Toxicology Mechanisms and Methods

ISSN: 1537-6516 (Print) 1537-6524 (Online) Journal homepage: https://www.tandfonline.com/loi/itxm20

Mitochondrial dysfunction in titanium dioxide nanoparticle-induced neurotoxicity

Nandini Nalika & Suhel Parvez

To cite this article: Nandini Nalika & Suhel Parvez (2015) Mitochondrial dysfunction in titanium dioxide nanoparticle-induced neurotoxicity, Toxicology Mechanisms and Methods, 25:5, 355-363, DOI: 10.3109/15376516.2015.1020183

To link to this article: https://doi.org/10.3109/15376516.2015.1020183

Published online: 16 Mar 2015.

Toxicology Mechanisms and Methods

http://informahealthcare.com/txm
ISSN: 1537-6516 (print), 1537-6524 (electronic)

Toxicol Mech Methods, 2015; 25(5): 355–363
 DOI: 10.3109/15376516.2015.1020183

informa healthcare

RESEARCH ARTICLE

Mitochondrial dysfunction in titanium dioxide nanoparticle-induced neurotoxicity

Nandini Nalika and Suhel Parvez

Department of Medical Elementology and Toxicology, Jamia Hamdard (Hamdard University), New Delhi, India

Abstract

Nanotechnology has emerged as a field of scientific innovation which has opened up a plethora of concerns for the potential impact on human and environment. Various toxicological studies have confirmed that nanoparticles (NPs) can be potentially hazardous because of their unique small size and physico-chemical properties. With the wide applications of titanium dioxide nanoparticles (TNPs) in day-to-day life in form of cosmetics, paints, sterilization and so on, there is growing concern regarding the deleterious effects of TNPs on central nervous system. Mitochondria is an important origin for generation of energy as well as free radicals and these free radicals can lead to mitochondrial damage and finally lead to apoptosis. The objective of our study was to elucidate the potential neurotoxic effect of TNPs in anatase form. Oxidative stress was determined by measuring lipid peroxidation and protein carbonyl content which was found to be significantly increased. Reduced glutathione content and major glutathione metabolizing enzymes were also modulated signifying the role of glutathione redox cycle in the pathophysiology of TNPs. Mitochondrial complexes were also modulated from the exposure to TNPs. The present study indicates that nanosize TNPs may pose a health risk to mitochondrial brain with the generation of reactive oxygen species, and thus NPs should be carefully used.

Keywords

Brain, *in vitro*, lipid peroxidation, nanoparticles, oxidative stress, protein carbonyl, titanium dioxide

History

Received 12 November 2014
Revised 5 February 2015
Accepted 8 February 2015
Published online 16 March 2015

Introduction

Nanotechnology is rapidly looming all over the industrial world with increasing production of nanomaterials (NMs). It has been evolved to create and manipulate the materials at nanoscale that exhibit innovative properties and can be used in various industries including construction, health care applications, food and consumer goods, e.g. cosmetics (Krystek et al., 2014). NMs, ranging from 1 to 100 nm, have been used to create peculiar-nanosized devices that possess certain physical and chemical properties (Suzuki et al., 2007). Different NMs including titanium dioxide nanoparticles (TNPs) have special and unusual properties and thus are widely used in many fields and bio-applications such as therapeutics, transfection vectors and fluorescent labels, in food industry (Rashidi & Khosravi-Darani, 2011). Development of nanotechnology leads to more production and release of the nanoparticles in the environment on a large scale.

The small size and poorly understood biological potential of NPs is emerging as an important public health issue (Oberdörster et al., 2005). The major toxicological concern is the fact that some of the manufactured NMs are redox active (Colvin, 2003), and some particles transport across cell membranes and especially into mitochondria (Foley et al., 2002). Human exposure may occur during both manufacturing and subsequent use of NPs like TNPs. Such widespread use and its potential entry in the body through dermal, ingestion and inhalation routes suggest that these NPs pose a potential exposure risk to humans, livestock and the ecosystem (Long et al., 2007). There have been attempts to address the safety of these materials by multidisciplinary teams. It is well-known that oxidative stress can lead to cell death: either by apoptosis or necrosis. Severe oxidative stress to cells causes necrosis and moderate oxidative stress causes apoptosis (Curtin et al., 2002).

The olfactory nerves of a rat brain carry the NPs directly from olfactory epithelium to the brain (Oberdörster et al., 2004). Many studies have reported that TNPs have potentially damaging effects on cells and tissues. Recent toxicological studies on NPs have confirmed that NPs can induce higher levels of cellular oxidative stress (Haase et al., 2012). TNPs are never distributed freely in the cytoplasm after being internalized by cells but are preferentially located in mitochondria. Despite the wide ranges of applications, there is a serious lack of information about the impact of the NPs on human health and the environment. Mitochondria have been known as the powerhouse of the cell (Correia et al., 2010;

Address for correspondence: Dr Suhel Parvez, Department of Medical Elementology and Toxicology, Jamia Hamdard (Hamdard University), New Delhi 110 062, India. Tel: +91-11-26059688/5573. Fax: +91-11-26059663. E-mail: drsuhelparvez@yahoo.com; sparvez@jamiahamdard.ac.in

Iwata et al., 2012). Mitochondria represent the most active cellular redox organelle and localization of these particles into such redox active centers is expected to cause alterations in various antioxidant enzyme systems that are functional in the region. Owing to the size of surface area, TNPs have greater ability to produce reactive oxygen species (ROS) (Moller et al., 2010). NP-induced mitochondrial damage has deleterious effects on the biological system, which includes the initiation of apoptosis and decreased adenine triphosphate (ATP) production (Hiura et al., 2000). As a result, NPs of various chemistries are taken up into cells via endocytic pathways and get localized mainly to mitochondria (Foley et al., 2002; Li et al., 2003). When TNPs enter into the brain, it can cause morphological changes of neurons in the cerebral cortex and significant disturbance of the monoamine neurotransmitter levels in the above sub-brain regions (Zhang et al., 2011).

In contrast, there is a paucity of literature on TiO_2-induced toxicity in brain mitochondria and the role of mitochondria in effecting the cascade of events leading to toxicity. Therefore, the potential risk of NPs, especially TNPs, to biological systems is needed to be investigated before it can cause some serious harm. To examine potential TNPs toxicity in brain mitochondria, we exposed the brain mitochondria at different concentrations of TNPs (anatase) at 37 °C.

Materials and methods

Characterization of TNPs using TEM

Transmission electron microscopy (TEM) characterization was performed to obtain the primary particle size and morphology of TNPs using a Tecnai G^2-20 Twin instrument (Eindhoven, Netherlands) at an accelerating voltage of 200 kV. TNPs were examined after dilution of NPs to 20 µg/ml suspensions in water and to prevent aggregation, the suspensions of nano-TiO_2 were ultrasonicated for 30 min (Teodoro et al., 2011) before subsequent deposition of 5 µl onto a TEM grid made of 2% colodion in amyl acetate which were then dried. Tecnai version 4.1 build 5722 software was then used to measure the size of NPs. The particle size was calculated from measuring NPs in random fields of view in addition to the images showing the general particle sizes of the NPs (Figure 1A and B).

