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Cytotoxic Effects of Titanium Dioxide Nanaoparticles Synthesized by Laser Technique on Peripheral Blood Lymphocytes and Hep-2 Cell Line

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Abstract

Objective: This study assay the toxic effects of titanium dioxide nanoparticles (TiO_2NPs) on peripheral blood lymphocytes and Hep-2 cell by measuring Mitotic Index (MI), Blastogenic Index (BI), Total Chromosomal Aberration (TCA) and growth rate of cancer cells.
Methods: TiO_2NPs were synthesized using laser technique by ablation of titanium target immersed in distilled de-ionized water (DDW) using Q-switched pulsed Nd:YAG laser with repetition rate of 1 Hz, wavelength of 1064 nm, 300 pulses and energy of 400 mJ. Chromosomal analyses were carried out at different concentrations of synthesized TiO_2 NPs (0.0, 45, 90, and 180) µg/mL.
Results: X-ray diffraction (XRD) exhibited anatase phase of prepared TiO_2NPs and showed that the crystalline structures of TiO_2NPs is tetragonal. Absorption spectrum of TiO_2 NPs was at ultraviolet-region 215 nm due to Surface Plasmon Resonance (SPR), and the particles size distribution of the prepared TiO_2NPs ranged from 40 to 110 nm. Finding showed that both MI and BI suffered from high reduction while TCA suffered from significant elevation represented by Chromatid Breaks (CB), Micronuclei (MN) and Ring Chromosome (RC). The highest proportion of (TCA) has been observed in 180 µg/mL of TiO_2NPs, (significant at $p \leq 0.05$). while the toxic effects of prepared TiO_2NPs on Hep-2 cell line represent decline in growth rate with an increase in concentration of TiO_2NPs with malformation in the cell structure and shape and placement of the nucleus.
Conclusion: The ablation via Q-switched Nd: YAG laser has adequacy to prepare TiO_2NPs with 215 nm absorption spectrum, nearly spherical particles and suitable particle size (40-110 nm). In vitro assay showed that prepared TiO_2 NPs have significant cytotoxic effects on peripheral blood lymphocyte and Hep-2 cell line by reduction in both Mitotic index (MI) and blastogenic index (BI) and growth rate of cancer cells.

Keywords: Q-switched pulsed Nd:YAG laser, Titanium Dioxide Nanaoparticles, Mitotic Index, Blastogenic Index, Chromosomal Aberration, Hep-2 cell line

Introduction

Metallic nanoparticles have unique biological, chemical, electronic and optical properties where the electrons conduction bands of nanoparticle interact with the different forms of energy (i.e., electrical, light, magnetic, etc.) due to their high surface to volume ratio and show very distinctive properties compared with their bulk counterparts, for example, diffusivity, electrical resistivity, electrical conductivity, hardness, strength, chemical reactivity and biological activity[1-4]. Therefore, nanomaterials are candidate in many applications, such as heterogeneous catalysis, microelectronics, medicine, gas sensor, nonlinear optics, biosensor cancer therapy and tumor detector[5-8]. In general, nanoparticles used in the field of biotechnology because of these particles communicate with biomolecules on the cell surfaces and within the cells in way that can be designated and decoded to various physiochemical and biochemical properties of these cells.

Titanium dioxide NPs (TiO_2NPs) are a appropriate material and used in several fields due to their rare properties such as high photo catalytic activity, high stability dielectric, and low cost. TiO_2 NPs have special magnetic, chemical and optical properties so used in

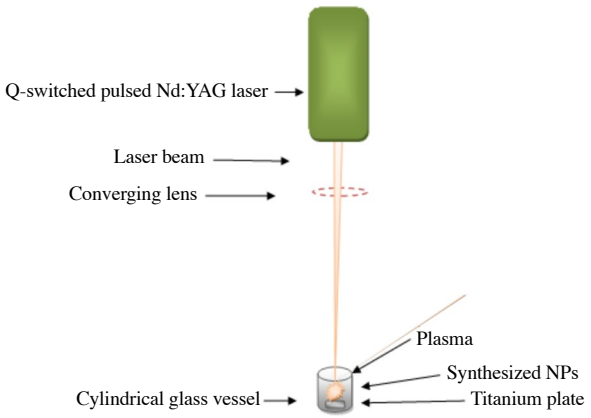

Figure 1. Laser set up system.

