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Research Article

The mTOR/GCLc/GSH Pathway Mediates the Dose-Dependent Bidirectional Regulation of ROS Induced by TiO_2 NPs in Neurogenic Cells

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Objective. The effect of TiO_2 NP exposure on the nervous system and the underlying mechanism remain unclear. The antioxidant effect of TiO_2 NPs at a low dose was newly found in our study, which was different from the effect at high dose. This study is aimed at exploring the mechanism underlying the antioxidant effects of TiO_2 NPs at low dose and the induction of ROS accumulation by TiO_2 NPs at high dose in neurogenic cell lines. *Methods*. We measured the changes in key molecules in the ROS regulation pathway by western blotting, flow cytometry, and commercial assay kits, and these key molecules were further evaluated to verify their interactions and roles using SH-SY5Y, U251, and SK-N-SH cell lines treated with TiO_2 NPs. *Results*. Our results showed that the weak antioxidant effect at low dose was caused by mTOR/GCLc-induced GSH overproduction and GSH-Px activity impairment. ROS accumulation at high dose was caused by a mTOR/GCLc-mediated decrease in GSH production, GSH-Px activity impairment, and dramatic ROS production. Furthermore, we found that the ROS species were mainly $O_2^{\cdot-}$, and that SOD played a crucial role in reducing $O_2^{\cdot-}$ levels before the mTOR protein was activated. *Conclusion*. We revealed the mechanism underlying the bidirectional regulation of ROS induced by TiO_2 NPs at different doses in neurogenic cell lines. Our study emphasized the potential neurotoxic effects of NPs at low dose, which should arouse concern about their safety.

1. Introduction

Titanium dioxide nanoparticles (TiO_2 NPs) are a traditional nanomaterial that can be found in many products. They were used as proper addictive in paint for their stable chemical characters, redox-reduction ability, and self-cleaning ability. The antibacterial ability and ultraviolet absorption capacity made them also good additives used in food and cosmetics. And the photocatalytic ability made them a preferred catalyzer in waste water treatment [1, 2]. Therefore, TiO_2 NPs can enter the human body via inhalation, skin, and digestion.

Previous animal studies showed that TiO_2 NPs harmed the nervous system [3, 4] as they can penetrate the blood-brain barrier (BBB) [4, 5], and can accumulate in the brain. However, the effect of TiO_2 NP exposure on the human nervous system remains unclear. TiO_2 NPs change the expression of genes associated with oxidative stress and affect the activity of key antioxidant enzymes [6, 7], thus leading to

the overproduction of reactive oxygen species (ROS) and eventually affecting brain function [8, 9]. Moreover, TiO_2 NPs could also induce ROS in many neurogenic cell lines [10, 11]. These studies collectively suggested that TiO_2 NPs could impair neurogenic cells via ROS.

ROS are a group of chemically reactive molecules that containing oxygen. ROS have dual roles in cells, as proper ROS level is essential for cell survival [12], while the overproduction of ROS induces cell death [13]. Recently, two top studies in *Cell* raised the concerns about the effects of antioxidants on cancer cell metastasis and reminded us of the potential adverse effects of antioxidant substrates [14, 15]. Most NPs could induce ROS [16, 17], including TiO_2 NPs [10], but the ROS induced by NP exposure mainly occurred at high treatment concentrations [18, 19]. Moreover, other studies have reported that modified TiO_2 NPs exert efficient antioxidant properties [20], and TiO_2 NPs could activate the antioxidant system in HepG2 cells [21] and could even protect cells from death by antioxidant effects [22]. In addition to from TiO_2 NPs, cerium oxide nanoparticles also showed antioxidant abilities, as they could modify the activities of enzyme that protect against oxidative stress and exert antioxidant effects by regulating the Nrf-2/HO-1 pathway [23]. Therefore, the effect of TiO_2 NPs on ROS seems complex, and TiO_2 NPs might cause neurotoxicity by upregulating ROS or disrupting antioxidant effects.

Mammalian target of rapamycin (mTOR) is an atypical Ser/Thr protein kinase that integrates extracellular signals and regulates cell growth, the cell cycle, and cell proliferation [24]. There is an interaction between mTOR and ROS; ROS can activate mTOR by activating the Ras-PI3K-Akt pathway [25] or inactivate mTOR by activating AMPK [26]. In contrast, the activation of mTOR/PI3K [27] or mTOR/GCLc [28] could also upregulate or downregulate ROS formation, respectively. The regulation of ROS regulated by mTOR is complex and might depend on the activation degree of the mTOR pathway. However, whether mTOR and ROS interact in neurogenic cells is unknown.

The human neuroblastoma SH-SY5Y cell line is widely used to investigate the mechanisms of neurotoxicity in humans and was therefore chosen and used in our research to study the mechanism of the ROS-related neurotoxicity caused by TiO_2 NP exposure. Interestingly, we found that TiO_2 NPs exerted weak antioxidant effects at a low dose (1 μg/ml), and this weak effect was significant when we repeated the independent assay up to 10 times. However, when the dose of TiO_2 NPs increased to 100 μg/ml, the ROS level was increased. ROS levels appeared unchanged at the dose of 10 μg/ml. Based on these results, the doses of 1 μg/ml and 100 μg/ml were used in the study. The range of doses was the same as that in previous papers addressing in vitro effects of NPs [29], which is related to the TiO_2 NP internal exposure level in humans [30].

The hypothesis of this study was that TiO_2 NPs cause toxicity through ROS in neurogenic cells via the mTOR pathway in a nonmonotonic dose-dependent manner. We further explored the mechanism underlying which TiO_2 NPs exert antioxidant effects at low dose and which induce ROS accumulation at high dose in the SH-SY5Y cell line, and we verified the findings in two independent human neurogenic cell lines (U251 and SK-N-SH).

2. Materials and Methods

2.1. Characteristics Measurement and TiO_2 NP Dispersion. TiO_2 NP is a commercial product (Sigma-Aldrich, CAS No. 13463-67-7). Characteristic measurement and cell treatment were performed according to a previous study [31]. Briefly, TiO_2 NPs were dispersed in the complete cell culture medium, vortexed at high speed, and sonicated for 30 min (100 W) before characteristic measurement. Stock solution (1 mg/ml) was gradually diluted and sonicated for another cycle before being administrated to cells.

2.2. Cell Culture and Treatment. SH-SY5Y cells (CRL-2266™) were purchased from ATCC; U251 (TCHu 58) and SK-N-SH (TCHu 51) were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). All these three cells were cultured in a 37°C, 5% CO_2 atmosphere with Dulbecco's modified Eagle's medium (DMEM) (HyClone, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The medium was replaced every day. TiO_2 NP stock solution was freshly prepared before cell treatment.

2.3. Transmission Electron Microscope (TEM) Analysis. Cells were cultured with 100 μg/ml TiO_2 NPs for 24 h and collected, and pellet was fixed with 1% glutaraldehyde. After washing with phosphate buffer, cell pellet was postfixed with 1% buffered osmium tetroxide and dehydrated in a graded acetone. After embedded in Araldite, samples were sliced (Leica EM UC7/FC7) and stained with lead citrate and uranyl acetate before TEM analysis.

2.4. Cell Viability Assay. The cytotoxicity of TiO_2 NPs was measured by MTT assay according to the standard protocol. Cells were seeded in 96-multiwell plates and treated with TiO_2 NPs (1, 10, 100 μg/ml) for 24 h. Then, 20 μl of freshly prepared MTT stock solution (5 mg/ml) was added to each well. After incubation for another 4 h, the medium was removed and 150 μl of the dimethyl sulfoxide (DMSO) was added. Supernatants were transferred to another 96-multiwell plates, and the absorbance was measured at 490 nm. The absorbance of the supernatants from the TiO_2 NP-treated groups was normalized to that from the control group. Each assay was repeated at least three times.

2.5. ROS Analysis. Cells were collected after treated with TiO_2 NPs for 24 h and incubated with dichloro-dihydro-fluorescein diacetate (DCFH-DA) (SS0033, Beyotime, China) for 30 min. Cells were washed three times before flow cytometry (FCM) analysis with excitation at 488 nm. The results were collected from at least three independent experiments.

