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Toxicology

Titanium dioxide nanoparticles induce endothelial cell apoptosis via cell membrane oxidative damage and p38, PI3K/Akt, NF-κB signaling pathways modulation

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ABSTRACT

Background: Titanium dioxide nanoparticles (TiO_2 NPs) are widely used nanoparticles. Despite, several studies investigated the toxic effects of TiO_2 NPs on HUVECs, the results are contradictory and the possible underlying mechanisms remain unclear.

Methods: In the present study, we conducted an *in vitro* study to re-evaluate the possible toxic effects of TiO_2 NPs on HUVECs including cell viability, lipids peroxidation, intracellular signaling pathways and nitric oxide syntheses enzymes.

Results: Our results demonstrated that, TiO_2 NPs were internalized to HUVECs and induce intracellular reactive oxygen species production and cell membrane oxidative damage at the higher concentration. TiO_2 NPs induce IKKα/β and Akt phosphorylation and p38 dephosphorylation. After 24 h treatment, pro-inflammatory cytokines, adhesion molecules and chemokine upregulated significantly. TiO_2 NPs have no significant effects on eNOS enzymatic activation and *iNOS* gene expression. At cellular level, apoptosis is the main process that occur in response to TiO2 NPs treatment. HUVECs pretreatment with *N*-acetyl-L-cysteine (NAC) ameliorate the toxic effects of TiO_2 NPs that indicate the oxidative stress is essential in TiO_2 NPs -induced toxicity. Total antioxidant capacity show a trend to increase in response to TiO_2 NPs exposure.

Conclusions: Taken together, this study confirmed the effects of TiO_2 NPs on endothelial cells and proposed multiple underlying mechanisms including cell membrane oxidative damage and intracellular processes.

1. Introduction

Titanium dioxide (TiO_2) is considered as a safe material and has been approved for use in widespread applications such as food additive, cosmetics and industry [1]. However, several studies showed, TiO_2 exerts toxic effects on bio-systems at nanoscale [2,3]. Endothelial cells are susceptible to the toxicity of engineered metal nanomaterial which result in endothelial and vascular dysfunction [4]. The toxicity of TiO_2 NPs on endothelial and possible underlying mechanisms were reported previously [5–9], on the contrary, others demonstrated that TiO_2 NPs are safe nanomaterial for endothelial cells [10–12]. Therefore this controversy should be resolved, considering the importance of endothelial injury in the pathogenesis of vascular diseases and widespread application of TiO2 NPs.

The earlier reports which supported the TiO_2 NPs toxicity suggested oxidative stress and subsequent NF-κB signaling pathways activation, pro-inflammatory cytokines and adhesion molecules overexpression as underlying mechanisms [5–9]. These molecular alterations are common feature of endothelial cells response to the metal nanoparticle including silver, iron and etc [10,13]. It is well-documented that metal nanoparticles- induced toxicity mediated via free radicals production which originated from metal ions by fenton like reaction and mitochondrial dysfunction [14–18]. Oxidative stress play initiator role and trigger subsequent pro-inflammatory response and other events. On the other hand, titanium is a transition metal with four stable valence electrons which exhibit fenton reaction quenching property [19,20]. Therefore, there is doubt that, whether, titanium dioxide and/or released titanium ions are capable to produce reactive oxygen species or not.

Oxidative stress induce cell death via direct lipid peroxidation-cell membrane disruption and or by indirect cell signaling pathways modulation [21]. While the effects of TiO_2 NPs on lipid peroxidation and cell membrane integrity remain unclear.

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Metal nanoparticles activate NF-κB signaling pathways via oxidative stress however PI3K/Akt and p38 signaling pathways are sensitive to oxidative stress [22]. Therefore, we investigated the effect of TiO_2 NPs on different signaling pathways simultaneously. Davalos et al showed nitric oxide increased as cellular response after TiO_2 NPs treatment in parallel to free radical production [23]. Nitric oxide is produced by three enzymes called nitric oxide synthase including iNOS, eNOS and nNOS [24]. In the present study, we evaluate the effect of TiO_2 NPs on NOS enzymes and upstream PI3K/Akt signaling phosphorylation.

This study aims to resolved controversial aspect of TiO_2 NPs toxicity on HUVECs including whether TiO_2 NPs induce cell death? Apoptosis or necrosis which one is mechanism of the cell death? How and where free radical is produced? Which signaling pathway are affected by TiO_2 NPs-oxidative stress. What is the effect of TiO2 NPs on nitric oxide producing enzymes? As well as, the expression of chemokine, adhesion molecule and pro-inflammatory cytokines secretion were evaluated as HUVECs response to TiO_2 NPs toxic effects.

2. Material and methods

2.1. Nanoparticle characterization

Titanium (IV) oxide (anatase) powder were purchased from Sigma–Aldrich (USA). Nanoparticles were suspended in phosphate-buffered saline at pH 7.4 and dispersed by sonication for 30 s at 40 W. The size distribution and zeta potential of the nanoparticles were analyzed by dynamic light scattering (Nanotrac Wave, Microtrac). The morphology and size of nanoparticles were confirmed by transmission electron microscopy (Philips BioTwin, the Netherlands) and electron micrographs were obtained. UV–vis spectra of TiO_2 NPs obtained by absorption in the region of 200–600 nm using spectrophotometer (Analytik Jena SPEKOL 2000).

2.2. Cell culture

HUVECs were purchased from Pastor Institute, Tehran, Iran. Cells were maintained in DMEM high glucose medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 100 IU/ml streptomycin-penicillin (Gibco, USA) at 37 °C in 5% humidified CO_2 incubator. Cell were treated with TiO_2 NPs at different concentration (0, 1, 5, 25, 50, 100 μg/ml) for 24 h. A group of cells were pretreated with *N*-acetyl-L-cysteine (NAC, Sigma, USA)) at 10 mM for 4 h and then were treated with TiO_2 NPs (100 μg/ml). NAC was used for confirmation of oxidative stress in TiO_2 NPs toxicity.

