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Toxicology

Examining the link between dose-dependent dietary iron intake and Alzheimer's disease through oxidative stress in the rat cortex

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ABSTRACT

Background: Neurodegenerative diseases such as Alzheimer's and Parkinson's disease are characterized by the progressive deterioration of the structure and function of the nervous system. A number of environmental risk factors including potentially toxic elements such as iron, lead to negative effects on many metabolic reactions as well as neuroprotection. The aim of this study is to reveal whether long-term iron overload is one of the underlying factors in the pathogenesis of Alzheimer's disease (AD).

Methods: 15 young-adult male rats were randomly divided into 5 groups treated with iron through drinking water for 4 months. Following feeding, the iron content, reduced glutathione (GSH), and hydrogen peroxide (H_2O_2) levels of cortex tissues were measured. Specific enzyme activities were determined spectrophotometrically. mRNA expression profiles were measured using real-time PCR (qPCR).

Results: Iron levels were elevated in case of non-toxic (0.87 and 3 μg/mL) iron administration. However, no changes were observed in toxic (30 and 300 μg/mL) iron administration. GSH and H_2O_2 levels altered with long-term iron overload. Glutathione peroxidase (GPx) enzyme activities significantly increased in all groups, while glutathione S-transferase (GST) activity increased only in case of 0.87 and 30 μg/mL iron administration. Expression levels of neuroprotective and AD-related genes were altered by 3 μg/mL iron overload in a dose-dependent manner. The expression and activity of acetylcholinesterase (AChE) were elevated at 3 μg/mL iron concentration.

Conclusion: The findings of the present study allow us to conclude that long-term dietary iron intake, especially at a dose of 3 μg/mL demonstrates negative effects on the rat cortex by provoking antioxidant metabolism and AD pathology in a dose-dependently.

1. Introduction

Although neurodegenerative diseases are characterized by the progressive deterioration of neuronal structures and functions, these diseases are different in terms of their etiology, pathologies, genetics, and treatments [1,2]. Alzheimer's disease (AD) is the most common type of age-associated dementia, resulting in massive neuronal death [3–6]. A wide variety of toxic pathways such as anomalous protein aggregation, inflammation, and reduced neurotransmitter level are involved in AD pathologies [7–9]. Among them, the aggregation of amyloid-β peptides (Aβ) in the extra-neuronal area and the abnormal hyperphosphorylation of the Tau protein within cells are regarded as the dominant hallmarks of AD [10]. Furthermore, DNA repair failures [11], synaptic transmission impairments [12], intracellular calcium level disruption [13], mitochondrial dysfunction [14], genetic factors [15,16], energy metabolism defects [17], and oxidative stress [18,19] may be involved in disease occurrence. However, as it can be understood from previous studies, AD is a multifactorial disorder and does not depend only on genetic abnormalities [20–22]. Therefore, attempts to understand the non-genetic basis of the disease have become of great importance in therapeutic interventions.

A number of environmental risk factors such as social conditions, nutrition, and hazardous chemical wastes are known to contribute to neurodegenerative processes [23]. Environmental contaminants can induce neuropathology together with bioaccumulation during the organism's lifetime, thus continuing to be a subject of discussion [24]. Exposure to metals in various ways, such as through the air, soil and contaminated water is a common case worldwide. Contamination of

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ground waters and dietary substances by potentially toxic elements is known to have negative effects on the human body since they are prone to oxidation [25]. Nevertheless, in recent years, as a result of anthropogenic activities such as industrial, technological, medical, and domestic applications, environmental metal exposure has increased dramatically. [26]. Indeed, in order to perform a series of missions in basic cellular processes, metal ions are essential [27]. Iron is an essential trace element naturally present in the environment. It has been recognized as a versatile cofactor of many metabolic reactions such as energy metabolism [28], oxygen transport [29], neurodevelopment [30,31], neurotransmitter synthesis [32], and growth [33]. Although it is essential for life, cellular or systemic abnormal iron homeostasis is believed to be associated with the pathology of multiple diseases [34]. Therefore, dyshomeostasis of transition metals has been suggested to be a risk factor for health hazards to humans and other organisms [35].

The i*n vivo* predominant transition metals including iron are classified as fundamental micronutrients and regarded as efficient catalysts of redox reactions, but fine-tuning of their concentrations is very important. Studies have shown that the free and/or excess form of these metals can be toxic because they are capable of stimulating the formation of reactive oxygen species (ROS) and can induce the cell redox homeostasis disruption which causes oxidative stress resulting in cellular macromolecule damage [36,37]. For this reason, dietary metal intake can be important for triggering the oxidative damage cascade. It is well known that the brain utilizes almost one-fourth of the body's total oxygen consumption [38]. This tissue is composed of easily oxidizable lipids, which makes make it the target of oxidation attacks. On the other hand, brain cells have a relatively low antioxidant defense system, which makes them vulnerable to metal toxicity [39]. Therefore, an assessment of the neurotoxicity induced by iron in the murine brain might provide precious insights into the disease pathology. The main aim of this study is to answer the following questions: Is long-term iron overload one of the underlying factors in the pathogenesis of AD? If yes, is there any possible relation between AD and iron dose? Furthermore, how is the antioxidant pathway stimulated at the gene and enzymatic level during long-term iron exposure?