2.6. Western Blotting Analysis. The protein concentrations were analyzed by BCA protein assay kit (P0012, Beyotime, China). Protein levels of mTOR, p-mTOR, and GCLc were detected by western blotting assay with an mTOR antibody (sc-1549, Santa Cruz Biotech), a p-mTOR antibody (S2448)

(sc-101738, Santa Cruz Biotech), and a GCLc antibody (BA1627, BOSTER), respectively. Each experiment was repeated at least three times.

2.7. Glutathione (GSH), Oxidized Glutathione (GSSG), and GSH/GSSG Ratio Measurement. After cells were treated with TiO_2 NPs for 24 h, they were collected and lysed. The supernatants were used to determine the total GSH and GSSG with a kit (S0052, Beyotime, China) according to the manufacturer's instructions. Total GSH was measured after GSSG reduction, and the total GSSG was measured after GSH was eliminated from the samples. GSH/GSSG ratio was then calculated. The data were obtained from three parallel groups of each treatment, and this assay was repeated at least three times.

2.8. H_2O_2 Concentration Measurement. H_2O_2 concentrations in the supernatants were measured with a hydrogen peroxide assay kit (A064-1-1, Nan Jing Jian Cheng Bioengineering Institute, China) according to the manufacturer's protocol. Briefly, cells were treated with TiO_2 NPs and then collected and lysed with lysis buffer. After centrifugation at 8000 g for 10 min of, cell lysate supernatants were collected, and 100 μl of the detection solutions was added. The supernatant absorbance was detected at 405 nm. Each assay was repeated three times.

2.9. Superoxide Anion ($O_2^{\cdot-}$) Measurement. Dihydroethidium (DHE) (S0063, Beyotime, China) was used to measure $O_2^{\cdot-}$ after cells were exposed to TiO_2 NPs. Briefly, cells were treated with TiO_2 NPs for 24 h and collected, and then the cells were incubated with 1 μM of DHE at 37°C for 30 min and protected from light. After the cells were collected and washed three times, cellular $O_2^{\cdot-}$ levels were detected by FCM for the fact that $O_2^{\cdot-}$ could redox DHE to ethidium with excitation at 610 nm.

2.10. Enzyme Activity Analysis. Cells were cultured and treated, and cell lysates were used to analyze the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) with a total SOD assay kit with nitro-blue tetrazolium (NBT) (S0109, Beyotime, China), a catalase assay kit (S0051, Beyotime, China), and a cellular GSH-Px assay kit (S0056, Beyotime, China), respectively, according to the manufacturer's instructions. Briefly, SOD activity was analyzed based on its ability to form formazan by reducing the NBT. CAT activity was determined based on its ability to decompose H_2O_2; the rest H_2O_2 could generate N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinonemonoimine, which reflecting CAT activity. GSH-Px activity was analyzed by detecting the decreasing amount of NADPH that consumed during the reduction of GSSG to GSH via GSH-Px. Each experiment was repeated for three times.

2.11. Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$). Cells were collected and washed three times with PBS and subsequently resuspended in buffer. After incubated with JC-1 (C2006, Beyotime, China) in the dark at 30°C for 20 min, the cells were washed twice and analyzed by FCM with an excitation at 610 nm. Monomeric JC-1 was measured at 530 nm, and aggregated JC-1 was measured at 590 nm emission. $\Delta\psi_m$ was calculated as the ratio of the fluorescence intensity at 590 nm/530 nm.

2.12. Statistical Analysis. All data are presented as the mean ± SE. Statistical differences between two groups were examined using the t-test, and the differences among three groups were examined by one-way ANOVA, followed by Dunnett's multiple comparison test. All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of TiO_2 NPs. The morphology of TiO_2 NPs is presented in Figure 1(a). TiO_2 NPs were nearly spherical with a diameter of ~21 nm, and the average hydrodynamic diameter was approximately 110 nm. The TiO_2 NP powder was consisted of approximately 80% anatase and 20% rutile with the purity of >99.5%, and the surface area was 35-65 m^2/g according to the manufacturer's report. Collectively, the TiO_2 NPs used in the following experiment were relatively well dispersed.

3.2. Uptake of TiO_2 NPs and Cytotoxicity. To explore whether TiO_2 NPs were located intracellularly, cells were cultured and treated with TiO_2 NPs for 24 h. The cells examined by TEM after pretreatment. As shown in Figure 1(b), TiO_2 NPs entered treated cells and accumulated in the cytoplasm. To determine the cytotoxicity of TiO_2 NPs, SH-SY5Y cells were cultured in the absence (vehicle control) or presence of different concentrations of TiO_2 NPs, and the cytotoxicity was measured with the MTT assay. As shown in Figure 1(c), there was no significant difference among the four groups, but the cell viability slightly increased in the low-dose group. Moreover, the cell density in the low-dose group was also slightly higher than that in the other groups (Figure 1(d)).

3.3. Effects of TiO_2 NPs on ROS Formation. The ROS levels were examined with FCM after cells were exposed to different doses of TiO_2 NPs. Figure 1(e) shows that the ROS level was slightly decreased after exposed to 1 μg/ml TiO_2 NPs. Then, the ROS level increased to the control level at the concentration of 10 μg/ml and continuously increased to a significantly high level ($P < 0.001$) at a concentration of 100 μg/ml. Repetition of this experiment showed the same results, and the change in the low-dose group became statistically significant ($P < 0.05$) until we repeated this experiment up to 10 times. Based on the current results, 1 μg/ml and 100 μg/ml TiO_2 NP treatments and the control group were used in the subsequent experiments to explore the underlying mechanisms.

3.4. Effects of TiO_2 NPs on mTOR Protein Expression. mTOR and p-mTOR protein levels were determined by western blotting. As shown in Figure 2(a), both mTOR and p-mTOR protein levels were increased in the low-dose (1 μg/ml) group and decreased in the high-dose group (100 μg/ml) compared with the control group. The changes in mTOR proteins were consistent with the changes in ROS levels.

