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Original Research Article

Plasmonic phototherapy of gold nanoparticles with Light Emitting Diode

Poorani Gananathan[*1], Aruna PrakasaRao[1] and Ganesan Singaravelu[1] and Elanchezhiyan Manickam[2]

[1]*Department of Medical Physics, Anna University, Chennai-600025, India*
[2]*Department of Medical Microbiology, University of Madras, Taramani, Chennai, India- 600 113*

***Correspondence Info:**
Dr. Poorani Gananathan
Department of Medical Physics,
Anna University, Chennai-600025, India
E-mail: poorani.sundaresan@gmail.com

Abstract

Plasmonic Photo therapy (PPT) is a developing treatment modality for premalignant and malignant conditions. In this study, Gold nanospheres of 30 nm chose as potential sensitizer based on their pros and cons. Green Light Emitting Diode (LED) with 530nm of 30mW used as irradiation source. The cytotoxic effect of 30 nm GNPs based on the concentration and time was studied with MTT assay. In PPT, we study the cell death mechanism with non-coherent low power LED with GNP by photo irradiation study in Vero, Hela and Hep 2 cell lines. Post therapeutic changes in normal Vero and malignant Hela and Hep 2 cell lines were also observed for 2, 24 and 48 hours. We observed localized apoptotic/secondary necrosis in irradiated spot, leading to irreversible cell. The viability and cell division of non-irradiated cells around irradiated spot was observed microscopically and by MTT assay. In PPT, fluence and GNP concentration were optimized since they perform crucial role in the mode of cell death. In this study we hypothesized that; plasmonic phototherapy leads to apoptosis with green LED of 30mW with 30nm GNPs.

Keywords: Plasmonic Photo therapy (PPT), GNP (Gold Nanoparticles), LED (Light Emitting Diode), Apoptosis, MTT.

1. Introduction

Photodynamic therapy (PDT) is a treatment modality for cancer where the targeted cells are selectively destroyed by a combination of light and light sensitive drug known as photosensitizers (PS) [1]. Efficiency of various PS and derivatives were explored, none of the existing PS meet all the ideal properties of a PS [2]. Even the most exciting sensitizers of current interest have their disadvantages like hydrophobicity, prolonged retention time, poor selectivity, etc. In this regard, increasing research efforts were devoted in developing a potential sensitizer that would overcome the limitations. Nanoparticles are being explored owing to the inherent advantages to become a potential photosensitizes [3]. Progress in nanomedical research offers the potential to specifically target metal nanoparticles to tumor cells [4]. The large surface to volume ratio of the nanoparticle would enable them to be more potent photosensitizers [5].

Moreover, unique properties like size/shape tunable nature, simple on surface modification and stability provide nanoparticles advantageous over conventional PS [6]. The primary advantage of nano scale is the enhancement of the cellular uptake by taking advantage of enhanced permeation and retention (EPR) effect by tumor cells [7]. Generally in tumors, due to the uncharacteristic growth of blood vessel, the blood vessel walls are more porous compared to the normal tissue and thus the nanosensitizers can easily move in to tumors [8]. In this regard, Gold nanoparticles (GNPs) are being presently explored for its efficacy as sensitizers in enhancing the Plasmonic Phototherapy (PPT). GNPs is having various distinctive advantages, make them a promising candidate for PPT. GNPs have advantages like stability in most physiological conditions and have very low cytotoxicity [9]. GNPs can be functionalized with bio fluids by replacing the weak chemical capping agents make it more biocompatible [10].

The size dependent optical property and cytotoxicity determines the role of GNP in cells. Previous reports in GNP states that, 1-2 nm sized GNPs were highly toxic and particles of size above 15 nm were non-toxic [11]. Reduction of particle size of GNPs lesser than 20 nm when irradiated while GNPs above 20 nm were found to be stable by [12]. Larger GNPs have higher scattering cross section; their labeling efficiency is smaller which may be due to steric hindrance [13]. GNPs have scattering and/or absorption, SERS by Surface Plasmon Resonance (SPR), inter band absorption and fluorescence [14]. The optical propertiesand cellular response of GNPs varies with size, shape and incident wavelength. Based on the experimental determination of the particle uptake efficiency; the light scattering and absorption

properties of the nanoparticles and by literature survey the average size of 30 nm GNPs were selected for Plasmonic Phototherapy.

Laser light has the characteristics of monochromaticity, coherence, and collimation [15]. These properties provide a narrow beam of high intensity light photons which transmits to the target tissue with minimal power loss and great precision [16]. When laser producing non ionizing radiation is applied, optical energy get converted to heat energy in metal nanoparticles by electron excitation and relaxation [17]. In Plasmonic photo thermal therapy, the biggest disadvantage is that lasers were available for selected wavelength. Nd: YAG -532nm; Ti: Sapphire laser around 800nm [16,18-21], between these two ranges GNPs are available for each nanometer, but our selection limited with light source. And also, Nd: YAG lasers can only be fibre coupled to reach the deep seated target tissue. Laser heating can result in thermal vaporization and shock waves production [18-21]. Gold Nanoparticles hit with high power laser releases energy of up to 200^{o}C to surrounding cells leading to carbonization or vaporization. GNPs with Continuous Wave laser produce shock waves in cells [22-24]. Precise control over the local temperature distribution is the key factor to be considered in the context of enhanced PPTT efficacy with laser. Moreover, coherent laser light causes speckles of local heating of in homogenous tissues [25]. Light emitting diodes (LED) are monochromatic non coherent light source that can be used in PPT to overcome the limitations of laser.

