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Anti tumour efficacy of curcumin/chitosan-coated iron oxide nanoparticles in human lung cell line (L132) In an *In Vitro* study

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Abstract---Cancer may be a major public ill health, with significant associated death and disability. It is the second leading explanation for death in developed countries and is one among the three leading causes of death for adults in developing countries. Cancer which causes the morbidity and mortality in worldwide, approximately 14 million new cases in 2012. The number of latest cases is predicted to rise by about 70% over the next 2 decades. The present work was aimed to investigate the effect of curcumin/chitosan-coated iron oxide nanoparticles for topical application on L132 cells, for example, cytotoxicity, Reactive oxygen species (ROS) and induction of apoptosis. Diseases resulting from dermal exposure may have a big impact on human health. Cytotoxic potential of curcumin/chitosan-coated iron oxide nanoparticles was measured by tetrazolium bromide (MTT assay) in L132 cells. ROS assay and cell cycle were used to confirm the basis of apoptosis. Apoptosis was investigated by Annexin V with the Propidium iodide method in Flow cytometry analysis.

Keywords---Apoptosis, Cell line, Reactive Oxygen Species, Cytotoxicity.

Introduction

Cancer is the abnormal growth of cells in our bodies that can lead to death. Cancer cells usually invade and destroy normal cells. These cells are born thanks to imbalance within the body and by correcting this imbalance, the cancer could also be treated. Billions of dollars have been spent on cancer research and yet we do not understand exactly what cancer is cell growth and cell multiply process is known as cell division. It must be extremely controlled that each one the cells within the body should grow at the proper place, and for all the organs and tissues to function properly. Cancer is one of the leading causes of death. In 2015, nearly 8.8 million peoples were died in cancer in worldwide. The previous report says nearly 1 people is affected in every 6 death is due to cancer. Malignancies are ordered by the kind of cell that the tumor looks like and is subsequently dared to be the cause of the tumor. Globally, there are 239,000 new cases and 152,000 deaths annually thanks to ovarian cancer, making this cancer the second commonest explanation for gynaecological cancer mortality (Reid et al., 2017). Curcumin may be a multifunctional polyphenolic phytochemical extract of turmeric (*Curcuma longa*). It is most commonly available in the form of a mixture with two othercurcuminoids, desmethoxycurcumin (~5%) and bis-desmethoxycurcumin (~18%), in whichcurcumin (~77%) is the major component. The international standard name ofcurcumin is 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene, 3,5-dione [Moniruzzaman*et al.*,2020]. Several preclinical and clinical studies proved the various medicinal activities of curcumin (Basnet, *et al.*,2011; Wanninger*et al.*, 2015; Karthikeyan*et al.*, 2020) Apoptosis is a programmed cell death activity, which plays a specific role in all therapy. It is a highly regulated process of cell death and activated by various stress-factors including cytokines and oxidative stress. The fast production of ROS could activate apoptotic pathways. The present study aims to investigate the anti-cancer efficacy of curcumin/chitosan-coated iron oxide nanoparticles in human cell line.

Methods

Cell culture and treatment of curcumin/chitosan-coated iron oxide nanoparticles

The Lung cell line (L 132) was obtained from National Centre for Cell Sciences, Pune, India, and cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 0.2% sodium bicarbonate and 10 ml/l antibiotic and antimycotic solution, respectively at 37°C under the humidified atmosphere of 5% CO₂. The samples were weighed and make a desired concentration. The mixture was dissolved in Dimethyl Sulfoxideand volume was made up with Dulbecco's Modified Eagle Mediumin order to obtain a stock solution (1 mg/ml). After sterilization of the stock solution by filtration, aliquots of 5 µg/ml to 250 µg/ml concentration were prepared from stock solution for carrying out analysis. Stock suspension of ZnflNs (200 µg/ml) in Dulbecco's Modified Eagle Medium (supplemented with 10% FBS) was serially diluted to concentrations ranging from 5 to 250 µg/ml for cytotoxicity assays (MTT Assay), ROS generation and cell cycle progression analysis. In every experiment, the particle suspension was prepared newly and immediately applied to the cells. For

different assays 96- and 6-well culture plates having a treatment volume of 0.1 and 2 ml, respectively were used. Culture medium without ZnflNs was served as the control in each experiment (Patel *et al.*, 2016).

