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RESEARCH ARTICLE

Acute cytotoxic effects of Titanium dioxide (TiO_2) and chronic exposure to safe dose of nanoparticle on Hepatocarcinoma cell line ($HepG_2$)

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ABSTRACT

Objective(s): To address cellular response to nanosized particles, we designed in vitro cytotoxic effects of titanium dioxide nanoparticle was assessed on hepatocarcinoma cell line (HepG2) with novel UVA exposure.

Methods: Cellular morphology, cell viability, and membrane leakage of lactate dehydrogenase were used to evaluate the acute cytotoxic effect of TiO2 after 24 hours of exposure.

To determine the chronic exposure effects, Hepatocarcinoma cell lines were treated with 125 and 250ppm of TiO2 in 4 consecutive passages lasting 25days.

Results: Obvious changes in cellular morphology like cell shrinkage and rounded appearance and cytoplasm granulation was observed at 2500ppm and higher concentration.Cell count decreased during four passages, while cellular oxidative responses such as nitric oxide production, and membrane lipid peroxidation, and total cell protein showed significant increases compared with controls.

Conclusions: These results suggest that chronic exposure even to the safe doses of nanosized particles can stimulate cellular oxidative and inflammatory responses.

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INTRODUCTION

Nanoparticles introduce novel analytical tools with various biological functional characteristics to life science [1-3]. Nanosized Titanium dioxide (TiO2), an odorless, noncombustible nanoparticle exists in different crystal types such as Anatase, Rutile, Brookits and amorphous with different physical and chemical properties[4-6].Titanium dioxide is used in the skeleton of various productions such as papers, plastic, paint, food colorant, cosmetics, sunscreens and recently widely in cancer treatment and drug delivery system [7,8].

For this widespread usage and possible acute and chronic exposure to titanium dioxide, there is a severe concern of human health and environmental implications of manufactured nanoparticles [9,10].

Some relevant studies evaluated the cellular cytotoxic effect of titanium dioxide nanoparticles with respect to different concentrations showed a remarkable toxic effect such as oxidative DNA damage, micromoles formation and increased hydrogen peroxide production in human bronchial cells depended on Anatase crystal form, higher doses and smaller size [11].

Some other studies had demonstrated that the cytotoxicity of TiO2 nanoparticles was as considerable as the other nanoparticles like nanosilver and nano silicons , and Ag nanomaterials [12-13].

Also, few studies indicated that TiO2 nanoparticle could produce H2O2 and hydrogen peroxide under Ultraviolet-A radiation (UVA)

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and increase cytotoxic effects of TiO2versus some studies which had reported TiO2 chronic inflammatory response in pulmonary system of rat without UVA activations [14].

To clarify in vitro cytotoxic effects of TiO2 after acute and chronic exposure, we designed a study in which Human Hepatocarcinoma cell line was exposed with various concentrations of titanium dioxide and then cellular toxicity was accessed.

METHODS

Chemicals

Ultrafine titanium dioxide (99% purity), was purchased from PlasmaChem CmbH company (www.plasmachem.com). The particles were suspended and ultrasonicated in culture medium to reach the final concentration of 200 μ g/m. TiO2 nanoparticle were analyzed for the size and shape using Zetasizer NanoZS, UK. It was homogenous, turbid solution with no precipitation, 50000ppm, Anatase crystal dominant and 4-8 nm in diameter [15].

Cell culture and extract

Human Hepatocarcinoma cell lines were obtained from the Pasteur institute (Tehran-Iran) in the exponential phase. They were cultured in RPMI cell medium supplemented with 5%-10% fetal bovine serum incubated in 5% CO2 and at 370 C. Sample cell were passaged 10-20 times during the study and in every passage; in 80% confluency, they were detached by using trypsinization enzyme.

After exposure, the cell samples were suspended in 500 μl chilled homogenizing buffer (250 mM sucrose, 12mM Tris-HCl, 0.1% Triton X-100, pH 7.4, and 5mM PMSF) and lysed. The solution was centrifuged at 8000 ×g for 10 min at 4°C. many biochemical assays were performed in cell extract medium.