2. Material and methods

2.1. Experimental design and animal care

Young adult (3–4 month old) male Sprague Dawley (SD) rats (*Rattus norvegicus,* n = 15) were obtained from Atatürk University Medical Experimental Application and Research Center. Animals were divided into five groups and kept for 1 week under standard conditions (diet, air conditions, humidity *etc.*) for acclimatization before use. After this short period, animals were fed with deionized water including fresh daily prepared iron (Fe^{3+} chloride hexahydrate, Sigma-Aldrich) for almost 4 months. Iron concentrations (0.87, 3, 30, and 300 μg/mL) were administered orally according to the World Health Organization's data [40,41]. Body weights and water consumption of rats were recorded daily during the treatment. No significant differences were observed in the treatment groups compared to the control group (data not shown). No animal died and exhibited any behavioral abnormalities during the experiments. After administering anesthesia with the ketamine/xylazine cocktail, animals were sacrificed. Brain tissues were quickly removed from the skull. The cortex parts were dissected and separated in ice-cold 1X PBS from the whole brain using a binocular microscope. Animal experiments were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee of Atatürk University (Protocol no: 36643897-80).

2.2. Determination of GSH, iron content, and H_2O_2 levels

Homogenate preparation, determination of GSH levels, tissue iron content, and H_2O_2 measurement experiments were performed as previously described in [42]. Briefly, the amount of GSH (nmol) for each homogenized sample was determined using a Glutathione Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. For the measurement of the total iron (Fe^{2+} and Fe^{3+}) content, an Iron Assay Kit (Abcam, UK) was used following the manufacturer's instructions. Briefly, tissues (10 mg) were harvested in Iron Assay Buffer using a homogenizer (Bead Blaster 24, Benchmark Scientific, USA) after being washed with cold PBS. Iron in the sample was reduced using a Fe reducer to produce a stable colored complex. The absorbance was measured at a wavelength of 593 nm, and iron contents of the samples (μg/g) were determined against a standard concentration curve. The concentration of H_2O_2 in the tissue homogenate was determined by using a hydrogen peroxide estimation kit (Abcam, UK) as described in the manufacturer's instructions. All assays were performed in triplicate, and measurements were made on a microplate reader (Multiscan GO, Thermo Scientific, USA). The results were averaged with triple independent experiments for each animal. The obtained data were used for analysis and discussion of the result.

2.3. Lipid peroxidation quantification

Malondialdehyde (MDA) content in the rat cortex tissue was determined by the thiobarbituric acid (TBA) method at the absorbance wavelength of 532 nm. Methods and procedures were applied using a Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The absorbance was measured using a microplate reader (Multiscan GO, Thermo Scientific, USA).

2.4. RNA isolation and cDNA synthesis

RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The isolated RNAs was checked using RNA gel electrophoresis and a 96-well plate spectrophotometer (Multiskan GO, Thermo Scientific USA). cDNA was synthesized using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis Kit (Invitrogen, USA) according to the manufacturer's protocol.

2.5. Primers and quantitative gene expression analysis

Gene expression profiles were measured using the SYBR Green (Qiagen, Germany) based qPCR method. PCR reactions were performed in a Rotor Gene Q (Qiagen, Hilden, Germany). Primers were selected to bind specifically to the target genes (Table 1). Primer sets were designed using the Primer3 software program (v. 0.4.0) [43] and purchased commercially from Metabion (Martinsried, Germany). Their gene symbols and GenBank accession numbers were as follows: amyloid precursor protein; *App* (NM_019288.1), presenilin 2; *Ps2* (NM_031087.2), *Ache* (NM_172009.1), nicastrin; *Nct* (NM_174864.3), forkhead transcription factor 3a; *Foxo3* (NM_001106395.1), and glyceraldehyde-3-phosphate dehydrogenase; *Gapdh* (NM_017008.4). Specific primers for antioxidant system genes were purchased commercially from Roche Diagnostics (Mannheim, Germany). Their gene symbols and GenBank accession numbers were as follows: glutathione S-transferase; *Gsta5* (NM_001010921) and glutathione peroxidase; *Gpx2* (NM_183403). The reaction mixture was prepared for 25 μl and amplification reactions were performed as follows: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 10 s, and annealing/extension at 60 °C for 1 min. The expression results were analyzed using the ΔCT method [44].

2.6. Homogenate preparation and enzyme assays

Tissue homogenates were prepared using a Heidolph Silent Crusher M (Germany) mixer in 50 mM Tris HCl buffer, pH 7.6, containing 1 mM

Table 1
Primer pairs for genes used in qPCR.