Chemicals

ATP, benzylamine hydrochloride (BAHC), bovine serum albumin (BSA), 2,6-dichloroindophenol (DCIP), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide (NADH), oxidized glutathione (GSSG), reduced glutathione (GSH), reduced NADPH and thiobarbituric acid (TBA) were purchased from Sigma Chemicals Co. (St. Louis, MO). 4-Amino-3-hydroxy-1-naphtalenesulfonic acid (ANSA), butylated hydroxyl toluene (BHT), 1-chloro-2,4-dinitrobenzene (CDNB), 2,4-dinitrophenylhydrazine (DNPH), epinephrine, EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), orthophosphoric acid (OPA), sodium azide, sulphosalicylic acid (SSA) and trichloroacetic acid (TCA) were purchased from Merck Limited (Mumbai, India). TNPs were obtained from SRL Chemicals Pvt. Limited (Mumbai, India).

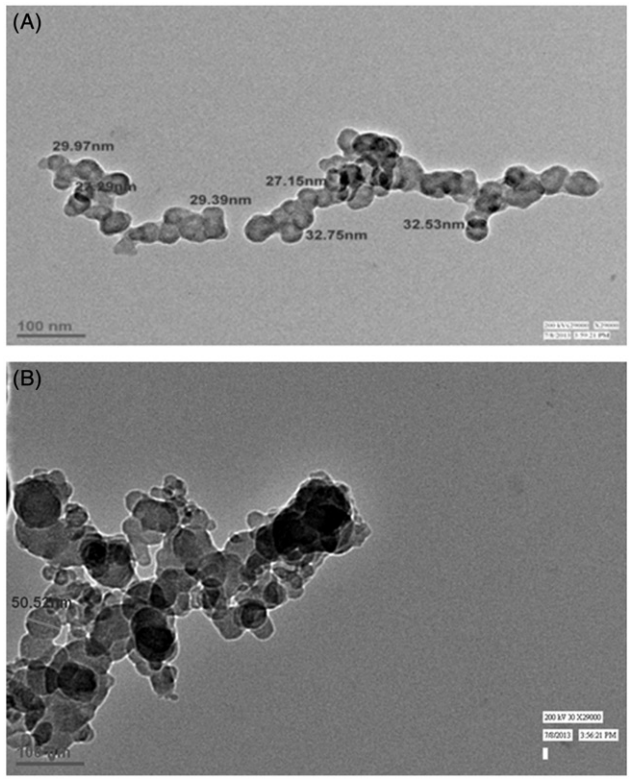

Figure 1. TEM images of TNPs (A and B), scale bars 100 nm. TNPs dispersion was observed with advanced high-illuminating TEM microscopy.

Animals used

Male adult Wistar rats weighing between 120 and 150 g were procured from the Central Animal House of Jamia Hamdard (Hamdard University), New Delhi, India. The rats were acclimatized for a week before starting the experiments. They were kept at 25 ± 1 °C with relative humidity at $65 \pm 10\%$ and at a photoperiod of 12 h light/dark cycle. Standard pellet rodent diet and water were provided to the animals *ad libitum*. All the experiments were carried out according to the standard guidelines of Institutional Animal Ethics Committee of our University.

Isolation of mitochondrial brain

Rat brain mitochondria were isolated by the differential centrifugation method (Liu et al., 2002; Parvez et al., 2010). Briefly, the brain from the adult rat were excised and homogenized using IKA T25 homogenizer (Staufen, Germany) in an ice-cold isolation buffer (A) containing 250 mM sucrose, 10 mM HEPES, 1 mM EGTA and 0.1% fat-free BSA adjusted by Tris to pH 7.4, and centrifuged at 1000*g* for 8 min at 4 °C. The supernatant was collected and centrifuged at 10 000*g* for 10 min at 4 °C. Thereafter, the obtained pellet was re-suspended and washed twice with washing buffer (B) containing 250 mM sucrose, 10 mM HEPES and 0.1 mM EGTA adjusted by Tris to pH 7.4, and centrifuged at 12 300*g* for 10 min. Finally, the pellet was re-suspended in a buffer (C) containing 250 mM sucrose, 10 mM HEPES and 0.1% fat-free BSA adjusted by Tris to pH 7.4.

Experimental design

To investigate the toxicity of TNPs in brain under *in vitro* conditions, the brain mitochondria was isolated and was incubated with different concentrations of TNPs (5–50 μg/ml) for 1 h at 37 °C in a temperature-controlled water bath. The concentrations and incubation time used for TNPs were based on *in vitro* study with NPs (Sekar et al., 2014). Phosphate buffer was added to controls instead of TNPs at the same volume. The stock and working solutions were prepared in such a way that the same volume was added in the isolated brain preparation of rats for incubation.

Biochemical estimation

Oxidative stress biomarkers

Determination of mitochondrial lipid peroxidation. Lipid peroxidation (LPO) was measured using the procedure of Tabassum et al. (2007). The mixture consisted of 10 mM BHT, 0.67% TBA, 1% chilled OPA (*o*-phosphoric acid) and mitochondrial preparation (4–6 mg protein/ml). The mixture was incubated at 90 °C for 45 min. The absorbance of supernatant was measured at 535 nm. The rate of LPO was determined as micromoles of TBA reactive substances (TBARS) formed per gram of tissue using a molar extinction coefficient of $1.56 \times 10^5\,M^{-1}\,cm^{-1}$.

Determination of mitochondrial protein oxidation (PC). Protein oxidation in brain and liver mitochondria was measured as the concentration of protein carbonyls (PCs) using DNPH assay. PC content was assayed by the procedure of Floor and Wetzel (1998). The mitochondrial preparations were treated with 10 mM DNPH in 2 M hydrochloric acid for 1 h at room temperature and precipitated with 6% TCA. The pelleted protein was washed thrice by re-suspension in ethanol:ethyl acetate (1:1). Proteins were then solubilized in 6 M guanidine hydrochloride, 50% formic acid and centrifuged at 16 000*g* for 5 min to remove any trace of insoluble material. The carbonyl content was measured spectrophotometrically at 360 nm. Results were expressed as nanomoles of DNPH incorporated per milligram protein using molar extinction coefficient of $2.1 \times 10^4\,M^{-1}\,cm^{-1}$.

Non-enzymatic antioxidants

Estimation of mitochondrial reduced glutathione (GSH). Reduced glutathione content was estimated according to the method of Tabassum et al. (2010). In this method, DTNB was reduced by –SH groups to form 2-nitro-5-mercaptobenzoic acid per mole of –SH. The nitromercaptobenzoic acid anion released had an intense yellow colour and can be used to measure –SH groups at 412 nm. Mitochondrial suspension was precipitated with 1 ml of SSA (4%). The samples were kept at 4 °C for 1 h and then centrifuged at 1200*g* for 15 min at 4 °C. The assay mixture contained 0.4 ml of mitochondrial suspension, 2.2 ml of sodium phosphate buffer (0.1 M, pH 7.4) and 0.4 ml DTNB in a total volume of 3 ml. The absorbance of reaction product was read at 412 nm on a double beam spectrophotometer. The GSH content was expressed as micromole GSH per gram of tissue.

