CHAPTER 3

IN VITRO ANTICANCER ACTIVITY OF HYDROXYAPATITE AND TITANIUM DIOXIDE NANOPARTICLES IN HUMAN HEPATOMA CELLS

3.1 INTRODUCTION

Nanomaterials are important materials due to their novel characteristics, and unique physical and chemical properties. Certain identified nanoparticles have anticancer activity through the accumulation of the nanoparticles in the cell from the enhanced permeability and retention effect (Sheng et al 2005, Lee et al 2010). Due to these changes the nanoparticles achieve new biological properties (Kasemets et al 2009). Hepatocellular carcinoma (HCC), the most common primary malignant tumor of the liver, is a serious threat to human life. Progress in diagnosis and chemical and radiation therapies are poor. Tumor cells are capable of rapid proliferation, local invasion and distance migration. It is almost impossible to excise malignant tumor completely.

Treatment of patients with HCC remains a clinical challenge due to the disappointing effects of most chemotherapies. The efficacy of chemotherapy is limited and patients have to suffer from serious side effects, some of which are life-threatening. Therefore, focus is now towards controlled and targeted drug delivery systems. Nanoparticles can provide a controlled and targeted way to deliver the encapsulated anticancer drugs and thus result in high efficacy with low side effects (Lifeng et al 2007). Hence
we need to find new materials for treatment and cure. Nanoparticles and molecules are a potential alternative for treatment of disease because of their unique biological effect. Zinc oxide nanoparticles have anticancer activity against human myeloblastic leukemia cells (Premanathan et al 2011). The anticancer activity of chitosan is well studied (Lifeng et al 2007). Nanocrystalline fullerenes produce anticancer activity against human glioma cell line (Harhaji et al 2007). Nickel oxide nanoparticles are found to have anticancer activity against human lung carcinoma cells (Horie et al 2009). Genetic material is delivered into the cell by gene delivery to modify the cell (Hosseim 2006). DNA nanoparticles were used for the delivery of genetic materials (Hossein and Tabata 2011). Thus, due to their unique structural character and properties, inorganic nanoparticles are used in medical field. The effect of inorganic nanoparticle’s morphology, size and ions substitution on the cancer cells is very important. The cellular uptake of the nanoparticles and their anticancer activity depends upon the particle size. The anticancer activity of the nanoparticles varies with the size and surface area of the nanoparticles (Liu et al 2003, Yin et al 2006, Li et al 2008).

HAp nanoparticles can be used as anti-cancer drug carriers, and they have a better therapeutic effect than anti-cancer drugs alone. Also, by themselves they have an inhibitory effects on a variety of cancer cells (Yin et al 2010). Pathi et al (2011) reported that the nanoscale properties of HAp play a key role in regulating breast cancer cell behavior. They have been shown to inhibit the proliferation of human osteoblastlike cells (MG-63) and various tumors, such as hepatoma, colon cancer, gastric cancer, and osteosarcoma (Fu et al 2005, Liu et al 2003, Hu et al 2005, Li et al 2008, Shi et al 2009, Yuan et al 2010). Moreover, the anti-proliferation effect of nano HAp has been shown to be the result of the induction of apoptosis.
The photocatalytic properties of TiO$_2$ have led to extensive research into its potential uses as a disinfectant, antibiotic, biological sensor, tumor cell–killing agent, and gene targeting device. Bulk forms of TiO$_2$ are generally biologically and chemically inert. However, the surface of TiO$_2$ nanoparticles smaller than 20 nm is reactive because of surface defects and such TiO$_2$ nanoparticles readily bind enediol bidentate ligands including dopamine, ascorbic acid, alizarin, and Alizarin Red S (ARS) (Thurn et al 2011). The endocytosis of nanoparticles depends on the size, shape, and charge of the nanoparticles, as well as the cell type being treated. The unique character of nanoparticles due to their small size and quantum size effect could make HAp and TiO$_2$ nanoparticles to exhibit biocompatibility.

This chapter deals with the influence of the particle size of the HAp and TiO$_2$ nanoparticles on cell viability, cell morphology, DNA fragmentation and cell cycle analysis of HCC cells in vitro. This cell line was selected since hepatoma is one of the most common tumors found worldwide. Furthermore, in the primary malignancy of the liver this cell line has been widely used as the human hepatoma model cell line in the development of new anti-tumor medicines. The aim of the study is to use in vitro studies to find the size at which HAp and TiO$_2$ nanoparticles have higher anticancer activity. This further helps to find the appropriate particle size for in vivo studies.