2.3. Cell viability assay

For cell viability assay, cells were seeded in 96-well plates at 10,000 cells per well. Cell were treated with TiO_2 NPs for 24 h. Serum starved medium (%1 FBS) and DMSO (10%) used as negative and positive control, respectively. The WST-1 tetrazolium salts were added to wells. Viable and metabolically active cells were determined based on the tetrazolium salt/formazan system. After 4 h incubation with WST-1, optical density was measured at 490 nm using a micro plate reader.

2.4. Lactate dehydrogenase assay

Lactate dehydrogenase is a ubiquitous enzyme that release into the cell supernatant after cell membrane damage. Cells were treated with TiO_2 NPs and Lactate dehydrogenase activity were determined using Cytotoxicity Detection Lactate Dehydrogenase kit (Roche Applied Science, IN, USA) following the manufacturer's instructions. Serum starved medium (%0.1 BSA) and Triton –X- 100 (2%) used as negative and positive control, respectively.

2.5. Nanoparticle uptake

For nanoparticle internalization assessment, intracellular titanium content was determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 730-ES). Briefly, the cells were seeded in 6 well plate (1×10^5 cells per well) and treated with different concentration of TiO_2 NPs. The nanoparticle-containing culture media was removed after 24 h incubation and cells were washed 3 time with PBS. Cells were trypsinized and harvested. The lysate were obtained by incubation of the harvested cells in 45% HNO_3 for 1 h at 96 °C. Titanium concentration was measured by ICP-OES. The standard curve and calculations performed using TraceCERT® reference materials. All samples diluted in the 5 ml as final volume and injected into ICP –OES. A group of cells which treated with nanoparticles free media used as control.

2.6. Cell apoptosis assay

The effect of TiO_2 NPs on cell apoptosis was assessed by annexin V-FITC and propidium iodide (PI) kit (Roche Applied Science, Germany). Briefly, cells were seeded in 6-well plate and were treated with TiO_2 NPs for 24 h. Serum starved medium (%1 FBS) and TNF-α (2 ng/ml) used as negative and positive control, respectively. Cells were trypsinized and collected by centrifugation. The cells were washed twice time with PBS and were resuspended in reaction buffer containing annexin V fluorescein and PI. After 15 min incubation, the samples were transferred into flow cytometer tube and were analyzed by a flowcytometry (Partec PAS) using 488 nm excitation and 515 nm and 600 nm band pass filters for fluorescein and PI detection, respectively.

2.7. DNA fragment assay

DNA fragmentation is biochemical feature of apoptotic cells occur result in the caspase cascade activation leading to cleavage of DNA to ˜200 base pairs fragments that known as DNA ladder which examined via 2% agarose electrophoresis. Cell were treated and DNA extraction were performed via phenol–chloroform extraction and ethanol precipitation. The DNA samples were separated by electrophoresis (Elchrom Scientific ORIGINS 2100U) on 2% agarose gel. The DNA pattern visualized by safe stain under UV geldoc instrument (Bioer, China).

2.8. Intracellular ROS measurement

The effects of TiO_2 NPs on intracellular ROS content was assessed by 2, 7-dichlorofluorescein diacetate activation (DCFH-DA) (Sigma, USA). Briefly, HUVECs were treated with TiO_2 NPs for 24 h. Serum starved medium (%1 FBS) and H_2O_2 (3%) used as negative and positive control, respectively. Then, the cells were incubated with PBS containing 10 mM DCFH-DA for 30 min at CO_2 incubator. After incubation, the cells were washed 3 time with PBS and fluorescence intensity was measured by flowcytometry and observed by fluorescent microscope (japan MT 6000).

2.9. Lipid peroxidation

Lipid peroxidation were evaluated by spectrophotometrically measuring of MDA in cell supernatant and cell lysate. Briefly, samples (500 μl) and trichloroacetic acid (500 μl, 10% w/v) were added to in 1.5 ml microfuge tube. After centrifugation (3000 rpm, 10 min) 0.67% thiobarbituric acid were added to acidified samples, mixed and boiled in water bath (96 °C for 30 min). Then 200 μl of heated mixture were transferred to ELISA plate and optical density were measured at 531 nm wavelength. The amount of lipid peroxidation were calculated using molar extinction coefficient of MDA (1.56×10^5) and dilution coefficient. For cell lysate samples, calculated MDA concentration were adjusted to the protein concentration.

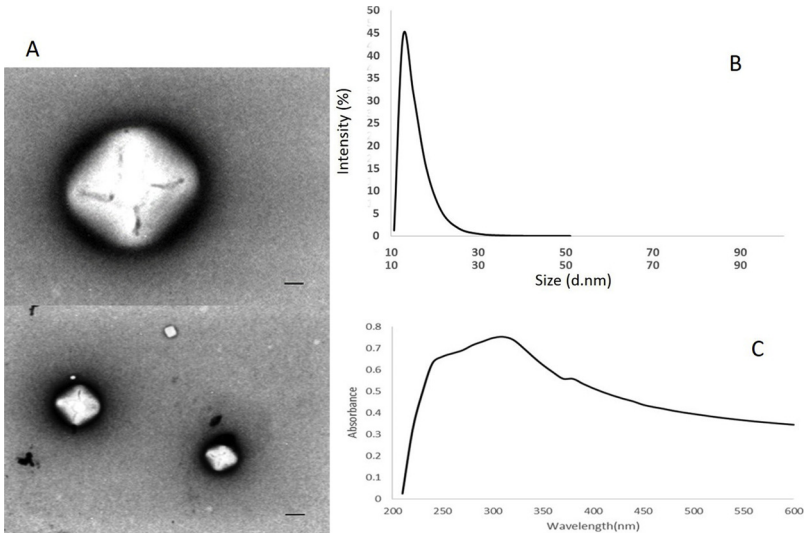

Fig. 1. (A). TEM microscopy image of tetragonal deuteropyramid structure of anatase TiO2 NPs. (B). The particle-size distribution of TiO2 NPs (C). The UV–vis absorption spectrum of TiO2 NPs.