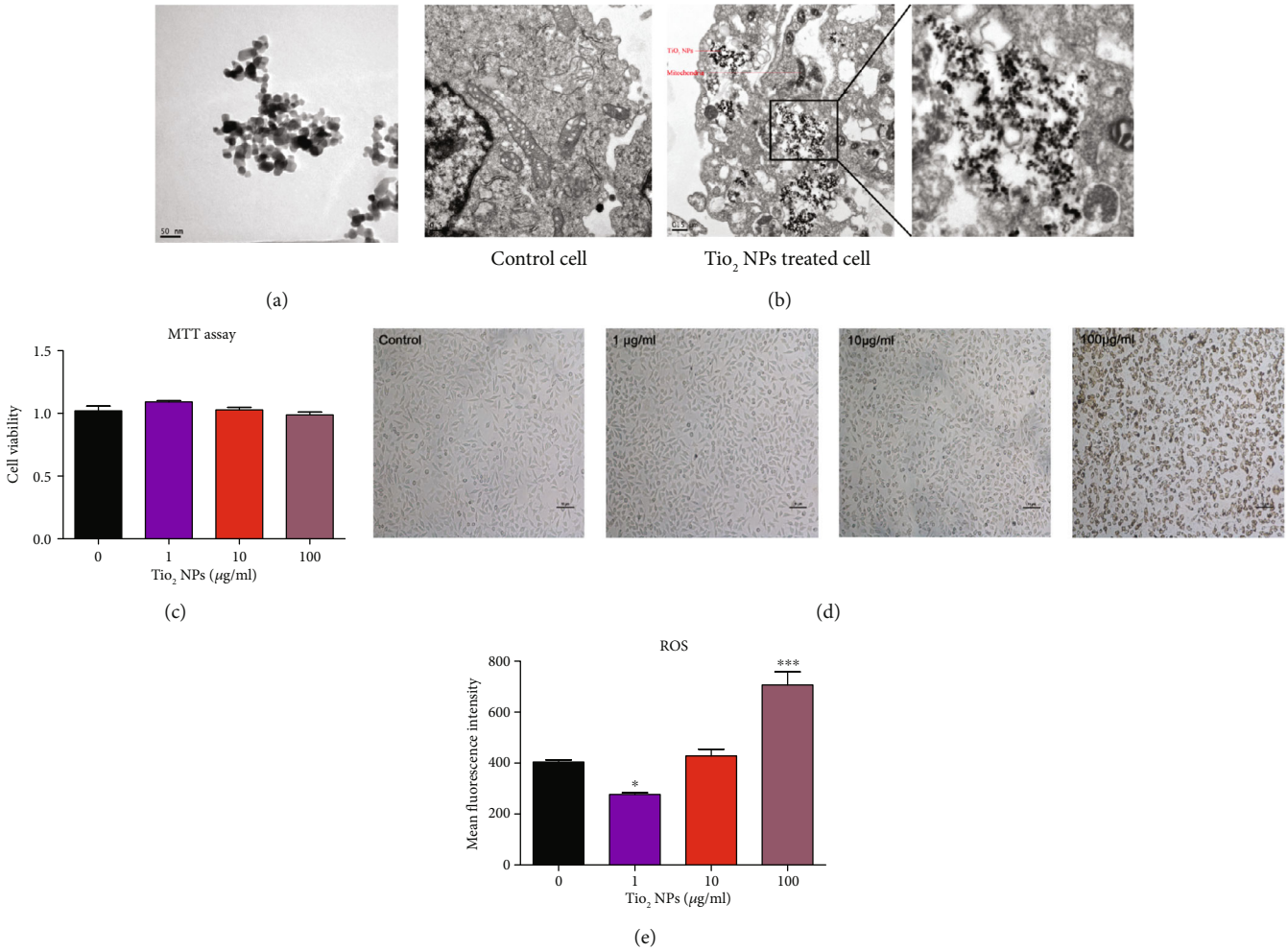

Figure 1: (a) Image of TiO_2 NPs obtained by transmission electronic microscope (TEM), bar = 50 nm. (b) TEM image of control and TiO_2 NP-treated SH-SY5Y cell, and the TiO_2 NPs and mitochondria are indicated in red, bar = 0.5 μm. (c) Cytotoxicity of TiO_2 NPs was determined by MTT assay after 24 h exposure. (d) Cell densities and conditions observed by light microscope after 24 h incubation. (e) Intracellular ROS were measured by FCM after 24 h treatment, and values are presented as mean ± SE. Independent experiment was repeated ten times. $^{*}P < 0.05$, $^{***}P < 0.001$.

3.5. Association of mTOR Proteins and ROS Formation. To explore whether the reduction in ROS was associated with mTOR protein expression, we used rapamycin (RAPA), a specific inhibitor of mTOR proteins, which can directly bind to the FRB domain of mTOR to inhibit the protein activity. As shown in Figure 2(b), ROS level was decreased slightly in 1 the μg/ml of the TiO_2 NP-treated group when compared with the control group. When 100 nM of RAPA was added, the ROS level was significantly increased ($P < 0.01$) when compared with the ROS level of the 1 μg/ml TiO_2 NP group.

3.6. Effects of TiO_2 NPs on Glutamate-Cysteine Ligase Catalytic (GCLc) Expression. GCLc is the first rate-limiting enzyme in GSH synthesis and is the downstream of mTOR. Therefore, we examined the protein levels of GCLc in three groups by western blotting. In the treated groups, the changes in GCLc protein expression of were similar to the change in mTOR protein expression relative to the control group. The GCLc expression level was increased in the 1 μg/ml group and decreased in the 100 μg/ml group (Figure 2(c)).

3.7. Association of mTOR Proteins and GCLc Expression. To clarify whether GCLc expression was associated with mTOR proteins, RAPA was added to inhibit the activity of mTOR proteins, and then GCLc expression levels were measured. As shown in Figure 2(d), the GCLc expression level was increased after cells were treated with 1 μg/ml of TiO_2 NPs compared with the control cells. However, when 100 nM RAPA was added, the expression level of GCLc protein was decreased.

3.8. Effects of TiO2 NPs on GSH and GSSG Concentration. GSH is the downstream product of GCLc, so the GSH and GSSG concentrations were then measured in the three groups. As shown in Figure 2(e), the concentration of GSH was increased significantly ($P < 0.01$) in the 1 μg/ml of the TiO_2 NP group, while decreased significantly ($P < 0.01$) in the 100 μg/ml TiO_2 NP group compared with the control group. These results were consistent with the changes in GCLc protein, but the magnitude of these changes was relatively small. The GSSG concentrations and the ratio of

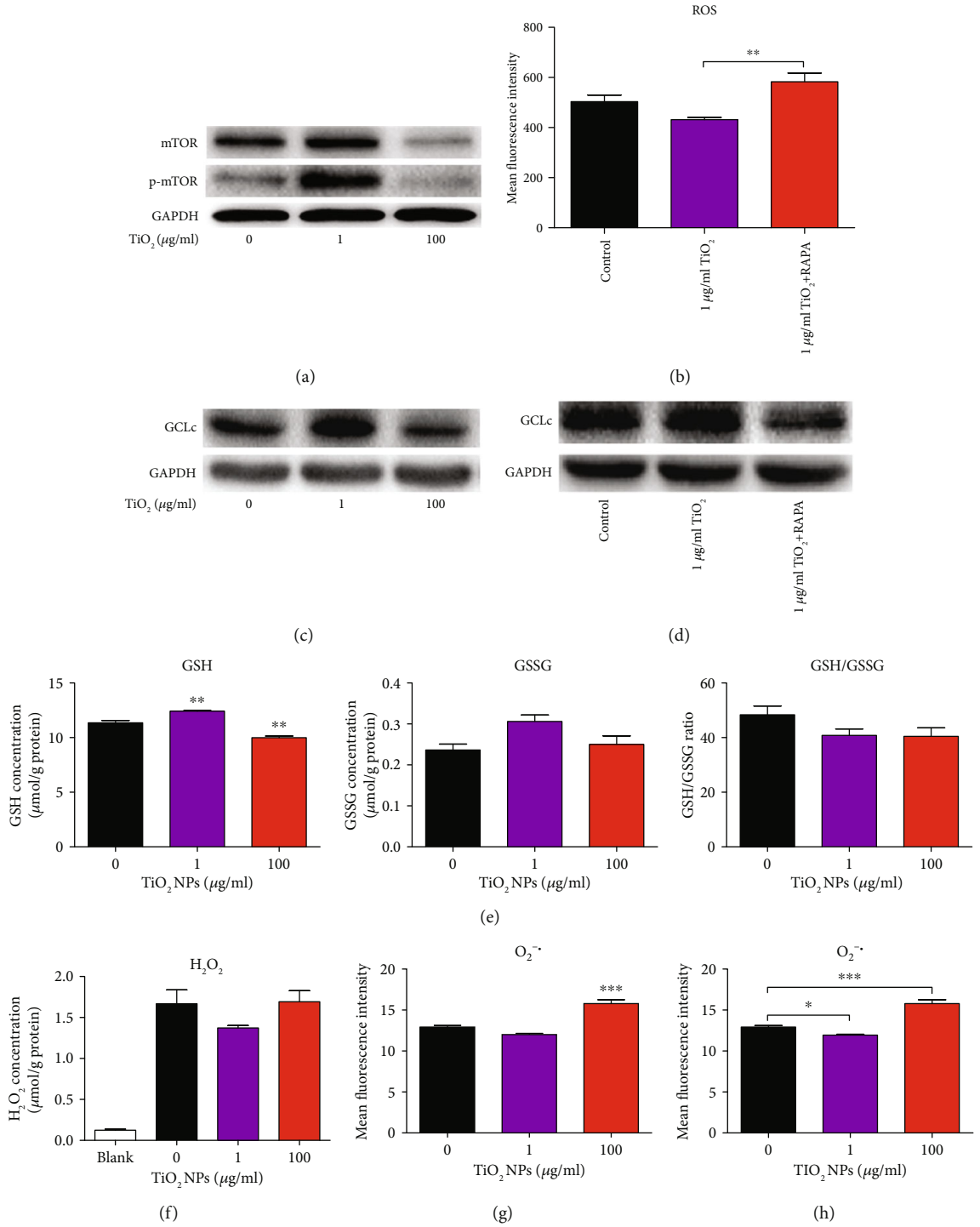

FIGURE 2: (a) mTOR and p-mTOR (Ser2448) protein expressions were measured by western blotting experiment after cells were treated with 1 μg/ml and 100 μg/ml of TiO_2 NPs for 24 h. (b) ROS levels after cells were treated with 1 μg/ml TiO_2 NPs and 1 μg/ml TiO_2 NPs+RAPA. Mean fluorescence intensity was measured by FCM. (c) GCLc protein expression levels after cells were treated with 1 μg/ml and 100 μg/ml of TiO_2 NPs for 24 h. (d) GCLc protein expressions after cells were treated with 1 μg/ml TiO_2 NPs and 1 μg/ml TiO_2 NPs+RAPA. (e) GSH, GSSG levels, and the ratio of GSH/GSSG were measured by a commercial assay kit after cells were treated with 1 μg/ml and 100 μg/ml TiO_2 NPs for 24 h, and the results were from three panel samples of each experiment. Each assay was repeated at least three times. (f) The H_2O_2 concentrations after cells were treated with TiO_2 NPs for 24 h. Total protein was used as an internal control. (g, h) Intracellular superoxide anion was measured by DHE after cells were treated with TiO_2 NPs for 24 h. The independent experiment was repeated three times and five times, respectively. GAPDH was used as protein loading control. Blank means blank control; "0" means control group; RAPA means rapamycin. All the values are presented as mean ± SE, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

GSH/GSSG showed no significant differences among three groups after TiO_2 NP treatment.