Further this study has utilized LED, which is easily portable, feasible for in vivo applications for deep seated tumors via coupling with optical fibers and economically feasible yet efficient light source. Tina Karu (1998: The Science of Low-Power Laser Therapy) states that the coherence of light is of no importance in low-power laser clinical effects [26].Light Emitting Diode (LED) found to be a successful alternate for LASER in PPT. There are several papers published in various cell lines experiments concerning application of nanosensitisers to Plasmonic photo thermal therapy of cancer cells [16-22,24]. In vitro and In vivo reports on the utility of GNPs for PPTT applications are available; there remain several areas still need to study in detail. Plasmonic Phototherapy (PPT) is a combined effect with light photons and GNPs, 30mW green noncoherent monochromatic light source (LED) were designed in this study.

2. Materials and Methods

30nm GNPs were prepared from reducing Chloroauric acid with Trisodium citrate by modified Turkevich method [27].UV –Vis absorption spectra and TEM images were taken to confirm the Gold nanoparticles. Light source LED chosen with 530nm peak wavelength with 30mW power. The spectral width of LED is 10-20nm. Nonmalignant Vero and malignant Hela and Hep 2 cell lines were chosen for in vitro cell cytotoxicity and Plasmonic Phototherapy studies.

Vero HeLa and Hep -2 cell lines were studied in this study. 80% confluence cells were cleaved from bottom of the culture flask by trypsinisation. The cell dislodgement was checked under microscope to make sure all the cells detached from bottom of the plate. Minimum Essential Medium (MEM) of 5ml with 10% FCS media was added to the culture flask and the cell suspension was collected into 15 ml centrifuge tubes. The cells were then centrifuged for 1000 rpm at room temperature, for 5 mins. The cell pellet resuspended in MEM medium containing 10% FCS. Cells were then seeded in a 96 tissue culture plate and cell concentration was adjusted to 4000 cells /well. 96 well plate were allowed to grow in CO_2 incubator (5% CO_2, 37^{o}C). After 48 hours, the 96 well plate were viewed under microscope for confluence and free from contamination. The 96 well plate were ready to use for cytotoxicity and Plasmonic Phototherapy study.

2.1 Cytotoxicity Assay for 30nm GNPs

To determine cytotoxicity, 30nm GNPs of 1-100 µl in Minimum Essential Medium (MEM) added to each well in cell culture plate and incubated for 2h, 24h and 48h at 37^{o}C in CO_2 incubator. Controls were maintained without GNPs. After incubation, MTT reagent added to each well and again incubated for 4 hours. 100µl of 0.1N isopropanol was added to each well and kept in dark for 30 mins at room temperature. The well plates then kept on a shaker for 1 min and read at 630 nm wavelength in ELISA plate reader.

2.2 Light Emitting Diode and Plasmonic Phototherapy

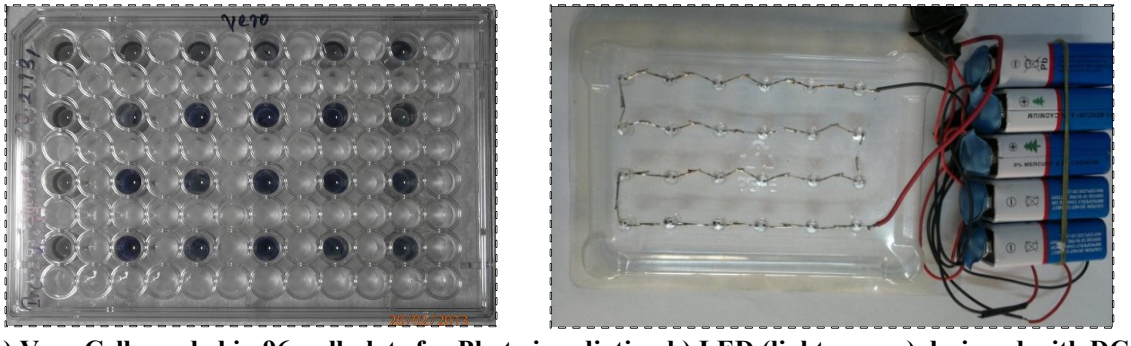

Figure 1: (a) Vero Cells seeded in 96 well plate for Photo irradiation b) LED (light source) designed with DC power for plasmonic photo therapy

Plasmonic Photothermal property of GNPs in conjunction with LED for Vero and Hela and Hep -2cell lines

were explored. Cells were grown in 96 well plate and 30 nm GNPs of 5-25 µl in MEM were added to the wells. Cells with GNP incubated for 2 hours at 37°C in CO_2 incubator. After incubation, GNPs were replaced with fresh MEM and cells were exposed to LED. Green light source of 530 nm wavelength with power 30 mW were applied to cells for two minutes. The morphological changes in irradiated spot were observed with light microscopy. To achieve therapeutic dose GNP concentration of5-25 µl, LED with power density of 30mW to obtain 3.6 mW/cm^2, and the treatment exposures between 1 and 5 min were adjusted and optimized. Such a technology implies a precise control of local temperature within cells.

