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Enhancement of cancerous cells treatment by applying cold atmospheric plasma and photo dynamic therapy simultaneously

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ABSTRACT

Cold atmospheric plasma (CAP) has recently emerged as a novel approach to treat cancer as well as Photo Dynamic Therapy (PDT). The Produced ionized gas (i.e. helium, here) involves highly reactive species which are cell killing responsible in CAP treatments. The photosensitizer 5-Aminolevulinic acid (ALA) was utilized for PDT process. In this paper, the effectiveness of these two techniques separately and also together was examined. The cell killing rates for six groups (i.e. Control, ALA, CAP, ALA & CAP, ALA & LED or PDT, and their combination technique PDT & CAP) on the cancerous human lung carcinoma cells (A549) were investigated using their cell's viabilities obtained from MTT assay. Viability analysis for different time durations of irradiation (plasma and/or light) also showed a decrease in a dose-dependent manner. It was also showed that PDT & CAP treatment could have an enhancement of about 37% relative to PDT and about 41% relative to CAP method (for 60 s irradiation). Hence, combined technique could be known as a promising method for treatment of the cancerous cells which are accessible to light and CAP (e.g. skin cells). Moreover, applying a photosensitizer (e.g. ALA) before CAP therapy could also enhance the treatment process.

1. Introduction: novel techniques of cancer treatment

In addition to traditional methods for cancer therapy such as chemotherapy, other techniques might apply photons (from low-frequency electromagnetic waves till x- and gamma-rays), accelerated massy particles (e.g. electrons, neutrons, protons and atoms) and/or mechanical waves (e.g. ultrasound beam). Such rays or beams should transfer energy into the tissues so strong that maximally kill the cancerous cells (through necrosis or apoptosis) whilst minimally harm surrounding healthy cells. In order to optimize the absorbed energies, a proper treatment plan is required. Using a treatment planning system, physicians try to perform an optimized treatment strategy based on the anatomical and functional information of the tumor and the beam/ray specifications (e.g. its type, energy, field size and incident angle). As well as the energy, the type of the beam/ray is selected depending on its interaction with the tissues and the tumor's position and depth within the body. For example, the electron beam is proper for skin and superficial cancers because of its low penetration depth (below a few cm). Although, such beams lead to apoptosis of the cells, unfortunately, generation and applying of them are relatively expensive, complicated and time consuming in addition to their ionization problems for healthy cells.

In contrast, some relatively low cost and accessible techniques are just applying non-ionization waves such as the laser or ultrasound (in the form of high intensity focused) to provide hyperthermia and necrosis in cancerous cells.

Recently, a lot of low power techniques provide some 'killer agents' between cancerous cells using a substance which would be toxic after radiation e.g. Photo Dynamic Therapy (PDT), or through directly particular irradiation e.g. plasma therapy. Since irradiation range and energy transmission within the tissues for such techniques relatively happen at short distances, they are usually applied for skin and low-depth diseases.

PDT is a promising treatment for cancer and other localized disease based on some interactions between light, photosensitizer (PS) and oxygen [1,2]. After distributing the absorbed PS within the tumor, it is activated by emitted light and prepared to react with oxygen and produce the singlet oxygen ($^1O_2$) which is highly reactive and ready to

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kill tumor cells.

The plasma, known as the fourth state of the matter is a partially ionized gas including of electrons, ions, electromagnetic radiations (e.g. UV, blue light and near infra-red) and reactive chemical species (e.g. Reactive Oxygen/Nitrogen Species or ROS/RNS) [3]. In general, plasma is classified into two kinds, thermal and non-thermal. In thermal plasma, ions and electrons are in equilibrium with the same temperature, whilst in non-thermal plasma because of slightly ionization of carrier gas (e.g. Helium or Argon), the ions temperature cool rapidly down to room temperature leading to be not in equilibrium with electrons temperature [4]. This type of plasma which is named Cold Atmospheric Plasma (CAP) has the same properties of high-temperature plasma without heat production [5]. In the past, only thermal properties of plasma have biomedical applications in cauterization, cutting of tissue, and coagulation of blood [6]. Recent investigations in medical applications of CAP emerged a new field of study in medicine. Some of the medical applications of CAP included bacterial and fungal sterilization [7,8], cell detachments [9], wound healing [10], dentistry techniques [11–13] and inducing apoptosis in cancer cells [14].