dental implants, pharmaceutical products, packaging, cosmetics and catheters[9-11], in spite of wide uses of such nanomolecules but their toxic effects still unmarked yet. Generally, there is many studies refer to generate of reactive oxygen species (ROS), this oxidizing agents have deep damage effects on DNA and protein molecules, causes serious health problems[12,13]. Interaction of cells with nanoparticles induces pro-oxidant effects leading to ROS formation, so the present study aims to investigate the toxic actions TiO_2 NPs by measuring one of most uses bio indicators (MI, BI and TCA) by exposing blood lymphocytes to graduated concentrations of TiO_2 NPs that prepared using laser ablation that considered one of green and safe method that give pure colloidal of nanoparticles with one-step without needing of surface active agents (surfactant)[14-16]. Characterization of the prepared nanoparticles can be affected using laser parameters, such as width of laser pulse, pulse energy, pulse repetition rate and laser wavelength, moreover the confined liquid[6].

Results and Discussion

Optical Properties of TiO_2NPs

The absorption spectra, type and concentration of the prepared TiO_2NPs were determined by Ultraviolet-Visible spectrophotometer. The laser beam was focused at the immersed target in 5 mL of DDW. The focal length, the repetition rate, and the number of applied pulses were 10 cm, 1 Hz and 300 pulse respectively.

The laser beam was absorbed by the metal and exited with electrons. High temperature and pressure of plasma created due to rapid ionization and vaporization in the liquid-target interface[19-21]. Strong shockwave was generation and the plasma expanded adiabatically and

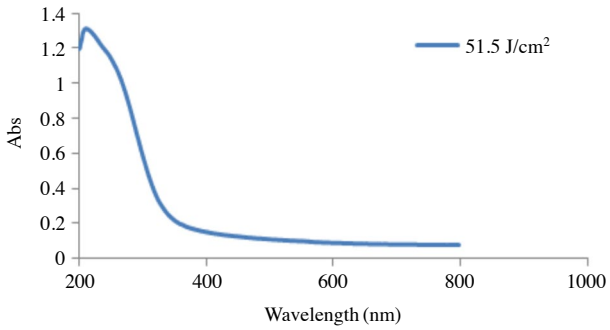

Figure 2. UV-Visible absorption spectrum of TiO_2 NPs.

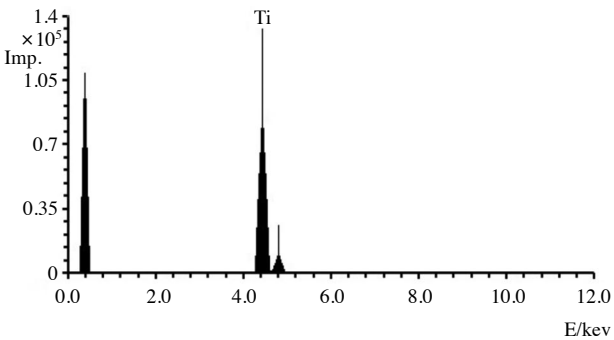

Figure 3. X-ray fluorescence spectrum of titanium metal.

mixed with the surrounding liquid[19]. Thus, visible vapor of metal created upon the surface of metal and TiO_2 NPs colloidal. The peak position of the absorption spectrum of TiO_2NPs was at ultraviolet-region (around 215 nm) due to SPR as shown in Figure (2).

Structural Properties

The purity of titanium plate (98.4%) was investigated using Energy Dispersive X-Ray Fluorescence (EDXRF) as shown in Figure (3).

X-ray Diffraction (XRD) was used to clarify the phase formation of TiO_2 NPs. All the reflections were well indexed to the tetragonal crystalline of TiO_2 NPs and matched with the standard values of anatase phase of TiO_2 crystal planes according to (JCPDS file Card No 96-900-9087) as shown in Figure (4). All XRD parameters of TiO_2NPs were tabulated in Table (2). The astounding crystalinity can be construed as a result of sharpness and correct number of peaks in the XRD pattern. The crystalline size of NPs (C.s) was calculated according to Scherrer formula and according to (JCPDS file Card No 96-900-9087). Table (2) shows all XRD results of experimental anatase TiO_2NPs including peak position (2θ), full width half maximum (FWHM), crystalline size variation, the interplanar spacing (d_{hkl}) and