Gene Symbols	Accesion Number	Primer	Sequence (5'-3')
Foxo3a	NM_001106395.1	Forward	ACTGAGGAAAGGGGAAATGG
		Reverse	TGCTGGGTTAGGAAGATGGC
App	NM_019288.1	Forward	TGATCTACGAGCGCATGAAC
		Reverse	TAGAAGGCATGAGAGCATCG
Ps2	NM_031087	Forward	TCTCCACAGACAACCTGGTG
		Reverse	AAGCGTCCTACAGCTCTCAG
Ache	NM_172009	Forward	ATGAGTGCGGTGGTAGGC
		Reverse	ACGGTGTTCAAAGATGTAGGC
Nct	NM_174864.3	Forward	ACCAGGAAGAGGGTGTGAAAG
		Reverse	TGGGATACCCACCTAACAGCA
Gpx	NM_183403	Forward	TCCCTTGCAACCAGTTCG
		Reverse	CTTGAGGCTGTTCAGGATCTC
Gst	NM_001010921	Forward	GGGAAGCCAGTGCTTCACTA
		Reverse	CTCTGTATAAGCTTCTCTTCAAACTCC
Gapdh	NM_017008.4	Forward	TGGACCTCATGGCCTACATG
		Reverse	AGGGAGATGCTCAGTGTTGG

DDT (dithiothreitol), 1 mM EDTA (ethylenediaminetetraacetic acid), and 1 mM PMSF (phenylmethanesulfonyl fluoride) of 1/5 (w/v). The lysate was centrifuged at 13.000 × g for 1 h, and then the precipitate was removed. Quantitative protein determination was performed according to Bradford's method [45]. Bovine serum albumin was used as standard. GST activity with different substrates was determined as described by [46]. GPx enzymatic activity was measured by Wendel's method [47].

2.7. Statistical analysis

All the measurements were triplicated for each group (three animals). Statistical comparison of the results was performed by one-way ANOVA and Tukey's post-hoc test using Prism software (GraphPad Software, San Diego, CA). Statistically significant differences are presented as follows: $p > 0.05$ (not significant, ns); $*p < 0.05$ (significant); $**p < 0.01$ (very significant); $***p < 0.001$ (extremely significant).

3. Results

3.1. Redox-sensitive and AD-related genes are altered by iron administration

In the brain, the ferric iron content of the rat cortex tissues was measured after 4 months of non-toxic and toxic iron treatment. The results showed that iron levels were elevated in the cortex only by non-toxic iron administration. No statistically significant differences were observed in the toxic iron overload (30 and 300 μg/mL) groups compared to the control group (Fig. 1A). To confirm whether iron exposure is associated with oxidative stress in the rat cortex, the expression of *Foxo3a* which is key a factor involved in neuroprotection, was determined by qPCR after long-term iron overload. The results demonstrated the induction of *Foxo3a* expression at 3 μg/mL in a dose-dependent manner (Fig. 1B). To determine whether iron exposure alters the expression of App and γ-secretase components (Ps2 and Nct), which play essential roles in AD progression, the related factors were measured in the brain cortex. The presented results showed that the expression of *Ps2* and *App* increased and *Nct* expression decreased in the presence of 3 μg/mL in a dose-dependent manner (Fig. 1C–E).

3.2. GSH and H_2O_2 levels are affected by iron overload

The decreased the GSH level is a marker of oxidative stress demonstrating whether iron accumulation in the cortex induces oxidative stress in cells [48]. Therefore, the GSH level of the rat cortex tissues was investigated in the presence of non-toxic (0.87 and 3 μg/mL) and toxic (30 and 300 μg/mL) ferric iron. The rats were treated with ferric iron, which caused the depletion of GSH either in the non-toxic or toxic groups (Fig. 2A). Since H_2O_2 is a central messenger molecule for the physiological oxidative stress, the level of H_2O_2 was also measured in all groups. The results clearly showed that the production of H_2O_2 increased significantly in the presence of iron in the cortex (Fig. 2B).

3.3. Iron overload affects the expression and specific enzyme activities of GPx and GST

Highly toxic ferric iron is oxidized to ferrous iron via the Fenton reaction, which induces the production of H_2O_2 [49]. GPx metabolizes H_2O_2 to water by using GSH as a substrate. GST also reacts with GSH directly which leads to the detoxification and excretion of harmful compounds from the cell. Here, the impacts of long-term iron overload on *Gpx* and *Gst* expression and specific activities were investigated in the rat cortex. The results showed that while the expression of *Gpx* increased only in the presence of 0.87 μg/mL iron concentration (Fig. 3A), *Gst* expression was significantly repressed in the presence of non-toxic iron (Fig. 3C). Although GPx enzyme activities significantly increased in all groups (Fig. 3B), GST activity increased only in the presence of 0.87 and 30 μg/mL iron (Fig. 3D).

3.4. Increasing the expression and enzymatic activity of AChE protects lipid peroxidation against iron-induced oxidation

Lipids are susceptible biologic targets of oxidative stress due to their structural properties [50]. Therefore, MDA, as a lipid peroxidation marker, was investigated in all groups to explain the effects of long term dietary iron intake on the generation of lipid peroxides in the rat cortex (Fig. 4A). As shown in Fig. 4B, the AChE mRNA transcript level was elevated in the presence of 3 μg/mL iron concentration after a 4 month period. However, the enzyme activity of AChE increased in the presence of 3 and 30 μg/mL (Fig. 4C). In parallel to the activity, the MDA level was not affected by this concentration.

