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Cytotoxic Effects of Titanium Dioxide Nanoparticles on Rat Embryo Fibroblast REF-3 Cell Line *in vitro*

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) is an important industrial material that is widely used as an additive in cosmetics, pharmaceuticals, and food colorants. Although the small size of the TiO_2 nanoparticles is useful in various applications, the biosafety of this material needs to be estimated .For this purpose this study was designed to evaluated the cytotoxic effects of TiO_2nanoparticle on Rat Embryo Fibroblast cell line (REF-3) in vitro. Seven concentrations of TiO_2 nanoparticles (0.5,1,5,10,25,50 and100) µg/ml were prepared and tested on cell line with three replicates for each concentration ,The optical density of cell growth read by Elisa reader 492 nm by used Tetra zolium Bromide (MTT).The result of REF-3cellgrowth assay had shown that the TiO_2nanoparticles had cytotoxic inhibition ,This inhibition was time and dose dependent. the inhibitory effect of TiO_2nanoparticles on the proliferation of REF-3started from concentration 1 µg/ml during 24 hour .The CC50% of TiO_2nanoparticles on REF-3 cell line were more than 100µg/ml for all period of time .

Keywords: cytotoxicity ofTiO_2 Nanoparticles, nanotoxicity.

INTRODUCTION

Nanotechnology is all about making products from very small constituents, components or subsystems to gaingreatly enhanced material properties and functionality[1].The industrial use of metallic oxide nanoparticles in a wide variety of applications has been rapidly expanded in the last decade. Such applications include the use of silicon, titanium, iron, and other metallic oxide nanoparticles, thereby increasing the occupational and other environmental exposure of these nanoparticles to humans and other species [2].

Nevertheless, the health effects of exposure of humans and other species to metallic oxide nanoparticles have not been systematically investigated as their impact on the environment has not been under the scrutiny of regulatory control [3,4].

Titanium dioxide (TiO_2) has been increasingly employed in a variety of industrial applications including production of paper, plastics, cosmetics, and paints [5]. This lead to increasing human exposure to these nanoparticles. The pleural disease was present in 17% of the workers exposed to titanium dioxide and was associated with the duration of work in titanium manufacturing, even though the health risk resulting from this increased exposure to such nanoparticles both in natural as well as industrial environments has not been comprehensively or systematically

assessed[6]. Recently in vivo studies in animals have pointed to the possibilities that inhalation and other lung exposure to titanium dioxide particles can induce inflammatory responses in lung tissue and even cytotoxicity in lung cells although thedegree of inflammatory responses and cytotoxicity elicited depends critically on particle size and its surface chemistry [7,8,9,10,11,12].human epithelial A549 cells were able to take up titanium dioxide nanoparticles by endocytosis[13].The inflammatory responses elicited from A549 cells by exposure to titanium dioxide nanoparticles and this also induced dose-related apoptotic damage in such cells [14]. The human lung epithelial (A549) cells could take up a range of titanium dioxide nanoparticles (20–300 nm) and exposure to these nanoparticles triggered inflammatory responses from the cells[15].The anatase titanium dioxide nanoparticles were more cytotoxic than rutile titanium dioxide nanoparticles to the A549 cells [16]. Exposure of human bronchial epithelial cell line BEAS-2B cells to titanium dioxide nanoparticles likewise resulted in oxidative damage to those cells [17].

The aim of the present Study is to determine the cytotoxic effects of titanium dioxide nanoparticles on Rat Embryo fibroblast cell line(REF-3)viability at various concentrations and for various treatment periods

MATERIALS AND METHODS

Titanium (IV) dioxide nanoparticle was obtained from (sigma ,USA), Rosswell Park Memorial Institute (RPMI)-1640 Medium , fetal bovine serum (FBS) were purchased from (sigma, USA). Benzyl penicillin(1g) and streptomycin (1g) was purchased from Ajanta pharm (India) Trypsin –versin, MTT dye, Free PBS(without Ca+2 and Mg+2)and dimethyl sulfoxide (DMSO)

Titanium(IV) Oxide nanoparticle Preparation

One thousand µg /ml of Titanium (IV) oxide with partial size ~21 nm as a stock solution was prepared by dissolving 0.02gm Titanium (IV) oxide in 20 ml sterile D.I. water, which was then sonicated for 15 min according [18]. Working solution were made by serial dilution in culture media without serum, followed by vigorous vortexing as and when required [19].