In one of the usual CAP sources in biomedical applications, a high voltage electrode wrapped around an insulating tube carrying a gas produces an ionized gas plume jetting out through a nozzle so named as the plasma jet. Generally, the exiting plume contains free radicals (i.e. ROS and RNS), charged particles, ultraviolet (UV) and visible radiations. The ROS and RNS known as the most important of plasma products (i.e. singlet oxygen, atomic oxygen, superoxide, nitric oxide and hydroxyl radical) could damage the membrane and DNA through affecting on the lipids and proteins [15].

There are several methods to produce CAP such as dielectric barrier discharge (DBD), atmospheric pressure plasma jet (APPJ), plasma needle, and plasma pencil. Plasma needle was the first microplasma device used for generating a millimeter size of CAP for localized treatment in dentistry applications (e.g. root canal). Some researchers have reviewed a lot of CAP production techniques and their applications in dentistry and oncology [11]. In one in vivo study, APPJ has been applied on visible tumor surface of head and neck cancer and shown apoptotic cell kill and no growth in tumor [16].

DBD generates microsecond-long, high-voltage-pulsed CAP between two parallel metal plates when at least one of them is covered by a dielectric layer [11].

Typically DBD device has large (several centimeters) size of plates and a few millimeters gap distance. Between different kinds of DBD structures, the floating electrode DBD (FE-DBD) which designed by Fridman et al. is the most popular source for biomedical applications [17].

CAP could be applied from sterilization (inactivate bacteria by DBD) [18] to cancer treatment that could be found by 2013 in a review research [19]. Some researchers have demonstrated that plasma-activated medium (aqueous) could have some treatment effects on even chemoresistant ovarian cancerous cells [20]. It was also demonstrated that there is no increased risk of CAP application and no indications for genotoxic effects [21].

Experience shows that applying some parallel techniques in cancer treatment could operate more effective than single one did; such as the combination of PDT with radiotherapy [22], PDT with hyperthermia [23], PDT with sonodynamic therapy [24], PDT with nanoparticles [25], and CAP with nanoparticles [26].

Fortunately, recently, the light with low penetration depth are transmitted to high depth tissues within the body using optical fibers [27], as well as CAP transmission using miniature probe [28]. Since PDT and CAP might be applied for treatment of some cancerous cells anywhere within the body; the lung cancerous cells were selected in this research based on the future view.

Since the main killer agent of PDT the ROS could also be one of the CAP's components, it seems that applying simultaneously both techniques CAP and PDT might enhance therapeutic effects. In addition to ionization UV with known effects on the cells, another CAP's components which are chemical reactive species in combined with PDT's singlet oxygen might intensify the treatment process. Hence, the objective of this paper is to investigate the effects of CAP in combined with PDT on cancerous cells in vitro.

2. Materials and methods

In order to investigate and compare therapeutic effects of the PDT, CAP and the combined techniques on the cancerous cells, six groups each containing 15 samples were provided named respectively as Control, ALA, CAP, ALA-CAP, PDT, PDT & CAP clusters. After execution of any treatment process on the cancerous cells, the number of death cells is measured by MTT assay.

2.1. Cell line and culture conditions

The human lung carcinoma cell (A-549) was supplied by Iranian Biological Resource Center and cultured in DMEM: Ham'SF12 + 2Mml-Glutamine + 10% FBS in a 5% CO2 incubator at 37 °C.

A549 cell lines were seeded into 96-well plates at the concentration of 1×10^4 cells per well and were incubated for 24 h for proper attachment to the substratum.

2.2. PDT conditions

A common administrated drug named 5-AminoLevulinic Acid (ALA; from 'Sigma Chemical Co') was dissolved in distilled water to obtain the stock solution (1 mg/ml). The PDT was carried out with LED light source at the wavelength of 632 nm and a constant fluence rate of 35mw/cm^2.

2.3. CAP configuration

As shown in Fig. 1 (in real picture and schematic), the applied CAP source device consists of a cylindrical Pyrex tube with inner diameter of 4 mm and the outer diameter of 6 mm. A copper wire was assembled around the tube as a power electrode supplied by a 10 kV rectangular signal with frequency 18 KHZ. The plasma jet was supplied by 99.999% pure helium gas with the constant flow rate of 4 L/min. The distance between the nozzle tip and cells was fixed at 1 cm during actuating.