4. Discussion

Iron is an essential cofactor for critical metabolic reactions. However, its excess accumulation in the cell can stimulate different deleterious events such as DNA fragmentation, organelle dysfunction, and apoptosis [51,52]. Furthermore, it has been especially implicated as an important generator of a pro-oxidative microenvironment [53]. Of course, all cells can suffer from oxidative damage that may be formed naturally, but the brain tissue cells are often mentioned to be sensitive to oxidative stress. Iron metabolism-related disorders are among the common diseases and encompass a broad spectrum ranging from anemia to neural diseases [54]. The observations have shown that the identified significant neuronal alterations in AD are associated with cortical changes [55]. The experimental evidence also indicates that oral iron loading causes significant neuronal loss in the cortex and alteration in the neurobehavioral activity of adult rats [56,57]. The World Health Organization (WHO) reported that iron intake through food is the major source of iron exposure [58]. By moving in this direction, firstly we established an oxidative stress model induced by oral administration of iron (once a day, uninterrupted for 4 months) [59]. The results showed that the oxidative status was influenced by exposure and significant alterations that prove whether oxidative stress occurs, in the antioxidant system markers and metabolites were observed in the model groups compared to the control group.

It has been more than a century since AD was first described, but scientists are still working on the underlying causes of AD. Extensive studies have demonstrated that chronic environmental exposure to metals such as mercury, iron, and aluminum is an important risk factor for AD [60]. In the oxidative stress hypothesis in dementia, which is

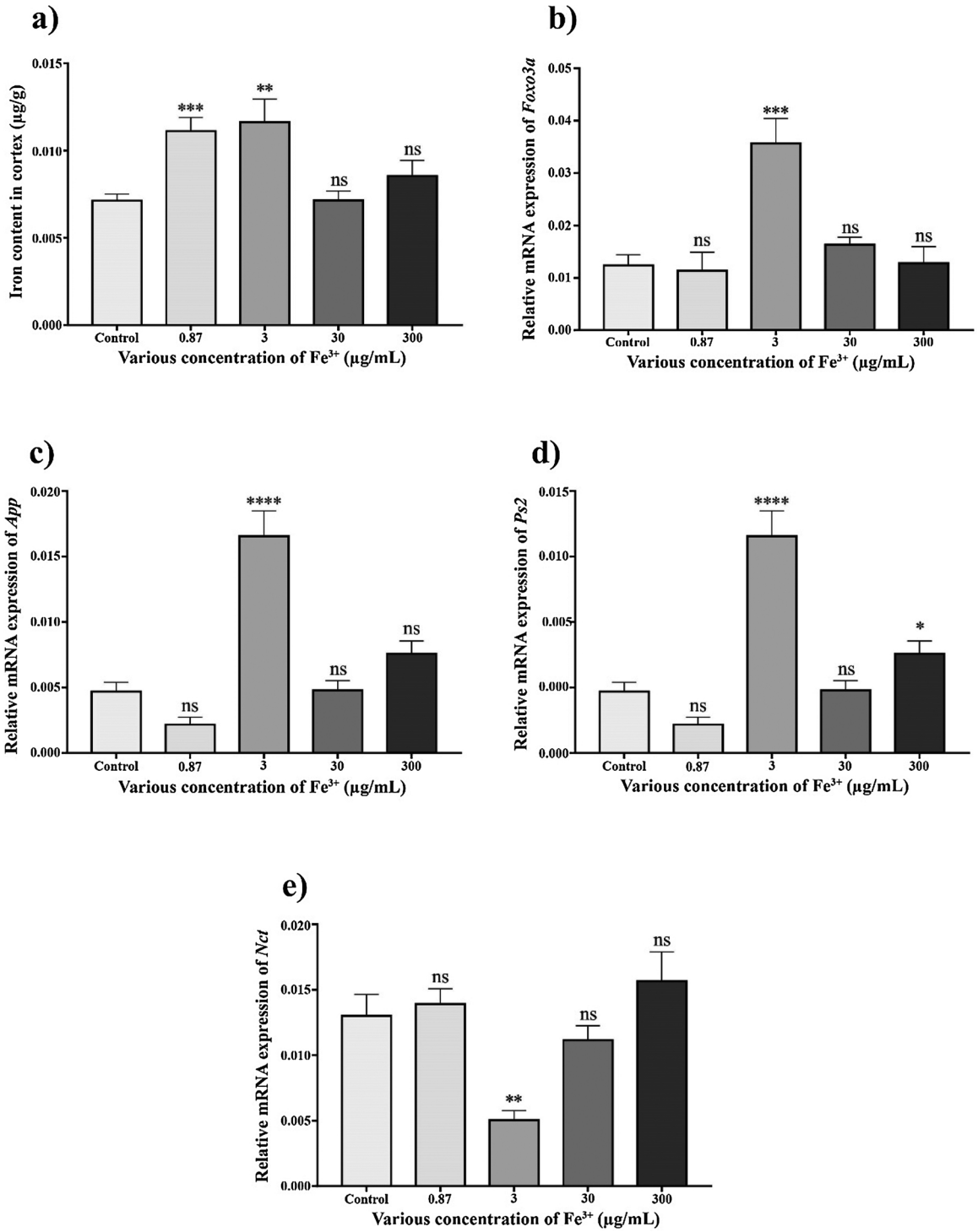

Fig. 1. Effect of long-term iron treatment on iron accumulation in the cortex tissues (a). Various concentrations of iron changed the transcription levels of the *Foxo3a* (b), *App* (c), *Ps2* (d) and *Nct* (e) genes in dose-dependent manner. The data are presented as mean ± SEM (n = 3). Gapdh was used as a housekeeping gene.