In vitro cytotoxicity assay:

Tetrazolium salt (Sodium 3,3′-(1((phenylamino) carbonyl)-3, 4-tetrazolium) XTT are quantification of viable cells. Cells were cultured in 96-wells plates (106 cell/200μl medium). After 24 hours of TiO2 treatment, a mixture contains 5ml of Sodium 3, cells cultured in 96-wells plates were mixed by 3′-XTT labelling and Electron coupling reagent and after 4 hours incubation in room condition, the absorbance was read in 495nm with ELISA plate-reader. Half maximal (50%) inhibitory concentration (IC) of was calculated as IC50.

Biochemical assays

LDH enzyme activity was evaluated in the cell culture media after 24 hours -TiO2 treatment according to Gayman's LDH cytotoxicity assay [16].

After four consecutive passages; protein concentration of the cell extracts was estimated by Bradford method, Nitric oxide concentration in cell media was measured according to their instruction in NO assay kits(Chimazzim company) [17].Melanodialdehyde (MDA), end-product and an index of lipid peroxidation was assessed in cell extracts as explained by Ohawa [18].

Cell morphology

Human Hepatocarcinoma cell lines were treated with TiO2 by different concentration for 24 hours and then were washed with PBS for cleaning attached TiO2 particles (TiO2 made cell medium turbid and not suitable for evaluating morphology). Cells were visualized with Light microscope (×10, ×40, and ×100 magnification).

TiO2 treatment protocol

Acute exposure

IC50 assessed acute cytotoxicity of nanoparticle (is defined as the concentration, which reduces the viability of uninfected cell by 50%), monolayer cell line became confluent in a 96-wells plate with a density of 10000. HepG2 cells were c with different concentration of TiO2 (0-17000ppm) as shown in *figure (6)* for 24 hours. After 24 hours incubation, cell medium was removed and toxicity endpoints such as cell viability (XTT assay), Lactate dehydrogenase enzyme leakages (LDH assay) were accessed. IC 50 was calculated according to results of XTT assay and clarified as a dose measured cell count decreased approximately 50% compared with control sample based on Optic density.

We evaluated ultraviolet-A (UVA) (320-400nm) radiation effect on TiO2 cytotoxicity by designing three groups as follows [19]:

1) Cell lines were treated with TiO2 without UVA radiation

2) TiO2 was radiated by UVA for 40 minutes and then added to cell samples.

3) Cell lines were treated with TiO2 nanoparticle and all contents received 5 minutes UVA radiation.

Chronic exposure

To estimation the oxidative stress response to

chronic exposure, HepG2 cell lines were cultured in 6 flasks in 106 counts, two samples as control, two of them exposed to 5% and 2 of them were treated with 10% of titanium dioxide toxic dose (2500ppm). To make a more homogenous medium, we mixed titanium dioxide with 5ml of cell media in the sterile condition and added it to remains. When the control groups reached 80% confluency, the cell cultures in all the flasks were renewed again. This process followed in four consecutive passages. The cell medium was collected, centrifuged at 1000 rpm 5 minutes for TiO2 separation and then frozen. Also, cell samples after washing with PBS were trypsinized centrifuged at 1000 rpm and frozen in -70 C. This test repeated two times.

After completing these processes, we conducted oxidative stress tests such as lipid peroxidation, and total cell protein content in cell extracts and Nitric oxide production in cell mediums.

Statistical analysis:

We used software SPSS version 16 for analysis of data. The experiment was carried out twice in duplicate. Our results were expressed by means ± S.D and three groups' results were accessed by One-way ANOVA and also Univariate analysis of variance. $P<0.05$ was considered statistically significant.

RESULTS

Cell morphology

HepG2 cell line had no apparent changes in the presence of TiO2 up to a concentration of 1500-2500 pmm. However, obvious changes in cellular morphology like cell shrinkage and rounded appearance and cytoplasm granulation were observed at higher level which might suggest the cytotoxic effect of TiO2, although macroscopic evaluation of cell morphology got more difficult, for cell media turbidity made by TiO2 precipitation. Also no noticeable difference was detected between groups received UV-A radiation and not (*figure 1-2*).

