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Research Paper

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

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ABSTRACT

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

Methods: Herein cytotoxic effects of Fe_2O_3-NP with a size of 50 nm on the primary culture of neonatal rat hippocampus were investigated using 2,5-diphenyltetrazolium bromide (MTT) assay. Pathophysiological signs of Alzheimer's disease such as amyloid precursor protein (APP) expression, Aβ aggregation, soluble APPα, and APPβ secretion were also investigated in hippocampal cells treated with various concentrations of nanoparticle (NP) for different exposure times.

Results: Our results revealed that Fe_2O_3-NP treatment causes oxidative stress in cells which is accompanied by upregulation of the APP and Aβ in a concentration-dependent manner. NP exposure also leads to more secretion of sAPPβ rather than sAPPα, leading to increased activation of β-secretase in NP-received cells. All the harmful effects accumulate in neurons that cannot be renovated, leading to neurodegeneration in Alzheimer's disease.

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

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Highlights

- Fe_2O_3-NP induced oxidative stress in hippocampal cells in a concentration dependent manner.
- Fe_2O_3-NP imposed up-regulation of APP in hippocampal cells.
- Fe_2O_3-NP activated β-secretase and elevated sAPPβ/sAPPα ratio.
- Cumulative effects of Fe_2O_3-NP damages increased cell death in neurons.

Plain Language Summary

The most common type of dementia is Alzheimer's disease (AD), which is characterized by chronic neurodegeneration, impairment of memory, and disturbed planning, language, and thinking ability. In recent years, the use of nanoparticles has been increased in all aspects of life. Among these nanoparticles, iron oxide nanoparticles (Fe_2O_3-NP) are vital in biological sciences, medicine, magnetic resonance imaging, ultrasound, and optical imaging. Considering the general application and high reactivity of iron, growing concerns exist about the Fe_2O_3-NP application harms, especially in the central nervous system. Hippocampus tissue is one of the affected tissues in AD, which is widely investigated in recent years. This study aimed to investigate the cytotoxic effects of Fe2O3-NP on the primary culture of the hippocampus as one of the main tissues damaged in patients with AD. Our results revealed that treatment with different concentrations of Fe_2O_3-NP caused cellular damage in hippocampal cells. Exposure to Fe_2O_3-NP also caused oxidative stress. Our results showed a close association between oxidative stress and AD's pathological symptoms. The Fe_2O_3-NP application in medicine and biology should be limited.

1. Introduction

According to World Health Organization (WHO) report, brain disorders constitute 12% of total deaths 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's disease (AD), which is characterized by chronic neurodegeneration, impairment of memory, and disturbed planning, language, and thinking ability (Dolan et al., 1997). Modern life can cause AD due to stressor conditions, poor eating habits, and the 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 et al., 2008). Iron, cobalt, and nickel-fabricated NPs have specific features, such as stability, magnetic, and high surface area to volume ratio (Lu et al., 2007). These NPs, especially iron oxide nanoparticles (Fe_2O_3-NP) are vital in biological sciences, medicine, magnetic resonance imaging (MRI), ultrasound, optical imaging, and X-ray imaging as the main molecular detection methods that exposed humans to Fe_2O_3-NP (Indira & Lakshmi, 2010). Considering the general application and high reactivity of iron, growing concerns exist about the Fe_2O_3-NP application harms, especially in the central nervous system (CNS). Our previous study confirmed that Fe_2O_3-NP with a size of 50 nm creates harsh oxidative damage and decreases cell viability in the Hep G2 cell line (Sadeghi et al., 2015). While the nervous system is more susceptible to oxidative stress than other tissues due to more consumption of oxygen and a weak antioxidant barrier (Salim, 2017). This study aimed to investigate the cytotoxic effects of Fe_2O_3-NP on the primary culture of the hippocampus as one of the main tissues damaged in AD. By considering the high reactivity and high surface-to-volume ratio of Fe_2O_3-NP, the main harmful effect is oxidative stress, which can be assessed by standard biochemical methods (Sadeghi et al., 2015). By considering the pathophysiological signs of AD (Habib et al., 2017), the activity of beta-site amyloid precursor protein (APP) cleavage enzyme-1 (BACE-1) was compared in hippocampal cells that were cultured in the presence or absence of Fe_2O_3-NP by measurement of secreted soluble amyloid precursor α and β (sAPPα and sAPPβ). The immunoblotting method also assessed APP expression and intracellular amyloid β (Aβ) accumulation in hippocampal cells were treated

