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Title: Investigation of the Iron Oxide Nanoparticle Effects on Amyloid Precursor Protein Processing in Hippocampal Cells

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To appear in: Basic and Clinical Neuroscience

Received date: 2019/08/24

Revised date: 2020/10/4

Accepted date: 2020/10/10

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Please cite this article as:

Sadeghi, L., & Marefat, A. (In Press). Investigation of the Iron Oxide Nanoparticle Effects on Amyloid Precursor Protein Processing in Hippocampal Cells. *Basic and Clinical Neuroscience*. Just Accepted publication Aug. 15, 2020. Doi: <http://dx.doi.org/10.32598/bcn.2021.2005.1>

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Abstract

Introduction: Iron oxide nanoparticles (Fe_2O_3 -NPs) are small magnetic particles that widely used in different aspects of biology and medicine in modern life. Fe_2O_3 -NP accumulated in the living cells due to absence of active system to excrete the iron ions so damages cellular organelles by highly reactivity.

Method: Herein cytotoxic effects of Fe_2O_3 -NP with 50 nm size were investigated on primary culture of neonatal rat hippocampus by MTT assay. Pathophysiological signs of Alzheimer disease such as amyloid precursor protein (APP) expression, $\text{A}\beta$ aggregation, soluble $\text{APP}\alpha$ and $\text{APP}\beta$ secretion also were investigated in hippocampal cells treated by various concentration of NP for different exposure time.

Results: Our results revealed, Fe_2O_3 -NP treatment causes oxidative stress in cells that accompanied by upregulation of the APP and $\text{A}\beta$ in a concentration dependent manner. NP exposing also leads to more secretion of $\text{sAPP}\beta$ rather than $\text{sAPP}\alpha$ that concluded to increased activation of β -secretase in NP received cells. All of the harmful effects accumulate in neurons that could not be renovated so lead to neurodegeneration in Alzheimer disease.

Conclusion: This study approved iron-based NPs could help to develop the Alzheimer and related neurological disorders and explained why some of the iron chelators have therapeutic potential in Alzheimer disease.

Keywords: $\text{A}\beta$ deposition, Neurodegeneration, Oxidative stress, Hippocampal cells, Amyloid precursor protein

Introduction

According to World Health Organization (WHO) report, brain disorders constitute 12 % of total deaths in worldwide (WHO, 2006). Brain pathologies and dementia are strictly related to people aging (Dolan et al., 1997) and 24.3 million people suffer from dementia today, with 4.6 million new cases annually (WHO, 2006). The most common type of dementia is Alzheimer disease (AD) that characterized by chronic neurodegeneration, impairment of memory and disturbed planning, language, and thinking ability (Dolan et al., 1997). Modern life could be making AD due to stressor condition, poor eating habits and widespread application of cytotoxic agents such as nanomaterials (Cai et al., 2012). In recent years, the use of nanoparticles (NPs) (particles with less than 100 nm size in at least one dimension) has been developed in all aspects of life (Sun, Lee, & Zhang, 2008). Iron, cobalt and nickel-fabricated NPs have specific features such as stability, magnetic and high surface area to volume ratio (Lu, Salabas, & Schuth, 2007). These NPs especially iron oxide nanoparticle (Fe_2O_3 -NP) play important role in biological sciences, medicine, Magnetic Resonance Imaging (MRI), ultrasound, optical imaging, and X-ray imaging as main molecular detection methods that exposed human to Fe_2O_3 -NP (Indira & Lakshmi, 2010). By considering general application and high reactivity of iron, there are growing concerns about the Fe_2O_3 -NP application harms especially in central nervous system (CNS). Our previous study confirmed Fe_2O_3 -NP with 50 nm size creates harsh oxidative damages and decreases cell viability in Hep G2 cell line (Sadeghi, Tanwir, & Yousefi Babadi, 2015). While nervous system is more susceptible against oxidative stress in comparison with other tissues due to more consumption of oxygen and weak antioxidant barrier (Salim, 2017). This study aimed to investigate Fe_2O_3 -NP cytotoxic effects on primary culture of hippocampus as one of the main tissues damaged in AD. By considering high reactivity and high surface area to volume ratio of Fe_2O_3 -NP, the main harmful effect is