3. Results and Discussion

30 nm GNPs prepared by citrate reduction technique were used in this study [27]. The cytotoxic effects of 30 nm GNPs were analyzed in normal Vero and cancerous HeLa and Hep2 cell lines. GNPs synthesized by Trisodium Citrate reduction procedure showed excellent reproducibility with narrow size distribution. Gold Nanospheres were around 30 nm in diameter as revealed by Transmission Electron Microscopy (TEM) image (Figure 1a, b). Size distribution analysis using TEM clearly showed that particles mostly reside within 30nm size range. This average particle size come in a very good agreement with the calibration curve performed on nanoparticle size as a function of peak wavelength of the SPR in comparison with Mie theory [28]. The characteristic absorption of metal nanoparticles corresponds to the wavelength of the light source used for irradiation. The absorption maximum of 30nm GNPs was at 530 nm referred as Plasmon Resonance peak recoded by Perkin Elmer Lambda-2 UV-Vis spectroscopy (Figure 1c).

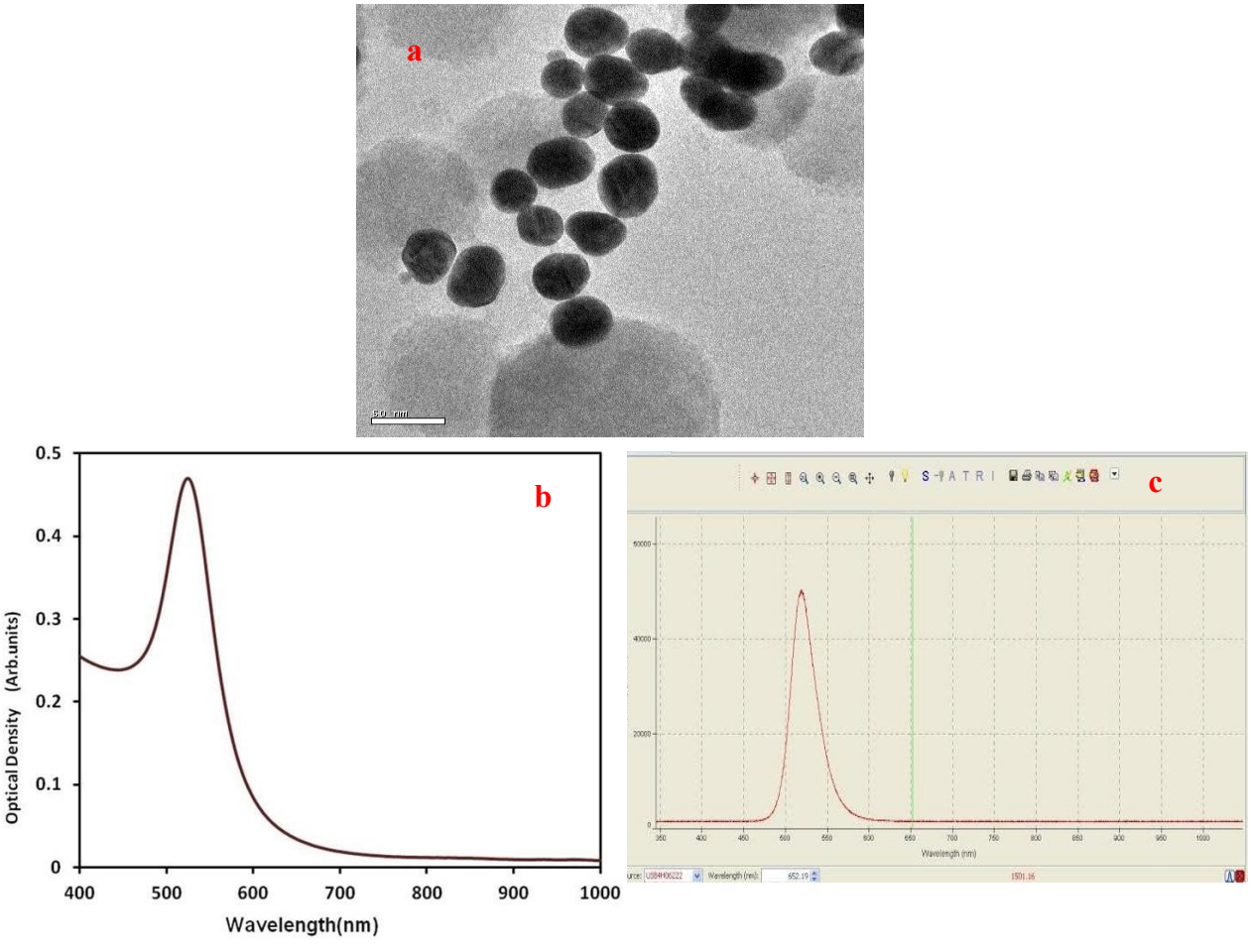

Figure 2: a. TEM image of GNP 30nm b. UV-Vis SPR spectra of GNPs c. Green LED spectra

3.1 Cytotoxicity Assay

Cells incubated with GNPs shows significant viability up to 72 h incubation for GNPs concentration ranging from 25-100µl. Vero, HeLa and Hep 2 cell line treated with GNPs were observed for 2, 24 and 48 hours to observe the complete effect of cytotoxicity. Citrate capped GNPs of 30nm alone were nontoxic to all the three cell lines when observed microscopically.