2.10. Total antioxidant capacity

The effect of TiO_2 NPs on total antioxidant capacity of cell supernatant were determined according to Benzie and Strain method [25]. The reduction of ferric to ferrous and formation of ferrictripyridyltriazine complex in acidic condition indicate antioxidant capacity of cell culture media. Acetate buffer (300 mM, pH = 3.6), tripyridyltriazine (10 mM) and $FeCl_3$ (20 mM) used for FRAP reagent reconstitution and were added to cell culture supernatant (160 µl buffer for 80 µl sample) in ELISA plate and optical density were measured at 600 nm. The cell supernatant obtained from cells treated with TiO_2 NPs, NAC and 1% H_2O_2 and untreated control cell. The obtained optical density of treated sample were divided by untreated control sample and considered as relative total antioxidant capacity.

2.11. Western blot analysis

For analysis of IKKα/β, p38, Akt and eNOS phosphorylation, cell lysate was prepared using RIPA lysis buffer (50 mM Tris − HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton. × 100, 0.1% SDS 1% Sodium deoxycholate) containing anti-protease (PMSF, aprotinin, leupeptin at 1 µg/ml concentration) and phosphatase inhibitors (50 mM NaF, 0.5% Sodium Orthovanadate). The concentration of the lysate protein was determined using Bradford method. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membrane. Blocking was performed with 3% BSA in TBST for 2 h and the PVDF membrane were incubated with anti-IKKα/β, anti-P- IKKα/β, anti-AKT, anti-p-AKT, anti-p-eNOS and anti -eNOS for overnight at 4 °C. For primary antibodies detection, horseradish peroxidase conjugated anti-rabbit IgG and anti-mouse IgG secondary antibody were used. After each step, PVDF membrane were washed with TBST (3 time for 5 min) and TBS (1 time for 5 min) buffers. The bands were visualized by enhanced chemiluminescence and X-ray film and quantified by densitometry using ImageJ software (https://imagej.nih.gov/ij/).

2.12. Pro-inflammatory cytokines measurement

The effect of TiO_2 NPs on proinflammatory cytokine secretion were evaluated. HUVECs were treated with TiO_2 NPs and LPS (Sigma-Aldrich Lipopolysaccharides Escherichia coli) for 24 h and cell supernatant was collected. The IL-6 and TNF-α were measured using ELISA Kits (IBL International GMBH, Germany) according to manufacturer's instructions. Briefly, standards were prepared by serial dilution. Samples and standards were added to each well and incubated for 1 h at room temperature. Primarily and secondary antibodies and tetramethylbenzidine substrate used for detection of target antigens. Stop solution was added and optical density was measured by micro plate reader at 450 nm.

2.13. RT-PCR

RT-PCR was used to adhesion molecule, chemokine and *iNOS* gene expression analysis. The HUVECs were treated with TiO_2 NPs at different concentration and LPS as positive control. After 24 h incubation, total RNA was extracted by a commercial RNA extraction kit (GeneAll Biotechnology, South Korea) according to manufacturer's protocol. RNA was quantified by spectrophotometer (NanoDrop, Thermo Fischer Scientific) and RNA quality was assessed using agarose gel electrophoresis and visualization of 28S and 18S ribosomal RNA bands. For cDNA synthesis, equal amounts of RNA transcripted into cDNA using the cDNA Synthesis kit (GeneAll Biotechnology, South Korea). *MCP-1*, *ICAM* and *iNOS* mRNA relative expression were measured by semi-quantitative RT-PCR through gel electrophoresis of the amplicons, β-actin as an internal standard and measuring the density of the bands by the ImageJ software.

2.14. Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS 16.0). The statistical comparisons of means were performed using a one-way analysis of variance (ANOVA). A value of $p < 0.05$ was accepted as significantly different.

3. Results

The experiments were performed in triplicate independently. All data were expressed as the mean ± SEM where * show significant difference between treated and untreated control ($p < 0.05$, $n = 3$) and # indicate significant difference between 100 µg/ml TiO2 NPs with and without NAC, $p < 0.05$, $n = 3$)

3.1. Nanoparticle size and zeta potential and UV–vis spectra

The size distribution of particles, UV–vis absorption spectra and TEM microscope image of TiO_2 NPs are shown in Fig. 1(A, B, C). The UV–vis absorption spectra show a peak at 310 nm indicating that TiO2

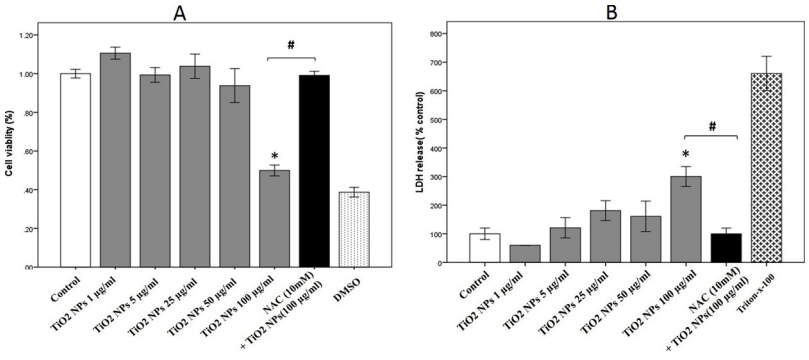

Fig. 2. (A). The toxicity of TiO2 NPs on HUVEC cell line measured by WST-1 assay. 2 (B). LDH activity in cell supernatant as cell membrane indicator (Bar: mean ± SEM, the * show significant difference treated vs untreated control and # significant difference between TiO_2 NPs (100 µg/ml) with vs without NAC ($p < 0.05$, $n = 3$).

