Potential Neuroprotective Effect of Clopidogrel on Aluminum Chloride-Induced Alzheimer Disease in Rats.

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Abstract

Background and aim: Alzheimer's disease (AD) is a common progressive disease characterized by neurodegeneration. Multiple molecular mechanisms such as amyloid β ($A\beta$) formation, tau protein hyperphosphorylation, reduced cholinergic neurotransmission, oxidative stress, and neuroinflammation are involved in the disease pathophysiology. The purpose of this study was to assess potential neuroprotective effect of clopidogrel in AD model induced by aluminum chloride ($AlCl_3$) in rats

Methods:

Forty adult male Sprague-Dawley rats were haphazardly separated into four groups of ten rats each: Control, $AlCl_3$ (100 mg/kg orally), $AlCl_3$ + Memantine (10 mg/kg orally), and $AlCl_3$ + Clopidogrel groups (20 mg/kg orally). $AlCl_3$ and drugs were administered once every day for 42 days. The spatial learning and memory were evaluated using Morris Water Maze (MWM) test. After euthanization, hippocampal acetylcholinesterase (AChE) activity, tumor necrosis factor alpha ($TNF-\alpha$), and interleukin-1beta ($IL-1\beta$) concentrations were biochemically assessed in all groups. Moreover, amyloid precursor protein (APP) mRNA gene expression was analyzed in the hippocampus of all groups with Real-time quantitative polymerase chain reaction (qPCR). Histopathology for amyloid plaques was done in all groups' hippocampus using hematoxylin and eosin and Congo stain.

Results: $AlCl_3$ and clopidogrel co-treatment significantly ameliorated the cognitive deficits induced by $AlCl_3$ in rats. Moreover, $AlCl_3$ and clopidogrel co-treatment significantly reduced AChE activity, $TNF-\alpha$ and $IL-1\beta$ concentrations, and APP mRNA gene expression in the rat hippocampi compared to $AlCl_3$ -treated rats. $AlCl_3$ and clopidogrel co-treatment significantly reduced $TNF-\alpha$ and $IL-1\beta$ concentrations in the rat hippocampi compared to $AlCl_3$ and memantine co-treated rats. In addition, $AlCl_3$ and clopidogrel co-treatment alleviated amyloid plaque deposition in the rat hippocampal tissues stained with hematoxylin and eosin and Congo stains compared to $AlCl_3$ -treated rats.

Conclusion: These results showed that clopidogrel could improve cognitive deficits triggered by $AlCl_3$ in rats. The neuronal protection influence of clopidogrel in $AlCl_3$ -triggered AD might be mediated through its anti-inflammatory effect as demonstrated by its ability to decrease hippocampal $TNF-\alpha$ and $IL-1\beta$ concentrations. It might also be mediated through its lowering effect on AChE activity and/or decreasing mRNA gene expression of APP gene in the hippocampus

Keywords: Alzheimer's disease, Neuroinflammation, Clopidogrel, Aluminum chloride

Introduction

Alzheimer's disease (AD) is a rapidly deteriorated disease prevalent all over the world, characterized by neurodegeneration, but yet with no effective cure (**Chen et al, 2018**). The pathogenesis of this disease is still unclear. Many hypotheses have been proposed for AD, involving amyloid β ($A\beta$) deposition, tau protein hyperphosphorylation, cholinergic nerve cell destruction, neuroinflammation, oxidative stress, etc. (**Du et al, 2018**). The pathological hallmarks observed in AD brain include extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs). Clinically, AD patients suffer from learning and memory deficits, and neuropsychiatric and behavior changes (**Chen et al, 2018**).

Cholinesterase inhibitors donepezil, galantamine and rivastigmine and the N-methyl-D-aspartate (NMDA) receptor blocker memantine are the only food and drug administration (FDA) approved AD treatments (**Kim et al, 2017**). These approved drugs provide only symptomatic relief and haven't shown to prevent or delay the progression of disease (**Deardorff & Grossberg, 2016**). Therefore, seeking effective disease-modifying therapeutics with multiple targets is highly desirable (**Chen et al, 2018**).

The current study aimed to evaluate potential neuronal protection effect of clopidogrel in aluminum chloride ($AlCl_3$)-triggered AD in rats and compare it with the effect of memantine. It also aimed to understand its possible mechanism(s) for neuroprotection.

Material and Methods

Chemicals and drugs

Aluminum chloride anhydrous (molecular weight: 133.332 g/mol) was purchased from **El-Gomhouria Chemical Company, Mansoura, Egypt**. Memantine hydrochloride was used in the form of Ebixa tablets (manufactured by **Lundbeck**

company, Denmark). Clopidogrel bisulphate was used in the form of Plavix tablets (manufactured by **Sanofi- Aventes company, France**).

Experimental animals

Adult male Sprague-Dawley rats were utilized at the study beginning with a weight of 200-250 g. They were obtained from the Medical Experimental Research Center (MERC), Mansoura Faculty of Medicine. For seven days before the study, the animals were adapted to ordinary laboratory circumstances; adjusted temperature ($23 \pm 2^\circ C$), humidity ($50 \pm 5\%$) and 12 hours cyclic exposure to light and dark. The animals were housed in plastic cages lined with sawdust that was renewed daily and were freely fed on food and water ad libitum. These entire manipulations were performed in the daylight between 09.00 a.m. and 5.00 p.m.

