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The Neurotoxicity of Titanium dioxide Nanoparticles in Mice.

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ABSTRACT

Titanium dioxide (TiO_2) nanoparticles (NPs) are the second NPs in the industry worldwide.TiO_2 is widely used in chemical, electrical and electronic industries. TiO_2NP penetrate directly into the brain through the olfactory bulb and can be deposited in the hippocampus region consequently, the present study was investigated the toxic effect of TiO_2 NPs on brain of mice. Male mice were exposed to intraperitoneal injection (i.p) with (50 and 100 mg/kg body weight (BW) TiO_2 NPs for 21 successive days. At the end of experiment animals were sacrificed, brains were isolated for estimation of Neurotransmitters (dopamine (DA) and norepinephrine (NE)), Malondialdehyde (MDA), Antioxidant enzymes (superoxide dismutase (SOD) and glutathione peroxidase (GPx), Inflammatory mediators (interlukine-6 (IL-6) and tumor necrosis factor-α (TNF-α)) and Apoptosis biomarker (Bcl-2). The results showed that exposure to these nanoparticles induced a significant increase of DA and NE, decrease of SOD and GPx, raise of IL-6 and TNF-α and down regulation of Bcl-2 level. In conclusion , TiO_2 nanoparticles could be translated to the brain and in turn caused the brain damage includes oxidative stress that leads to an change in the level neurotransmitters, lipid peroxidation, reduce the activities of antioxidative enzymes and activation of inflammatory cytokines resulted the stimulation of apoptosis.

Keywords: Nanoparticles, Brain, Oxidative stress, Inflammation,Apoptosis.

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INTRODUCTION

Nanoparticles are substances that measured approximately 1–100 nm in at least one dimension of scale, when particlesize is decreased below this dimension , the resulting material showed a significant different in physical and chemical properties that are diverse from the properties of macroscale materials composed of the similar material [1]. Recently, nanoparticles are issues in many fields such as industrial applications, environmental studies and human health impacts, due to their unique physical and chemical characteristics [2,3]..In the early 20th century, TiO2 goods had already appeared, and it is widely used in paints, printing ink, rubber, paper, cosmetics, sunscreens, car materials, cleaning air products, industrial photocatalytic processes, and moldering organic matters in waste [4].

Given the widespread use of nano-TiO2 its safety has gained even more attention. Nano TiO_2 able to penetrate the body by different ways, for example throughout the respiratory and digestive systems or the skin, and circulate via the blood or lymphatic system, finally accumulating in various organs [5] and tissues including the brain [6,7]. Nano-TiO2 produces hazards effects in several organs of the body, causing deleterious effects such as neurotoxicity, immunotoxicity, reproductive toxicity and respiratory toxicity, although these nanoparticles are often eliminate from the body by sweat or the metabolism.

Previous studies have shown that the effect of nanomaterials on the central nervous system is not negligible,especially for the workers working in nanomaterials manufacturing factories [8]. Brain is the most important and complex organ of the human body. However, it is also highly vulnerable to adverse effects especially oxidative stress. In reality it faces continuous exposure of the high level of oxidative stress produced from oxidative metabolism further adds its venerability. Now it's well documented that NPs are able to cross the blood–brain barrier [9,10] and enter (in low numbers) the central nervous system(CNS) of the exposed animals [11,12] Moreover, TiO2 nanoparticles were revealed to stimulate of ROS generation in the brain microglia and lead to neuron damages in vitro [13]. On the other hand, the oxidative toxicity of TiO2 nanoparticles in the brain has not been well considered *in vivo* to date.

Furthermore, the exposure to TiO2 NPs was confirmed to cause deposition of calcium in neurocytes, proliferation of ependyma and all glial cells, and disturb the homeostasis of trace elements, neurotransmitters,and enzymes in mouse brain, thus leading to reduction in spatial recognition memory in mice [14,15]. Besides these, some *in vitro* studies revealed that the stimulation of inflammatory responses was significantly increased, in truth some neurons turned into inflammatory cells following nano-TiO2 exposure [16]. In proteomic studies, nano-TiO2 has shown to significantly alter the expression of brain proteins, even if NPs were beyond the detection range in the tissue [17].

