the presence of ICG with pre-treatment Crt leads to the death of MCF-7 cells, whereas minimum survival in this group is obtained after 10 min irradiation (60.3 ±7.4**)** %. The maximum reduction in cell viability is observed in the groups receiving the PLGA-Crt with 10 min NIR laser irradiation in the presence of ICG **(**75.5 ±5.3**)**%. In general, there is significantly differences in cell survival among the receiving groups of PLGA, PLGA-Crt or Crt and ICG with similar laser exposure time in comparison with the control group ($p<0.001$). The use of PLGA NPs without ICG with 10 min of NIR laser irradiation not only reduces cell death, but lead to the proliferation of cells (103.1 ±14.9) % (Figs. 4-B, 5).

Coefficient of laser

To consider the outcome of laser exposure on the cells with the different agents, a laser index was calculated as coefficient of laser. It was obtained from the ratio of cell survival percentage in the treatment groups without laser to cell survival in the similar groups with laser radiation (Table 4).

ED $_{50}$ calculation

Fig.6 shows the ED_{50} of PLGA-Crt-ICG group is ~1125 J/cm^2 and it is created in other groups at a later time. According to the obtained curve, there is a significant difference between the PLGA-Crt NPs in the presence of ICG with control, PLGA-Crt and Crt groups after 10 min of radiation ($p<0.001$).

Table 4. The coefficient of laser calculated for the groups receiving Crt-ICG, PLGA-Crt-ICG, PLGA-Crt and ICG with laser exposure

Laser exposure time (min)	No agent	ICG	Crt+ ICG	PLGA-Crt +ICG	PLGA+ICG
3	1.2	1.47	1.4	0.98	1.04
5	1.24	1.34	1.41	0.85	1.47
10	0.99	1.53	1.81	2.45	1.12
Coefficient of Laser (mean ±SD)	1.1±0.17	1.4±0.09	1.2±0.13	1.3±074	1.2±0.22

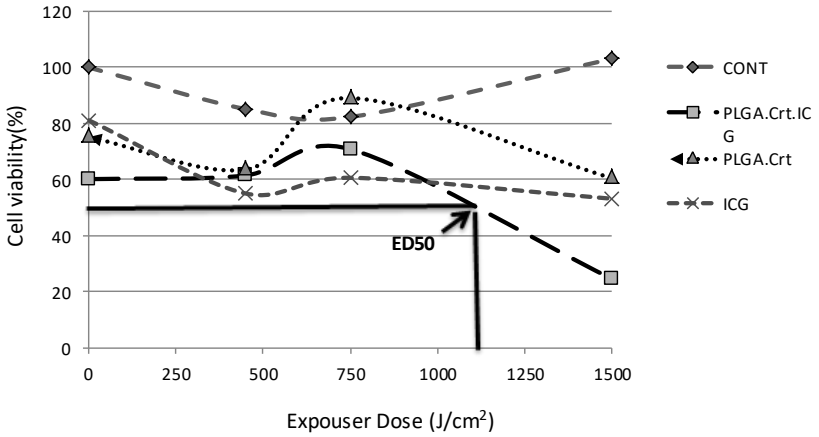

Figure 6. Cell survival variations against NIR exposure dose (ED) in the groups receiving PLGA-Crt-ICG, PLGA-Crt and ICG. The data present the mean of at least three repetitions ± SD.

Discussion

In a PDT process, selecting a proper PS is critical to the successful eradication of malignant tumor cells [28], an appropriate PS should be nontoxic before light activating [3, 29]. For systemic application and activation (activating) by an appropriate wavelength, it's better to have a hydrophilic PS [3]. ICG has been reported as a photothermal and photodynamic agent, that is activated by Near-infrared (NIR) laser 808 nm (suitable for tissue penetration) [18]. This agent converts optical energy into thermal energy and has proposed it as an effective NIR absorber for laser-mediated photothermal therapy [15, 18]. According to experiments, the amount of ICG used is about 12.9 µM, with increasing concentration of ICG, the cell viability decrease. In this study, the survival rates at minimum and maximum concentrations of ICG 62% and 12 **%** respectively Fig. 10. Abadi et al. investigated, toxicity of ICG with concentrations (5, 10, 32 and10 µM) on the MCF-7 cells. Therefore, after performing the MTT assay, it was concluded that concentration of more than 100 µM in the cell causes toxicity, the results of this study is similar to our study.