related to the neurotoxic trace element hypothesis, there is the remarkable evidence arguing that iron is mainly responsible for selective neuron death since it catalyzes the formation of toxic free radicals via the Fenton reaction. [61–63]. Numerous studies have also revealed the importance of pathogenic triggers, such as deteriorated iron metabolism, leading to impaired brain function in AD patients' brains [64,65]. However, the mechanism(s) behind the disease progression and how oxidative stress initiates the activation of the redox-sensitive cascade in neuronal death are still unclear. For these reasons, iron content and iron distribution metabolism become important in the brain [65]. Different brain regions of AD patients were examined, and the results demonstrated that a striking increase in the iron level [66]. Our results are consistent with these studies (Fig. 1A). However, the iron content of the cortex increased with iron overload but then began to decline. It is well known that, depending on the cell type, multiple factors such as transferrin saturation, ferroportin/hepcidin or ferritin/hepcidin ratios affect iron uptake or export [67,68]. Therefore, the distribution and storage of iron can differ from tissue to tissue or in some regions of an organ [69–71]. Excess iron is stored mainly in the liver. However, other organs such as heart, pancreas, testes, and even skin may also be

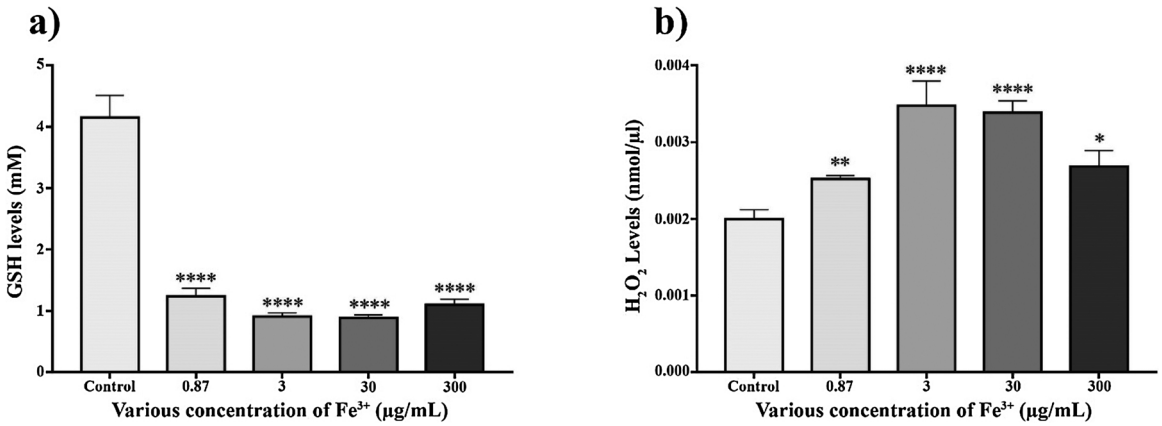

Fig. 2. GSH (a) and H_2O_2 level (b) in the rat cortex treated for 4 months with distilled water, and four different iron concentrations.