Acute cytotoxicity of TiO2

As illustrated in *figure 3*, according to the results of cell viability assay (XTT) after 24 h treatment, a considerable decrease in the viability of HepG2 was

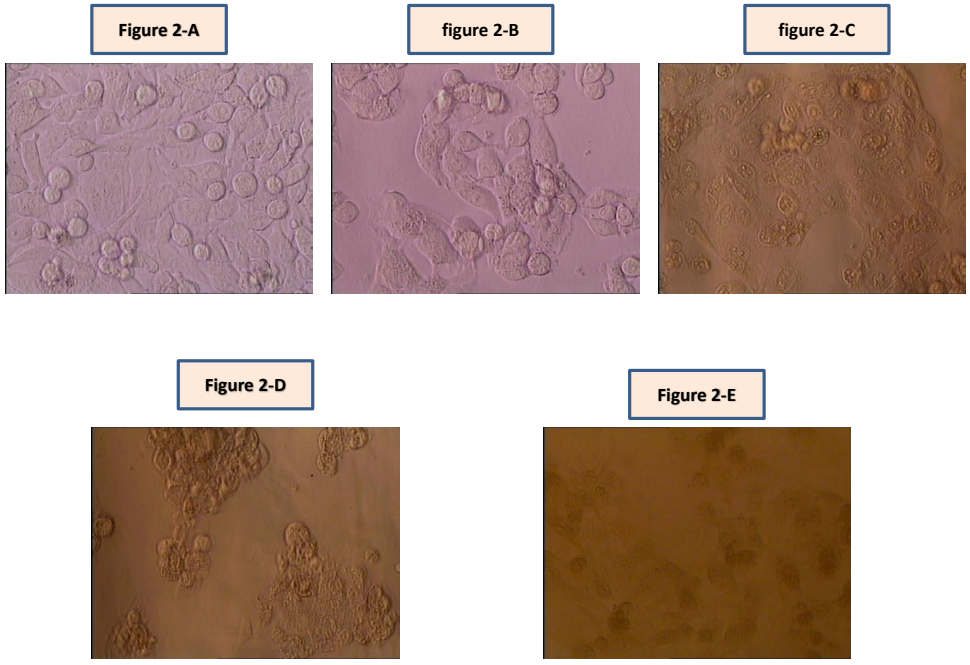

Fig. 1. Morphological characterization of Hepatocarcinoma cells in the second group in which TiO2 after40 minutes UV-A radiation was added to cell Culture (Magnification 100×). At lower doses up to 1000ppm. No cellular morphology changes were observed but at higher dose, cell shrinkage, cytoplasm granulation and rounded appearance were seen. Control cell (A); Cells were treated with 250ppm (B); Cells treated with 2500ppm (C); at higher dose than 4500ppm, titanium dioxide produced turbidity confounded cell appearance. Cell lines were exposed to 8500ppm (D), cell exposed to high TiO2 concentration 10000ppm (E).

