Evaluating Photodynamic Therapy Efficacy Using Laser Induced Breakdown Spectroscopy

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Abstract. Laser-induced breakdown spectroscopy (LIBS), is an excellent tool for trace elemental analysis, was exploited for a detecting concentrations of calcium and magnesium in malignant tissues before and after PDT. Calcium and magnesium concentrations are known to be high in malignancy. Tissues were injected with methylene blue photosensitizer with concentrations 0.5%, 1% and 2%. Two different light sources were used with two different energy densities/each light sources. The results showed a decrease in tissue elements content after PDT application for both calcium and magnesium compared to before PDT application as shown in the tissue spectral lines’ intensities which has been reflected in. Type of light source showed no effect on tissue elements content which showed slight differences among the different energy densities. It has been shown that LIBS technique can be adopted method to monitor tumor photodynamic therapy applications.

Keywords: Photodynamic therapy, Methylene blue, Diode laser, non-coherent light, LIBS

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INTRODUCTION

PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) involves the generation of reactive oxygen specie after activation of a photosensitiser by light [1,2] Optical microscopy techniques are widely applied in biology, medicine and industry. Laser-Induced Breakdown Spectroscopy (LIBS) is a type of atomic emission spectroscopy which utilizes a highly energetic laser pulse as the excitation source. LIBS can analyze any matter regardless of its physical state, be it solid, liquid or gas. Even slurries, aerosols, gels, and more can be readily investigated. Because all elements emit light when excited to sufficiently high temperatures LIBS can detect all elements, limited only by the power of the laser as well as the sensitivity and wavelength range of the spectrograph and detector. Operationally, LIBS is very similar to arc/ spark emission spectroscopy [3].

A typical LIBS system consists of a (Nd:YAG) laser and a spectrometer with a wide spectral range and a high sensitivity, fast response rate, time gated detector. This is coupled to a computer which can rapidly process and interpret the acquired data. As such LIBS is one of the most experimentally simple spectroscopic analytical techniques, making it one of the cheapest to purchase and to operate. The Nd:YAG laser generates energy in the near infrared region of the electromagnetic spectrum, with a wavelength of 1064 nm. The pulse duration is in the region of 10 ns generating a power density which can exceed 1 GW·cm−2 at the focal point. Other lasers have been used for LIBS mainly Excimer laser which generates laser light in the ultraviolet region [4].

LIBS principle is simple (Fig 1). First, a high-power laser pulse is focused onto the sample. Nearly instantaneous absorption takes place then local ablation of thin layer of material, which subsequently gains energy from the laser beam and is heated into the ionic state, creating the laser-induced plasma, or “spark.” The excited atoms and ions relax, in part, by emitting light at characteristic atomic emission lines, and spectral analysis of the plasma can be used to determine the ablated sample’s elemental composition. Emission from the plasma is directed to a spectrograph, and an attached
Ca and K metabolism in an intimate but poorly understood way [7].

During chemical carcinogenesis it is possible to observe Mg cellular deficit in preneoplastic and neoplastic states.

It seems, therefore, that an established cancer induces Mg disturbances which cause Mg load in tumoral tissue, possibly due to Mg mobilization through blood cells, with Mg depletion in nonneoplastic tissue [10].

**CALCIUM**

Calcium (Ca) is required for the proper functioning of numerous intracellular and extracellular processes, including muscle contraction, nerve conduction, hormone release, and blood coagulation. In addition, the Ca ion plays a unique role in intracellular signaling and is involved in the regulation of many enzymes [11].

Calcium is an essential element in intercellular regulation and metabolism. Extracellular calcium is an essential component of cofactors required for bone formation, blood clotting, adhesions molecules, and as a first messenger in signaling functions. Many of its actions within cells are dependent [12] on a constant extracellular pool of available calcium. The parathyroid hormone-Vitamin D-calcitonin system and the parathyroid gland “set point” tightly regulate extracellular calcium concentrations. Magnesium is involved in neuromuscular transmission and is a cofactor in various enzyme reactions. It is important in ribosomal protein synthesis and ATP energy transfer. Phosphorus is ubiquitous throughout the body [13].

The objective of the present work is to monitor the efficacy of photodynamic therapy (PDT) with intralesional methylene blue photosensitizer and two light sources. The light sources were a diode laser (l = 650 nm with fluencies 108 and 162 J/cm²) and tungsten lamp with red filter 650 nm (fluencies 120 & 180 J/cm²). In this paper, the basic processes of the laser-induced breakdown spectroscopy are discussed in respect to our experiments with spectroscopic analysis of normal and malignant tissues elements content.

