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Full Length Research Paper

Biochemical responses of nanosize titanium dioxide in the heart of rats following administration of idepenone and quercetin

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Concerns with the environmental and health risk of widely distributed, commonly used nanoparticles are increasing. As titanium dioxide nanoparticles (TiO_2 NPs) are widely used commercially, their potential toxicity on human health has attracted particular attention. In this paper the *in vivo* acute toxicity of nano-sized TiO_2 particles (80 nm) to adult rats was investigated before and after treatment with either idebenone or quercetin. Rat groups were orally administered at low (600 mg/kg bw) and a high dose (1 g/kg bw) n-TiO_2, and the effect of these nanoparticles before and after treatment with either antioxidants was evaluated through measurement of serum tumour necrosis factor (TNF)-α, C-reactive protein, vascular endothelial growth factor (VEGF), immunoglobulin G (IgG), interleukin 6 (Il-6), troponin, myoglobin, creatine kinase-MB (CK-MB) and nitrite levels. In addition, Ca and caspase-3 were measured in the hearts of these animals. DNA damage was also detected using Comet assay. The results showed that both doses of n-TiO_2 caused an elevation in all serum and tissue parameters, which increased with increasing dose. Treatment with either idebenone or quercetin significantly reduced these elevated levels, the improvement being more pronounced with the lower dose. In conclusion, this study aims to give some insight on the toxicity and tissue distribution of orally administered TiO_2 nanoparticles through measurement of an extended set of biochemical parameters in serum and heart tissue to gain information on potential pathological changes after administration of NP-TiO_2. In addition, the protective role of idebenone and quercetin, as therapeutic agents to ameliorate these changes were evaluated.

Key words: TiO_2 nanoparticles, idebenone, quercetin, oxidative stress, cardiovascular diseases.

INTRODUCTION

Although, the benefits of nanotechnology, the nano-material hazards is just beginning to emerge (Horie et al., 2010). The novel physical and chemical properties of these nanoparticles make them attractive for use in medical, agricultural, industrial, manufacturing, and military sectors. Although more and more nanoparticles enter the environment with the increasing development of nanotechnology, little is known of their interactions with biological systems. The small size and large surface area endow them with an active group or intrinsic toxicity. Since nanoparticles diameter does not exceed a hundred nanometers at maximum, they are able to penetrate cells and interfere with several subcellular mechanisms (Park et al., 2007). Indeed, some studies show that some nanoparticles can penetrate into cell nuclei and hence may directly interfere with the structure and function of

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genomic DNA (Chen and von Mikecz, 2005). Several studies have reported that inhaled or injected nanosize particles enter systemic circulation and migrate to various organs and tissues (Wang et al., 2007) where they could accumulate and damage organs and biological systems that are especially sensitive to oxidative stress (Møller et al., 2010).

Due to its excellent optical performance and electrical properties, titanium dioxide (TiO_2) has a wide range of applications in many fields for diagnostic or therapeutic tools (Shen et al., 2010). Titanium dioxide nanoparticles ($TiO_{(2)}$ NPs) are now in daily use including popular sunscreens, toothpastes, and cosmetics (Hu et al., 2010) and in the environmental decontamination of air, soil, and water (Esterkin et al., 2005; Choi et al., 2006).However, some studies have reported that nano-sized TiO_2 are widely used commercially and may generate potential harm to the environment and humans (Colvin, 2003; Chen et al., 2009; Bu et al., 2010). Such widespread use and its potential entry through dermal, ingestion, and inhalation routes suggest that nanosize TiO_2 could pose an exposure risk to humans, livestock, and eco-relevant species that are sensitive to oxidative stress (OS) damage (Warheit et al., 2007a; Warheit et al., 2007b). In addition, numerous *in vitro* studies have reported OS-mediated toxicity by TiO2 in various cell types (Wang et al., 2007; Gurr et al., 2005).

When mitochondrial bioenergetic pathways involving oxidative phosphorylation, glycolysis, among others are severely depressed, this would create levels of reactive oxygen species (ROS) and ultimately OS in the cell. ROS have been known to play an important role in the pathogenesis of atherosclerosis and several other cardiovascular diseases. It is now apparent that ROS induce endothelial cell damage and vascular smooth muscle cell (VSMC) growth and cardiac remodeling, which are associated with hypertension, atherosclerosis, heart failure, and restenosis (Long et al., 2007).

Idebenone is an analogue or synthetic equivalent of life's most essential bio-chemicals Coenzyme Q10, (CoQ10) also known as Ubiquinone. In nature, CoQ10 is a vitamin-like compound found in all aerobic organisms that plays a pivotal role in cellular energy production. It is also an effective antioxidant, and has been known for almost thirty years for a number of therapeutic effects including its ability to improve the effects from heart failure, reduce free radical damage, enhance general cerebral metabolism and slow down specific neurological conditions (Fariss et al., 2005). Notably, idebenone is more effective than CoQ10 in protecting cells from the free radical damage resulting from reduced blood flow and resultant low-oxygen environment, and more beneficial than CoQ10 to certain types of noxious free-radical leakage in the cell's mitochondria. One of the superior features of Idebenone is that it performs well in low oxygen environments, protecting the cells from free-radical damage (Orsucci et al., 2011).

