International Journal of Medicine and Medical Sciences Vol. 2(1), pp. 053-058, January, 2012. Available online at http://internationalscholarsjournals.org (ISSN: 2167-0404) © International Scholars Journals

Full Length Research Paper

Cytotoxic Effects of Zinc Oxide Nanoflakes (ZNO NFS) in Human Muscle Carcinoma

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Received October 29, 2011; Accepted January 24, 2012

Cytotoxicity of bare and conjugation of Tween-80 capsulated nanomaterials e.g. zinc oxide nanoflakes (ZnO NFs), iron oxide nanoparticles (Fe_2O_3 NPs) was examined in dark as well as under light exposure in cell model of immature human muscle carcinoma (RD) via microinjection and free standing drug delivery system. ZnO NFs were grown on the tip of a capillary and characterized by applying atomic force microscopy (AFM) technique and the tip was used as pointer to insert chemicals into cell to visualize/assess the emission of reactive oxygen species (ROS) especially from mitochondria. Bare and conjugated ZnO NFs with δ-aminolevulinic acid (ALA) were irradiated /excited with UV light after cellular uptake, reactive oxygen species were generated. We deduct that ROS is damaging mitochondria resulting in cell necrosis within few minutes. ZnO NFs are not biosafe and have significant toxic effects for both normal as well as cancer cell especially for rhybdomyosarcoma cell line (RD).

Keywords: atomic force microscopy (AFM), δ-aminolevulinic acid (ALA), reactive oxygen species (ROS), human muscle carcinoma (RD cells), Zinc Oxide nanoflakes ZnO NFs.

INTRODUCTION

Current research suggests that photodynamic therapy (PDT) is encouraging, minimally invasive treatment modality for premalignant, malignant lesions requiring the interaction of light (UV-Visible), photosensitizer, and singlet oxygen [1]. The basic principle of PDT is to take a chemical and excite with light (Laser) of specific wavelength matchable to absorption peak of chemical drug, leads to the energization of chemicals causing cell death [2]. Photosensitizer (PS) complexed with nanomaterials (NMs) activated by UV light (240 nm of light wavelength) results in tissue necrosis by direct tumor killing effect, vascular blockade, most importantly singlet oxygen release from mitochondria. [3-5]. Nanotechnology or nanoscience is “the design, characterization, production, and application of structures, devices, and systems by controlled manipulation of size and shape at the nanometer scale (atomic, molecular, and macromolecular scale) which produces structures, devices, and systems with at least one novel/superior characteristic or property” [6]. In addition, Nanomedicine and nanotechnology, have introduced numerous NPs of variable chemistry and architecture for cancer diagnostics and treatment, involving engineering multifunctional devices with dimensions at the nanoscale [7]. Zinc oxide nanoflakes (ZnO NFs) and zinc oxide nanowires (ZnO NWs) are emerging milestones in PDT ongoing research with tremendous multiple clinical applications, diagnostic as well as antitumoricidal, in many microbial nonmicrobial