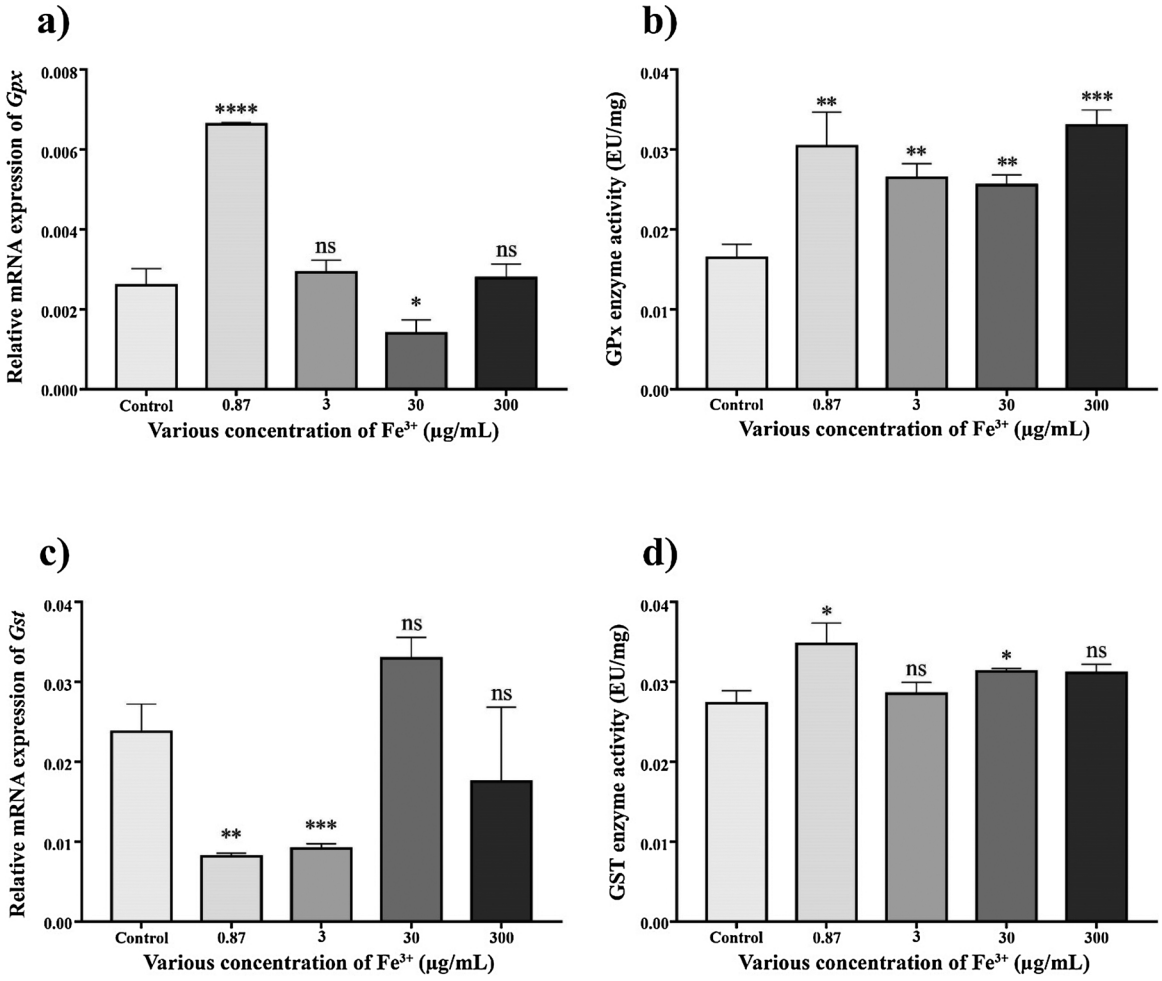

Fig. 3. *In vivo* effects of various concentrations of iron on the expression (a–c) and specific activities (b–d) of the antioxidant metabolism enzymes in the cortex tissues of rats.

involved in excessive iron deposition [72,73]. When evaluated in this respect, the results of this study are similar to the previous studies on the kidney [41]. Similarly, some brain regions, especially associated with motor functions, are particularly enriched in iron [74,75]. The blood-brain barrier (BBB) limitations can cause this situation. The disrupted BBB has been proposed as a reason for increased iron-mediated neurotoxicity [76,77], and the experimental evidence indicates that the intact BBB prevents the brain from iron overload even at elevated iron levels [78,79]. Therefore, we suggested that, besides oxidative stress, dietary iron exposure may trigger BBB disruption. Moreover, neurodegenerative disorders associated with the brain iron level have been known to correlate in a dose-dependent manner, and this gradual accumulation seems to be a feature of normal aging [80]. However, the intake of iron assisted nutrients in the early stages of life, resulting in the increased brain iron level, can accelerate the early Ab_{42} deposition [81]. This kind of situations, as in this study, can also amplify the risk of iron-associated disorders such as AD in later life by altering the nature of brain iron uptake and multiple regulatory mechanisms. [82].

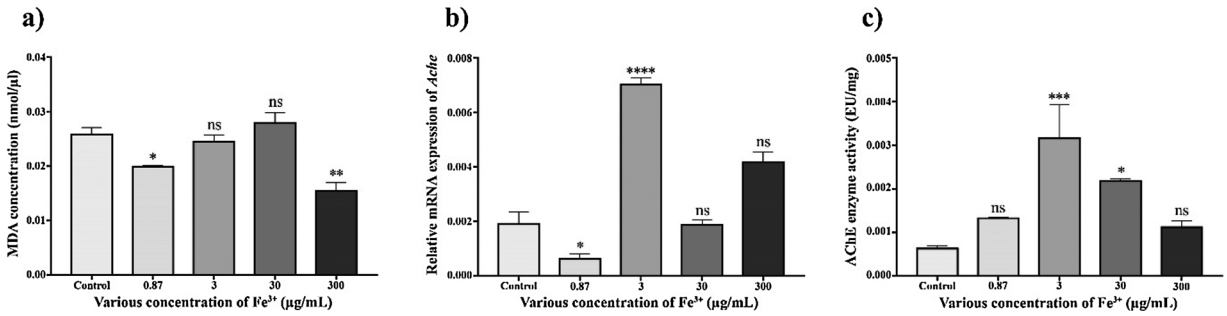

Fig. 4. Changes in the MDA content after long-term iron administration (a), and effects of various concentrations of iron on the expression (b) and specific activities (c) of AChE.