2.3.1. Optical emission spectroscopy

To identify the output components of CAP, an optical emission spectroscopy (OES), Ocean Optics HR 2000 was applied in the wavelength range of 200–900 nm.

2.4. Setup of LED & plasma jet combination

In order to irradiate simultaneously plasma and LED light with uniform light, three LEDs were installed around the plasma nozzle with 120 degrees distances (not shown in the figures). They all were focused at a common point 1 cm far from the nozzle tip. In order to provide identical light intensity with PDT alone, the distances of three LEDs from the focal point were set at 1.73 cm (i.e. root square of 3).

2.5. Preparation, irradiation and evaluation of the samples

The magnitude of 1×10^4 cells/well were seeded into 96-well flat bottomed microplates in 100 µl growth medium and incubated overnight (for 24 h) until the cells adhered to the bottom of the plates. After 70–75% cell confluence, the media of wells was removed then phosphate buffered saline added to them to prepare the cells for plasma irradiation.

After adding 10 µl 5-ALA per well for 60 samples of the prepared cells (15 samples for each ALA, ALA-CAP, PDT and PDT & CAP groups),

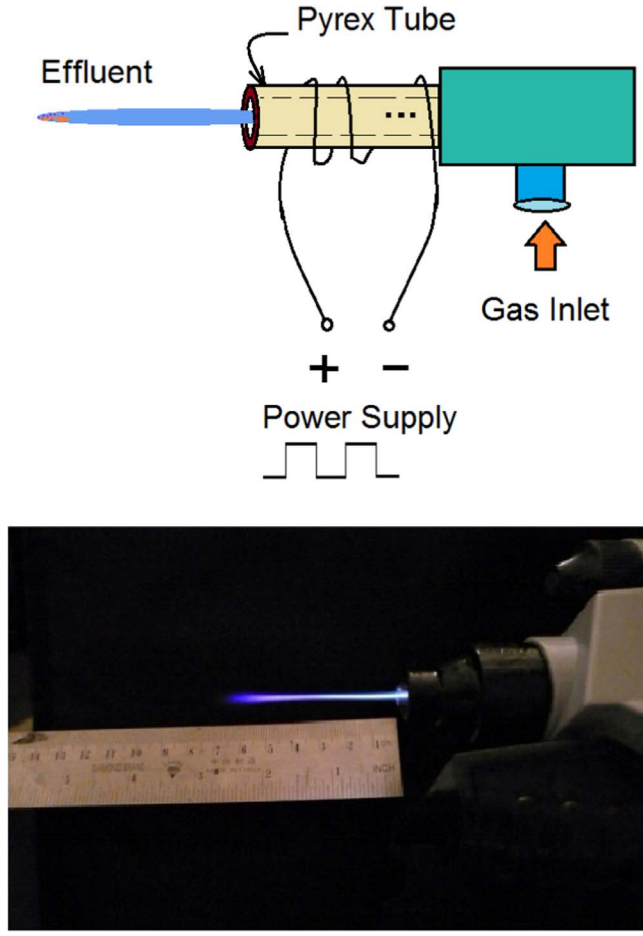

Fig. 1. Applied atmospheric gas plasma jet device: (up) structural schematic diagram with two gas and RF signal inputs; (down) photography at actuating instant with about 6 cm flare.

they were incubated for 3 h. Then, except ALA group, other three groups were irradiated with LED light (PDT and PDT & CAP groups) and/or plasma (CAP, ALA-CAP and PDT & CAP groups) at identical distance 1 cm for 60 s duration.

In order to evaluate the irradiation, 24 h after the treatment, the media was aspirated from the wells and 10 µl MTT added to each well for 4 h incubation. The triplicated wells (3 by 5 i.e. 15 samples) were run for each group by adding 100 µl DMSO to wells to dissolve formazan crystals.

Hence, Cell cultures were divided into six study groups:1. Control group including untreated cells without applying PDT and CAP, 2. ALA group including cells applied with 10 µl ALA and 3 h incubation without any irradiation, 3. CAP group including cells irradiated with plasma, 4. ALA-CAP group including cells received 10 µl ALA then after 3 h incubation, irradiated by CAP, 5. PDT group including cells received 10 µl ALA then after 3 h incubation, irradiated by LED, 6. PDT & CAP group, after applying 10 µl ALA and 3 h incubation, irradiated by plasma and LED light simultaneously. It was tried to perform all of the experiments in identical environmental light and conditions.