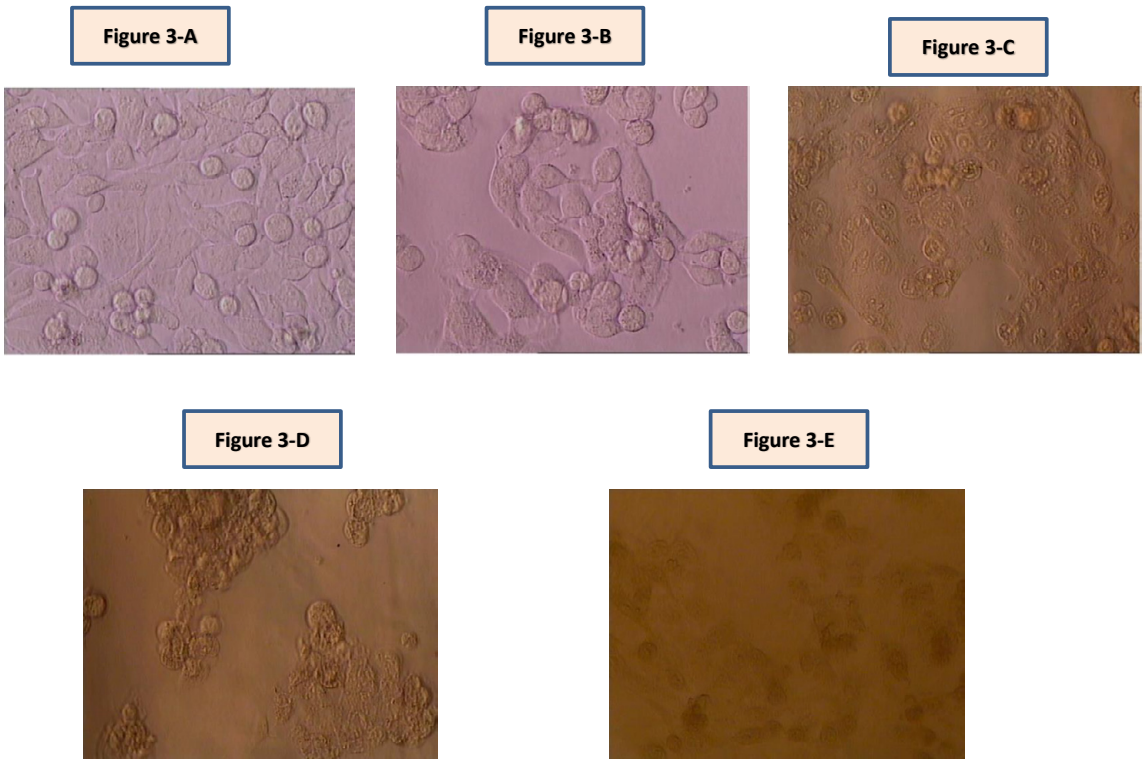

Fig. 2. Morphological characterization of Hepatocarcinoma cells in the third group in which UV-A was radiated 5 minutes to cell lines treated with TiO2 (Magnification 100×). From lower doses up to 1000ppm. No cellular morphology changes were observed but at higher dose, cell shrinkage, cytoplasm granulation and rounded appearance were seen and no significant different in morphology was detected with second group. Control cell (A); Cells were treated with 250ppm (B); Cells treated with 2500ppm (C); at higher dose than 4500ppm, titanium dioxide produced turbidity confounded cell appearance. Cell lines were exposed to 8500ppm (D), cell exposed to high TiO2 concentration 10000ppm (E).

seen in exposure to a different, accumulative dose of titanium dioxide. This decrease wasn't remarkable in the 500ppm and 1000ppm TiO2 concentration but, at the higher doses, it was showed a dose-dependent cytotoxicity .Approximately half-maximal (50%) inhibitory concentration (IC) of a TiO2 was observed in 2500ppm which was statistically significant compared with control ($p<0.05$). So it was accounted as IC50 value on HepG2 cells. Optic density was affected by cell medium turbidity at the higher concentration and assessment was impossible.

We designed three groups for evaluation of UV-A radiation on TiO2 cytotoxicity as described in part 6. Our results showed a significant difference in cell viability in group 3 in which cell lines after TiO2 treatment received UV-A radiation. But in the other 2 groups, cell counts didn't have any significant difference with each other (*figure 3)*.

Chronic cytotoxicity of TiO2

After four consecutive passages (approximatly25days), cell count decreased from 9×105 to 6.8×105 in the control group versus exposed samples (125ppm and 250ppm) which reached to 5 and 4 ×105 respectively.

NO production changes are illustrated in (*figure 4*).After four consecutive passages, NO production as an inflammatory indicator increased in the HepG2 cell medium in both 125 and 250ppm TiO2-exposed samples compared with control after second passage ($p<0.05$). The nitric oxide content increased from 0.4 µM to 0.354 µM in the 125ppm group. This increase was significantly dominant from 0.9 µM to 0.925 µM in exposed 250ppm group rather than 125ppm ($p<0.05$).

Considerable increase in total cell protein content versus decrease in the cell count of both 125 and 250ppm groups were recorded in comparison with control groups after the second passage (*figure*