Cell culture

Rat embryo fibroblast cell line was a secondary culture of 10 days rat embryo. It was kindly provided by Iraqi center for cancer and medical genetics research(ICCMGR). Cells of this normal murine cell line were a mixture of fibroblastic and epithelial cells with normal chromosome picture. The cells were cultured in RPMI-1640 with 10% FBS, 0.5 ml penicillin and0.5 ml streptomycin at 37 °C in a 5% CO2 humidified environment.

Cytotoxicity assay

For the MTT assays, the cells were seeded in 96-well plates(set one) at a density of 45000 cells/ml cells per well in 200 µl culture medium. All cells were exposed to titanium dioxide nanoparticles after 70-80% confluence. Titanium dioxide nanoparticles were freshly dispersed in the cell culture medium and diluted toappropriate concentrations (0.5-100µg/mL). REF-3 cells were cultured in media containing different concentrations of titanium dioxide nanoparticles for 24 ,48 and 72 hour .six well of Culture media and cell without titanium dioxide nanoparticles served as the control in each experiment.28 microliters of MTT dye 2mg/ml was added to each well, and the plates were incubated at 37 °C in 5% CO2/air for 2 h. The medium was then carefully removed, and the purple products were dissolved in 130µL dimethyl sulfoxide (DMSO). The plates were shaken for 10 min [20] .to prevent the nanoparticles from interfering with this assay (data not shown), the formazan material dissolved in DMSO in each well of each plate was quantitatively transferred to an empty well in another plate (set two) [21].The optical density of each well in each plate (set two) was read using Enzyme Linked Immunosorbent Assay (ELISA) Reader at a transmitting wavelength of 492 nm [20,22].Data are represented as the mean ±SE

RESULTS

Cytotoxic Effect of Titanium Dioxide Nanoparticles on REF-3 Cell Line *in vitro:*

The result of the present study shows that the effect of Titanium dioxide nanoparticles on REF-3 cell line. It was highly significant ($P \leq 0.001$) among all concentrations in all periods 24,48 and 72 h. of the treatment, the interaction between time and concentrations was highly significant ($P \leq 0.001$) also after 24 ,48 and 72 h.

After 24 h the interaction between concentrations and time revealed that the effect of titanium dioxide nanoparticles was started at 1 µg/ml up to 100 µg/ml, the value of OD was 0.290 ± 0.005 and0.264±0.002, respectively, the concentrations from1up to 100 µg/ml had the same effect 0.290±0.005, 0.282± 0.005 ,0.272±0.007 ,0.265±0.004,0.259±0.006 and 0.264±0.002 (Table 1), (Figure 1a). The concentration 100 µg/ml had shown more effect on the viability of REF-3cell line (Figure 2b) as a comparison with the control group (Figure 2a).

After48 the Interaction between concentrations and time revealed that the effect of titanium dioxide nanoparticles was started at 25 µg/ml the value of OD was 0.274± 0.004 , then the inhibition activity of titanium dioxide nanoparticles was increased in concentration 50 µg/ml the OD value was 0.256±0.0006 .Whereas The concentrations 50 and100 µg/ml had the same effect 0.256±0.0006 and 0.248± 0.001 on proliferation of REF-3cell line (Table 1), (Figure 1b). There was a clear difference in REF-3 cell line proliferation between control (figure 3a). and high concentration 100 µg/ml (figure 3 b).