3.9. Effects of TiO2 NPs on Intracellular H_2O_2 and $O_2^{\cdot-}$. H_2O_2 and $O_2^{\cdot-}$ are the major ROS in cells, and the DHE and hydrogen peroxide assay kits were used to detect $O_2^{\cdot-}$ and H_2O_2, respectively. As shown in Figure 2(f), there were no significant changes in the H_2O_2 levels among three groups, except a mild decrease in the 1 μg/ml group. The $O_2^{\cdot-}$ level was increased significantly ($P < 0.01$) with 100 μg/ml of TiO_2 NPs, while $O_2^{\cdot-}$ level was decreased slightly in the 1 μg/ml group compared with the control group (Figure 2(g)). This small change was similar to the changes of ROS in each assay and became significant when the assay was independently repeated up to five times (Figure 2(h)).

3.10. Reverse Effects of GSH on ROS. To clarify whether $O_2^{\cdot-}$ was the main ROS, and whether the intercellular GSH was abundant, additional GSH was added after cells were exposed to the high-dose TiO_2 NPs. The results showed that the cellular ROS increased significantly ($P < 0.01$) in the high-dose group (Figure 3(a)), but remained significantly higher than the ROS level in the control group ($P < 0.01$) with only a mild decrease after additional GSH was added.

3.11. Effects of TiO_2 NPs on Activities of Key Enzymes in ROS Regulation Pathway. Cells were treated with TiO_2 NPs and collected. Total SOD activity, CAT activity, and GSH-Px activity were measured. After treated with TiO_2 NPs, there was no change in both SOD activity and CAT activity among three groups (Figures 3(b) and 3(c)). However, the activity of GSH-Px was decreased significantly in both 100 μg/ml ($P < 0.001$) and 1 μg/ml ($P < 0.05$) of the TiO_2 NP groups compared with the control group (Figure 3(d)).

3.12. Effects of TiO_2 NPs on the Permeability of Inner Mitochondrial Membrane. Mitochondria is known as a source of $O_2^{\cdot-}$, so we examined the mitochondrial membrane potential, which reflects mitochondrial membrane permeability. Figure 3(e) shows that after treated with 1 μg/ml and 100 μg/ml TiO_2 NPs, the aggregated JC-1 was decreased significantly ($P < 0.05$) while the monomeric JC-1 was increased significantly ($P < 0.05$), and the aggregate/-monomer ratio decreased significantly ($P < 0.001$). However, ATP production surprisingly was not different among the three groups (Figure 3(f)).

3.13. Interaction between $O_2^{\cdot-}$ and mTOR Protein Expression. As the results indicated that the main ROS was $O_2^{\cdot-}$, we further clarified the relationship between mTOR proteins and $O_2^{\cdot-}$. We inhibited mTOR activity with RAPA in the low-dose group and neutralized $O_2^{\cdot-}$ with 50 μM of Mn(III)TBAP in the high-dose group. After $O_2^{\cdot-}$ level was decreased (Figure 3(g)), the protein level of mTOR was increased in the high-dose group (Figure 3(h)). As shown in Figure 3(i), $O_2^{\cdot-}$ was decreased after cells were exposed to 1 μg/ml TiO_2 NPs ($P < 0.01$) compared with the control group. When 100 nM of RAPA was added, the $O_2^{\cdot-}$ level was increased to control group level.

3.14. Effects of SOD Activity on $O_2^{\cdot-}$ Level prior to mTOR Upregulation with Low-Dose Treatment. After 3 h of incubation, the mitochondrial membrane potential (aggregates/-monomer ratio) was decreased (Figure 3(j)), and the mTOR protein level remained similar to the control group (Figure 3(k)). However, SOD activity was increased significantly ($P < 0.01$) (Figure 3(l)), and $O_2^{\cdot-}$ level was also significantly ($P < 0.05$) decreased (Figure 3(m)).

3.15. Validation of the Changes and Associations of Key Molecules in Two Independent Cell Lines. To verify the findings in SH-SY5Y cell lines, we examined the changes and associations of key molecules in two independent human neurogenic cell lines (U251 and SK-N-SH). The results showed that $O_2^{\cdot-}$ was decreased in the low-dose group and was increased in the high-dose group in both cell lines (Figure 4(a)). Figures 4(b) and 4(c) show that the protein expression levels of mTOR, p-mTOR, GCLc, and GSH levels were increased in the low-dose group and decreased in the high-dose group in both cell lines, while H_2O_2 levels were not different among the three groups (Figure 5(a)). Further investigation found that (Figures 5(b)–5(d)) in the low-dose group, SOD activity was increased, but the CAT activity and GSH-Px activity showed no change. In the high-dose group, SOD and CAT activities showed no change, but GSH-Px activity was significantly decreased. Figure 6(a) shows that when the mTOR activity was inhibited by 100 nM of RAPA in the low-dose group, the expression of GCLc was decreased and was followed by a significant increase of the $O_2^{\cdot-}$ level in both cell lines. When $O_2^{\cdot-}$ was neutralized with 50 μM of Mn(III)TBAP in the high-dose groups, the expression of mTOR was increased (Figure 6(b)). These results were generally consistent with the findings in SH-SY5Y cells, emphasizing the potential neurotoxic effects of TiO_2 NPs at low dose.

4. Discussion

TiO_2 NPs are the most widely manufactured nanoparticles worldwide, and many studies had raised concerns about their potential hazards. To date, the ability of TiO_2 NPs to induce ROS in various cells and organs had been extensively reported, especially in neurogenic cells. Therefore, we employed neurogenic cell lines to explore the mechanism underlying the regulation of ROS after TiO_2 NP exposure, and we observed an interesting phenomenon in which TiO_2 NPs caused a nonmonotonic dose-dependent effect on ROS induction in SH-SY5Y cells. The low dose of TiO_2 NPs (1 μg/ml) showed a weak antioxidant effect, and the dose of 10 μg/ml results in ROS level similar to the control group, while high dose of TiO_2 NPs (100 μg/ml) induced ROS accumulation.

