

HYDROXYAPATITE (HA) ATTENUATE TiO₂ TOXICITY IN BIO-SYSTEM TRIGGERING *E. COLI* AND MOUSE BONE MARROW MONO-NUCLEAR CELLS (BMMNC'S)

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ABSTRACT

The purpose of this in-vivo study was aimed to first prepare and characterize Titanium dioxide (TiO₂) and Hydroxyapatite (HA) nanoparticles (NPs). Further analyzed the toxicological effect of TiO₂ NPs on Bacteria *E. coli*, and furthermore determined the effect of TiO₂ NPs, HA NPs and their applicable mingling ratio to Mouse bone marrow mononuclear cells (BMMNCs). Chemical precipitation method was used for NPs synthesis, TiO₂ and HA both nano particles were characterized by X-ray diffraction (XRD) technique. Surface study of both NPs were done using Scanning Electron Microscope (SEM). Bacteria *E. coli* toxicity was determined by NPs incubation and optical density (O.D.) measurement. The methodology for BMMNCs toxicity determination for nano particles was first separate incubation of BMMNCs with TiO₂ and HA in concentration range of 0.4 - 2mg/ml and then combined incubation of BMMNCs with TiO₂ and HA in ratio 1:1 with concentration 0.4mg/ml. TiO₂ and HA nano particles synthesized and characterized along with SEM surface study, TiO₂ NPs were toxic for *E. coli* growth. The BMMNCs analysis revealed the viability of BMMNCs were very less in medium containing HA or TiO₂ while it were maximum in medium containing blend of HA and TiO₂. TiO₂ has antibacterial property also it is toxic for BMMNCs survival but HA attenuate TiO₂ cellular toxicity when applied in suitable mingling ratio of HA and TiO₂ NPs. Future studies are necessary to analyze the potential application of HA and TiO₂ NPs blend in cancer treatment and bone regeneration which may be useful in both cancer growth prevention and restoration of bone at the interface of implant and tissue.

Key Words: *Bone Marrow Mononuclear Cells (BMMNCs), Hydroxyapatite (HA), Titanium dioxide (TiO₂), Nanoparticles (NPs).*

INTRODUCTION

Nano materials and Nano particles have been widely used in the field of cosmetics, biomedicine, food technology and modern chemistry. Their wide applications are attributed to their numerous properties such as their size, surface structure, shape and composition. The advantage of nanoparticles over outmoded diagnostic and therapeutic agent is due to their unique property of

high surface area to volume ratio^[1]. Previous research analysis demonstrate TiO₂ and HA NPs have used as a potential material in discovering the therapy and treatment of human disorders. Further, Numerous application of TiO₂ NPs have been reported such as anticancer drug efficacy enhancer, drug delivery^[2], bone implant material^[3] and antibacterial materials^[4]. Since, previous research demonstrated that the acute health concern and challenges of using TiO₂ nanoparticle as a

therapeutic agent therefore, TiO₂ NPs safety have high interest in the scientific community due to its high potency as a drug carrier in cancer therapy^[5]. Although there are a number of published study on the toxicity of TiO₂, however, there are no details on dose and time gradient toxicity. In this study growth curve analysis of TiO₂ treated Bacteria (*E. coli*) was done and Mouse Bone marrow cells toxicity were analyzed after treatment with various concentration of (a) Hydroxyapatite (HA) (b) Titanium dioxide (TiO₂) and (c) Blend of TiO₂ and HA respectively. Hydroxyapatite (HA) is the chief inorganic mineral constituent of natural teeth and bones. HA displays excellent bioactivity, biocompatibility, affinity and osteo conductivity. Orthopedic implants and dental treatments is widely based on the use of HA, due to having highly active surface and used as a drug delivery

MATERIALS AND METHOD

TiO₂-nanoparticle Preparation

NH₄OH, TiCl₄, and distilled water were used in the preparation of TiO₂-nanoparticles. TiCl₄ was dispensed to distilled water of temperature 2–3 °C and stored in similar conditions for 12 hours. NH₄OH solution was added to the stored solution thereafter and stirred well until the precipitate of ammonium chloride and Ti(OH)₂ appeared, moreover, the pH of a reaction mixture was kept constant at 8. The removal of chloride ions present in the precipitate was done by washing twice, first with normal distilled water and then using hot distilled water. The Furnace maintained at temperature 200°C was used to dry the washed precipitate for 24 hrs to remove Hydroxyl ion. The obtained solid after drying contain only TiO₂. XRD was used for characterization (size determination and phase conformation) of obtained TiO₂ precipitate.