After 72h All concentrations had shown significant effect on growth of REF-3 cell line (Table 1) ,(Figure 1c).The effect of titanium dioxide nanoparticles was started from concentration 0.5 µg/ml up to 100 µg/ml, the value of OD were 0.291 ± 0.003and 0.247±0.003 respectively. The concentrations from 1 µg/ml up to 100 µg/ml had the same effect, The high concentration of titanium dioxide nanoparticles 100 µg/ml was more effect on viability of REF-4 cell line (Figure 4 b) as compared to control group (Figure 4 a).

The exposure times had a highly significant effect (P≤0.001) on the growth of REF-3 cell line treated with titanium dioxide nanoparticles. Table (1) demonstrated that titanium dioxide nanoparticles was more toxic after 72 h than 24 and 48h.

Table (1): Mean ± SE for the effect of different concentrations of TiO_2nanoparticles on the proliferation of REF-3 cell line after 24 ,48 and72 h. treatments *in vitro*: (Observations of O.D).

Concentration µg/ml	Time			Over all concentration
	24h.	48h.	72h.	
0	0.322 ±0.002	0.326 ± 0.002	0.333 ± 0.003	0.327 ± 0.002
0.5	0.314 ± 0.009	0.315 ± 0.001	0.291 ± 0.003	0.306 ± 0.004
1	0.290 ± 0.005	0.308 ± 0.001	0.273 ± 0.005	0.290 ± 0.005
5	0.282 ± 0.005	0.299 ± 0.005	0.268 ± 0.004	0.283 ± 0.005
10	0.272 ± 0.007	0.293 ± 0.003	0.261 ± 0.001	0.275 ± 0.005
25	0.265 ± 0.004	0.274 ± 0.004	0.254 ± 0.006	0.264 ± 0.003
50	0.259 ± 0.006	0.256 ± 0.0006	0.252 ± 0.003	0.256 ± 0.001
100	0.264 ± 0.002	0.248 ± 0.001	0.247 ± 0.003	0.253 ± 0.003
Over all time	0.283 ± 0.004	0.290 ± 0.005	0.272 ± 0.004	0.282 ± 0.005
Effectors	Concentration***	Time***	Concentration & time***	
LSD	0.00722	0.0044	0.0125	