Animal care and ethical approval

Laboratory animal handling was done according to the "Guide for the Care and Use of Laboratory Animals" organized by the institute of Laboratory Animal Research and issued by the National Research Council, USA, 2011 (**Albus, 2012**). The study design and protocol were reviewed and accepted by Mansoura Faculty of Medicine, Institutional Research Board (IRB) under the code of MD15.08.06.

Experimental design

Forty adult male Sprague-Dawley rats were haphazardly separated into four groups (ten rats each): **Control group**: administrated the vehicle for $AlCl_3$ (distilled water) daily orally for 42 days. **$AlCl_3$ group**: received $AlCl_3$, 100 mg/kg daily orally for 42 days. $AlCl_3$ + Memantine group: received $AlCl_3$, 100 mg/kg and memantine hydrochloride, 10 mg/kg (**Ahmed et al, 2014**) daily orally for 42 days. **$AlCl_3$ + Clopidogrel group**: administrated $AlCl_3$, 100 mg/kg and and clopidogrel bisulphate, 20 mg/kg (**Tu et al, 2008**) daily orally for 42 days.

The drugs were introduced by mouth 1 h after AICl₃ treatment and continued for 42 days (6 weeks) (Kumar et al, 2009). The AICl₃ dose used was chosen depending on an earlier study (Thippeswamy et al., 2013) and a pilot study in our laboratory that showed AICl₃ in a dosage of 100 mg/kg was associated with the most obvious pathological characteristics of AD especially amyloid plaques. The mortality rate with AICl₃ (100 mg/kg) was about 20%. Other groups showed mortality rates of 0-10%.

Evaluation of learning and memory by Morris Water Maze (MWM) test

Morris Water Maze is formed of a big circular pool (its diameter is 150 cm, its height is 45 cm) filled with water at $28 \pm 1^\circ\text{C}$ to 30 cm depth and divided into four equivalent quadrants (N, S, E, and W) by two strands fixed at right angles. The pool was positioned in an illuminated test chamber. In any pool quadrant, a circular platform (its diameter is 4.5 cm) was positioned, 1 cm above the water level by 1 cm during the acquisition stage. This platform was positioned below the water level by 1 cm during the retention stage. The platform location was constant in the same quadrant during evaluation of both stages (Kumar et al, 2011). The water was made opaque during the retention phase by adding a nontoxic dye e.g. starch. During the test the rats were trained to avoid swimming by jumping onto the platform and by time the rats seemingly learn the spatial platform site from any beginning site at the pool circumference (Prashar et al, 2014).

a) Maze acquisition phase (training)

On day 20, animals were trained four times with 5 minutes interval in between. During the four training sessions, dissimilar beginning sites (N, S, E, and W) were used. A trial was initiated by introducing the animal into the maze fronting the pool wall and the delay to discover and jump onto the platform was noted to a 90 second maximum. If

the rat did not jump onto the platform within 90 seconds, it was carried and directed to the platform and was kept there for 20 seconds. The initial acquisition latency (IAL) was described as the time consumed by the animal to jump on the platform (Ramachandran et al, 2013; Thippeswamy et al, 2013).

b) Maze retention phase (testing for retention of learned task)

After the four training sessions, the time consumed to discover and reach the hidden platform (retention latency "RL") was recorded and evaluated on day 21 (1st RL) and day 42 (2nd RL). The alteration in RL from day 21 to day 42 was utilized to assess the acquired skill or memory (Ramachandran et al, 2013; Thippeswamy et al, 2013).

Brain Tissue Sampling and Preparation

When the study was finished, rats were deprived from food and water in the whole night, then euthanized by decapitation following intraperitoneal thiopental sodium injection (40 mg/kg) (IACUC Guidelines: Anesthesia. The university of Iowa, Office of Animal Resources, Institutional Animal Care and Use Committee, 2017). The skull was opened with cautious and each rat's entire brain was quickly taken out and cut mid-sagittally into two hemispheres. Hippocampus was micro-separated out from each hemisphere according to procedure documented previously (Carleton et al, 1980).

Hippocampal halves from rats of each group were used as follows:

They were further equally divided to be processed either: 1) cleaned with ice-cold saline to eliminate blood, quickly kept in eppendorf tubes, embedded in liquid nitrogen and stored at -80°C till its usage for Real-time quantitative polymerase chain reaction (qPCR) (Balgoon et al, 2015) or 2) cleaned with isotonic saline, desiccated on filter

paper, and weighed then immediately homogenized in frozen phosphate-buffered saline at pH 7.4. Centrifugation of the homogenate was done at 2000-3000 rpm for 20 min at 4°C and the supernatant was separated and stored at -20°C until its usage for biochemical estimations.

Other hippocampal halves were kept in 10% neutral buffered formalin to be fixed and then examined histopathologically by hematoxylin and eosin and Congo stains (**Balgon et al, 2015**).

