Relationship between the monoamine oxidase gene overactivity and the other pathophysiological and behavioral parameters implicated in memory deficiency in albino Winstar rats as Alzheimer’s disease model

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Abstract
The current study aimed to assess the pathophysiology mechanisms that mediate the effect on albin winstar rats’ memory induced by the co-administration of fluoride and aluminum sulfate, as a model of Alzheimer’s disease. This was done by assessing monoamine oxidase-A (MAO-A) activity, antioxidant activity, H$_2$O$_2$ and amyloid-β concentration in the hippocampus, embedded deep into the brain’s temporal lobe, and level of cytokines in serum. The polymerase chain reaction approach was used to genotyping MAO-A, followed by single-stranded conformational polymorphism (SSCP) coupled with sequencing technique. The experimental animals were divided into two groups: control and treated groups. The uptake of heavy metals led to significantly increased MAO-A activity, amyloid-β deposition, H$_2$O$_2$ and cytokines levels in the treated group. However, the finding showed a significant decrease in antioxidant activity in the treated group. The results indicated that metals caused memory and learning impairments. PCR -SSCP genotyping showed many SNPs and haplotypes of the MAO-A exon 2 region, which showed the MAO-A gene polymorphism changes associated with Alzheimer’s disease. The overall results indicated a role of metals to induce oxidative stress stimulating pathophysiological hallmarks in the hippocampus due to an increase in the influx of monoamine oxidase expression, which has been implicated in impaired memory, this study focused on the genetic variation of the exon 2 in monoamine oxidase-A gene and its relationship to Alzheimer's disease with the presence of several single nucleotide polymorphisms that may be related to Alzheimer's disease model in rats.

Keywords: Alzheimer’s disease, Amyloid –β. Antioxidant enzyme, Cytokines, MAO–A gene

INTRODUCTION
Alzheimer's disease (AD) is a serious progressive neurological illness, widely prevalent in the world (Prema et al., 2017). It primarily infects aging men and women and can affect younger people due to industrial development and increased environmental pollution (Zhou et al., 2016). AD is characterized by progressive damage of cognitive domains, learning processes, especially memory loss and behavioral disorders such as mood swings, aggression and social isolation (Hussien et al., 2018). The features of AD that have been reported include stimulating oxidative stress leads to the production of free radicals and neuronal damage, which are considered efficient factors causing behavior and memory disturbances (Zandi et al., 2021). It also contributes to the deposition of the extracellular amyloid-beta peptide and the formation of neurofibrillary tangles (NFTs) in the hippocampus (Kalra et al., 2016; Nobakht et al., 2017 and Promyo et al., 2020). Moreover, AD was also shown to induce inflammatory responses through microglial activation and liberation of proinflammatory cytokines such as IL -1, IL -12, IL -6, TNF - α, and COX -2 (Abd el-Rady et al., 2021). The monoamine oxidase-A (MAO-A) gene provides guidance for creating a monoamine oxidase- A enzyme. This enzyme is a portion of a family of enzymes that destroy certain molecules called monoamines (neurotransmitters), which convey signals between nerve cells in the brain (Chester et al., 2015). Genetic variations affecting the MAOA gene have been linked to some disorders such as memory deficiency. Some of these genetic
differences include removing DNA pieces (deletion mutations) from the MAOA gene. Deletion mutations have been detected in individuals with delayed motor skills and mental development. Individuals missing the MAO-A gene have a sharp intellectual disability and difficulty with social interactions (Endo et al., 2014). It has also been observed several common genetic changes (polymorphisms) in or near the MAOA gene affect the activity of the gene increase or decrease (Hajipour et al., 2016). There are studies that suggest that polymorphism of the MAO-A gene and increase activity of the monoamine oxidase-A (MAO-A) enzyme lead to mitochondrial dysfunctions, a synthetic impairment and ion dyshomeostasis, cause dysfunction in the brain implicated in happening Alzheimer's disease and neurological disorders such as senile dementia (Azad et al., 2011; Wojtunik-Kulesza et al., 2016 and Abd el-Rady et al., 2021).