The Cell viability was assessed using an optical densitometry technique at 570 nm measuring the activity of mitochondria and cellular dehydrogenase enzymes. The data were analyzed by *one-way ANOVA* statistical method in SPSS software.

The morphology of the cells was also observed with the inverted microscope for some samples before and after the treatment but since such morphological variations were not applied to the quantitative evaluation process, their pictures were not presented here.

3. Results

Fig. 2 shows the spectrum emission along the axis of CAP in the range of 200–900 nm representing OH (309 nm), N_2^+ (371, 379, 381 and 419 nm), helium (708, 720 and 740 nm), oxygen (880 nm) and many weak emissions of nitric oxide (NO) species appeared in the UV region (not marked).

At first, different timing combinations for applying T seconds (e.g. 60 s) combined technique CAP (T1 seconds) and PDT (T2 seconds) were tested (T1 + T2 = T) for some cells samples. Based on our findings, applying 'PDT then CAP' showed better results than 'CAP then PDT'. Finally, using simultaneous PDT and CAP showed the most effective result from the view of viability through MTT assay. These effects seem to be mediated by the interaction of applied photosensitizer with the light of CAP. Of course, if LED is also applied (PDT and CAP at the same time), the irradiated light intensity and hence killing rate are increased.

Fig. 3 shows cell viability 24 h after 60 s treatment of six groups in the form of column bar (mean) and error bar (standard deviation: sd). It could be noticed that for combining technique PDT & CAP, enhancements of about 37% relative to PDT, and about 41% relative to CAP method were obtained. It shows that the dual technique PDT & CAP has more cytotoxic effects on the cell viability compares to other groups (including CAP and ALA-PDT alone).

All of the applied data here were significant ($p < 0.05$) through *one-way ANOVA* statistical method in SPSS software. The group indexes were purposely selected according to the effectiveness of any technique to show viability in a descending manner. It shows that PDT acted better than CAP in killing the cancer cells whilst the mixed technique is the best.

In order to compare mixed technique with basic groups CAP and PDT, the cell viability was measured for some irradiation time durations

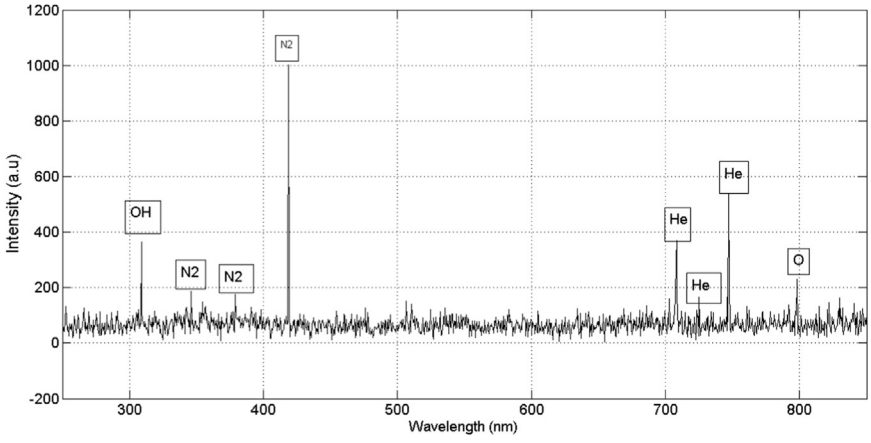

Fig. 2. Measured spectrum in the range of 200–900 nm for He plasma jet.

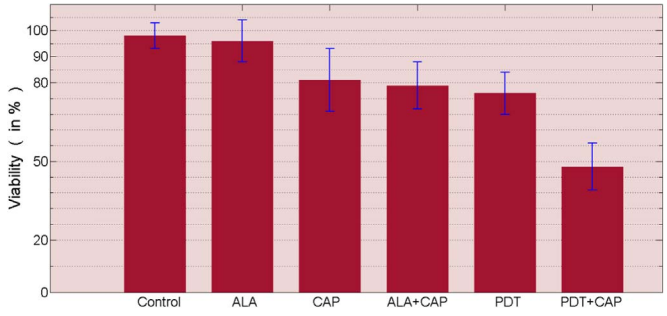

Fig. 3. Viability findings of six groups (in percent): Control (98 ± 5), ALA (96 ± 8), CAP (81 ± 12), ALA & CAP (79 ± 9), PDT (76 ± 8), and PDT & CAP (48 ± 9).