**MATERIALS AND METHODS**

**ANIMALS**

The animals selected for the study comprise 200 mice (Albino mice) – National Cancer Institute NCI experimental animal center -- of average weight 25±5 gm, which were housed in National Institute of Enhanced Laser Sciences NILES, Cairo University’s animal house.

**TUMOUR SOURCE**

Tumor source and Method of Implantation:

The source of tumor used in the study was Ehrlich’s Tumor, which originally derived from breast carcinoma transplanted in mice. It grows in solid and as cites forms.(Tumor source prepared in NCI experimental animal center labs)

Ehrlich’s Tumor cells were injected intraperitoneally in a healthy mouse to keep the source of tumor cells available, the ascites fluid was collected by aspiration and kept for further use. The aspirated ascites fluid’s cells is diluted with 4.5 normal saline for each 0.5 ml prior to subcutaneously inoculation. Each animal received 0.5 ml diluted cell line so that animal received 2x10^6 cells in 0.2 ml of inoculums, in the thigh of the hind limb.
Photosensitizer and Photodynamic therapy

Methylene blue injected interalesionally, then exposed to the two different light sources with two different energies per each light source. Laser Induced Breakdown Spectroscopy was used to monitor the efficacy of tumor PDT by means of tumor tissue elements content.

Sample preparation

Tumor and normal thick (~ 3-4 mm thickness) cut tissue samples were taken from the hind limb of mice before and after PDT.

LIBS Application

The laser beam will irradiate normal / tumor tissue samples before and after PDT, the photoluminescence emitted by the tissue biopsies due to the ablation and plasma formation will be collected by a one meter long and 600 μm diameter fused silica optical fiber. The collected light was coupled into a spectrometer. An ICCD (Intensified Charge Coupled Device) is connected to the monochromator detector port. The specified ICCD gating capability was 5ns and it was controlled by a build in delay generator. The obtained spectra of tumor tissues including the Ca²⁺ and Mg²⁺ spectral lines will be compared with that of normal tissue samples [14].

RESULTS AND DISCUSSION

Photodynamic therapy (PDT) is an effective local cancer treatment in which a photosensitizer is administered and the tumor is irradiated with light. Date M, 2004 [15]. Cancer diagnosis and classification is extremely complicated and for the most part, relies on subjective interpretation of biopsy material. Such methods are laborious and in some cases might result in different results depending on the histo-pathologist doing the examination. Real-time diagnostic procedures would greatly facilitate cancer diagnosis and classification.

Laser-induced breakdown spectroscopy (LIBS) is used for the first time to our knowledge to distinguish normal and malignant tumor cells from histological sections. To take variations in the radiation intensity due to material properties or fluctuations in the laser energy into account normalization is necessary. Normalization for the obtained spectra of the different tissues was performed against carbon spectra to avoid any experimental errors. The carbon content in the mortar samples was assumed to be constant. The samples were measured with the described LIBS set-up in materials and methods.

From the LIBS spectra obtained in the present study it is quit clear that Ca²⁺ and Mg²⁺ content is higher in malignant tissues than normal tissues. The spectra obtained from the different tissues treated with PDT and different Methylene blue concentrations showed that the Ca²⁺ and Mg²⁺ content varied according to photosensitizer concentration and light exposure time (see Figures 2-7).
FIGURE 2. Calcium content in laser groups

FIGURE 3. Calcium content in non-coherent light groups
FIGURE 4. Magnesium content in laser groups

FIGURE 5. Magnesium content in non-coherent light groups
FIGURE 6. LIBS spectra, showed Ca$^{2+}$ content in PDT treated with MB 0.5%, 1% & 2% comparing to normal tissue spectra

FIGURE 7. LIBS spectra, showed Mg$^{2+}$ content in PDT treated with MB 0.5%, 1% & 2% comparing to normal tissue spectra
Spectra shifted to normal tissue spectral line rather than malignant tissue spectral line. This shift was varied and clears in 2% MB² that exhibit more shift to normal tissue spectral line then 1% MB² and at last 0.5% MB² that showed least shifting behavior compared to the normal tissue. The light systems used to activate the photosensitizer gave rise to slightly differences in their spectral

Niemz, 1996 [16] reported that numerous trace elements in the range from below the part-per-billion concentration up to the regime of percent are encountered in calcified tissue. The actual concentrations often provide information on deficiency or disease states, or whether poisoning or contamination has occurred. This agreed with our results estimates that the Ca²⁺ and Mg²⁺ concentrations obtained from the present study are actually represent the tumor tissue element content.