Cytotoxicity assay

The effect of curcumin/chitosan-coated iron oxide nanoparticles (1, 5, 25, 50, 75, 100, 200, 300, 400 and 500 µg/ml) on cell viability was assessed by MTT assay. The study was performed on L132 cells. Cells were purchased from National Centre for Cell Science, Pune, Maharashtra, India. Cell cytotoxicity of L132 cells after exposure to curcumin/chitosan-coated iron oxide nanoparticles (was determined by MTT [3-(4, 5-dimethylthiazoyl-2-yl) 2, 5-diphenyltetrazolium bromide] assay. MTT assay is predicated on the mitochondrial activity and was done consistent with the tactic of Mosmann (1983). Briefly, L132 cells was suspended in DMEM to 5×10^4 cells per ml and aliquots (5×10^3 cells/100 µl/well) were seeded in 96-well microtitre plates and were maintained in culture media for 24 h or and incubated for 24 and 48 h at 37°C, 5% in a CO₂ incubator. Cells were further exposed to different concentrations (5–250 µg/ml) of curcumin/chitosan-coated iron oxide nanoparticles for 0, 24 and 48 h. After exposure, MTT (5 mg/ml in PBS medium) was added to each well and incubated for 4 hours in CO₂ incubator. After incubation of 4 h at 37°C in a 5% CO₂ atmosphere, the supernatant was removed. 100 µL of DMSO was added to the collected supernatant. The optical density of each well was read at 590/540 nm with a microplate reader (model 450). The percentage growth inhibition was determined and the concentration of the test drug needed to inhibit cell growth by 50% (IC₅₀ values) was generated from the dose-response curves.

Cell cycle progression analysis

The distribution of DNA in the cell cycle was studied in the flow cytometer. L 132 cells were cultured in six-well cell culture plates to 30% confluence and then exposed to different concentrations of curcumin/chitosan-coated iron oxide nanoparticles (100 and 250 µg/ml) for 24 h. The treatment was removed and cells were harvested and centrifuged at 135g for 10 min at 37°C. The cells were collected and fixed in ice-cold ethanol (70% in 1× PBS) and incubated at -20°C for 30 min with regular mixing. Cells were centrifuged and resuspended in lysis buffer (0.2% triton in 1× PBS) and incubated at 4°C for 30 min. Cells were further centrifuged and resuspended in 20 µg/ml RNase prepared in 1× PBS, incubated at 37°C for 30 min. The cells were centrifuged and resuspended in 1× PBS and stained with 10 µl PI (1 mg/ml) followed by incubation at 4°C until analysis by flow cytometry. A histogram of cell distribution was obtained using the FL2 channel (595nm) of the flow cytometer and the distribution of cells in the different cell cycle phases was analyzed using the CellQuest Pro software (Patel *et al.*, 2016).

Quantification of Intracellular Reactive Oxygen Species (ROS) Assay

ROS, key signaling molecules during cell signaling and homeostasis, are reactive species of molecular oxygen. ROS generation was measured by using ROS Assay Kit. The intracellular ROS generation level was determined.(Wan *et al.*1993)then

modified by Shukla *et al.* (2011) using dichlorofluorescein-diacetate (DCF-DA) dye. L132 cells (1.10^4 cells/well) were cultured in 6/96-well black plates. After incubation with various doses of curcumin/chitosan-coated iron oxide nanoparticles for 24 and 48 h, cells were rinsed twice with $1\times$ PBS /HBSS. Cells were harvested using 0.25% trypsin-EDTA and then centrifuged at 135000rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml of PBS containing DCF-DA (20 μ M) and incubated for 30 min at 37°C. The level of intracellular ROS was measured both by flow cytometer (FACSCalibur, BD Bioscience, CA, USA) and fluorescent microscope (Model DM 2500 Leica, Wetzlar, Germany).