Quercetin, on the other hand, belongs to a group of plant pigments called flavonoids that give many fruits, flowers, and vegetables their color. Flavonoids have been proposed to have anti-atherogenic, anti-inflammatory, and anti-hypertensive properties leading to the beneficial effects against cardiovascular diseases (Becker et al., 2010; Ishizawa et al., 2011). Flavonoids, such as quercetin, are antioxidants -- they scavenge damaging particles in the body known as free radicals, which damage cell membranes, tamper with DNA, and even cause cell death. Antioxidants can neutralize free radicals and may reduce or even help prevent some of the damage they cause. Quercetin acts like an antihistamine and an anti-inflammatory, and may help protect against heart disease and cancer. Quercetin can also help stabilize the cells that release histamine in the body and thereby have an anti-inflammatory effect (Singh et al., 1998; Long et al., 2006). Previous studies suggest that flavonoids such as quercetin, may help reduce the risk of atherosclerosis. These nutrients appear to protect against the damage caused by low density lipoproteins (LDL) ("bad") cholesterol and may help prevent death from heart disease. However, most human studies have looked at flavonoids in the diet, not as supplements (Donaldson et al., 2004). Thus, the nanosize TiO_2-induced toxicity was demonstrated on certain biochemical parameters in mice heart as well as the effect of both idebenone and quercetin in ameliorating these parameters was investigated.

MATERIALS AND METHODS

Chemicals

The 80 nm TiO_2 powders were purchased from Sigma Co. (USA). All chemicals used were of high analytical grade, product of Sigma and Merck companies.

Animals and treatments

Ninety wistar albino rats weighing 180-200 g were used. The rats were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. Animals were kept in special cages, and maintained on a constant 12 h light/12 h dark cycle with air conditioning and temperature ranging 20–22°C and humidity (60%). Rats were fed with standard rat pellet chow with free access to tap water *ad libitum* for one week before the experiment for acclimatization. Animal utilization protocols were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the King Saud University, College of Pharmacy. After one week acclimation, the rats were kept fasting over night before treatment and randomly divided into two classes according to the dose of TiO_2-nanoparticle administration to rats. Class I, consists of five groups (each of ten rats): G1, Normal healthy animals; G2-G4 groups of animals administered orally low dose (600 mg/ Kg body weight/day) n- TiO_2 for 5 constitutive days (Wang and Feng, 2008) and divided as follows: G2, $nTiO_2$ intoxicated animals with oral low dose (600 mg/ Kg/day) daily for 5 constitutive days; G3, n-

TiO_2 intoxicated animals co-administered quercetin (200 mg/Kg) daily (Kambe et al., 2010); G4, n- TiO_2- intoxicated animals co-administered idebenone (200 mg/Kg) daily (Kiyota et al., 2003). Class II consists of 4 groups (G5-G7), each of ten rats, administered orally high dose 1 g/Kg body weight/ day n-TiO2 for 5 constitutive days high (4) and divided as follows: G5, n- TiO_2 treated animals with an oral high dose(1 g/Kg/day) daily for 5 days; G6, n- TiO_2intoxicated animals co-administered quercetin (200 mg/Kg) daily; G7, n- TiO_2- intoxicated animals co-administered idebenone (200 mg/Kg) daily. Quercetin and idebenone were orally administered daily for three constitutive weeks from the beginning of the experiment. The body weights of rats were recorded before and after the administration period. Three weeks later, and after 24 h of the last dose administration, rats were fasted overnight then sacrificed and the blood was collected. Serum was separated by centrifugation at 3000 r.p.m. for 10 min and kept at -80°C for different biochemical estimations. Four hearts were rinsed in cold isotonic saline, homogenized, and frozen at −80°C.

Serum biochemical analyses

Determination of troponin, myoglobin and CK-MB

Serum troponin T and myoglobin concentrations were determined using a Siemens Dimension Xpand® Plus instrument (IL, USA). Serum CK-MB, level was measured with an auto-analyser (ILab-300 bioMérieux Diagnostics, Milan, Italy).

Determination of TNF-α level

TNF-α in serum was determined using commercially available enzyme-linked immunosorbent assay (ELISA) assays following the instructions supplied by the manufacturer (DuoSet kits, RandD Systems; Minneapolis, MN, USA).). The results are shown as pg of cytokine per ml.

Determination of C-reactive protein (CRP) level

CRP was measured using the method of Kim et al. (2010). CRP was measured with latex-enhanced immunonephelometry on a Behring BN II Nephelometer (Dade Behring). In this assay, polystyrene beads coated with rat monoclonal antibodies bind CRP present in the serum sample and form aggregates. The intensity of the scattered light is proportional to the size of the aggregates and thus reflects concentration of CRP present in the sample. The intra-assay and interassay coefficients of variation for CRP were 3.3 and 3.2%, respectively. The lower detection limit of the assay was 0.15 mg/L.

Determination of vascular endothelial growth factor (VEGF) level

The level of VEGF in serum was determined at 492 nm by quantitative colorimetric sandwich ELISA; (R and D systems, UK) in accordance with the manufacturer's instructions (Wang et al., 2008). Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer.

