Effect of Titanium Dioxide Nanoparticles on the Stereological Parameters of the Dentate Gyrus and the Morphology of Granular Hippocampal Neurons in Mice

Efecto de las Nanopartículas de Dióxido de Titanio sobre los Parámetros Estereológicos del Giro Dentado y Morfología de las Neuronas Granulares del Hipocampo en Ratones

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RAHNAMA, S.; HASSANPOUR, A.; YADEGARI, M.; ANVARI, M.; HOSSEINI-SHARIFABAD, M. Effect of titanium dioxide nanoparticles on the stereological parameters of the dentate gyrus and the morphology of granular hippocampal neurons in mice. Int. J. Morphol., 38(6):1623-1630, 2020.

SUMMARY: This study aims to investigate the Effects of Titanium dioxide nanoparticles (TiO2 NPs) on the stereological parameters in the dentate gyrus and the morphology of granular hippocampal neurons in adult mice. Adult male mice (n=20, weight average: 45 g) were randomly divided into four groups including: group receiving saline (controls), low-dose (LD) 2.5 mg/kg TiO2 NPs, medium-dose (MD) 5 mg/kg TiO2 NPs and high-dose (HD) 10 mg/kg TiO2 NPs, daily using gavage for 35 days. To estimate the volume of the hippocampus, dentate gyrus, and sub-layers of dentate gyrus the Cavalieri principle was used. The physical dissector was used to determine the numerical density of dentate gyrus granular cells. For analyzing the morphology of dentate gyrus granular cells the qualitative Golgi staining was used. Our data showed that the total volume of the hippocampus, dentate gyrus and its sublayers including molecular, granular and polymorph in TiO2 treated mice decreased significantly compared to the control group. Moreover, the total number and numerical density of dentate gyrus granular sub layer cells showed a significant reduction in all three experimental groups compared to the control group. The granular cells of the dentate gyrus had shorter dendritic length and decreased dendritic branches in the TiO2-treated in comparison with the control mice. These data can justify the disorders related to memory, learning and hippocampus neurons damages due to using of TiO2 NPs.

KEY WORDS: TiO2; Stereology; Hippocampus; Dentate gyrus.

INTRODUCTION

Titanium dioxide nanoparticles (TiO2 NPs) have been widely used, including the cosmetics industry, medicine, food additives, paint industry, confectionary products, agriculture, weather purification, etc. (Powell et al., 2010; Skocaj et al., 2011; Weir et al., 2012). However, many studies have investigated the disadvantages of TiO2 NPs. Several reports showed the negative effects of TiO2 NPs on the mice's brain and hippocampus. It has been proven that the exposure of pregnancy/lactating mice to nano-TiO2 lead to thinning of cerebral and cerebellar cortex, and pyramidal cell layer in hippocampus, decrease in number of neurons per unit area of cerebrum, dysplasia of neurites in hippocampal pyramidal cells, and decrease in learning and memory of mice offspring (Ze et al., 2014; Mohammadipour et al., 2016; Hong et al., 2018). Also, investigation of the hippocampus in mice offspring that exposed to TiO2 NPs during pregnancy showed a decrease of dendritic length in hippocampal CA1 neurons and TiO2 NPS accumulation in neurons of hippocampus neurons, also caused apoptosis, severe autophagy and ROS (reactive oxygen species) production (Zhou et al., 2017). Another study showed similar data in which TiO2 NPs can penetrate the blood-brain barrier and accumulate in the cortex of the brain and the hippocampus, causing activation of microglia neuroinflammation and signaling pathway of brain inflammation, ROS generation, toxicity and cell death in vitro and in vivo (Czajka et al., 2015; Song et al., 2015; Hong et al., 2017). Moreover, studies demonstrated that TiO2 NPs caused apoptosis in the hippocampus and impaired the spatial recognition memory ability (Hu et al., 2010; Bideskan et al., 2017; He et al., 2018).

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Despite several studies that examine the adverse effects of TiO$_2$ NPs on the structure of the hippocampus qualitatively, there are a few studies that quantitatively examine its effects on the structure of the hippocampus. So, this study aimed to provide estimates of the total number of granular neurons, the volume of the hippocampus, dentate gyrus and sublayers of dentate gyrus and morphology of granular neurons of the dentate gyrus influenced by TiO$_2$ NPs exposure in mice by stereological methods.