oxidative stress that could be assessed by standard biochemical methods (Sadeghi et al., 2015). By considering the pathophysiological signs of AD (Habib, Sawmiller, & Tan, 2017), activity of beta-site APP cleavage enzyme-1 (BACE-1) was compared in hippocampal cells that were cultured in the presence or absence of Fe₂O₃-NP by measurement of secreted soluble amyloid precursor α and β (sAPP α and sAPP β). Immunoblotting method also assessed amyloid precursor protein (APP) expression and intracellular amyloid β (A β) accumulation in hippocampal cells were treated by different concentration of Fe₂O₃-NP. We tried to find a significant accompaniment between oxidative damages, iron nanoparticle accumulation and pathophysiological signs of AD in hippocampal cells.

Material and Methods

Chemicals

Neurobasal Plus Medium were purchased from Thermo Fisher Scientific. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7 dichlorofluorescein diacetate (DCFHDA), o-phthalaldehyde (OPT), and 5,5'-dithiobis (2-nitrobenzoic acid) were obtained from Sigma Chemical Company. Anti-beta amyloid 1-42 antibody (ab10148), rat specific anti-amyloid precursor protein (APP) antibody (ab2072) prepared from Abcam Company. Nano ferric oxide (Fe₂O₃) particles in crystalline phase with 50 nm size prepared from Sigma Aldrich Company.

Cell culture and treatment

Rat hippocampal primary culture was prepared according to previous studies with some modifications (Seibenhener & Wooten, 2012). Neonatal rat brain (embryonic day 19) was isolated; striatal, thalamic, and midbrain was removed to expose hippocampus tissue in medial surface of the brain (Seibenhener & Wooten, 2012). Dissected hippocampus tissue disrupted mechanically

and triturated by trypsin enzyme. Separated hippocampal cells were plated on 24-well plates (5×10^5 cells per well) containing Neurobasal medium and differentiated in Neurobasal medium improved by B27 and FGF2 during 7 days (Brewer, 1995). Prepared cells were treated with 50 and 100 $\mu\text{g/ml}$ concentration of $\text{Fe}_2\text{O}_3\text{-NP}$ for 8 and 16 h according to previous experiments (Naqvi et al., 2010). Before treatment, $\text{Fe}_2\text{O}_3\text{-NP}$ was dispersed by sonication (10 min, 750 W and 20 kHz) in phosphate buffer solution (PBS) and filtered through a 0.2 μm membrane. Exposure of cells was performed with approximately 80 % confluence of cell in 25 cm^2 flasks and 24-well plates in a humidified atmosphere at 37 $^\circ\text{C}$ and 5 % CO_2 . Cells received PBS without $\text{Fe}_2\text{O}_3\text{-NP}$ were considered as control in each experiment.

MTT assay

Cell viability was assessed by using the MTT assay, which evaluated reduction of the MTT dye to formazan crystals, an insoluble intracellular blue product, in living cells. The procedure was done according to our previous work (Sadeghi et al., 2015).

Oxidative stress assessment

Reactive Oxygen Species (ROS) concentration measurement

The concentrations of ROS inside the hippocampal cells were determined by calculating the oxidative conversion of DCFH-DA to dichlorofluorescein (DCFH) which is fluorescent compound (Wan, Myung, & Lau, 1993). Briefly, primary hippocampal cells were cultured in 24-well plates, then treated with 50 and 100 $\mu\text{g/ml}$ of $\text{Fe}_2\text{O}_3\text{-NP}$ for 8 and 16 h. Treated and control cells incubated with DCF diacetate in culture medium for 15 min and washed with cold PBS three times. Oxidized DCFH resulted green fluorescence that measured by using a microplate fluorometer (LB 941, Berthold Technologies, Bad Wildbad, Germany) (excitation and emission were done in 488 and

530 nm respectively). The cell-free wells containing Fe₂O₃-NP in PBS and DCFH were used to assess nonspecific particle-induced fluorescence. The total protein concentration was evaluated using standard Bradford method (Bradford, 1976). Fluorescence was reported as percentage in comparison with untreated control cells.

