Modulatory Role of Quercetin in Mitochondrial Dysfunction in Titanium Dioxide Nanoparticle-Induced Hepatotoxicity

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ABSTRACT: Background: Titanium dioxide (TiO2) nanoparticles are among the largely manmade nanomaterials worldwide and are broadly used as both industrial and user products. The primary target site for several nanoparticles is the liver, including TiO2 nanoparticles (TNPs), exposed directly or indirectly through ingestion of contaminated water, food, or animals and elevated environmental contamination. Oxidative stress is a known facet of nanoparticle-induced toxicity, including TNPs. Mitochondria are potential targets for nanoparticles in several types of toxicity, such as hepatotoxicity. Nevertheless, its causal mechanism is still controversial due to scarcity of literature linking the role of mitochondria-mediated TNP-induced hepatotoxicity.

Aim: The objective of the current study was to evaluate the relation of mitochondrial oxidative stress and respiratory chain mechanisms with TNP-induced mitochondrial dysfunction in vitro, and explore the hepatoprotective effect of quercetin (QR), which is a polyphenolic flavonoid abundant in fruits and vegetables with known antioxidant properties, on TNP-induced mitochondrial oxidative stress and disturbance in respiratory chain complex enzymes in the liver of rats.

Results: Enzymatic and non-enzymatic antioxidant levels, oxidative stress markers, and mitochondrial complexes were assessed with regard to TNP-induced hepatotoxicity. The depleted lipid peroxidation levels and protein carbonyl content, in mitochondria, induced by TNPs were restored significantly by pretreatment with QR. QR modulated the altered non-enzymatic and enzymatic antioxidants and mitochondrial complex enzymes.

Conclusion: Based on the findings, we conclude that QR, which mitigates oxidative stress caused by mitochondrial dysfunction, holds promising capability to potentially diminish TNP-induced adverse effects in the liver.

INTRODUCTION

The rapid breakthrough in nanoscience research has resulted in growth in the manufacture and usage of nanoparticles, which are less than 100 nm in dimension.1,2 Conventionally, nanoparticles of sizes ranging to as low as several nanometers are often produced as byproducts of anthropogenic activities, such as simple combustion, chemical manufacturing, welding, smelting, vehicular combustion, airplane engines, and ore refining.3 TiO2 nanoparticles are widely used because of their unique properties in various biological and allied domains, such as medicine, drug delivery, transfection vectors, food industry, and fluorescent labels.4 It has been known from long that mitochondria acts as a powerhouse of the cell. Mitochondria is the most active organelle for cellular redox reactions in a cell, and build-up of these particles into such redox active centers is expected to cause alterations in several antioxidant systems, both enzymatic and non-enzymatic, as these markers are vital for cellular functions to occur accurately. Because of its properties such as surface area, TiO2 possesses a great capacity to produce reactive oxygen species (ROS).5−8 Nanoparticle (NP)-mediated mitochondrial damage has harmful results, which involves events like...
commencement of apoptosis, inflammation, membrane permeability, decrease in membrane potential, and reduction of adenosine 5′-triphosphate (ATP) levels. Thus, mitochondrial dysfunction may play a pivotal role in the toxicity resulting from exposure to NPs.

In vitro studies have shown variations in the mitochondrial structure and function of hepatic cells exposed to TNPs. It seems that these variations may play an important role in explicating various aspects of hepatotoxicity induced by TNPs.