Biochemical Estimations

Rat hippocampal tissue homogenate was used to evaluate acetyl cholinesterase (AChE) activity by AChE Assay Kit (Colorimetric) (**Abcam Co., UK, Cata. No.: ab138871**) according to their manufacturer's instructions. In addition, tumor necrosis factor alpha (TNF- α) and interleukin-1beta (IL-1 β) concentrations were estimated in rat hippocampal tissue homogenate using rat enzyme linked immunosorbent assay (ELISA) kits (**Bionevan Co. Ltd., China, Catalog. No. In-Ra1371 for TNF- α , Catalog. No.: In-Ra0668 for IL-1 β**) according to their manufacturer's instructions.

Real-time quantitative reverse transcription-polymerase chain reaction (Real-time qRT-PCR) for amyloid precursor protein (APP) gene expression determination in rat hippocampus

Entire RNA was taken out from rat hippocampal tissue samples in all groups by RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instruction. NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) was used to detect quantity and purity of every RNA sample using 260 and 260/280 nm ratio respectively. RNA sample purity was detected and it ranged from 1.8 to 2.1 indicating its high quality. RNA was reverse-transcribed into complementary DNA (cDNA) using Arktik Thermal Cycler (Thermo Fisher Scientific Inc., MA, USA) and quantified using infra-red

specific primer by Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The primers were manufactured by Primers Biosearch technologies (Primers Biosearch technologies, CA, USA) and the specific gene sequences were obtained from Pubmed (Entrez Gene). The primers utilized in this experiment included β -actin (the control gene) F: 5'-CCCATCTATGAGGGTTACGC-3'; β -actin R: 5'-TTTAATGTCACGCACGATTTC-3' (**Zhou et al, 2009**); APP (the studied gene) F: 5'-TGGGTTGACAAACATCAAGACAGAA-3'; APP R: 5'-GCACCTTTGTTTGAACCCACATC-3' (**Ying-Cai et al, 2007**). The thermal cycling conditions were adjusted as follow: 15 min at 95 °C for DNA polymerase stimulation and subsequently 40 cycles of 15 seconds at 95 °C, 20 seconds at 60 °C and 20 seconds at 72 °C.

mRNA expression's relative quantification (RQ) was detected with the $2^{-\Delta\Delta Ct}$ method (**Schmittgen & Livak, 2008**). The data were presented as target mRNA relative quantity, normalized respect to β -actin mRNA and relative to a calibrator sample. Normal control samples were used as calibrators. Where: $\Delta Ct = (Ct \text{ of target gene} - Ct \text{ of reference gene})$; $\Delta\Delta Ct = (\Delta Ct \text{ of sample} - \Delta Ct \text{ of control})$. Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

Histopathological examination

Rat brain hippocampus specimens were kept in 10% formalin for 24 hours to be fixed and subsequently cleaned with tap water. Sequential alcohol dilutions were used for desiccation. Specimens were cleaned in xylene immersed in paraffin in hot air oven at 56°C for 24 hours. Paraffin bees wax tissue block preparation was performed to be divided by microtome at 4 microns thickness. The resulting tissue slices were put on glass slides, cleared from paraffin and stained with hematoxylin and eosin stain for histopatho-

logical examination under light microscopy (Ali et al, 2016). Congo red staining was done consistent with the manufacture's strategy. The hippocampal slice was put a slide and desiccated in air. The slice was embedded first in 80% ethanol containing 4% sodium chloride for 1 hours, then embedded in 80% ethanol containing 0.2% Congo red at room temperature for 1 hours. The sections were cover slipped and examined under light microscope (Guo et al, 2015).

Statistical Analysis

The results were statistically analyzed using Statistical Package for Social Science (SPSS) program, version 23.0, for windows 10. Charts were done using SPSS and/ or Excel program (Microsoft Excel, version 14.0.4734.1000, 2010). The parametric results were expressed as Mean $\pm$ SD. One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparisons were used for statistical analysis between groups. Repeated measures ANOVA followed by post-hoc Bonferroni test were used for analyzing MWM data (IAL, 1st RL, and 2nd RL) within groups. P value < 0.05 indicate statistical significance.

Results :

Effect of memantine or clopidogrel on memory function in spatial navigation task of Morris Water Maze (MWM) test in aluminum chloride (AlCl₃)-treated rats

In the spatial navigation task of MWM test, control rats were educated to directly swim to the visible platform rapidly on the day 20. AlCl₃-treated rats demonstrated a significant rise in mean IAL to jump onto the visible platform when compared to control rats on the day 20 ($p < 0.001$). On the other hand, concomitant administration of memantine or clopidogrel with AlCl₃ significantly reduced the IAL to jump onto the visible platform when compared to AlCl₃-treated rats on the day 20 ($p < 0.001$), **Figure 1**.

After the four training sessions, the visible platform was hidden. AlCl₃-treated rats demonstrated a significant increase in mean 1st and 2nd RLs when compared to control rats on the days 21 and 42, respectively. ($p < 0.001$). However, concomitant administration of memantine or clopidogrel with AlCl₃ demonstrated a significant decrease in the 1st and 2nd RLs when compared to AlCl₃-treated rats on the days 21 and 42, respectively ($p < 0.001$), **Figure 1**.

