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Effect of maghemite nanoparticles in the induction of apoptosis on human hepatocellular carcinoma (Hep G2) cell line

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The present study focuses on the effect of maghemite nanoparticle in the induction of apoptosis in human hepatocellular carcinoma (Hep G2) cells. The antiproliferative effect of maghemite nanoparticle was analysed by cell viability assay and results showed less cytotoxicity at 50 to 250 µg/mL concentration. However, further increase in particle concentration exhibited higher cytotoxicity. Dysfunction of cell was noted by shrinking and small size of the cells in morphological analysis. The induction of apoptosis was revealed with live/dead assay and DNA fragmentation assay results by noticing increasing apoptotic fluorescent cells and fragmented DNA pattern, respectively. The cell cycle analysis of Hep G2 cells illustrated that they were arrested at G2/M phase transition and favoured induction of apoptosis. The expression of inflammatory cytokines interferon-γ (IFN-γ) and interleukin-12 (IL-12) were analysed by semi quantitative polymerase chain reaction that showed altered expression compared to the control. Thus the present study shows that maghemite nanoparticles induced apoptosis in Hep G2 cells through arresting the cell cycle at G2/M phase transition mediated by IFN-γ pathway.

Keywords: Apoptosis, G2/M phase arrest, Hep G2 cells, IFN-γ, maghemite nanoparticles

Introduction

Hepatocellular carcinoma is a commonly occurring and 5th most leading cancer world-wide[1]. Every year, more than 7 lakh new patients are estimated irrespective of ethnic groups and geographical location. The most commonly used drugs for its treatment are floxuridine, cisplatin, mitomycin C and doxorubicin. Even though chemo drugs are used to target mainly the dividing cells, but sometimes along with this other cells like bone marrow, the lining of mouth, intestine and hair follicles are also getting affected. The side effects of drugs include hair loss, mouth sores, nausea, vomiting, diarrhoea, loss of appetite, increased chance of infection, fatigue and easy bleeding, however it depends on the type of drug. During chemotherapy, pharmacologically active cancer drugs reach the tumour tissue with poor specificity and induce the dose-limiting toxicities. The gene MDR1 (multi drug resistant gene 1) has been found responsible for multi drug resistant in human primary liver cancer and cirrhosis[2]. Tyrosine kinase inhibitors (TKI)[3] have been used as the effective drug for several types of tumour. Among TKI, the notable drug is sorafenib[4], which has also failed to treat liver cancer effectively and expands life of liver cancer patients only for very few months with more side effects.

Now-a-days, nanoparticles are used in many areas of biology for its advantages over other conventional methods. Nanoparticle drug delivery may provide a more efficient and less harmful solution to overcome these problems. One of the nanoparticles, which widely used in molecular medicine, is iron oxide nanoparticles (IONP). This nanoparticle has large surface area to mass ratio that facilitates its novel application. It diffuses across the biological membrane and tissue barrier. The maghemite has the super paramagnetic property and are called as SPIONs (superpara iron oxide nanoparticles)[5]. IONPs are reported to be biodegradable[6], clinically approved and compared to other nanoparticles have more biomedical application, such as, tissue engineering, hyperthermia, magnetic resonance image, iron detection, targeted drug delivery and targeted destruction[7]. The toxicity, cellular uptake, biodistribution and its metabolism firmly depends upon the size, surface property and route of administration of IONP[8]. For different intracellular uptake pathways and its degradation *via* Fenton reaction, it directly damages DNA, protein and lipid molecules, and leads to cell death[5]. In the present study, we have made an attempt to analyse the cytotoxicity effect and apoptotic potency of maghemite nanoparticles on hepatocellular carcinoma cells.

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Materials and Methods

Cell Culture

The human hepatocellular carcinoma (Hep G2) cell line (ATCC HB-8065) was obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM (Dulbecco's modified eagle medium) supplemented with 2 mM L-glutamine, 1% of penicillin/streptomycin and 10% of foetal bovine serum (FBS). The cultures were maintained at 37ºC with 5% (v/v) CO_2 and subcultured periodically. Maghemite nanoparticles were obtained from Sigma-Aldrich (size ≤ 50nm).