NPs absorbed light in ultraviolet range (200–350 nm). DLS results showed the size of nanoparticles ranged from 10.74 to 30.41 nm that confirmed company's product data and the zeta potential of TiO_2 NPs in PBS was +2.6 mv that indicate instable colloidal dispersions. Therefore, for preparation a stable dispersed working solution, dilution performed immediately after sonication in 1% FBS-contained media.

3.2. Cell viability

WST-1 assay showed that the treatment of HUVECs with TiO_2 NPs at the concentration of 100 µg/ml for 24 h reduced cell viability but had no significant effect at the lower concentration (Fig. 2A). The TiO_2 NPs-induced cell death were ameliorated by NAC pretreatment. LDH assay confirmed WST-1 results that demonstrated cell membrane integrity were affected by TiO_2 NPs in similar pattern. The LDH activity were increased in supernatant of TiO_2 NPs (100 µg/ml) treated cells than untreated cells. TiO_2 NPs at the lower concentration (< 100 µg/ml) had no significant effect on LDH release. The protective effect of NAC against TiO_2 NPs-induced toxic effects was also confirmed in LDH assay (Fig. 2B).

3.3. Cell apoptosis

The effects of TiO_2 NPs on cell apoptosis were evaluated by DNA fragmentation assay and flowcytometric analysis. The results of DNA fragmentation assay showed a typical DNA ladder pattern which indicated that TiO_2 NPs induced apoptosis at the higher concentration (50 and 100 µg/ml) and NAC pretreatment prevents apoptotic DNA fragmentation (Fig. 3A). Fig. 3B–D) depicts flowcytometry results that showed TiO_2 NPs increased early and total apoptosis in concentration dependent manner. As shown in figures, NAC pretreatment reduced TiO_2 NPs -induced apoptosis significantly.

3.4. Nanoparticle internalization

Fig. 4 represent the intracellular titanium content measured by ICP-OPE after TiO_2 NPs treatment. The results showed that TiO_2 NPs internalization occurred in concentration dependent manner. Intracellular titanium content was 0.038, 0.186, 0.170, 0.561 and 0.807 ppm after treatment with TiO_2 NPs at the 1, 5, 25, 50, 100 µg/ml, respectively.

3.5. Intracellular ROS measurement

The fluorescence probe, DCFDA activated by hydrogen peroxide, peroxynitrite and/or hydroxyl radical which considered as intracellular redox state [26]. The fluorescence intensity of DCFDA were observed by fluorescence microscopy that showed TiO_2 NPs increased intracellular ROS Levels (Fig. 5A). The flowcytometric analysis of DCFDA indicated that intracellular ROS levels increased after treatment with TiO_2 NPs at the 100 µg/ml concentration and NAC pretreatment reduced TiO_2 NPs -induced ROS production significantly (Fig. 5B).

3.6. Lipid peroxidation

Cellular membrane lipids are susceptible target molecules for oxidative damage and MDA is widely used biomarker for lipid peroxidation [27]. Intracellular MDA levels (adjusted to total protein concentration), increased after treatment with TiO_2 NPs (100 µg/ml) and NAC pretreatment reduced MDA levels (Fig. 6A). MDA measurement in cell supernatant also showed TiO_2 NPs (50 and 100 µg/ml) lead to a significant increase in MDA levels which were ameliorated by NAC pretreatment (Fig. 6B).

3.7. Total antioxidant capacity

Total antioxidant capacity were measured in cell supernatant which were obtained from TiO_2 NPs treated and untreated cells. There is no significant change in total antioxidant capacity of cell supernatant after 24 h treatment with TiO_2 NPs (Fig. 7). However the antioxidant capacity of cell supernatant have a trend to increase after TiO_2 NPs treatment. The effect of TiO_2 NPs on total antioxidant capacity of cell free media were measured after overnight incubation that showed any significant change in total antioxidant capacity (data not shown).

3.8. IKKα/β, Akt/eNOS and p38 signaling pathways

Several signaling pathways were investigated in order to identify possible underlying mechanisms of TiO_2 NPs -induced HUVECs toxicity. Our results showed IKKα/β and Akt proteins were phosphorylated and p38 were dephosphorylated by TiO_2 NPs in concentration dependent manner. NAC pretreatment reversed the TiO_2 NPs-induced signaling pathways regulation but exacerbated the dephosphorylation of p38 (Fig. 8A–E). Despite the activation of Akt, the phosphorylation of eNOS were not changed after treatment with TiO_2 NPs.

3.9. Gene expression profiling

A critical point for regulation of *ICAM*, *MCP-1* and *iNOS* is gene expression regulation. The effect of TiO_2 NPs on genes expression showed the *ICAM* expression induced by TiO_2 NPs in concentration dependent manner and NAC pretreatment were not reversed the induction of this gene. Although previous studies demonstrated that *iNOS* gene did not expressed in HUVECs but for precise evaluation of TiO_2 NPs on nitric oxide system, we evaluated *iNOS* expression after treatment with TiO_2 NPs that indicted *iNOS* were not expressed in treated and untreated cells. *MCP-1* gene expression were induced by TiO_2 NPs and effects reversed by NAC pretreatment (Fig. 9 A–C)