Effect of memantine or clopidogrel on acetylcholinesterase (AChE) activity, and tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) concentrations in hippocampus of aluminum chloride (AlCl₃)-treated rats

Continuous AlCl₃ administration to rats demonstrated a significant rise in rat hippocampal AChE activity when compared to control rats ($p < 0.001$). However, concomitant administration of memantine or clopidogrel with AlCl₃ significantly attenuated the rise in AChE activity when compared to AlCl₃-treated rats ($p < 0.001$). Rat hippocampal AChE activity in AlCl₃ + memantine group or AlCl₃+clopidogrel was still significantly raised when compared to control group ($p < 0.001$); but not significantly changed when compared to each other ($p = 0.96$), **Table 1**.

Continuous AlCl₃ administration to rats demonstrated a significant rise in the rat hippocampal TNF- α and IL-1 β concentrations when compared to control rats ($p < 0.001$). However, concomitant administration of memantine or clopidogrel with AlCl₃ significantly decreased rat hippocampal TNF- α and IL-1 β concentrations when compared to the AlCl₃-treated rats ($p < 0.001$). In addition, AlCl₃ and clopidogrel co-treatment significantly decreased rat hippocampal TNF- α and IL-1 β concentrations when compared to AlCl₃ and memantine co-treatment ($p < 0.01$). Rat hippocampal TNF- α and IL-1 β concentrations in AlCl₃ + memantine group were still significantly raised when compared to control group ($p < 0.001$); while,

TNF- α and IL-1 α concentrations in AlCl₃ + clopidogrel group were not significantly raised when compared to control group (**p = 0.75 and p = 0.07 respectively**), **Table 1**.

Effect of memantine or clopidogrel on relative quantification (RQ) of amyloid precursor protein (APP) mRNA gene expression in hippocampus of aluminum chloride (AlCl₃)-treated rats

Continuous AlCl₃ administration to rats demonstrated a significant rise in the rat hippocampal APP gene expression when compared to control rats (**p < 0.001**). However, concomitant administration of memantine or clopidogrel with AlCl₃ significantly decreased rat hippocampal APP gene expression when compared to the AlCl₃-treated rats (**p < 0.001**). The rat hippocampal APP gene expressions of AlCl₃ + memantine group or AlCl₃ + clopidogrel group are still significantly raised when compared to control group (**p < 0.001**) but not significantly different when compared to each other, **Table 1**.

Effect of memantine or clopidogrel on histopathological examination in hippocampus of aluminum chloride (AlCl₃)-treated rats

Examination of the hippocampus of AlCl₃ group by hematoxylin and eosin revealed areas of brain cell apoptosis (**Figure 4**). Congo red-stained

slides examined under light microscopy revealed orange brown plaque-like structures (**Figure 5 A**) in addition to orange stained thickened wall of the cerebral blood vessels (**Figure 5 B**) raising the possibility of amyloid deposition. When the Congo-red stained slides examined under the polarized light, it showed birefringence both in hippocampal brain tissue (**Figure 5 C**) as well as the wall of the hippocampal blood vessels (**Figure 5 D**) confirming the presence of amyloid plaques.

Examination of the hippocampi of AlCl₃+ Memantine group by hematoxylin and eosin revealed more or less normal hippocampal brain tissue with normal brain cellularity and normal thickness of the hippocampal blood vessels when compared to AlCl₃ group (**Figure 6 A, B**). No amyloid plaques were detected neither in hematoxylin and eosin-stained slides (**Figure 6 A, B**) nor in Congo red-stained slides examined both under light microscopy and polarized light (**Figure 7 A, B**). Histopathological results similar to AlCl₃+Memantine group were demonstrated on examination of the hippocampi of AlCl₃+Clopidogrel group with no amyloid plaques detected neither in hematoxylin and eosin-stained slides (**Figure 8 A, B**) nor in Congo red-stained slides when examined both under light microscopy and polarized light (**Figure 9 A, B**).

Table 1: Effect of memantine (10 mg/kg) or clopidogrel (20 mg/kg) on AChE activity, tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) concentrations, and relative quantification (RQ) of amyloid precursor protein (APP) mRNA gene expression in aluminum chloride (AlCl₃)-treated rats (100 mg/kg).