with different concentrations of Fe_2O_3-NP. We tried to find a significant accompaniment between oxidative damages, iron nanoparticle accumulation, and pathophysiological signs of AD in hippocampal cells.

2. Materials and Methods

Chemicals

Neurobasal Plus Medium was purchased from Thermo Fisher Scientific. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7 dichlorofluorescin diacetate (DCFHDA), o-phthaldialdehyde (OPT), and 5,5´-dithiobis (2-nitrobenzoic acid) were obtained from Sigma Chemical Company. Anti-beta amyloid 1-42 antibody (ab10148), rat-specific anti-APP antibody (ab2072) was prepared from Abcam Company. Nano ferric oxide (Fe_2O_3) particles in crystalline phase with a size of 50 nm were prepared from Sigma Aldrich Company.

Cell culture and treatment

Primary rat hippocampal culture was prepared according to previous studies with some modifications (Seibenhener & Wooten, 2012). The neonatal rat brain (embryonic day 19) was isolated; the striatal, thalamic, and midbrain were removed to expose hippocampus tissue on the medial surface of the brain (Seibenhener & Wooten, 2012). Dissected hippocampus tissue was mechanically disrupted and triturated by the trypsin enzyme. Separated hippocampal cells were plated on 24-well plates (5×1055 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 a concentration of 50 and 100 µg/mL Fe_2O_3-NP for 8 and 16 h according to previous experiments (Naqvi et al., 2010). Before treatment, Fe_2O_3-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 cells in 25 cm^2 flasks and 24-well plates in a humidified atmosphere at 37˚C and 5% CO_2. Cells receiving PBS without Fe_2O_3-NP were considered as a control in each experiment.

MTT assay

Cell viability was assessed by using the MTT assay, which evaluated the 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 DCFH, a fluorescent compound (Wan et al., 1993). Briefly, primary hippocampal cells were cultured in 24-well plates, then treated with 50 and 100 µg/mL of Fe_2O_3-NP for 8 and 16 h. Treated and control cells were incubated with DCF diacetate in the culture medium for 15 min and washed with cold PBS three times. Oxidized DCFH resulted in green fluorescence, which was measured 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 Fe_2O_3-NP in PBS and DCFH were used to assess non-specific particle-induced fluorescence. The total protein concentration was evaluated using the standard Bradford method (Bradford, 1976). Fluorescence was reported as a percentage compared to untreated control cells.

Intracellular reduced glutathione (GSH) evaluation

Cellular levels of GSH were measured according to the previous method (Hissin & Hilf, 1976). This method evaluates the GSH content of cells based on a reaction between GSH and o-phthaldialdehyde (OPT) which produces a fluorescent agent. The primary culture of the 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 minutes 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 minutes and the supernatant was used for GSH evaluation in the presence of 100 µL OPT (10 mg/mL). Fluorescence was measured at 360 nm excitation and 420 nm emission using a 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 was assessed using the Bradford method.