**MATERIAL AND METHOD**

**Animal and treatment.** In this experimental study, Twenty adult male mice were randomly assigned from the animal house of the institute of reproductive sciences that age average 10-12 weeks and weight average 45 g were used. Animals were maintained in the same cages under standard conditions, 12 h light/dark cycle and at temperature 23 °C ± 2 °C and humidity of 55 % ± 5 %. Water and food were easy to access ad libitum. The mice were randomly divided into four groups including a control group and three experimental groups were treated with TiO$_2$ NPs as a suspension via gavage for 35 days and then dehydrated in 50 %, 70 %, 80 % and 96 % ethanol for 5 min and mounted in Entellan (Merck, Germany) (Cardiff et al., 2014).

**Stereolementary procedures.** For animal anesthesia, ketamine (Merk, Germany), phosphate-buffered solution of 10 % formaldehyde were used for perfusion and were fixed in 1 % glutaraldehyde. After perfusion, the brains were removed carefully from the skull by craniotomy and each brain was separated from the midsagittal line and divided into hemispheres. For estimating the volume of the hippocampus, dentate gyrus and sublayers of the dentate gyrus and the numerical density of dentate gyrus granular cells, one hemisphere was chosen accidentally, and the other was utilized for morphological analysis of dentate gyrus granular neurons. After the samples were embedded in paraffin, the serial coronal sections (5 μm for estimating the volume, 30 mm for the number of neurons and 50 μm for the morphology of neurons) were sliced from the full thickness of the hippocampus using a rotatory microtome (litez Germany). From the tissue sections cut, 12 pair sections were systematically and uniformly sampled with a random start in the first 80 sections. For estimating the number of neurons and the volume, the slide-mounted sampled sections were stained with routine hematoxylin and eosin (Sigma, USA). For estimating the morphology of neurons in the granular layer of dentate gyrus, the tissue sections were stained with silver nitrate staining (Merck, Germany).

**Hematoxylin and Eosin staining.** Before staining, the slide-mounted sampled sections were placed in two xylene containers each one for 10 min to deparaffinized. In the following, rehydration in 96 %, 80 %, 70 % and 50 % ethanol each one for 2 min and distilled water for 1 min. Subsequently, the sections were stained in hematoxylin solution for 5 min and then rinsed quickly in running water. For decolorization, 75 % ethyl alcohol was used for a few seconds. The sections were then stained in eosin solution for 2 min and then dehydrated in 50 %, 70 %, 80 % and 96 % ethanol for 5 min and mounted in Entellan (Merck, Germany) (Cardiff et al., 2014).

**Estimation of tissue volume.** For volumetric analysis of the hippocampus, dentate gyrus and sublayers of the dentate gyrus, the photography was performed under the 5× objective lens (Olympus, Japan), and total magnification was 50×. The volume of the hippocampus, dentate gyrus, and sublayers of the dentate gyrus were estimated by using Cavalieri’s principle method (Sarbishegi et al., 2016; Dortaj et al., 2018). Cavalieri’s Principle (Gundersen et al., 1988) is a method for determining the reference volumes of the hippocampal, dentate gyrus and sublayers of dentate gyrus by using the point-counting grid. A transparent grid was randomly placed over the sublayers of each sampled section. Points that hit each layer of the dentate gyrus were counted. The reference volume was calculated by the following formula:

\[
Nv = \sum Q/N (dis) \times V (dis)
\]

Where \( \Sigma pi \) is the total number of grid points appeared in sections, \( A (pi) \) is the area associated with each grid point, \( t \) is the known distance between sections and \( M2 \) is the magnification.

**Estimation of cell density.** The granular cell density in the granular layer of the dentate gyrus was determined via the physical dissector method (Miki et al., 2005; Hadizade Asar et al., 2016) by the following formula:

\[
Nv = \sum Q/N (dis) \times V (dis)
\]
Where $\sum Q$ is the number of granular cells counted (nucleolus as counting units) in each dissector frame of sampled sections, $N_{\text{dis}}$ is the sum of all counted dissector frames, $V_{\text{dis}}$ is the volume of dissector frame: $V_{\text{dis}} = \text{A(frame)} \times h$.

Where A (frame) is the known area associated with each dissector frame and h is the height of section and was equal to the section thickness. For the estimation of the density of granular cells of the dentate gyrus, two serial sections were observed with a 40× objective lens and total magnification was 400×. If the nucleolus of a cell was located entirely inside the counting frame or contact with the inclusion lines of the one section (reference section) were counted, whereas those in touch with the exclusion lines of the adjacent serial section (look-up section) were excluded (Fig. 1). The total number of pyramidal cells was estimated by multiplying the measures of the reference volume $V_{\text{ref}}$ of the sublayers by the measures of the numerical density, $N_v$.