Studies have been done on natural antioxidants to explore their properties as potential nutraceuticals that might help ameliorate toxicity and its side effects. QR (3,3′,4,4′,5,7-pentahydroxyflavone) is a ubiquitously present polyphenolic flavonoid in plant food sources such as fruits, vegetables, tea, aromatic plants, and red wine. Usage has been reported to have the therapeutic potential to mitigate various kinds of toxicity, such as cardiotoxicity, nephrotoxicity, neurotoxicity, and hepatotoxicity. Studies have reported that mitochondrial dysfunction in rodents can be modulated by pretreatment with QR, and the rationale behind its ameliorative effect on mitochondrial toxicity is based on its antioxidant properties. Due to the scarcity of literature on TNP-mediated hepatotoxicity in the mitochondria, it is still not clear what role the mitochondria play in triggering the chain of events that eventually lead to subcellular toxicity. Hence, the present study was aimed at mounting the therapeutic aspects of QR to control the hepatotoxic properties of TNP exposure in isolated rat liver mitochondria.

**RESULTS**

**Surface Area and Size Distribution of Titanium Dioxide Nanoparticles.** The TNPs used in this study were examined by transmission electron microscopy (TEM). The results obtained show that our TNP solution contained a combination of nano-sized compounds as well as agglomerates (Figure 1).

**Effect on Oxidative Stress Biomarkers.** In the preliminary study, TNPs evoked lipid peroxidation (LPO) and protein carbonyl (PC) levels. In the 10 and 50 μg/mL TNP-exposed groups, there was a noteworthy elevation (p < 0.01–0.001) in TBARS levels when equated to the control.
group (Figure S1A). There was no significant alteration in LPO levels in the 5 μg/mL TNP-exposed group when compared to the control group. Figure S1B characterizes the effect of TNPs on PC level in the liver mitochondria of rats. In the 10 and 50 μg/mL TNP-exposed groups, the PC contents were suggestively increased ( \( p < 0.01 \) ) in the liver mitochondria in comparison to the control group. However, QR treatment diminished the LPO and PC elevated levels in the TNP-treated group. TNP treatment at a dose of 50 μg/mL (group IV) led to remarkable elevation in LPO (Figure 2A) and PC (Figure 2B) content ( \( p < 0.05 \) − 0.001) when compared to the control group (group I). The TNP dose of 50 μg/mL was chosen based on the results depicted in Figures S1−S5. Prior exposure to QR (group III) significantly ( \( p < 0.001 \) ) prevented enhancement in LPO and PC products in the liver mitochondria in comparison with the TNP-treated (50 μg/mL; group IV) group (Figure 2).

Effect on Non-enzymatic Antioxidant Levels. The preliminary results reflected that TNP treatment diminishes non-enzymatic antioxidant capacity. On exposure to TNPs at a dose of 5−50 μg/mL, a significant reduction ( \( p < 0.05 \) − 0.001) was encountered by way of reduced glutathione (GSH) levels in the rat liver mitochondria when compared to the control group (Figure S2A). For non-protein thiol (NP-SH) determination, a significant decline ( \( p < 0.001 \) ) in NP-SH contents was seen in the TNP groups (5−50 μg/mL) in comparison to the control group (Figure S2B). Later, the results showed that QR treatment ameliorated reduction in GSH and NP-SH levels in the TNP-exposed group. The GSH (Figure 3A) and NP-SH (Figure 3B) levels in rat liver mitochondria were significantly diminished ( \( p < 0.05 \) − 0.01) in comparison with the TNP-exposed (50 μg/mL) group (group IV). Only the QR-treated group (group II) showed no significant change in GSH and NP-SH levels as compared to the control group (Figure 3).

Effect on Enzymatic Antioxidant Levels. With the preliminary studies, it has been seen that TNPs modify antioxidant enzyme kinetics and provoke the glutathione-S transferase (GST) and manganese-superoxide dismutase (Mn-SOD) activity. Figure S3A signifies the GST activity in rat liver mitochondria. There was a noteworthy decline in Mn-SOD activity for the 10−50 μg/mL TNP-treated group ( \( p < 0.05 \) − 0.001) in comparison with the control group. An important modification ( \( p < 0.01 \) ) was also seen in Mn-SOD activity for 5−50 μg/mL ( \( p < 0.05 \) − 0.01) of the TNP-exposed group as