Measurement of secreted soluble amyloid precursor protein α and β (sAPPα) and (sAPPβ)

Following the exposure of hippocampal cells to the concentrations of 50 and 100 µg/mL, the amount of APP cleavage products, soluble amyloid precursor protein α and β (sAPPα and sAPPβ) level, released into the culture medium were determined using commercially available solid phase sandwich ELISA (IBL 27419) according to the manufacturer's protocol. The concentration of secreted sAPPα and sAPPβ in the culture medium was evaluated according to a standard curve based on recombinant sAPPα, and sAPPβ, respectively, and data were expressed as ng/mL. The protein content was determined by the Bradford method in each sample (Bradford, 1976), which 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 µg/mL of NP for 8 and 16 h. After exposure times, cells were harvested by centrifugation (700 g, 5 min), and intracellular APP and Aβ were measured by the 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 minutes using the semi-dry transfer system. Blocking buffer containing 0.1% tween-20 in Tris-buffered saline (TBS), pH 6, 4% fat-free milk was used to block the polyvinylidene difluoride (PVDF) membrane at room temperature for 1 h then the membrane was incubated with the primary antibody (dilution 1:1000) in TBS/Tween-20 (TBST) containing 5% bovine serum albumin overnight at 4°C, and then incubated with the secondary antibody (dilution 1:1000) at room temperature for 1 h. Immunoreactive bands were detected by a chemiluminescence detection kit (Millipore Corporation, Billerica, MA, USA) according to the manufacturer's guidelines. β-actin was used as a 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±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 the control. A value of $P<0.0001$ was considered significant.

3. Results

Fe_2O_3-NP significantly reduced hippocampal cell viability

Hippocampal cell culture was treated with 50 and 100 µg/mL of Fe_2O_3-NP and analyzed by inverted microscope during the experiment. Figure 1 shows degeneration and abnormal morphology of neurons in the presence of NP. Treatment with 100 µg/mL of Fe_2O_3-NP has more toxic effects and reduced normal cell number in the culture medium. The majority of cells were deformed by consisting of the condensed cell body in the presence of NP. Short dendrites and axons could also be observed in NP-treated cells, especially in cells that received 100 µg/mL. Some cell debris was also visible in a 100 µg/mL dose-containing medium. According to Figure 1, rounded and shrieked cell body, abnormal arbors and short dendrites are hallmarks of Fe_2O_3-NP toxicity, especially in high doses. The cytotoxic effect of Fe_2O_3-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 µg/mL Fe_2O_3-NP, respectively in 8 h exposure time compared to the control ($F_{(2, 9)}=283.1$, $P<0.0001$, one-way analysis of variance (ANOVA), control vs. 8 h treated cells, Dunnett post-test, $^{*}P<0.0001$). The viability of cells that grew in the presence of NPs at more time duration (16 h) was estimated as follows, cells that received 50 µg/mL showed 48.15% viability and cells treated with 100 µg/mL revealed 32.94% viability that is significantly different compared to the control ($F_{(2, 9)}=543.5$, $P<0.0001$, one-way ANOVA, control vs. 16 h treated cells, Dunnett post-test, $^{\#}P<0.0001$).

Iron oxide nanoparticles (Fe_2O_3-NP) caused acute oxidative stress in hippocampal cells hippocampal cells

Reactive oxygen species (ROS) content of cells increased in the presence of iron oxide nanoparticles (Fe_2O_3-NP)

ROS are chemically reactive molecules containing oxygen, such as oxygen ions, superoxide, and peroxides presenting in the cells with very low concentrations but harmful stress can increase and damage the cellular organelles (Cabiscol et al., 2000). Therefore, measuring the ROS will provide useful information about the oxidative condition of the cells (Wang et al., 2014). The concentration of intracellular ROS generation induced by Fe_2O_3-NP was measured by DCF fluorescence intensity