3.2 EXPERIMENT
3.2.1 Cell Line and Culture

Human HCC cells HepG2 (GDC055) were obtained from National Centre for Cell Sciences, Pune (NCCS), India. The cells were maintained in RPMI-1640 supplemented with 10% Fetal Bovin Serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 50 μg/ml CO$_2$ at 37°C.
3.2.2 In Vitro Assay For Cytotoxicity Activity (MTT Assay)

The cytotoxicity of samples on HepG2 cells was determined by the MTT assay (Mosmann et al 1983). Cells (1 × 10^5/well) were plated in 100 μl of medium/well in 24-well plates (Hi media chemicals Ltd, Mumbai, India). After 48 hours of incubation, the cell reached the confluence. Then cells were incubated in the presence of various concentrations of the samples in 0.1% dimethyl sulfoxide (DMSO) for 48 h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200 μl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide cells (MTT) phosphate- buffered saline solution was added. After 4 hours of incubation, 0.04 M HCl/isopropanol were added. Viable cells were determined by the absorbance at 570 nm with reference at 655 nm. Measurements were performed, and the concentration required for 50% inhibition of viability (IC_{50}) was determined graphically. The absorbance at 570 nm was measured with a UV spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of Human Liver cancer cells (HepG2) was expressed as the % cell viability, using the following formula:

\[
\text{% cell viability} = \left( \frac{A_{570 \text{ of treated cells}}}{A_{570 \text{ of control cells}}} \right) \times 100 \% \ (3.1)
\]

3.2.3 Statistical Analysis

Statistical analysis was performed on Software Statistica 6.0. Significant difference between two groups was evaluated by one way analysis of variance (ANOVA) and the level of significance was set as P < 0.05 or P < 0.01 (Zar 1996).
3.2.4 DNA Fragmentation

After treatment with 10 nm (49.02 µg.ml⁻¹), 14 nm (37.58 µg.ml⁻¹), 60 nm HAp nanoparticles (NPs) (123.45 µg.mL⁻¹) of HAp NPs and 6 nm TiO₂ NPs (26.77 µg.ml⁻¹), 12 nm TiO₂ NPs (61.25 µg.ml⁻¹) for 48 hours, human hepatoma cells (HepG2) were collected, washed with PBS, and lysed with a solution containing 10 mmol.l⁻¹ Tris-HCl pH 7.4, 10 mmol.l⁻¹ EDTA and 0.5% Triton X-100. The lysates were incubated with 200 mg.ml⁻¹ RNase A (Sigma Aldrich Co, USA) for 1 hour followed by 200 mg.ml⁻¹ proteinase K (GIBCO) also for 1 hour at 37°C. These samples were then extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) followed by chloroform. DNA was precipitated in two volumes of ethanol in the presence of 0.3 mol.L⁻¹ sodium acetate. The DNA samples thus obtained were run on 1.5% agarose gel at 50 V and visualized by ethidium bromide staining under UV light (Qi et al 2005).

3.2.5 Flow Cytometry Analysis

HepG2 cells (2 x10⁵ cells/well) were cultured in 60-mm Petri dishes and incubated for 48 hours. The cells were then harvested, washed with phosphate buffered saline (PBS), then resuspended in 200 µl of PBS, and fixed in 800µl of ice-cold 100% ethanol at 4°C. After being left to stand overnight, the pellets of cell were collected by centrifugation, resuspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5l g/ml RNase), and incubated at 37°C for 30 min. Next, 1 ml of propidium iodide solution (50 µg/ml) was added, and the mixture was kept in ice for 30 min. Fluorescence emitted from the propidium iodide–DNA complex was evaluated after excitation of the fluorescent dye by FACScan cytometry.
3.3 RESULTS AND DISCUSSIONS

3.3.1 Cell Viability Analysis of Hydroxyapatite Nanoparticles (MTT Assay)

In this study, the exponentially grown human HCC cells HepG2 were treated with various concentrations of HAp nanoparticles ranging from 1.953 to 1000 µg.ml\(^{-1}\), and the cell viability was measured by the MTT assay. The significant inhibition (P < 0.05; P < 0.01; P < 0.001) of cell viability by nanoparticles was clearly observed in a dose dependent manner.