HA-nanoparticle Preparation

The wet chemical method was used for the preparation of Hydroxyapatite nano particles. The materials were (NH₄)₂HPO₄, CaCl₂·2H₂O, and NH₄OH. The calcium chloride dehydrate solution of concentration 1.0 M slowly added at 70°C to form the HA precipitate. The pH was monitored and adjusted to 11 by an addition of NH₄OH to the medium. While maintaining the temperature at 70°C, the mixture was thoroughly stirred for 8 hrs, then dried for one day at room temperature. Finally isopropyl alcohol and water were used to wash the precipitate. XRD was used for characterization

system. Multiplication of cancerous cells can also retarded by HA nano particles^[6]. HA nano particles can prove as an important therapeutic material in cancer treatment and orthopedic implants. Here, we demonstrated the role of HA NPs in attenuation of TiO₂ NPs toxicity. We have performed this experiment on Bone marrow cells because Bone marrow mononuclear cells is the source of adult stem cells, and these adult stem cells have the potential to trans differentiate into the lineage of their choice and used in no. of cell-based therapy. TiO₂ nano particles used in the therapy of cancer and as a carrier of targeted drug while HA is worthwhile substance in bone implants and other orthopedics applications. Our purpose of undertaking this study was to discover the separate and the combined effect of TiO₂ and HA nano particle on mouse BMMNCs.

(Size determination and phase conformation) of obtained HA^[7].

X-ray diffraction (XRD)

The XRD pattern of synthesized nanoparticles TiO₂ and HA were investigated by X-ray diffraction (XRD), Cu K α radiation ($\lambda = 0.1548$ nm), a monochromatic source was used and the samples were scanned from 0° to 80° at a scanning rate of 0.5°/min using PHILIPS X-ray diffractometer (*BRUKER, Germany*). The protocol for synthesis and characterization has been followed as shown in no^[7].

Scanning Electron Microscopy (SEM)

Surface analysis of TiO₂ and HA nano particles were done with *SEM Hitachi 3700 N* at 15.0 KV accelerating potential of electron beam.

Toxicity on bacterial cells

Growth characteristics of the bacteria *E. coli* was checked using a colorimeter, O.D. was measured at 600 nm after inoculating and incubating along with TiO₂ in concentration of 0, 40, 50, 70, 100 mg in the LB broth for overnight (24 hr) on a shaker^[8].

Bone marrow mononuclear cell isolation and toxicity

The first mice was sacrificed and then cervical dislocation procedure was used to remove Femur and Tibias. Phosphate-buffered saline (PBS) solution, whose pH was maintained at 7.4 utilized with 10 mM ethylene diamine tetra acetic acid (EDTA) solution to obtain Bone marrow by flushing through the amputated ends of the bone.

Density centrifuge Histopaque-1083 (*Sigma-Aldrich, St. Louis, MO, USA*) was used to separate the BMMNCs. Red blood corpuscle (RBC) lysis buffer solution was deployed to remove the erythrocyte contamination. The counting of total no of viable cells were done by using trypan blue dye exclusion assay (*Sigma-Aldrich, St. Louis, MO, USA*). The viable BMMNCs were cultured in Dulbecco's modified Eagle's medium (DMEM) (*Invitrogen*). Cultured cells were treated with TiO₂, HA and blend of TiO₂ and HA respectively which were dissolved in the Dimethyl sulfoxide (DMSO) solvent^[9].

RESULTS

Synthesis and characterization of TiO₂ and HA Nano particles

Synthesis and XRD pattern of HA and TiO₂ nano particles shown in figure 1. The XRD characterization of both nano particles HA and TiO₂ shown in Table 1a. The XRD pattern of synthesized HA nanoparticle compared with JCPDS # 9-432 and JCPDS #21-839, which established the occurrence of hydroxyapatite along with small amount of Calcium Phosphate. JCPDS # 88-175 for rutile and JCPDS # 84-1286 for anatase were employed to compare with XRD pattern of TiO₂, the obtained sharp peak confirmed the polycrystalline structure of TiO₂ nano particle. The size of both nano particles were calculated using Scherer's formula ($d = 0.9\lambda/\beta \cos \theta$, where λ – wavelength of X-rays, β – FWHM of diffraction peak, the peak corresponding to angle θ). the calculated size of HA and TiO₂ were 35 nm and 65 nm respectively