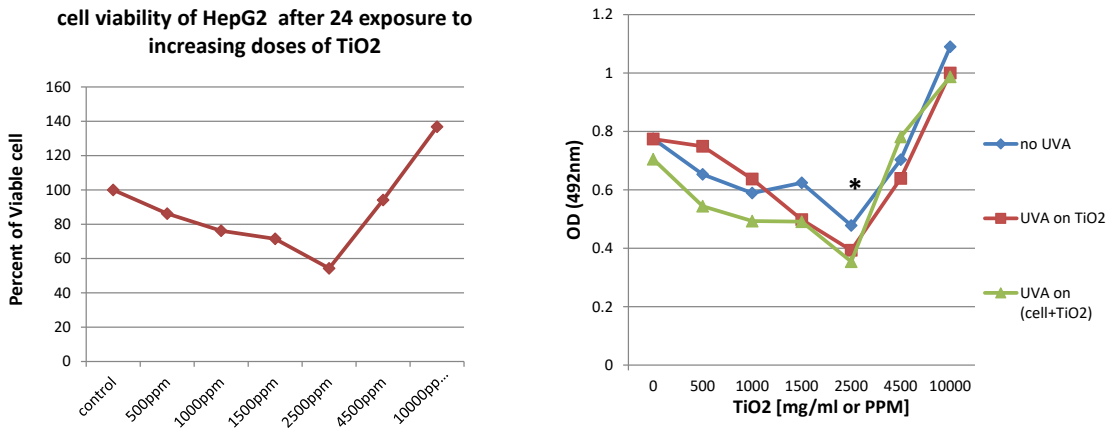

Fig. 3. titanium nanoparticle effect on cell viability of hepatocarcinoma cells.

$HepG_2$ cells were treated with different concentrations of nanoparticle after 24 h incubation period, and cell viability was determined by the XTT reduction assay as described. The average OD value of control cells was taken as viability index and compared with OD in nanoparticle- exposed cells. The data are expressed as mean of two independent experiments (*) indicates a statistically significant difference compared to controls ($p < 0.05$).

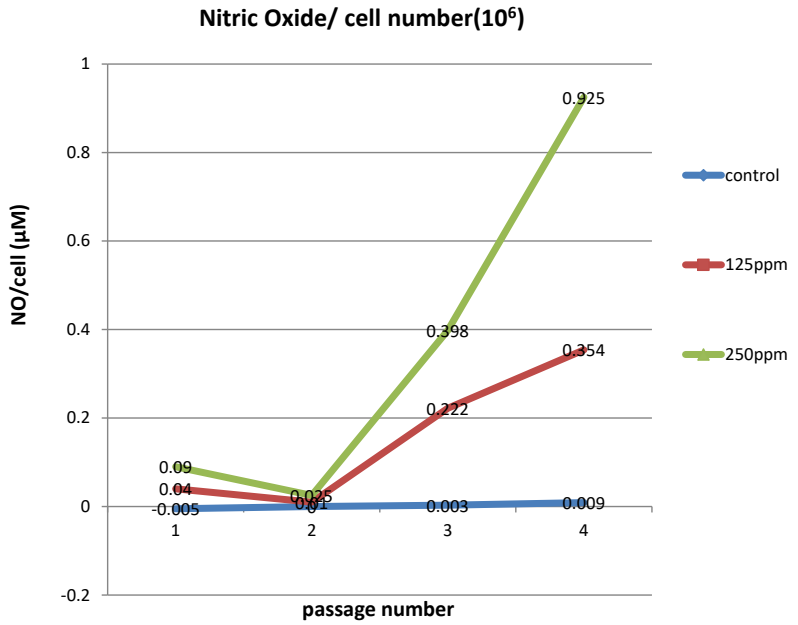

Fig. 4. NO production after 4 consecutive passages exposed to subtoxic dose of titanium dioxide. Increase in NO production after every following passage in the exposed samples compared with control one. Sample exposed to 250ppm of TiO2 produced more NO compared with 125ppm exposed.

5). In the first exposure, cell protein content was calculated 0.316 mg/ml and 0.361mg/ml in 125ppm in 250ppm group respectively without significant difference compared with control group (0.281 mg/ml) but, it rose to 0.687 mg/ml and 0.85 mg/ml after second passages (13 days) compared with control content of 0.432 mg/ml ($p<0.05$)and confirmed that cell protein content was more statistically dominant in the 250ppm rather than 125ppm ($p<0.05$).

Our results represented that membrane lipid peroxidation products as the other indicator of oxidative cell stress was significant increase in the TiO2-exposed cells and also this difference was more evident in 250ppm TiO2 exposed cells