Figure 2. Effect of pretreatment with QR (50 μM) and TNP (50 μg/mL) on (A) LPO and (B) PC content in rat liver mitochondria. The values are stated as nmol of TBARS formed/h/g tissue and nmol of DNPH incorporated/mg protein. All values are represented as mean ± SE (n = 6). Significant alterations are indicated by * \( p < 0.05 \) and *** \( p < 0.001 \) in comparison with the control group, and substantial differences are shown by ### \( p < 0.001 \) in comparison with the TNP-treated group.
compared to the control group (Figure S3B). When TNP-exposed mitochondrial samples were treated with QR, the diminished activity of GST and Mn-SOD gets alleviated. TNP (group IV) exposure (50 μg/mL) remarkably (p < 0.01–0.001) reduced GST (Figure 4A) and Mn-SOD (Figure 4B) activity in liver mitochondria in comparison to the control (group I). QR pre-exposure (group III) resulted in a significant increase (p < 0.05–0.01) in GST and Mn-SOD activity when compared to the TNP-exposed group (50 μg/mL). QR alone treatment (group II) showed no significant change in GST and Mn-SOD activity as compared to the control group (Figure 4).

**Effect on Mitochondrial Complex Enzymes. NADH Dehydrogenase (Complex I) and Succinate Dehydrogenase (Complex II) Activity.** TNP treatment inflicts mitochondrial complex I (nicotinamide adenine dinucleotide (NADH) dehydrogenase) and complex II (succinate dehydrogenase) activity. The TNP-exposed groups (10–50 μg/mL) demonstrated a significant drop in NADH dehydrogenase activity (p < 0.01–0.001) in comparison to the control group (Figure S4A). However, a minimal dose of TNPs (5 μg/mL) showed negligible significant change in NADH dehydrogenase activity when compared to the control group. The succinate dehydrogenase activity in the liver mitochondria of the rat is shown in Figure S4B. The 10–50 μg/mL TNP-exposed group exhibited a notable decline in succinate dehydrogenase activity (p < 0.01) as compared to the control group. The 5 μg/mL dose TNP group demonstrated no significant change in succinate dehydrogenase enzyme activity in comparison to the control group. QR ameliorated the drop in NADH dehydrogenase and succinate dehydrogenase enzyme activity against the TNP-exposed group. Complex I (Figure 5A) and complex II (Figure 5B) activity was markedly depleted (p < 0.001) in group IV (TNPs) when compared to the control group. QR pre-exposure (group III) resulted in a significant elevation (p < 0.01–0.001) in the activity of NADH dehydrogenase and succinate dehydrogenase in rat liver mitochondria as compared to the TNP-exposed group (50 μg/mL). QR (group II) exposure caused no significant alteration in NADH dehydrogenase and succinate dehydrogenase activity in comparison to the control group (Figure 5).

**MTT Ability (Complex III) and F1 – F0 Synthase (Complex V) Activity.** TNP treatment obstructs mitochondrial complex III and complex V (F1 – F0 synthase) activity. The 10–50 μg/mL TNP exposure group had a significant reduction in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) ability (p < 0.01–0.001) in liver mitochondria in comparison to the control group (Figure S5A). There was no significant alteration in MTT ability in the 5 μg/mL group when compared to the control group. The F1 – F0 synthase was markedly declined (p < 0.05) on TNP exposure with the dose of 10–50 μg/mL in comparison to the control group (Figure S5B). A minimal dose of TNPs (5 μg/mL) did not reflect any significant alteration in F1 – F0 synthase activity when compared to the control group. The results showed that QR modulated the TNP-mediated inhibition in complex III and F1 – F0 synthase activity. TNP exposure (50 μg/mL) (group IV) led to a significant decline (p < 0.01–0.001) in MTT ability (Figure 6A) and the F1 – F0 synthase activity (Figure 6B) when compared to the control group. QR pre-exposure (group III) caused a significant increase (p < 0.05–0.01, respectively) in MTT ability in mitochondrial dehydrogenase and F1 – F0 synthase activity when compared to the TNP-exposed group. QR exposure (group II) showed no significant alteration in