SE=Standard Error

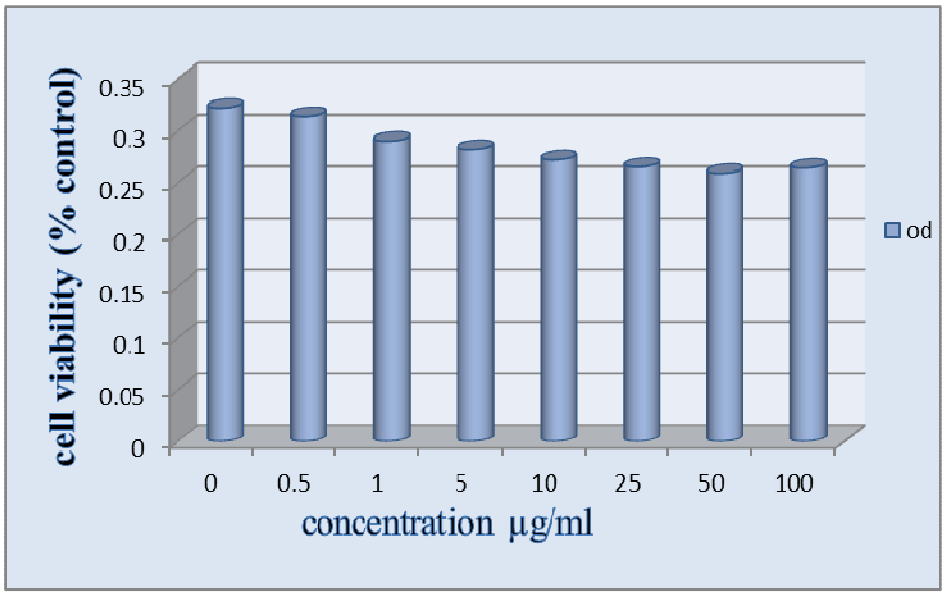

Figure (1a) : REF-3 Cells viability after 24 h

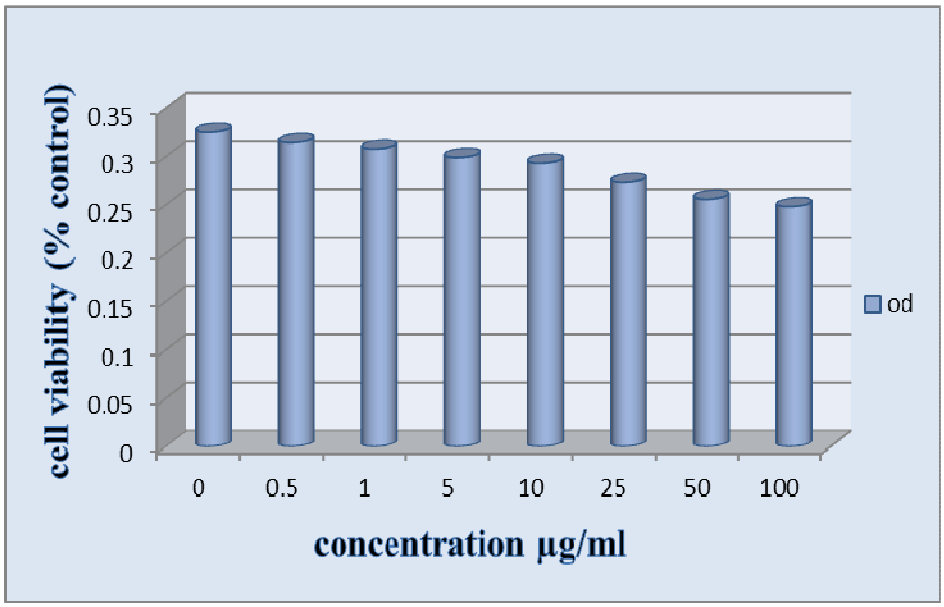

Figure (1b) : REF-3Cells viability after 48h

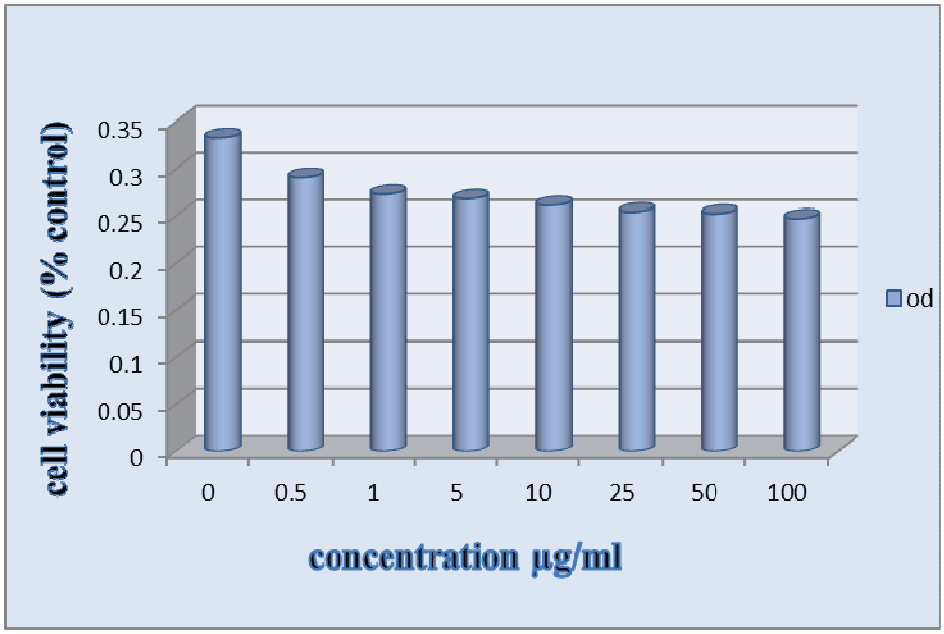

Figure (1c) :REF-3Cells viability after 72h

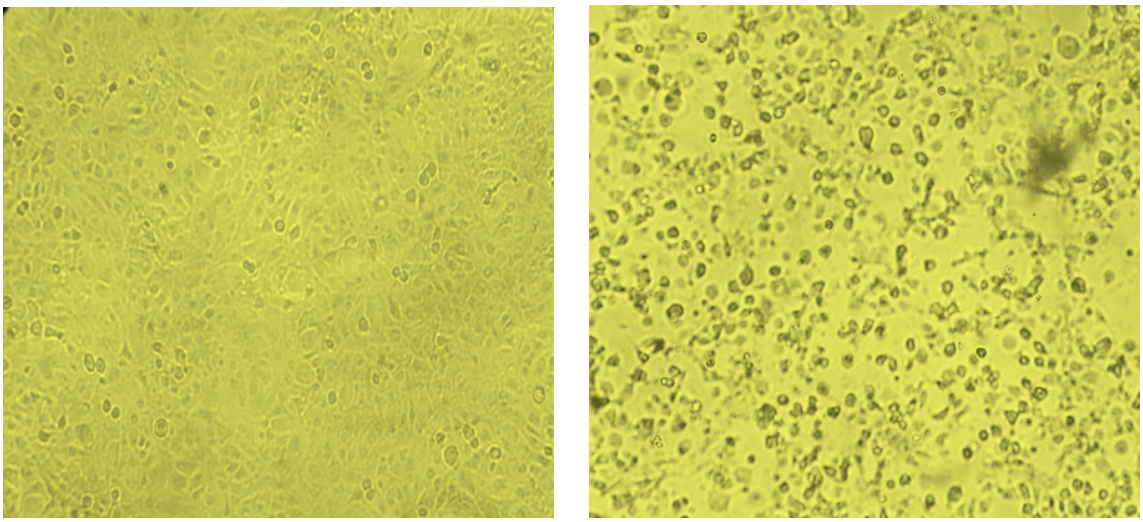