Cell Proliferation Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was a simple colorimetric assay to measure cell cytotoxicity. Metabolic active cells were able to convert this tetrazolium salt into a water-insoluble dark blue formazan. The resultant value was related to the number of living cells. To determine cell cytotoxicity/viability, the Hep G2 cells were plated at a density of 1×10^4 cells per well in a 96-well plate at 37ºC in 5% (v/v) CO_2 level. After cell attachment, the fresh medium containing with and without maghemite nanoparticles in varying concentrations was added. After 24 h treatment, nanoparticles were removed and medium was added along with MTT dye solution (0.5 mg/mL) to the cells. After 4 h of incubation at 37ºC in 5% (v/v) CO_2, the medium was removed and formazan crystals were solubilized in 200 µL of DMSO (dimethyl sulfoxide). The absorbance was read on a microplate reader at 575 nm[9]. The relative cell viability (%) compared to control was calculated by $[A]_{test}/[A]_{control} \times 100$.

Morphological Analysis

Hep G2 Cells were seeded with and without nanoparticles at various concentrations for 24 h at 37ºC in 5% (v/v) CO_2. Then cells were rinsed with PBS (Phosphate buffer solution) and images were taken by inverted phase contrast microscope (Nikon, Japan) with 10× magnification. Hep G2 cells without nanoparticles were used as control.

Live/Death Assay

Hep G2 cells were treated with and without maghemite nanoparticle in different concentration for 24 h, then cells were washed with PBS for 3 times and stained with acridine orange/propidium iodide with the concentration of 1 µg/mL for 5 min. This was examined under a fluorescent microscope with 20× magnification (Zeiss, Germany).

DNA Fragmentation Assay

Hep G2 cells were seeded at 5×10^5 cells per well in 6 well plates. Cells were treated with and without nanoparticles at various concentrations for 24 h at 37ºC in 5% (v/v) CO_2. After treatment, cells were harvested and lysed in 500 µL of DNA lysis buffer (20 mM EDTA, 10 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.2% Triton X-100, 100 µg proteinase K, 100 µg of RNase) and incubated at 37ºC for 90 min and centrifuged to get supernatant. DNA was isolated by adding equal volume of iso-propanol to the supernatant and then 25 µL of 4 M NaCl. After overnight at –20°C, it was centrifuged to get DNA pellet, which was washed with 70% ethanol. DNA pellets were dissolved in 20 µL of TE (Tris-EDTA) buffer and then separated by electrophoresis in a 0.8% (w/v) agarose gel.

Cell Cycle Analysis

Cells were seeded in a 35 mm dishes at a density of 5×10^5 cells and then were treated with 500 µg/mL of maghemite nanoparticle. After 24 h, cells were collected in 1 mL of extraction buffer (45 mM Na_2HPO_4, 2.5 mM citric acid, 0.1% Triton X-100, 1 mg/mL of RNase and propidium iodide 50 µg/mL, pH=7.8) and analysed using BD FACS Aria III and the data were analysed with Flow Joe software. Cells without nanoparticles served as control.

Semi Quantitative PCR Analysis

Total RNA was isolated from Hep G2 cells using TRIZOL reagent. For DNAase treatment 3 µg of RNA was incubated with DNAse buffer (1×) and DNAse enzyme (3 units) in 30 µL of volume for 15 min at room temperature. Reaction was stopped by adding EDTA 3 µL (1 mM) and heat inactivated at 65ºC for 10 min. After DNAse treatment, total RNA (1 µg) was reverse transcribed (RT) using M-MuLV RT enzyme and 0.5 µg of Oligo dT primers in a 20 µL reaction volume. Amplified cDNA were subjected to PCR (polymerase chain reaction) and amplified products were resolved in 2% (w/v) agarose gel electrophoresis. The digital image of gel was captured by using gel documentation system and then dentiometric analysis was performed with Lab image platform 2.1 software. The targeted gene expressions were normalized to β-actin as an internal control. The targeted genes analysed by semi quantitative PCR are shown in Table 1.