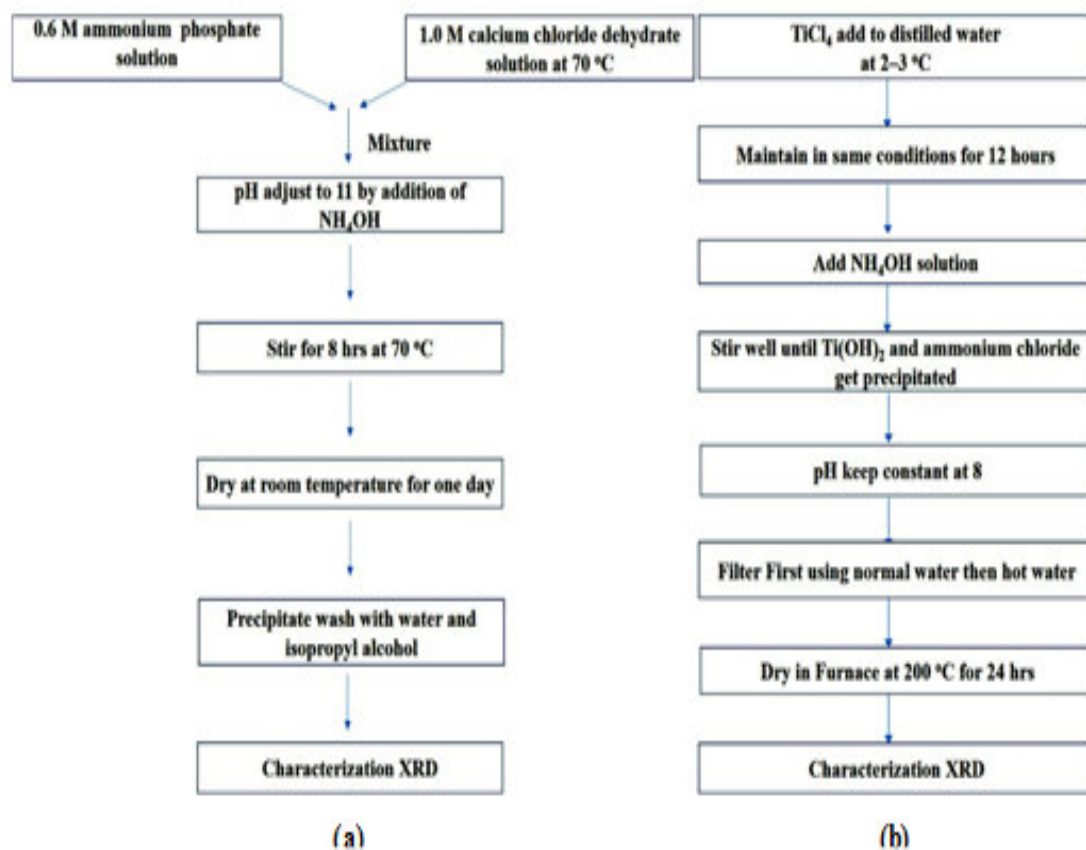
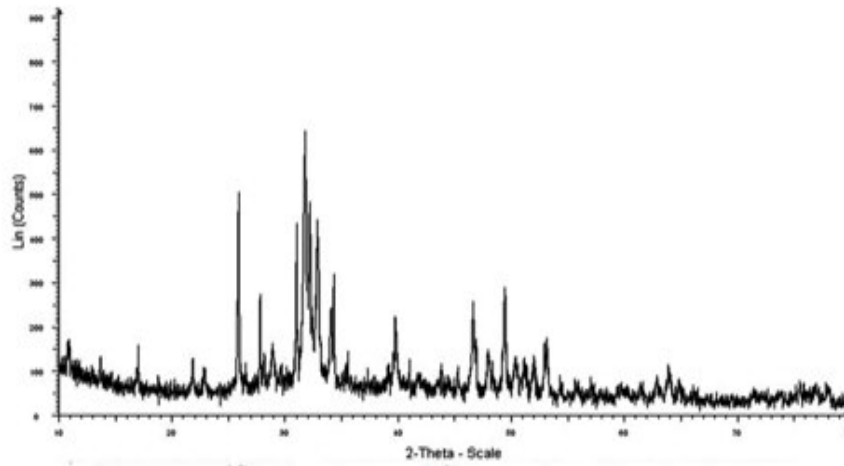
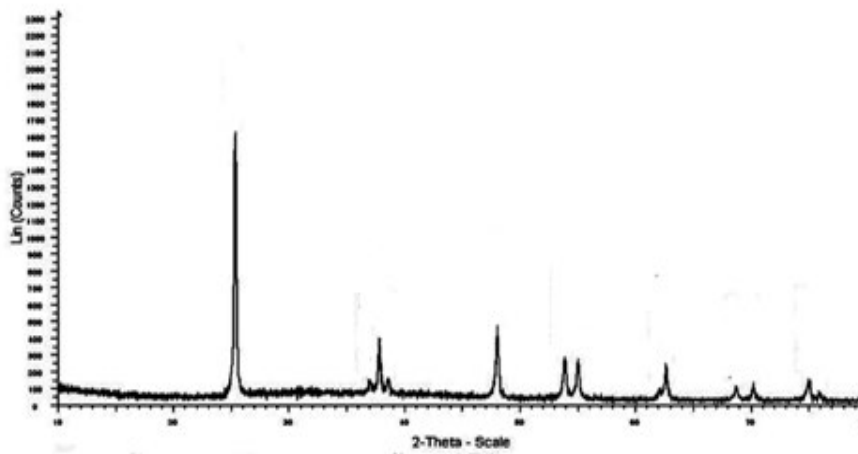


Figure 1
(a) Synthesis of HAN Nanoparticles (b) Synthesis of TiO₂ Nanoparticles



(c)