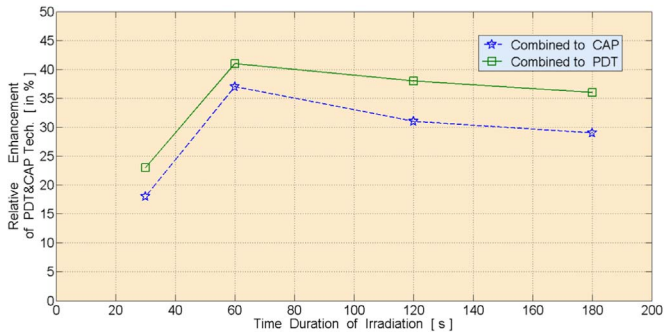

Fig. 4. Enhancement of combined PDT & CAP technique (for 30, 60, 120 and 180 s irradiation, respectively) relative to PDT (dashed-star: 18%, 37%, 31% and 29%) and CAP (solid-rectangle: 23%, 41%, 38% and 36%) from viewpoint of viability.

(30, 60, 120 and 180 s). The mean of cells viability of PDT, CAP and PDT & CAP respectively versus irradiation times for **30 s** are 88, 93 and 72 for **60 s** are 76, 81 and 48, for **120 s** are 65, 73 and 45 and for **180 s** are 59, 66% and 42% (all sd under 18). The obtained cell viability for any group showed a decrease in a dose-dependent (or irradiation time-dependent) manner. Based on such data, the enhancement of dual technique PDT & CAP relative to each single method (i.e. relative viabilities in percent) versus the times were obtained and showed in Fig. 4.

An enough long time irradiation could kill all of the cancerous cells which are the only living objects under irradiation of CAP and/or PDT in vitro study. Nonetheless for short time irradiation, the combined method is more effective than individual technique PDT or CAP; as seen in the Fig. 4 the maximum enhancement appeared about 60 s.

In real conditions (e.g. for skin cancer in vivo) although the irradiation time enlargement could increase the number of killed cancerous cells too but unfortunately might also harm or even kill the neighbored healthy cells, depending on the validity of the applied treatment plan.

Fortunately, in order to destroy only the cancerous region and to increase the efficiency and the selectivity, there are some maneuvers which could be performed as follows: localizing the ALA distribution in the cancerous region; making the field of view of irradiation similar to cancer spreading; selecting the proper instants of the start/end of irradiation relating to the magnitude and distribution of the diffused PS within the target tissue.

4. Discussion

This study demonstrated that a combination of CAP and PDT can be useful for enhancing cytotoxic effects on cancer cells. The ability of CAP in inducing death for a number of cancer cells has been studied by several groups and in all of them the ROS/RNS known as a major responsible for killing cells [19,29–32].

As shown in Fig. 2, some exogenous reactive species such as N_2^+, OH and O were detected in the emission spectrum of CAP. Exogenous ROS can activate membrane receptors and subsequent signaling cascades. For example, OH is very reactive and can effectively damage all types of macromolecules such as the lipid and amino acids in the proteins [33]. In addition to reactive species, light photons are also emitted by plasma plume toward the biologic target. Though Plasma included required photons for PDT to convert PS to 'toxin', because of their low intensities, it was needed to irradiate such photons for a long time. Hence, it was preferred to add LED on plasma jet device in order to provide identical photon intensities and irradiation times for groups including PDT. Applying of some other gases (e.g. Ar or Xe) for plasma jet could cause to generate more photons intensity [34], and maybe to execute the combined method without using LED. It is needed to evaluate different types and doses of PS against different types and/or mixes of gases for plasma to find the most effective technique of CAP therapy (with no need to LED) after PS administration.