Determination of immunoglobulin G (IgG) level

IgG level was measured using ELISA. 1 µg/ml of goat anti-rat IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersberg, MD) was used as capture antibodies. Standards were prepared from rat IgG (Sigma Chemical Co., St. Louis, MO) Goat anti-rat IgG peroxidase conjugates diluted 1:250 in PBS/BSA (from Kirkegaard and Perry Laboratories, Inc., Gaithersberg, MD) were used as detecting antibodies. The chromogenic substrate used was 2,2′-azino-di[3-ethyl-benzthiazoline sulfonate] (ABTS; Kirkegaard and Perry Laboratories, Inc., Gaithersberg, MD). Color development was detected as optical density at 405 nm using an automated ELISA plate reader (Bio-tek Instruments, Inc., Winooski, VT) and immunoglobulin concentrations were determined by comparison of sample color development to standard curves (Kineticalc, Bio-tek Instrumentsmeasured in, Inc., Winooski, VT

Determination of* IL-6 *level

IL-6 was measured by ultra-sensitive ELISA (Quantikine HS Human IL-6 Immunoassay; R and D Systems, Minneapolis, MN) with an analytical CV of 6.3% and a detection level of 0.04 pg/ml (Kaden, 2007).

Determination of nitrite level

Nitrite level concentration (an indirect measurement of NO synthesis) was assayed using Griess reagent (sulfanilamide and N-1-naphthylethylenediamine dihydrochloride) in acidic medium (Moshage et al., 1995).

Determination of glucose level

Glucose level was estimated using the method of Trinder (1969).

Biochemical assay of heart tissue

Calcium concentration

Calcium level were measured with an auto-analyser (ILab-300 bioMérieux Diagnostics, Milan, Italy).

Caspase 3 activity assay

To assay for caspase-3-like protease, the ipsilateral and contralateral hemispheres of NMDA-injected rat pups were separately pulverized over dry ice and portions of the samples were lysed in a buffer containing 20 mM Tris-HCl (pH 7.4 at 48°C), 150 mM NaCl, 1 mM dithiothreitol (DTT), 5 mM EDTA, 5mM ethylene glycol-bis (h-aminoethyl ether (EGTA) and 1% (w/ v) Triton X-100 for 90 min at 48C. The centrifugation- cleared lysates were mixed with 50% (v/v) glycerol. Cell lysates (30 mg protein) were assayed with 100 mM acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (ac-DEVD-MCA; Bachem Bioscience), 100 mM Hepes, 10% glycerol, 1 mM EDTA, 10 mM DTT and 10 mM Z-D-DCB (optional). Fluorescence (excitation 380 nm215 nm and emission 460 nm215 nm) was measured at 60 min with Cyto¯or 2300 (33).

Comet analysis

The Comet assay or single cell gel electrophoresis (SCGE) is a widely used technique for measuring and analysing DNA breakage in individual cells. The method of Singh et.al (1988) include the unwinding DNA under alkaline conditions is used. The parameters measured to analyze the electrophoretic patterns were: Tail length

measured from the middle of the head to the end of the tail and relative DNA content in the tail.

RESULTS AND DISCUSSION

The present results reveal that, the biochemical markers for myocardial infarction were measured and demonstrated that troponin T, myoglobin and CK-MB were increased in mice sera whereas caspase-3 was elevated in the heart tissue of n-TiO_2 intoxicated rats, the effect was more pronounced with the higher dose (Tables 4A and B and Figures 1 and 2). In addition, increase in TNF-α, Il-6 and C-reactive reactive protein in a dose dependent manner of n-TiO_2 intoxicated rats Tables 2A and B). The present work also show an increase in NO, Ig G, VEGF, serum glucose and calcium post n-TiO2 NPs (Tables 1A and B).

The present work reveal also an increase in apoptosis as confirmed by the increase in the activity of caspase-3 enzyme and the administration of the nanoparticles induced DNA damage as shown by the increase in tail length (Tables 3A and B; Figures 1 and 2). The results showed that both idebenonne and quercetin greatly ameliorated the increased levels of the studied parameters, the improvement being more pronounced with the lower dose which reveals that toxicity with these nanoparticles is a more threat as the dose increases.

Inhaled or injected nanoparticles enter systemic circulation and migrate to various organs and tissues, raising concern that they may cause damage to biological systems through OS pathways. Nano-sized TiO_2, for example, can produce free radicals (that is, reactive species of molecules) and exert a strong oxidizing ability. A potential exposure route for general population is the oral ingestion because TiO_2 is used as a food additive in toothpaste, capsule, cachou, and so on. Thus, in the present paper, the purposes of testing for acute oral TiO_2 toxicity are to obtain information on the biological response of a chemical and to gain insight into the targets of its action.

In the present work, the biochemical markers for myocardial infarction were measured and demonstrated that troponin T, myoglobin and CK-MB were increased in mice sera whereas caspase-3 was elevated in the heart tissue of n-TiO_2 intoxicated rats, the effect was more pronounced with the higher dose. Recent *in vivo* studies (Liu et al., 2009; Wang et al., 2009) have confirmed that TiO_2 nanoparticles accumulate in the heart (Mallik et al., 2011) and that there is a close association between nanoparticle toxicity and cardiovascular adverse effects such as myocardial infarction (Peters et al., 2001). The latter results from rupture of an atherosclerotic plaque in the coronary artery, followed by rapid thrombus growth caused by exposure of highly reactive subendothelial structures to circulating blood, thus leading to additional or complete obstruction of the blood vessel. Epidemiologic studies have provided valuable information on the adverse health effects of nanoparticles indicating that the latter act as an important environmental risk factor for cardiopulmonary mortality. Particle-induced pulmonary and systemic inflammation, accelerated atherosclerosis, and altered cardiac autonomic function may be part of the patho-physiological pathways, linking particulate air pollution with cardiovascular mortality. In accordance with this, histopathological examinations by Chen et al. (2009) showed that some TiO2 particles had entered the spleen and caused the lesion of spleen. Thrombosis was found in the pulmonary vascular system, which could be induced by the blocking of blood vessels with TiO_2 particles.