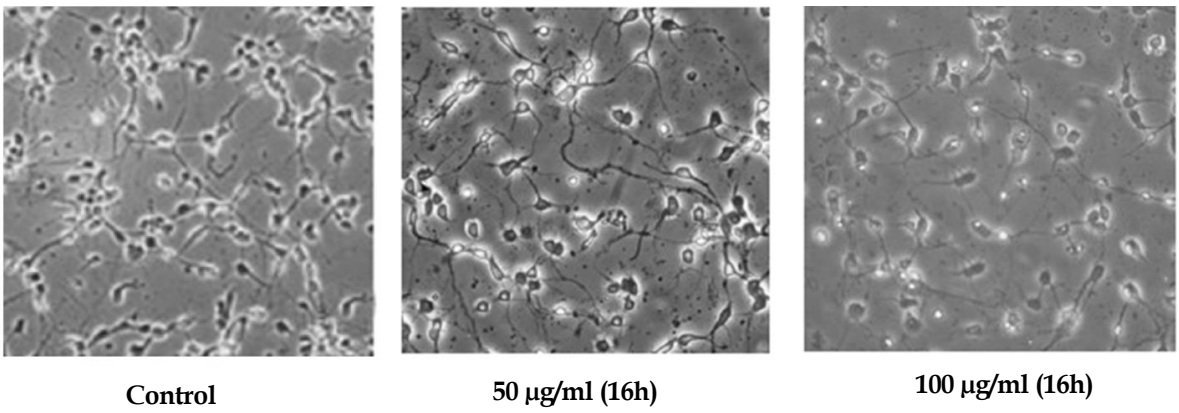

Figure 1. Effect of Fe_2O_3-NP on the morphology of hippocampal neurons (scale bars: ×100)

Control cells cultured in the absence of nanoparticles showed normal number and shape. Hippocampal cells revealed abnormal morphology (shrieked cell body and short arbors) and degeneration in the presence of nanoparticles. 100 µg/mL of Fe_2O_3-NP decreased the normal cell number in the medium.

in cells. As shown in Figure 2 A, a concentration-dependent increase in ROS generation was observed after 8 h exposure to 50 and 100 µg/mL of Fe_2O_3-NP. 50 µg/mL imposed 1.7 folds and 100 µg/mL induced 3.2 folds increase in DCF fluorescence that is 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, Figure 2 A). Sixteen 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, Figure 2 A).

Iron oxide nanoparticles (Fe_2O_3-NP) treatment reduced GSH content significantly reduced GSH is the best antioxidant barrier in the biological systems especially in the CNS (Miura & Shinohara, 2009). Therefore, comparing the reduced GSH level between the control and treated cells verify the oxidative damage. We assessed the GSH content of hippocampal primary culture by standard method. Figure 2 B showed the treatment of hippocampal cells by Fe_2O_3-NP reduced GSH content. According to the results, 50 µg/mL of NP exposure during 8 and 16 h decreased GSH content by more than 2.5 and 3.4 folds compared to the control. While 100 µg/mL treatment of NP induced GSH decline of about 3.5 and 7.7

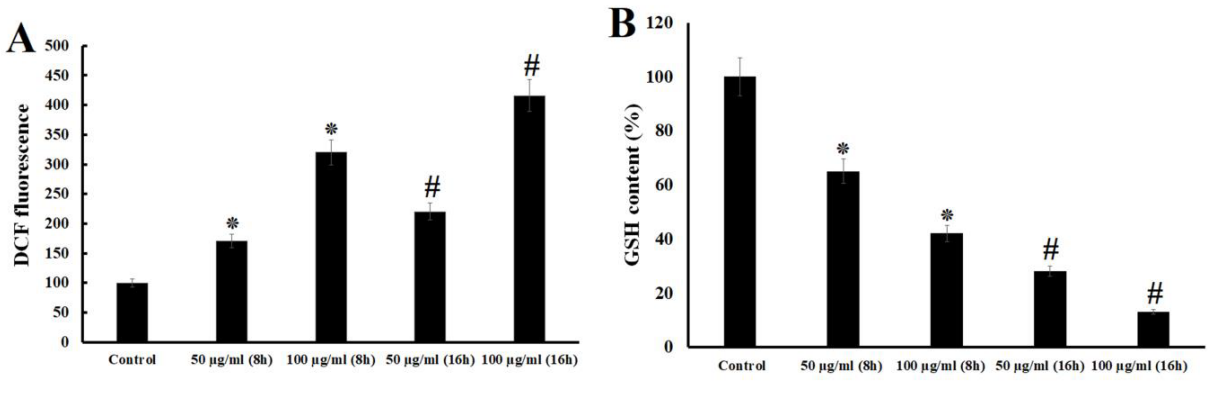

Figure 2. Oxidative stress estimation in primary culture of hippocampus in the presence and absence of nanoparticles

A) Increasing the DCF fluorescence in nanoparticle-received cells revealed ROS overproduction in the presence of Fe_2O_3-NP (n=4).