Figure (2) : REF-3 Cell line after 24 h.(70x).(a)control confluent monolayer (b)cells treated with 100µg/ml of TiO_2nanoparticles

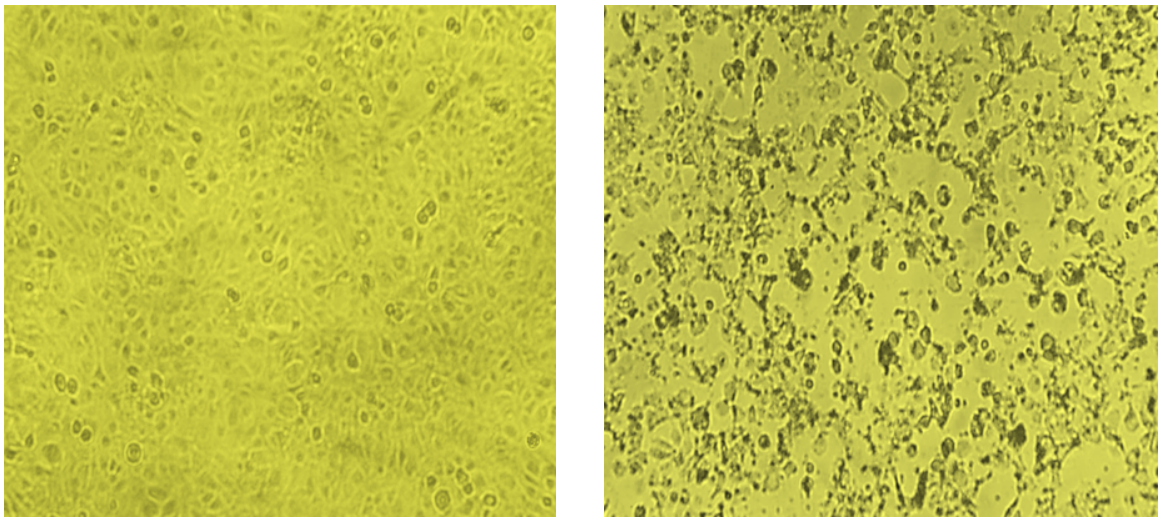

Figure (3) : REF-3 cell line after 48 h.(70x).(a)control confluent monolayer (b)cells treated with 100µg/ml of TiO_2nanoparticles