Figure 1(c)
XRD:HA



(d)

Figure 1 (d)
XRD:TiO₂

Source	PHILIPS X-ray diffractometer (BRUKER, Germany), Monochromatic source (Cu K α radiation ($\lambda = 0.1548$ nm), Angle: 0 $^\circ$ to 80 $^\circ$, Scanning rate: 0.5 $^\circ$ /min	
XRD Analysis	HAP	TiO ₂
	1-XRD pattern of synthesize HAP compare with JCPDS#9-432 (Hydroxyapatite) and JCPDS#21-839 (Calcium Phosphate)	1-XRD pattern of synthesize TiO ₂ compare with JCPDS#88-175 (Rutile) and JCPDS#84-1286 (Anatase)
	2-Size calculation using Scherer's formula ($d = 0.9\lambda/\beta \cos \theta$)	2-Size calculation using Scherer's formula ($d = 0.9\lambda/\beta \cos \theta$)
Result	1-Presence of HAP along with some amount of Calcium Phosphate	1-Sharp peak, Polycrystalline
	2-Size 35 nm	2-Size 65 nm

(a)

TiO ₂ NPs/ TiO ₂ NPs derivatives	Use in Biological system	Reference
TiO ₂ NPs	Antibacterial	4
TiO ₂ NPs codoped Ag and Nitrogen	Antibacterial	13
TiO ₂ NPs-NiFe ₂ O ₄ biomaterial	Antimicrobial	14
Daunorubicin-TiO ₂ NPs nanocomposite	Drug delivery system	16
Polylactic acid-TiO ₂ NPs nanocomposite	Drug releasing	17
TiO ₂ NPs	prevention of Osteosarcoma and Chondrosarcoma	18
Pt-TiO ₂ NPs nanocomposite	Cancer treatment	19
Nitrogen doped TiO ₂ NPs	Photokilling of Cancer cells	20
TiO ₂ -chondroitin-4-sulfate nanocomposite	Bone regeneration	21
Ag-TiO ₂	Antileishmanial	22

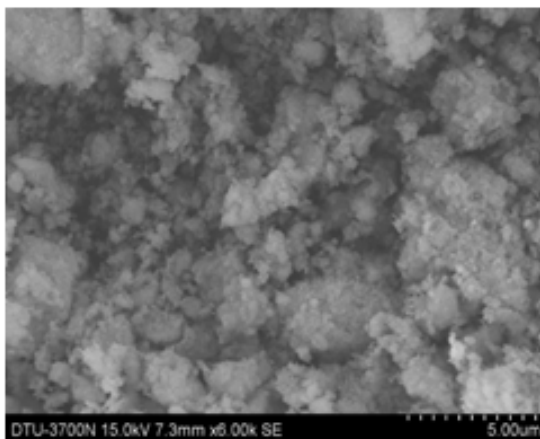
(b)

Table 1(a)XRD-Characterization of HA and TiO₂ (b) TiO₂/TiO₂ derivative use in biological system

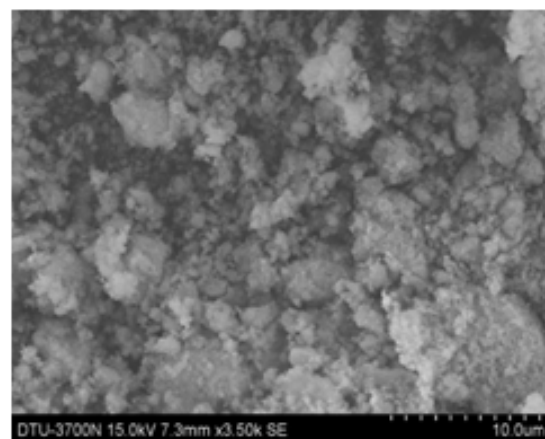
SEM s view of TiO₂ and HA nanoparticles

Aggregated clusters type pattern observed on surface view of HA NPs at magnification 6000X (Figure 2a) and 3500X (Figure 2b). Similar kind of aggregated pattern observed on surface analysis of

TiO₂ NPs at magnification of 6500X (Figure 2c) and 4200X (Figure 2d). The size difference of TiO₂ and HA NPs were observed by comparing Figure 2 (a, b) to 2 (c, d) TiO₂ particle size was bigger than the HA NPs.