Disturbance of neurotransmitters and enzymes,oxidative stress and inflammatory response have been described as neurotoxic effects after nasal instillation, intrapritoneal injection, oral administration or prenatal exposure [8,18]. These findings raised the question of the entry of TiO2 NPs into the brain and of their interactions with the BBB. Nowadays, most of the studies on the toxicity of nano-TiO2 in mammals focused on the pulmonary impact of inhaled nano-TiO2 or dermal and oral exposure, but little is known about the impact of intrapritoneal (i.p.) exposure of nano-TiO2 in brain.

Hence the present study was designed to explore the effect of i.p injection of TiO2 Nanoparticles on brain of mice. This goal could be achieved through evaluation of neurotransmitter (dopamine (DA) and norepinephrine(NE)), Malondialdehyde (MDA), antioxidant enzymes (superoxide dismutase (SOD) and glutathione peroxidase (GPx), inflammatory mediators (interlukine-6 (IL-6)), tumor necrosis factor-α (TNF-α) and apoptosis bio-marker (Bcl-2).

MATERIALS AND METHODS

Experimental Animals

30 male Swiss albino mice (*Mus musculus*), aging (9-12) weeks old and weighting 25-30 g, were acquired from the Helwan Breeding Farm , Cairo .Egypt. Animals have been breed and maintained in an air-conditioned of animal house with specific pathogen- free conditions and were subjected to a 12:12 h daylight/darkness and allowed limitless access to chow and water.The animals were acclimated for a period of

2 weeks before the beginning of the experiments. The experiment was carried out in agreement with the care and use of laboratory animals guidelines permitted by Research Ethical Committee (Medical Research of Bilharizia Center , Faculty of Medicine , Ain Shams University).

Chemicals

Titanium dioxide nanoparticles TiO2NPs (< 100 nm particle size) Purchase from Sigma Aldrich .Com (U.S.A), the TiO2-NPs were suspended in a double distilled water at stock concentration 100 mg/kg body weight and ultrasonicated (ultrasonic cleanear CD-4831 , Ac 220-240 v, 50 HZ, 170 W) for 30 min. The freshly prepared working solution was made by serial dilution of the stock solution, two concentrations of 50 and 100 mg/ kg b.wt.

Animal Grouping

The animals were randomly divided into three groups, as follows:

Group (I) received 0.5 ml of distilled water and served as a control
Group (II) received 50 mg/kg b.w of TiO_2NPs.
Group (III) received 100 mg/kg b.w of TiO_2NPs.

TiO_2NPs was injected intraperitoneally daily for 21 successive days. At the end of the experiment animals were sacrificed, and brain was dissected out, washed with PBS, frozen in liquid nitrogen, and stored at -80 C. Then the tissues were rinsed in cold Tris-KCl buffer(0.15 M pH 7.4) and made 10 % homogenates (1g tissue in 10 ml Tris-KCl buffer). After that they were centrifuged at 10,000xg for 30 min at $4C^0$. The Supernatant was used for biochemical assay.

Brain homogenate of each animal in all groups was subjected to the following analysis.

Biochemical Analysis

Neurochemicals Assay(dopamine and norepinephrine) (DA&NE)

Dopamine and norepinephrine levelassayed by ELISA techniqueusing DA and NE assay kit purchased from My biosource Co., according to the manufacturer's instructions provided with DA and NE assay kit.

Malondialdehyde(MDA)Content was determined in brain by using TBARS assay kit purchased from Biodiagnostic, Co.according to the method described by Ohkawa *et al* [19].

The content of superoxide dismutase (SOD) was estimated by using SOD assay kit purchasedfrom Biodiagnostic,Co., according to the method described by Nishikimi *et al* [20]. **Glutathioneperoxidas(GPx)** was determined by using GPx assay Kit purchased from Biodiagnostic, Co., according to the method described by Paglia and Valetine [21].

interlukine-6 and tumornecrosis factor- α assayed by ELISA techniqueusing IL-6 and TNF-α assay kit purchased from Ray Biotech, Inc. Co., according to the manufacturer's instructionsprovided with IL-6 and TNF-α assay kit.
Bcl-2 content was assayed by ELISA technique using Bcl-2 purchased from Cusabio, Wuhan Hi-tech Medical Devices, China.

Statistical Analysis

The data have been analyzed by using SPSS for windows software version 20.0 statistical program. Differences among experimental groups were determined by One way ANOVA (analysis of variance). Data was expressed as mean ± Standard Error (SE).