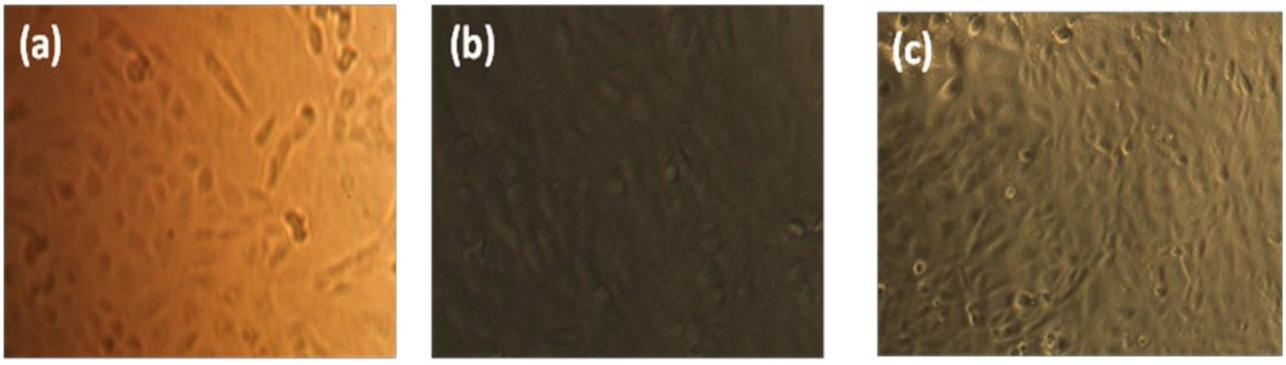

Figure 3: Hela cells a. Control b. Cells incubated with GNPs c. Cells with fresh MEM after incubation

MTT assay were carried out to quantify the cell viability. 90-100% cell viability was observed for Vero cell line whereas the cell viability for HeLa cells were 70-90% and Hep 2 cells were 85-95%. This depicts that 30 nm GNPs had negligible toxicity to HeLa cells in comparison to Vero and Hep2 cells. This cytotoxicity was checked for varying concentration of GNPs from 0.1µl to 100µl. The microscopically image of Hela cells incubated with GNP is shown in Figure 3.

Cell lines were monitored for 2 -72 hours and GNP concentration of 25 -100 µl were taken. The 80% confluent cells with 2 h incubation with GNP show no cell death. In figure 4; 1-Control; 2-5 GNP incubated for 2 hours;6-9 and 10-13 incubated for 24 hours; 14-17 incubated for 48 hours and 18-21 for 72 hours, 22 Control without GNPs.

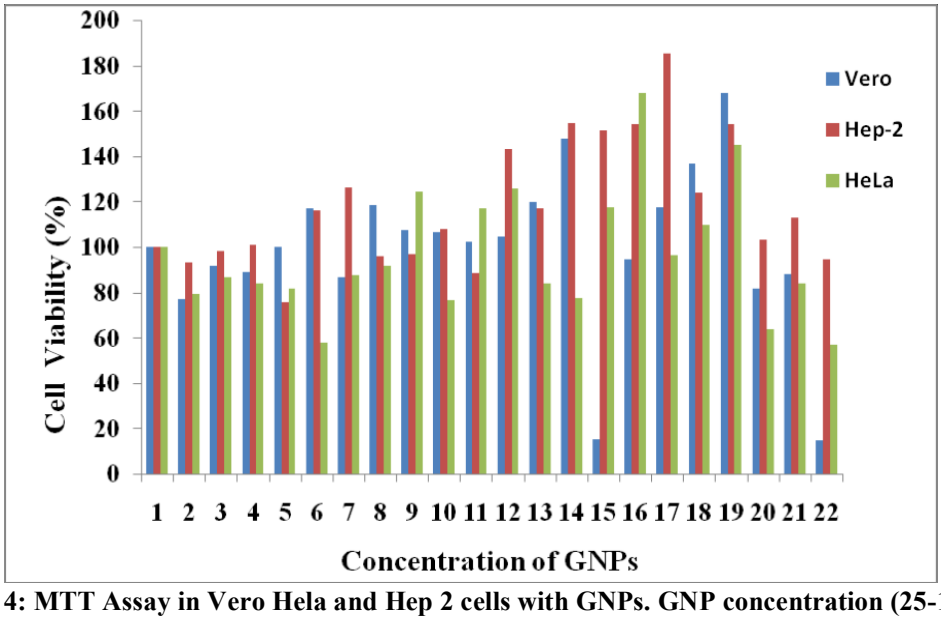

Figure 4: MTT Assay in Vero Hela and Hep 2 cells with GNPs. GNP concentration (25-100µl)