HAp showed size-dependent cytotoxicity against HepG2 cells. The cell viability for HAp nanoparticles of different sizes are given in Figure 3.1.

![Figure 3.1: Cytotoxicity effect of HAp nanoparticles of different particle size at different Concentration Vs control](image)

**Figure 3.1** Cytotoxicity effect of HAp nanoparticles of different particle size at different Concentration Vs control
The cell viability decreases with the increase in the concentration of the nanoparticles. As the concentration of HAp nanoparticles is increased from 1.953 to 1000 µg.ml\(^{-1}\), the cell viability decreased. This is observed in HAp nanoparticles of different sizes. The variation in IC\(_{50}\) values as a function of the size of nanoparticles is given in Figure 3.2. The IC\(_{50}\) value for HAp nanoparticles of size 10 nm is 49.02 µg.ml\(^{-1}\), 14 nm is 37.58 µg.ml\(^{-1}\), 24 nm is 61.62 µg.ml\(^{-1}\), 31 nm is 95.24 µg.ml\(^{-1}\) and for 60 nm is 123.45 µg.ml\(^{-1}\). When the particle size increases, the IC\(_{50}\) value also increases. But the IC\(_{50}\) value for HAp nanoparticles of size 14 nm is less than that of the 10 nm size HAp nanoparticles. The cell viability of HepG2 cells significantly decreased after exposure to HAp nanoparticles of sizes 10, 24, 31 and 60 (P < 0.01) and (P < 0.001) for 14 nm size particle after 48 h. In particular, when the HepG2 cells were treated by HAp nanoparticles of size 14 nm, the loss of cell viability attained is as high as 62.42%. HAp nanoparticles of size 60 nm exerted a weak inhibitory activity in HepG2 cells. This clearly shows that the anticancer activity of HAp nanoparticles against HepG2 cells depend significantly on the particle size.

![Figure 3.2 Dependence of the cell viability on HAp nanoparticle size](image-url)
The impact of particle size on cellular uptake and ensuing functional impact is well known. The HAp nanoparticles used in this study ranges between 10 to 60 nm. They are synthesised by wet chemical method and by modulating the calcination temperature in the range of 773 to 1073 K. Results obtained showed that NPs induced significant growth inhibition and the strength of the anti-proliferative effect was in the order of 14 nm > 10 nm > 24 nm > 31 nm > 60 nm for HAp NPs. Nanoparticles interact with the biomolecules on the surface or within the cells (Jiang et al 2008). So cellular trafficking plays an important role in the size dependence of HAp nanoparticles. Particle size also plays an important role in determining the nuclear penetration of HAp nanoparticles. Due to the passive mechanism, nanoparticles of different size have different biodistribution.

The cellular uptake of the nanoparticle is a two step process. First, the uptake of the nanoparticles from surface of the cell to cytoplasm and then cellular trafficking of the nanoparticle. Nanoparticles of size ~ 50 nm are more readily taken up by the cells than the particles of other sizes by endocytotic mechanism (Bansal and Chaudhary 2005). Particles smaller than 50 nm tend to form clusters and aggregate during uptake. This is due to the higher surface curvature of smaller nanoparticles which restricts the binding and the mutual absorption between the nanoparticles and cells. For nanoparticles larger than 50 nm, the slower receptor diffusion rate is the reason for fewer nanoparticles taken up by the cells (Chithrani and Chan 2007). In this study it is found that compared to other sizes, more HAp nanoparticles of size 14 nm had been taken up by the cells.

After absorption into the cancer cell, a part of the HAp nanoparticles is digested. HAp particles dissolving would release calcium ions into the cytoplasm, which disturb the intracellular calcium homeostasis. Homeostasis of intracellular calcium is crucial because of its major role as a
secondary messenger, regulating many vital cellular functions, cell proliferation, and apoptosis. Calcium is regulated to <200 nM in the cytosol and to 400-600 mM within lysosomes. Even relatively minor changes in intracellular calcium concentration can cause profound effects on cellular metabolism. Increase of \( \text{Ca}^{2+} \) and phosphate ions within lysosomes and endosomes will cause their pH to rise. The compartment will supersaturate with respect to \( \text{Ca}^{2+} \) and further dissolution of HAp will consequently be inhibited. This may be the cause of the relatively slow dissolution of HAp. Due to high endosomal or lysosomal calcium ions, HAp NPs escapes the phagocytic pathway and enters the nucleus to transfect cells. The particle escaping the phagocytic pathways and their enhanced transport through nuclear pores are up regulated by an increase in calcium ions.