Results

Cytotoxicity Effect of Maghemite Nanoparticles on Hep G2 Cells

The MTT assay for cell viability assessment has been described as a suitable method for detection of

Table 1 — Primer details for genes analysed in the present study

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)	T_a	Amplicon size (bp)
IFN-γ	GTCGCCAGCAGCTAAAACAG	CCACAGCTAAGAAGACTCCCC	54°	91
IL-12	AGCTCTGGGGGAGTCTTAGG	CCTTTCTGGCCAGCATACCA	54°	154
β-actin	CCTTGCACATGCCGGAG	GCACAGAGCCTCGCCTT	53°	112

biomaterial toxicity. The proliferation/viability of Hep G2 was measured by MTT assay. The MTT assay relies on the mitochondrial activity of Hep G2 and represents a parameter for their metabolic activity. The cytotoxicity of Hep G2 cells exposed to maghemite nanoparticles were increased in comparison to the control in relation to increasing concentration (50-5000 µg/mL) as shown in Fig. 1. The less cytotoxic effect was noted at 50-250 µg/mL of maghemite nanoparticle concentrations, while half maximal cytotoxicity (IC_{50} value) was observed at 3750 µg/mL concentration.

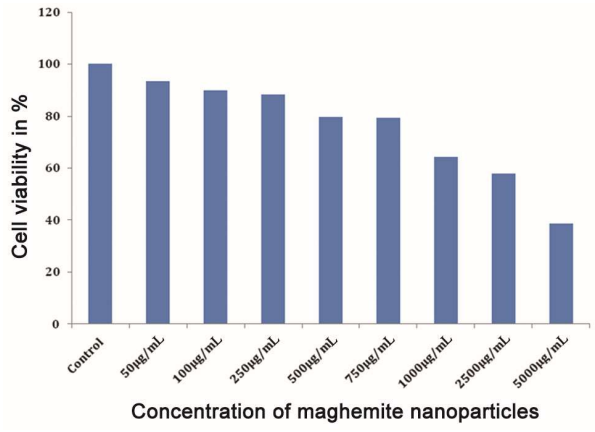

Fig. 1 — Effect of different concentrations of maghemite nanoparticles on HepG2 cells.

Morphological Analysis of Hep G2 Cells

Hep G2 cells were exposed with different concentrations (500-5000 µg/mL) of maghemite nanoparticles for 24 h. Increasing number of shrinked cells with a round shape that lost their fibroblast morphology was observed with increasing concentrations (500-5000 µg/mL) of maghemite nanoparticles (Fig. 2). Moreover, increasing size and shape of lysosome in nanoparticle-exposed Hep G2 cells as compared to the control showed the cellular stress. The cell morphology of nanoparticle-exposed Hep G2 was irregular in shape and the numbers of small shrinked cells increased with the increasing concentration of maghemite nanoparticles. The cells lost their contact because of less proliferation and spreading pattern due to cytotoxicity in comparison to the control. In HepG2 cells, the highest cytotoxicity was observed when exposed to 5000 µg/mL maghemite nanoparticles.

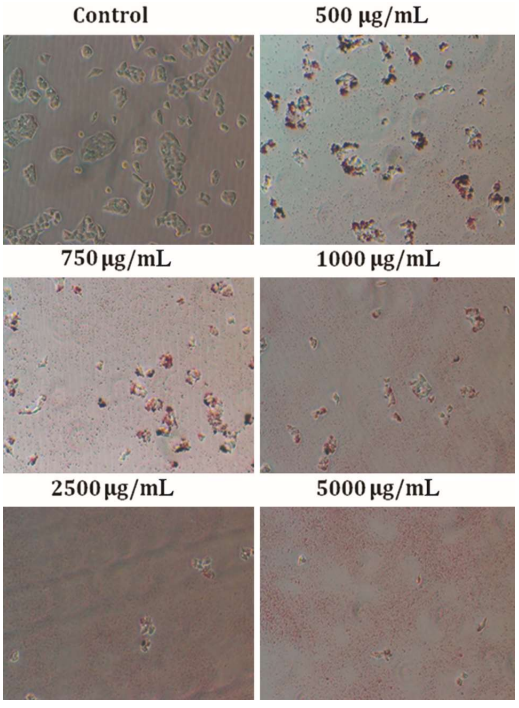

Fig. 2 — Phase contrast images of Hep G2 cell line after the exposure to different concentrations of maghemite nanoparticles for 24 h.