(a)



(b)

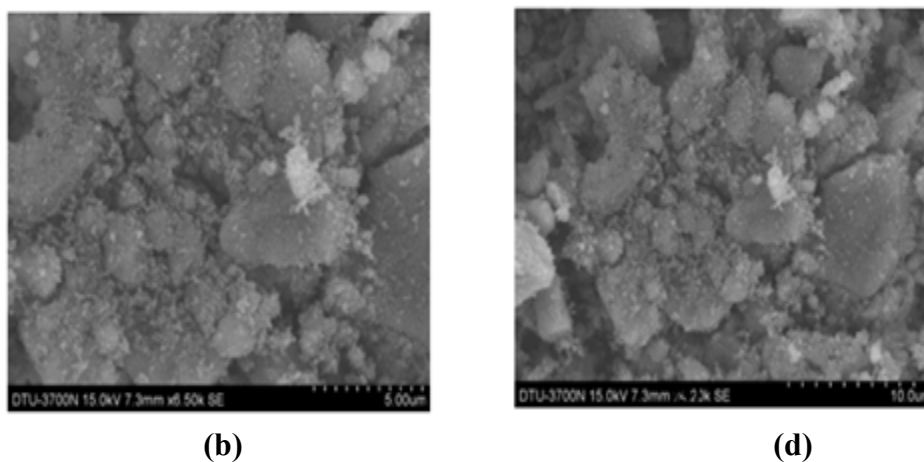


Figure 2(a) Demonstrates SEM of HANPs at 6000X magnification (b) Demonstrates SEM of HANPs at 3500X magnification (c) Demonstrates SEM of HANPs at 6500X magnification (d) Demonstrates SEM of HANPs at 4200X magnification

Solubility of TiO₂

The solubility of TiO₂ were checked in various solvents, it was insoluble in HCl, Human Saliva,

Human blood plasma and BSA while partially soluble in Propanol, Toluene, Dichloromethane and DMSO (Table 2).

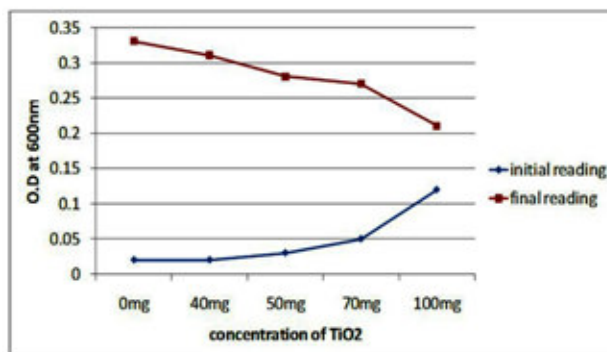
Reagent	Titanium dioxide
Propanol	Partially soluble
Toluene	Partially soluble
HCl	Insoluble
Human saliva	Insoluble
Dichloromethane	Partially soluble
DMSO	Partially soluble
Human blood plasma	Insoluble
BSA	Insoluble

**Table 2
Solubility of TiO₂ in various solvents**

Toxicity of nanoparticle in Bacterial Culture

Bacterial (*E. coli*) growth was affected by increasing concentration of TiO₂ nanoparticle in bacterial growth medium (LB broth), Figure 3a red line illustrate the decrease in optical density of

bacteria after overnight growth culture and blue line indicate initial optical density which was increasing by addition of increasing concentration of TiO₂. The growth of *E. coli* was inhibited by TiO₂



**Figure 3- (a)
Bacterial Toxicity**

Isolation of bone marrow and Dose and time-dependent experiment

Bone marrow of mice was isolated and suspended in PBS and NH_4Cl solution, the pictorial representation of bone marrow isolation depicted in figure 3b. Since the dose and time-dependent toxicity of TiO_2 on BMMNCs have not determined yet, here we have determined the concentration and

exposure time of BMMNCs to TiO_2 which significantly affect the viability of cells. Figure 4a shows the concentration range of 0 to 15 mg/ml and exposure time 60 minutes is less toxic while concentration beyond 15 mg/ml and exposure more than 60 minutes have severe effect on the viability of cells