Enzymatic antioxidants

Activity of mitochondrial glutathione peroxidase (GPx). GPx activity was assayed according to the method of Haque et al. (2003). The assay mixture consisted of 1.44 ml sodium phosphate buffer, 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 0.1 ml GSH (1 mM), 0.1 ml NADPH (0.02 mM), 0.01 ml H_2O_2 (0.25 mM) and 0.15 ml mitochondrial suspension (4–6 mg/ml) in a total volume of 2 ml. Oxidation of NADPH was recorded kinetically at 340 nm. The enzyme activity was calculated as nanomoles of NADPH oxidized per minute per milligram protein, using a molar extinction coefficient of $6.22 \times 10^3\,M^{-1}\,cm^{-1}$.

Activity of mitochondrial glutathione reductase (GR). GR activity was assayed by the method of Tabassum et al. (2007). The assay system consisted of 1.7 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml GSSG (1 mM), 0.1 ml NADPH (0.1 mM) and 0.05 ml of mitochondrial preparation in a total volume of 2.0 ml. The enzyme activity was quantitated at 25 °C by measuring the disappearance of NADPH at 340 nm, and was calculated a molar extinction coefficient of $6.22 \times 10^3\,M^{-1}\,cm^{-1}$.

Activity of mitochondrial manganese-superoxide dismutase (Mn-SOD). Mn-SOD activity was measured according to the method of Govil et al. (2012). The assay was based on the ability of SOD to inhibit the auto-oxidation of epinephrine at alkaline pH. The mitochondrial suspension (0.2 ml) was treated with 0.8 ml of 50 mM glycine buffer (pH 10.4) and 0.02 ml epinephrine. Mn-SOD activity was measured kinetically at 480 nm. The activity was measured indirectly by the oxidized product of epinephrine, i.e. adrenochrome. Mn-SOD activity was expressed as nanomoles of (−) epinephrine protected from oxidation per minute milligram protein using a molar extinction coefficient of $4020\,M^{-1}\,cm^{-1}$.

Mitochondrial markers

Complex-I (NADH dehydrogenase) activity. NADH dehydrogenase activity was assayed by method of Waseem et al. (2015). The assay mixture consisted of 0.6 mM DCIP, 2 mM glycine–glycine buffer, 0.6 mM NADH and mitochondrial suspension (4–6 mg/ml). The reaction was followed by recording the absorbance change at 600 nm, against blank. The enzyme activity was expressed as micromoles of NADH oxidized per minute per millgram protein using a molar extinction coefficient of $21\,000\,M^{-1}\,cm^{-1}$.

Complex II (succinate deydrogenase) activity. The method of Waseem et al. (2015) was used to measure the succinate dehydrogenase activity. The method involved oxidation of succinate by an artificial electron acceptor, potassium ferricyanide. The mitochondrial suspension was treated with 15 μM potassium ferricyanide, 15 mM sodium succinate, 1% BSA and sodium phosphate buffer (7.4 pH). Succinate dehydrogenase activity was measured kinetically at 420 nm. The enzyme activity was calculated as micromoles of succinate produced per minute per milligram protein using molar extinction coefficient of $1 \times 10^3\,M^{-1}\,cm^{-1}$.

Complex III (MTT ability). The MTT reduction rate was used to assess the activity of the mitochondrial respiratory chain in isolated mitochondria by the procedure of Waseem et al. (2015). Briefly, 100 µg mitochondria was incubated with 37 °C in the presence of 20 µl of MTT (0.1 mg/ml). After 30-min incubation period, tubes were centrifuged at 1000*g* for 10 min and the blue formazan crystals were solubilized in 1 ml DMSO and the absorbance was measured at 595 nm. The results were expressed as microgram formazan formed per minute milligram protein using a molar extinction coefficient of $5.1 \times 10^4 M^{-1}\ cm^{-1}$.

Complex V (ATP synthase) activity. The activity was measured as the hydrolysis rate of ATP to ADP plus inorganic phosphate (Pi), as described by Waseem et al. (2015). Mitochondria were incubated in ATPase buffer (50 mM Tris and 5 mM $MgCl_2$, pH 7.5) at 37 °C with 5 mM ATP for 10 min. The reaction was halted by adding 10% (*w*/*v*) TCA. Each assay was centrifuged at 3000*g* for 20 min, and supernatant were mixed with distilled water. The enzyme activity was expressed as microgram Pi liberated per minute milligram protein.

Determination of protein

The protein content was determined in brain mitochondria by the method of Lowry et al. (1951) using BSA as a standard.

Statistical analysis

Results were expressed as mean ± standard error (SE). All data were analysed using analysis of variance (ANOVA) followed by Tukey's test. Values of $p < 0.05$ were considered as significant. All the statistical analyses were performed using GraphPad Prism 5.

Results

Surface area and size distribution of TNPs

The TNPs employed in this study were measured by TEM. We observed that the TNPs (spherical) ranged in size from ~29.29 nm as small and ~34.90 nm as maximum (32.34 ± 2.37). These results show that our TNP solution prepared for further experiments contained a mixture of nanosized material as well as agglomerates (Figure 1A and B).

Oxidative stress markers

Effect on LPO

The effect of TNPs on mitochondrial brain was investigated by assessment of TBARS level in brain mitochondria which resulted in a significant ($p < 0.001$) enhancement in the mitochondrial LPO of brain when compared with the control group (Figure 2A).

Effect on PC content

Oxidative damage and protein oxidation was detected by formation of PC when exposed of brain mitochondria to TNPs