3.10. Proinflammatory cytokine secretion

The measurement of TNF-α and IL-6 in cell supernatant by ELISA

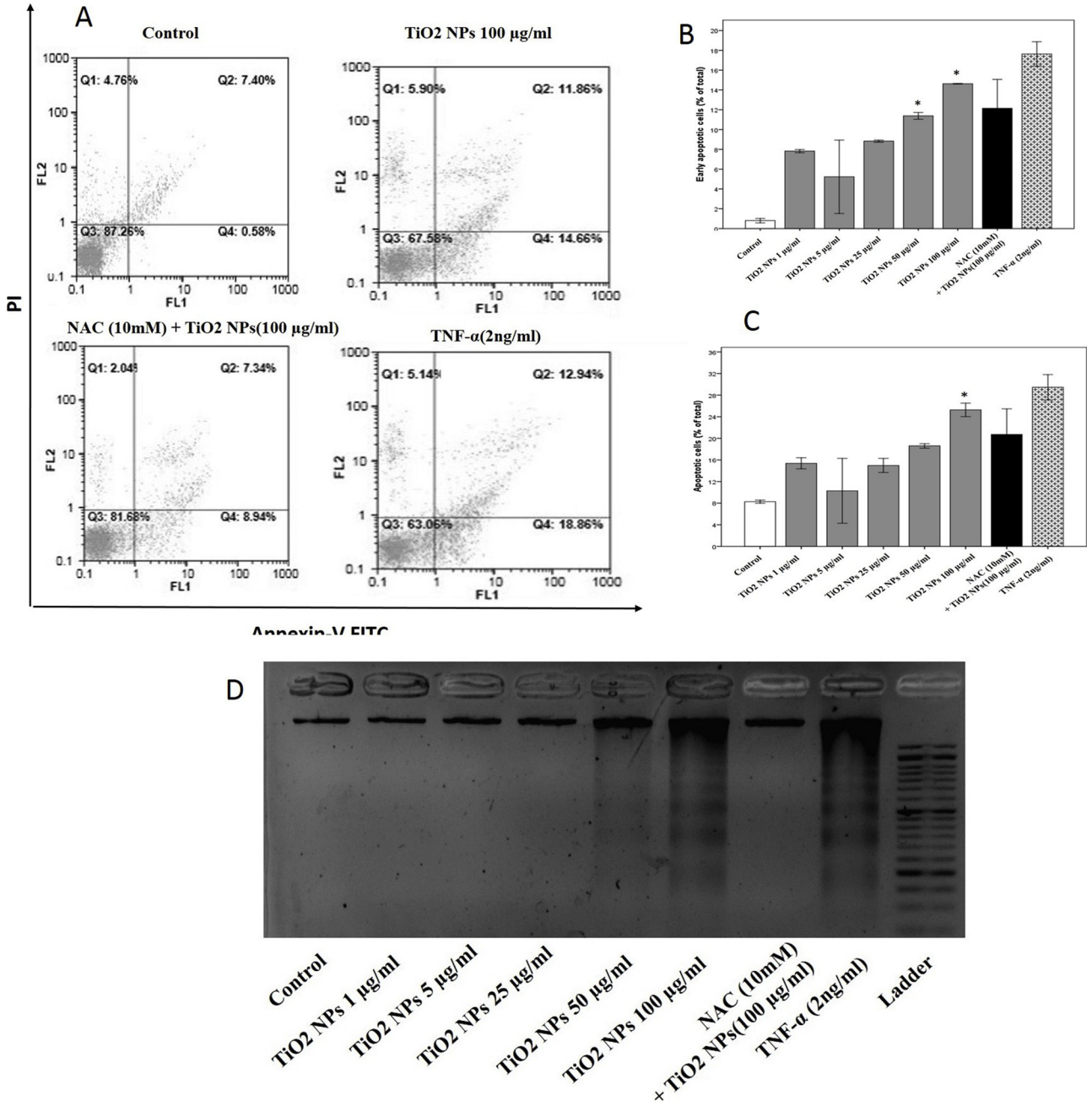

Fig. 3. (A). HUVECs apoptosis detection after exposure to TiO2 NPs via Annexin V/propidium iodide stinging. Annexin V positive with and without propidium iodide positive indicate late apoptosis and early respectively, (B) the effect TiO2 NPs of early HUVECs apoptosis, (C) the effect TiO2 NPs of total HUVECs apoptosis (early + late apoptosis), (D) The effect of TiO2 NPs on DNA fragmentation. TiO2 NPs (50–100 µg/ml induce apoptosis and the effects reversed by NAC. (Bar: mean ± SEM, the * show significant difference treated vs untreated control and # significant difference between TiO_2 NPs (100 µg/ml) with vs without NAC ($p < 0.05$, n = 3).

demonstrated that TiO_2 NPs induce proinflammatory cytokine production significantly. Inflammatory cytokine levels were increased in concentration dependent manner and were reduced by NAC pretreatment that depicted in Fig. 10A, B.

4. Discussion

At the present, the TiO_2 NPs toxicity on endothelial cells remain controversial and the precise underlying molecular and cellular mechanisms are still not well understood. Our results support the potential toxicity of TiO_2 NPs on HUVECs. We suggest that TiO_2 NPs affect HUVECs in two distinct way including membrane associated events and intracellular processes. Nanoparticles may disrupt the cell membrane by interfering with the hydrophobic interaction or oxidation of double bonds of polyunsaturated fatty acids in cell membrane lipid bilayer and subsequent disturbance in the membrane fluidity-permeability and ion gradient distribution [28]. The results of LDH assay and increased late apoptosis demonstrate that cell membrane permeability may increase after treatment with TiO_2 NPs. Although we did not know the share of each lipid peroxidation and hydrophobic interfering in lipid bilayer disruption but increased MDA levels and ameliorative effects of NAC on LDH activity confirm the pivotal role of lipids peroxidation on TiO_2 NPs –induced HUVECs death. The DCFDA analysis demonstrate that the intracellular ROS elevate after treatment with TiO_2 NPs. Therefore the

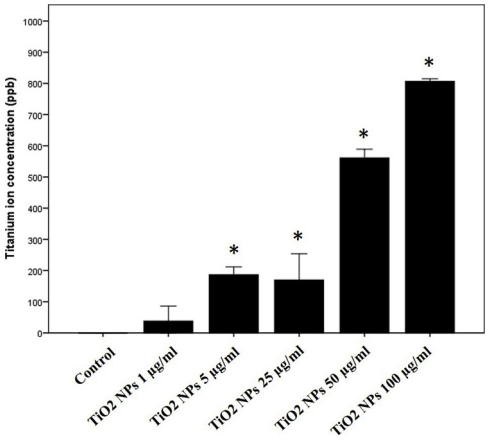

Fig. 4. Nanoparticle internalization were obtained by ICP- OES. (Bar: mean ± SEM, the * show significant difference treated vs untreated control ($p < 0.05$, $n = 3$).

oxidative stress may occur in intracellular space and probably induce oxidative damage in the inner side of the cell membrane.