The intracellular \( \text{Ca}^{2+} \) concentration increases the rate of programmed cell death. \( \text{Ca}^{2+} \) activates calcium-dependent endonucleases that cleave the DNA. \( \text{Ca}^{2+} \) activated proteins which induce apoptosis can be activated by high intracellular \( \text{Ca}^{2+} \) concentration. Microtubules usually functions more during conditions of high intracellular calcium concentration in an ATP-dependent manner, which leads to depletion of ATP and is a well-known signal of apoptosis. In addition, the vacuoles containing HAp nanoparticles in cytoplasm are the scene of cytoskeleton disruption. In other words, the cytoskeleton around nanoparticles is disrupted, and it is assumed that the presence of the calcium concentration activates the calpain, a kind of \( \text{Ca}^{2+} \) dependent protein kinases, which can induce cytoskeleton disruption. Due to high \( \text{Ca}^{2+} \) ions, the HAp nanoparticle has a high surface energy, which can activate some molecules and form many free radicals. ROS also can damage actin filament, resulting in cytoskeleton disruption, and ultimately disrupting the integrity of cytoskeleton (Meena et al 2012).
3.3.2 Cell Viability Analysis of Titanium Dioxide Nanoparticles (MTT Assay)

Cell proliferation in response to different concentrations of TiO$_2$ nanoparticles present in the culture media was evaluated by the MTT assay. There were slight changes in the cell viability at lower concentrations of TiO$_2$ nanoparticles, and as the concentration increased, cell viability decreased drastically. In comparison with the control group, cells cultured in medium containing TiO$_2$ nanoparticles for 48 hours showed a decrease in viability. The variation in cell viability with different concentration of TiO$_2$ is given in Figure 3.3. TiO$_2$ also showed size-dependent cytotoxicity against HepG2 cells.

![Cytotoxicity effect of TiO$_2$ nanoparticles of different particle size at different Concentration Vs control](image)

**Figure 3.3** Cytotoxicity effect of TiO$_2$ nanoparticles of different particle size at different Concentration Vs control

The IC$_{50}$ for TiO$_2$ nanoparticles of different sizes is given in Figure 3.4. The cell viability significantly decreased after exposure to TiO$_2$ nanoparticles of sizes 6 nm (P < 0.001) and 8, 9, 10 and 12 nm (P < 0.01) size
particle after 48 hours. The IC_{50} value for TiO_2 particle of 6 nm is 26.77 µg.ml^{-1} and it is 31.48 µg.ml^{-1}, 49.88 µg.ml^{-1}, 60.23 µg.ml^{-1} and 61.25 µg.ml^{-1} for 8 nm, 9 nm, 10 nm and 12 nm respectively. The cell viability is very low for 6 nm size particle and it increases with an increase in particle size. At 6 nm the loss of cell viability is as high as 68.53%.

![Figure 3.4 Dependence of the cell viability on TiO_2 nanoparticle size](image)

TiO_2 particle-mediated anticancer activity is due to particle-cell interactions. It is due to the surface properties of the TiO_2 particles. TiO_2 particles have a net negative charge (at pH = 7) and also bind preferentially to amino acids containing -OH, -NH, and -NH_2 in their side chains. These observations indicate that TiO_2 particles may react with cell membrane proteins and contribute to cell particle interaction (Tran 2006).

The cytotoxicity of TiO_2 is due mainly to protein adsorption. The adsorption of the components of the culture media onto the metal oxide nanoparticles induces a starvation state and subsequent enervation of cells in
in-vitro. The coating of the metal oxide nanoparticles with proteins may change their biological activities. Protein adsorption to metal oxide depends on the surface charge of the protein and the particle. Thus, the isoelectric point of the metal oxide is important for protein adsorption (Horie et al 2009).

The experiments with MTT assay after 48 hours showed significantly lower cell proliferation compared to the control as shown in Figure 3.5. In 14 nm HAp nanoparticles the value is less than that of those treated with 10 nm HAp NPs. The maximum reduction in cell proliferation was observed at 6 nm. Taken together, these results demonstrate that maximum intake of NPs was observed in 14 nm HAp and 6 nm TiO$_2$ NPs.