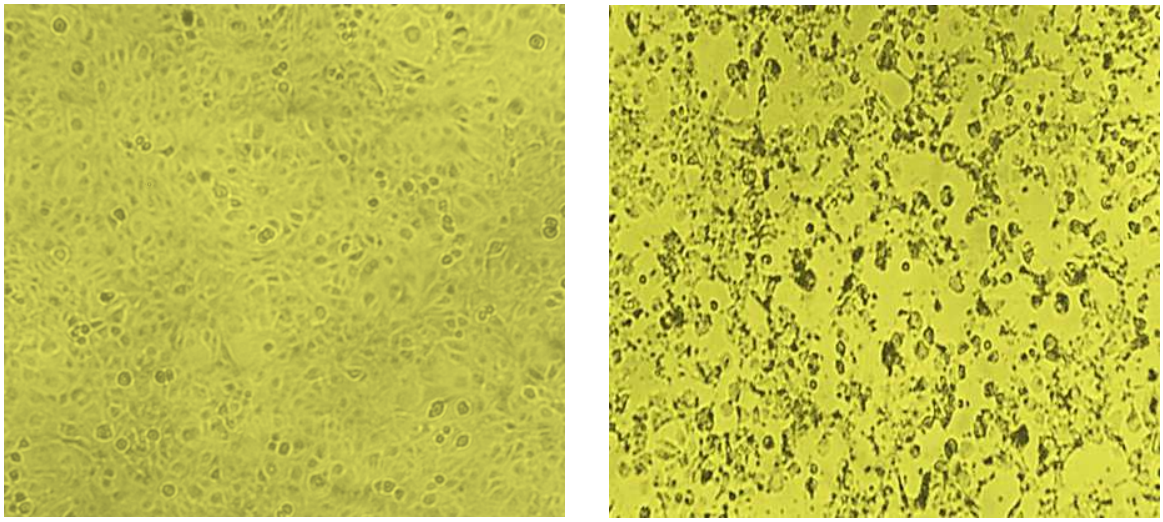

Figure (4): REF-3 cell line after 72 h.(70x).(a)control confluent monolayer (b)cells treated with 100µg/ml of TiO_2nanoparticles

Table (2) shows the values of CC50 of TiO2 nanoparticles on the REF-3cell line after 24,48 and 72h. , this table revealed that the Tio2nanoparticles had CC50% values higher than 100 µg/ml in all periods of times (Figure 5 a, b, c)

Table(2) :Cytotoxicity TiO_2NP on REF-3 cell line expressed as CC50 values obtained in 24, 48 and72 h.

CC50 µg/ml		
24h	48h	72h
(>100)	(>100)	(>100)