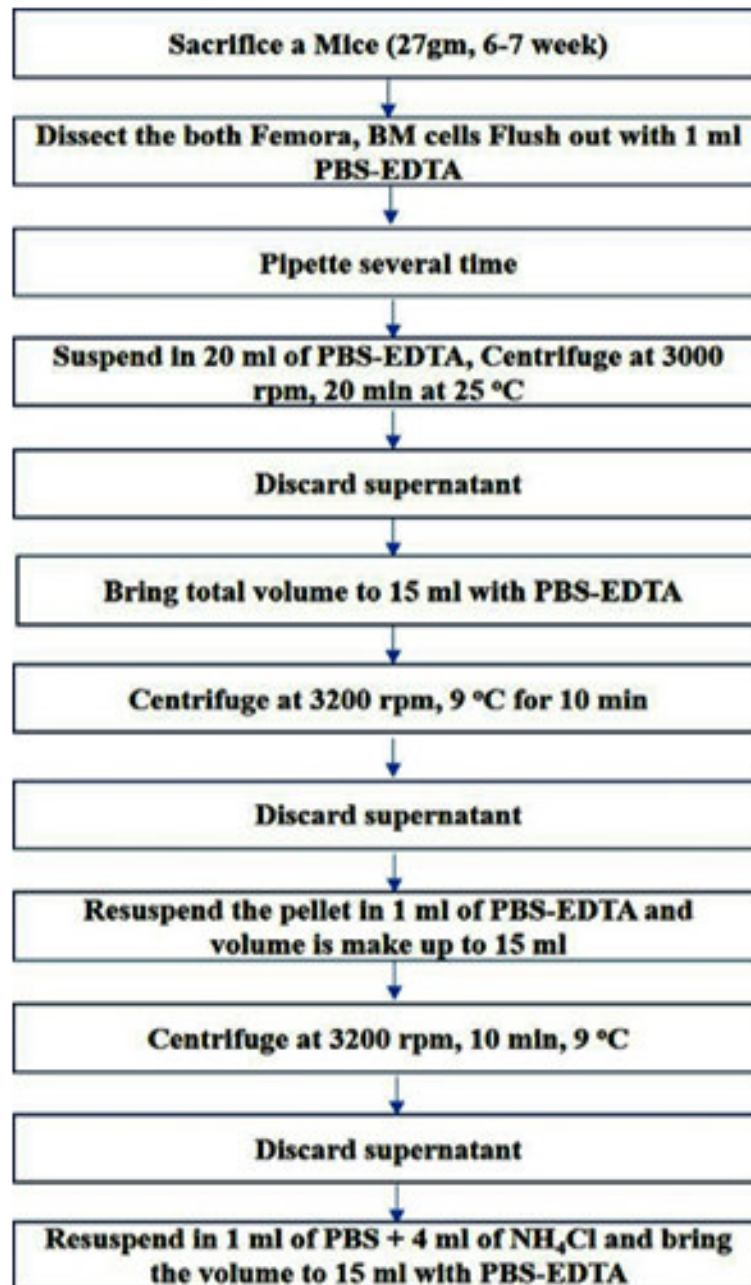


Figure 3(b)

Bone marrow isolation flow diagram

TiO_2 effect on BMMNCs

We have determined the dose and time-dependent toxicity of TiO_2 (Figure 4a). Here, we used concentration range of 0.1 to 0.4mg/ml. (figure 4c)

demonstrate the concentration range 0.1 to 0.4mg/ml for 10 to 30 minutes time duration is toxic for BMMNCs based on cell viability test