**Statistical analysis.** Data were expressed as mean±standard deviation (SD). Statistical comparisons were performed using one-way ANOVA followed by Tukey post hoc test. All statistical analysis was carried out using graph pad prism software, version 19. Differences were defined as $p<0.05$.

**RESULTS**

Data showed TiO$_2$-treated mice had significantly thinner volumes than controls in the total volume of the hippocampus, dentate gyrus and subfields of the dentate gyrus (Figs. 2 to 6 and 9). A comparison of the five groups showed a decrease in both total number (Fig. 8) and the numerical density of granular cells of the dentate gyrus of the hippocampus in experimental groups compared with controls (Fig. 7). The results of the qualitative analysis of granular cells of dentate gyrus also demonstrated that in these neurons, the dendritic length was shorter in the TiO$_2$-treated in comparison with the control mice (Fig. 10).

**Total volume of the hippocampus in mice.** Stereological investigation indicated that the total volume of the hippocampus in TiO$_2$-treated groups was decreased compared with the control group. The Low dose group was decreased compared to the control group with $P<0.05$, mediate dose group was decreased compared to control group with $P<0.001$, high dose group was decreased compared with the control group ($P<0.001$) (Fig. 2).

**Total volume of the dentate gyrus in mice.** Stereological investigation indicated that the volume of the dentate gyrus in TiO$_2$-treated groups was decreased compared with the control group. The mediate dose group ($P<0.01$) was decreased compared to the control group. High dose group $P<0.001$ was decreased compared with the control group (Fig. 3).

**Volume of the molecular layer of the dentate gyrus.** Stereological investigation indicated that the volume of the dentate gyrus in TiO$_2$-treated groups was decreased compared with the control group. The mediate dose group ($P<0.05$) was decreased compared to the control group. High dose group was $P<0.001$ decreased compared with the control group (Fig. 4).
Fig. 2. Bar graphs showing the effect of TiO2 NPs on the total volume of the hippocampus. # Low dose group compared to the control group with P<0.05. &&& mediate dose group compared to the control group with P<0.001. $$ high dose group compared to the control group with P<0.001.

Volume of the granular layer of the dentate gyrus.
Stereological investigation indicated that the volume of the granular layer of the dentate gyrus in TiO2-treated groups was decreased compared with the control group. The mediate dose group (P<0.01) was decreased compared to the control group. High dose group was (P<0.001) decreased compared with the control group (Fig. 5).

Fig. 3. Bar graphs showing the effect of TiO2 NPs on the volume of the dentate gyrus. && mediate dose group compared to the control group with P<0.01. $$ high dose group compared to the control group with P<0.001.

Volume of polymorph layer of the dentate gyrus.
Stereological investigation indicated that the volume of the polymorph layer of the dentate gyrus in TiO2-treated groups was decreased compared with the control group. The low dose group (P<0.001) was decreased compared with the control group. The mediate dose group (P<0.001) were decreased compared to the control group. High dose group was (P<0.001) decreased compared with the control group (Fig. 6).

Fig. 4. Bar graphs showing the effects of TiO2 NPs on the volume of the molecular layer. & mediate dose group compared to the control group with P<0.05. $$ high dose group compared to the control group with P<0.001.

Fig. 5. Bar graph showing the effect of TiO2 NPs on the granular layer. && mediate dose group compared to the control group with P<0.001. $$ high dose group compared to the control group with P<0.01.

Fig. 6. Bar graph showing the effects of TiO2 NPs on the polymorph. ### Low dose group compared to the control group with P<0.001. &&& mediate dose group compared to the control group with P<0.001. $$ high dose group compared to the control group with P<0.001.
Numerical density of dentate gyrus granular cells. The results showed that TiO\textsubscript{2} affected the hippocampus and decreased the number of granular cells. The Low dose group (P<0.001), mediate dose group (P<0.001) and high dose group (P<0.001) were decreased compared with the control group (Fig. 7).

Total neuron of the granular layer of the dentate gyrus. The results showed that TiO\textsubscript{2} affected the hippocampus and decreased the total neuron of the granular layer of the dentate gyrus. The low dose group (P<0.001), mediate dose group (P<0.001) and high dose group (P<0.001) was decreased compared with the control group (Fig. 8).

Fig. 7. Bar graphs showing the effect of TiO\textsubscript{2} NPs on the numerical density of dentate gyrus granular cells. ### Low dose group compared to the control group with P<0.001. &&& mediate dose group compared to the control group with P<0.001. $$$ high dose group compared to the control group with P<0.001.