**Figure 3.** Effect of pretreatment with QR (50 μM) and TNP (50 μg/mL) on (A) GSH and (B) NP-SH levels in rat liver mitochondria. The values are stated as μmol of GSH/g tissue and μmol of NP-SH/g tissue. All values are represented as mean ± SE (n = 6). Substantial alterations are indicated by **p < 0.01 in comparison with the control group, and significant differences are indicated by *p < 0.05 and **p < 0.01 in comparison with the TNP-treated group.
both MTT and F$_1$ − F$_0$ synthase activity when compared to the control group (Figure 6).

**DISCUSSION**

Studies exploring nanoparticle toxicity have focused on oxidative stress, which has been identified to have a significant role in the toxicity of numerous chemicals and drugs.$^{32-35}$ Several in vivo studies have strongly indicated that nanoparticles tend to accumulate in the liver.$^{32-35}$ Besides a large number of applications of TNPs, there are some hepatotoxic actions that are mediated by mitochondrial impairment.$^1$

Mitochondria have long been regarded as the cell’s powerhouse.$^{5,6,27}$ Mitochondria have been implicated in the pro-oxidative consequences of TNPs.$^{36,37}$ There is direct connection between mitochondrial dysfunction and the toxic indexes of nanoparticles.$^{38}$ The TNP treatment in liver mitochondria aggravates numerous responses, including membrane peroxidation and mitochondrial dysfunction.$^{39}$ The objective of the current study was to evaluate the TNP-induced mitochondrial hepatotoxicity in vitro. We also explored the modulatory effect of QR on TNP-mediated hepatotoxicity.

The lipids present in the mitochondrial membrane are rich in polyunsaturated fatty acids (PUFA) and are prone to oxidation. LPO and PC are excellent biomarkers of oxidative stress that have been most extensively investigated in the processes induced by free radicals. Generation of free radical or oxidative stress advances with imbalance in the pro-oxidants and antioxidants ratio, which leads to the generation of ROS.$^{27,40}$ It has also been noticed that increased LPO or diminished antioxidant levels are linked with the complexes’ activity deprivation, which consequently leads to mitochondria-mediated apoptosis.$^{41}$ Major fatty acids in the mitochondrial membranes might be depleted on account of the rise in the LPO levels in the mitochondria of the TNP-exposed group. As a result, vital phospholipids (such as cardiolipin) that are crucial for mitochondrial enzymes and their function might be exhausted.$^{42,43}$

By virtue of being a natural flavonoid ubiquitously available in our immediate environment, and owing to its widely accepted antioxidant properties, QR has long been heralded as a nutraceutical because of its potential to alleviate various types of toxicity. QR also possesses the ability to scavenge ROS and reactive nitrogen species. These properties render QR as an important therapeutic agent that can be used to alleviate the mitochondrial hepatoxicity induced by TNPs. Our results suggest that QR pretreatment ameliorates mitochondrial perturbation and alleviates the oxidative stress induced by TNPs. Taken together, pretreatment with QR thus holds potential as a promising agent to reverse TNP-induced mitochondrial toxicity.

In our study, pretreatment of QR significantly overturned the LPO and diminished the antioxidant status. It has been stated that QR decreases the LPO, an oxidative stress marker,
Our results reflected that QR has the potential to ameliorate LPO, which may thus act as a compensatory mechanism that helps in maintaining cell integrity and provide defense against free radical damage. Diminished LPO levels might be due to the deactivation and glycation of the enzymes having antioxidant features by free radicals. PC is employed universally as a biomarker for accumulation of protein carbonyl and oxidation of proteins. In our study, TNP treatment to the liver mitochondria resulted in considerable increase in PC content. Oxidative damage is generally linked with a damage in different protein functions and an apparent upsurge in the PC content in liver mitochondria, probably because of overproduction of superoxide radicals. This substantiates our preliminary findings that TNP's induce oxidative stress. Additionally, alteration of PC status may lead to adverse functional outcomes. QR pretreatment amended the increased PC caused by TNP's in liver. QR has been reported as an electrophilic scavenger, as well as an antioxidant. Our results, QR pretreatment prevented the increase of liver PC content instigated by TNP's. QR played a potential role in the regulation of PC content in the mitochondria of rat liver in the TNP-treated group.