Detection of Apoptosis

In addition to cell-cycle arrest and repair machinery, the damaged cells, where damage is beyond repair, may induce an apoptotic (programmed cell death; PCD) response that is highly cell-specific and is the most common form of physiologic cell death in multicellular forms. Apoptosis of L 132 cell lines were evaluated by flow cytometry on FACSCanto II cytometer (BD, San Diego, CA, USA). The cells (2.0×10^5) were seeded in 2 mL of DMEM in six-well plates. After 24 h, the DMEM was removed, replaced with the 5–15 nm ZnO NPs suspension in DMEM, at 50 or 100 μ g/mL concentrations. Both cell lines were incubated for 24 and 48 h. The cells were detached, resuspended in DMEM and then in binding buffer. Subsequently, the cells were stained with FITC Annexin V and PI (FITC Annexin V apoptosis detection Kit I, (BD Pharmingen™, San Diego, CA, USA) at room temperature, in the dark, for 15 min. Data were analyzed using FACSDiva software. In the present study, fluconazole - zinc was loaded on curcumin/chitosan-coated iron oxide nanoparticles using an emulsion solvent evaporation technique. The MTT assay demonstrated that the curcumin/chitosan-coated iron oxide nanoparticles showed significant cytotoxicity to L132 cells while no influence on the L132 normal cells. Intracellular reactive oxygen species (ROS) and glutathione (GSH) measurement ROS production were monitored by flow cytometry using 2,7-dichlorodihydrofluorescein-diacetate (DCFH-DA). The dye is a stable and nonpolar compound that easily diffuses into cells and is hydrolyzed by intracellular esterase to yield the DCHF, which is trapped within the cells.

Results

In vitro Cell Cytotoxicity Studies

In vitro cytotoxicity of curcumin/chitosan-coated iron oxide nanoparticles were evaluated on the L132 human lung cell line under the following variable concentrations such as 1, 5, 25, 50, 75, 100, 200, 300, 400 and 500 μ g/ml for 24 and 48 hrs time interval. Cell viability rates of all the curcumin/chitosan-coated iron oxide nanoparticles against the L132 cell line are shown in Figure 1. Treatment with curcumin/chitosan-coated iron oxide nanoparticles at maximum value 250 μ M for 24 hrs recorded cell viability of up to 53% which declined to 50 % at the end of 48 hrs treatment. From Figure, it is clear that the curcumin/chitosan-coated iron oxide nanoparticles demonstrate higher cell viability at the end of 24 hrs than after 48 hrs. Hence the concentration of 100 μ M & 250 μ M was taken for further studies because of the values of IC₅₀ that

show more than 340 μM which will amount to greater concentrations leading to cell death much due to starvation from nutrition than from the treatment. The curcumin/chitosan-coated iron oxide nanoparticles were further tested for the impact on cell cycle, generation of ROS and induction of Apoptosis to confirm the toxicity studies.

Cell Cycle Arrest

The phases of cell cycle were considered as major events of cell proliferation and inhibition of cells. The effect of curcumin/chitosan-coated iron oxide nanoparticles on the cell cycle progression is determined by using L132 normal lung cell line was treated for 24 hrs with curcumin/chitosan-coated iron oxide nanoparticles at a concentration of 100 μM & 250 μM before analysis in flow cytometer. Untreated cells served as the control and it exhibited 82.51%, 10.22% and 7.69% in G0/G1, S and G2/M phase respectively. Cells under the treatment of curcumin/chitosan-coated iron oxide nanoparticles in 100 μM & 250 μM demonstrated decrease in G0/G1 phase to the level of 77.7 %, 77.5% respectively. At the same time, there was significant increase in the S phase and G2/M phase with 13.2% & 9.3% at 100 μM concentration. Whereas, treatment under 250 μM for 24 hrs did not present significant change for the G0/G1 phase (77.5%) and S (9.81%) phase while increased the G2/M (12.69%) phase. These results evidently bring out the fact that curcumin/chitosan-coated iron oxide nanoparticles do not arrest or significantly change the cell cycle in normal lung cells.