The effects of GNPs in cells were studied by post-irradiation in detail. The concentrations were varied from 25 to 100 µl. Cell viability of HeLa cell line was always higher compared to Vero and Hep 2 cells depicting the aggressive nature of Hela cells (Figure 4). Further at concentration of 70 µl the cancerous cell lines, HeLa and Hep2 cell lines were found to grow even in the absence of media (MEM). It was notable that the Vero cells showed a cell viability of 10% without MEM. In 72 hours, the cell viability decreased by overgrowth of cells.

3.2 Plasmonic Phototherapy by Light Emitting Diode

The effect of LED irradiation on GNPs were examined in Vero Hela and Hep 2 cell lines with 30 mW /530nm green LED for 2 min exposure time. Figure 2c and figure 2d shows absorption spectra of GNPs and spectral pattern of LED source. GNPs of 30nm has absorption around 530nm and LED with 530 nm output found to be suitable for irradiating GNPs (Figure 5).

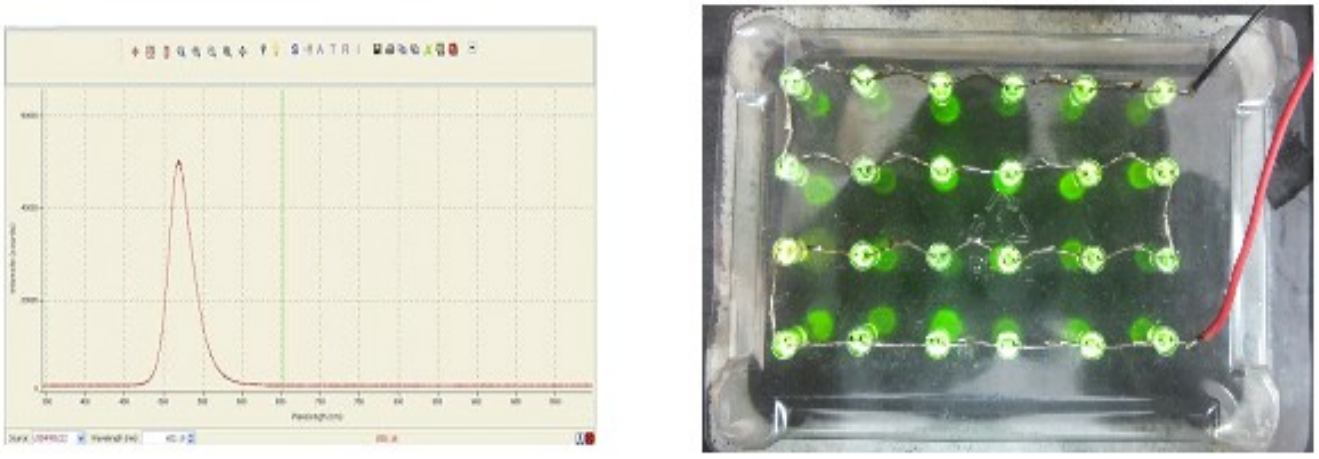

Figure 5: LED spectra and Green LED designed for 96 well plate

Fluence was optimized by irradiating the cells with LED upto 5 minutes in equal intervals without GNPs. During observation for the first 3 minutes (1, 2 and 3 min) there was no change in the cell. At 4, 5vminutes irradiation, monolayer rupture was seen as shown in Figure 6. On repeated trials with different cell lines incubated with GNPs, it was found that 2 min irradiation was found to contribute effect. For one minute irradiation we didn't find any photo irradiation effect in cells (Figure 6).