TiO_2 NPs did not induce cell death even at a relatively high dose in our study, which was also reported in other studies [32, 33], indicating that TiO_2 NPs might be relatively safe. Therefore, people may neglect the potential risks of TiO_2 NPs at low dose. However, in our study, we noticed that TiO_2 NPs could decrease ROS level and slightly promote cell proliferation in neurogenic cells at low dose, indicating the

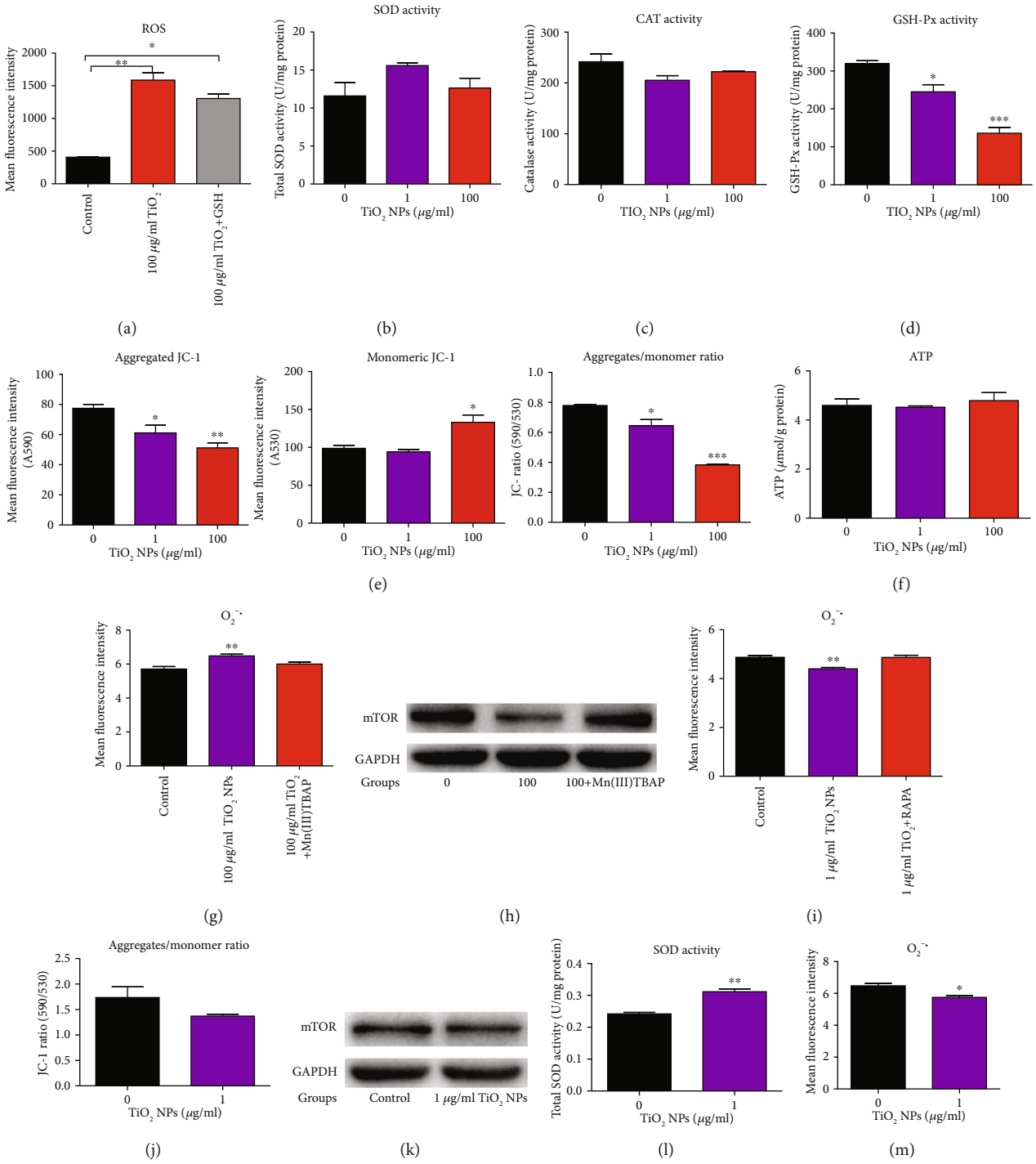

FIGURE 3: (a) ROS levels were measured by FCM after cells were treated with 100 μg/ml TiO_2 NPs and 100 μg/ml TiO_2 NPs+GSH for 24 h. (b–d) Key enzyme activities in the ROS regulation pathways (including SOD, CAT, and GSH-Px) were measured after cells were treated with TiO_2 NPs. Total protein was used as an internal control. (e) Mitochondrial membrane potential was determined by JC-1. Aggregated and monomeric JC-1 concentrations were measured by average fluorescence intensity at 590 nm and 530 nm, respectively. The ratio of 590/530 nm indicated the changes of mitochondrial membrane potential. (f) ATP levels were measured after cells were treated with TiO_2 NPs for 24 h. (g, h) Intracellular superoxide anion and mTOR protein expression levels were measured after cells were treated with 100 μg/ml of TiO_2 NPs and 100 μg/ml of TiO_2 NPs+50 μM of Mn(III)TBAP for 24 h. (i) Intracellular superoxide anion levels were measured after cells were treated with 1 μg/ml of TiO_2 NPs and 1 μg/ml of TiO_2 NPs+RAPA for 24 h. (j, k) Mitochondrial membrane potential and mTOR protein expression levels were measured after cells were treated with 1 μg/ml of TiO_2 NPs for 3 h. (l, m) SOD activities and superoxide anion concentrations were detected after cells were treated with 1 μg/ml of TiO_2 NPs for 3 h. Values are presented as mean ± SE, and each assay was repeated three times. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

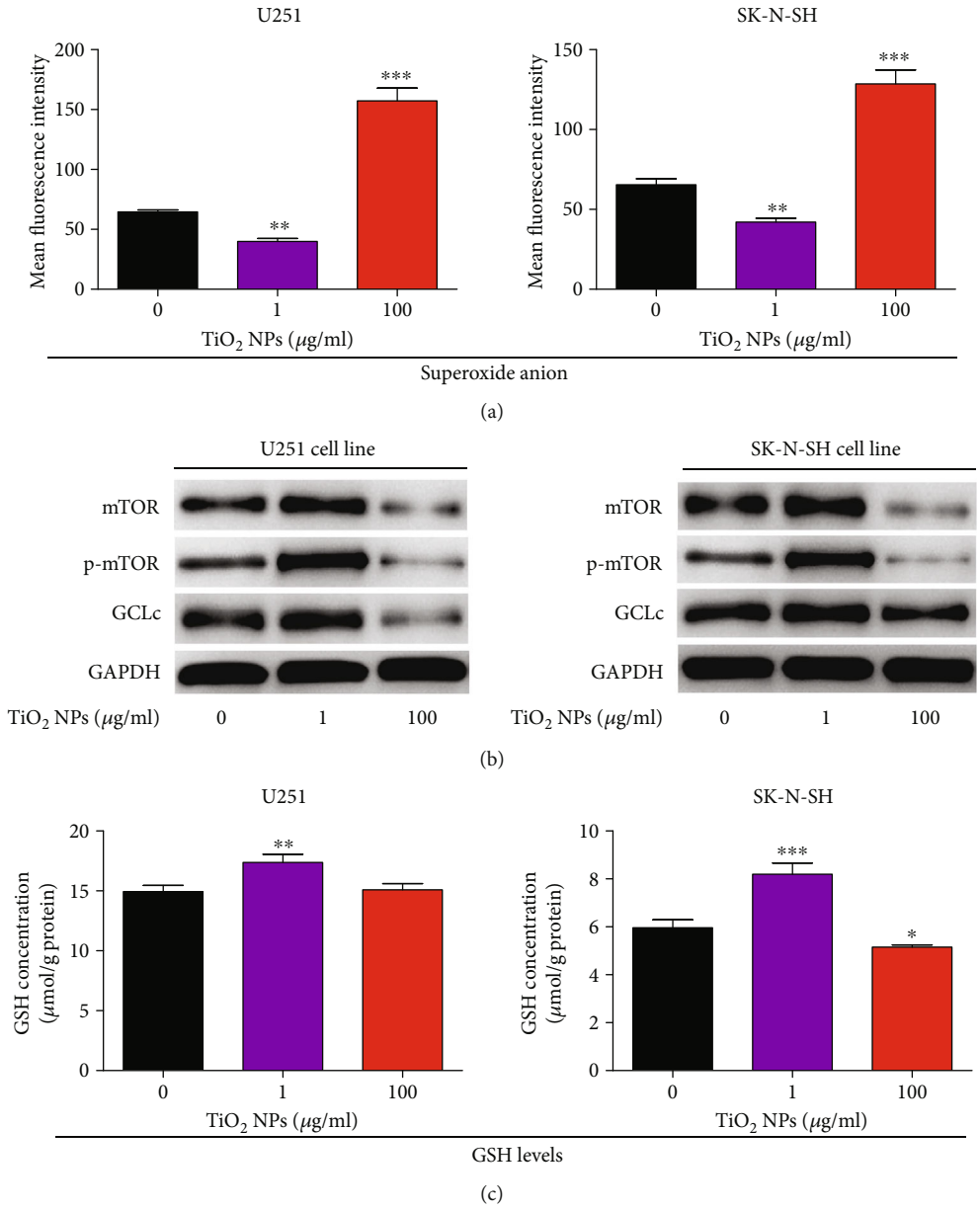

Figure 4: (a) Intracellular superoxide anion was determined by DHE after U251 cells, and SK-N-SH cells were treated with 1 μg/ml and 100 μg/ml of TiO_2 NPs, respectively. (b) mTOR, p-mTOR, and GCLc protein levels were determined by western blotting assay after two kinds of cells were treated with 1 μg/ml and 100 μg/ml of TiO_2 NPs. (c) GSH levels were determined after two kinds of cells were treated with 1 μg/ml and 100 μg/ml of TiO_2 NPs. The results were from three panel samples of each experiment. Values were presented as mean ± SE; each assay was repeated three times. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

nonnegligible neurotoxic effects of TiO_2 NPs even at low dose. Based on these results, three groups including the control group and 1 μg/ml- and 100 μg/ml-treated groups were used in the subsequent experiments to explore the underlying mechanisms.