ROS

All the living cells constantly produce Reactive Oxygen Species (ROS) during aerobic metabolism. Some of the ROS are associated with apoptosis which can be detected using oxidizable fluorescent dyes such as acetylated forms of 2',7'-dichlorofluorescein (DCFH-DA). Fluorescence exhibited can be detected in flow cytometer and correlated with the generated amount of ROS that might have implication in apoptosis. Study on flow cytometer demonstrated that ROS levels increased (107.2%,109.5%,110.31%) in the cells treated with curcumin/chitosan-coated iron oxide nanoparticles at 100 μM concentration at 3hrs, 2hrs and 1hr time interval. Similarly, ROS levels increased (116.56%,118.12%,120.65%) when treated with 250 μM concentration at 3hrs, 2hrs and 1hr time interval respectively considering the untreated cells to be at 100%. Therefore, ROS results project only 20% increase under 250 μM concentration, which represented minimal apoptosis due to a minimal oxidative stress.

Apoptosis

L132 cells were treated with 100 μM and 250 μM concentrations curcumin/chitosan-coated iron oxide nanoparticles for 24 hrs. Then the treated cells were stained with annexin V and propidium iodide. The apoptotic effect of curcumin/chitosan-coated iron oxide nanoparticles on L132 cells was detected by flow cytometer. The result analysis revealed that untreated (control) cells exhibited 99.7% cell viability while that of 100 μM concentration exhibited 96.07% of cell viability; 1.56% of necrosis; 1.5% of late apoptosis and 0.87% of early apoptosis. Similarly, curcumin/chitosan-coated iron oxide nanoparticles at 250

μM treatment on L132 cell line demonstrated 7.93 % of early apoptosis, 8.42% in late apoptosis and 1.51% of the cell population underwent necrosis while 82.14% cells survived normally. So it has been concluded that the curcumin/chitosan-coated iron oxide nanoparticles at maximum concentration of 250 μM induced only 18% of cell death.

Figure 2: Photomicrograph represents morphological changes in L132 human lung cell line such as shrinkage, detachment, membrane blebbing and distorted shape induced by curcumin/chitosan-coated iron oxide nanoparticles treatment as compared with control. Control cells showed normal intact cell morphology and their images were captured by light microscope.

Anderson et al., (2015) reported that the cytotoxic effect of silver is the result of active physicochemical interaction of silver atoms with the functional groups of intracellular proteins as well as with the nitrogen bases. Sriramet *al.*, (2010) demonstrated that AgNPs serve as antitumor agents by decreasing progressive development of tumor cells.

Now a days, the chemotherapy and radiotherapy is used to treat the cancer, but they are high cost and it causes adverse effect, including such minor ones as vomiting, alopecia, diarrhoea, constipation, and major ones such as myelosuppression, neurological, cardiac, pulmonary and renal toxicity. In addition, many of these treatments present limited anti-cancer activity. Because of this reasons the researchers are try to find the alternate anticancer drugs which have high potential and less toxic to use.

Nanoparticles are particles of approximately 1–100 nm in diameter possess unique physical, chemical, and biological properties that can be useful for drug delivery. Nanoparticles are 1,000 times smaller than the average human body cell and consist of materials engineered at the atomic or molecular level. They are also suitable for both controlled and targeted drug delivery systems (Rudramurthy *et al.*, 2016). The encapsulation of drug inside the nanoparticle, favor the pharmacokinetics and solubility of drugs, provide targeted delivery and controlled release of drugs. So far, polymer, solid lipid, magnetic, gold, and albumin-based nanoparticles are extensively used to improve the curcumin therapeutic applications.

Due to the smaller in size and biocompatible nature, it can circulate long time in blood circulation in the body. Many synthetic and natural polymers have been identified and utilized for the synthesis of curcumin nanoparticles such as N-isopropylacrylamide, polyvinyl alcohol, polyethylene glycol monoacrylate, silk fibronin and chitosan. The previous study of Chang et al., (2002) proves the molecular mechanisms activated by curcumin loaded-poly lactic co-glycolic acid nanoparticles controlled the activity of multiple drug resistance protein 1 and the development of reactive oxygen species in CAR cells by activating the intrinsic apoptotic pathway. The higher activity of curcumin-loaded poly lactic co-glycolic acid nanoparticles is identified in the treatment of CAR cells along with enhanced bioactivity and bioavailability is noted in both in vitro and in vivo conditions compare to the native curcumin.