The restoration of the cell redox state by the transcriptional activation of genes encoding transcription factors (TFs) involved in the clearance of ROS is a consequence of oxidative stress [83,84]. The activity of TFs is the major component of the initiation of transcription in gene regulation [85]. The class O forkhead (FOXO) family members, a set of TFs, are activated in various physiological processes. FOXOs act as a sensor in stress stimuli and are triggered by increased cellular stress or decreased vital signals and directly regulate the transcription of antioxidant and cell cycle regulatory protein genes [86,87]. Recent studies have shown that one of them, FoxO3, protects cells by elevating the expression of manganese superoxide dismutase (MnSOD) [88] and catalase (CAT) [89] encoding genes of which products target ROS elimination [90]. Furthermore, knockdown and transfection studies have also clearly demonstrated that *FoxO* increases cell vitality in response to oxidative stress by regulating the expression of ROS scavengers [91,92]. On the other hand, experiments using cell models have shown that Foxo3a activation initially promotes the activation of antioxidant enzymes and rescued cells from oxidative stress, but prolonged exposure promotes apoptotic genes leading to cell death [93]. Therefore, we investigated addressed whether iron treatment would alter Foxo3a expression. Relative mRNA analysis showed a significant increase in *Foxo3a* expression at a non-toxic dose of iron (Fig. 1B). Previous reports have shown that Akt (protein kinase B) inhibits the activation of pro-apoptotic signaling components such as FoxO3a [94]. Akt-mediated phosphorylation promotes the nuclear exclusion of FoxO (inactive form), resulting in the alteration of FoxO-dependent gene expression [95]. Thus, we concluded that the elevated iron content contributes to the progression of neurodegeneration by regulating Foxo3a activation through the inhibition of the PI3K-Akt signaling pathway, and our findings are consistent with previous studies [96].

Since it is associated with aberrant Aβ processing, we next examined whether the expression of some major genes, which are the known risk factors for AD, would also be regulated by iron treatment. Familial AD is caused by mutations in APP, presenilin 1 (PS1), and presenilin 2 (PS2) genes [97]. Extracellular amyloid plaques, that are composed of Aβ peptides generated from specific endoproteolysis of APP by the action of β (beta) and γ (gamma) secretase cleaving, are accepted as the main pathological hallmark of AD [98,99]. The γ-secretase complex consists of four integral membrane proteins that are essential for complex activity: presenilin (PS), presenilin enhancer 2 (PEN-2), NCT, and anterior pharynx-defective 1 (APH-1) [100]. The catalytic core of the secretase complex resides on PS1 and PS2. Other factors are required for amyloidogenic actions and binding with the substrates [101,102]. Therefore, alterations in secretase components, in particular PS2, can influence the proper maturation of the secretase complex and cleavage specificity resulting in AD pathology [103]. The studies have suggested that oxidative stress-induced products do not change γ-secretase activity by increasing the levels of secretase components, which means that they have an impact on the protein structure. In consistency with previous findings, recent reports have indicated that the stability of γ-secretase components, especially NCT is modulated by AKT1 kinase activity. Through AKT mediated modification, the interaction between NCT and APP is interrupted. Consequently, γ-secretase dependent cleavage is reduced [104]. Additionally, it was also found out that γ-secretase activity significantly increased with iron administration [105]. Intriguingly, although previous studies have verified the main function of FoxO in tuning cellular response to stress, recent studies have revealed that FoxO also acts as a critical factor involved in AD pathogenesis [106]. It was found out that APP promoter activity and neurotoxic Aβ processing were increased by nuclear Foxo3a [107]. In accordance with *Foxo3a* expression, AD-related gene expression significantly altered at 3 µg/mL iron concentration in a dose-dependent manner (Fig. 1C–E). Taken together, we concluded that a certain amount of iron exposure might accelerate AD pathology progression through the alteration of protease activity and translocation of Foxo3.

Biological systems exhibit an endogenous response to protect cells against oxidative damage via the antioxidant defense system [108]. SOD, CAT, and GPx are considered as three primary enzymes involved in removing active oxygen species [109]. In addition, because of their inducible activities, GPx and GST have been accepted as reliable toxicity biomarkers for their rapid and sensitive responses in comparison with glucose-6-phosphate dehydrogenase (G6PD) in protecting cells against free radical damage [110–112]. Therefore, in this study, only GPx and GST were selected, and their alterations were studied.

It is known that GST catalyzes the detoxification of metal ions from the cell by using reduced GSH as a substrate. GSH, a key intracellular antioxidant, is critical for defending the brain from oxidative stress, and its either increased or decreased level has been accepted as a marker of oxidative stress [113–115]. GSH reacts directly with oxidants to inhibit oxidative damage and reacts enzymatically with GPx and GST against neurodegeneration in the cell [116]. Indeed, a number of studies have claimed that GSH depletion occurs in neurodegeneration. However, it is unclear whether this depletion is an outcome or a cause of disease [117]. The postmortem analysis of pre-symptomatic Parkinson's disease (PD) subjects showed decreased GSH levels, whereas other antioxidants levels remained unchanged [118]. Furthermore, it was also reported that the levels of pro-oxidants, such as iron, did not show any difference in the brain [119]. These results suggest that GSH depletion is not only a result of oxidative stress but also the primary cause of neurodegeneration. Another opinion, is that it could just be because glutathione does not cross the BBB or the possibility of its transport from the blood to the brain is limited. Therefore, GSH depletion may also demonstrate a failing attempt to protect from ROS stress in AD [120]. As shown in our results (Fig. 2A), the GSH level was markedly reduced in the presence of iron. When cells are faced with endogenous factors of oxidative stress, this supports the regulation of antioxidant enzymes such as GPx, GSH, and CAT as a defense mechanism to protect them from free radicals. CAT is involved in the detoxification of high concentrations of H_2O_2, whereas GPx is more sensitive to lower concentrations. Moreover, the brain has fewer CAT, levels and hence GPx plays a critical role in attenuating H_2O_2 [121]. Many studies conducted on aging and age-