Genetic and environmental factors have been proven to be implicated in AD progression and neurodegenerative diseases. Deposition and abnormal aggregation of heavy metals such as zinc, lead, copper, fluoride, and aluminum major playing role to a contributory in this disease (Justin Thenmozhi et al., 2017), because these metals can be pass through the blood-brain barriers and deposited in different regions of the brain such as the hippocampus and cerebral cortex (Mustafa, 2020). Aluminum sulfate and fluoride are toxicity materials for brain cells. \( Al_2(SO_4)_3 \) is the material used as a coagulant in drinking water treatment and purification in many areas of the world (Abd El-Rahman, 2003), while fluoride is overlapping industrial pollutants, some foods (such as sardines), pesticides, dietary supplements, and residues (Grandjean, 2019).

The hippocampus region is a limbic structure associated with memory and learning and the site of memory and cognitive disorders initiation as a model of Alzheimer's disease in both humans and rodents. Therefore, aggregation of the heavy metals in this region affects memory and learning (Baranauskaite et al., 2020). This study aimed to assess the likely correlation between exposure to toxicity of heavy metals (by co-administration of fluoride and aluminum) and pathological mechanisms in hippocampus of Wistar rats. It was carried out through the investigation of the activity of the monoamine oxidase-A (MAO-A) enzyme which is related to polymorphism of MAO-A gene, the concentration of amyloid-\( \beta \), levels of inflammatory cytokines and \( H_2O_2 \), and activity of the antioxidant enzymes, all these linked to memory deficits in rats as a model of Alzheimer's disease.

**MATERIALS AND METHODS**

**Ethics approval**

Experiment protocols were approved by the Department of Biology, College of Sciences, University of Babylon (Protocol No. 516 /18-May-2020). According to the National Committee for Research Ethics in Science and Technology (NETNT) (https://www.etikkom.no/en/ethical-guidelines-for-research/ethical-guidelines-for-the-use-of-animals-in-research/). Written approval was obtained from all participants before study participation.

**Experimental animals**

Fifty adult male albino Wistar rats were used obtained from the animal house of the Department of Biology, University of Babylon, Iraq. Animals were kept in a standard condition of temperature and humidity with 12-h dark-light cycle, and with free access to water and food for two weeks. The male rats were housed four per cage.

**Alzheimer’s disease model**

This model was designed by exposure of rats to a mixture of aluminum sulfate (\( Al_2(SO_4)_3 \)) and fluoride. This model simulates environmental Alzheimer's disease. The metals were obtained from the laboratories of the Department of Chemistry, College of Science, University of Babylon. Dilute mixture solutions of aluminum sulfate and fluoride were prepared (150 mg/ kg and (20) mg/ kg body weight, respectively, based on knowledge of the recorded half-lethal dose (LD50) (Han and Choi, 2009). The animals were randomly divided into two main groups. The first group (15 male rats weighing 200–240 g as a control) were orally administered 1 mL of distilled water for three consecutive months. The second group (35 male rats weighing 200–235 g as treated group) were orally administered a mixture of aluminum and fluoride (150 mg/kg and 20 mg/kg, respectively) for three consecutive months.

**Samples preparation and biochemical analysis**

**Preparation of tissues**

After the end period of the experiment, the rats were anesthetized with chloroform, the blood samples were collected and divided into two parts. The first part was used for genetic studies, and the second part was used for estimating IL-6 and TNF-\( \alpha \) in the serum. The hippocampal cortex was quickly isolated from animals and immediately stored at -20°C. Each sample was homogenized (2% M - pH 7.0 - 7.2) in the ice-cold phosphate buffer solution (PBS) by homogenizer (Potter-Elvehjem Tissue Grinder consist of PTFE smooth pestle for soft tissues, china). The hippocampal tissue homogenates were centrifuged at 4 °C, and 1500 g for 20 min. and the supernatants were used to estimate the activity of monoamine oxidase, antioxidants activity, \( H_2O_2 \) level and amyloid \( \beta \) concentration.
Monoamine oxidase-A (MAO-A) activity analysis
The activity of the MAO-A enzyme was determined using Kit (Fluorometric) manufactured by (Arigo Biolaboratories Corporation /Taiwan). "Principle of the test provides a convenient fluorimetric suitable means for measure MAO-A enzyme activity. In the test, MAO-A reacts with p -tyramine, resulting in H₂O₂ formation of, which is determined by a fluorimetric method (λem/ λex = 585/530nm)". Measure the fluorescence (FLU) of the samples and standards at λex = 530/λem = 585 nm. MAO enzyme activity is calculated in the samples:

\[ \text{MAO Activity (units/g)} = \frac{\text{FLU sample} - \text{FLU control}}{\text{Slope} \times t} \]  

….Eq.1

FLU sample = Absorbance measure in an unknown samples. FLU control = Absorbance measure in control samples (samples in presence of inhibitor). Slope = Determine from the calibration curve (μM−1). t = incubation time (20 minutes).