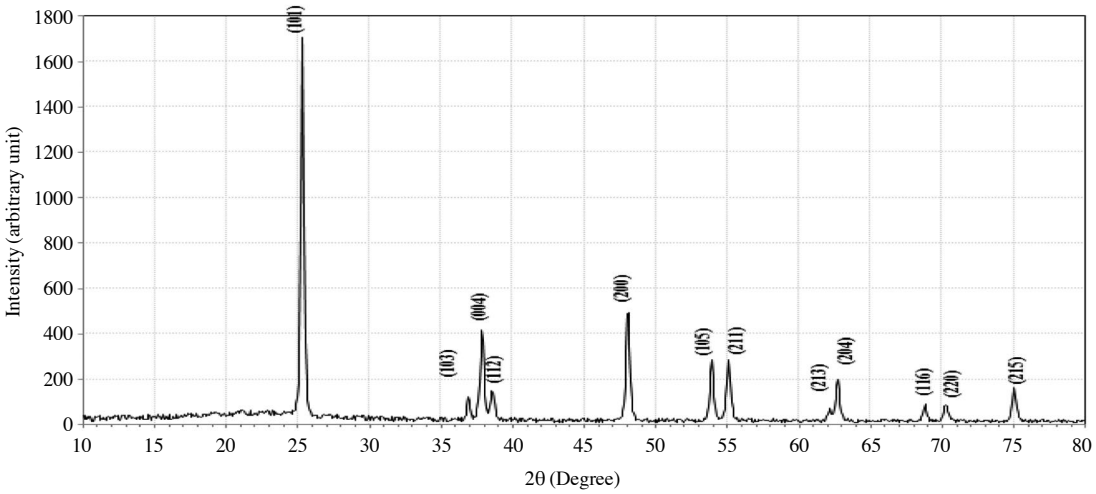

Figure 4. XRD pattern of TiO_2 NPs.

Table 1. Laser parameters of nanosecond pulsed Nd:YAG.

Laser parameters	Details
Wavelength	1064 nm
Laser energy	400 mJ
Repetition rate	1 Hz
Pulse duration	10 ns
Number of pulses	300 pulses
Focal length	10 cm

Table 2. XRD parameters of TiO_2 NPs.

2θ (Deg.)	FWHM (Deg.)	d_{hkl} exp. (Å)	G.S (nm)	hkl
25.3321	0.2245	3.5131	36.3	(101)
37.0065	0.2526	2.4272	33.2	(103)
37.8204	0.2525	2.3768	33.3	(004)
38.6062	0.2526	2.3302	33.3	(112)
48.1759	0.2807	1.8873	31.0	(200)
53.9850	0.3087	1.6972	28.9	(105)
55.1356	0.2807	1.6644	31.9	(211)
62.3199	0.3087	1.4887	30.1	(213)
62.7970	0.3367	1.4785	27.7	(204)
68.8587	0.3367	1.3624	28.6	(116)
70.4022	0.3367	1.3363	28.9	(220)
75.1731	0.3648	1.2629	27.5	(215)
76.1833	0.3649	1.2486	27.7	(310)

miller index (hkl), respectively.

Morphological Properties of TiO_2 NPs

Morphological properties and particle size distribution of synthesized TiO_2NPs were characterized using Transmission Electron Microscopy (TEM) as shown in Figure (5) where the crystalline shape and particle size distribution of synthesized TiO_2NPs using 1064 nm ranged from 40 to 110 nm. The morphological results of synthesized TiO_2NPs by laser technique in liquid media were agreed with Boutinguizaa results[15].

Cytogenetic Effects of Synthesized TiO_2NPs

Practically, Mitotic index (MI) and blastogenic index (BI) are important indicators for inspection for several types of chemical and physical factors because of their sensitive to such variables and the possibility of measuring the influence on their occurrence and repetition rate[21]. From results which were shown in table (3), both Mitotic index (MI) and blastogenic index (BI) suffered from significant reduction with increasing in TiO_2 NPs concentration, while total chromosomal aberration (TCA) suffered from significant elevation represented by chromatid breaks CB, micronuclei MN and ring chromosome RC. The highest proportion of total chromosomal aberration (TCA) has been observed specially in 180 µg/mL of TiO_2 NPs (significant at $p \leq 0.05$). The cytotoxic effects of TiO_2 NPs attributed to their special properties such as high surface area, small size of particles which is similar to the biomolecules size, shape, coatings and charge of particles surface. Chemical reactions occur between NPs and biomolecules cause reactive oxidative Stress (ROS) caused by hydrogen peroxide and hydroxyl radicals and contain superoxide radical (O_2–), induce an imbalance in the macrobiomolecules leading to disruption in the cell membranes, proteins, enzyme, phospholipids, nucleic acids and DNA[6,4,21,22].