![Figure 3.5 Observations of HepG2 cell morphological changes after treated with nanoparticles for 48 hours. The cells were observed by optic microscope directly](image)
3.3.3 DNA Fragmentation

The DNA damaging effect of HAp and TiO$_2$ nanoparticles in human hepatoma cells (HepG2) were evaluated using DNA fragmentation studies (Figure 3.6). The concentrations of NPs used in this study were based on the results of IC$_{50}$ values obtained from cytotoxicity assay. Control DNA, 10 nm HAp NPs, and 14 nm HAp NPs, 60 nm HAp NPs, 6 nm TiO$_2$, and 12 nm TiO$_2$ NPs were examined for DNA fragmentation. Control, 10nm HAp NPs and 60 nm HAp NPs showed a presence of undamaged genomic DNA represented by a thick band on the agarose gel. The highest extent of DNA damage was observed for 14 nm HAp-NPs, 6 nm TiO$_2$ NPs and 12 nm TiO$_2$ NPs. At 6 nm of TiO$_2$ and 14 nm of HAp-NPs, large number of fragments, less than 1 kb was observed when compared to control group.

![Figure 3.6 DNA fragmentation induced by HAp and TiO$_2$ NPs in human hepatoma cells (HepG2). Lane C – Control, M – Marker L1 – 10 nm HAp NPs (49.02 µg.ml$^{-1}$) L2 – 14 nm HAp NPs (37.58 µg.ml$^{-1}$) L3 – 60 nm HAp NPs (123.45 µg.ml$^{-1}$) L4 – 6 nm TiO$_2$ NPs (26.77 µg.ml$^{-1}$) L5 – 12 nm TiO$_2$ NPs (61.25 µg.ml$^{-1}$)](image-url)
The nanoparticles trigger the changes of expression of the apoptotic proteins, and thereby induce the apoptosis and cytotoxicity only within the cell nucleus of the HepG2 cells. The 14nm HAp NPs, with the most efficient cellular uptake and nuclear trafficking led to the greatest changes of the apoptotic proteins, the strongest anti-tumor activity, and apoptosis in HepG2 cells. For 60nm HAp NPs, although taken up by HepG2 cells, they cannot penetrate into cellular nucleus. Thus, the weakest cytotoxicity and apoptosis induction were observed. During cellular trafficking of the nanoparticles, the nanoparticles need to cross the double nuclear membrane, which separates the nucleoplasm from the rest of the cell. This nuclear membrane contains specialized channels called nuclear pore complexes (NPCs), through which nuclear import and export processes occur. The nanoparticles should be smaller than the NPCs to enter the nucleus. The sizes of NPCs vary from 20 to 50 nm, and vary with cell type (Wente 2000).

HAp nanoparticles are not taken up by HCC cells by simple diffusion, or by membrane protein transportation, or by pinocytosis because they are not micromolecules or materials with <10 nm diameter, or dissolved matter. HCC cells are not specialized cells like neutrophils or macrophages practicing phagocytosis. Phagocytosis is not the mechanism used by the HCC cells to taken in HAp nanoparticles. That the HAp nanoparticle was taken up into the HCC cells by the caveolae is well established by Yin et al (2010). The size of the caveolae plays an important role in the uptake of the substance. The results of present study suggest TiO$_2$ nanoparticles generate a large amount of hydroxyl free radicals thereby leading to DNA damage (Reeves et al 2007). The HAp particles, only within the cell nucleus of the HepG2 cells, can trigger the changes of expression of the apoptotic proteins, and thereby induce the apoptosis and cytotoxicity. Thus the cytotoxic and genotoxic
mechanism of HAp and TiO$_2$ NPs is related to their membrane penetration and apoptosis activity.

Several studies have indicated that the phenomenon of cellular uptake is of crucial importance in governing a range of cellular behaviours including cell growth, apoptosis, adhesion, migration, differentiation, survival and tissue organization (Absolom et al 1987). Current studies in this area have demonstrated that physicochemical properties of NPs, including particle size, chemical composition and surface chemistry, can affect NPs nonspecific uptake ability. NP size and concentration-dependent effects are important. Metal oxide nanoparticles spontaneously produce ROS based on material composition and surface characteristics. But other nanomaterials produce ROS only in the presence of selected cell systems. When the production of ROS is higher and the cellular antioxidant defense system is not capable of controlling them, oxidative stress occurs. This results in the damage of cellular components like lipids, proteins, and DNA (Lovric et al 2005, Xia et al 2006, Long et al 2006).