Fig. 8. Bar graphs showing the effect of TiO\textsubscript{2} NPs on the numerical density of dentate gyrus granular cells. ### Low dose group compared to the control group with P<0.001. &&& mediate dose group compared to the control group with P<0.001. $$$ high dose group compared to the control group with P<0.001.

Fig. 9. Representative photomicrographs of a hematoxylin-eosin stained that showing the total volume of the hippocampus, dentate gyrus and sublayers of dentate gyrus decreased in experimental groups compared to the control group. A. Control group; B. Sham group; C. Low dose group; D. Mediate group; E. High dose group. ML. Molecular layer; GL. Granular layer; PL. Polymorph layer. H & E staining, 50×.
In the current study, the effects of titanium dioxide nanoparticles on the stereological parameters of the dentate gyrus and the morphology of granular hippocampal neurons in mice were evaluated. In this study 2/5, 5 and 10 mg/kg TiO$_2$ NPs (5 nm) suspensions were given to the mice every day for 35 days. The results of this study by using the stereological methods were: administration of TiO$_2$ NPs reduced total hippocampal volume, dentate gyrus and dentate gyrus sublayers in experimental groups compared to the control group. Also, Golgi staining demonstrated that dendritic length and dendritic branching number of granular cells of the dentate gyrus in the nano-TiO$_2$ exposed groups with increasing dose were decreased. Also, Golgi-stained dentate gyrus granular neurons revealed that dendritic length and dendritic branching number with increasing dose were decreased in TiO$_2$-treated groups compared with control. The results of our study have corresponded with the results of a study conducted by Hong et al. (2018). They suggested that exposure to TiO$_2$ NPs has led to thinning of the cerebral cortex and cerebellar cortex, decreasing the number of neurons of the brain, nerve dysplasia in hippocampal pyramidal cells, decreasing the layer of hippocampal pyramidal cells and decreasing learning and memory in mice (Hong et al., 2018). In the study of Hong et al. (2018), the number of cells in the hippocampal pyramidal layer was studied but in the present study, the number of granular cells of the hippocampal dentate gyrus was investigated.

Reduction in the volume of the molecular layer of the dentate gyrus in experimental groups may be the result of shortening of the neuronal fractions in this layer, because the molecular layer is composed of neuronal fractures.

Zhou et al. (2017) reported exposure to TiO$_2$ NPs due to the absence of axonal outgrowth and decreased dendritic filament length, dendritic branching number, and dendritic spine density in mice hippocampal pyramidal neurons (Hu et al., 2010). This study confirms this.

Reduction in the volume of the granular layer of dentate gyrus may be due to the decrease in the number of neurons in this layer. Based on previous studies exposure to TiO$_2$ NPs can cause nerve dysplasia in hippocampal pyramidal cells and decrease the layer of hippocampal pyramidal cells (Hong et al., 2018). Reduction in the volume of the polymorph layer of dentate gyrus can be attributed to the decrease in proliferating neurons in this layer. Consistent with our results, it was described by Mohammadipour et al. (2014) that TiO$_2$-administrated mice showed TiO$_2$ NPs have a negative effect on neurogenesis in the mice offspring hippocampus. Shortening of dendritic length and reduction of dendritic branching number of granular neurons of mice hippocampal dentate gyrus are probably due to apoptosis, ROS production, and oxidative stress. According to previous studies, TiO$_2$ NPs due to shortening of the dendritic length of mice hippocampal pyramidal neurons (Zhou et al., 2017). Also, TiO$_2$ NPs due to the absence of axonal outgrowth, and decreased dendritic filament length, dendritic branching number, and dendritic spine density in mice hippocampal pyramidal neurons (Zhou et al., 2019). This may be the reason for the decrease in Neuronal consequences in our study.

Fig.10. photomontages of Golgi-impregnated dentate gyrus granular cells. Observe the decrease in dendritic length in TiO$_2$-treated mice compared to controls. Golgi staining, 100×.