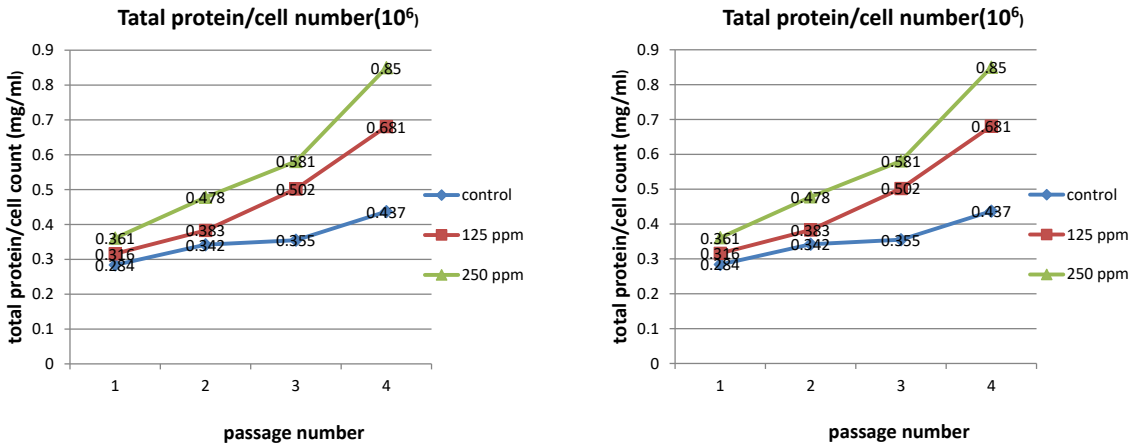

Fig. 5. Protein content after 4 consecutive passages exposed to subtoxic dose of titanium dioxide. Increase in protein content versus decrease in cell count after every following passage in the exposed samples compared with control one. Sample exposed to 250ppm of TiO2 produced more protein compared with 125ppm exposed.

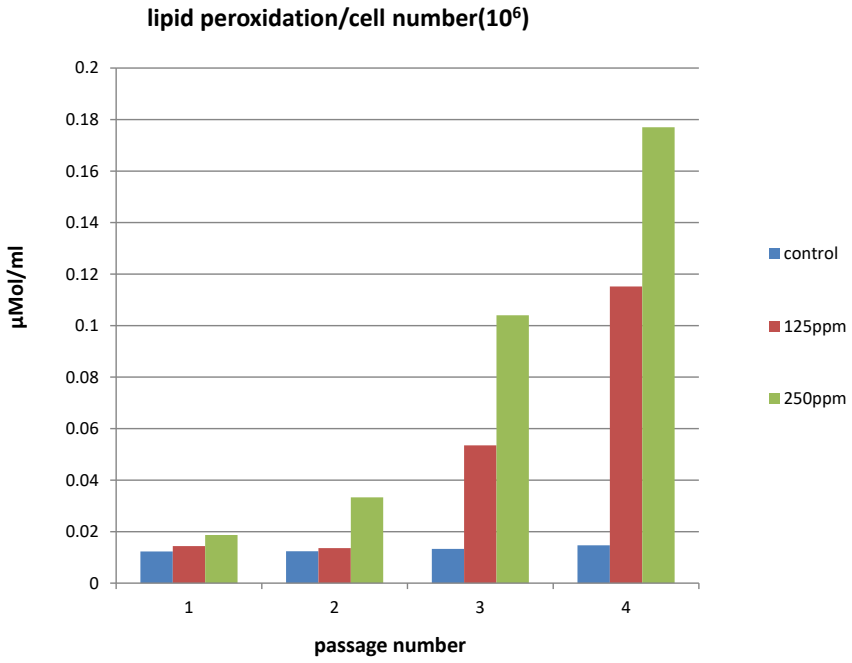

Fig. 6. lipid peroxidation products after 4 consecutive passages exposed to subtoxic dose of titanium dioxide. Increase in lipid peroxidation products after every following passage in the exposed samples compared with control one. Sample exposed to 250ppm of TiO2 produced more lipid peroxidation compared with 125ppm exposed.

($p<0.05$)(*figure 6*) and also, our results showed that chronic exposure to TiO2 during four passages induced peroxidation products.

DISCUSSION

In the present study, we tried to investigate the cytotoxic effects of TiO2 nanoparticle in a precise focus on crystal type, size, dose-dependency and UV-A radiation.

Although the increased use of nanobiomaterials, such as TiO2nanoparticles (TiO2 NPs), our results demonstrated that Anatase -crystal TiO2 (4-8 nm) has toxic effects on human cells. After 24h-exposure to titanium dioxide nanoparticle, IC50 was accounted for approximately2500ppm and UV-A radiation to complex TiO2-exposed

cells demonstrated more cell toxicity compared with other conditions.

Cytotoxic effect of titanium dioxide was discussed in different concentration, crystal type and sizes in various other researches.