Intracellular reduced glutathione (GSH) evaluation

Cellular levels of GSH were measured according to previous method (Hissin & Hilf, 1976). This method evaluates GSH content of cells based on a reaction between GSH and o-phthaldialdehyde (OPT) which produces fluorescent agent. Primary culture of hippocampus was exposed to 50 and 100 µg/ml for 8 and 16 h. After incubation the cells were centrifuged at 5000 rpm for 5 min and washed in PBS. The cells were homogenized in 200 µL of phosphate-EDTA buffer pH 8.0 and 80 µL of 20 % metaphosphoric acid. The cell homogenate was centrifuged at 16000 rpm at 4 °C for 30 min and supernatant used to GSH evaluation in the presence of 100 µL OPT (10 mg/ml). Fluorescence was measured at 360 nm excitation and 420 nm emission using Bio-Tek Synergy HT-I plate reader (LB 941, Berthold Technologies, Bad Wildbad, Germany). Results were calculated as µmol of reduced glutathione per mg of protein and presented as a percentage of the control group. Protein concentration assessed using Bradford method.

Measurement of secreted sAPP α and sAPP β

Following the exposure of hippocampal cells to 50 and 100 µg/ml, the amount of APP cleavage products, soluble amyloid precursor protein α and β (sAPP α and sAPP β) level, which released into the culture medium were determined using commercially available solid phase sandwich ELISA (IBL 27419) according to the manufacturer's protocol. Concentration of secreted sAPP α and sAPP β in the culture medium was evaluated according to a standard curve based on recombinant

sAPP α , and sAPP β , respectively, and data was expressed as ng/ml. The protein content was determined by Bradford method in each sample (Bradford, 1976) that was used as a control to minimize the standard error of the data between experimental repeats.

Immunoblotting analysis

Hippocampal cells were treated with 50 and 100 μ g/ml of NP for 8 and 16 h. After exposure times, cells were harvested by centrifugation (700 g, 5 min) and intracellular amyloid precursor protein (APP) and A β were measured by western blotting method. Cell lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Sadeghi et al., 2017). After separation, proteins were transferred to polyvinylidene difluoride (PVDF) membrane by semi-dry electrophoretic transfer at 20 V for 60 min using the semi-dry transfer system. Blocking buffer containing 0.1% tween-20 in Tris-buffered saline (TBS), pH 6, 4% fat free milk were used to block PVDF membrane at room temperature for 1 h then membrane was incubated with the primary antibody (dilution 1:1000) in TBS/Tween20 (TBST) containing 5% bovine serum albumin overnight in 4 °C, and then incubated with the secondary antibody (dilution 1:1000) at room temperature for 1 h. Immunoreactive bands were detected by an chemiluminescence detection kit (Millipore Corporation, Billerica, MA, USA) according to the manufacturer's guideline. β -actin was used as control for the total protein concentration in each well.

Statistical evaluation

Results are representative of at least three independent experiments with a minimum of two technical replicates per experiment. Data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis by one-way analysis of variance (ANOVA) followed by Dunnett post-test was

used to compare 8 h and 16 h treated rats rather than control. A value of $P < 0.0001$ was considered significant.