In another experiment, curcumin loaded with Eudragit RE 100 cationic copolymer produces the higher binding affinity as the cellular uptake of polymeric nanoparticles, it favors the cytotoxic activity. Taken together, this formulation of nanoparticles reduces the tumor growth and reported a 19-fold higher growth inhibition of colon-26 cells than curcumin alone. Xi et al., (2017) found that the curcumin silk fibroin (CUR-SF) nanoparticles are delivered to colon cancer, and it stimulates the anti-cancer effect than its free form in HCT 116 cells. Finally, they concluded that the release of CUR-SF able to enhance the curcumin cellular uptake into the carcinogen cells and it reduces the cytotoxicity to normal cells.

Solid lipid nanoparticles are produced through the dispersion of natural or synthetic lipids in water or surfactants. These solid lipid nanoparticles are stable and biocompatible drug delivery. It also favors the solubility of poorly soluble drugs. The solid lipid curcumin nanoparticles favors the dissolving nature over native curcumin and reduces the activity of the lipopolysaccharides induced pro-inflammatory mediators, NO, interleukin-6 by prohibit the activation of NF- κ B.

Sun et al., (2013) proved the curcumin solid lipid nanoparticles displayed the increasing of cellular uptake and it reduces the growth of cancer cells because of the increase dispersibility and the stability of the drug. In recently, FathyAbd-Ellalef et al., (2020) studied the curcumin solid lipid nanoparticles coupled with doxorubicin and it used to overcome the Pgb-mediated chemo-resistance in triple negative breast cancer cells. These combination to be more effective and safe because of its biocompatibility and lower toxicity nature.

Fig 3: CS/Fe complex induced DNA damage is oxidative stress mediated. Sybr Green-stained comets of a typical comet from nuclei of control and cells treated with increasing concentration of complex material. The magnetic nanoparticles contains metal or its metal oxides and a polymer. The metallic coating confirms the stability and biocompatibility of the magnetic nanoparticles. The magnetic nanoparticles have a specific properties in the human body. They may produced with low cost (Roacho-Pérez et al., 2020). Iron oxide nanoparticle core covered by CD and pluronic polymer (F68) with curcumin showed enhanced uptake in cancer cells. This formulation may retards the mitochondrial membranes potential and induces the ROS than unformulation of curcumin. Also, it exhibited a strong anticancer effect together with resonance imaging characteristics and magnetic targeting abilities (Yallapuet al., 2012b).

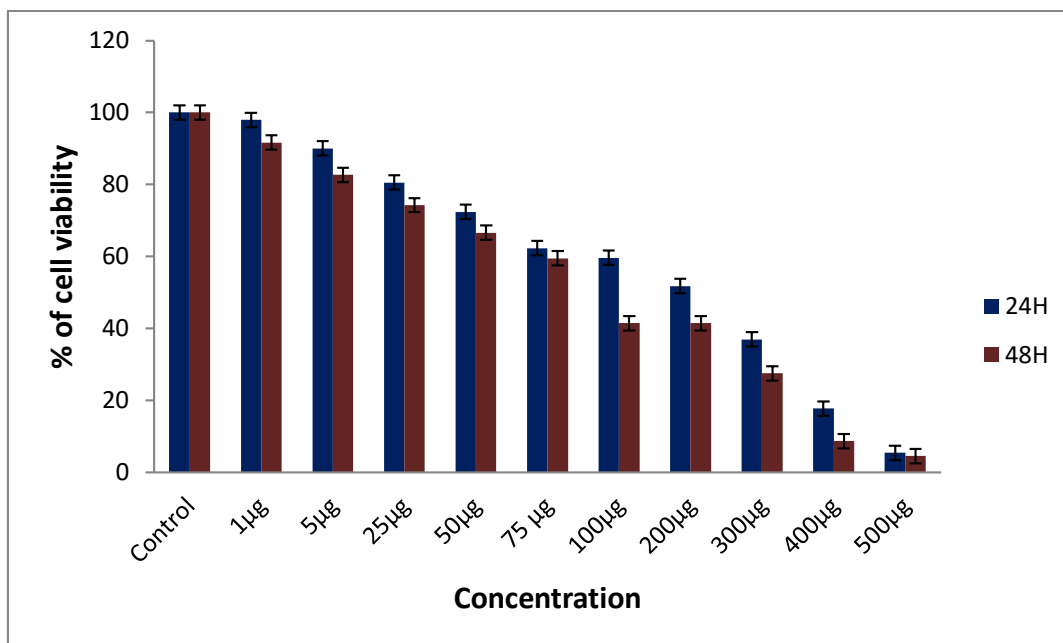


Figure 1: Anti-proliferative effects of curcumin/chitosan-coated iron oxide nanoparticles on the activity of cytotoxicity in L132 human lung cell line