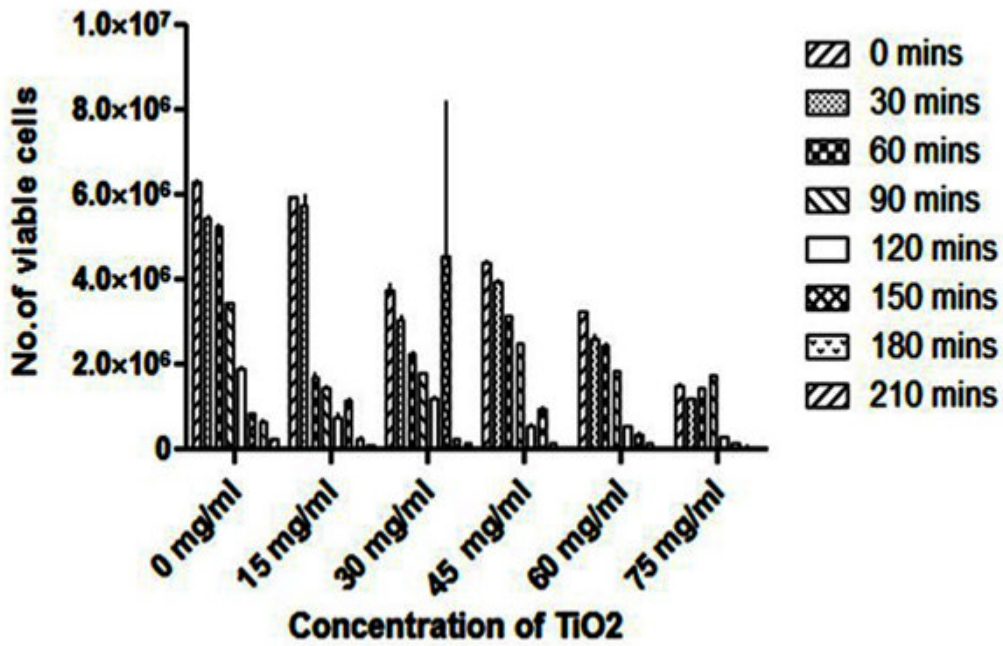


Figure 4-(a)
Dose and time dependent TiO₂ effect on BMMNCs,

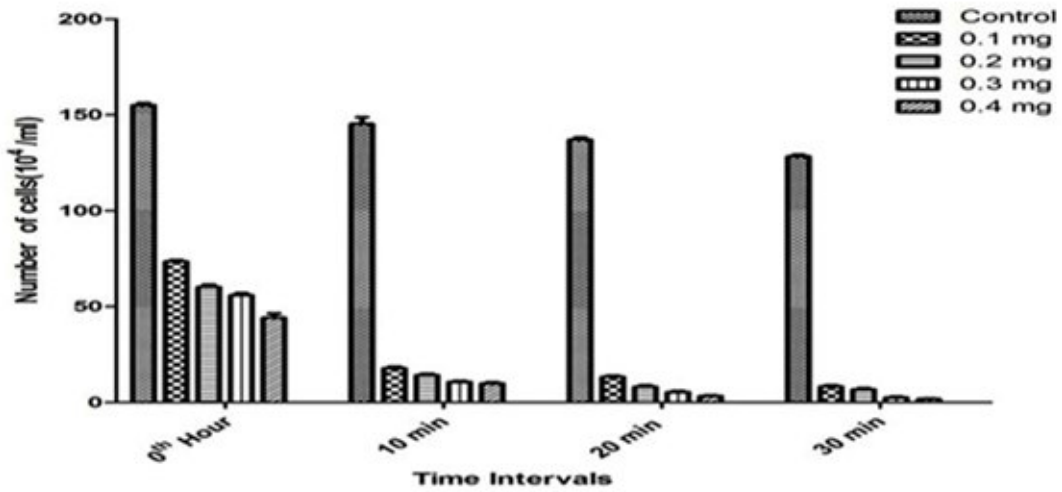


Figure 4(c)
TiO₂ effect on BMMNCs

HA effect on BMMNCs

We have used concentration range of 0.4 to 2mg/ml to see the toxic effect of HA on BMMNCs (Figure

4b), concentration range of 1 to 2 mg/ml for time duration of 10 to 40 minutes is toxic based on cell viability test

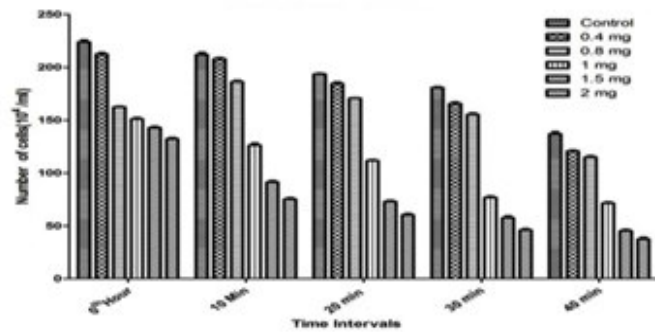


Figure 4(b)
HA effect on BMMNCs

TiO₂ and HA blend on BMMNCs

Toxicity effect of HA, TiO₂ Concoction were observed on BMMNCs with the concentration of 0.4mg/ml in a ratio of 1:1 (Figure 4d). The toxicity effect of TiO₂ was less in the presence of HA based

on cell viability test. There was a gradual increment in toxicity of blend while moving from 0 to 50 minutes on the time scale.

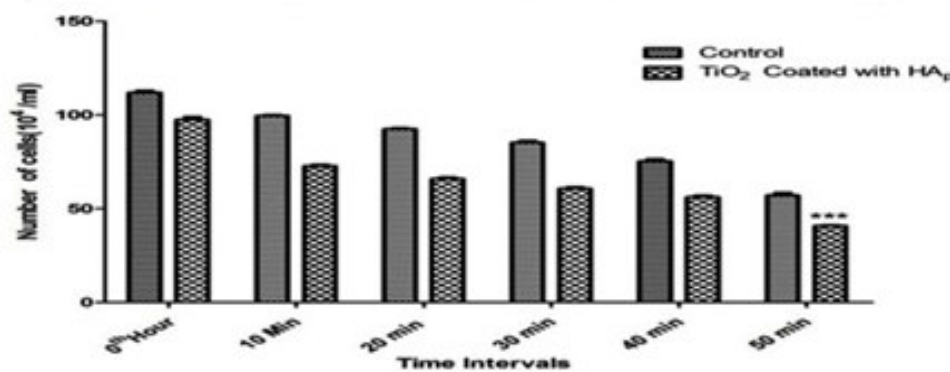


Figure 4(d)
HA and TiO₂ blend effect on BMMNCs.

DISCUSSION

Titanium dioxide (TiO₂) is a handy material with novel properties apt for a number of technically important applications, for example dye-sensitized solar cells white pigment for paints or cosmetics, catalyst, electrodes in lithium batteries, photocatalyst^[10] and biomedical application^[11]. Titanium dioxide (TiO₂) Possesses low-cost high stability, and safety toward both the environment and humans, therefore preferred in biological research and other application^[12]. Transition and Nobel metal can nobbled with TiO₂ and TiO₂ also complexes with Variety of compounds in order to achieve desired outcome. Table-1b summarizes the use of TiO₂ nanoparticles and their derivatives use in the biological system. Silver and nitrogen co-doped TiO₂ increase the antibacterial property of TiO₂. Ag- and N-doped TiO₂ nanoparticles were