Cytotoxic Effects of TiO_2NPs on Hep-2 Cell Line

As we mentioned before, the prepared TiO_2 NPs were examined in two biological systems normal cells

Table 3. Cytotoxic effects of synthesized TiO_2 NPs on peripheral blood lymphocytes.

Concentration µg/mL	BI	IP%	MI	IP%	TCA	SP%
0.0	64.16 ± 0.84bcd		0.66 ± 0.012 bcd		0.172 ± 0.005 bcd	
50	44.2 ± 0.29acd	31.08	0.51 ± 0.016 acd	22.72	0.22 ± 0.008 acd	27.90
90	32.56 ± 0.92abd	49.23	0.43 ± 0.041 abd	34.84	0.34 ± 0.02 abd	99.67
185	22.46 ± 0.93abc	64.98	0.22 ± 0.008 abc	66.66	0.52 ± 0.008 abc	213.95
Correlation factor	−0.94835		−0.99489		0.994475	

Each number represent (M ± SD) for three replicate.
Different letters refer to significant differences at ($p \leq 0.05$).
(BI) Blastogenic Index (MI) Mitotic Index (TCA) Total Chromosomal Aberration (IP) Inhibition Percentage (SP) Stimulation Percentage.

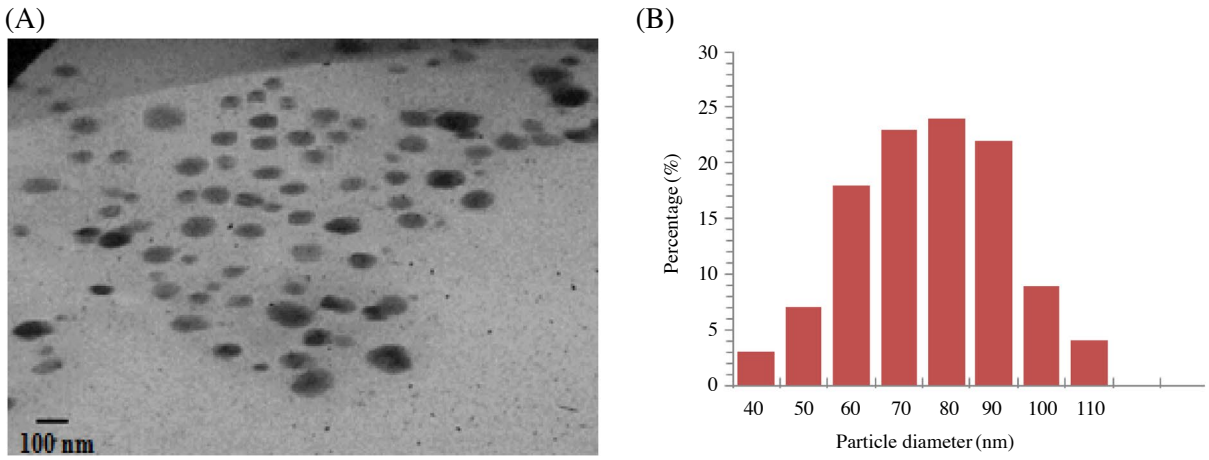

Figure 5. TEM image (A) TiO_2 NPs morphology (B) particles size distribution of TiO_2 NPs with 300 pulses at 400 mJ, 1 Hz and 1064 nm.

represented by PBLs and cancer cells represented by Hep-2 to understand the toxic behavior of these modern compounds on life cells, results in Figure (6) show decreasing in Hep-2 growth by increasing in inhibition rate gradually with concentration and exposure time, highest inhibition rate obtained at 200 µg/mL and 72 hrs. of exposure (68%), nanoparticles have a promising future in the therapeutic sector for its unique properties and to mediate in important chemical pathways within cells especially when mixed with other compounds have therapeutic features by strengthening the synergistic impact or by reducing resistance of pathogenic cells towards chemical therapies.

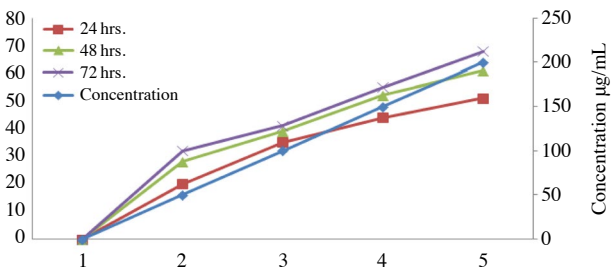

Figure 6. Cytotoxic effects of TiO_2 NPs on viability of Hep-2 cell line at 24, 48, and 72 hrs. of exposure time.