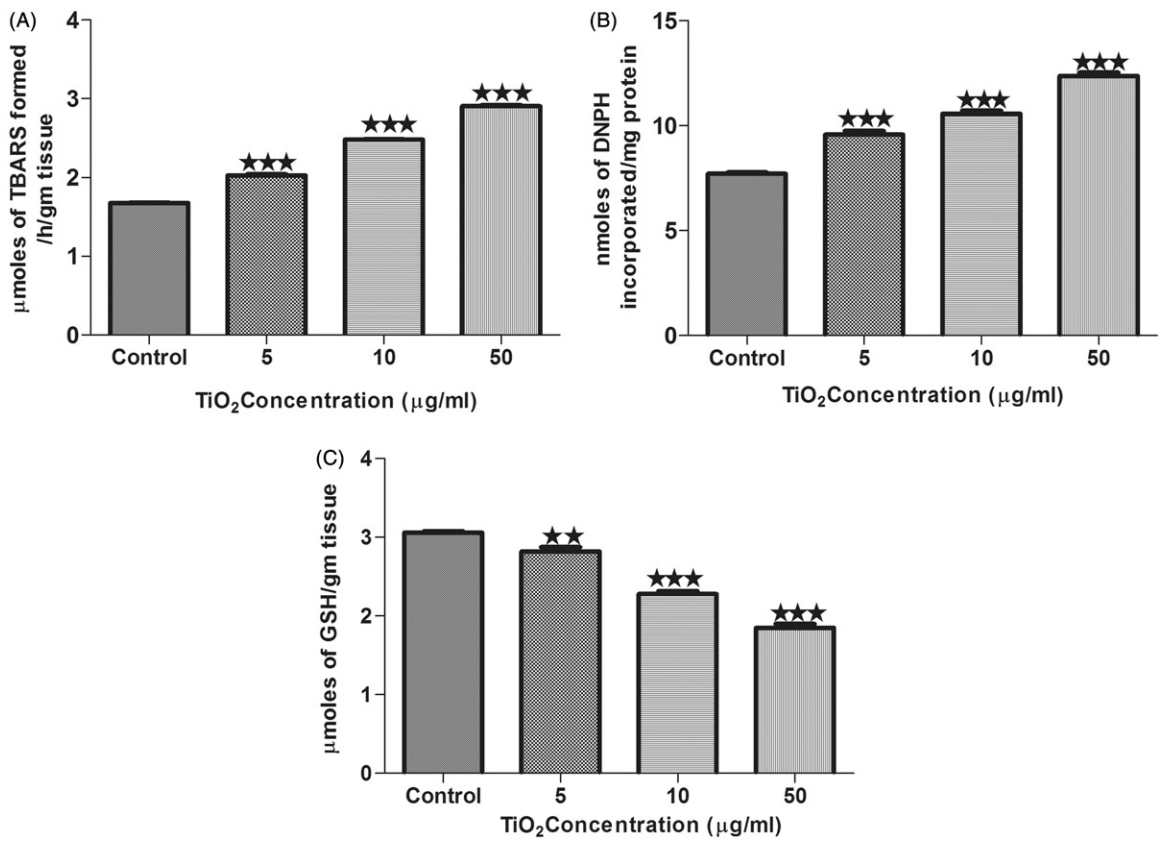

Figure 2. Effects of TNPs on (A) LPO level (B) PC content and (C) GSH levelin isolated brain mitochondria of rat. Each value represents mean ± SE ($n = 6$). LPO was measured as µmoles of TBARS formed /h/gm tissue, PC was measured as nmoles of DNPH incorporated/mg protein and GSH was measured as µmoles of GSH/gm tissue. Significant differences were indicated by (**$p < 0.01$ and ***$p < 0.001$) was used to show significant difference when compared to the control group.

which consequently resulted in a significant ($p < 0.001$) increase in PC content in the brain mitochondria when compared with control (Figure 2B).

Non-enzymatic antioxidant effects

Effect on GSH

GSH is a major antioxidant which scavenges free radicals in brain tissue and protects brain cells from oxidative damage. Exposure of brain mitochondria to TNPs caused a significant ($p < 0.01$–0.001) decrease in GSH content in brain mitochondria of rats with increasing concentration of TNPs (Figure 2C).

Enzymatic antioxidant activities

Effect of GPx

Exposure of TNPs with brain mitochondria have shown significant effect on GPx which plays crucial role in dealing with peroxides in mitochondria resulted in a significant ($p < 0.001$) increase in the activity of GPx as compared with control (Figure 3A).

Effect of GR

GR, which is a glutathione metabolizing enzyme, showed significant ($p < 0.001$) increase in the activity in a dose-dependent manner in brain mitochondria as compared to control (Figure 3B).

Effect on activity of Mn-SOD

Mn-SOD which is known to be the most important defence enzyme against the oxidative damage was found to be significantly ($p < 0.001$ and $p < 0.01$) increased upon exposure with TNPs when compared with control group (Figure 3C).

Mitochondrial markers

NADH dehydrogenase is one of the starting enzymes of oxidative phosphorylation in mitochondria. Exposure of TNPs with brain mitochondria have shown effect on NADH dehydrogenase activity which resulted in a significant ($p < 0.001$) decrease in the activity when exposed to different concentration of TNPs in brain mitochondria as compared with control (Figure 4A). Succinate dehydrogenase plays an important role in citric acid cycle and oxidative phosphorylation. TNP treatment with increasing concentration significantly ($p < 0.001$) decreased the activity of succinate dehydrogenase in brain mitochondria when compared with control (Figure 4B).

The mitochondrial dehydrogenases activity via MTT ability was detected by the formation of formazan when exposed to different concentration of TNP. The mitochondrial dehydrogenases activity via MTT ability was found to be significantly decreased ($p < 0.001$) when the brain mitochondria were exposed to different concentration of TNPs as compared to control (Figure 4C). ATP synthase enzyme

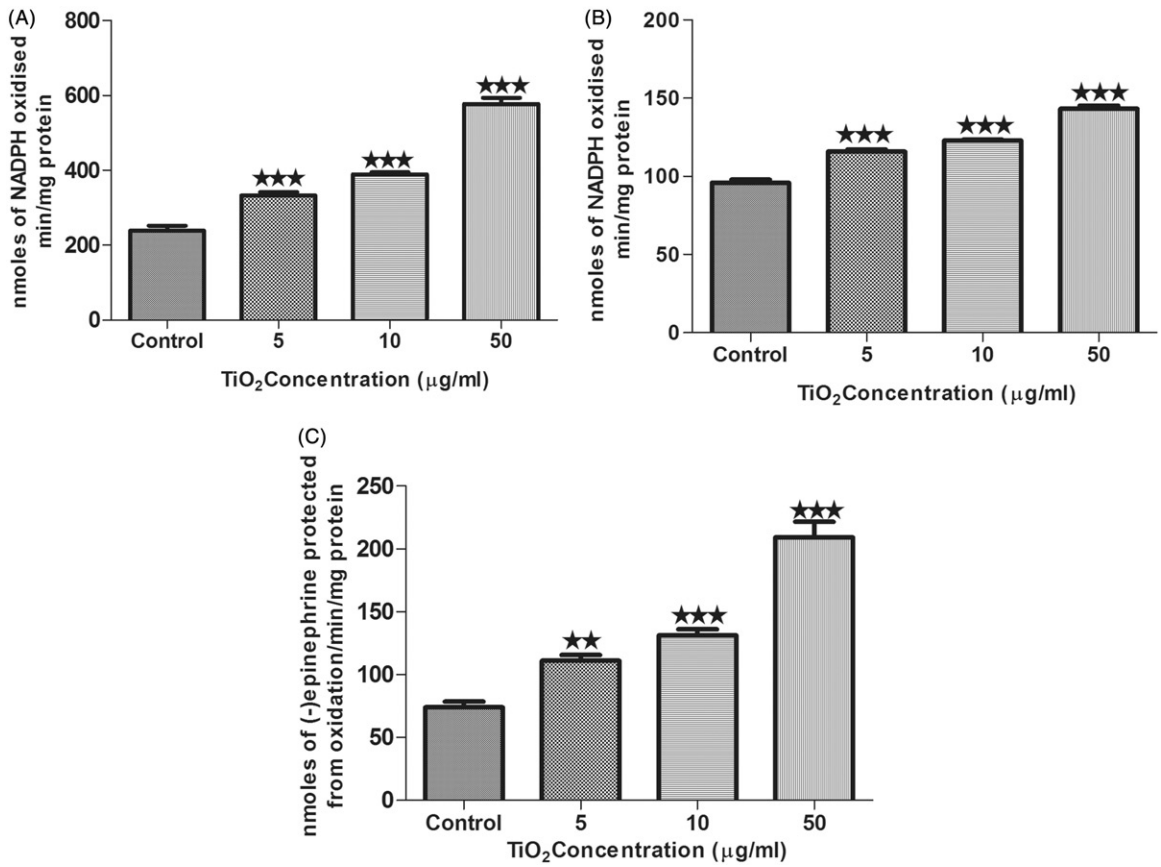

Figure 3. Effects of TNPs on (A) GPx (B) GR and (C) Mn-SOD activity in isolated brain mitochondria of rat. Each value represents mean ± SE ($n = 6$). GPx activity was measured as nmoles of NADPH oxidized/min/mg protein, GR activity was measured as nmoles of NADPH oxidized/min/mg protein and Mn-SOD activity was measured as n moles of (–) epinephrine protected from oxidation/min/mg protein. The TNPs exposed group were significantly different from control group (**$p < 0.01$ and ***$p < 0.001$).