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Figure Captions

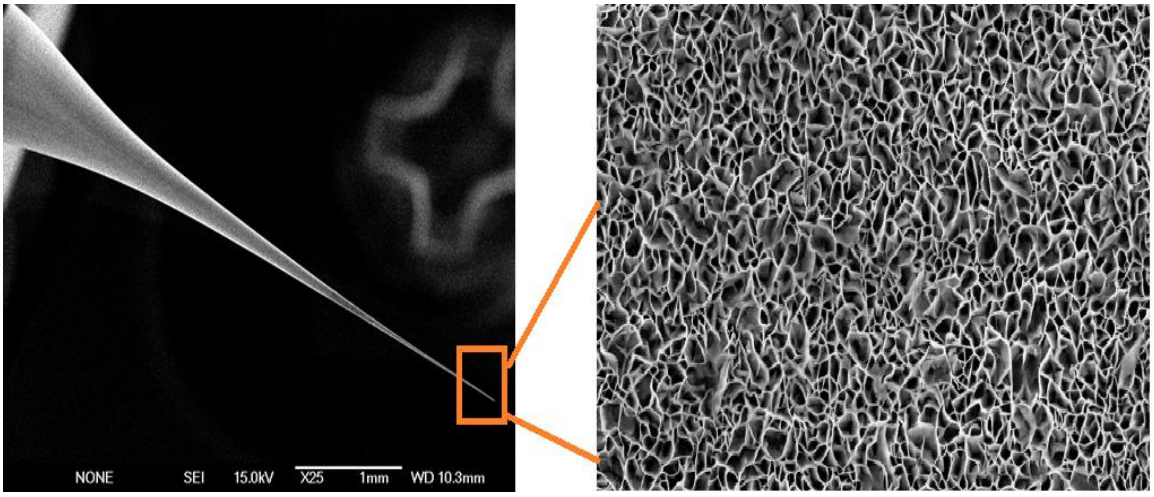

Fig.1. (a) A typical scanning electron microscopy (SEM) image of bare femtotip (b) Magnified SEM image of Zinc Oxide nanoflakes (ZnO-NFs) structures grown on borosilicate glass capillary femto tip using low temperature chemical growth.

treatment purposes and are front runners in such applications due to their high quantum yield, size dependent tunable emission of wavelength over wide spectrum of light. Currently, nano-dependent PDT technique involving zincoxide nanoparticles (ZnO NPs) and ZnO NRs is simple biosafe, biocompatible in dark, enhances endogenous fluorescence, noninvasive, fast with their least permeability in normal cells but ZnO NRs with high surface to volume ratio and biocompatibility can be used as an efficient photosensitizer carrier system and at the same time providing intrinsic white light needed to achieve cancer cell necrosis. Moreover, zinc oxide nanomaterials (ZnO NMs) having multistructures are prominent semiconducting and piezoelectric materials that have multiple applications in the field of optoelectronics, biosensors, resonators, electric nanogenerators, energy scavenging and nanolasers [8-14]. Zinc Oxide (ZnO) with its semiconducting and piezoelectric properties exhibits bio-safety and biocompatibility [15]. ZnO being a wide band gap (3.37 eV) semiconductor, having large excitation binding energy (≈ 60 meV) at room temperature, have applications in Optronics [16, 17]. Moreover, because of its electronic and optical properties ZnO nanostructures e.g. NRs, NTs, NPs, NWs and NFs are dominantly found in transparent electronics devices [18]. ZnO nanostructures have attracted the attention of researchers because of their potential applications in nanodevices e. g. nanobiotechnology one dimensional nanostructures such as nanotubes, nanowires and nanoribbons [18-20]. In addition, the role of nanoparticles in biomedical applications cannot be forgetful e.g. targeted drug delivery, hyperthermic cancer treatment, gene therapy, ultra-sensitive bio-agent detection and magnetic resonance imaging (MRI) and overcoming of multidrug resistance [21].

In this current work, author tried to demonstrate the toxicity of ZnO NFs alone and complex with different photosensitizers (PS) e.g. aminolevulinic acid (ALA), Photofrin® by using human muscle carcinoma (RD) as an experimental model by applying multiple techniques.

MATERIALS AND METHODS

Experimental Procedure

Deposition of ZnO nanoflakes (ZnO NFs) on the tip of Borosilicate glass capillary and its conjugation with photosensitizer (ALA) is the focusing part of our current conducted experimental work (microinjection drug delivery technique). Borosilicate glass capillaries (sterile Femtotip® II with tip inner diameter of 0.5 µm, an outer diameter of 0.7 µm, and a length of 49 mm, Eppendorf AG, Hamburg-Germany) were used for deposition of ZnO NFs by applying the low temperature aqueous chemical growth technique [19, 22]. The morphology of ZnO NFs structure is shown in the SEM images in Figure.1 (a and b). Its characterization snapshot is depicted in Fig. 2 by atomic force microscopy (AFM). The grown ZnO NFs on the capillary glass tip were functionalized by conjugating the ALA layer through a manual process. Powder form of ALA was purchased from Sigma Aldrich. Its molecular structure is shown in Fig. 3 [23]. The given drug has been used for PDT treatment of skin cancer and research based technology described in published data [24]. ALA was dissolved in phosphate buffered saline (PBS, pH 7.4) to obtain stock solution (300 µg/ml) [25-28] and was stored in dark. The ZnO NFs coated tips were dipped five times into the prepared working solution which has been stored in dark 4 ^{o}C. After each dip, the tip was allowed to dry at room