Apoptotic Potency of Maghemite Nanoparticles in Hep G2 Cells

After 24 h of incubation, the nanoparticles-exposed cells were stained with acridine orange/propidium iodide and analysed with blue filter. The live cells were observed with green fluorescence, which were stained only with acridine orange, and the death cells (apoptotic cells) were observed with red fluorescence, which were completely stained by propidium iodide. However, cells observed with yellow colour and orange fluorescence (with highly acidic vesicles) was pro-apoptotic and late-apoptotic cells, respectively. In the control, most of the cells were stained only with acridine orange, which had intact nucleus and DNA. At 50 µg/mL concentration of maghemite nanoparticles, the treated cells showed the initial stage of pro-apoptotic and apoptotic death cells, which were noted with yellow and red cells, respectively. The number of apoptotic cells was more at 500 µg/mL

concentration of maghemite nanoparticles (Fig. 3). Thus the results revealed that increase in concentration of maghemite nanoparticles increases the cell death of Hep G2 cells. From DNA fragmentation assay, it was observed that untreated control had intact DNA, while maghemite nanoparticles treated cells showed fragmented DNA as faint sheared pattern (Fig. 4). An increase in the concentration of maghemite nanoparticles increased Hep G2 cell apoptosis.

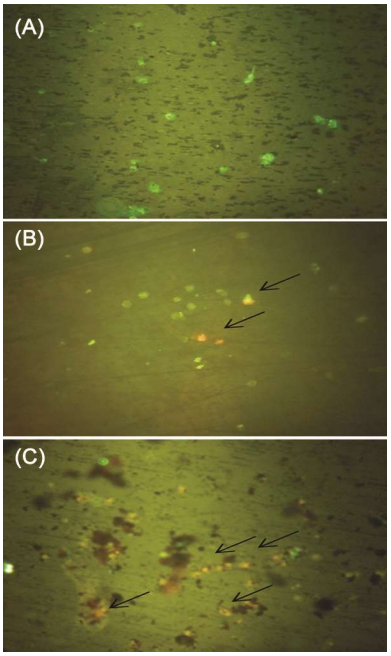

Fig. 3 (A-C) — A. Control cells without maghemite nanoparticles; B & C. Cells treated with maghemite nanoparticles at 50 and 500 µg/mL, respectively for 24 h.

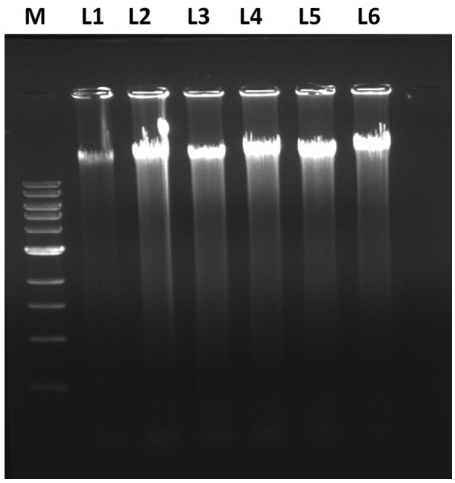

Fig. 4 — Results of DNA fragmentation assay after the exposure of maghemite nanoparticles: M, 1 kb ladder; L1, Control (without maghemite nanoparticles); L2, 500 µg/mL; L3, 750 µg/mL; L4, 1000 µg/mL; L5, 2500 µg/mL; L6, 5000µg/mL).