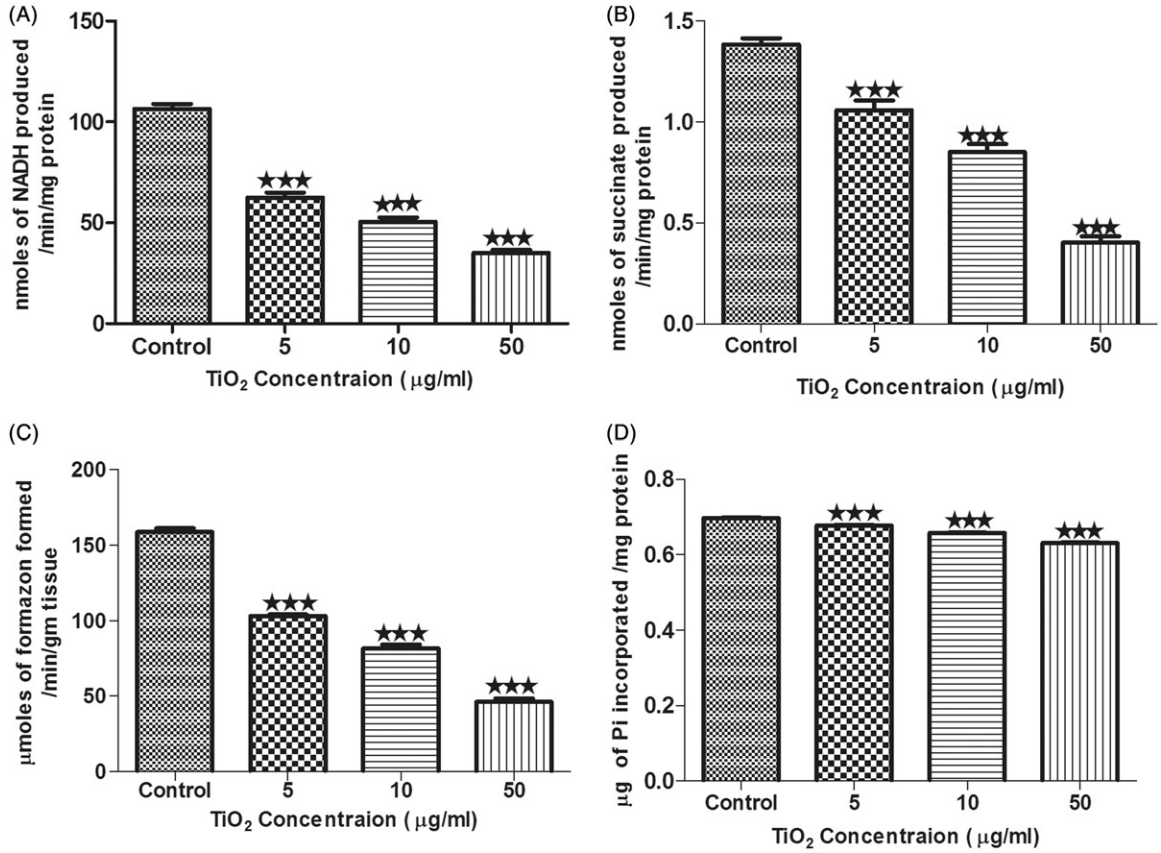

Figure 4. Effects of TNPs on (A) NADH dehydrogenase, (B) succinate dehydrogenase, (C) MTT ability and (D) ATP synthase activity in isolated brain mitochondria of rat. Each value represents mean ± SE ($n = 6$). Values were expressed as nmoles NADH oxidized/min/mg protein, nmoles succinate oxidized/min/mg protein, μmoles formazan formed/min/mg protein and μg Pi liberated/min/mg protein respectively. Significant differences were observed in TNPs exposed group when compared to the control group as indicated by ***$p < 0.001$.

promotes the synthesis of ATP from ADP and Pi. The ATPase synthase activity was found to be significantly decreased ($p < 0.001$) when different concentrations of TNPs were exposed to brain mitochondria (Figure 4D).

Discussion

The purpose of this investigation was to evaluate potential neurotoxicity of TNPs and the possible mechanisms being implicated by employing isolated rat brain mitochondria as an *in vitro* model. There has been a paucity of studies directly or indirectly investigating the toxic effects of TNPs and no clear guidelines are presently available to determine these effects. TNPs (<100 nm in diameter) have been widely used in pharmaceutical and cosmetic products (Nohynek et al., 2008; Wolf et al., 2003). The important characteristic of small size and large surface area per mass provide NPs more biological activity (Wang et al., 2008).

When the mitochondria are invaded by the TNPs, the antioxidant defense ability could be altered (Long et al., 2006). Brain is most vulnerable to oxidative stress, in comparison to other organs, due to its low oxidative capacity (Uttara et al., 2009). It has been shown that the total anti-oxidation capacity decreased with increasing nanoparticulate anatase doses (Liang et al., 2009). Several works concerning the toxicity of TNPs (Federici et al., 2007; Gramowski et al., 2010; Johnston et al., 2009; Moore et al., 2006) have shown that oxidative stress is a prominent feature of the response to TNPs.

LPO is the principal biomarker of oxidative stress and is known to be the most substantial investigated activity which is induced by free-radical production and generation of ROS (Hussain et al., 2005). Generation of free radicals or oxidative stress develops when there is an imbalance between pro-oxidants and antioxidants ratio, leading to the generation of ROS (Dalle-Donne et al., 2002; Rezvanfar et al., 2010). The superoxide radical produced by TNPs can get across the cell membranes and form the highly reactive hydroxyl radical by interaction with transition metal ions (Ahsan et al., 2003), and the hydroxyl radicals attack membrane phospholipids in a free-radical process, resulting in phospholipid peroxidation and loss of membrane integrity (Blaisdell, 2002). The overproduction of ROS would break down the balance of the oxidative/antioxidative system in the brain, resulting in LPO, which is closely related to the reduction of the antioxidative enzymes (Ma et al., 2010). TNPs induced the oxidative damage which results in elevated MDA levels (Wang et al., 2008). In the cell membrane, the abundant lipids, polysaccharides and proteins are the targets of ROS. The free-radical attack at the membrane lipids would result in the accumulation of a complex mixture of products at the cell surface, consisting of the initial lipids and their fragmentation products, as well as peroxidation products of both former and latter. TNP induces

oxidative stress which is a common mechanism that damages cell and a wide range of NM species have been shown to create ROS. The enhanced LPO in TNP-treated brain mitochondria may result in loss of major fatty acid components from mitochondrial membranes resulting in the loss of critical phospholipid-like cardiolipin that are critical to mitochondrial enzymes/function (Kamboj & Sandhir, 2011). Our results demonstrate induction of LPO levels of brain mitochondria on exposure to TNPs at different concentrations. The increase in LPO levels in our study, after exposure of brain mitochondria to TNPs may be the result of increased production of ROS or a reduction in antioxidant status.