related dysfunction have shown an increase in H_2O_2 levels [122–124]. Additionally, it has been suggested that antioxidant enzymes are also targets for ROS and can be inactivated by ROS. As shown in Figs. 2B and 3 B, under the conditions when H_2O_2 is increased, GPx activity is repressed slightly. This inverse association between H_2O_2 and GPx activity may be because of the increased peroxides, which inactivate antioxidant enzymes. Pigeolet et al. verified that the reason for the reduced specific activity of GPx, SOD, and CAT might be the inactivation of the enzyme by the overproduction of peroxides [125]. It has previously been shown that the active site of GPx contains a tryptophan residue involved in the binding of peroxides [126]. However, GPx is surrounded by glutathione molecules which protect it from peroxides. Thereby, a decrease in the concentration of such antioxidants makes the enzyme sensitive to hydroxyl radicals. A significant decrease in the GSH level has already been reported above. Therefore, we hypothesized that iron-induced oxidative stress could accelerate AD progression by either disrupting the redox state of the cell and passivating the antioxidant system at advanced levels.

MDA content is another sign of oxidant/antioxidant balance in a cell [127]. Increased MDA levels in the brain of different strains of rats treated with a high dose of iron were reported [77,128]. In contrast, in our study, iron-treated SD rats did not show such abnormalities (Fig. 4A). Similar results were obtained for MDA content using SD rats [76]. These results suggest that several factors such as dose, exposure route, age, strain, and the form of administered iron (ferrous or ferric) may alter the effects of iron. Interestingly, lipid peroxidation reduction in the cortex after iron exposure can be associated with AChE activity. Normally, AChE terminates nerve impulses by hydrolyzing acetylcholine at the synaptic cleft [129]. In this regard, it is accepted as critical for AD therapy. However, the enzyme can exist in various molecular forms. Therefore, it is believed to play different roles unrelated to cholinergic neurotransmission [130,131]. This suggestion is further supported by previous studies. A recent study has revealed the capability of AChE to protect LDL against metal-induced oxidation. The results suggest that AChE can abolish the onset of lipid oxidation, and hence, inhibits the early oxidative product formation, such as lipid peroxides [132]. As shown in (Fig. 4C), the AChE activity increased initially, especially at non-toxic 3 μg/mL treatment, and then decreased with the increasing dose. The decreased enzyme activity may be associated with oxidative deactivation. This possibility has been further supported by previous studies showing the relationship between the oxidative status and AChE enzyme activity. It was found out that oxidative molecules might have an impact on the activation/deactivation of the enzyme activity by causing structural changes in the active site in a dose-dependent manner [133,134]. Taken together, we may speculate that the present study can be another report on the antioxidant effect of AChE against lipid oxidation. Furthermore, our results also supported the possibility that the decreased enzyme activity augments the redox state and this complicates the adaptation to oxidative injury.

Stress-based alterations can occur during a lifetime, but these changes may offer excellent non-genetic information traces about age-associated diseases. Nutrition, one of them, plays a prominent role in disease circumstances and directly affects systemic functions [135,136]. A number of studies have shown that nutrition and nutrient contents might be significant for AD. On the other hand, they are accepted as modifiable risk factors for disease development [136,137]. Therefore, improving the quality of life and reversing diet-related neuronal distortions are possible by rectified nutrition.

In conclusion, our findings allow us to conclude that long-term iron administration has demonstrated negative effects on the rat cortex by provoking antioxidant metabolism and AD pathology. We have highlighted here that prolonged iron exposure, especially at a 3 μg/mL iron dose, increases the possibility of creating a hazardous environment for the cognitive system in a dose-dependent manner by the following findings; (i) expression changes in AD-related genes, (ii) increase in the AChE activity, (iii) elevation of mRNA expressions of critical TFs involved in the regulation of stress metabolism in the brain tissue. The results of the current study may be an answer to whether iron toxicity is one of the underlying factors of AD and may offer suggestions to further studies on potential targets for neuroprotective and neurorestorative approaches.

Declaration of Competing Interest

The authors declare that there is no potential conflict of interest with respect to the research, authorship, and/or publication of this article. All authors read and approved the final manuscript.

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Conceived and designed the experiments: HC, HB and OE. Performed the experiments: HC, HB, EFK, and NGB. Analyzed the data: HC, HB, and EFK. Contributed reagents/materials/analysis tools: HB and OE. Wrote the paper: HC and HB. This research has been supported by Atatürk University Scientific Research Projects Coordination Commission [Grant Numbers: PRJ2010/277, PRJ2015/97, and FAD-2018-6352].

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