Similar to other metal nanoparticles such as gold, silver and iron nanoparticles the internalization of nanoparticles or internalization of nanoparticles-shedding metal ions, is a key factor in the toxicity of TiO_2 NPs [29–31]. Our results show, both nanoparticles internalization and the toxicity occur in concentration dependent manner. Therefore, probably the intracellular ions are responsible for oxidative stress and toxicity. It is well documented that the nanoparticle internalization is a function of concentration, size, and surface charge [32]. Also Shi et al, demonstrated that NAC prevent silver nanoparticle internalization but we did not observed any significant reduction in nanoparticle internalization via 10 mM NAC pretreatment [13,33]. Therefore, the reduction of TiO_2 NPs by NAC confirm the oxidative stress as underling molecular mechanism.

Currently, there are several mechanisms to describe free radical production by metal nanoparticles; first, surface functional group mediated radical generation via fenton like reaction [34]. The second mechanism is prooxidant and antioxidant enzymes regulation [35]. The induction of mitochondrial dysfunction and endogenous ROS production considered as third possible mechanism [36]. In the case of TiO_2 NPs, the first mechanism is less possible because Tengvall et al's study showed Ti and TiO_2 could not induce oxidative stress and free radical formation due to TiOOH adduct formation and quenching of the Fenton reaction [20]. Our study showed, there is increasing but insignificant trend in total antioxidant capacity in the cell supernatant. We suggest that the increasing trend of antioxidant capacity may due to induction of antioxidant enzymes in the first hours of TiO_2 NPs treatment when the produced ROS is not sufficient to result in cell death [37]. Therefore the event priority order of TiO_2 NPs toxicity is ROS production, antioxidant enzymes induction, excess ROS production and oxidative stress because ROS serve as a force for induction of antioxidant enzymes. In this regards Kim et al demonstrate that TiO_2 NPs induce oxidative stress via induction of prooxidant enzymes and suppression of antioxidant enzymes [38]. Although our study did not analyze the TiO_2 NPs effects on mitochondrial structure and function but we suggest the mitochondrial dysfunction as initial source of ROS and oxidative stress.

Nitric oxide is a double edged sword in endothelial system which induce and inhibit the cell apoptosis through different pathways [39]. As well as, nitrosative stress alongside oxidative stress involve in cardiovascular system pathogenesis [40]. In pathologic condition, NOSs enzymes activity enhance nitrosative stress by uncoupling of superoxide production from nitric oxide production [41]. Davalos et al's study showed TiO_2 NPs lead to nitric oxide overproduction while our results showed TiO_2 NPs have no significant effect on eNOS phosphorylation and *iNOS* gene expression [23]. In the HUVEC line, the basal *iNOS* expression is very low and undetectable but eNOS activity is significant and is controled by Akt signaling [42,43]. In our study, Akt undergo phosphorylation without eNOS activation. We evaluated p38 phosphorylation that indicated TiO_2 NPs lead to a down regulation of p38 signaling pathway. Anter et al, reported that p38 activation is essential and require for Akt mediated eNOS activation [44]. Therefore, inactive eNOS in presence of active Akt may be due to inactivation of p38 signaling.

The RT-PCR analysis for *iNOS* gene expression showed no baseline expression of *iNOS* without any change after TiO_2 NPs treatment. Taken together, these results suggest that nitric oxide over production or nitrosative stress may not mediate the TiO_2 NPs toxicity in HUVECs. Meanwhile the possible catastrophic effects of nitric oxide down regulation and/or un-bioavailability remain unclear and need further comprehensive study [23].

Flowcytometric apoptosis analysis and DNA fragmentation assay showed, apoptosis is main process occur at cellular levels. Although WST-1 and LDH assay did not showed significant cell death at the 5–50 µg/ml concentration but 15–25% apoptosis were observed at this concentration that may be due to the sensitivity and accuracy of techniques [45]. Here we propose some mechanism for TiO_2 NPs-induced apoptosis. Mitochondrial membrane oxidative damage may initiate the intrinsic apoptosis pathway and/or signalling transduction mediated extrinsic apoptosis activation [46]. Davalos et al and Han et al

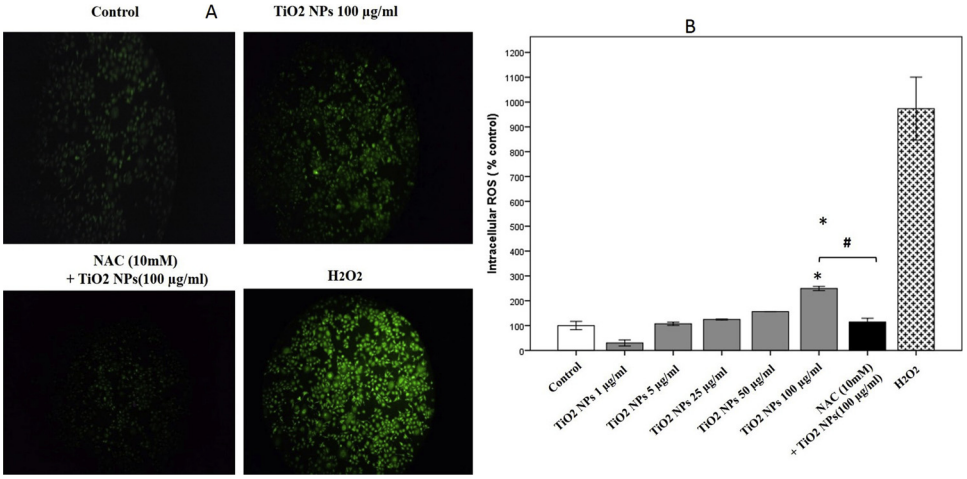

Fig. 5. (A). The image of DCFDA activation obtained from fluorescent microscope (B) Intracellular ROS in HUVECs measured by flowcytometry. (Bar: mean ± SEM, the * show significant difference treated vs untreated control and # significant difference between TiO_2 NPs (100 µg/ml) with vs without NAC ($p < 0.05$, $n = 3$).