Groups	Acetylcholinesterase (AChE) activity (U/mg tissue)	Tumor necrosis factor- α (TNF- α) concentration (pg/mg tissue)	Interleukin-1 β (IL-1 β) concentration (pg/mg tissue)	Relative quantification (RQ) of amyloid precursor protein (APP) gene expression
Control group	0.15 $\pm$ 0.035	114.8 $\pm$ 6.91	30.34 $\pm$ 3.14	1.01 $\pm$ 0.11
AlCl ₃ group	0.34 $\pm$ 0.037 #	165.1 $\pm$ 11.18 #	67.99 $\pm$ 5.02 #	2.5 $\pm$ 0.17 #
AlCl ₃ +Memantine group	0.25 $\pm$ 0.032 # \$	136.6 $\pm$ 8.37 # \$	50.54 $\pm$ 7.49 # \$	1.5 $\pm$ 0.14 # \$
AlCl ₃ +Clopidogrel group	0.24 $\pm$ 0.033 # \$	119.8 $\pm$ 6.25 \$ &	38.20 $\pm$ 6.67 \$ &	1.58 $\pm$ 0.15 # \$
<i>p</i> value of ANOVA	<0.001	<0.001	<0.001	<0.001

Data were expressed as means $\pm$ SD (n=8) and were tested by one-way ANOVA followed up by Tukey post hoc test. *p* value < 0.05 is considered to indicate statistical significance.

#: significance relative to Control group

\$: significance relative to AlCl₃ group

&: significance relative to AlCl₃ + Memantine group

Figure (1): Effect of memantine (10 mg/kg) or clopidogrel (20 mg/kg) on memory function in Morris Water Maze (MWM) test in aluminum chloride (AlCl₃)-treated rats (100 mg/kg).

Figure (2): Hippocampus of control group stained with hematoxylin and eosin.

Control group's hematoxylin and eosin-stained section (x100) revealed normal hippocampal brain tissue with no detected microscopic abnormalities.

Figure 3: Hippocampus of control group stained with Congo red.

Control group's Congo red-stained sections (x100) both under light microscopy (A) and polarized light (B) revealed unremarkable pathological changes.

Figure 4: Hippocampus of AlCl₃ group stained with hematoxylin and eosin.

AlCl₃ group's hematoxylin and eosin-stained section (x100) revealed multiple apoptotic bodies (tip of black arrows).

Figure 5: Hippocampus of AlCl_3 group stained with Congo red.

AlCl_3 group's Congo red-stained sections (**x200**) examined under light microscopy revealed **(A)** orange brown plaque-like structures (thin black arrows) within the hippocampal brain tissue; **(B)** orange stained thickened wall of the hippocampal blood vessels (thick black arrows). AlCl_3 group's Congo red-stained sections (**x200**) examined under the polarized light showed **(C)** birefringence (white arrows) in hippocampal brain tissue and **(D)** the wall of the hippocampal blood vessels confirming the presence of amyloid plaques.

Figure 6: Hippocampus of AlCl₃+Memantine group stained with hematoxylin and eosin.

AlCl₃+Memantine group's hematoxylin and eosin-stained sections (x200) revealed (A) more or less normal hippocampal brain tissue with normal cellularity and (B) normal thickness of the blood vessels (white arrows). No amyloid plaques were detected.

Figure 7: Hippocampus of AlCl₃+Memantine group stained with Congo red.

AlCl₃+Memantine group's Congo red-stained sections (x200) revealed absent amyloid plaques when examined (A) under light microscopy and (B) polarized light.

Figure 8: Hippocampus of AICl3+Clopidogrel group stained with hematoxylin and eosin.

AICl3+Clopidogrel group's hematoxylin and eosin-stained sections (A, x100 and B, x200) revealed (A) more or less normal hippocampal brain tissue with normal cellularity and (B) normal thickness of the blood vessels (black arrows). No amyloid plaques were detected.

Figure 9: Hippocampus of AICl3+Clopidogrel group stained with Congo red.

AICl3+Clopidogrel group's Congo red-stained slides (x200) revealed absent amyloid plaques when examined (A) under light microscopy and (B) polarized light.

Discussion

The current study's results demonstrated that continuous AICl₃ administration in rats for 42 days led to spatial learning and memory impairment as assessed by MWM. AICl₃-treated rats showed a significant prolongation in time taken to jump onto the platform in both maze acquisition and retention phases compared to control rats. These results are in consistent with earlier experimental studies that demonstrated cognitive deficits caused by the chronic AICl₃ administration in rats (Qusti, 2017; Rather et al, 2018), and mice (Jangra et al, 2015; Kasbea et al, 2015). Cognitive decline after aluminum (Al³⁺) exposure may be caused by numerous Al³⁺ actions on the nerve cells. Exposure to Al³⁺ can result in a dramatic accumulation of Al³⁺ in the brain leading to a severe impairment in learning and memory abilities and in long term potentiation (LTP) in the hippocampus (Liang et al, 2012). This impairment is caused by Al³⁺ capacity to inhibit downstream effector mediators e.g. cyclic guanosine monophosphate (cGMP) participating in LTP and disrupt the glutamate-nitric oxide-cGMP pathway in the rat brain (Canales et al, 2001).

In contrast, co -administration of memantine or clopidogrel with AICl₃ for 42 days to rats significantly improved spatial learning and memory deficits as shown by a significant decline in the MWM test's 1st and 2nd RLs compared to AICl₃-treated rats.