One of the most universally accepted biomarker is PC and has been frequently used so far (Tabassum et al., 2007). It has been observed that production of ROS leads to high level of protein carbonylation (Tabassum et al., 2007). There is a rapid progress in the identification of carbonylated proteins that would provide new diagnostic biomarkers for oxidative stress (Tabassum et al., 2007). Oxidative damage to protein results in generation of superoxide radicals by modifying the symmetrical arrangement via aggregation, fragmentation, and formation of cross-linkages in the polypeptide chain (Chen et al., 2005). The usage of PC groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins. We can measure the amount of the stable dinitrophenyl (DNP) hydrazone product which has been formed from DNPH and can be detected by various means, such as spectrophotometric assay (Dalle-Donne et al., 2002). It is well-known that TNPs have more biological activities to produce ROS (Wang et al., 2008). Increase of PC content in brain mitochondria, may be due to increased production of superoxide radicals. Our results show that TNPs induce oxidative stress by elevating the levels of protein oxidation. It has been reported that modification of PC status results in adverse functional of the cells (Guo et al., 2011; Obrenovich et al., 2011).

Reduced glutathione (GSH), a ubiquitous tripeptide thiol, and is a vital intracellular and extracellular protective antioxidant (Zitka et al., 2012). GSH is considered to be one of the most important scavengers of ROS, and its ratio with oxidized glutathione (GSSG) may be used as a marker of oxidative stress. Within cells, total GSH exists in free form and bound to proteins. Since the enzyme GR reverts free glutathione from its oxidized form (GSSG), the free glutathione is constitutively active and inducible upon oxidative stress, and thus free glutathione may exist almost exclusively in its reduced form (Zitka et al., 2012). GSH is a ubiquitous sulfhydryl containing molecule in cells that are responsible for maintaining cellular oxidation reduction homeostasis (Sies, 1999). GSH contributes around 90% of the intracellular NP-SH. The remaining 10% is made up of other small thiol compounds such as cysteine and methionine (Ghezzi et al., 2002). It has been investigated that mitochondria contains a distinct GSH/NP-SH pool important in maintaining a reduced matrix environment and detoxifying H_2O_2 produced in the matrix. The depletion of mitochondrial GSH has been shown to be associated with xenobiotic-induced toxicity (González-Salazar et al., 2011). GSH is one of the essential compounds for maintaining cell integrity against ROS and as being a non-enzymatic free-radical scavenger, it participates in the detoxification of ROS and reduces H_2O_2. Mitochondrial GSH plays a key role in mitochondria functionality and a more significant role than cytosolic GSH in determining cellular function and viability (Garcia et al., 2010). The reduction of GSH content demonstrated in our study may be attributed to the direct conjugation of TNPs and its reactive metabolites with thiol groups, thereby interfering antioxidants status (Oberdörster et al., 2004). Several questions arise as to how TNPs could induce toxicity? It is not known, for instance, how TNPs depletes GSH levels, whether it binds directly to GSH, or inhibits enzymes involved in GSH synthesis.

GR is another important enzyme helpful for maintenance of intracellular concentration of GSH (Ahmed et al., 2006). GR serves to regenerate reduced GSH from oxidized GSSG by the activation of GPx (Srinivasan & Pari, 2012). GR requires NADPH for its activity, which is maintained at high level in the cell. In our study, TNP exposure showed increased activity of GR in brain mitochondria. It may be due to the protection from oxidative stress formed by overproduction of free radical. GPx is an enzyme that plays an important role in removing excess free radicals and hydroxides and catalyses the conversion of H_2O_2 into water in the presence of GSH. In the present investigation, there is an increased GPx activity. GPx plays a crucial role in dealing with peroxides in mitochondria because of lack of catalase (Tabassum et al., 2010). In our results, TNP treatment showed increased activity of GPx in brain mitochondria. SOD destroys the superoxide radical and GSH-PX detoxifies H_2O_2 especially (Liang et al., 2009). Mn-SOD presents in the matrix of mitochondria and it behaves as the first line of defense against deleterious effects of oxyradicals in cells by catalyzing the dismutation of endogenous cytotoxic superoxide radicals to H_2O_2 (Mruk et al., 2002). In our study, TiO_2 treatment showed an enhanced activity of Mn-SOD in brain mitochondria. Many investigators have found an elevation in Mn-SOD activity in the isolated mitochondria of the brain (Bhagwat et al., 1998; Kanbagli et al., 2002; Ranjbar et al., 2014). Reports indicate that this increase may play an important role in the protection of mitochondria against ethanol-induced oxidative stress (Kanbagli et al., 2002). Mn-SOD, unlike CuZnSOD, is an enzyme which can be induced by its substrate and/or some other factors. According to this, we can say that an increase in MnSOD activity may play an important role in the regulation of mitochondrial susceptibility against TNPs-induced oxidative stress, in spite of the decrease in GSH. This elevation may play a major role in the regulation of mitochondrial susceptibility against TNPs toxicity.

Mitochondria appear to be important cellular organelles targeted to the pro-oxidative effects of NPs (Li et al., 2003) and an important source that contribute significantly for the production of ROS after some toxicity or an injury (Allen & Bayraktutan, 2009). The mitochondrial complexes that are selected in the current study represent vital biological functions of the brain mitochondria as well as provide a general sense of toxicity in a relatively short time (Hussain et al., 2005). The results described in this study provide a range of concentration that were toxic to the cells and data

pointing to a general toxicity in brain mitochondria by TNPs. Complex I catalyzes the transfer of electrons from NADH to coenzyme Q10 (CoQ10) and it is located in the inner mitochondrial membrane (Andreyev et al., 2005). It has been widely accepted as a major source of mitochondrial oxygen radical generation. Thus, it has been postulated that the one-electron transfer to molecular oxygen could happen from the flavin mononucleotide, the semiubiquinone-binding site of complex I (Kudin et al., 2004). Our results using TNPs have shown the ability of TNPs to induce oxidative stress by decreasing NADH dehydrogenase activity.