The mTOR pathway interacts with ROS. For example, low level of ROS could increase the expression of p-mTOR in H4IIE cells [34], and high level of ROS could inhibit the mTOR pathway [35], which indicated that mTOR protein was a key factor that regulates ROS. The results showed that the changes in mTOR proteins were consistent with the changes in ROS, indicating that ROS might have a correlation with mTOR proteins. To further verify whether ROS had associations with mTOR proteins, we used RAPA, a specific inhibitor of mTOR proteins for further study. RAPA inhibited mTOR activity and increased ROS level. This preliminary result confirmed the association between ROS and mTOR protein, and was in accordance with the result of a previous study [36].

GCLc is the first rate-limiting enzyme in GSH synthesis and is the downstream of mTOR. Therefore, we examined the protein levels of GCLc in three groups by western blotting. The results showed that the trend in GCLc protein levels was similar to that of both ROS and mTOR protein

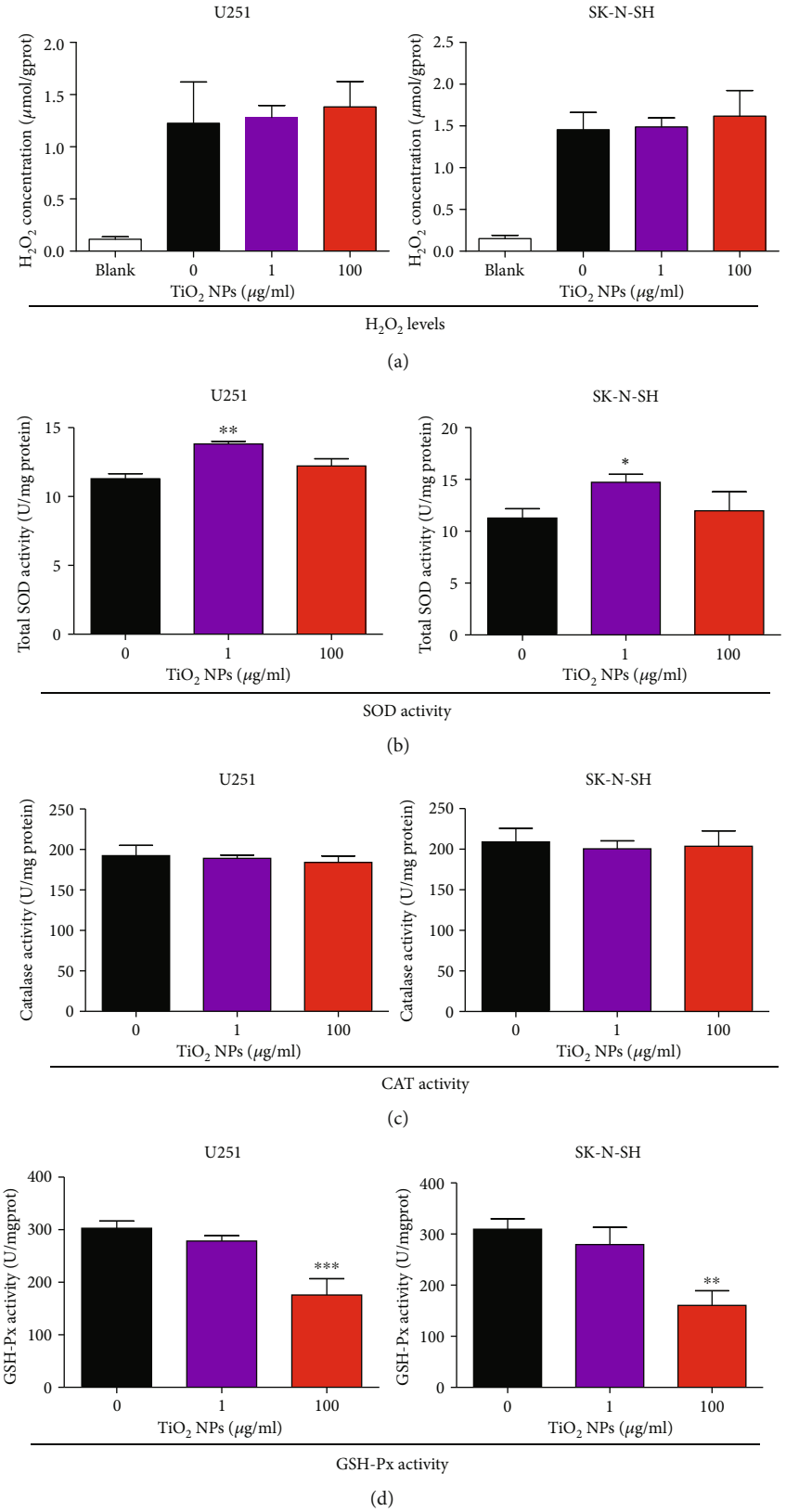

Figure 5: Intracellular H_2O_2 levels (a), SOD activities (b), CAT activities (c), and GSH-Px activities (d) were determined after U251 cells, and SK-N-SH cells were exposed to 1 μg/ml and 100 μg/ml of TiO_2 NPs, respectively. All data are presented as mean ± SE, and each assay was repeated three times. Blank means blank control, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