Previous studies also supported that memantine improved memory and cognition assessed by MWM test (Abdel-Aal et al, 2011; Ahmed et al, 2014). Memantine could prevent Al³⁺-induced neuronal toxicity and cognitive deficit by several mechanisms. Memantine unexpectedly is a more potent blocker when glutamate levels rise due to its low affinity and non-competitive antagonism for NMDA receptor, so causing memantine to favora-

bly decrease pathological NMDA receptor stimulation without disrupting the physiological activity important for cognition (Lockrowa et al, 2011). Memantine also decreased elevated AChE enzyme activity (Ahmed et al, 2014; Al-Bishri et al, 2017). Moreover, memantine prevent cognitive deficits induced by neuroinflammation (Rosi et al, 2009) and enhanced cognitive functions in A β -induced AD in rats (Nyakas et al, 2010). In the current study, memantine significantly decreased hippocampal AChE activity, TNF- α and IL-1 β concentrations, APP mRNA gene expression and A β deposition.

To our knowledge, this is the initial study to test clopidogrel neuroprotective influence on AICl₃-triggered AD in rats. Clopidogrel cognitive enhancing effect might be due to its ability to decrease AChE activity and hence improving cholinergic neurotransmission as shown in the current study. Other mechanisms for cognitive enhancement produced by clopidogrel might be due to its ameliorating effect on inflammation by significantly decreased hippocampal TNF- α , IL-1 β levels or its lowering effect on APP mRNA gene expression and A β as shown in the current study. Several previous studies demonstrated an anti-inflammatory effect of clopidogrel in different animal models (Patel et al, 2012; Webster et al, 2013; Suh et al, 2016).

In the current study, AICl₃-treated group exhibited a significant elevation of AChE activity in the hippocampus as compared to control rats. Information about the AChE activity alteration in AD animal models are not constant, with some showing elevated, and others showing suppressed activity. These divergences may be caused by the variations in animal models and experimental methods, involving timing of sample taking for assessing activity (Xiao et al, 2011). In consistency with our results, previous studies reported increased AChE activity with AICl₃-treated rats (Lin

et al, 2015; Prema et al, 2016; Qusti, 2017; Rather et al, 2018). The increased AChE activity in AlCl_3 -treated rats might be caused by direct Al^{3+} -induced neuronal toxicity. Prolonged Al^{3+} administration changes AChE kinetics and its elevated activity may be attributed to Al^{3+} ability to allosterically interact with the enzyme peripheral anionic site, causing modification of its secondary structure, thereby enhancing its activity (Gulya et al., 1990; Zatta et al, 1994). Another possible mechanism for increased AChE is due to IL-1 β overproduction as shown in the current study that promotes the activity and expression of AChE via interaction of IL-1 β with muscarinic acetylcholine receptors (AChRs) (Schliebs et al, 2006). Additional explanation might be due to A β deposition as shown in the current study. A β combines with nicotinic AChRs causing enhanced AChE activity (Arendt et al, 1984).

Contrarily, other studies reported that AlCl_3 administration inhibited AChE activity compared to control groups (Lakshmi et al, 2014; Lakshmi et al, 2015; Taïr et al, 2016; Singla & Dhawan, 2017). The reduction in AChE activity may be attributed to Al^{3+} attachment to SH-groups of AChE catalytic active site, which ultimately have hindered the activity of enzyme in various chemical reactions (Singla & Dhawan, 2017).

Co-administration of memantine or clopidogrel with AlCl_3 to rats results in significant decrease of hippocampal AChE activity compared to AlCl_3 -treated rats. Memantine had shown to decrease AChE activity in several studies (Ahmed et al, 2014; Al-Bishri et al, 2017). The decreased AChE activity with memantine might be due to ability of memantine to lower IL-1 β concentration or APP mRNA gene expression and A β deposition as shown in the current study. This study is the first study to show clopidogrel inhibitory effect on AChE activity in AlCl_3 -induced AD model. Similarly, the decreased AChE activity with clopidogrel

might be due to its ability to lower IL-1 β concentration, APP mRNA gene expression and/or A β deposition as shown in the current study.

The current study's results demonstrated that continuous AlCl_3 administration in rats significantly elevated hippocampal TNF- α , IL-1 β concentrations compared to control rats. These data are consistent with other studies in rats (Cao et al, 2016; Qusti, 2017; Rather et al, 2018; Ravi et al, 2018) and mice (Jangra et al, 2015; Kasbea et al, 2015). In humans, brains and plasma of AD patients showed elevated TNF- α concentrations (Chang et al, 2017). Similarly, IL-1 β was elevated in both the brains (Cacabelos et al, 1994) and plasma of AD patients (Forlenza et al, 2009).

Aluminum is strongly implicated in neuroinflammation induction and inflammatory cytokine production (Cao et al, 2016). Al^{3+} can activate the glial cells and initiate the macrophages responsiveness (Campbell et al, 2002). Microglial stimulation by Al^{3+} leads to marked proinflammatory cytokine generation e.g. TNF- α , IL-1 β , IL-6 (Cao et al, 2016). Moreover, Al^{3+} activates the transcription factor nuclear factor-kappa B (NF- κ B), mitogen-activated protein kinase/activator protein-1, and hypoxia inducible factor-1 (HIF-1), which are implicated in inflammation (Lukiw et al, 2005; Verstraeten et al, 2008). In addition, accumulation of Al^{3+} in the hippocampus causes abnormal deposition of A β (Wang et al, 2014) which induces microglial chemotaxis and provide a chronic stimulus to microglial cells contributing to the ongoing inflammatory process (Lue et al, 2001). Also, A β itself may act as an inflammation inducing factor leading to stimulation of several inflammatory components (Ghavami et al, 2014).