Effect of Maghemite Nanoparticles on Cell Cycle Regulation in Hep G2

This study analysed whether maghemite nanoparticles had any impact on cell cycle regulation. Cell cycle analysis was performed by using FACS (fluorescence assisted cell sorting). At 500 µg/mL concentration of maghemite nanoparticles, the cell cycle phase transition arrested at G2/M phase with decreased G1 phase and DNA synthesis S phase (Fig. 5).

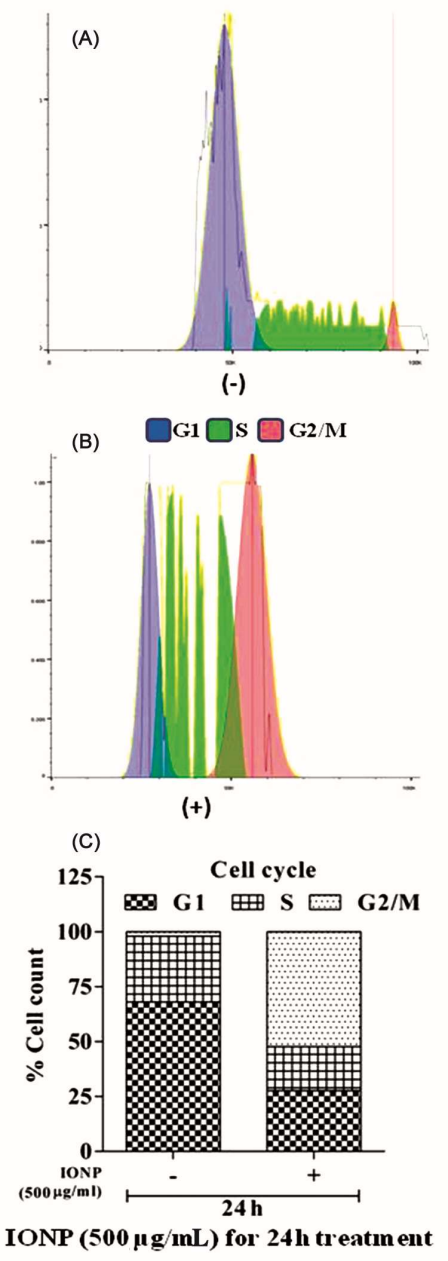

Fig. 5 (A-C) — A. Control, HepG2 cells without maghemite nanoparticles; B. HepG2 cells treated with 500 µg/mL of maghemite nanoparticles; C. Graphical representation of cell cycle analysis in Hep G2 cells.

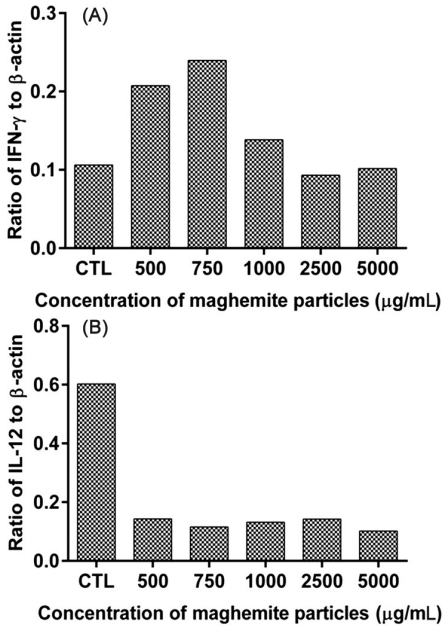

Fig. 6 (A & B) — Expression profiles of IFN-γ (A) and IL-12 (B) in HepG2 cells in response to various concentrations of maghemite nanoparticles for 24 h. [CTL=Control]

Thus, the maghemite nanoparticles arrested the Hep G2 cells at the G2/M phase transition to inhibit cell proliferation and further leading to apoptosis pathway.

Effect on Inflammation Cytokines of Hep G2 Cells

The expression of cytokines like IFN-γ and IL-12 in Hep G2 cell was altered when treated with maghemite nanoparticles. IFN-γ and IL-12 transcriptional expression was compared with housekeeping β-actin gene in treated Hep G2 cells. The results showed an increased expression of IFN-γ when cells were treated at 500 and 750 μg/mL concentrations of maghemite nanoparticles, while further increase in concentration decreased IFN-γ expression in comparison to the control. An increasing concentration of maghemite nanoparticles also decreased the expression of IL-12 as compared to the control (Fig. 6).