Succinate dehydrogenase or respiratory Complex II is an enzyme complex, bound to the inner mitochondrial membrane of living cells (Andreyev et al., 2005). It is the only enzyme that participates in both the citric acid cycle and the electron transport chain (Oyedotun & Lemire, 2004). Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. This occurs in the inner mitochondrial membrane by coupling the two reactions together. Results showed decreased activity of succinate dehydrogenase when exposed to TNPs in brain mitochondria. NAD(P)H-dependent cellular oxidoreductase enzymes or mitochondrial dehydrogenases under defined conditions can reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium yellow dye MTT bromide to its insoluble formazan. Our results showed decrease number of viable cells present when the brain mitochondria was exposed to MTT and TNPs. Complex V (ATP synthase) is the final enzyme in the oxidative phosphorylation pathway. ATP synthase is a massive protein complex (Nakamoto et al., 2008). The enzyme uses the energy stored in a proton gradient across a membrane to drive the synthesis of ATP from ADP and inorganic phosphate (Morin et al., 2002). ATP synthase function is to regulate the flow of protons from intermembrane space to matrix of the mitochondria. ATP content is closely related to maintenance of membrane potential; thus, a reduction in ATP would produce an irreversible drop in membrane potential, opening the permeability transition pore and can trigger mechanisms of cell death. Our data showed decreased activity of ATP synthase activity with increasing concentrations of TNPs. The special character of small size and large surface area per mass render TNPs more biological activity. The inferences drawn from this study highlight the markers of toxicity in experimental rat model. Our data using TNPs have reported the ability of TNPs to induce oxidative stress.

In summary, to evaluate the toxicity of TNPs, we studied the oxidative stress of the particles in rat brain mitochondria. It is important to study the effects and ROS status by evaluation of various enzymes and oxidations. TNP is widely used in many fields related to our daily life. Thus, more attention is needed on the impact of nano-TiO_2 on human health and the environment. Among various mechanisms in which the effect of nano-TiO_2 exposure is involved, oxidative stress seems to be an important mechanism which involves interaction between nano-TiO_2 particles and living organisms. The oxidative stress markers, mitochondrial complexes, non-enzymatic and enzymatic antioxidants were modulated in our study, suggesting that nano-TiO_2 could induce oxidative stress in brain mitochondria.

Acknowledgements

This work was supported by Science and Engineering Research Board (SERB), Department of Science and Toxicology, Government of India under the Fast Track Young Scientist Program to S.P. (No. SB/FT/LS-152/2012), and Senior Research Fellowship from CSIR, Government of India to N.N. (No. 09/591(0112)/2009-EMR-1).

Declaration of interest

The authors report no conflicts of interest.

References

Ahmed RS, Suke SG, Seth V, et al. (2006). Impact of oral vitamin E supplementation on oxidative stress and lipid peroxidation in patients with polymorphous light eruption. Indian J Med Res 123:781–7.

Ahsan H, Ali A, Ali R. (2003). Oxygen free radicals and systemic autoimmunity. Clin Exp Immunol 131:398–404.

Allen CL, Bayraktutan U. (2009). Oxidative stress and its role in the pathogenesis of ischaemic stroke. Int J Stroke 4:461–70.

Andreyev AY, Kushnareva YE, Starkov AA. (2005). Mitochondrial metabolism of reactive oxygen species. Biochemistry (Mosc) 70: 200–14.

Bhagwat SV, Vijayasarathy C, Raza H, et al. (1998). Preferential effects of nicotine and 4-(N-methyl-N-nitrosamine)-1-(3-pyridyl)-1-butanone on mitochondrial glutathione *S*-transferase A4-4 induction and increased oxidative stress inthe rat brain. Biochem Pharmacol 56: 831–9.

Blaisdell FW. (2002). The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg 10: 620–30.

Chen YR, Chen CL, Zhang L, et al. (2005). Superoxide generation from mitochondrial NADH dehydrogenase induces self-inactivation with specific protein radical formation. J Biol Chem 280:37339–48.

Colvin VL. (2003). The potential environmental impact of engineered nanomaterials. Nat Biotechnol 21:1166–70.

Correia SC, Carvalho C, Cardoso S, et al. (2010). Mitochondrial preconditioning: a potential neuroprotective strategy. Front Aging Neurosci 138:1–13.

Curtin JF, Donovan M, Cotter TG. (2002). Regulation and measurement of oxidative stress in apoptosis. J Immunol Methods 265:49–72.

Dalle-Donne I, Rossi R, Giustarini D, et al. (2002). Methionine oxidation as a major cause of the functional impairment of oxidized actin. Free Radic Bio Med 32:927–37.

Federici G, Shaw BJ, Handy RD. (2007). Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects. Aquatic Toxicol 84: 415–30.

Floor E, Wetzel MG. (1998). Increased protein oxidation in human substantia nigra parscompacta in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. J Neurochem 70:268–75.

Foley KF, Van Dort ME, Sievert MK, et al. (2002). Stereospecific inhibition of monoamine uptake transporters by meta-hydroxyephedrine isomers. J Neural Transm 109:1229–40.

Garcia J, Han D, Sancheti H, et al. (2010). Regulation of mitochondrial glutathione redox status and protein glutathionylation by respiratory substrates. J Biol Chem 285:39646–54.

Ghezzi P, Romines B, Fratelli M, et al. (2002). Protein glutathionylation: coupling and uncoupling of glutathione to protein thiol groups in lymphocytes under oxidative stress and HIV infection. Mol Immunol 38:773–80.

González-Salazar A, Molina-Jijón E, Correa F, et al. (2011). Curcumin protects from cardiac reperfusion damage by attenuation of oxidant stress and mitochondrial dysfunction. Cardiovasc Toxicol 11:357–64.

Govil N, Chaudhary S, Waseem M, Parvez S. (2012). Postnuclear supernatant: an *in vitro* model for assessing cadmium-induced neurotoxicity. Biol Trace Elem Res 146:402–9.

Gramowski A, Flossdorf J, Bhattacharya K, et al. (2010). Nanoparticles induce changes of the electrical activity of neuronal networks on microelectrode array neurochips. Environ Health Perspect 118: 1363–9.

Guo J, Zhu S, Chen Z, et al. (2011). Sonochemical synthesis of TiO_2 nanoparticles on graphene for use as photocatalyst. Ultrason Sonochem 18:1082–90.

Haque R, Bin-Hafeez B, Parvez S, et al. (2003). Aqueous extract of walnut (*Juglans regia* L.) protects mice against cyclophosphamide induced biochemical toxicity. Hum Exp Toxicol 22:473–80.

Hiura TS, Li N, Kaplan R, et al. (2000). The role of a mitochondrial pathway in the induction of apoptosis by chemicals extracted from diesel exhaust particles. J Immunol 165:2703–11.

Hussain SM, Hess KL, Gearhart JM, et al. (2005). *In vitro* toxicity of nanoparticles in BRL 3A rat liver cells. Toxicol In Vitro 19:975–83.

Haase A, Rott S, Mantion A, et al. (2012). Effects of silver nanoparticles on primary mixed neural cell cultures: uptake, oxidative stress and acute calcium responses. Toxicol Sci 126:457–68.

Iwata T, Nishiyama N, Nagano K, et al. (2012). Role of pulmonary resection in the diagnosis and treatment of limited-stage small cell lung cancer: revision of clinical diagnosis based on findings of resected specimen and its influence on survival. Gen Thorac Cardiovasc Surg 60:43–52.

Johnston HJ, Hutchison GR, Christensen FM, et al. (2009). Identification of the mechanisms that drive the toxicity of TiO_2 particulates: the contribution of physicochemical characteristics. Part Fibre Toxicol 6: 33–9.