Lipid peroxide, produced when fatty acid is oxidized, initiates a chain reaction resulting in the disruption of plasma and organelle membranes and subsequent cell death. HAp and TiO$_2$ start the apoptosis process with the fragmentation of DNA. Apoptosis is the programmed death of cells by the fragmentation of DNA, and cell shrinkage, followed by more cell fragmentation and the formation of membrane vesicles called apoptosis bodies. Dysregulation of apoptosis is linked to the development of most cancers. Thus, an induction of apoptosis in cancer cells is considered as a new focus in the discovery of anticancer drugs. DNA cleavage at the internucleosomal linker sites yielding DNA fragments is regarded as a biochemical hallmark of apoptosis. The primary mechanism of these
nanoparticles and anticancer activity might proceed by inducing the
generation of ROS, which then are responsible for the induction of apoptosis.

3.3.4 Flow Cytometry Analysis of Hydroxyapatite Nanoparticles

Apoptosis is programmed cell death which is a normal physiologic process that occurs during embryonic development and in the on-going process of tissue homeostasis in the adult animals. Any dysregulation of apoptosis can result in abnormality, disease and death (Wilson 1998). Cancer is a result of the dysregulation of apoptosis as well as uncontrolled cell proliferation (Jia et al 1999). Recently, inducers of apoptosis have been used in cancer therapy. Activation of apoptosis pathways is a key mechanism by which cytotoxic drugs kill tumor cells. It is now considered an important method of assessment for the clinical effectiveness of many anti-tumor drugs.

Cell cycle arrest analysis for HAp nanoparticles is given in Figure 3.7. When HepG2 cells were treated with various concentrations of HAp for 48 hours, G2/M phase arrest was observed in a concentration dependent manner. When the concentration of HAp nanoparticles added was increased from 15.625 µg/ml to 31.25 µg/ml an increase in the percentage of cells in the G2/M phase from 25.67% to 45.17% respectively is observed compared to that of the control (14.56%).
HAp-inhibited HepG2 cells grow by arresting the cell cycle at the G2/M phase and inducing apoptosis. Cyclin A, cyclin B1, Cdc2, Cdc25C and p21 proteins have been reported to influence G2/M transition in the cell cycle (Molinari 2000, Coqueret 2003). CDKs play an important role in cell cycle progression and have been considered as drug targets (Molinari 2000). Among these CDKs, Cdc2 (CDK1) activation requires phosphorylation on Thr-161 and dephosphorylation on Thr-14 and Tyr-15 (Millar et al 1997,
Activated Cdc2 interacts with cyclin A and/or cyclin B1 to form an active heterodimer to participate in the progression from G2 to M phase. Cdc25C is required for entry into mitosis and is believed to be the major phosphatase that dephosphorylates Thr-14 and Tyr-15 of Cdc2 (Millar et al 1997). Therefore, G2/M phase arrest by PMF might be due to the decrease of Cdc2 complex activity, which may be mediated through downregulation of the Cdc25C protein. This downregulation may inhibit the dephosphorylation of Cdc2 at Thr-14 and Tyr-15 as well as the cyclin A expression, to decrease the formation of cyclin A/Cdc2 complex. p21 has been found to contribute to the arrest cell cycle at G2 phase by blocking the activating phosphorylation of Cdc2 on Thr-161 (Smits et al 2000). Upregulation of the p21 protein may be involved in the PMF-mediated G2/M arrest in the HepG2 cells by blocking the phosphorylation of Cdc2 at Thr161.