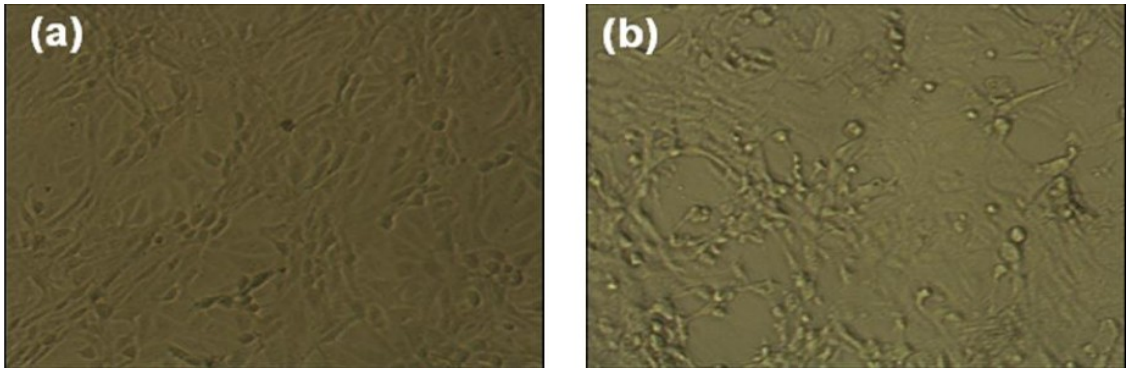

Figure 6: (a) Cell Control (b) Cells irradiated with LED for 5 mins

Photo irradiation effect of GNPs in combination with LED found to be remarkable and this work concentrates on optimizing concentration of GNPs and fluence. The photo irradiation effect was analyzed using 10 to 50µl of GNP since the concentration was found to be suitable for photo irradiation effect. The concentrations above 50 µl of GNPs were found to produce shielding effect in cells which occurs when the GNPs are excess in the medium blocking the entry of incident light into the cell. The microscopically image of Vero, Hela and Hep 2cells irradiated with 10 µl to 50 µl concentration shows cell killing effect in the laser irradiated spot which was observed 2 and 48 hrs after irradiation. The media was changed after 24 h to support the cell growth and to avoid starvation of cells. With 2h of post irradiation the irradiated spot showed morphological disturbance with undisturbed cells in the non-irradiated area. Floating cells were not observed which confirms that there is no necrotic cell death. After 24 h, the irradiated spot with morphological variation at 2 h time interval has now developed clear apoptotic bodies. Cell growth in non-irradiated area was observed and found that the cells were not spreaded to the irradiated spot. Thus PPT in cells is completely localized. After 48 hrs, irrespective of the cell lines, there is no rejuvenation of the cells in the apoptotic region and the growth of cells in the non-irradiated region shows growth beyond monolayer. Hence this modality can be considered as a promising tool for highly localized treatment. For effective comparison of the treatment MTT assay was performed in cell lines .We have compared the cytotoxicity, phototoxicity, post irradiation effect for a period of 24 and 48 hrs with varying concentrations (5-25 µl) intra and inter cell lines.

Figure 7 shows the cell monolayer with membrane blebbing and cell swelling in the irradiated spot. This membrane blebbing is caused by the influx of Ca^{2+} into the cell [32].When cells are observed at 2 hours after irradiation, the morphological changes observed in the irradiated spot was not specific. However after 48 hrs, apoptotic growth at the irradiated spot was clearly observed as in Figure 6. In figure 6 iv. c - 50 µl concentration of GNP, the vaporization in cells may be due to the less interparticle space leading to cavitational damage. The cell debris is found on the irradiated spot which would not be seen as when irradiated with high

power laser. After 48 hours there was no further change in the morphology of the irradiated spot. The non-irradiated area showed unaffected cells which were growing continuously, comparable to the cells in the control well. Even after 24 and 48 hours of post irradiation, the cells in the non-irradiated area continued to grow in a normal rate. Meanwhile the irradiated spot did not rejuvenate the cells. In conventional hyperthermia therapy cells regenerate after irradiation, in PPT hyperthermia the cell death is irreparable and irreversible.