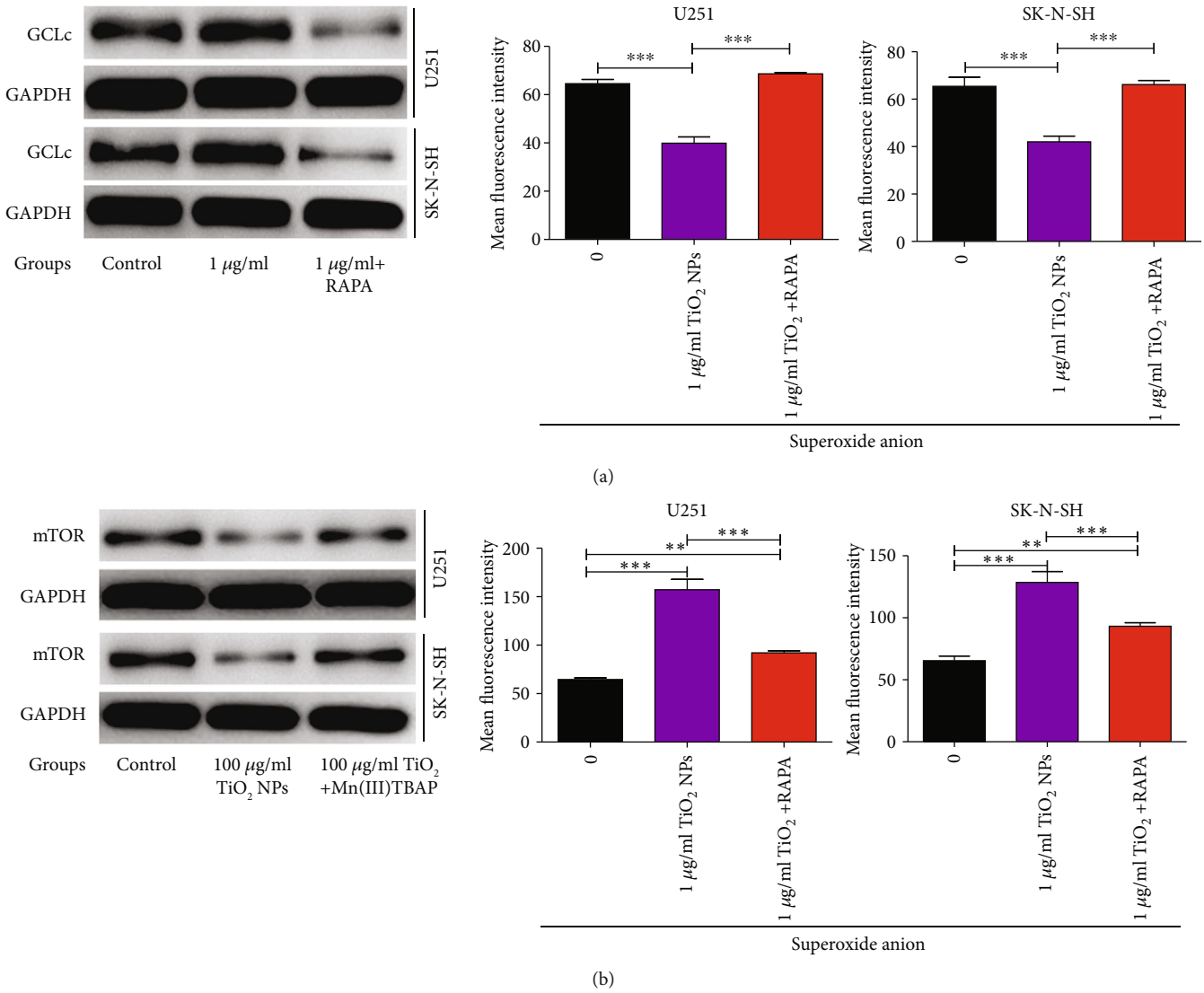

Figure 6: (a) GCLc protein levels and superoxide anion levels were examined after U251 and SK-N-SH cell lines were treated with 1 μg/ml of TiO_2 NPs and 1 μg/ml of TiO_2 NPs+RAPA. (b) mTOR protein levels and superoxide anion levels were determined after two kinds of cells were treated with 100 μg/ml of TiO_2 NPs and 100 μg/ml of TiO_2 NPs+50 μM of Mn(III)TBAP; GAPDH was used as a protein loading control. The values are presented as mean ± SE, and each assay was repeated three times. $^{**}P < 0.01$, $^{***}P < 0.001$.

levels, which suggested an association between mTOR and GCLc proteins. To clarify whether GCLc expression was associated with mTOR proteins, RAPA was added to inhibit the activity of mTOR proteins, and we found that the expression of GCLc protein was decreased. These results confirmed our hypothesis that GCLc was regulated by mTOR in neurogenic cells.

GSH is the product of GCLc; thus, the concentrations of GSH and GSSG were then measured. The concentration of GSH was increased significantly in the low-dose group and decreased significantly in the high-dose group. These changes were consistent with the changes in GCLc proteins. The GSSG concentrations and the ratio of GSH/GSSG showed no significant changes among three groups. Although GSH concentration was significantly increased in low dose, the magnitude of these changes was relatively small, with only an increase of ~8%, which might be explained by the fact that GSH was relatively abundant and feedback-inhibited the activity of GCLc [37], a phenomenon that was also reported by Langston et al. [38]. After exposed to high dose of TiO_2 NPs, the ROS level in the cells was extremely high, but GSH showed only a decrease of ~15% in value. Previous studies showed that GSH could decrease 50% or more to exert antioxidant effect [39, 40], so the remaining GSH in the high-dose group was abundant.

To explore why GSH could accumulate under high ROS level, we examined the ROS regulation pathway. H_2O_2 and $O_2^{\cdot-}$ are the major ROS in cells. We observed no significant changes in the H_2O_2 levels among three groups. The level of $O_2^{\cdot-}$ was slightly decreased in the low-dose group and was increased significantly in the high-dose group.

To further verify whether $O_2^{\cdot-}$ was the main ROS, additional GSH was added after cells were exposed to the high dose of TiO_2 NPs. The results showed that GSH could only

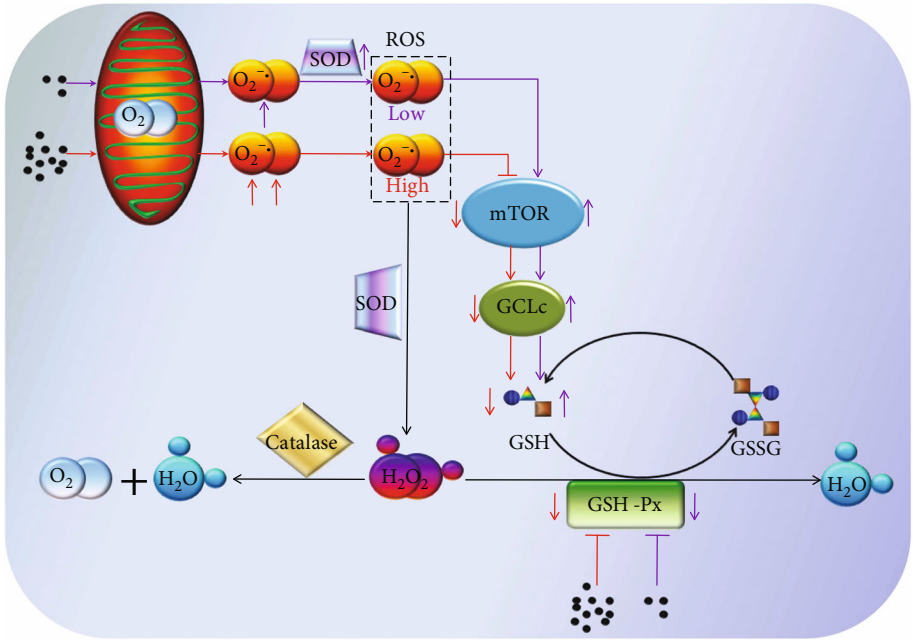

Figure 7: The mTOR/GCLc/GSH pathway mediated the dose dependently bidirectional regulation of ROS induced by TiO2 NPs in neurogenic cells. When low dose (1 μg/ml) of TiO_2 NPs entered cells, they affected mitochondrial membrane permeability and produced a small amount of superoxide anions ($O_2^{\cdot-}$). The low dose of $O_2^{\cdot-}$ activated the expression of mTOR protein and increased GSH production. However, TiO_2 NPs also impaired GSH-Px activity, so the antioxidant effect was weak. When cells exposed to high dose (100 μg/ml) of TiO_2 NPs, the expression of mTOR protein was inhibited by massive $O_2^{\cdot-}$ from mitochondria. Moreover, high dose of TiO_2 NPs impaired the GSH-Px activity. Although GSH production was reduced, the pathway that responsible for GSH consumption was inhibited, so the intercellular GSH was still abundant.

mildly decrease the ROS level. If GSH was lacking, the application of additional GSH might more obviously decrease ROS. The results further suggested that GSH was sufficient in the high-dose group, which might be explained by altered consumption. The results also suggested that $O_2^{\cdot-}$ was the main ROS. Therefore, the key enzymes involved in the GSH metabolic pathways were then studied.

Our results showed no change in SOD activity and CAT activity, but the activity of GSH-Px was decreased significantly with both the high-dose and the low-dose treatment, which indicated that the activity of GSH-Px was impaired and that it could not efficiently catalyze GSH to neutralize $O_2^{\cdot-}$, resulting in the GSH accumulation. The difference in the enzyme activities may be caused by the different absorption abilities of TiO_2 NPs [41], as different enzymes possess different surface charges. The impaired activity of GSH-Px might be due to the replacement of $O_2^{\cdot-}$ by TiO_2 NPs, which is supported by the fact that GSH-Px activity was also decreased in the low-dose group with low level of $O_2^{\cdot-}$. Collectively, the impaired activity of GSH-Px might be the reason for the coexistence of abundant GSH and a high burden of ROS and $O_2^{\cdot-}$.