3.3.5 Flow Cytometry Analysis of Titanium Dioxide Nanoparticles

Cell cycle arrest analysis for TiO$_2$ nanoparticles is given in Figure 3.8. When HepG2 cells were treated with various concentrations of TiO$_2$ for 48 hours, a significant increase in sub-G1 population was observed. The percentage of cells increases from 31.29% to 59.11% for 31.25 µg/ml and 62.50 µg/ml respectively compared to that of the control (12.45%).
Figure 3.8  Fluorescence activated cell sorting (FACS) analysis of HepG2 cells with TiO$_2$ after 48 h

TiO$_2$ treated cells have shown sub G1 phase arrest. Cells undergoing apoptosis were found to have an elevation of cytochrome c in the cytosol,
with a corresponding decrease in the mitochondria (Yang et al 1997). After the release of mitochondrial cytochrome c, the cysteine protease 32 kDa proenzyme CPP32, a caspase 3, is activated by proteolytic cleavage into an active heterodimer (Nicholson et al 1995). Activated caspase 3 is responsible for the proteolytic degradation of poly (ADP-ribose) polymerase, which occurs at the onset of apoptosis (Laizebnik et al 1994, Tewari et al 1995). Inter-nucleosomal DNA fragmentation is not essential for apoptotic cell death. Some necrotic cell death is accompanied by inter nucleosomal DNA fragmentation, suggesting the possibility that this fragmentation may not be sufficient as an indicator of apoptotic cell death (Cohen et al. 1992, Schulze-Osthoff 1994). The central mechanism of apoptosis is evolutionarily conserved, and that caspase activation is an essential step in this complex apoptotic pathway (Thornberry and Lazebnik 1994). The Bcl-2 family plays an important regulatory role in apoptosis, either as activator (Bax) or as inhibitor (Bcl-2) (Rao and White 1997, Green and Reed 1998, Adams and Cory 1998). It has also been demonstrated that the gene products of Bcl-2 and Bax play important roles in apoptotic cell death (OlTVai et al 1993, Jacobson and Raff 1994, Jacobson et al 1994). Of the Bcl-2 family members, the Bcl-2 and Bax protein ratio has been recognized as a key factor in regulation of the apoptotic process (Rao and White 1997, Green and Reed 1998, Adams and Cory 1998).

TiO$_2$ may induce apoptosis and sub -G1 arrest by inhibiting the protein expression of anti-apoptosis proteins, survivin and Bcl-xL. It may disturb the Bcl-2/Bax ratio and, therefore, lead to apoptosis. Many of the agents that induce apoptosis are oxidants or stimulators of cellular oxidative metabolism, while many inhibitors of apoptosis show antioxidant activity (Buttke and Sandstrom 1994). Indeed, factors for oxidative stress, such as ROS production (Coyle and Puttfarcken 1993, Loo et al. 1993, Albrecht et al 1994, Garcia-Ruiz et al 1997, Krumen et al 1997), lipid peroxidation
(Hockenbery et al 1993), downregulation of the antioxidant defenses characterized by reduced glutathione levels (Marchetti et al 1996), and reduced transcription of superoxide dismutase, catalase, and thioredoxin, have been observed in some apoptotic processes (Briehl and Baker 1996). Moreover, ROS can also play an important role in apoptosis by regulating the activity of certain enzymes involved in the cell death pathway (Coyle and Puttfarcken 1993, Loo et al 1993, Albrecht et al 1994, Garcia-Ruiz et al 1997, Kruma et al 1997). All these factors point to a significant role for intracellular oxidative metabolites in the regulation of apoptosis. ROS production was probably the cause of this apoptic cell death.

3.4 CONCLUSIONS

In this chapter, the influence of particle size of HAp and TiO$_2$ on HCC cells in vitro was investigated. The size of nanoparticles had obvious effect on the anti-proliferation activity. HAp and TiO$_2$ particles have the potential to treat cancer. In HAp, 14 nm size HAp nanoparticle have higher activity. In TiO$_2$, 6 nm size particles have high activity. The cytotoxic and genotoxic mechanism of HAp and TiO$_2$ NPs is related to their membrane penetration and apoptosis activity. An induction of apoptosis in cancer cells is considered as a new focus in the discovery of anti-cancer drugs. Cell cycle arrest analysis for HAp and TiO$_2$ nano particles revealed the influence of HAp and TiO$_2$ nanoparticles on the apoptosis of HepG2 cells. HAp nanoparticles of size 14 nm and TiO$_2$ nanoparticles of size 12 nm will be further processed with surface modification for targeted drug delivery. HAp nanoparticles with higher anticancer activity were selected for targeted drug delivery. But in TiO$_2$, 12 nm size particles were taken for surface-modification, because particles less than 10 nm in size will have high renal clearance. Hence, to avoid high renal clearance, particles of size 12 nm were selected for surface modification.