Studies have shown an association between reduced GSH and NP-SH levels and xenobiotic toxicity. The GSH and NP-SH levels that were altered by TNP exposure were also reinforced to the standard level by pretreatment of QR. GSH is involved in ROS detoxification and helps in reduction of $\text{H}_2\text{O}_2$ as it has non-enzymatic free radical scavenger property. Thus, GSH safeguards the cell integrity against ROS.

Mitochondria have a GSH/NP-SH pool that helps maintain a diminished matrix environment and is involved in cleansing the $\text{H}_2\text{O}_2$ generated in the matrix. It has been established that mitochondrial GSH is pivotal in maintaining mitochondrial functionality and is reported to have greater significance than cytosolic GSH in maintaining cellular functioning and viability. Previous research had demonstrated that QR supplementation evidently shunted the GSH or thiol ratio in nanoparticle-mediated oxidative stress. In our study, the reduced GSH levels may be linked to the TNP (and its reactive metabolites) treatment with thiol groups, thereby altering the antioxidant status.

GSTs belong to a complex of enzymes that can conjugate GSH with varied electrophilic compounds. GST catalyzes the conjugation of GSH via a sulphydryl group to electrophilic centers on an extensive variety of substrates. According to our results, the activity of liver mitochondrial GST was decreased by TNP's. Mitochondrial GST activity was persuaded by ROS due to sulphydryl group oxidation. Studies have demonstrated that reduced mitochondrial GST activity is attributed to increased ROS generation in case of tissue injury. Pretreatment with QR helped restore reduced mitochondrial GST activity in our study. Hence, QR is capable of providing protection from oxidative stress that is brought about due to excess $\text{O}_3$ and $\text{H}_2\text{O}_2$. It is well known that GST can potentially inactivate the cytotoxic effects of oxidative stress.

Studies have reported that the mitochondrial matrix houses Mn-SOD. In mitochondria, about 1−2% of the breathed $\text{O}_2$ may be converted to superoxide anions. In cells, Mn-SOD is considered as the first line of defense against the toxic effects of superoxide anions.

**Figure 5.** Effect of pretreatment with QR (50 μM) and TNP (50 μg/mL) on the activity of (A) NADH dehydrogenase and (B) succinate dehydrogenase in rat liver mitochondria. The values are stated as n mol of NADH oxidized/min/mg protein and μmol of succinate produced/min/mg protein. All values are represented as mean ± SE (n = 6). Substantial differences are shown by ***p < 0.001 in comparison with the control group. The significant differences are indicated by ##p < 0.01 when compared to the TNP-treated group.
oxyradicals, by catalyzing the dismutation of endogenous cytotoxic superoxide radicals to H$_2$O$_2$. In our study, TNPs treatment showed a decreased activity of Mn-SOD in liver. Studies have reported deviations in the activity of Mn-SOD in liver mitochondria. Our results also highlight a decrease in Mn-SOD activity. Intensification in superoxide radical formation is thought to be the rationale for the same. In our study, Mn-SOD activity was restored in the liver mitochondria in the QR pretreatment group. These findings signify the ability of QR to improve the scavenging and deactivation of H$_2$O$_2$ and the hydroxyl radical.