Results

Fe₂O₃-NP significantly reduced hippocampal cell viability

Hippocampal cell culture was treated by 50 and 100 $\mu\text{g/ml}$ of Fe₂O₃-NP and analyzed by inverted microscope during the experiment. Fig. 1 showed degeneration and abnormal morphology of neurons in the presence of NP. Treatment by 100 $\mu\text{g/ml}$ of Fe₂O₃-NP has more toxic effects and reduced normal cell number in the culture medium. The majority of cells were deformed by consisting the condensed cell body in the presence of NP. Short dendrites and axons also could be observed in NP treated cells specially in cells that received 100 $\mu\text{g/ml}$. Some cell debris was also visible in 100 $\mu\text{g/ml}$ dose containing medium. According to Fig. 1, rounded and shrieked cell body, abnormal arbors and short dendrites are hallmarks of Fe₂O₃-NP toxicity specially in high dose. Cytotoxic effect of Fe₂O₃-NP on the hippocampal cell culture was also evaluated by MTT assay. Exposure of cells to NP significantly reduced cell viability in a concentration dependent manner. Cell death increased up to 37.75 % and 56.5 % in the presence of 50 and 100 $\mu\text{g/ml}$ Fe₂O₃-NP respectively in 8 h exposure time in comparison with control ($F(2,9) = 283.1$, $P < 0.0001$, one-way ANOVA, control vs. 8 h treated cells, Dunnett post-test, * $P < 0.0001$). Viability of cells that grown in the presence of NPs at more time duration (16 h) estimated as follow: cells that received 50 $\mu\text{g/ml}$ showed 48.15 % viability and cells were treated with 100 $\mu\text{g/ml}$ revealed 32.94 % viability that are significantly different in comparison with control ($F(2,9) = 543.5$, $P < 0.0001$, one-way ANOVA, control vs. 16 h treated cells, Dunnett post-test, # $P < 0.0001$).

Fe₂O₃-NP caused acute oxidative stress in hippocampal cells

ROS content of cells increased in the presence of Fe₂O₃-NP

ROS are chemically reactive molecules containing oxygen such as oxygen ions, super oxide and peroxides that present in the cells with very low concentration but harmful stress can increase it so damage to the cellular organelles (Cabiscol, Tamarit, & Ros, 2000). Therefore, measurement of the ROS will give useful information about the oxidative condition of the cells (Wang et al., 2014). The concentration of intracellular ROS generation induced by Fe₂O₃-NP was measured by DCF fluorescence intensity in cells. As shown in Fig. 2A, a concentration dependent increase of ROS generation was observed after 8 h exposure to 50 and 100 µg/ml of Fe₂O₃-NP. 50 µg/ml imposed 1.7 folds and 100 µg/ml induced 3.2 folds increase in DCF fluorescence that are significantly more than control ($F(2,9)=4006$, $P<0.0001$, one-Way ANOVA, control vs. 8 h treated cells, Dunnett post-test, * $P<0.0001$, Fig. 2A). 16 h exposure to same concentrations of NP imposed more production of reactive molecules in the hippocampal cells ($F(2,9)=7103$, $P<0.0001$, one-Way ANOVA, control vs. 16 h treated cells, Dunnett post-test, # $P<0.0001$, Fig. 2A).

Fe₂O₃-NP treatment reduced GSH content significantly

Reduced GSH is a best antioxidant barrier in the biological systems especially in CNS (Miura & Shinohara, 2009). Therefore, comparison of reduced GSH level between the control and treated cells verify the oxidative damage. We assessed GSH content of hippocampal primary culture by standard method. Fig. 2B showed treatment of hippocampal cells by Fe₂O₃-NP decreased reduced GSH content. According to the results, 50 µg/ml of NP exposure during 8 and 16 h decreased GSH content more than 2.5 and 3.4 folds rather than control. While treatment by 100 µg/ml of NP induced GSH decline about 3.5 and 7.7 folds in cells were treated for 8 and 16 h respectively in comparison with cells received saline. Administration of NP in various times and concentrations

causes significant difference in GSH content ($F(2,9)=583.4$, $P<0.0001$, one-way ANOVA, control vs. 8 h treated cells, Dunnett post-test, * $P<0.0001$. $F(2,9)=1139$, $P<0.0001$, one-way ANOVA, control vs. 16 h treated cells, Dunnett post-test, # $P<0.0001$, Fig. 2B).