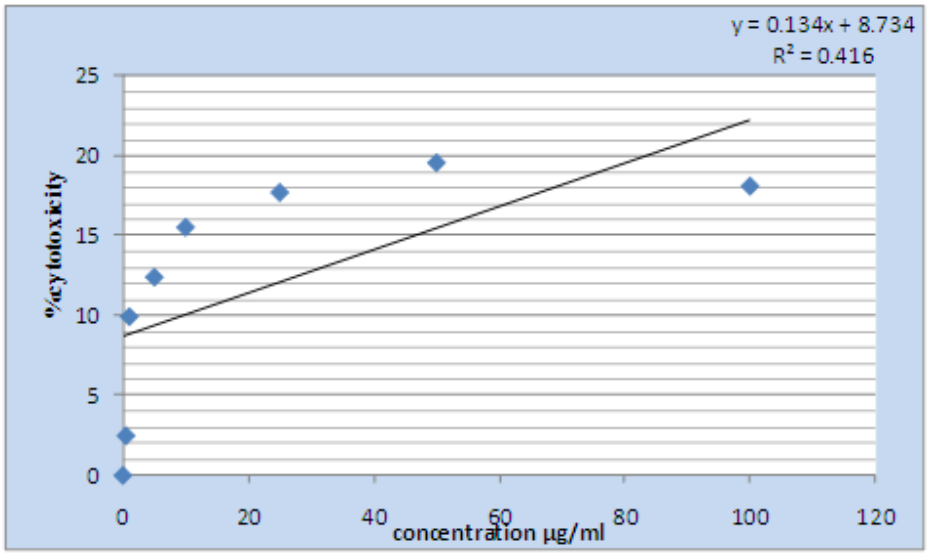

Figure (5 a) :The CC50% of TiO_2nanoparticles at 24h.

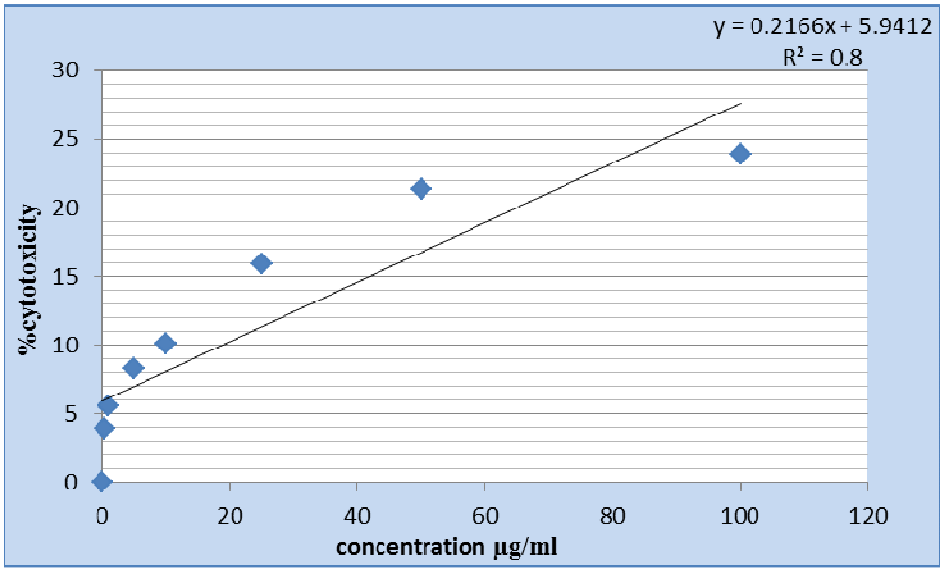

Figure (5b) :The CC50% of TiO_2nanoparticles at 48h.

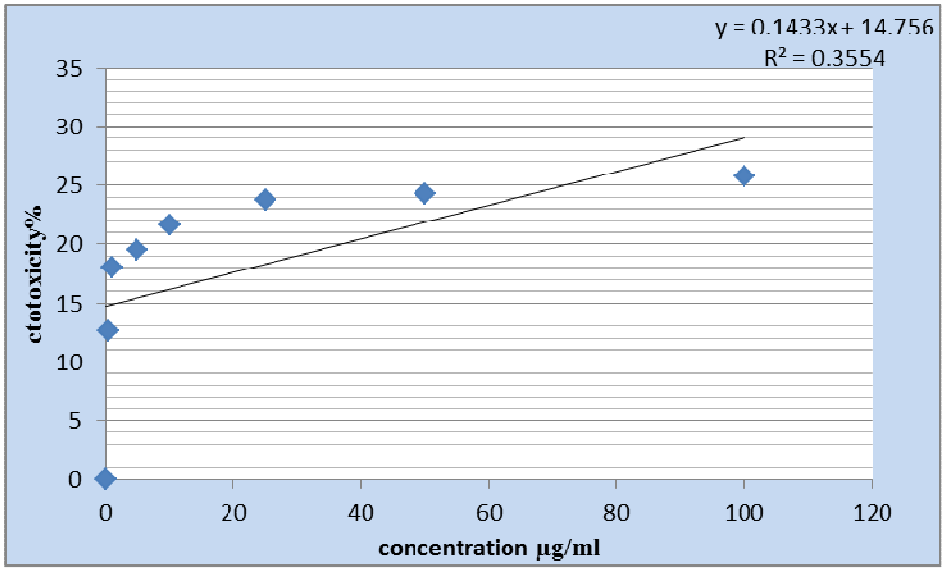

Figure (5c) :The CC50% of TiO_2 nanoparticles at72h.