Estimation of the inflammatory cytokines (TNF -α and IL -6)
The tumor necrosis factor Alpha (TNF -α) and Interleukin -6 (IL -6) levels (pg/ ml) in rats serum were evaluated using an ELISA kit from (Elabscience, China) following the manufacturer’s protocol. The absorbance was read at 450 nm that is uses for the immunoassay techniques of the quantitative sandwich enzymes.

Determination activity of the antioxidant enzymes
The activity of the catalase (CAT), glutathione S -transferase (CAT), and superoxide dismutase (SOD) enzymes were analyzed in hippocampal tissue using an ELISA kit from (Elabscience, China).

Estimation of amyloid-β (Aβ) and hydrogen peroxide (H₂O₂) level in hippocampus
The Aβ concentration was determined using the Sandwich ELISA kit principle and H₂O₂ level was measured using the fluorescence assay Kit according to the manufacturer’s instructions (Elabscience, China).

Behavioral analysis
The behavioral tests were measured near the experiment’s end period to assess the effect of heavy metals that induced Alzheimer’s disease on the memory and cognition of the rats by the radial arm maze test (RAM) according to the method of Justin Thenmozhi et al. (2017). The following parameters such as working memory and reference memory, were determined. The working memory is a calculation of the numbers of duplicated entries to baited arms, indicated as (short term memory) and reference memory is the calculation of the number of entries to unbaited arms, indicated as the knowledge required for a particular situation that remains constant over time (long -term memory). The time required to complete the test in all experiments was calculated. All the behavioral tests were achieved from 09.00 a.m. to 12.00 p.m. for 10 min for three days.

Genetic analysis
Animals were sacrificed with chloroform anesthetic, blood samples were collected from the left ventricle by needle stab and 2 ml of blood was put in the EDTA tubes. Genomic DNA was obtained from the white blood cells of both the treatment and control groups were extracted using a DNA extraction kit (Favorgen). The PCR -SSCP approach was examined after the target site for MAO-A genotyping was expanded. The specific primer used during the present study was designed.

PCR primer design.
PCR primer pair of rat monoamine oxidase-A (MAO-A) gene was designed by the NCBI primer BLAST server to consolidate the targeted SNPs in the designed fragments that are localized in MAO-A exon 2 region haplotypes dependent on Primer3 plus MAO-A gene ID reference sequences: NC -00 5120.4. The genetic fragments length of (120 bp) was made to set the recommended amplicon length in the PCR -SSCP protocol (Hashim & Al-Shuhaib, 2019). The sequence forward and reverse primer was Forward 5’- CATCCCCACCAGTTTTTGACTGC3’ , Reverse5’TCCCTTGGAATACACCATGCACT3’. Statistical Analysis
Statistical significance was measured by analysis by an independent t -test between two groups. The value of p<0.05 was considered to be statistically significant for all exams. Analysis correlation between parameters done by Pearson’s test (SPSS version 23).

RESULTS
Biochemical analysis
Fig. 1(A, C, D) show that the activity of the MAO-A enzyme, H₂O₂ level and amyloid -β concentration in the hippocampus of the experimental group were significantly (P < 0.05) increased compared to the control group. The activities of antioxidant enzymes (SOD, CAT and GSH) were significantly (P < 0.05) decreased in the hippocampus of the treated rat’s group as compared to the control group (Fig. 1B). Long-term exposure to metals in the rats of the treated group significantly (P < 0.05) increased the generation of serum proinflammatory cytokines such as IL -6 and TNF -α compared to the control group, Fig. 1(E).
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**Behavioral analysis**

The analysis of the radial arm maze tests data, the rats treated with a mixture of aluminum and fluoride exhibited more errors in both the working memory and reference memory tests and needed more time to complete this task as compared to the control group, Fig. 2.

**Correlations between MAO-A enzyme and the other pathophysiological parameters of Alzheimer's disease:**

The major pathological features of Alzheimer's disease involve not only amyloid -β plaques deposition but also stimulation oxidative stress, altered activities of antioxidants, and production of proinflammatory cytokines. These pathological features have complex interactions with the increased activity of MAO-A. Table (1) shows the relationship of pathological parameters (IL6, TNF, SOD, CAT, GSH, H₂O₂, and amyloid -β) with MAO-A. There were positively significant relationships of IL -6, TNF-α, H₂O₂ and amyloid -β with the MAO-A enzyme. The antioxidant enzymes (CAT, GSH and CAT) were negatively related to the MAO-A enzyme. There were positively significant relationships between memory test (working, reference) and activity of MAO-A enzyme.