Recently, derivatives of traditional medicinal plants have been used as effective medicinal agents in modern medicine. [24]. Drugs derived from some plants as photodynamic agents cause cell death through various mechanisms such as apoptosis, necrosis, cell cycle interference, and various cell signaling pathways [9, 30]. There are several natural products which have been extensively explored as PSs such as L. Racemosa, C. Odorant and A. Procera [3, 31]. Crocetin, a carotenoid compound isolated from saffron, has shown promising effects as an anti-tumor agent [24]. Crt inhibits DNA, RNA, and protein synthesis in malignant cells [21]. However, the biological applications of Crt have been limited due to its hydrophobic nature and low aqueous solubility. Encapsulation of Crt in NP systems could be improved water solubility, pharmacokinetics and bioavailability of Ctr [24-26].

In the present study, Crt was used as pretreatment, according to experimental observations, the appropriate concentration of Crt is for the therapeutic process approximately 15 µM, which leading to in 10% cell death, and in high concentration (200 µM) results in 70% cell death. Ying-Zhong et al (2011), show that the appropriate concentration of Crt depends on the behavior and type of the cell [22]. The use of Crt with concentration of (60-240 µM) after 48h leads to a decrease in cells proliferation for three cell lines (HeLa, SKOV3 and A549) [22].

On the other hand, it has demonstrated that NPs could increase PDT cancer treatment by improvement of PS delivery efficiency through enhancement of cellular uptake of PS drugs in targeted tumor cells reduce toxicity, as well as increase stability and solubility of PSs [32].

PLGA NPs are considered as one of the exciting candidates for drug delivery applications due to their unique features such as high biocompatibility, optimal drug loading capacity, and controlled drug release [25, 26]. PLGA NPs are being considered as a nano-delivery system for photosensitizers in PDT.

In the study of Saxena et al., NPs was used as a delivery agent for ICG. On the basis of their report, when ICG was administered as PLGA NPs, more uptakes were recorded by various organs in compared to free ICG. ICG Recovery from different organs showed

efficiency above 80% [33]. In another study, PLGA NP was utilized as a model to deliver a hydrophobic photosensitizer of Zn (II)-tetraphenylporphine (ZnTPP) to the HeLa cells. The results showed that ZnTPP-PLGA NPs coated with poly ethylene glycol (PEG) provide a high potential as a delivery system in photodynamic applications [13]. In this study, influence of a pre-treatment with free Crt and PLGA-Crt NPs before PDT mediated by ICG was evaluated on MCF-7 cell line. Our data showed in the group of ICG receiving in the presence of PLGA-Crt NPs has been induced more cytotoxicity compared to Crt after 10 min irradiation (Fig. 10). This finding is in agreement with our previous studies [25, 26]. Pre-treatment of the cells with free Crt or PLGA-Crt showed more cytotoxicity compared to ICG alone after NIR laser irradiation. However, PLGA-Crt and ICG received group showed the highest cytotoxicity compared to the other groups. Our results indicated that Crt can be considered as enhancer agent along with ICG in PDT. Therefore, encapsulation of Crt into PLGA NPs as a pretreatment agent can improved the efficiency of PDT in the presence of ICG [13].

Conclusion

In this study, in order to utilize from ICG properties in PDT and PTT, 3 radiation doses of NIR laser with the power of 2.5 W with and without a pre-treatment of PLGA, PLGA-Crt or Crt were performed on the independent groups of the cells. Our findings confirmed photosensitizing effect of ICG on the cells. Also, pre-treatment of PLGA-Crt NPs and Crt with ICG and NIR laser leads to a significant reduction in the survival of cancer cells of MCF-7. The use of PLGA NPs without ICG with 10 min of NIR laser irradiation not only reduces cell death, but lead to the proliferation of MCF-7 cells.

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