B) Reduced glutathione also decreased in the presence of nanoparticles 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.

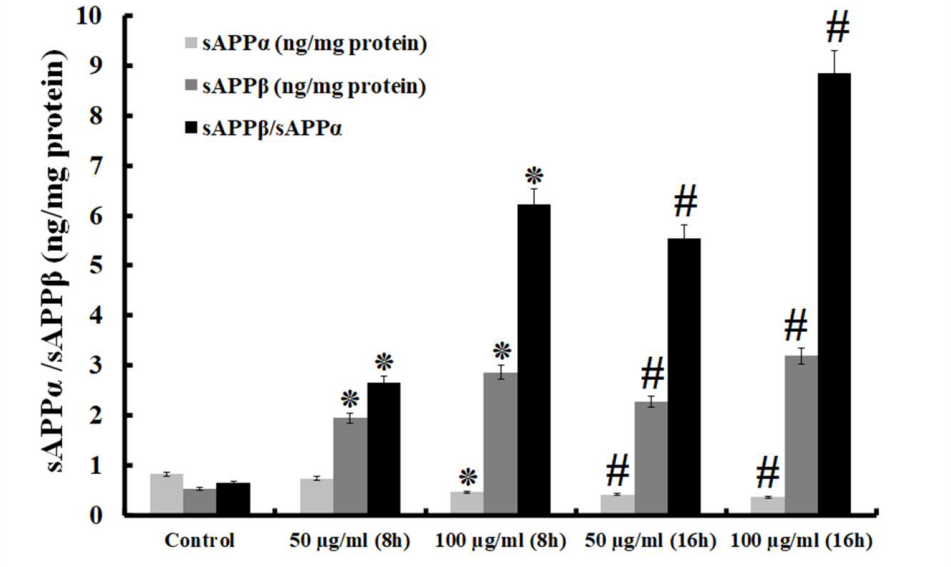

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Figure 3. Secreted Soluble amyloid precursor protein α and β (sAPPα and sAPPβ) evaluated by standard ELISA method in nanoparticles received cells and control

Results showed that Fe_2O_3-NP increased released sAPPβ in the cell medium accompanied by the reduction of sAPPα content. Increased sAPPβ/sAPPα 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.

folds in cells were treated for 8 and 16 h, respectively compared to the cells receiving saline. Administration of NP in various times and concentrations caused a 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, Figure 2 B).

Nanoparticles (NPs) exposure imposed soluble amyloid precursor protein β (sAPPβ) secretion from the hippocampal cells

To understand whether NP toxicity affects the metabolism of APP, the primary culture of the hippocampus was exposed to Fe_2O_3-NP and released sAPPα and sAPPβ assessed by a specific ELISA kit. sAPPα and sAPPβ are two crucial products resulting from different secretase enzyme catalytic functions on the APP (Chow et al., 2010). Results showed NP treatment causes increased sAPPβ level in cell culture medium and reduced level of sAPPα according to exposure time and NP concentration (Figure 3). Eight h incubation with 50 and 100 µg/mL NP increased sAPPβ up to 1.94±0.01 and 2.86±0.15 ng/mg protein, respectively which are significantly more than sAPPβ 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, Figure 3). During 16 h incubation with 50 and 100 µg/mL NP increased sAPPβ 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, Figure 3). Incubation with NPs reduced extracted sAPPα, time, and concentration-dependently. According to the results, sAPPα concentration in cells treated with 50 µg/mL of NP increased up to 0.04±0.73 and 0.02±0.41 ng/mg protein after 8 and 16 h, respectively while cells that received 100 µg/mL of NP showed 0.02±0.46 and 0.02±0.36 ng/mg protein after 8 and 16 h, respectively, sAPPα content in control cells was 0.05±0.82 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, Figure 3). Therefore, the amount of sAPPβ/sAPPα ratio increased in the presence of Fe_2O_3-NP. Sixteen h incubation in the medium with 100 µg/mL increased sAPPβ/sAPPα ratio close to 14 folds compared to the control. Figure 3 revealed sAPPα, sAPPβ, and sAPPβ/sAPPα ratios in different exposure times and NP concentrations.