An accelerated cytokine production was observed by the increase in TNF-α, Il-6 and C-reactive reactive protein. These data suggest that macrophage uptake and accumulation of large amounts of TiO_2 aggregates may result in cytokine release and potential cytotoxicity in cells and tissues responsible for clearance of TiO_2 from circulation. Available data in the literature showed that TiO_2-NPs can cause several adverse effects on mammalian cells such as increase of ROS production and cytokines levels (Iavicoli et al., 2011). In agreement with our data, (Miller et al. (2007) reported that cytokine analysis of treated cells revealed an increased release of TNF-, IL-1ß, IL-6, and MIP-2 with increasing dose and time of exposure to TiO_2. Also, it has been shown that particles deposited in the alveoli lead to activation of cytokine production by alveolar macrophages and epithelial cells and to recruitment of inflammatory cells. An increase in plasma viscosity, fibrinogen and C-reactive protein has been observed in samples of randomly selected healthy adults in association with particulate air pollution (Peters et al., 1997). Bu et al. (2010), studied the biochemical effects of n-TiO_2 by an NMR-based metalobomic approach and found that aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH) were elevated and mitochondrial swelling in heart tissue was observed in TiO_2 NP-treated rats. These findings indicate that disturbances in energy and amino acid metabolism and the gut microflora environment may be attributable to the slight injury to the liver and heart caused by TiO_2 NPs. It should also be pointed out that the increase in the circulating antibody production is the result of production of different inflammatory cytokines including TNF-α with potential impact on immunoglobulin production during inflammation These result may indicate that TiO_2-NPs induced inflammatory injury through production of the inflammatory mediators associated with an increase in IgG level as previously reported by Davis et al. (1998).

The present work also revealed an increase in NO, which is an important class of signalling agents in the heart. It has been shown that nanoparticles can induce

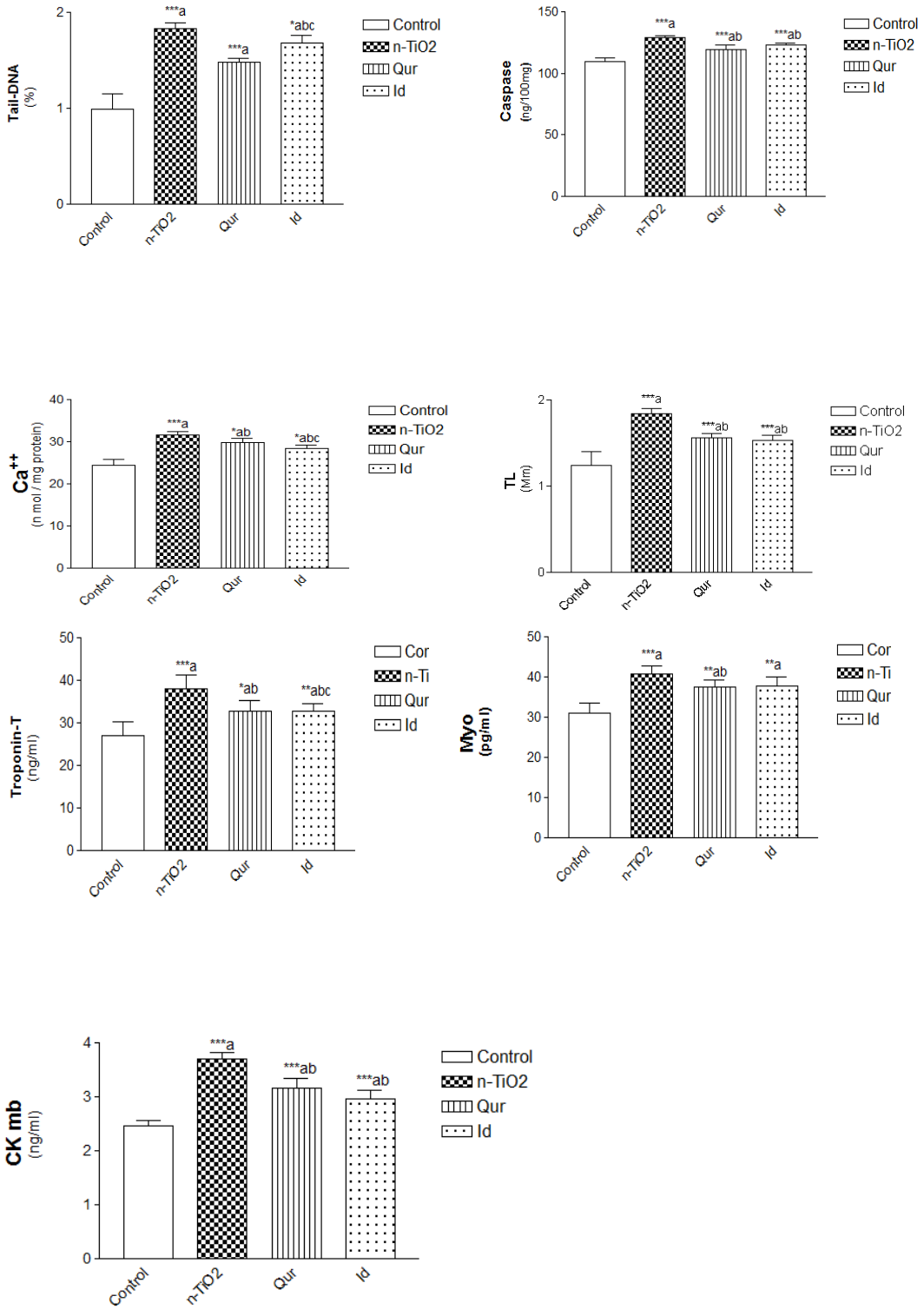

Figure 1. Low dose for heart parameter.