If only the activity of GSH-Px was impaired, $O_2^{\cdot-}$ might not accumulate, because the activity of CAT activity was normal and cells could scavenge $O_2^{\cdot-}$ by CAT. Therefore, the accumulation of $O_2^{\cdot-}$ might be due to increased production, which is related to mitochondria [42]. A previous study showed that $O_2^{\cdot-}$ level presented a time-dependent increase after exposed to Triton X-100, which increases the permeability of the mitochondrial membrane [43]. To study the induction of this cascade, we examined the membrane potential, as decreased mitochondrial membrane potential reflects increased inner mitochondrial membrane permeability. The results showed that the permeability of inner mitochondrial membrane was increased, so the release of $O_2^{\cdot-}$ was increased. Although the permeability of the inner mitochondrial membrane was increased, we surprisingly found that ATP production did not differ among three groups, which may be explained by the fact that the change in the inner mitochondrial membrane permeability was more sensitive than the changes in ATP production change, and TiO_2 NPs did not cause severe mitochondrial damage.

As the results indicated that the main ROS was $O_2^{\cdot-}$, we further clarified the relationship between mTOR proteins and $O_2^{\cdot-}$. We considered the uncertain effects of mTOR proteins overexpression of on ROS and inhibited mTOR proteins in the low-dose group and neutralized $O_2^{\cdot-}$ in the high-dose group to examine the $O_2^{\cdot-}$ levels and the expression of mTOR proteins, respectively. To demonstrate that the $O_2^{\cdot-}$ level could affect mTOR protein expression, we examined mTOR protein levels after $O_2^{\cdot-}$ was scavenged by 50 μM of Mn(III)TBAP, an $O_2^{\cdot-}$ scavenger [44]. The protein level of mTOR was increased after $O_2^{\cdot-}$ was scavenged in the high-dose group. After mTOR protein was inhibited, $O_2^{\cdot-}$ was increased in the low-dose group. These results indicated the interaction between $O_2^{\cdot-}$ and mTOR protein expression.

Our results showed that the potential of the mitochondrial membrane was decreased after treated with low dose of TiO_2 NPs for 24 h. Therefore, similar to the high-dose group, the $O_2^{\cdot-}$ level should have increased in the low-dose

group. However, it decreased. To explore how the low oxidative stress condition formed, we detected the $O_2^{\cdot-}$ level after cells were treated for 3 h, when mTOR protein level of the treated group was still similar to that of the control group. After 3 h of incubation, the mitochondrial membrane potential decreased, but SOD activity was increased significantly in the low-dose group compared with the control group. Meanwhile, $O_2^{\cdot-}$ level was significantly decreased, which indicated that SOD responded rapidly in the first 3 h of $O_2^{\cdot-}$ release, and that SOD downregulated the $O_2^{\cdot-}$ level, and created a low oxidative stress condition to activate the upregulation of mTOR proteins. When the low oxidative stress condition developed, SOD activity gradually decreased to the control group level at 24 h. Previous studies indicated that SOD activity could be activated by $O_2^{\cdot-}$, but no change was found in the high-dose group. Although SOD activity was activated by $O_2^{\cdot-}$, it might also be inhibited by TiO_2 NPs [45, 46], so no significant change was detected by the enzyme activity assay kit.

In this work, we explored the mechanism underlying the regulation of ROS by TiO_2 NPs (Figure 7). When the low dose of TiO_2 NPs entered cells, they increased the permeability of mitochondrial membrane and produced low concentration of $O_2^{\cdot-}$ to rapidly activate SOD. In turn, the decreased $O_2^{\cdot-}$ activated the mTOR/GCLc/GSH pathway and finally overproduced GSH. Additionally, TiO_2 NPs impaired GSH-Px activity which hindered the neutralization of $O_2^{\cdot-}$ by GSH and therefore caused a weak antioxidant effect. When high dose of TiO_2 NPs entered cells, they induced very large $O_2^{\cdot-}$ levels and decreased the protein expression of mTOR to inactivate the mTOR/GCLc/GSH pathway, which finally led to a decrease in the GSH concentration. In addition, TiO_2 NPs impaired the activity of GSH-Px and resulted in the accumulation of GSH. Moreover, we also demonstrated that the decreased $O_2^{\cdot-}$ gradually activated mTOR upregulation after 3 h of exposure to TiO_2 NPs. Although, a low dose of NPs, including TiO_2 NPs, is considered safe under most conditions, our study revealed the antioxidant effects of TiO_2 NPs at low doses via upregulation of mTOR protein, which is closely related to adverse effects including tumor development, emphasizing the potential neurotoxic effects of NPs at low doses. The antioxidant effect may be just one of the important effects caused by low-dose TiO_2 NPs in neurogenic cells; thus, the biological safety of TiO_2 NPs should be evaluated more comprehensively in the future.

Data Availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Conflicts of Interest

The authors declare no competing financial interest.

Authors' Contributions

MZ and LS designed the study, ZL and YM carried out the study, ZZ was responsible for language editing, and CM and MZ are the corresponding authors. Zhilei Mao, Shushu Li, and Lina Zhang contributed equally.

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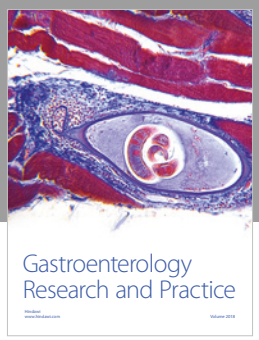

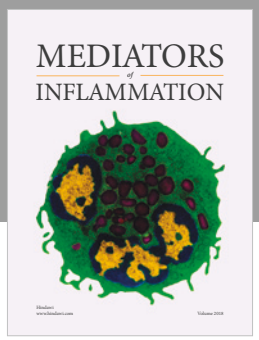

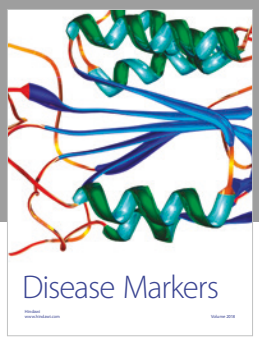

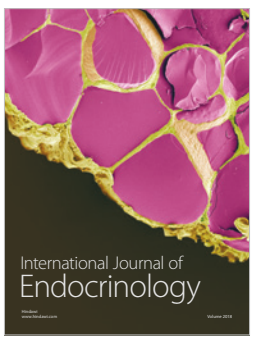

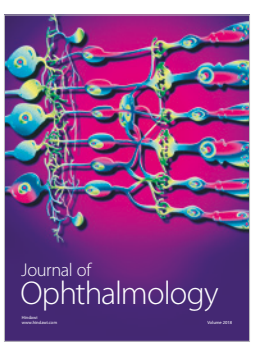

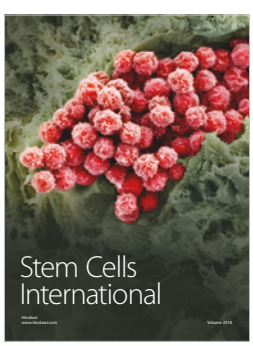

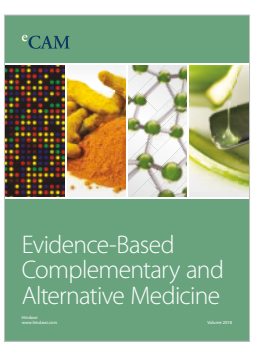

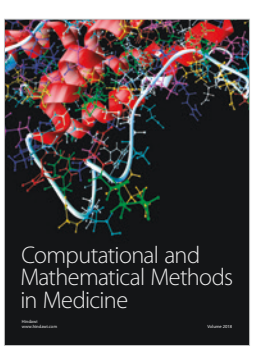

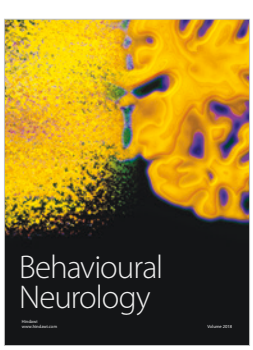

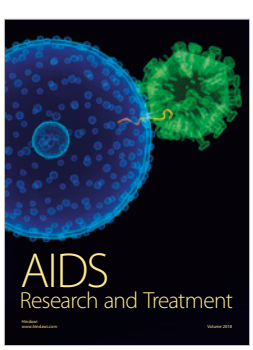

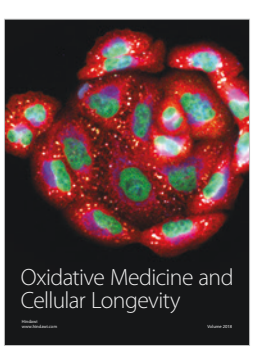

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