![Graphs showing changes in the activity of MAO-A, antioxidant enzymes, concentrations of amyloid-β, hydrogen peroxide level, and serum inflammatory cytokines levels in the rats treated by a mixture of aluminum and fluoride for 3 months.](image)

**Fig. 1.** Showing changes in the activity of MAO-A (A), antioxidant enzymes (catatase (CAT), glutathione S-transferase (GSH), superoxide dismutase (SOD)) (B), concentrations of amyloid- β (C), and hydrogen peroxide level (H₂O₂) (D) in hippocampus tissue, and serum inflammatory cytokines levels (tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)) (E) in the rats treated by a mixture of aluminum and fluoride (as model Alzheimer’s disease in the rats) for 3 months. Data were represented as M ± S.E. (*)represent significantly different at (p<0.05) between the control group (n = 15) and treated group (n = 35).

| Biochemical    | IL-6 | TNF-α | SOD | CAT  | GSH   | H₂O₂ | Amyloid-β | Working memory | Reference memory |
|----------------|------|-------|-----|------|-------|------|-----------|----------------|-----------------|
| MAO-A Pearson Correlation | 0.373** | 0.463** | -0.422** | -0.617** | -0.531** | 0.463** | 0.814** | 0.224 | 0.188 |
| Sig. (2-tailed) | 0.008 | 0.001 | 0.002 | 0.000 | 0.000 | 0.001 | 0.000 | 0.118 | 0.191 |

**Table 1.** Correlations between the MAO-A enzyme overactivity and the other pathophysiological and behavioral parameters of Alzheimer's disease, n=50.

*Correlation is significant at the 0.01 level (2-tailed).
MAO-A gene

Fig. 3 shows agarose gel electrophoresis of an amplified product patterns of MAO-A gene (120bp) for treatment and control groups, Fig. 4 shows PCR-SSCP for momamine oxidase-A gene. The results indicated that there was the existence of two different conformational haplotypes in treated groups, including the 1st haplotype with two bands, the 2nd haplotype patterns with 3 bands, table (2). These DNA polymorphisms must therefore be verified by in silico and DNA sequence, Fig. 5.

Fig. 6 position of methylated cytosines in CpG occurrence in the sequences of the MAO-A gene shows that these sequences contained 30 methylated CpG-dinucleotides.

DISCUSSION

Aluminum and fluoride are among the heavy metals that cause neurotoxicity through several mechanisms that lead to changes in the behavioral and biochemical parameters (Hussien et al., 2018). The results in Fig. 1. indicated that MAO-A enzyme overactivity, amyloid -β deposition, inhibition of antioxidants enzymes in the hippocampal brain of treated rats, and activated inflammatory cytokines play important roles in inducing the pathophysiology of Alzheimer's disease. In addition to the significant elevations in H2O2, which mainly lead to increased oxidative stresses in the treated group, these results are consistent with (Xie et al., 2015; Wojtunik-Kulesza et al., 2016; Hussien et al., 2018 and Grandjean 2019). Accumulation of fluoride in the brain causes neurotoxicity through changes in the nucleic acid metabolism and proteolytic enzymes that cause DNA damage may be due to these metals create oxidative stress and free radicals in the hippocampus responsible for neuronal destruction and death, DNA damage and mitochondrial dysfunctions (Kalra et al., 2016). Oxidative stress plays a critical role in memory disorders induces the development of ageing and Alzheimer's disease due to the imbalance between the removal and production of reactive oxygen species (ROS) (Weinreb et al., 2016). The free radical may be targeting the cellular proteins, polysaccharides and lipids, and increases the activities of the mitochondrial enzymes MAO-A which catalyze the oxidative deamination of many amines, such as monoamine neurotransmitters (dopamine, serotonin, and noradrenaline), resulting in a decrease their levels in the hippocampus which are linked to the progression of Alzheimer's disease and memory deficits (Naoi et al., 2016).