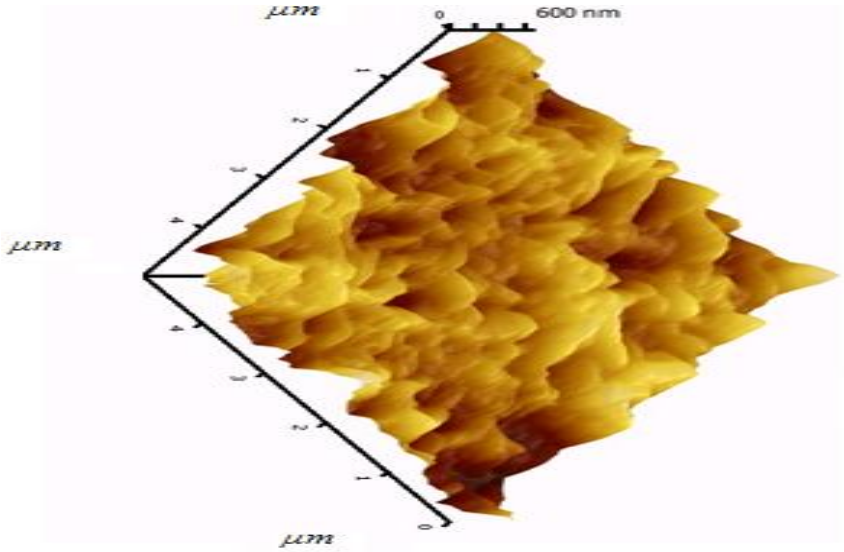

Fig. 2 Atomic force microscopic (AFM) image of ZnO-NFs structure.

temperature [4]. A sub-micrometer glass pipette covered with bare grown ZnO-NFs was also used as reference PDT devices to allow the separation of the contribution to fluorescence effect of the NFs and the NFs-photosensitizer conjugated tip. The fluorescence spectrum of same nature experimental work having ZnO NWs-ALA conjugated with femtotip excited at 240 nm of UV light was shown in our published data [29]. These bare and conjugated ZnO-NFs devices were used as local PDT intracellular photosensitizer delivery system for Human Rhybdomyosarcoma cells (RD) treatment. During above investigations, the treated cells were under examined online using Nikon microscope attached with CCD camera.

Cell Culture

Human muscle cancer cell line (RD) were used as an in vitro model, suggested cells were seeded out in 25 cm^2 plastic tissue-culture flasks (Nunc Wiesbaden Germany) individually, in Minimum Essential Medium (MEM) with Hanks salts, containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine along with some non-essential amino acids and were incubated for 24 hours for proper attachment to the substratum. Cells were maintained at 37 ^oC in a moist environment as a sub-confluent monolayer and were routinely sub-cultured twice or thrice weekly. The cell culture with 70-80% confluence was harvested using 0.25% trypsin [25, 26]. After tipsinization some of these cells were transferred to a regular petri-dish and incubated for 48 hours again for the proper attachment to the bottom of the Petri-dish at 37 ^oC in CO_2 atmosphere. By mechanical manipulation the ALA-conjugated femtotip-ZnO NFs were inserted gently inside the cultured cells and visualized online by Nikon microscope, contrast images were captured by CCD camera. Finally, cell death was confirmed by detection of ROS production.

During the experiments, firstly the cells were treated with 0-300 µg/ml of ALA in the absence of light via free standing drug delivery. After 18 hours of incubation cells were tested for determination of cellular viability [25-28]. In second step of current experiment RD cells were photosensitized with 100 µg/ml of ALA, in parallel non-photosensitized (controlled with serum) cells were cultured in 96 well plates. Both biological samples (PS treated and non-treated cells) were exposed to 0-160 J/cm^2 of diode laser light (635 nm) by changing time of irradiation (0-20 minutes) data not shown. After 18 hours of incubation time, light treated cells were also tested for the determination of cell viability.