NPs exposure imposed sAPP β secretion from the hippocampal cells

To understand whether NP toxicity affects the metabolism of APP, primary culture of hippocampus was exposed to Fe₂O₃-NP and released sAPP α and sAPP β assessed by specific ELISA kit. sAPP α and sAPP β are two important products resulted from different secretase enzyme catalytic function on the APP (Chow et al., 2010). Results showed NP treatment causes increased sAPP β level in cell culture medium and reduced level of sAPP α according to exposure time and NP concentration (Fig. 3). 8 h incubation with 50 and 100 $\mu\text{g/ml}$ NP increased sAPP β up to 1.94 ± 0.01 and 2.86 ± 0.15 ng/mg protein respectively which are significantly more than sAPP β content in control culture medium (0.52 ± 0.03 ng/mg protein) ($F(2,9)=909.6$, $P<0.0001$, one-way ANOVA, control vs. 8 h treated cells, Dunnett post-test, * $P<0.0001$, Fig. 3). While 16 h incubation with 50 and 100 $\mu\text{g/ml}$ NP increased sAPP β up to 2.27 ± 0.12 and 3.19 ± 0.17 ng/mg protein respectively which are significantly more than control ($F(2,9)=3721$, $P<0.0001$, one-way ANOVA, control vs. 16 h treated cells, Dunnett post-test, # $P<0.0001$, Fig. 3). Interestingly incubation with NPs reduced extracted sAPP α , time and concentration dependently. According to the results, sAPP α concentration in cells were treated with 50 $\mu\text{g/ml}$ of NP increased up to 0.73 ± 0.04 and 0.41 ± 0.02 ng/mg protein after 8 and 16 h respectively while cells that received 100 $\mu\text{g/ml}$ of NP showed 0.46 ± 0.02 and 0.36 ± 0.02 ng/mg protein after 8 and 16 h respectively, sAPP α content in control cells was 0.82 ± 0.05 ng/mg protein ($F(2,9)=210.6$, $P<0.0001$, one-way ANOVA, control vs. 8 h treated cells, Dunnett post-test, * $P<0.0001$ and $F(2,9)=664.0$, $P<0.0001$, one-way ANOVA, control vs. 16 h treated cells, Dunnett post-test, # $P<0.0001$, Fig. 3). Therefore, amount

of sAPP β /sAPP α ratio increased dramatically in the presence of Fe₂O₃-NP. 16 h incubation in the medium with 100 μ g/ml increased sAPP β /sAPP α ratio near to 14 folds in comparison with control. Fig. 3 revealed sAPP α , sAPP β and sAPP β /sAPP α ratio in different exposure times and NP concentrations.

Immunoblotting analysis confirmed NP causes accumulation of A β and APP in hippocampal cells

After 8 and 16 h incubation time, hippocampal cell lysates were subjected to immunoblotting analysis to evaluate the A β accumulation and APP expression inside the cells. Fig. 4 compared expression of the APP and A β proteins in NP received and control cells. Densitometric evaluation of the blots demonstrated that toxicity imposed by NP gradually increased the level of APP and A β expression with incubation time. Exposure of cells to 50 μ g/ml of NP for 8 and 16 h increased APP expression more than 2.4 and 3 folds respectively rather than control ($F(2,9)=466.3$, $P<0.0001$, one-way ANOVA, control vs. 8 h treated cells, Dunnett post-test, * $P<0.0001$ and $F(2,9)=1395$, $P<0.0001$, one-way ANOVA, control vs. 16 h treated cells, Dunnett post-test, # $P<0.0001$, Fig. 4). While treatment of cells by 100 μ g/ml of toxicant NP during 8 and 16 h enhanced APP expression more than 3.1 and 3.6 folds respectively. In addition, cells that received NP showed increased expression of A β according to NP concentration ($F(2,9)=337.9$, $P<0.0001$, one-way ANOVA, control vs. 8 h treated cells, Dunnett post-test, * $P<0.0001$ and $F(2,9)=311.3$, $P<0.0001$, one-way ANOVA, control vs. 16 h treated cells, Dunnett post-test, # $P<0.0001$ Fig. 4).