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RESULTS

As shown in (Table 1) the level of dopamine and norepinephrine (DA&NE) was detected after administration of TiO2 NPs at two doses. The results revealed a significant (p< 0.001) elevate in DA level (45.93± 1.28 and 51.62 ± 2.52 pg/mg) in 50 and 100 mg/kg treated groups,respectively as compared to control group (27.28± 2.04 pg/mg). The brain NE level also showed significant (P< 0.001) increase in both doses (67.00 ±1.75 and 87.10 ±2.16 pg/mg),respectively, when compared to control group (52.07± 2.62 pg/mg) and it was showed that there was a significant increase between the low and high dose.

Table 1: Effect of TiO2 nanoparticles on neurotransmitters levels in mice brain.

Groups	Control	Low dose	High dose
DA (pg/mg)	27.28 ± 2.04	45.93 ± 1.28	51.62 ± 2.52
NE (pg/mg)	52.07 ± 2.62	67.00 ± 1.75	87.10 ± 2.16

Data are represented as Mean ± SE for 10 mice/group.

MDA level was significantly (P< 0.001) enhanced in a dose dependent manner after exposure to nano-TiO_2 as compared to control group the levels were (33.65 ± 2.51 and 74.93 ± 3.28 nmol/g) in low and high dose, respectively,as compared to control group (17.2±1.11 nmol/g) as represented in (Table 2).

Table 2: Effect of TiO2 nanoparticles on oxidative stress variables in mice brain.

Groups	Control	Low dose	High dose
MDA (nmol/g)	17.2 ± 1.11	33.65 ± 2.51	74.93 ± 3.28
SOD (U/mg)	6.42 ± 0.53	5.15 ± 0.14	2.18 ± 0.07
GPx (U/mg)	59.17 ± 4.96	48.27 ± 0.76	27.68 ± 1.24

Data are represented as Mean ± SE for 10 mice/group.

The activity of antioxidative enzymes (SOD & GPx) in the brain of mice treated with TiO_2 NPs was significant reduce in both low and high dose as compared to control group. Where, SOD levels were significant (P< 0.01) decrease in low dose at (5.15 ± 0.14 U/mg) but highly significant decrease recorded in high dose (2.18±0.07 U/mg) as compared to control group (6.42 ± 0.53). Similarly, GPx levels also showed significant (P< 0.001) decease with increasing the dose of nano-TiO_2 (48.27±0.76 and 27.68± 1.24 U/mg) at low and high dose,respectively, as compared to control group (59.17 ± 4.96) (Table 2).The present study illustrated that inflammatory markers (IL-6 & TNF-α) levels were significant (P< 0.001) increase after exposure to TiO_2 NPs in a dose dependent manner, the levels of IL-6 were (59.47 ± 4.65 and 95.07 ± 0.82 pg/mg) when compared to control group (34.28 ± 1.15).While TNF-α levels were (80.97 ± 5.74 and 114.83 ± 5.02 pg/mg) when compared to control group (52.37 ± 2.16) (Table 3).

Table 3: Effect of TiO_2 nanoparticles on inflammatory mediators and apoptic marker in mice brain.

Groups	Control	Low dose	High dose
IL-6 (pg/mg)	34.28 ± 1.15	59.47 ± 4.65	95.07 ± 0.82
TNF-α (pg/mg)	52.37 ± 2.16	80.97 ± 5.74	114.83 ± 5.02
Bcl-2 (ng/g)	154.53 ± 4.82	125.10 ± 2.76	88.68 ± 3.34

Data are represented as Mean ± SE for 10 mice/group.

Bcl-2 content in the brain of animals treated with 50 and 100 mg/ kg nano-TiO_2 was significantly (P< 0.001) lower than the control group with the increasing the dose of nano-TiO_2. It was (125.10 ± 2.76 and 88.68 ± 3.34 ng/g) against control group (154.53 ± 4.82) (Table 3).

DISCUSSION

Nano-toxicology has only been studied for several decades.At present, studies on the toxicity of nano-TiO2 are still in their infancy [22]. Although TiO2 was thought to be a non-toxic material,several studies have suggested that TiO2 nanoparicles may be toxic to living system [23].

Thus, an attempt was made in this study to further investigate the toxic effects of TiO2 nano-particles at doses 50,100 mg/kg for a period of 21 days on mice brain.