RD cells survival was assessed using neutral red assay (NRA). This assay is based on the ability of living cells having active mitochondria show the countable optical density counts as compared to dead cells. NRA protocol was published in our prior data [27, 28]. After photodynamic treatment of above cell lines, the cellular viability was assessed by means of the mentioned assay. The percentage of viable cells in the cell population at each concentration of the test agent was calculated by means of standard method [25-28].

RESULTS AND DISCUSSION

The assessment of possible toxic/photodynamic effects of ZnO NFs via microinjection as well as free standing drug delivery towards RD cell line is the main focusing part of current experimental study. For this purpose

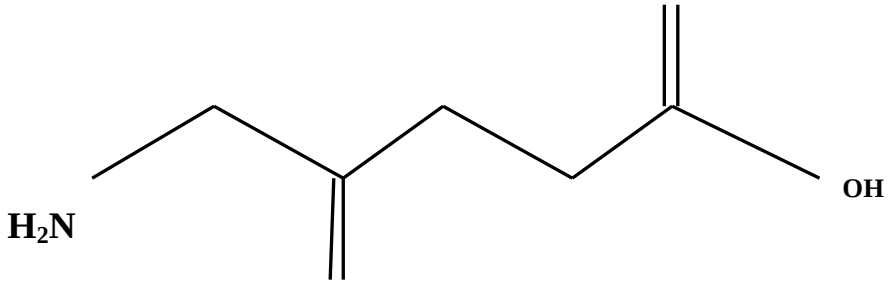

Fig. 3 Molecular structure of δ-aminolevulinic acid (ALA)

borosilicate glass capillaries femtotip II, coated with silver is the SEM image of bare femtotip as depicted in Fig. 1(a), while Fig.1 (b) indicates the magnified SEM image of the tip with the grown ZnO NFs. All online investigated/captured images were taken by coupled charged camera device (CCD) connected to the inverted microscope, but as a control experiment, the excitation of a bare and conjugated ZnO NRs was first performed. Fig.2 shows the atomic force microscopy (AFM) image of suggested ZnO NFs grown on the tip of femtotip borosilicate pointer. These photographs were taken when a filter (DAP1) for UV excitation was used. Moreover, the purpose of introducing this novel technique was to activate marvelous photodynamic reactions (might be direct or indirect), which are responsible for the significant amount of reactive oxygen species (ROS) leads to cell death. In our published data it has been already proved that microinjection drug delivery is more efficient and responsible for countable cytotoxicity towards muscle carcinoma [29]. In addition it was analyzed that the fluorescence is relatively high using both the bare ZnO NFs and conjugated ZnO NFs with ALA. ZnO NFs with a large surface area to volume ratio are not only attractive for more drug delivery to carcinogenic tissues also might be act as efficient drug/photosensitizer carrier for multiple malignant cell models [30]. Various steps/different stages of mechanical manipulation of the PDT device inside the cancer cell until the cell necrosis has been already discussed in our published data [29]. The basic strategy of our data collections is that when the ZnO NFs grown borosilicate femtotip were used as pointer under 10 minutes exposure time of UV light irradiation. Liberation of ROS which resulted in cell death was found in few minutes, because of enough quantity of ROS/singlet oxygen production which produces cell necrosis and responsible for killing of cell as described in Fig. 4. It was investigated during experimental proceeding that malignant/tumor cell is rapidly dividing cell, with destroyed outer barrier. These are the basic properties of tumor cell along with other such as altered enzymes and increased blood perfusion [27]. Then the cancer cell is exposed to UV laser light for 10 minutes by delivering light dose of 20 J/cm^2, while the PDT device is inserted inside the cell. This is already discussed in our recorded data [31] data now shown again. The basic needs for PDT is the availability of the molecular oxygen, through a photosensitizer excited by white light, which in our case can be emitted by ZnO NFs [21-22]. There is an energy exchange process between the ZnO NFs conjugated with ALA tip and the molecular oxygen inside the labelled cell. The phototoxic reaction involves the formation of singlet oxygen in the cell which causes initial damage to mitochondria leading to cell necrosis. As a resultant femtotip which act as pointer caused the cell necrosis via mitochondria damaging activity. After mechanical manipulation of pointer the necrosed part of the treated cell was visualized by using inverted microscope coupled with CCD camera (Online Image). The tip appears thicker after the manipulation inside the cell because some remnants' of the cell are attached to it. C. Hanley publicized in his data that ZnO nanoparticles show a strong potency to kill cancerous cells as compare to noncancerous [32]. Some other researchers quoted that nanoparticles can have induced oxidative stress in alveolar epithelial cells because of interaction with different organelles of cellular components [33]. S. M. Hussain demonstrated that different nanoparticles e.g. Ag, cdo, Al and Fe_3O_4 exhibit significant cell toxicity uptill 80% loss in cellular viability with the effective concentration of 50 µg/ml of Ag [34]. Optimal concentration of MnO_2 nanowires can produce the countable ROS production and depletion of glutathione (GSH) which are responsible for DNA damaging effects and cell apoptosis resulted in HeLa cell death [35]. Fig.4 shows the schematic of PDT algorithm in the form of different reactions [36]. When femtotip grown ZnO NFs conjugated with ALA were irradiated with optimal dose of 240 nm of UV light were depicted by I^{ST} reaction/first step. As a resultant white light spectrum with broad wavelength were emitted by