Morphological Changes on Hep-2 Cell Line

Hep-2 cell line was exposed to four concentrations of synthesized TiO_2NPs from 50-200 µg/mL the development and growth of cells were monitored during 24, 48 and 72 hours and morphological retardation was studied in both exposed and unexposed cells. In the control group, the monolayer Showed one continuous adhesive layer of malignant cells, homogeneous pigmentation, prominent exterior features, will differentiated nucleus and high nucleus/cytoplasm ratio, in control group can be distinguish two types of cells bold small proliferative cells and bright giant cells with double nuclei Figure (7A, B). At low concentrations (50 and 100) µg/mL of TiO_2NPs the cellular growth showed a small inhibition ratio, the growth mat seemed to be fragmented in some areas, with non-homogenized of pigmentation outer cellular feature was distinctive and clear with vacuolated

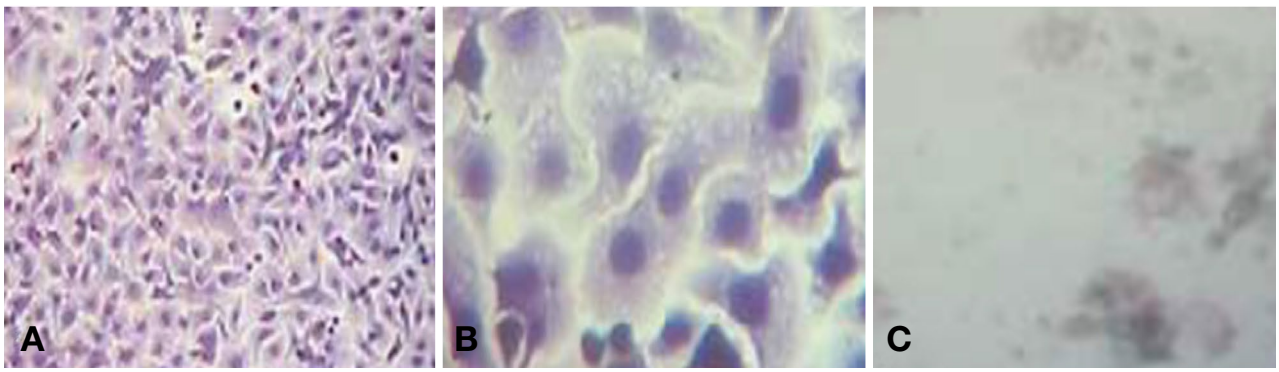

Figure 7. (A) and (B) unexposed Hep-2 cell line with continuous monolayer (C) total lysis of Hep-2 cell line exposed to 150 μg/mL for 72 hrs.

cytoplasmic and change in cell dimensions and can watch aggregated of exposed cells as dark patches, these changes in growth layer become more and more affluent with a period of exposure. In high concentrations (150 and 200) μg/mL the above changes become more extensive in this stage cell lysis was observed, large number of cells were dead and lost differentiation Figure (7C). TiO_2NPs with unique super-photocatalytic characteristics can be used to kill malignant cells by stimulation of valence electrons of TiO_2 and causes electronic holes that have ability to generate cellular reactive oxygen species (ROS) or promote autophagy[24,25].

Materials and Methods

Sample Preparation

Titanium plate was supplied by Danyang Xinli Alloy Company, China. The purity of titanium metal sheet (98.4%) was investigated using ED-XRF. Titanium plate was cut into square-shaped plates with 1 cm × 1 cm dimensions. Each plate was polished and cleaned using emery paper and DDW.

Preparation of TiO_2NPs

TiO_2NPs were synthesized using pulsed laser ablation (PLA) in liquid media. Square-shaped plate was immersed in the cylindrical glass vessel containing 5 mL from double distilled water. Q-switched pulsed Nd:YAG laser was used to irradiate the immersed target. The details of laser parameters are summarized in Table 1 and the schematic diagram of experimental set up are shown in Figure (1).

Characterization of TiO_2NPs

TiO_2NPs were characterized using optical, structural and morphological advanced techniques. UV-Vis spectrophotometer (Lambda 750, Perkin Elmer) was used to determine the optical spectra of TiO_2NPs colloid.