In vitro cytotoxicity assessment by Fin Dechsakultho on human skin fibroblast with two different nanoparticles, ZnO and TiO2 (50-70 nm and less than 150nm for four and 24h were showed that ZnO had higher toxicity after four and 24 h and by increasing the time of exposure, nanoparticle toxicity increased too. Also, they estimated IC50 for ZnO was 49.56± 12.89 and for TiO2 was 2,696± 667ppm [20].

The smaller sized titanium dioxide particles induced more cytotoxicity and oxidative damages and also photocatalytic cytotoxicity induced by Anatase of TiO2 was higher than Rutile form and slightly more oxidative DNA damage was founded after Anatase-Rutile mixture exposure compared with Anatase alone [21].

S.M. Hgussain and his colleagues investigated acute toxic effects of different nanoparticles *in vitro* rat liver cell. After 24 h treatment, TiO2 did not show any considerable effect on the cellular morphology and structure, mitochondrial function, membrane damage and leakage of lactate dehydrogenase and reduced glutathione level at lower doses (10-50µg/ml), in comparison, a significant cell toxicity was detected at the higher titanium dioxide doses (100-250µg/ml).

Toxicity evaluation of ultrafine (nanoparticle) and fine-sized TiO2 on lung epithelial cells (*in vitro* and rat lung *in vivo)* after 1, 4, 8 and 24h revealed LDH release, mitochondrial function (MTT) and total cell protein changes. This study showed ultrafine TiO2 increase LDH release in cell medium more than fine TiO2 but in the *in vivo* system, neither the fine nor ultrafine produced cytotoxicity. Finally, they suggested that cytotoxicological response to the nanoparticle in 48h weren't accurately reflected and more prolonged observation with developed and validated techniques will need [22].

M. Says in his study presented cytotoxic response correlation with nanoscale titanium dioxide structure at high concentrations (100µl/ml) and confirmed that cellular response had dose-response toxic effect increasing with time of exposure. They indicated that Anatase TiO2, 100 times more toxic than Rutile [21,22].

Cyto- and Wang and his colleagues investigated the genotoxicity of ultrafine TiO2 particles on Human lymphoblastoid cells. Considerable reduction in cell viability when TiO2 (130 _g/ml UF-TiO2) dose was increased; and 48-h exposure to this dose induced more than two folds increases in the mutation frequency [23].

The cytotoxic mechanism of nanoparticle is not clarified exactly. Some theories were hypothesized in this area and some of them were studied. The mechanism of nano-TiO2 was supposed to activate ROS system, such as superoxide radicals, hydrogen peroxide and free hydroxyl radicals [24].Geotoxic in Chinese hamster and mouse lymphoma cell lines [25] cytotoxic to cultured HeLa cells [26] human fibroblasts [27] and Chinese hamster ovary cells 28, and human colon carcinoma Ls-174-t cells 29are some examples of this mechanism. A study by T. Unchio [21] on hamster ovary indicated that titanium dioxide induced cytotoxicity by producing OH radical under UV-A irradiation and the formation of OH radical varies in both crystal size and crystal form. TiO2 toxicity depended on Ana crystal concentration but weakly on the intensity of UVA. In contrast, Rutile form didn't show any toxic effect on the target cell even with photoactivation [28,29].

It seems that, nano-TiO2 has no inflammatory effect or genotoxicity without ultraviolet (UV) irradiation [30], or DNA damages in human cells [27-31].In contrast, some others have shown a significant level of nano-TiO2 inflammation and cell apoptosis. [32, 33].Some other studies indicated that Anatase TiO2 (10-20nm) without photoactivation could induce oxidative damage DNA damage, lipid peroxidation and hydrogen peroxide production in human bronchial epithelial cells of human being generation, despite that Anatase dominant TiO2 in concentration of 200nm more did not show independent oxidative stress [11].

Chronic exposure even to subtoxic dose of nanoparticle was another striking and exciting aspect of our study. In our study, oxidative stress responses to a subtoxic dose of TiO2 were investigated after four consecutive passages prolonged approximately [25] days and it was shown that hepatocarcinoma cell lines endured the toxicological effect of subtoxic dose by increasing the time exposure. Increase in total cell protein, nitric oxide production and lipid peroxidation products as the indicators of cell injury responses reinforced the effect of dose-dependent and time-exposure relation with cytotoxicity of nanoparticles.