DISCUSSION

In the current study, the effects of titanium dioxide nanoparticles on the stereological parameters of the dentate gyrus and the morphology of granular hippocampal neurons in mice were evaluated. In this study 2/5, 5 and 10 mg/kg TiO$_2$ NPs (5 nm) suspensions were given to the mice every day for 35 days. The results of this study by using the stereological methods were: administration of TiO$_2$ NPs reduced total hippocampal volume, dentate gyrus and dentate gyrus sublayers in experimental groups compared to the control group. Also, the results of this study indicated that the numerical density and the total number of granular neurons in the granular layer of hippocampal dentate gyrus decreased in the three experimental groups compared to the control group. Also, Golgi staining demonstrated that dendritic length and dendritic branching number of granular cells of the dentate gyrus in the nano-TiO$_2$ exposed groups with increasing dose were decreased. Also, Golgi-stained dentate gyrus granular neurons revealed that dendritic length and dendritic branching number with increasing dose were decreased in TiO$_2$-treated groups compared with control. The results of our study have corresponded with the results of a study conducted by Hong et al. (2018). They suggested that exposure to TiO$_2$ NPs has led to thinning of the cerebral cortex and cerebellar cortex, decreasing the number of neurons of the brain, nerve dysplasia in hippocampal pyramidal cells, decreasing the layer of hippocampal pyramidal cells and decreasing learning and memory in mice (Hong et al., 2018). In the study of Hong et al. (2018), the number of cells in the hippocampal pyramidal layer was studied but in the present study, the number of granular cells of the hippocampal dentate gyrus was investigated.

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Based on previous studies, exposure to TiO$_2$ NPs can cause inflammation in microglia, ROS production and activation of signaling pathways involved in brain inflammation (Czajka et al.). Also, these nanoparticles can be accumulated in the brain, causing oxidative stress and overproduction of glial cells, tissue necrosis, and apoptosis in the hippocampus (Ze et al., 2013). Furthermore, Renping Hu et al. (2011) investigated the molecular mechanism of apoptosis in the hippocampus of mice exposed to TiO$_2$ NPs for 60 days orally and reported that these nanoparticles induced apoptosis in the hippocampus and can impair spatial memory recognition. A previous study reported that exposure to TiO$_2$ NPs resulted in the upregulation of Cyt C, caspase-3, and cJun expression in neuronal apoptosis and a decrease in neuronal dendrite length in offspring mice (Zhou et al., 2019).

CONCLUSION

We have demonstrated that exposure to TiO$_2$ NPs has significant effects on the total volume of total hippocampal volume, dentate gyrus and dentate gyrus sublayers and the granular cell numbers in the dentate gyrus. This data can justify the disorders related to memory, learning and hippocampus neuron damage due to the use of TiO$_2$ NPs.

ETHICS APPROVAL. All experiments were conducted according to relevant guidelines and were approved by the Ethics Committee of the Shahid Sadoughi University of Medical Sciences (Registration number: IR.SSU. MEDICINE.REC.1398.327).

ACKNOWLEDGMENTS. This article was extracted from a thesis written by Sharare Rahnama, MSc. candidate of Anatomy and financially supported by Shahid Sadoughi University of Medical Sciences and Health Services, Yazd, Iran. We would like to thank the Department of Anatomy and Cell Biology for their official cooperation.

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RESUMEN: En este estudio se analizaron los efectos de las nanopartículas de dióxido de titanio (TiO$_2$ NP) sobre los parámetros estereológicos en el giro dentado y la morfología de las neuronas granulares del hipocampo en ratones adultos. Se divieron aleatoriamente ratones machos adultos (n = 20, promedio de peso: 45 g) en cuatro grupos: grupo que recibió solución salina (controles), dosis baja (LD) 2,5 mg/kg NP de TiO$_2$, dosis media (MD) 5 mg/kg de NP de TiO$_2$ y dosis altas (HD) de 10 mg/kg de NP de TiO$_2$, por vía utilizando sonda durante 35 días. Para estimar el volumen del hipocampo, el giro dentado y las subcapas del giro dentado se utilizó el principio de Cavalieri. Se utilizó el disector físico para determinar la densidad numérica de las células granulares del giro dentado. Para analizar la morfología de las células granulares del giro dentado se usó la tinción cualitativa de Golgi. Nuestros datos mostraron que el volumen total del hipocampo, el giro dentado y sus subcapas, incluyendo la molecular, granular y polimorfas, en ratones tratados con TiO$_2$ disminuyó significativamente en comparación con el grupo de control. Además, el número total y la densidad numérica de las células de la subcapa granular del giro dentado mostró una reducción significativa en los tres grupos experimentales en comparación con el grupo control. Las células granulares del giro dentado tenían una longitud dendrítica menor y ramas dendríticas disminuidas en los ratones tratados con TiO$_2$, en comparación con los ratones del grupo control. Estos datos pueden justificar los trastornos relacionados con la memoria, el aprendizaje y los daños en las neuronas del hipocampo debido al uso de NP de TiO$_2$.

PALABRAS CLAVE: TiO$_2$ Estereología; Hipocampo; Giro dentado.
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Received: 07-03-2020
Accepted: 23-07-2020