In the view of the present results,nanopaticles administration resulted in significant elevation in brain neurotransmitters dopamine (DA) and norepinephrine (NE) in a dose dependent manner compared to control group indicating that cognitive function of the mice brain may be altered by TiO2 NPs. Neurotransmitters are small and polar molecules and known to play a key role in memory, awareness, thought, and consciousness and allow the organism to become alert and guards against the intensification of reflex reactions and other behavior by mediate signal transmission between neurons and other cells around the synapse [24].

Administration of chemicals disturbs the spontaneous activity of the cells and influences neurotransmitter turnover.Thus the level of neurotransmitters in brain of mice has been evaluated. Nanoparticles led to an increased level of norepinephrine (NE) and dopamine (DA) in cortex region of mouse brain suggesting weakened ability to maintain an appropriate state of activation in the central nervous system due to toxicity [18].They suggest that accumulation of these nanoparticles in brain resulted in altered synthesis and release of certain neurotransmitters and receptors in nerve cells,leading to neural damage. Previous reports also indicate that exposure to TiO2 NPs through various routes lead to enhanced oxidative stress and also alteration of neurotransmitters in brain [25,26]. Moreover,Long et al [13] reported that TiO2 NPs were cause oxidative stress and impair dopaminergic function in brain microglia in vitro. Dysregulation of neurotransmitters levels, and synaptic plasticity may contribute to the neurotoxicity of TiO2 NPs [27].

In this line, Takahashi et al [23] found that significant increase in the amount of DA and DA metabolites were observed in the striatal and prefrontal area of the Tio2-exposed group compared to the control animals.

The data showed that increase the production of MDAcontent which is the mark of lipid peroxidation levels occurred in the brains of the mice treated with TiO2 NPs in a dose dependent manner indicating that these nanoparticles treated mice brains underwent sever oxidative stress. Also, Ze et al [8] showed that the brains from TiO2 NP-treated mice exhibited greater vulnerability to oxidative stress, with significant increases in the generating rate of $O.^{2}$ and H_2O_2, and higher levels of MDA. In accordance with the present results Meena et al [28] postulated the effect of (TiO2 nanoparticles) on the brain of rats administrated intravenously with various doses of nano-TiO2 (21 NM) once a week for 4 weeks showed significant increase of MDA content as compared to control groups.

Oxidative stress due to nano-TiO2 is believed to be atoxic mechanism in brain. Long et al [13] showed that nano-TiO2(25 nm) has the capacity to cause neurological damages by stimulating ROS in BV2 microglia cells even at low concentrations. A similar study conducted by Ma et al [29] on ICR mice had showed that oxidative stress induced by nano-TiO2 played a significant role in induction of brain injuries. Further enhancement in the ROS following nano-TiO2 treatment found to induce acascade of reactions such as lipid peroxidation, DNA damage, and reduction in the activities of key antioxidative enzymes (SOD, GPx, and CAT) involved inthe oxidative defense mechanism.

The current study revealed that the levels of antioxidant enzymes(SOD &GPX) are significantly decreased by administration of TiO2 NPs in mice brain tissue with increase a dose .The results are consistent with other studies in which noted that further enhancement in the ROS following TiO2 treatment found to induce a reduction of key antioxidative enzymes (SOD &GPX) involved in the oxidative defense mechanism [28]. Also, Shrivastava et al [18] stated that the activities of SOD and GPx, were significantly inhibited in the groups exposed to the nanoparticles.Similarly, Ma et al [29] reported decreased activities of antioxidant enzymes in rat brain exposed to TiO2 nanoparticles.These enzymes can decrease the damage produced by ROS throughout can be transformed to other less toxic molecules. Therefore, the reduction in the antioxidative enzymes appears to be due to either mass death of the brain cells or oxidation of the proteins by nano-TiO2-induced ROS.

The entrance of nano-TiO2 into the cell causes a variety of biochemical reactions, including the expression of inflammatory cytokines and the production of large amounts of ROS.

The present result reveled a significant increase the expression levels of inflammatory markers (IL-6 & TNF-α) indicating that TiO2 NPs causes brain inflammation that results in brain injury. In this line, Grissa et al [30] noted that the cerebral IL-6 level showed a statistically dose dependent increase in 100 and 200 mg/kg bw of anatase TiO2 NPs. Moreover, Long et al [13] showed that in Cell culture experiments in murine glial cells, nano-TiO2 may exacerbate inflammation and apoptosis, inhibit the cell cycle and energy metabolism, and cause the production of ROS in the brain.