Discussion

Iron is one of the essential elements in DNA synthesis, mitochondrial respiration, and oxygen transport in human and animal body but overexposing of the metal causes harsh oxidative stress

in whole body especially immune system and CNS due to ability in giving and receiving of the electron (Patil et al., 2015). Iron-based nanoparticles could pass through cell membrane and penetrate from blood brain barrier (Saraiva et al., 2016). Upon cell absorption, Fe_2O_3 -NP localizes in the acidic medium of lysosomes and metabolizes to free iron ions inside the cells (Malvindi et al., 2014). Iron accumulation causes aseptic inflammation that finally leads to cell degeneration (Kozlowski et al., 2012). By considering AD as most prevalent neurodegenerative disease, this study investigated molecular and pathophysiological effects of iron oxide nanoparticle on hippocampal cells. Hippocampus tissue is one of the affected tissues in AD that widely being under investigation in recent years (Sarnyai et al., 2000). Our results revealed treatment by different concentration of Fe_2O_3 -NP causes harsh cellular damages in hippocampal cells (Fig. 1). NP imposed abnormal shape in hippocampal cells, short dendrites and reduced cell numbers. According to Fig. 1A ROS molecule overproduction inside the cells directly was affected by NP concentration and exposure time, these results are in agreement with our previous experiments on hepatoma-derived Hep G2 cells (Sadeghi et al., 2015). Iron reacts with hydrogen peroxide (H_2O_2) in biological system and produces $\text{OH}^\bullet$ radical that could attack to organic substance (Barbusinski, 2009). Accumulation of iron ions interrupts electron transfer of mitochondria and causes ROS molecule overproduction (Zorov, Juhaszova, & Sollott, 2014) that approved by our results (Fig. 1A). Decrease in GSH content of cells (Fig. 1B) also causes limited cell ability to neutralize ROS molecules (Hissin & Hilf, 1976). Our results showed a close association between oxidative stress and pathological signs of Alzheimer. According to Fig. 4 APP expression increased in NP received cells also. Upregulation of APP can be caused by iron accumulation inside the cells, oxidative damages resulted from NP toxicity or synergism effects of both reasons. Earlier results approved expression of APP increased in oxidative stress induced by H_2O_2 treatment (Muche, Arendt, &

Schliebs, 2017). Previous studies confirmed positive effects of iron chelators in Alzheimer treatment (Liu et al., 2010) that confirmed iron accumulation triggered Alzheimer pathophysiology and neurodegeneration. Our results revealed cleaved APP that secreted to the medium and also deposited A β inside the cells increased significantly in NP received cells according to the NP concentration. Control cells were treated with solvent without NP showed low concentration of sAPP β in cell medium and also deposited A β inside the hippocampal cells. Therefore, in control cells APP was mainly cleaved by α -secretase that lead to small value of sAPP β /sAPP α ratio. But presence of Fe₂O₃-NP activated β -secretase and increased sAPP β , therefore sAPP β /sAPP α ratio increased in NP received cells. Fig. 3 revealed sAPP β /sAPP α ratios directly depend on NP concentration in cell medium that concluded to cumulative effects of Fe₂O₃-NP damages in hippocampal cells. Cell viability test confirmed negative effects of NP on cell viability based on concentration. Our previous results also confirmed Fe₂O₃-NP increased cell death in Hep G2 cells that are in agreement with the results on hippocampal cells (Sadeghi et al., 2015). Hippocampus tissue is very sensitive to oxidative stress such as other tissues in CNS due to higher rate of oxygen consumption and limited antioxidant barrier therefore the main goal of toxicant agents are neuronal cells (Salim, 2017). Accumulation of iron ions inside the cells causes a specific type of cell death called ferroptosis (Dixon et al., 2012) that confirmed by Fig. 1. Our results approved oxidative damages, mitochondrial dysfunction and A β aggregation as a result of Fe₂O₃-NP toxicity that possibly induced ferroptosis in hippocampal cells. Therefore, AD was considered as neurodegenerative disease that is sensitive to oxidative stress (Chen, Guo, & Kong, 2012). Taken together our results revealed a positive feedback-loop between Fe₂O₃-NP accumulation, oxidative stress and A β aggregation. This loop seems as an effective risk factor in developing of the Alzheimer and related neurodegenerative disorders in today's life that extensively exposes human