A study by A. Koeneman evaluated whether at 10µg/mL and above, TiO2 nanoparticles can cross the intestinal epithelial line by transcytosis and distrust membrane integrity or not. They controlled and examined the epithelial integrity by transepithelial electrical resistance (TEER) and detected that low concentrations (10 or 100µg/mL) of TiO2 did not cause any disruption of epithelial integrity and cell death after acute exposure.

Whereas six days chronic exposure of TiO2chronic dropped the TEER on the1000µg/mL significantly ($p<0.05$) below the control value and the cells did begin to recover, after time from this treatment[34].

Muhle conducted a chronic inhalation assessment. A 1.5, 6, and 24 mg/3m test toner, 40 mg/m3 for TiO2, and 3 mg/m3 for crystalline silica exposure of groups of Syrian golden hamsters for six h/day, five days/ wk for 18 mo were investigated.

Primary lung tumors were not detected in either control or toner-exposed groups. One small-sized bronchioloalveolar adenoma was found in a female group exposed TiO2 and one in a female of the SiO2-exposed group. Slight degree of interstitial fibrosis and chronic inflammation were detected after exposure to toner high (83%), to TiO2 (82%), and SiO2 (91%). [35].

A new investigation showed a disrupted the tight junctions-permeability barrier after an immediate exposure to 42 µg/mL TiO2 nanoparticles and after 4 h incubation time and an extensive effect was seen on membrane integrity at 24 h exposure [36].

Other new study in Argentina showed that neuroblastoma cell response to TiO2 NPs exposure is associated with increases in oxidative metabolism and cell apoptosis and ROS function where endoplasmic reticulum-mediated signal pathway supposed to be the main neurotoxic mechanism[37].

The new other results of Brandao and colleagues showed a concentration- time- and cell-type- dependent increase in cell uptake of TiO2 NPs but no considerable micronuclei induction and apoptosis was found in any of the tested conditions [38].During 90 days, percentage of cell viability after higher doses of TiO2-NPsexpousure was reduced both *in vitro* and *in vivo* [39] or exposure of TiO2 NPs can lead irreverisvle affects on to cytoskeleton of lung epithelial cells but not in cumulative dose [40].

It is clear that *in vitro* cytotoxic effect of nanoparticles on cellular systems will need to be further information , standardized and valid *in vivo* assays to provide useful screening data about nanoparticles cell toxicity.

Limitation

As we added Anatase crystal to hepatocarcinoma cell medium in 96 wells plates, we observed a non homogenous precipitation of TiO2 which made a layer on cell medium without complete cell surface coverage.

We tried to solve this problem by an excellent mixing of TiO2 in cell medium with a micro sampler.

Also, cell medium color was changed from red to white especially at higher dose than 3500-4500ppm and affected optic density read by plate reader. Thus, higher concentration (>3500-4500ppm) toxicity remained unclear

To solve the problem, we designed a protocol in which 5×104 cell were cultured in 6 wells plates and treated with adjusted doses of TiO2 for 24h , the cell samples after washing with PBS were trypsinized, centrifuged at 1000 rpm and accounted by Hemocytometer.

This process was conducted to estimate the exact number of cell viability after TiO2 exposure. Although a significant decrease of cell count was observed, titanium dioxide precipitation confounded our assessment by changes in the nanoparticle size and similar appearance to cell samples under a light microscope. So, we had to stand on the results of XTT assay.

The other problem was detected in the LDH assay performed on the collected cell medium. The results were in contrast to cell viability assay, and the amount of LDH released was gradually decreased at higher concentration of titanium dioxide.

To clarify the problem, we designed another assay. Pure LDH enzyme activity was assessed in the presence of a different concentration of TiO2. The precipitation of nanoparticle was appeared again and changed sample color, and the same results appeared. So we supposed that LDH assay might be interrupted by TiO2percipitation. This idea requires a precise evaluation.

CONCLUSION

These results suggest that chronic exposure, even to the safe doses of nanosized particles, can stimulate cellular oxidative and inflammatory responses.

When we exposed HepG2 samples with subtoxic

doses of TiO2, we had a significant challenge with the mentioned problem. In every passage, TiO2 was added to 5ml of cell media and were mixed by mixture to make a more homogenous form and then added to flasks. But we couldn't omit this problem completely, which might affect our data.

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DISCLOSURE/DECLARATION

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