oxidative stress locally and implicate that the oxidative species most probably were produced by activated macrophages, after nanoparticle phagocytosis. Uptake of polymeric nanoparticles by cells induces modifications in

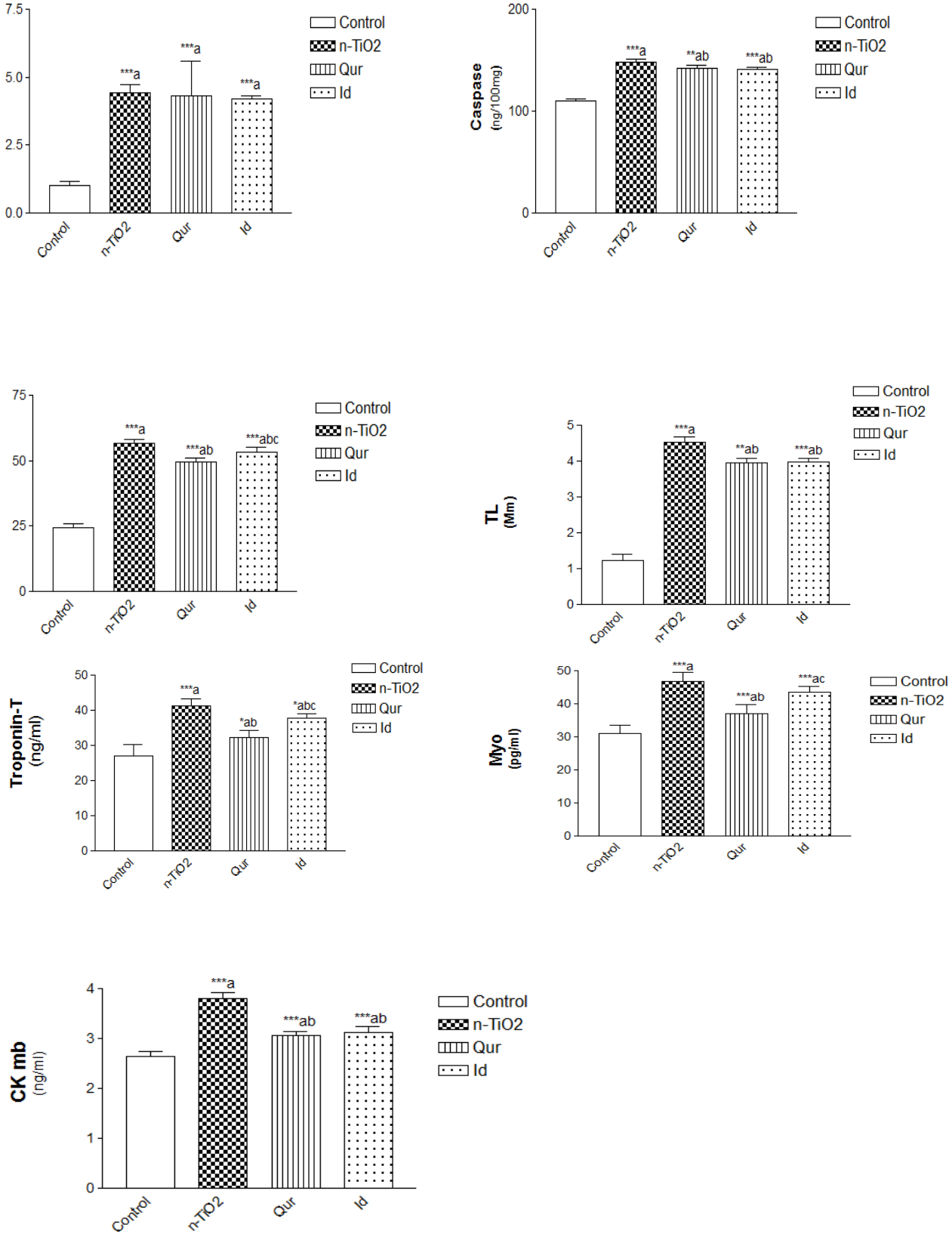

Figure 2. High dose for heart parameter.

Table 1A. Effect of idepenone and/or quercetin and treatment on blood glucose level, VEGF, NO and IgG with low dose of n-TiO_2.

Group	Glucose (mg/dl)	VEGF (pg/ml)	NO (µmol/l)	IgG (ng/ml)
Control	115.8±1.5	174.56 ± 4.46	31.8 ± 2.39	126.42±3.98
n-TiO_2	126.2±3.03^{***a}	215.14±10.19^{***a}	65.68 ± 2.07^{***a}	151.362±2.92^{***a}
Qur	115±1.58^{***b}	200.26±3.26^{**ab}	41.6± 2.07^{***ab}	125.7±2.69^{***b}
Id	118.8±1.9^{***abc}	202.82±3.09^{*ab}	38.2 8 ± 2.86^{***ab}	133.2±2.92^{*abc}

n-TiO_2, Titanium dioxide; Qur, quercetin; Id, idepenone; VEGF, vascular endothelial growth factor; NO, nitric oxide; IgG, immunoglobulin G. Values are expressed as mean ± SD. ***p , **p , a: Compared to control group, b: compared to n-TiO_2 group, c: compared to Qur.