Our results also exhibit a reduction in the activities of NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), and mitochondrial dehydrogenase (complex III) through MTT ability and F$_1$-F$_0$ synthase activity (complex V) in rat liver mitochondria with TNP treatment. Succinate dehydrogenase participates only in transporting electrons to the electron transport chain (ETC), whereas NADH dehydrogenase is linked with translocation of protons and transfer of electrons. The defective functioning of any of the enzyme complexes accountable for oxidative metabolism may result in mitochondrial cytopathy and also opening of the mitochondrial permeability transition pore, which permits the membrane potential to disintegrate and could lead to uncoupling of oxidative phosphorylation, and subsequently, diminished cellular production of ATP.

Studies have indicated that nanoparticle (including TNPs)-induced mitochondrial toxicity leads to mitochondrial dysfunctions. A drop in the complex-I, II, III, V activities, along with ATP depletion, has been observed in various disease models of animals. Such metabolic stress could result in increased ROS production and subsequently, cell damage. Studies have reported that ROS-induced mitochondrial protein oxidation affects ATP synthase and respiratory chain enzymes. Thus, the decrease in NADH dehydrogenase, succinate dehydrogenase, F$_1$F$_0$ ATP synthase activity, and MTT indicate an inclusive agitation of the electron transfer pattern as a result of TNP treatment. Pretreatment of QR to TNP-exposed mitochondria restored the usual functioning of ETC enzymes, which could be because of its antioxidant potential. Numerous studies have highlighted that QR plays a role in ameliorating mitochondrial oxidative damage by reducing ROS production.

Several lipophilic compounds, including QR, have been reported to breach the inner mitochondrial membrane. The results showed that QR not only prevents the mitochondrial oxidative stress reactions, but also dynamically mounts up in the mitochondria in biologically active form in cells when treated with the dosages that have been used in the present study. We hypothesize that the useful effect of QR observed in our study may be facilitated by similar mechanisms, thereby ameliorating the TNP-mediated oxidative stress, which in turn results in defense against the mitochondrial dysfunction produced by the TNPs’ toxicity. Although QR has potential antioxidant effects, it is crucial to remember that at higher concentrations, QR can be a pro-oxidant. Thus, it is imperative to choose the appropriate concentration of QR that allows it to function as an antioxidant. Preclinical studies provided in-depth information comprising the development of progressively specific and targeted clinical studies. With the advancement of distinct techniques, the clinical use of QR has been widely accepted.
against various triggering agents like TNPs as a preventing agent, and in combination with other drugs that potentiate its efficacy synergistically or additively.

**CONCLUSIONS**

In light of the above results, we conclude that TNPs induce oxidative stress in the liver mitochondria of rats. Due to the ubiquitous nature of TiO2 in our daily lives, it is important to place sufficient emphasis on examining the effect of TiO2-induced oxidative stress and ROS on the environment and our health clinically. Among the several mechanisms by which NP exposure is involved, mitochondrial stress is considered to be a crucial mechanism, which involves an interface between TiO2 and living organisms. Our results signify that QR has a beneficial effect in modulating the mitochondrial perturbation induced by TNPs. These findings are of added importance owing to the widespread consumption of QR locally and its use as a therapeutic agent to improve numerous hepatic disorders. Forthcoming studies should inspect the hypothesis of QR as an antioxidant therapy targeted at mitotoxicity, and further research on the mitochondrial damage induced by TNPs might help shed light on newer approaches to mitigate subcellular hepatotoxicity.