Kamboj SS, Sandhir R. (2011). Protective effect of N-acetylcysteine supplementation on mitochondrial oxidative stress and mitochondrial enzymes in cerebral cortex of streptozotocin-treated diabetic rats. Mitochondrion 11:214–22.

Kanbagli O, Balkan J, Aykaç-Toker G, Uysal M. (2002). Hepatic mitochondrial prooxidant and antioxidant status in ethanol-induced liver injury in rats. Bio Pharm Bull 25:1482–4.

Krystek P, Tentschert J, Nia Y, et al. (2014). Method development and inter-laboratory comparison about the determination of titanium from titanium dioxide nanoparticles in tissues by inductively coupled plasma mass spectrometry. Anal Bioanal Chem 406:3853–61.

Kudin AP, Bimpong-Buta NY, Vielhaber S, et al. (2004). Characterization of superoxide-producing sites in isolated brain mitochondria. J Biol Chem 279:4127–35.

Li M, Xia T, Jiang CS, et al. (2003). Cadmium directly induced the opening of membrane permeability pore of mitochondria which possibly involved in cadmium-triggered apoptosis. Toxicology 194: 19–33.

Liang G, Pu Y, Yin L, et al. (2009). Influence of different sizes of titanium dioxide nanoparticles on hepatic and renal functions in rats with correlation to oxidative stress. J Toxicol Environ Health A 72: 740–5.

Liu Y, Fiskum G, Schubert D. (2002). Generation of reactive oxygen species by the mitochondrial electron transport chain. J Neurochem 80:780–7.

Long TC, Saleh N, Tilton RD, et al. (2006). Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity. Environ Sci Technol 40:4346–52.

Long TC, Tajuba J, Sama P, et al. (2007). Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons *in vitro*. Environ Health Perspect 115:1631–7.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951). Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–75.

Ma L, Liu J, Li N, et al. (2010). Oxidative stress in the brain of mice caused by translocated nanoparticulate TiO_2 delivered to the abdominal cavity. Biomaterials 31:99–105.

Moller P, Jacobsen NR, Folkmann JK, et al. (2010). Role of oxidative damage in toxicity of particulates. Free Radic Res 44:1–46.

Moore MN, Allen JI, McVeigh K. (2006). Environmental prognostics: an integrated model supporting lysosomal stress response as predictive biomarkers of animal health status. Mar Environ Res 61:278–304.

Morin C, Zini R, Simon N, Tillement JP. (2002). Dehydroepiandrosterone and -estradiol limit the functional alterations of rat brain mitochondria submitted to different experimental stresses. Neuroscience 115:415–24.

Mruk DD, Silvestrini B, Mo MY, Cheng CY. (2002). Antioxidant superoxide dismutase – a review: its function, regulation in the testis, and role in male fertility. Contraception 65:305–11.

Nakamoto RK, Baylis Scanlon JA, Al-Shawi MK. (2008). The rotary mechanism of the ATP synthase. Arch Biochem Biophys 476:43–50.

Nohynek GJ, Dufour EK, Roberts MS. (2008). Nanotechnology, cosmetics and the skin: is there a health risk? Skin Pharmacol Physiol 21:136–49.

Oberdörster G, Maynard A, Donaldson K, et al. (2005). Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol 2:8.

Oberdörster G, Sharp Z, Atudorei V, et al. (2004). Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol 16:437–45.

Obrenovich ME, Li Y, Parvathaneni K, et al. (2011). Antioxidants in health, disease and aging. CNS Neurol Disord Drug Targets 10: 192–207.

Oyedotun KS, Lemire BD. (2004). The quaternary structure of the *Saccharomyces cerevisiae* succinate dehydrogenase. Homology modeling, cofactor docking, and molecular dynamics simulation studies. J Biol Chem 279:9424–31.

Parvez S, Winkler-Stuck K, Hertel S, et al. (2010). The dopamine-D2-receptor agonist ropinirole dose-dependently blocks the Ca^{2+}-triggered permeability transition of mitochondria. Biochim Biophys Acta 1797:1245–50.

Ranjbar A, Sharifzadeh M, Karimi J, et al. (2014). Propofol attenuates toxic oxidative stress by CCl_4 in liver mitochondria and blood in rat. Iran J Pharm Res 13:253–62.

Rashidi L, Khosravi-Darani K. (2011). The applications of nanotechnology in food industry. Crit Rev Food Sci Nutr 51:723–30.

Rezvanfar MA, Farshid AA, Sadrkhanlou RA. (2010). Benefit of *Satureja khuzestanica* in subchronically rat model of cyclophosphamide-induced hemorrhagic cystitis. Exp Toxicol Pathol 62:323–30.

Sekar D, Falcioni ML, Barucca G, Falcioni G. (2014). DNA damage and repair following *in vitro* exposure to two different forms of titanium dioxide nanoparticles on trout erythrocyte. Environ Toxicol 29: 117–27.

Sies H. (1999). Glutathione and its role in cellular functions. Free Radic Biol Med 27:916–21.

Srinivasan S, Pari L. (2012). Ameliorative effect of diosmin, a citrus flavonoid against streptozotocin-nicotinamide generated oxidative stress induced diabetic rats. Chem Biol Interact 195:43–51.

Suzuki H, Toyooka T, Ibuki Y. (2007). Simple and easy method to evaluate uptake potential of nanoparticles in mammalian cells using a flow cytometric light scatter analysis. Environ Sci Technol 41: 3018–24.

Tabassum H, Parvez S, Pasha ST, et al. (2010). Protective effect of lipoic acid against methotrexate-induced oxidative stress in liver mitochondria. Food Chem Toxicol 48:1973–9.

Tabassum H, Parvez S, Rehman H, et al. (2007). Catechin as an antioxidant in liver mitochondrial toxicity: inhibition of tamoxifen-induced protein oxidation and lipid peroxidation. J Biochem Mol Toxicol 21:110–17.

Teodoro JS, Simões AM, Duarte FV, et al. (2011). Assessment of the toxicity of silver nanoparticles *in vitro*: a mitochondrial perspective. Toxicol In Vitro 25:664–70.

Uttara B, Singh AV, Zamboni P, Mahajan RT. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol 7:65–74.

Wang J, Chen C, Liu Y, et al. (2008). Potential neurological lesion after nasal instillation of TiO(2) nanoparticles in the anatase and rutile crystal phases. Toxicol Lett 183:72–80.

Waseem M, Bhardwaj M, Tabassum H, et al. (2015). Cisplatin hepatotoxicity mediated by mitochondrial stress. Drug Chem Toxicol 13:1–8.

Wolf R, Matz H, Orion E, Lipozencić J. (2003). Sunscreens-the ultimate cosmetic. Acta Dermatovenerol Croat 11:158–62.

Zhang L, Bai R, Li B, et al. (2011). Rutile TiO_2 particles exert size and surface coating dependent retention and lesions on the murine brain. Toxicol Lett 207:73–81.

Zitka O, Skalickova S, Gumulec J, et al. (2012). Redox status expressed as GSH:GSSG ratio as a marker for oxidative stress in paediatric tumour patients. Oncol Lett 4:1247–53.