against nanomaterials. The body has no physiologic mechanism for iron excreting; thus, tissue accumulation of iron rises with age (Kohgo et al., 2008). Based on previous results iron chelators prevent the progress of the mental diseases specially dementia (Liu et al., 2010). By considering all of the damaging agents in today's life and accumulation of harmful effects, Alzheimer considered as an age-related disease. Cytotoxic effects of Fe_2O_3 -NP and its role in AD developing suggest NPs application in medicine and biology should be limited.

Acknowledgements

This research was supported by University of Tabriz.

Funding information

No specific funding has been provided for the research.

This manuscript does not contain in vivo human and animal studies.

Conflict of interest

The authors declare that there is no conflict of interest.

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Figure legends

Fig. 1 Effect of Fe₂O₃-NP on the morphology of hippocampal neurons (scale bars: × 100). Control cells that cultured in absence of nanoparticle showed normal number and shape. Hippocampal cells revealed abnormal morphology (shrieked cell body and short arbors) and degeneration in the presence of nanoparticle. 100 µg/ml of Fe₂O₃-NP decreased normal cell number in the medium.

Fig. 2 Oxidative stress estimation in primary culture of hippocampus in the presence and absence of nanoparticle. (A) Increasing of the DCF fluorescence in nanoparticle received cells revealed ROS overproduction in the presence of Fe₂O₃-NP (n=4). (B) Reduced glutathione also decreased in the presence of nanoparticle in concentration and exposure time dependent manners (n=4). Each data indicates the mean ± SD. One-way ANOVA, Dunnett post-test, * P<0.0001, control vs. 8 h treated cells; # P<0.0001, control vs. 16 h treated cells.

Fig. 3 Secreted sAPP α and sAPP β evaluated by standard ELISA method in nanoparticle received cells and control. Results showed Fe₂O₃-NP increased released sAPP β in the cell medium that accompanied by reduction of sAPP α content. Increased sAPP β /sAPP α ratio by increasing NP concentration refers to β -secretase activation in NP received cells. Each data indicates the mean ± SD. One-way ANOVA, Dunnett post-test, * P<0.0001, control vs. 8 h treated cells; # P<0.0001, control vs. cells were treated for 16 h.

Fig. 4 Nanoparticle effect on APP and A β contents in hippocampal cells. Western blotting analysis confirmed upregulation of APP in the presence of nanoparticle. Fe₂O₃-NP also increased A β accumulation inside the hippocampal cells. (1) control cells, (2 and 3) 50 and 100 µg/ml nanoparticle received cells for 8 h, (4 and 5) cells received 50 and 100 µg/ml NP for 16 h.

Quantification of bands confirmed upregulation of APP and A β are dependent on NP concentration and exposure time. Each data indicates the mean $\pm$ SD. One-way ANOVA, Dunnett post-test, * P<0.0001, control vs. 8 h treated cells; # P<0.0001, control vs. cells were treated for 16 h.

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Fig. 1

Fig. 2

Fig. 3

Fig. 4