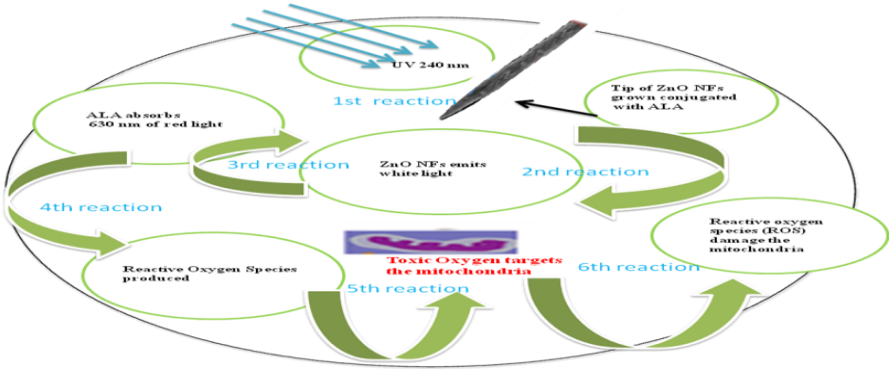

Fig. 4 PDT schematic diagram

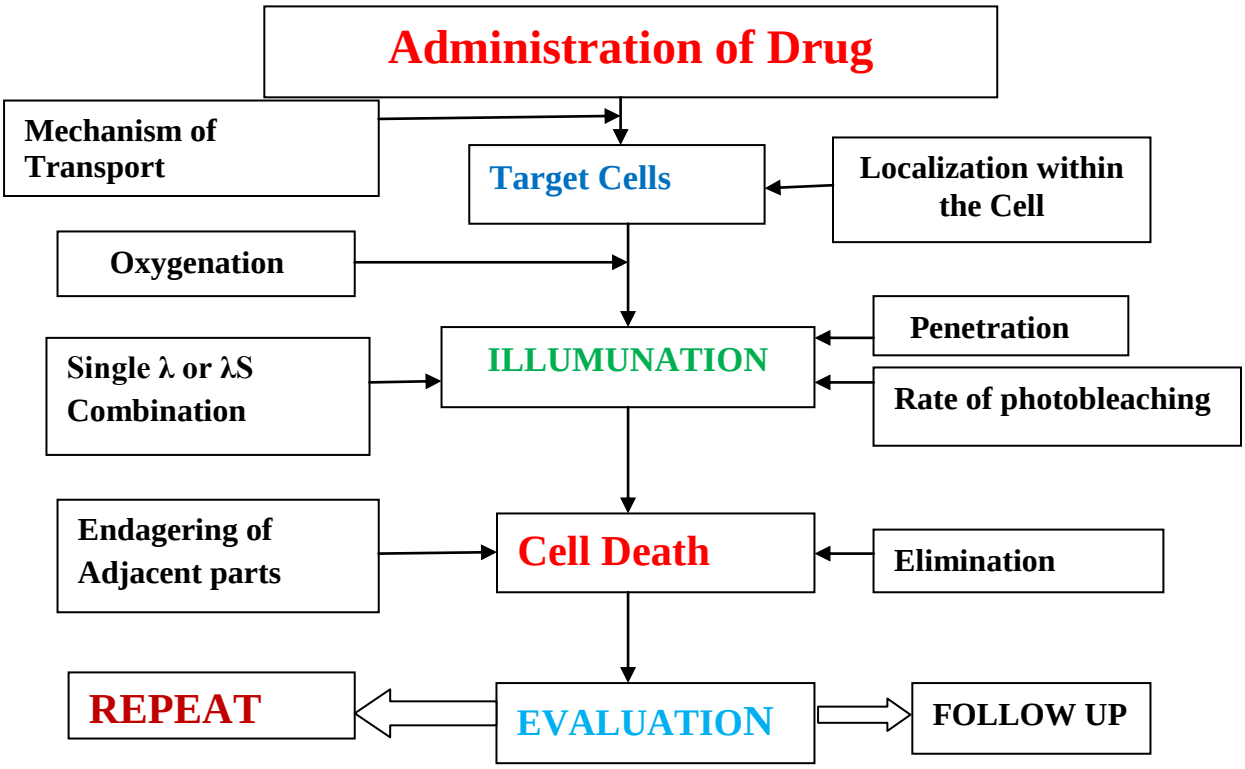

Figure 5: Administration of Drug Delivery

ZnO NFs, this white light spectrum also consist of red light with 630 nm of wavelength which is feasible for the excitation of ALA which is matchable to the absorption peak of ALA absorbance spectrum as demonstrated by S. Kishwar et al [4]. The discuss steps are shown by 2nd and 3rd reactions in Fig.4. In multiple published data we have discussed that enough quantity of molecular oxygen along with optimal dose of light and drug are responsible of the countable amount of ROS fluorescence which leads to mitochondria damaging effectiveness describes by 4th and 5th reactions in Fig.4. In the last step at last due to mitochondria damaging effect, cell necrosis occurred within few minutes as mentioned in Fig.4. But in case of free standing drug delivery case of ZnO NFs bare or conjugated with ALA, no significant toxicity was recorded in labelled RD cells. When the cultured cells were labelled with suitable concentration of ZnO NFs and ZnO NFs conjugated with ALA were irradiated with suitable laser light only 20% loss in cellular viability were counted which is not significant (data not shown) as our previous published data exhibit the marvelous loss in viability different malignant cell lines. In Fig. 5, we tried to focus/demonstrate the administration of drug delivery along with message conveying that how the combination of drug and suitable wavelength of light is valuable for