DISCUSSION

Specific properties of nanoparticles, such as their small size, shape, high surface area, and special structure, make these compounds promising candidates in both industrial and biomedical applications [23,24]. However, in recent years, there has been increasing evidence of the adverse effects of nanoparticles, such as increase in respiratory and cardiovascular mortality and morbidity and worsening of asthma [25,26].

Assessment of human health and environmental safety with respect to the use of nanoparticles is urgently required. Many types of commercial nanoparticles, such as silica, nano titanium dioxide ,silver, chrysotile asbestos, carbon nanotubes, as well as some magnetic particles, have been investigated for their biosafety, and these nanoparticles exhibited various levels of cytotoxicity in different cell lines [27, 28].

Measuring of the optical densities (O.D.) for the stained cell line plate, after treatment with different concentrations of Tio_2nanoparticlesduring the time of incubation revealed that, Tio_2 of high concentration gave low value of O.D., which indicate maximum response, because the affected (dead) cells are removed by washing during staining procedure leaving a light color represented the attached viable cells. In contrary, the low concentration gave high value of O.D., which indicates minimum response in proportional to high percentage of viable cells. The classical method for evaluating the effect of deleterious treatments on cell is based on proportion of inhibition [29], which indicate the rate of inhibition of cell growth [30] or percentage of toxicity [31]. These parameters were used in present study for evaluation of cytotoxic effect of titanium dioxide nanoparticles. Moreover, the cytotoxic effect of titanium dioxide nanoparticles on REF-3 cell line varied with different time and concentration levels.

The inhibition activity of titanium dioxide nanoparticles against the cell line may be explained by , titanium dioxide had capacity to induced the inflammatory response as a result reactive oxygen level . There was a relationship between the inflammatory and genotoxic potential of several particles; namely Ultrafine carbon black, titanium dioxide and α quartz in Rats were exposed to particles via intratracheal instillation. All particle types induced the infiltration of neutrophils into the lungs[32].

Titanium dioxide nanoparticles responsible for DNA damage and prompted cell death that was due to it stimulated the oxidative stress, titanium dioxide nanoparticles were generated of reactive oxygen species (ROS) and depletion cellular antioxidant such as glutathione and vitamin E [33], Reactive oxygen levels are physiological products generated by mammalian cells in the mitochondria during aerobic metabolism, Intracellular ROS levels were kept in a balance by metabolism offset by cellular antioxidant enzymes and scavengers, Several possible signaling

pathways have been described linking ROS to apoptosis these include cell surface death receptors (extrinsic) and mitochondria (intrinsic) pathways[34].There are contribution between oxidative stress and the neurotoxicity of titanium dioxide nanoparticles in mouse brine microglial BV2 cells were as a result of rapid production of ROS [35].

The study revealed that the effect of titanium dioxide nanoparticles , on the proliferation of REF-3 cell line was dose and time –dependent .This result was in agreement with reported by [36] showed that the cytotoxic effect of TiO_2nanoparticles on human dermal fibroblast ,HaCaT keratenocytes,SZ95 sebaceous gland cell , primary human melanocytes and BEAS-2B cell line was dose and time –dependent . The cytotoxicity effect of titanium dioxide nanoparticles on human colon carcinoma cells in the presence of UVA light was dose and time –dependent[37].The photocatalytic activity of TiO_2 is greater in the presence of solar light as compared to UV light[38].

CONCLUSION

The results concluded that TiO_2 nanoparticles had cytotoxic effect on Rat Embryo Fibroblast REF-3 cell line and the inhibition activity of titanium dioxide nanoparticles was time and dose dependent .

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