In agreement with the present study results Durlach et al., 1986 [9] reported that at a later cancerous stage, disturbances in Mg distribution in tumor tissues are even more complex. They proved that, the disturbance in Mg distribution associates an increased Mg level in the tumor tissue.

In consistency with results obtained in the present present study Günther et al. 1986 [17] mentioned that the uptake of cellular Mg is possible when the normal cells are moderately depleted and are growing. But tumor cells, e.g., Ehrlich or Yoshida ascites tumor cells, or tumorigenic pancreatic B cells or thymocytes rapidly reaccumulate Mg.

Agreed with our results Nasiatek et al., 2005 [18] investigated the cadmium (Cd), copper (Cu), zinc (Zn), iron (Fe), magnesium (Mg) and calcium (Ca) concentrations in uterine cancer and uterine myoma. Tissue levels of six elements in 15 uterine cancers and 28 uterine myomas were measured by atomic absorption spectrometry. The results showed that the tissue Cd concentration was significantly lower in myoma than in non-lesion tissue. In uterine cancer, however it was statistically significant, but only slightly lower than controls (the non-lesion uterine tissue).

In the investigated tissues, the correlation between Cd concentration and age was found, but no effect of menopausal status or smoking habits on Cd level was detected. In uterine cancer tissue, a significant increase in Ca concentration and an insignificant increase in Mg level was observed when compared to normal uterine tissue. In uterine myoma, a significant increase of Mg and Mg / Ca ratio is pronounced.

In accordance with the present study results Nasulewicz et al., 2004 [19] examined the relationship between Mg status and tumour growth in mice. The results showed a significant retardation of primary tumour growth in mice receiving Mg-deficient diet by 60% for Lewis lung carcinoma (LLC), 57% for colon adenocarcinoma (C38), 75% for mammary carcinoma (16/C) and 30% for melanoma (B16) comparing to the control diet fed group. Mg depletion followed by repletion leads to the significant increase of primary LLC tumour burden (142% of the control tumor weight). Even if long-term Mg deficiency results in marked decrease of Mg concentration in plasma, Mg concentration in tumour cells is weakly affected. Moreover, they have shown that Mg depletion enhances the efficacy of antitumour treatment. The life span of P388 leukaemia-bearing mice, kept on Mg-deficient diet and treated with doxorubicin, was higher then those of identically treated animals, which were fed control diet (133% vs. 99% as compared to the control untreated group kept on Mg-sufficient diet).

Cells undergoing apoptosis typically show DNA fragmentation, condensation of chromatin, membrane blabbing, cell shrinkage, and finally, disassembly into membrane-enclosed vesicles. A hallmark of this type of cell death is the fragmentation of nuclear DNA into multiples of 200 base pairs through the activation of endogenous nucleases that cleave the DNA between nucleosomes (Zhao et al., 1999) [20]. Kumar et al., 2004 [21] proved that LIBS can be used to distinguish between normal and malignant tissues by analyzing their LIBS spectra. They proved also that concentration of calcium and magnesium are indicative in this issue. This has been also dealt with recently, but on human tissues by El-Hussein et al. [6] as motioned above.

**CONCLUSIONS**

PDT is a local treatment modality, which in its nature has several potential benefits. For laser induced breakdown spectroscopy the results obtained showed that tissues specimens from each group were examined for Ca²⁺ and Mg²⁺ content using LIBS technique.

The obtained spectra showed and emphasized that Ca²⁺ and Mg²⁺ content is higher in malignant tissues than normal tissues. The spectra obtained from the different tissues treated with PDT and different Methylen blue concentrations showed that the Ca²⁺ and Mg²⁺ content varied according
to photosensitizer concentration and light exposure time. There was a difference in elemental spectra for the groups subjected to 10 min PDT protocol and groups subjected to 15 min PDT protocol. Calcium and Magnesium spectra shifted to normal tissue spectral line rather than malignant tissue spectral line. This shift was varied and cleared in 2% MB that exhibit more shift to normal tissue spectral line then 1% MB and at last 0.5% MB that showed least shifting behavior compared to the normal tissue. The light systems used to activate the photosensitizer gave rise to slight differences in their spectral lines. Diode laser system showed better results in respect to non-coherent light system.

REFERENCES