the efficiency of significant PDT outcome. In addition, the researcher tried to reveal the importance of drug and laser light of optical window for liberation of reactive oxygen species resulting of cell necrosis [36-37, 23].

CONCLUSIONS

The given analysis of current conducted experiment nullifies the effectiveness of ALA or ALA with ZnO NFs in case of free standing drug delivery. But in current analysis it was seen that ZnO NFs bare or conjugated with ALA show excellent anticancer effect via microinjection drug delivery, which are responsible for the enough amount of reactive oxygen species (ROS) liberation resulted in cell necrosis. But rather is the case with free standing. We are of the opinion that least concentration of ALA or ZnO NFs entering into the RD cells, a selective property of photosensitizer's insignificant cell toxicity were analyzed via free standing drug delivery. While ZnO NFs, being classical anticancer drugs are maximally production of singlet oxygen into the RD cells, thus having maximum toxic effects for given cells can enhance cell necrosis due to possibly effectivity of their shape and size. While microinjection drug delivery technique is having astonishing antitumor effects on RD cells, implying that ALA complexed with ZnO NFs can be an appealing candidate for treatment of muscle carcinoma.

ACKNOWLEDGEMENTS

I acknowledge the Higher Education Commission, Pakistan, for providing financial support for completion of my PhD study/work and Abdullah Azam Ms student for helping reference template arrangement.

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