Table 1B. Effect of idepenone and/or quercetin treatment on blood glucose level, VEGF, NO and IgG with HIGHdose of n-TiO_2.

Group	Glucose (mg/dl)	VEGF (pg/ml)	NO (µ mol/l)	IgG (ng/ml)
Control	115.8±1.5	174.56 ± 4.46	31.8± 2.39	126.42±3.98
n-TiO_2	128±3.4***a	249.8±3.55***a	74.4±3.51***a	183.28±4.81***a
Qur	120±1.6***ab	245.12±3.77***a	46.4± 2.07***ab	165.18±3.93***ab
Id	130±1.6***ac	244.12±4.45*ab	40.8± 2.28***abc	162.64±2.97***ab

n-TiO_2, Titanium dioxide; Qur, quercetin; Id, idepenone; VEGF, vascular endothelial growth factor; NO, nitric oxide; IgG, immunoglobulin G. Values are expressed as mean ± SD. ***p , **p , a: Compared to control group, b: compared to n-TiO_2 group, c: compared to Qur.

Table 2A. Effect of idepenone and/or quercetin treatment on TNF- α , VEGF, CRP and IL-6 with LOW dose of n-TiO_2.

Group	TNF-α (pg/ml)	CRP (ng/ml)	IL-6
Control	231.68 ± 7.99	2.918 ± 0.09	30.6± 2.07
n-TiO_2	297.58 ± 6.31***a	3.88 ± 0.31***a	45 ± 1.58***a
Qur	270.28 ± 5.84***ab	3.46 ± 0.19***ab	39.2 ± 4.32*ab
Id	278.66 ± 3.33***abc	3.6 ± 0.22***a	36 ± 3.39*ab

n-TiO_2, titanium dioxide ; Qur, quercetin ; Id, idepenone; VEGF, vascular endothelial growth factor; CRP, C-reactive protein; Il-6, interleukin-6. Values are expressed as mean ± SD. ***p , **p , a: Compared to control group; b: Compared to n-TiO_2 group, c: compared to Qur.

Table 2B. Effect of idepenone and/or quercetin treatment on TNF- α , VEGF, CRP and IL-6 with high dose of n-TiO_2.

Group	TNF-α (pg/ml)	CRP (ng/ml)	IL-6
Control	231.68 ± 7.99	2.92 ± 0.09	30.6 ± 2.07
n-TiO_2	323.74 ± 4.82^{***a}	4.34 ± 0.32^{***a}	48.6 ± 2.61^{***a}
Qur	311.58 ± 5.61^{***ab}	3.61 ± 0.42^{**ab}	38.8 ± 1.92^{***ab}
Id	270 ± 3.47^{***abc}	3.40 ± 0.24^{***ab}	38.2 ± 3.11^{***ab}

n-TiO_2, titanium dioxide ; Qur, quercetin ; Id, idepenone; VEGF, vascular endothelial growth factor; NO, nitric oxide; IgG, immunoglobulin G. Values are expressed as mean ± SD. ***p , **p , a: Compared to Control group, b: Compared to n-TiO_2 group, c: compared to Qur.

Table 3A. Effect of idepenone and/or quercetin treatment on Caspase -3, Tail DNA TL and Ca^{++} with low dose of n-TiO_2.

Group	Caspase-3 (ng/100mg)	Tail-DNA (%)	TL (Mm)	Ca^{++} (n mol/mg protein)
Control	109.82 ±2.71	0.99 ± 0.16	1.24 ± 0.16	240.5 ± 1.42
n-TiO_2	128.84±2.03^{***a}	1.83 ± 0.06^{***a}	1.84 ± 0.06^{***a}	31.58±0.92^{***a}
Qur	119.72±3.06^{***ab}	1.48 ± 0.04^{***a}	1.56 ± 0.05^{***ab}	29.88±0.87^{*ab}
Id	123.022±1.85^{***ab}	1.68 ± 0.08^{*abc}	1.53 ± 0.06^{***ab}	28.42±0.77^{*abc}

n-TiO_2, titanium dioxide; Qur, quercetin; Id, idepenone. Values are expressed as mean ± SD. ***p , **p , a: Compared to Control group, b: Compared to n-TiO_2 group, c: compared to Qur.

Table 3B. Effect of idepenone and/or quercetin treatment on Caspase -3, Tail DNA , TL and Ca^{++} with HIGH dose of n-TiO_2.

Group	Caspase-3 (ng/100mg)	Tail-DNA (%)	TL (Mm)	Ca^{++} (n mol/mg protein)
Control	109.76±2.66	1.00 ± 0.16	1.24 ± 0.16	24.5 ± 1.42
n-TiO_2	148.522±2.27***^{a}	4.43 ± 0.29***^{a}	4.53 ± 0.15***^{a}	56.82±1.32***^{a}
Qur	142.64±2.48**^{ab}	4.33 ± 1.28***^{a}	3.95 ± 0.14**^{ab}	49.66±1.49***^{ab}
Id	141.44±1.27***^{ab}	4.19 ± 0.12***^{a}	3.97 ± 0.1***^{ab}	53.24±1.98***^{abc}

n-TiO2, titanium dioxide ; Qur, quercetin ; Id, idepenone. Values are expressed as mean ± SD. ***p , **p , a: Compared to Control group, b: Compared to n-TiO2 group, c: compared to Qur.