Some in vitro studies demonstrated the efficiency of ALA-PDT in cancer cells inhibitions [35,36]. ALA isn't known as an intrinsic photoactive but after usage; it is preferentially accumulated within the tumor cells and metabolized through heme biosynthesis to become a photosensitizer porphyrin [37]. Porphyrin could absorb photons from UV (most around 420 nm) till IR (around 650 nm) leading to transit toward excited electronic states [38]. The excited porphyrin has the ability to react directly with tissues or with an oxygen molecule to generate excited singlet oxygen [39]. Singlet oxygen can upset the cellular activities and cause abnormal cellular functions through impairing the structure of the cell. Such a biological destruction could be performed by several mechanisms such as oxidation of membrane lipids and amino acid of proteins, cross-linking of proteins and oxidative damage to nucleic acids [40–42].

PDT could be used by photons band in which fundamental wavelength might be known as relatively low penetrating photons (i.e. UV to the Red band) within the tissues. It could treat superficial cancer such as basal cell carcinoma [37,43] and squamous cell carcinoma of head and neck [27,44]. PDT could be performed by fiber optics to transmit photons to deep tumors at where recently cold plasma might also be delivered through endoscopic devices. Therefore, PDT & CAP could be properly applied for lesions with any depth within the body.

Some researchers demonstrated that by increasing the time of CAP exposure, the higher concentration of reactive species were created in cell medium causing greater cell damage [45] as well as other investigators that indicated ALA-PDT induced a significant cytotoxicity in cancer cells in a dose-dependent manner [46]. As shown here, MTT assay revealed that all therapy techniques decreased the viability of cells consistent with other researcher's findings, in a dose-dependent manner.

ALA-PDT & CAP technique could be expressed as the effects of a 'doubled' oxidative stress which obtained from the high concentration of reactive agents and singlet oxygen leading to cytotoxic effects and finally an enhancement in cancer treatment as shown in Fig. 4. In PDT alone, apoptosis is the main factor of cells killing, but by increasing light irradiation time other factor necrosis could also kill more cells. During irradiation time, because of lowering magnitudes of PS and oxygen concentration, this killing rate usually decreases. Nevertheless, by concurrent applying CAP, some enhancements appear so that all cancerous cells might be killed by continuing CAP irradiation (even with vanished PS).

Oxygen plays a key role in PDT. The successful treatment of cancer by PDT is influenced by oxygen condition [1]. Tissue oxygenation decreases during PDT by two reasons [2]:

First, tissue oxygen is depleted by photochemical consumption and thus oxygen availability lowered. Second, at high fluence rates of light irradiations in PDT, vessel damage occurs and oxygen supply compromised. Therefore PDT can create hypoxia in the tumor by two reasons mentioned above. Therefore, since oxygen is prerequisites for PDT, hypoxia during irradiation could cause to poorer treatment outcome [4]. To overcome of tissue hypoxia during PDT and enhance tissue

oxygenation, some combinational methods such as PDT under hyper-oxygenation condition [4,47–49] have been applied. In a research, It was observed that tissue oxygen partial pressure (po2) in mouse skin rapidly increased (up to 4 times) after 5 min exposing to CAP [7]. Improvement of tissue oxygenation by plasma could be compensating oxygen consumption by PDT in combining these two techniques as presented here.

Some researchers have shown by optimizing plasma parameters it's possible to tune the interaction between plasma components and the cells of the immune system to treat many systemic diseases [50]. Other researchers showed that PDT can cause immunogenic apoptosis to induce an effective antitumor immune response through establishing a cancer vaccine [51]. However, recently, PDT and/or CAP are gradually being applied as routine therapy procedures and combined with immunotherapy and targeted therapy [52].

5. Conclusion

In this paper, cancerous cells of the human lung were treated by different combinations of applying ALA, LED's light and CAP irradiation in vitro. It was shown that the combined treatment PDT & CAP had the most killing rate of cancerous cells. Finding the best technique for cold plasma generation which provides the most effective in cancer therapy after administration a proper PS without LED irradiation could be studied in future. Execution of such techniques and comparison of their results on the live animal is our upcoming work. The effect of the execution time of such techniques on both healthy and cancerous skin tissues would be the main question to find the optimal duration. In addition, the start instant of irradiation is very important because of pharmacokinetic of PS. When administrated drug (i.e. ALA here) converts to PS which diffuses into the tissues and the target region, reaches to its maximum concentration, it's the best time to start irradiation process.

It seems that the combined method would be very effective and promising in deep cancer treatments using miniature applicator for CAP and visible light delivery as well as surface cancer therapy. Applying ALA-CAP technique in vivo study is our coming work in order to evaluate and model such treatment method.

Conflicts of interest

None.

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