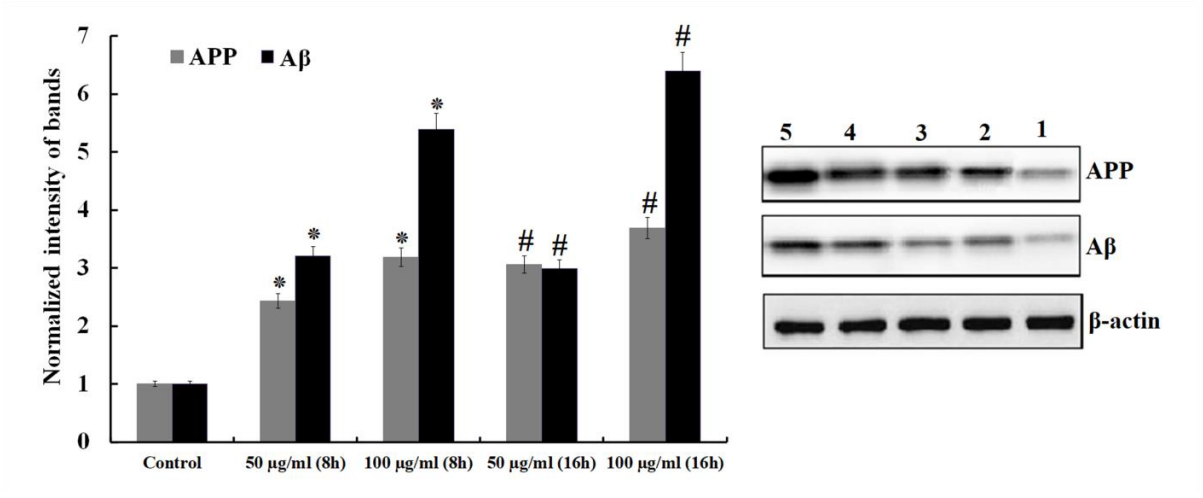

Figure 4. Nanoparticle effect on APP and Aβ contents in hippocampal cells

Western blotting analysis confirmed the upregulation of APP in the presence of nanoparticles. Fe_2O_3-NP also increased Aβ accumulation inside the hippocampal cells. 1) Control cells; 2 and 3) 50 and 100 µg/mL cells received nanoparticle 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 Aβ are dependent on NP concentration and exposure time. 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.

Immunoblotting analysis confirmed nanoparticle (NP) causing accumulation of amyloid β (Aβ) and amyloid precursor protein (APP) in hippocampal cells

After 8 and 16 h incubation time, hippocampal cell lysates were subjected to immunoblotting analysis to evaluate the Aβ accumulation and APP expression inside the cells. Figure 4 compared the expression of the APP and Aβ 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 Aβ expression with incubation time. Exposure of cells to 50 µg/mL of NP for 8 and 16 h increased APP expression by more than 2.4 and 3 folds, respectively compared to the 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, Figure 4). While treatment of cells by 100 µg/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 Aβ 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 Figure 4).