Another point of view was manged by Song et al [31] that permeability of BBB can be malformed by TiO2 NPs, which could encouragement influx of exogenous substances into the brain. As a result, NPs caused inflammation, edema, and cell damage or even cell death in brain regions. Local or systemic inflammation can change BBB selectivity properties, affecting the transport and inducing physiological and mechanical changes in specialized endothelial cells of BBB comprise enhanced levels of cytokines secretion [32].

TiO2 NPs was confirmed to support an exaggerated neuro inflammatory response via enhancing microglial activation in the pre-inflamed brain of mice [33], that in turn causes release of chemokines and proinflammatory cytokines,producing further neurodegeneration and brain injury [34].The present study illustrated that inflammatory markers (IL-6 & TNF-α) levels were significant increase after exposure to TiO2NPs in a dose dependent manner IL-6 has dual roles in brain injury and disease. It is produced during reactive astroglios is as a response to neuronal damage acting as a neurotrophin promoting neuronal survival, while elevated levels of IL-6 have also been adversely associated with several brain diseases [35,36].

In agreement with the present results Disdier et al [37] noted a raise in expressions of interleukin 6 (IL6) at 28 days after IV administration, the persistent brain inflammation evidenced in this study at the brain micro vasculature level 28days after TiO2 NPs exposure raised the question of potential brain dysfunction.

Meena et al [28] detected the effect of nano-TiO2 administrated intravenously with various doses for 4 weeks on the rats brain and showed increases in the level of TNF-α. Also, park et al [38] mentioned that oxidative stress may activate inflammation signals By the generation of ROS, expressions of inflammation-related genes such as IL-I, IL-6, IL-8 and TNF-a were increased in a concentration-dependent manner. Consistent with other studies, Xue et al [39] reported that TiO2 NPs has been established to promote the secretion of cytokines IL-6 and TNF-α.

By using an in vitro human immune assemble,Schanen et al [40] found that nano-TiO2 lead to increase in the expression of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8,IFN-γ, and TNF-α) by 5–20-fold.

The data obtained allow suggestions that TiO2 NPs can provoke neuro inflammation within the brain by inducing IL-6 and TNF-α production in brain cells.

Mitochondria are the targeted organelle of intracellular nano-TiO2, which reduces the mitochondrial membrane proteins and leads to apoptosis [41].

The mitochondria are not only the major source of intracellular ROS generation, but also an important target for the damaging effects of ROS. Excessive ROS production can increase the permeability of mitochondrial membrane, decrease the MMP, and contribute to the cytochrome c release from the mitochondria into the cytoplasm and then activate downstream caspases, such as caspase-3 and eventually result in apoptosis [42]. Furthermore, the mitochondria-mediated apoptotic pathway is controlled by members of the Bcl-2 family consisting of pro-apoptotic (such as Bax) and anti-apoptotic members (such as Bcl-2), which play acentral regulatory role to decide the fate of the cells [43]. In a previous study, Long et al [44] showed that TiO2 particles can bind to the mitochondrial membrane, causing the disintegration of the mitochondrial membrane electrontransport chain and the generation of additional O2•-.

In the view of the current study detected that decline of Bcl-2 content in the brain of mice after demonstrating to TiO2 NPs for 21successive days in a dose dependent manner. Consistent with the present findings Hu et al [45] and Meena et al [28] also shown TiO2 NPs mediate apoptosis in the hippocampus in mice through the induction of ROS and inhibition of Bcl-2 expressionin hippocampus of mice.

Ze et al [15] had also showed the induction of apoptosis in nano-TiO2-treated primary cultured hippocampal neurons by altering the Bax/Bcl-2 ratio.

The present study confirms that interaction of absorbed nanoparticles with cellular components could generate ROS and lead to cellular toxicity if the magnitude of ROS production overwhelms the antioxidant defense status of the cell.

In this regard,it is important to point out that all the cellular alterations induced by TiO2 NPs observed in this work,including disruption the metabolism of neurotransmitters, lipid peroxidation, decrease of antioxidant enzymes,increase of proinflammatory markers and downstream of antiapoptic mediator, ultimately leading to brain damage and thereby indicating the neurotoxic potential of these NPs.

CONCLUSION

In conclusion, these findings imply that the exposure of brain cells to TiO2 NPs as food and drug additives could be harmful to health by inducing neuroinflammation and brain injury.

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