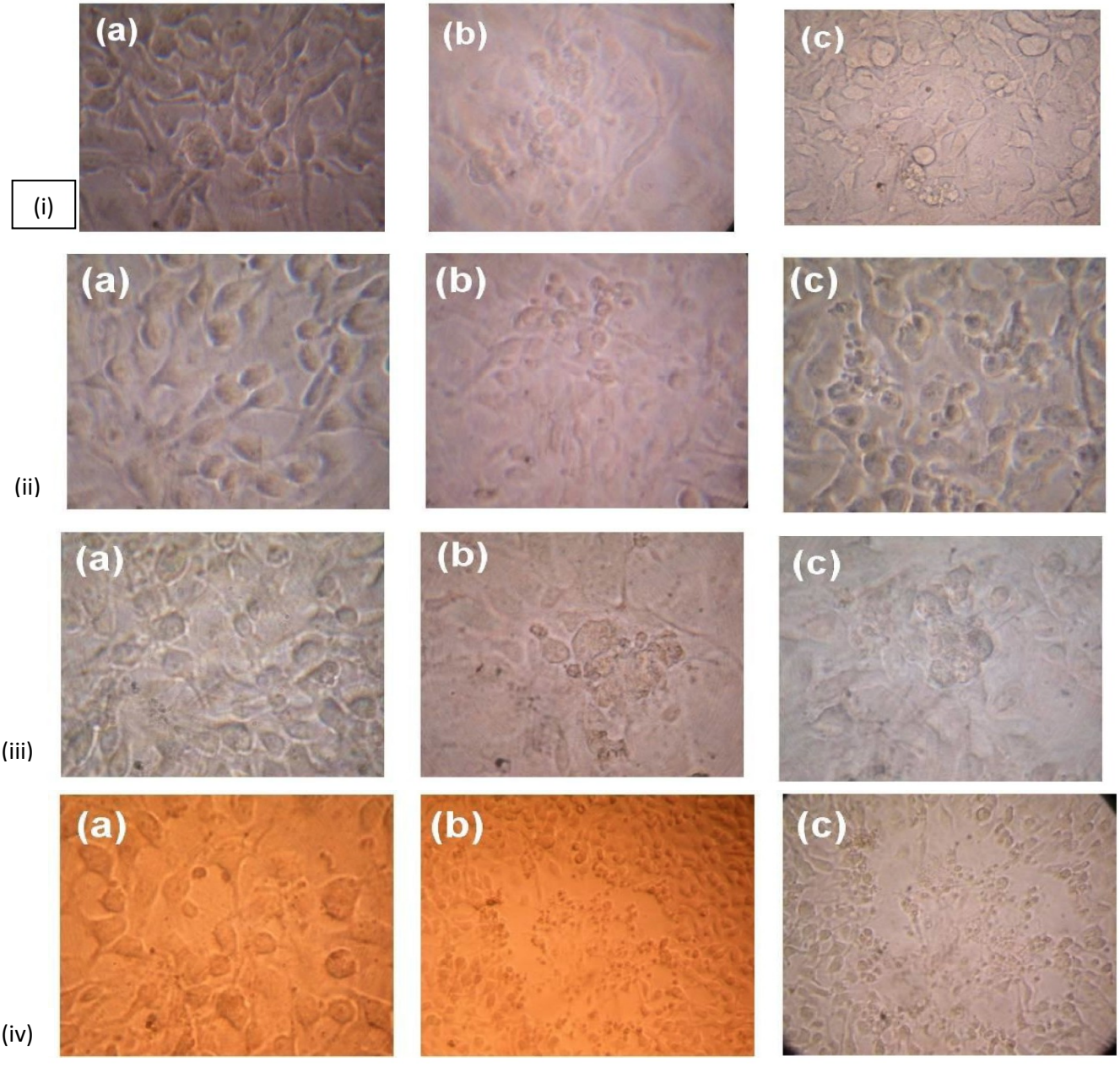

Figure 7: (i) GNP 10 (ii) 20 µl (iii) 30 µl (iv) 50 µl: a. Control b. 2 hours after irradiation c. 24 hours after irradiation

MTT assay also confirmed the same revealing that this technique is highly confined to the target area. In Vero cells MTT shows cell death upto 70%implying 30% of viable cells after 2 hrs of post irradiation period and further cell growth of 60 % after 24 hours post irradiation was observed.MTT results by cytotoxicity after 2 and 24 hours compared with photo irradiation and post irradiation after 24 hours and 48 hours. In Vero cell line, the MTT result shows 92 -95% of viability for 2 hrs incubation with GNP, while in photo-irradiation the cell viability was 75% for 5 µl concentration and decreased successively to 58% for 25 µl concentration. After 24 and 48 hrs of irradiation, the cell viability rate was 73% and 74% respectively for 25 µl concentration which had the least cell viability at zero hour. Comparing cytotoxicity and phototherapy post-irradiation after 24 hrs, result shows 81% viability and 73% implying that the non-irradiated cells in the well which were irradiated grow and this growth is now comparable to the non-irradiated well. Upon 48 hrs post-irradiation, the cell viability in the irradiated well shows an appreciable growth of 74%. This growth rate was found to be normal and there was no further need to observe the cells after 72 hours.

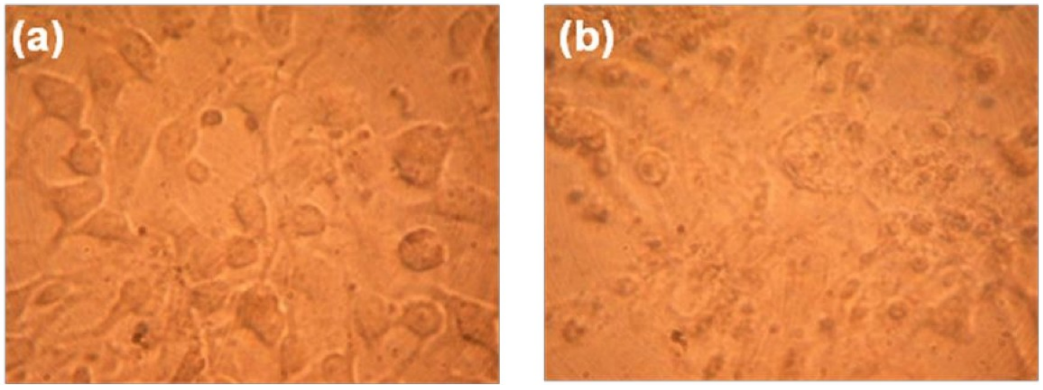

Figure 8: Hela cells a. Control b. 2 hours after PPT with LED of 530 nm and GNP

In HeLa cell lines the MTT result shows 86 -94% of viability for 2 h incubation with GNP, which is slightly lower than Vero cell lines, while in photo-irradiation the cell viability was 74% for 5 µl concentration and decreased successively to 53% for 25 µl concentration. The results were comparable to Vero cell lines. After 24 h of post irradiation, the cell viability rate was 90% and 77% respectively for 5 and 25 µl concentration which shows the growth rate of Hela cells are higher than Vero cells which is quiet normal that the cancerous cell lines grows faster than normal cell lines.