**MATERIALS AND METHODS**

**Chemicals.** Ethylenediaminetetraacetic acid (EDTA), 4-amino-3-hydroxy-1-naphthalenesulfonic acid (ANS), benzyl amine hydrochloride (BAHC), bovine serum albumin (BSA), dichlorophen indophenols (DCIP), butylated hydroxy toluene (BHT), 1-chloro-2,4 dinitrobenzene (CDNB), 2,6, 2,4-dinitrophenyl hydrazine (DNPH), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), e-phosphoric acid (OPA), ethylene glycol-O2,O'-bis, (2-aminoethyl) tetra acetic acid (EGTA) epinephrine, reduced glutathione (GSH), hydrogen peroxide (H2O2), nicotinamid adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate reduced tetra sodium salt (NADPH), persillic acid (PCA), thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were procured from Sigma Chemicals Co. (St. Louis, MO). TNPs and QR were attained from SRL Chemicals (Mumbai India) and Hi- Media Labs (Mumbai, India), respectively. Other routine chemicals were purchased from Merck Limited (Mumbai, India).

**Animals.** Male Wistar rats weighing 180–250 g were procured from the Central Animal House of Jamia Hamdard, New Delhi, India. The animals were acclimatized in the laboratory and were given ad libitum food and water for 7 days before initiation of the experiments. Rats were kept at an ambient temperature of 22 ± 2 °C with a relative humidity of 65 ± 10% and in a photoperiod of 12 h light/dark cycle. All experiments were executed according to the guidelines of the Jamia Hamdard Institutional Animal Ethics Committee (IAEC).

**Preparation of Nanoparticle Suspensions.** TNPs were suspended in sterile distilled water in an eppendorf tube and the suspension was vortexed for seconds, and ultrasonicated for 30 min to disperse it completely. Ultrasonication is done so that no agglomeration is present in the TNP solution. The TNP suspension was vortexed thoroughly before each experiment.

**Particle Characterization by Transmission Electron Microscopy.** Transmission electron microscopy (TEM) characterization of TNPs was carried out using a Tecnai G2-20 Twin instrument (Eindhoven, Netherlands) at 200 kV accelerating voltage to determine the primary particle size and morphology. TNPs were studied after dilution of the nanoparticles to 20 μg/mL (in distilled water), and to avert accumulation, the TNP suspensions were ultrasonicated for 30 min before a drop was deposited on a TEM grid comprising 2% colodion in amyl acetate, which was subsequently dried. Tecnai software (version 4.1 build 5722) was used to evaluate the size of nanoparticles with different diameters. The particle size was estimated by measuring the nanoparticles in random fields of view in addition to the images showing the general nanoparticles’ particle sizes.

**In Vitro Model, and Drug and Nutraceutical Treatment.** To evaluate the hepatic mitochondrial toxicity, TNPs were investigated under an *in vitro* environment. A previously reported procedure was followed to incubate the liver mitochondria with TNPs (at concentrations of 5, 10, and 50 μg/mL) for 1 h at 37 °C. Different concentrations of TNPs were utilized to evaluate the best dose for TNPs, and eventually a dose of 50 μg/mL was used. Identical volumes of stock and working solutions were mixed in hepatic mitochondrial samples for incubation. For *in vitro* estimation of TNP-mediated hepatic mitochondrial toxicity and its reversal by means of QR, mitochondrial preparations were divided into four groups, namely: group I (control), group II (QR), group III (TNPs with pre-exposure to QR), and group IV (TNPs). For this assessment, liver mitochondria were incubated at 37 °C for 1 h with QR (50 μM), to restore pre-protection before exposure to TNPs at a concentration of 50 μg/mL. Thereafter, the mitochondrial samples were treated with the TNPs for 1 h. The QR concentration in contrast to TNPs was based on the procedures reported previously.

**Mitochondrial Preparations.** The previously described method of differential centrifugation was used to isolate rat liver mitochondria. A mechanically driven homogenizer, Teflon-coated Potter-Elvehjem type, was used to separate and homogenize the liver from adult rats in an ice-chilled isolation buffer (0.25 M sucrose and 1 mM EDTA, which was adjusted to pH 7.4 by Tris), and centrifugation for 5 min at 800 g was done. Afterward, the ensuing supernatant was centrifuged at 5100g for 4 min. The consequent pellet was dissolved again in a 0.25 M sucrose medium maintained by Tris to pH 7.4, and centrifugation was done at 12 300g for 2 min. Lastly, the pellet was re-suspended in a 0.25 M sucrose medium adjusted by Tris to pH 7.4, centrifuged at 12 300g for 10 min, and re-suspended in a buffer containing 0.25 M sucrose, 0.5 mM EDTA adjusted by Tris to pH 7.4. The Lowry method was performed to determine the concentration of protein present in the stock suspension (4–6 mg/mL).