Discussion

Maghemite nanoparticles are able to bring morphological change in cells, which denotes that cells are under stress and often considered the initiation of cell death[8]. Further, they disturb the metabolic enzyme activity by causing dysfunction/malfunction of mitochondria and altering the signal mechanism, ultimately causing cell death[5]. In the present study, this phenomenon was also observed where maghemite nanoparticle distorted the cell structure maintenance probably by decreasing the tublin polymerization and depolymerising microtubules structural organization, which manifested in the morphological changes, such as, rounding, blebbing and reduced cell size, depending on the dosage of the nanoparticles. Furthermore, reduction in the mitochondrial activity and dose dependent increase in cell mortality was observed in maghemite treated Hep G2 cells.

The apoptotic cell death of Hep G2 cells was analysed with live/dead cell assay and study of DNA fragmentation. Propidium iodide enters into the damaged cell membrane that resulted as red fluorescence and this was evidenced from the results of our live/dead cell assay. Compared to the control, there was an increase in the number of propidium iodide positive cells based on the exposure of different concentrations of maghemite nanoparticle. Thus our results support the earlier research work where biosynthesized AgNPs were found to be involved with *in vitro* cytotoxicity by showing propidium staining of the breast cancer cell MCF-7[10]. DNA fragmentation is a well known technique to analyze apoptosis. The ladder like appearance of nuclear DNA on an agarose gel in our study suggests that increased cellular stress by maghemite nanoparticles might have induced apoptosis in Hep G2 cells *via* DNA damage.

The cell growth is endorsed with the progression of cell cycle, which was analysed in cancer cells by FACS. In our study, Hep G2 cells were arrested at G2/M phase transition. G2/M phase arrest was also caused by DNA damage with the agent γ-irradiation[11]. Hep G2 cellular DNA damage was the important mechanism to inhibit DNA synthesis and this phenomenon was considered as a biochemical characteristic of G2/M phase arrest mediated apoptosis. According to Ramatchandirin *et al*[12], the reactive oxygen species (ROS) generation increased based on the condition of inflammation and infection. In earlier studies, it was noted that maghemite nanoparticles also produced ROS[5]. Further, in human prostate cancer cells, it was reported that G2/M phase arrest was induced by ROS in the treatment of diallyl tri-sulfide[13] and Wentilactone B[14]. In addition, the cell cycle of G2/M phase arrest was also observed with assembly of mitotic spindle arrangement[15]. The results obtained here clearly show the increased potential of meghamite nanoparticle against cancer cells and this deserves further analysis.

When nanoparticles are exposed to cell line, it modulates inflammatory response, so the study of

cytokines on response to nanoparticle could help to understand it further. It has been reported that cytokine IFN-γ promoted apoptosis in ovarian cancer cells *in vivo* and also in *in vitro*[16]. Further, the cell cycle regulator gene cyclin D3 and antiapoptotic regulators Bcl-2 were upregulated by IL-12 along with down regulation of apoptotic cascade protein caspase-3[17]. In the present study, IL-12 transcriptional expression was decreased in maghemite nanoparticle-exposed Hep G2 cells, thus it pave the way of transcriptional induction of of pro-inflammatory cytokine IFN-γ and might induce other apoptotic signal.

Conclusion

Maghemite nanoparticles induce a cytotoxic effect by down regulation of cytokine IL-12 at the transcriptional level and ultimately decrease the cell proliferation. The maghemite nanoparticles influence the arrest cell cycle at G2/M phase transition. Further, our results have shown that DNA damage by maghemite nanoparticle reduces the expression of IL-12, with G2/M phase arrest and IFN-γ mediated apoptosis. Thus the application of maghemite nanoparticles induces the apoptosis in hepatocellular carcinoma Hep G2 cancer cells.

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