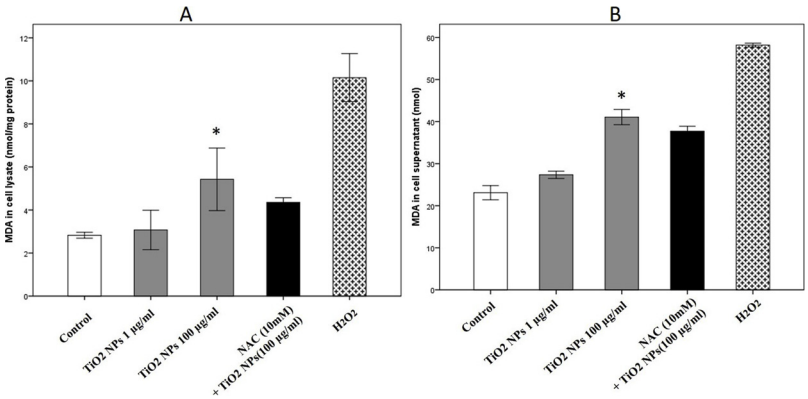

Fig. 6. (A). MDA levels in cell lysate adjusted to total protein, (B) MDA levels in cell supernatant adjusted cell count and media volume. (Bar: mean ± SEM, the * show significant difference treated vs untreated control ($p < 0.05$, n = 3).

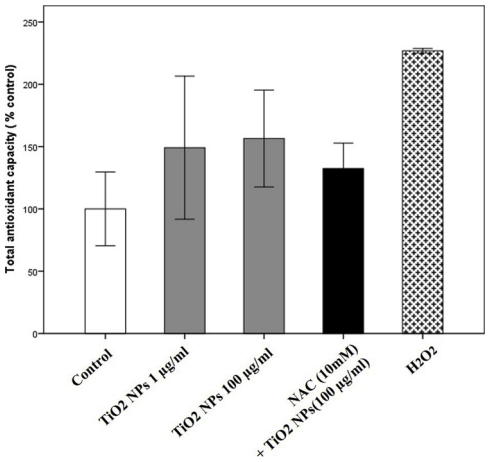

Fig. 7. The effect of Tio2 NPs on total antioxidant capacity of cell supernatant after 24 h exposure (no significant change).

investigated the effect of TiO_2 NPs on HUVECs previously [8,23]. There is consensus that post- TiO_2 NPs induced oxidative stress events are the induction of IKKα/β/NF-κB pathway, inflammatory factors and adhesion molecules expression. Our results showed that TiO_2 NPs upregulate the *MCP-1* as chemokine and *ICAM* adhesion molecule and TNF-α and IL-6 secretion as pro-inflammatory factors in consistent with previous studies. There is no doubt about the pro-apoptotic role of adhesion molecules and pro-inflammatory factors but the precise mechanisms remain unclear. Although, Han et al reported simultaneous activation of ERR1/2, p-38, JNK and Akt signaling by TiO_2NPs but our results showed Akt and IKKα/β were phosphorylated and the p38 were dephosphorylated in concentration dependent manner. There are reports that ROS-induced HUVECs apoptosis mediated via dephosphorylation of ERK1/2 and phosphorylation of p38 signaling pathway [47,48]. While Akt phosphorylation facilitate cell proliferation but activation of Akt is not sufficient for endothelial cell proliferation [49]. Therefore, we conclude TiO_2 NPs-induced p38 dephosphorylation in crosstalk with IKKα/β/NF-κB may lead to proapoptotic events. NF-κB signaling pathway play a double role in cell fate but in TiO_2 NPs- induced HUVECs apoptosis, NF-κB signaling exhibit anti-proliferative properties that probably mediated via of pro-inflammatory cytokine and adhesion molecules production [50]. As well as, we suggest that Akt activation as