Table 4A. Effect of idepenone and/or quercetin treatment on Myo, Troponin-T and CK with low dose of n-TiO_2.

Group	Myo (pg/ml)	Troponin-T (ng/ml)	CK mb (ng/ml)
Control	31.16 ±2.38	27.14 ± 3.15	2.46± 0.11
n-TiO_2	40.8±2.07***^{a}	38.1 ± 3.07***^{a}	3.71 ± 0.11***^{a}
Qur	37.64±1.67*^{ab}	32.8± 2.37*^{ab}	3.17 ± 0.17***^{ab}
Id	37.9±2.04**^{a}	32.86 ±1.77**^{abc}	2.97 ± 0.16***^{ab}

n-TiO_2, titanium dioxide ; Qur, quercetin ; Id, idepenone; Myo,myoglobin; CK, creatine kinase. Values are expressed as mean ± SD. ***p , **p , a: Compared to Control group, b: Compared to n-TiO_2 group, c: compared to Qur.

Table 4B. Effect of idepenone and quercetin and/or Qur treatment on Myo , Troponin-T and CK with HIGH dose of n-TiO_2.

Group	Myo (pg/ml)	Troponin-T (ng/ml)	CK mb (ng/ml)
Control	31.16±2.38	27.14± 3.15	2.64 ± 0.11
n-TiO_2	46.68±2.79***^{a}	41.18±2.23***^{a}	3.81 ± 0.12***^{a}
Qur	37.06±2.76***^{ab}	32.26±1.99*^{ab}	3.06 ± 0.09***^{ab}
Id	43.6±1.81***^{ac}	37.68±1.43*^{abc}	3.13 ± 0.12***^{ab}

n-TiO_2, titanium dioxide ; Qur, quercetin ; Id, idepenone; Myo,myoglobin; CK, creatine kinase. Values are expressed as mean ± SD. ***p , **p , a: Compared to control group, b: compared to n-TiO_2 group, c: compared to Qur.

antioxidant systems, probably due to the production of radical oxygen species (Fernandez-Urrusuno et al., 1997), and hence an increase in NO is observed. Besides NO known antioxidant property, NO which is produced by inducible nitric oxide synthase (iNOS) can be cytotoxic especially at higher local concentrations. Also, it can react with ROS or oxygen yielding reactive nitrogen species (RNS), which cause damage on biological molecules such as enzymes, lipids and DNA by nitrosation, oxidation and nitration. Mesangial and invading immune cells are capable of expressing iNOS upon stimulation with (TNF- α), (IL-1b) and, thus are likely to be responsible for the release of large amounts of NO during TNF α, IL-1b and triggered infammatory conditions in the glomerulus cells (Aiello et al., 1998). In the present study, n-TiO_2 produced an elevation of IL-6 and nitric oxide levels. Such elevation may be due to in-creased expression of neuronal NOS (nNOS) mRNA and NOS enzyme activity, which are expressed during inflammatory conditions (de Haar et al., 2009; Nath et al., 2006).

In the present study the level of VEGF was elevated post n-TiO_2 NPs treatment compared to normal group. VEGF, which is a potent mitogen for endothelial cells has been reported to be expressed in several tissues. Besides its mitogenic properties, VEGF is able to promote angiogenesis, induce proteases and increase vascular leakage (Drexler, 1997). This may result due to Ang II-induced glomerular and vascular damage associated with the increase in inflammatory markers.

In addition, the present work demonstrated an elevation in serum glucose which revealed that accumulation of the nanoparticles in organs induces metabolic disturbances in the different biochemical parameters. This was confirmed by Liu et al. (2009) who studied the toxicity of

nano-TiO(2) particles on organs and found that, with increasing doses of nano-anatase TiO(2), the indicators of liver function, such as alkaline phosphatase, alanine aminotransferase, leucine acid peptide, pseudocholinesterase, total protein, and albumin level, were enhanced significantly; the indicators of kidney function, such as uric acid and blood urea nitrogen, were decreased; the activities of aspartate aminotransferase, creatine kinase, lactate dehydrogenase, and alpha-hydroxybutyrate dehydrogenase, indicator of the myocardium function, were increased. Also, the contents of triglycerides, glucose, and high-density lipoprotein cholesterol were significantly elevated. Taken together, the authors concluded that nano-anatase TiO(2) in higher dose caused serious damage to the liver, kidney, and myocardium and disturbed the balance of blood sugar and lipid in mice. The accumulation of titanium in the organs might be closely related to the inflammatory responses of mice (Liu et al., 2009).

The rise in serum calcium may be explained on the basis that n-TiO_2 penetrate into and through the cells without disrupting junctional complexes. In addition, TiO_2 nanoparticles begin alteration of both microvillar organization on the apical surface of the epithelium as well as induce a rise in intracellular-free calcium. The latter is a mechanism cells use to respond to extracellular stimuli and may be linked to the alteration of the apical microvilli (Koeneman et al., 2010).

In a parallel study, Hu et al. (2010) found that the contents of Ca, Mg, Na, K, Fe and Zn in brain were significantly altered after TiO(2) NPs exposure possibly due to the disturbance of the homeostasis of trace elements. Because of their small size, nanoparticles can interact readily with biomolecules either on the surface of or within the cells. Cell/subcellular distributions of the particles have great influences on the protein aggregation, gene expression, and cell cytotoxicity as well (Jiang et al., 2008; Chen and Gerion, 2004).