Additional underlying mechanism for increased hippocampal TNF- α and IL-1 β concentrations by chronic AlCl_3 administration is Al^{3+} -induced excitotoxicity. Continued Al^{3+} administration was

shown to elevate brain glutamate concentration (**Abdel-Zaher et al, 2017**). Functional ionotropic glutamate receptors such as NMDA receptors in the microglia respond to glutamate release during synaptic activity or damage leading to inflammatory cytokine liberation such as TNF- α and IL-1 β (**Domercq et al, 2013**).

In contrast, the current study revealed that co-administration of memantine or clopidogrel with AICl₃ to rats resulted in significant decrease of hippocampal TNF- α , IL-1 β concentrations compared to AICl₃-treated rats. In addition, the decrease of hippocampal TNF- α , IL-1 α concentrations by clopidogrel was significant compared to its decrease by memantine.

Several studies using memantine reported similar results in AICl₃ induced model (**Alawdi et al, 2017**) and in other AD animal models (**Hemmati et al, 2013; Rai et al, 2013; Susmita et al, 2016; Budni et al, 2017**). Memantine was also shown to possess anti-inflammatory effects in vitro in microglia and astrocytes (**Wu et al, 2009**) and in T cells (**Kahlfub et al, 2014**), and in vivo against morphine addictive behavior in rats (**Chen et al, 2013**), and lipopolysaccharide (LPS)- induced acute lung injury in mice (**Ma et al, 2014**). Also, it was shown that memantine possess anti-inflammatory effects in clinical trials of bipolar depression (**Lee et al, 2014**).

One explanation for decreased hippocampal TNF- α and IL-1 β concentrations by memantine administration is due to its ability to inhibit NF- κ B pathway as shown in several studies in AICl₃-induced AD model (**Ahmed et al, 2014; Alawdi et al, 2017**). Another explanation is due to ability of memantine to activate cholinergic anti-inflammatory pathway by its inhibitory effect on AChE activity leading to increased acetylcholine (ACh) level. ACh is capable of suppression of the inflammatory cytokine liberation such as TNF- α

and IL-1 β (**Shytle et al, 2004**). Also, decreased hippocampal TNF- α and IL-1 β concentrations by memantine might be due to its lowering effect on APP mRNA gene expression and A β as shown in the current study. Additional possible mechanism might be due to its ability to block NMDA receptor and halt Al³⁺-induced excitotoxicity and glutamate release (**Abdel-Zaher et al, 2017**).

This is the initial study to demonstrate inflammation ameliorating influence of clopidogrel in AICl₃-induced AD model. Several studies showed inflammation ameliorating influence of clopidogrel in global cerebral ischemia (**Webster et al, 2013**) and other models of LPS-induced inflammation (**Hagiwara et al, 2011**), atherosclerosis (**Hadi et al, 2013**), renal injury (**Tu et al, 2008**), inflammatory bowel disease (IBD) (**Patel et al, 2012**), and asthma (**Suh et al, 2016**). Molecular explanation for decreased hippocampal TNF- α and IL-1 β concentrations by clopidogrel administration could be due to its ability to inhibit NF- κ B pathway as shown in a previous study of bilateral common carotid artery occlusion (global cerebral ischemia) where clopidogrel treatment decreases NF- κ B expression in hippocampal CA1 neuron and improved its viability and decreased immune responses (**Webster et al, 2013**). Another explanation is due to its ability to activate cholinergic anti-inflammatory pathway by its inhibitory effect on AChE activity as shown in the current study leading to increased ACh level. Also, decreased hippocampal TNF- α and IL-1 β concentrations by clopidogrel might be due to its lowering effect on APP mRNA gene expression and A β as shown in the current study.

Additional explanation for decreased hippocampal TNF- α and IL-1 β concentrations by clopidogrel is due to its ability to block P₂Y₁₂ receptors on the microglia leading to inhibition of microglial chemotaxis and consequently microglial phagocytosis. Chemokines and nucleotide adenosine tri-

phosphate (ATP) manage proper microglial migration to destructed region (Noda & Suzumura, 2012). Induction of apoptosis and necrosis by Al^{3+} was previously reported in rats and mice (Prakash & Sudhandiran, 2015; Said & Abd Rabo, 2017). Apoptosis and necrosis of neurons in AD result in release of ATP (Glass et al, 2010) which induces microglial migration (chemotaxis) through P_2Y receptors, especially P_2Y_{12} (Noda & Suzumura, 2012). P_2Y_{12} receptor is solely present in microglia in the central nervous system, is not present in peripheral macrophages and so, is a reliable marker to differentiate inhabitant microglia from penetrated macrophages (Tronel et al, 2017). The initial chemotaxis of microglial cells serves as a precursor to subsequent microglial activation and functional responses such as phagocytosis; however, chemotactic responses are also ongoing and could contribute to neuronal degeneration in environments of chronic neuroinflammation in brain and in the progression of disease (McLarnon, 2012).