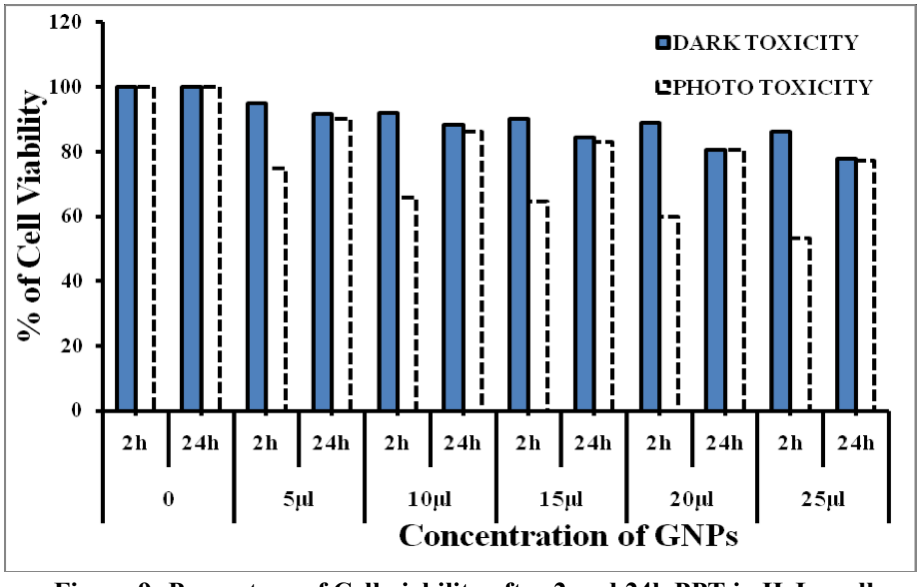

Figure 9: Percentage of Cell viability after 2 and 24h PPT in HeLa cells

Comparing cytotoxicity and phototherapeutic post-irradiation after 24 h, result shows 77% viability and 77% implying that the surrounding cells in the irradiated well were grow and this growth is now comparable to the non-irradiated well. From the graph it is observed that, the growth rate reaches similarity with cell control (figure 9).

In Hep-2 cell line, the MTT result shows 92-95% of viability for 2 h incubation with GNP, while in photo-irradiation the cell viability was 78% for 5µl concentration and decreased successively to 58% for 25 µl concentration. For 25 µl concentration which had the least cell viability at zero hour, the cell viability rate was 73% and 74% after 24 and 48 h respectively of irradiation. Comparing cytotoxicity and phototoxicity after 24 h post-irradiation, results shows 81% viability and 73% implying that the non- irradiated cells in the well which were irradiated grow and this growth is now comparable to the non-irradiated well. Upon 48 h post-irradiation, the cell viability in the irradiated well shows an appreciable growth of 74%.

3.3 Comparison of LED with LASER

In Figure 10, two normal cell lines were compared by irradiated with laser and LED. MDCK and Vero cells incubated with GNP and irradiated with Laser and LED is shown in Figure 10.

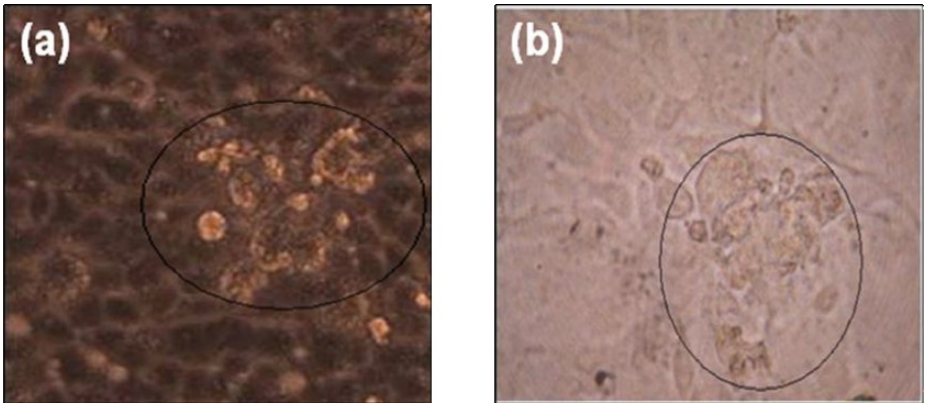

Figure 10: Comparison of mode of activity of light source in conjugation with GNPs against normal cell lines a) MDCK cells irradiated by Nd: YLF green laser of 532nm b) Vero cells irradiated by green LED source of 530 nm.

Both the light sources are of 532 nm; the power of the laser used was 7mW, irradiated for time duration of 10 minutes while that of LED was 30 mW for 2 minutes. From figure 10 it is evident that changes seen at the irradiated spot marked in the image (Figure 10). After 18 hrs post irradiation, apoptotic bodies had developed in the entire well of the laser treated MDCK cells whereas the LED treated Vero cells showed apoptotic bodies.

4. Conclusion

This study have confirmed that GNPs in combination with LED have many properties that are attractive for using in cancer therapy and have the ability to destroy cancer cells precisely if properly applied. PPT can be applied directly in cancer treatment with fiber coupled LED with increased effectiveness of cancer cure. GNPs can eradicates the cancer completely with harmless light source such as LED

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