The present work also revealed an increase in apoptosis as confirmed by the increase in the activity of caspase-3 enzyme. Apoptosis, or programmed cell death, is an important way to maintain the cellular homeostasis between cell division and cell death. It is well known that apoptosis can be triggered via two principal signalling pathways: the death receptor-mediated extrinsic apoptotic pathway and the recruitment of adaptor proteins, followed by activation of caspase-8, and the mitochondrion-mediated intrinsic apoptotic pathway. Previous investigations have revealed that TiO_2 NPs induced apoptosis (Ma et al., 2009; Li et al., 2010), and speculated that the apoptosis induction may be through the mitochondrion-mediated pathway, in which mitochondrial permeability transition is firstly promoted, followed by the release of apoptogenic factors such as cytochrome c and apoptosis-inducing factor, the activation of initiator caspase-9 and effector caspase-3. In studying DNA damage by comet assay, it was found that administration of the nanoparticles induced DNA damage as shown by the increase in tail length. This finding is supported by the reports of many authors that nanoparticles induce DNA breaks and damage. Trouiller et al. (2009) suggested the possible mechanisms for TiO_2 nanoparticles genotoxicity. TiO_2 nanoparticles might damage DNA directly or indirectly via oxidative stress and/or inflammatory responses. Two recent studies show a direct chemical interaction between TiO_2 nanoparticles and DNA, through the DNA phosphate group. On the other hand, other studies show that TiO_2 nanoparticles can cause DNA damage indirectly through inflammation (Chen et al., 2006; Grassian et al., 2007), and generation of reactive oxygen species (Kang et al., 2008; Federici et al., 2007). Also Petkovic et al. (2011) investigated the genotoxic responses of TiO_2 nanoparticles anatase: TiO(2)-An in human hepatoma HepG2 cells and reported that it induced a persistent increase in DNA strand breaks (comet assay) and oxidized purines (Fpg-comet). The authors added that TiO2-An was a stronger inducer of intracellular ROS.

The effect of orally administered antioxidants was studied using either idebenone or quercetin. The results showed that these antioxidants greatly ameliorated the increased levels of the studied parameters, the improvement being more pronounced with the lower dose which reveals that toxicity with these nanoparticles is a more threat as the dose increases. Both idebenonne and quercetin administration resulted in a decline in the inflammatory cytokines and in the oxidative stress markers. They also resulted in reduction in the levels of IgG, Ca and glucose suggesting their potent role in amelioration of the immune system and of the biochemical parameters underlying metabolic pathways. Furthermore these antioxidants were efficient in protecting against DNA damage induced by the nanoparticles by suppression of the induced oxidative stress Idebenone is a benzoquinone derivative which is structurally related to ubiquinone, a component of the respiratory chain. Preclinical studies suggest that idebenone may exert cytoprotective properties by acting as a free radical scavenger for a number of therapeutic effects including its ability to improve the effects from heart failure, by reducing free radical damage (Orsucci et al., 2011). It is theorized that because idebenone being very similar to Co Q10, it provides stimulating nourishment for the mitochondria, keeping the cells “awake” and therefore reducing this rate of degradation and decline. Because of this, idebenone is said to serve as a powerful mitochondrial free radical quencher and lessen the ever-increasing mtDNA damage that may occur. Hence, the body needs both CoQ10 and ascorbic acid to maintain healthy mtDNA, and there is ample evidence that high levels of ubiquinone, and ascorbic acid, slow the detrimental biochemical, structural and other changes

that acutely affects the systems with the highest energy demand (cardiovascular and immune). It is also believed that Idebenone works better than Co Q10 within the electron transport chain to keep energy production high, even under hypoxic (low oxygen) conditions. This is especially critical to brain and heart cells that may be rapidly damaged during low ATP (energy transfer) events that occur due to poor tissue oxygenation. In addition, idebenone is an antioxidant and can inhibit lipid peroxidation and may protect cell membranes and mitochondria from oxidative damage (James et al., 2005). Quercetin, on the other hand, can scavenge damaging particles in the body known as free radicals, which damage cell membranes, tamper with DNA, and even cause cell death. It possesses an antihistamic and an anti-inflammatory action, and may help protect against heart disease and cancer and reduce the risk of atherosclerosis (Ishizawa et al., 2011).

Conclusion

The results obtained in this study suggest that TiO2 NPs triggers oxidative stress, DNA damage and potentially change the apoptotic genes and their proteins expression and enhance apoptosis and the expression of inflammatory cytokines and markers of myocardial infarction in heart tissue. Administration of idebenone or quercetin greatly restores the induced disorders by reducing oxidative stress.

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ABBREVIATIONS

TiO(2) NPs, Titanium dioxide nanoparticles; **OS,** oxidative stress; **VSMC,** vascular smooth muscle cell; **CoQ10,** coenzyme Q10; **ELISA,** enzyme-linked immunosorbent assay; **CRP,** C-reactive protein; **VEGF,** vascular endothelial growth factor; **ABTS,** 2,2′-azino-di[3-ethyl-benzthiazoline sulfonate]; **EGTA,** ethylene glycol-bis (h-aminoethyl ether; **DTT,** dithiothreitol; **SCGE,** single cell gel electrophoresis; **AST,** aspartate aminotransferase; **CK,** creatine kinase; **LDH,** lactate dehydrogenase; **iNOS,** inducible nitric oxide synthase; **RNS,** yielding reactive nitrogen species.

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