investigated by the method of agar diffusion toward *Bacillus subtilis* and *Escherichia coli* under fluorescent light irradiation^[13]. TiO₂-NiFe₂O₄ biomaterial system has antimicrobial composite with the magnetic property that can be extracted from sprayed surface (human body) after exposure^[14]. Multifunctional porous TiO₂ nanoparticles used in targeted drug delivery and light controlled release^[15]. Daunorubicin-TiO₂ Nanocomposites are pH based drug delivery system for targeted cancer cells. Daunorubicin (DNR) is anticancerous molecule and its clinical application is limited due to its side effect. Its anticancer efficiency can be enhanced by DNR-TiO₂ Nanocomposites by inducing apoptosis in a caspase-dependent manner, increase intracellular concentration of DNR, thereby demonstrating that DNR-TiO₂ Nanocomposites could act as a Competent DDS importing DNR into target cancer

cells^[16]. Poly(lactic acid) based TiO₂ Nano-composite has biocompatible, high surface area and ease of surface chemistry modification. Further, Daunorubicin drug molecule can self-assemble on the surface of Poly(lactic acid) based TiO₂ Nano-composite^[17]. TiO₂ nanoparticle may be useful in prevention of malignant bone tumor chondrosarcoma and osteosarcoma as researchers proved it in *in-vitro* culture of two cancer Cell lines U-2 OS (osteosarcoma) and SW 1353 (chondrosarcoma), the TiO₂ nanoparticle killed cell lines in dose and time-dependent manner^[18]. Pt/TiO₂ Nano-composite effective in cancer cell killing, and Nobel metal Pt enhance the photocatalytic activity TiO₂ nanoparticles^[19]. Further, Aluminum phthalocyanine was used for photokilling of cancer cells in photodynamic therapy, when Nitrogen doped TiO₂ conjugated with it, and improvement was seen in photo-killing efficiency of aluminum phthalocyanine^[20]. TiO₂/chondroitin-4-sulfate Nanocomposites were biomimetic and useful in orthopedic application. It was advantageous mainly due to its nontoxicity and Osseointegration ability^[21]. Ag-doped TiO₂ possessed antileishmanial activities and it may be used in the treatment of Cutaneous Leishmaniasis (CL)^[22]. Particle size of TiO₂ play an important role in Bio medicinal therapy and research, the major factor that determine the toxicity is particle size. Many other factors are responsible for TiO₂ induced toxicity such as crystal phase, surface modification and particle aggregate. The diverse factors such as exposure method, species used, dose administered, cell type under investigation and light conditions also have the latent property to control the toxicity of TiO₂ particles^[23]. Widespread aquatic environment habitat of Bacteria, e.g., *Escherichia coli* (*E. coli*) is a virtuous model organism for studying the Cell/organism-nanoparticle interaction and ecotoxicity of nanoparticles. Extensive research work have investigated the toxicity of numerous TiO₂ nanoparticles on *E. coli*. The impelling factor for toxicity is crystal structure and size. It is reported that the toxicity of anatase TiO₂ nanoparticles is more than rutile nanoparticles by inducing greater oxidative stress. The small particle size of TiO₂ contribute towards greater toxicity^[24]. In our study

the dose-dependent toxicity of TiO₂ nanoparticles on the bacterial cell (*E. coli*) revealed, increasing the concentration of nanoparticles with constant time duration kill bacteria, show negative growth (Figure 3a). Many people performed different experiments on mouse organs and cells like brain, liver, mouse embryonic cells, Kidney cells, Fibroblast cells, Sertoli cells and Bone marrow cells^[25, 26, 27, 28], nobody reported the combined effect of TiO₂ and HA on bone marrow cells so far. Adult stem cells derived from Bone marrow. The potential of Adult stem cells is to replenish damaged cells of the body by its self-renewal capacity present in throughout body in the undifferentiated state. MSC (Mesenchymal stem cell) and HSC (Hemopoietic stem cell) is two main division of Adult stem cell. Plasticity of stem cells can be ruled by its ability to transdifferentiate to the lineage of its choice^[29]. The toxic effect of TiO₂ and HA nanoparticle on Bone marrow cells have analyzed (Figure 4d) and it is concluded that the HA reduce the toxicity of TiO₂ nanoparticle. HA and TiO₂ may use in targeted drug delivery to bone marrow cells and other cells of the body in a number of diseases. TiO₂ composite and doped TiO₂ have substantiated as a Prospective targeted drug carrier, based on our current research analysis a TiO₂ nanoparticle coated with HA and immobilized with cancer specific antibody may be used for the drug targeting and treatment of cancer.

CONCLUSION

The dose and time dependent effect of TiO₂ and HA nanoparticles on BMMNCs and their combined effect reveal that the TiO₂ and HA is more toxic when used it alone in incubation of BMMNCs while combination of HA with TiO₂ nanoparticles reduces the toxicity of TiO₂ on BMMNCs.

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