**Oxidative Stress Biomarkers.** *Estimation of Mitochondrial Lipid Peroxidation.* LPO was determined based on a previously described method. The mixture was composed of 0.01 M BHT, 6.7 mg/mL TBA, 1% chilled OPA, and the mitochondrial preparation. The resultant values were expressed in terms of the μmol of TBARS formed/h/g of tissue based on a molar extinction coefficient of 1.56 × 105 M−1 cm−1.

*Estimation of Mitochondrial Protein Carbonyl.* PC content was assessed on the basis of a previously described procedure. The PC content was calculated spectrophotometrically at 360 nm, and was determined as n mol of DNPH incorporated/mg protein based on a molar extinction coefficient of 21 000 M−1 cm−1.
Non-enzymatic Antioxidants. Estimation of Mitochondrial Reduced Glutathione. GSH was measured based on the procedure described previously. GSH level in the aforementioned mitochondrial preparation was calculated in terms of μmol of GSH/g tissue.

Estimation of Mitochondrial Non-protein-Bound Thiols. NP-SH was estimated based on a previously described procedure. Results were expressed in terms of μmol of NP-SH/g tissue using a molar extinction coefficient of 13 100 M⁻¹ cm⁻¹ at 412 nm.

Antioxidant Enzymes. Activity of Mitochondrial Glutathione-5 Transferase. GST was evaluated using a previously demonstrated method. The results were presented in terms of n mol of CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 × 10⁴ M⁻¹ cm⁻¹ at 340 nm.

Activity of Mitochondrial Manganese-Superoxide Dismutase. Mn-SOD activity was quantified based on a procedure previously employed. The results were determined in terms of n mol of (−) epinephrine protected from oxidation/min/mg protein using a molar extinction coefficient of 4020 M⁻¹ cm⁻¹.

Mitochondrial Complex Enzymes. Activity of Complex I (NADH Dehydrogenase). The activity of NADH dehydrogenase was estimated using the procedure of King and Howard. Enzymatic activity was calculated as μmol of NADH oxidized/min/mg protein (based on a molar extinction coefficient of 21 000 M⁻¹ cm⁻¹) was used to express the enzyme activity.

Activity of Complex II (Succinate Dehydrogenase). A previously described procedure was followed to determine the succinate dehydrogenase activity. Enzymatic activity was calculated as μmol of succinate produced/min/mg protein using a molar extinction coefficient of 1000 M⁻¹ cm⁻¹.

Activity of Complex III (Mitochondrial Dehydrogenase, MTT Ability). A previously described method was employed to determine the rate of MTT reduction so as to estimate the mitochondrial respiratory complexes’ activities in the isolated mitochondrial samples.

Activity of Complex V (Total ATPase). Complex V (Total ATPase) activity was determined using a previously described method.

Protein Determination. The mitochondrial protein contents of the samples were estimated by Lowry method.

Statistical Investigations. Values were expressed in terms of mean ± standard error of mean (SEM). Tukey’s test was done after analysis of variance (ANOVA) to analyze the data. Graph Pad Prism 5 software (Graph Pad Software, Inc., San Diego, CA) was used to perform all statistical analyses. The statistical values of P ≤ 0.05 were considered significant.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at http://pubs.acs.org/doi/10.1021/acsomega.1c04740.

Effect of different doses of TNPs on oxidative stress biomarkers, and enzymatic and non-enzymatic antioxidants level along with mitochondrial enzymes level (PDF)

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