A further increase in oxidative stress in the hippocampus may arise from the raised activities of the mitochondrial enzymes MAO-A. This enzyme is associated with neurodegeneration through oxidative stress (derived from the increased formulation of hydrogen peroxide) (Weinreb et al., 2016). The activated MAO-A enzyme raises the expression of g-secretase and b-secretase and improves Aβ formation from amyloid precursor proteins (APP). Activated MAO-A could also be connected with the production of neurofibrillary tangles (Zhou et al., 2016).

In general, the significantly increased hydrogen peroxide (H2O2) concentrations are inactivated by the Fe2+ ions and glutathione peroxidase (GPO). This increase in hydrogen peroxide was accompanied by a significant decrease in activities of antioxidant enzymes in the rat's brain, which is considered the first line of defence against the free radicals attack, and a decrease in their activities can contribute to the tissues damage (Kumar et al., 2017). Oxidative stress stimulating inflammatory cytokines (IL-6, TNF-α) and amyloid fragments may be due to a decrease in efflux neurotransmitters expression and an increase in influx monoamine oxidase expression, leading to a rising concentration of Aβ in the brain parenchyma, Table 1 (Church et al., 2014 and...
This study exhibits that amyloid-β accumulation is associated with markedly altered behavior and neurogenesis defect. Reference memory and working memory are estimated in rats by using radial arm maze tests (Fig. 2.). Working memory is estimated by taking into account the rat's entry into each arm once, and the reference memory is examined the number of times the rat enters the arms containing food. If the rats re-enter in the arms or fail to enter into the arm that is food-containing, it is considered a reference and working memory errors (Prema et al., 2017). In the present results, treatment with aluminum and fluoride decreased the working memory and reference memory in the radial arm maze tests that were implicated in the disruption of memory related functions due to the accumulation of heavy metals in the hippocampal region that led to memory impairment and neurodegenerative disorders including neuronal loss, cognitive deficits, and learning deficits. Chabuk et al. (2019) showed that aluminum chloride caused decrease levels of brain neurotransmitters and inhibited avoidance latency and escape latency in the elevated plus-maze tests indicating memory impairment in rats. This study suggested that heavy metals cause MAO-A overactivity in the hippocampus, which has been associated with cognitive and learning deficits.

Table 2. PCR-SSCP haplotype distribution of MAO-A gene

| Genotype | Treated group | Control group | P Value | OR=(95%CI) |
|----------|---------------|---------------|---------|------------|
| 2 bands  | 1 (2.8%)      | 5(33.3%)      |         |            |
| 3 bands  | 34(97.2%)     | 10(66.6%)     | 0.007*  | 0.05 (0.006-0.56) |
| total    | 35            | 15            |         |            |

P ≤ 0.05; OR=(95%CI).
Fig. 5. Sequence alignment of exon 2 region MAO-A gene accession number NC-005120.4 by Bio Edit program version 7.2.5

In total 15 sequences have been analyzed.

Position of methylated cytosines in CpG context in the alignment

Fig. 6. MAO-A gene methylation

In total these sequences contained 30 methylated CpG-dimucleotides. On average 2.00 CpG-dimucleotides have been found in each DNA Sequence corresponding to 1.67 methylated Cytosines in 100 nucleotides.
have been shown (Lau et al., 2009). Sequence analysis of MAO-A showed the presence of two CpG islands in the gene (Fig. 4.). The first CpG island is located in 9, and the second CpG island is located in 74. Methylation (Fig. 6.) can be detected and quantified by many methods, but all studies currently available have certain advantages. We have therefore tried to assess the region’s epigenetic potential using sequence-based analysis systematically. In fact, all bioinformatics analysis has its own features. Such as one advantage of the in silico analysis is that it is independent of the detection methods used. The sequence-based analysis is not dependent on the type of biological sample nature used for the analysis (Bock et al., 2007). Genetic variations and epigenetic variants of the MAO-A can be a solid indicator of the enzyme’s catalytic activity in question, possibly due to a variety of environmental factors. Nevertheless, this result can be viewed as an even more accurate and precise biological index for neurodegenerative disorders (Godar et al., 2016).

Conclusion

The current study obviously points to the role of heavy metals in inducing oxidative stress, the inhibition of antioxidant activity, the hyperactivity of the MAO-A enzyme linked with revealing the gene polymorphism MAO-A changes, and the amyloid-β deposition in the hippocampal tissues of the rats, which were associated with memory deficits in addition to stimulating inflammatory cytokines. These factors played a critical role in inducing the pathophysiology of the Alzheimer’s disease model in adult albino Wistar rats.

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Conflict of interest

The authors declare that they have no conflict of interest.

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