4. Discussion

Iron is an essential element in DNA synthesis, mitochondrial respiration, and oxygen transport in human and animal bodies, but overexposure to the metal causes harsh oxidative stress in the whole body, especially the immune system, and CNS due to the ability to give and receive electron (Patil et al., 2015). Iron-based nanoparticles could pass through the cell membrane and penetrate the 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, which finally leads to cell degeneration (Kozlowski et al., 2012). By considering AD as the most prevalent neurodegenerative disease, this study investigated the molecular and pathophysiological effects of iron oxide nanoparticles on hippocampal cells. Hippocampus tissue is one of the affected tissues in AD, which is widely investigated in recent years (Sarnyai et al., 2000). Our results revealed treatment with different concentrations of Fe_2O_3-NP caused harsh cellular damage in hippocampal cells (Figure 1). NP imposed abnormal shape in hippocampal cells, short dendrites, and reduced cell numbers. According to Figure 1 A, the overproduction of ROS molecules inside the cells was directly affected by NP concentration and exposure time, these results are in agreement with our previous experi-

ments on hepatoma-derived Hep G2 cells (Sadeghi et al., 2015). Iron reacts with hydrogen peroxide (H_2O_2) in the biological systems and produces OH° radical, which can attack organic substances (Barbusinski, 2009). Accumulation of iron ions interrupts electron transfer of mitochondria and causes ROS molecule overproduction (Zorov et al., 2014) approved by our results (Figure 1 A). A decrease in the GSH content of cells (Figure 1 B) also causes limited cell ability to neutralize ROS molecules (Hissin & Hilf, 1976). Our results showed a close association between oxidative stress and Alzheimer's pathological signs. According to Figure 4, APP expression increased in NP-received cells as well. Upregulation of APP can be caused by iron accumulation inside the cells, oxidative damages resulting 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 et al., 2017). Previous studies confirmed the positive effects of iron chelators in Alzheimer's treatment (Liu et al., 2010) that confirmed iron accumulation triggered Alzheimer's pathophysiology and neurodegeneration. Our results revealed that cleaved APP secreted to the medium and also deposited Aβ inside the cells increased significantly in NP-received cells according to the NP concentration. Control cells were treated with solvent without NP and showed a low concentration of sAPPβ in the cell medium and also deposited Aβ inside the hippocampal cells. Therefore, in control cells APP was mainly cleaved by α-secretase, leading to a low sAPPβ/sAPPα ratio. But the presence of Fe_2O_3-NP activated β-secretase and increased sAPPβ, therefore sAPPβ/sAPPα ratio increased in NP received cells. Figure 3 revealed that sAPPβ/sAPPα ratio directly depends on NP concentration in the cell medium, which leads to cumulative effects of Fe_2O_3-NP damage in hippocampal cells. Cell viability test confirmed negative effects of NP on cell viability based on concentration. Our previous results also confirmed Fe_2O_3-NP increased cell death in Hep G2 cells, which were consistent 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 the higher rate of oxygen consumption and limited antioxidant barrier, therefore, the main goal of toxic agents is neuronal cells (Salim, 2017). Accumulation of iron ions inside the cells causes a specific type of cell death called ferroptosis (Dixon et al., 2012) confirmed by Figure 1. Our results approved oxidative damages, mitochondrial dysfunction, and Aβ aggregation as a result of Fe_2O_3-NP toxicity that possibly induced ferroptosis in hippocampal cells. Therefore, AD was considered a neurodegenerative disease sensitive to oxidative stress (Chen et al., 2012). The results revealed a positive feedback loop between Fe_2O_3-NP accumulation, oxidative stress, and Aβ aggregation. This loop seems as an effective risk factor in the development of Alzheimer and related neurodegenerative disorders in today's life that extensively exposes humans to nanomaterials. The body has no physiological mechanism to excrete iron; thus, tissue accumulation of iron rises with age (Kohgo et al., 2008). Based on previous results, iron chelators prevent the progress of mental diseases, especially dementia (Liu et al., 2010). Considering all of the damaging agents in today's life and the accumulation of harmful effects, Alzheimer developing AD in the role of its Fe_2O_3-NP of effects of cytotoxic disease related to age and as considered suggest NPs application in medicine and biology should be limited.

Ethical Considerations

Compliance with ethical guidelines

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

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Authors' contributions

The both authors contributed equally to prepare this study.

Conflict of interest

The authors declared no conflict of interest.

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