Cytotoxicity

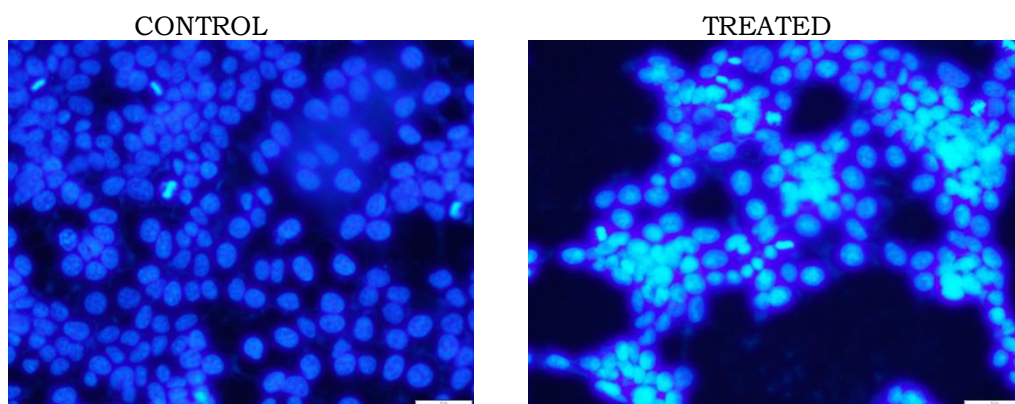


Figure 2: Photomicrograph represents morphological changes in L132 human lung cell line such as shrinkage, detachment, membrane blebbing and distorted shape induced by curcumin/chitosan-coated iron oxide nanoparticles treatment as compared with control. Control cells showed normal intact cell morphology and their images were captured by light microscope.

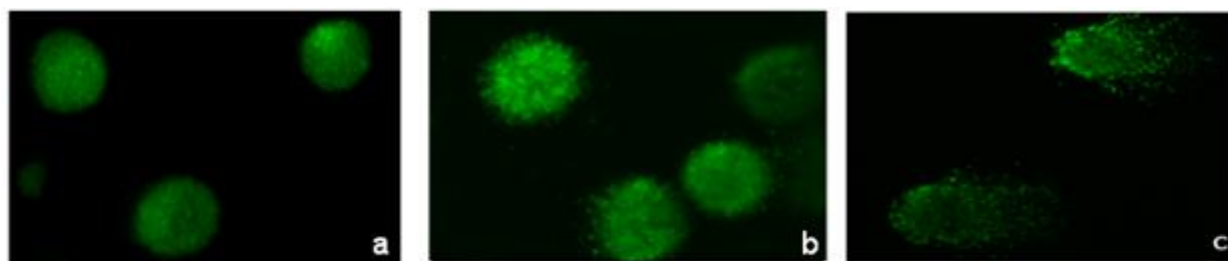


Fig 3: CS/Fe complex induced DNA damage is oxidative stress mediated

Sybr Green-stained comets of a typical comet from nuclei of control and cells treated with increasing concentration of complex material

Conclusion

Metal-oxide nanoparticles (MONs) are emerging as preferred nanocarriers in delivering several therapeutic drugs. They can offer additional functionalities besides carrying the payload, such as molecular probing, serving as contrasting agents in diagnosis, and providing additional therapeutic effects like photodynamic therapeutic effect and hyperthermia. Several studies have demonstrated the multifunctional benefit that metal oxides can offer in the biomedical fields. The use of metal oxides in drug delivery, specifically in the delivery of hydrophobic curcumin, can be referred to as limited compared to the overwhelming number of studies on the use of organic and other inorganic carriers. The in vitro release profile suggests that curcumin/chitosan-coated iron oxide nanoparticles is promising in drug delivery carriers in cancer therapy.

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