X-ray diffractions (XRD) type (Bruker Axs) made in Germany model (D8 Advance) was used to measure the structural properties of TiO_2NPs, X-ray radiation source was CuKα1 radiation with (1.54A°) wavelength at $2\theta = (3° - 80°)$. The crystal size was calculated by Scherrer equation [4]:

$$D = 0.94\ \lambda/\beta\ \mathrm{Cos}\ \theta \qquad (1)$$

Where λ: X-ray wavelength (1.54060 Å), β is the line broadening at half maximum and θ is the diffraction angle.

Transmission electron microscope (TEM) was used to investigate the morphology and particle size distribution of synthesized TiO_2NPs.

Blood Collection and Cultivation

Venous blood samples have been collected from healthy people, nonsmokers, unexposed to any direct pollution, didn't take any chemical or radiation therapy with age range from (25-40) years. Chromosomal analyses were carried out by adding 0.5 mL of whole blood to 4.5 mL of culture media (RPMI-1640) supplemented with 10 μg/mL of PHA (phytohemagglutinin) and different concentrations of synthesized TiO_2 NPs (0.0, 45, 90, and 180) μg/mL. The tubes were incubated in the CO_2 incubator (Memmert /Germany) at 37°C for 70 hrs., then 10 μg/mL of Colchicine solution was added to tubes and returned to the incubator at 37°C for 2 hrs. after that the mixture was centrifuged at 3000 RPM for 10 min, the supernatant was neglected while the pellet was re-suspended by 5 mL of Potassium. chloride (KCl), the mixture incubated at 37°C for 45 min to get rid of Red Blood Cells (RBCS). The mixture was centrifuged again at 3000 RPM for 10 min to get rid of the supernatant and the residual pellet was re-suspended

with cold fixative solution (3 : 1 volume to volume of absolute methanol and glacial acetic acid), the fixative solution added drop by drop with continuous shaking, this process was repeated for several times until the solution become colorless, the cloudy deposited cells were suspended in 1.5 mL of fixative and mixed by Pasteur pipette to become ready for dropping on cold and wet slides.

Cytotoxic Assay

Cytotoxic effects of five concentrations for synthesized TiO_2NPs (0.0, 50, 100, 150, and 200) µg/mL were investigated on laryngeal carcinoma cell line of human (Hep-2) using micro- titter plate technique 96-will with flat bottom for 24, 48 and 72 hrs. After exposure periods the culture media was removed, the micro- titter plates were washed in sufficient amount of phosphate buffer saline (PBS), then all plates were stained with Crystal Violate for 20 mins. the stain was removed and plates rinsed with PBS, all plates were examined by ELISA microplate spectrophotometer at 496 nm wavelength, the Inhibition rate was calculated by the equation below[17].

$$IR\% = A - B / A \times 100$$

Where IR = inhibition rate
A = absorbency of negative control
B = absorbency of tested

Morphological Changes

The cells of Hep-2 cell line was incubated with different concentration of TiO_2NPs and (0.0, 50, 100, 150, and 200) µg/mL for 24, 48 and 72 hrs. Cellular growth was fixed by 10% PBS/formaldehyde solution, then stained with Hematoxylin and Eosin to determine the morphological variations beyond the exposure time[18].

Statistical Analysis

All data were statistically evaluated using SPSS version 16, ANOVAI and presented as $M \pm SD$ with $p \leq 0.05$ being Least Significant Differences (LSD).

Conclusion

Findings showed that the ablation via Q-switched Nd: YAG laser has adequacy to prepare TiO_2NPs with 215 nm absorption spectrum, nearly spherical particles and suitable particle size (40-110 nm). In vitro assay showed that prepared TiO_2NPs have significant cytotoxic effects on peripheral blood lymphocyte and Hep-2 cell by decreasing both Mitotic index (MI), blastogenic index (BI) and growth rate of cancer cells while total chromosomal aberration (TCA) increased.

Conflict of Interest

Azhar M. Haleem declares that she has no conflicts of interest with the contents of this article. Ruaa H. Abbas declares that she has no conflicts of interest with the contents of this article. Mohammed Abed Jawad declares that he has no conflicts of interest with the contents of this article. Faiz Alberaqdar declares that he has no conflicts of interest with the contents of this article.

Ethical Approval

This article does not contain any studies on human participants or animals performed by any of the authors.

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