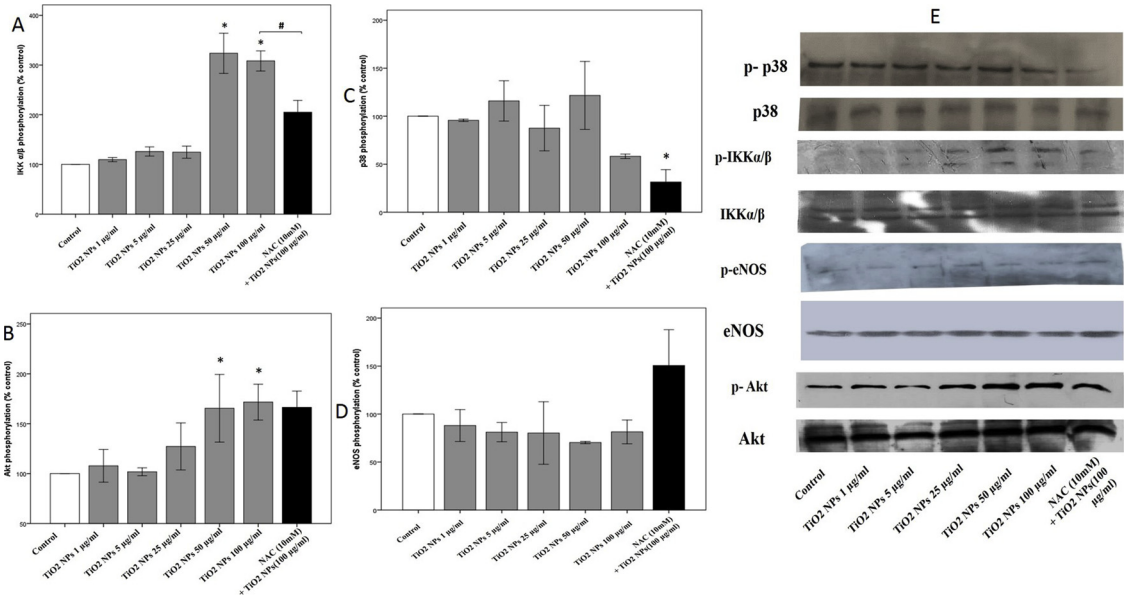

Fig. 8. The effect of TiO2 NPs on signaling pathways. (A) IKK phosphorylation (B) Akt phosphorylation (C) p38 phosphorylation (D) eNOS phosphorylation (E) western blot film (*(Bar: mean ± SEM, the * show significant difference treated vs untreated control and # significant difference between TiO_2 NPs (100 µg/ml) with vs without NAC ($p < 0.05$, n = 3).

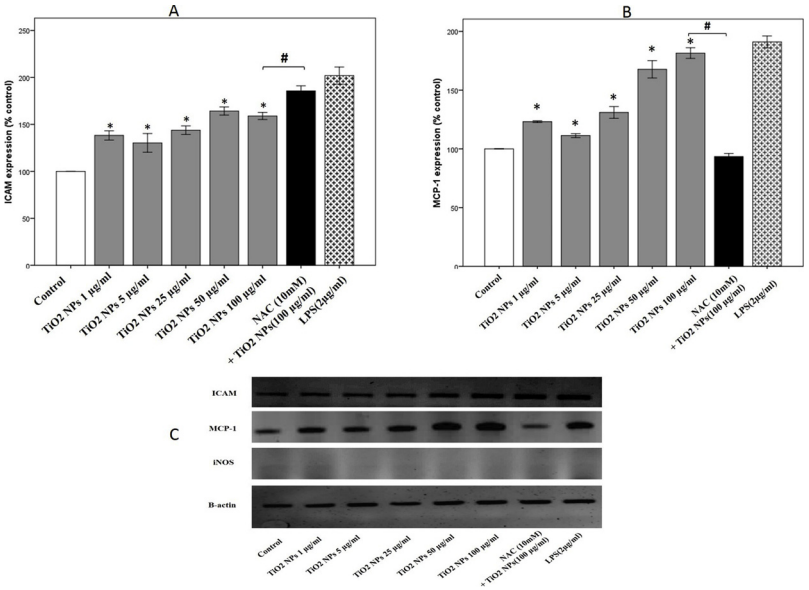

Fig. 9. The effect of TiO2 NPs on gene expression profile. (A) ICAM gene expression as adhesion molecule, (B) MCP-1 as inflammatory chemokine, iNOS gene expression involved in NO production and beta actin as housekeeping gene without significant change (C) RT PCR products (Bar: mean ± SEM, the * show significant difference treated vs untreated control and # significant difference between TiO_2 NPs (100 μg/ml) with vs without NAC ($p < 0.05$, n = 3).

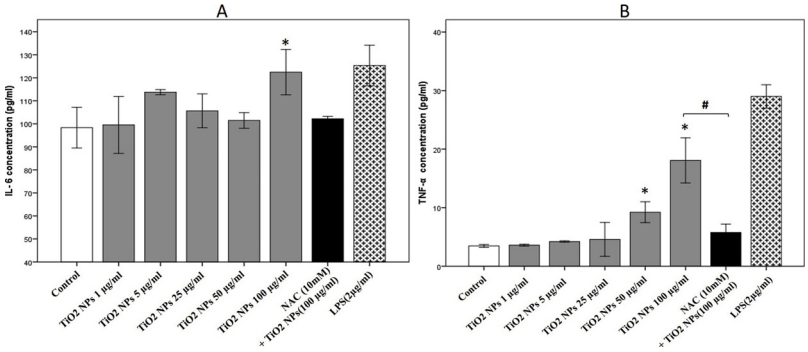

Fig. 10. (A). IL-6 levels in cell supernatant exposed to TiO2 NPs (B) TNF- α levels in cell supernatant exposed to TiO2 NPs. (Bar: mean ± SEM, the * show significant difference treated vs untreated control ($p < 0.05$, n = 3).

a cell survival signaling pathway, could not compensated total cell death forces including cell membrane associated process, inflammation and other intracellular processes.

5. Conclusions

In conclusion, our finding confirmed the results of previous studies which reported TiO_2 NPs exert toxic effects on endothelial cell line via nanoparticle internalization, oxidative stress, inflammation and ultimately apoptotic cell death. We also showed lipids peroxidation in inner semi-layer may contribute in the TiO_2 NPs toxicity. Titanium ion induce oxidative stress probably via mitochondrial dysfunction and pro-antioxidant enzymes regulation. TiO_2 NPs simultaneously upregulated NF-kB and Akt (proliferative signal) but downregulated p38 (anti-proliferative signal) which the total force of these changes lead to cell apoptosis. As well as nitric oxide and NOSs are not involved in TiO_2 NPs toxicity.

Competing interests

The authors declare no financial and non-financial conflict of interest.

Authors' contributions

Zafar gholinejad performed experimental tests and statistical analysis and Dr Yousef Rasmi contribute in study design and supervision. The results published here retrieved from Zafar gholinejad Ph.D thesis conducted at Urmia University of medical science.

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