Also, a unique explanation for decreased hippocampal $TNF-\alpha$ and $IL-1\beta$ concentrations by clopidogrel administration is due to its antiplatelet effect. A number of studies demonstrated that platelet activation is strongly linked to inflammation, and platelet activation can stimulate or exacerbate inflammation in vivo (Weyrich et al, 2003; Gawaz et al, 2005; Patel et al, 2012). Clopidogrel can suppress platelet activation by P_2Y_{12} receptor blockade (Tu et al, 2008). Several studies showed that clopidogrel reduced inflammation by its antiplatelet effect in various animal models (Evangelista et al, 2005; Angiolillo et al, 2006; Tu et al, 2008; Patel et al, 2012).

The current study's results demonstrated that continuous $AlCl_3$ administration in rats significantly increased mRNA expression of APP gene compared to control rats. Earlier studies have demonstrated parallel results with increased mRNA

expression (Khalaj et al, 2016; Yang et al, 2016) and protein expression of APP gene (Thenmozhi et al, 2016; Singla & Dhawan, 2017; Rather et al, 2018) in $AlCl_3$ -treated rats compared to control rats. Explanation for increased mRNA expression of APP gene is that the APP gene promoter region has numerous binding sites selective for $NF-\kappa B$ and $HIF-1$; two transcription factors linked to stress. They are implicated together in AD pathophysiology and pathology and are triggered by Al^{3+} (Lukiw et al, 2005). It was also reported that $IL-1$ enhance APP promoter transcriptional function (Lahiri and Nall, 1995). In addition, $IL-1$ significantly enhance APP mRNA translation (Rogers et al, 1999). Moreover, $IL-1$ ($IL-1\alpha$ and $IL-1\beta$) and $TNF-\alpha$ upregulate $A\beta PP$ transcription and translation in the astrocytic cells via regulatory elements present in the $A\beta PP$ promoter and in 5'-UTR, respectively (Lahiri et al, 2003).

Co-administration of memantine with $AlCl_3$ in rats significantly decreased mRNA expression of APP gene compared to $AlCl_3$ -treated rats. Previous studies showed similar results with decreased protein expression of APP by memantine through inhibiting internal ribosomal entry site (Wu & Chen, 2009; Tasi et al, 2015). Other studies showed that memantine therapy decreased the membrane-bound APP entire cortical concentrations (45%–55%) in transgenic and non-transgenic mice (Unger et al, 2006). An explanation for the decreased APP mRNA gene expression by memantine is due to its ability to lower $TNF-\alpha$ and $IL-1\beta$ concentrations as shown in the current study. Moreover, APP is produced by glutamate releasing nerve cells (Ouimet et al, 1994); so, NMDA receptor antagonism by memantine may affect the total APP formation (Unger et al, 2006). $NF-\kappa B$ enhanced APP promoter transcriptional function in glutamate-treated neurons (Grilli et al, 1996).

In the current study, concomitant clopidogrel

treatment with AlCl_3 in rats significantly decreased mRNA expression of APP gene compared to AlCl_3 -treated rats. An explanation for decreased mRNA expression of APP gene by clopidogrel is due to its ability to lower $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ concentrations as shown in the current study.

All the current study's results were confirmed by histopathological examination of the hippocampi from different rat groups stained with hematoxylin and eosin and Congo red stains. Examination of hippocampal tissue of AlCl_3 treated rats stained with hematoxylin and eosin revealed amyloid plaques and degenerative changes compared to control rats. Similar earlier studies had shown similar results of amyloid plaques in AlCl_3 model of AD (**Lin et al, 2015; Ali et al, 2016; Babu et al, 2016; Singh et al, 2018**). Congo red-stained hippocampal tissue examined both under light microscopy and polarized light confirmed the presence of amyloid plaques. These results are consistent with earlier studies (**Balgoon et al, 2015; Kaur & Sodhi, 2015; Abdel-Salam et al, 2016**).

Examination of hippocampal tissue of rats concomitantly treated with AlCl_3 and memantine stained with hematoxylin and eosin revealed more or less normal hippocampal brain tissue when compared to AlCl_3 -treated rats. In addition, no amyloid plaques were detected. These results are in accordance with earlier studies that showed memantine lowering effect on amyloid plaques (**Ahmed et al, 2014; Babu et al, 2016; Alawdi et al, 2017; Al-Bishri et al, 2017**). Congo red stain of hippocampal tissue examined both under light microscopy and polarized light confirm the absence of amyloid plaques. These results are in consistent with earlier studies utilized memantine in other AD models (**Sil et al, 2016**).

Examination of hippocampal tissue of rats concomitantly treated with AlCl_3 and clopidogrel

stained with hematoxylin and eosin stain or Congo red stain showed similar results to AlCl_3 and memantine co-treated rats. To our knowledge, this study is the initial one to show clopidogrel potential neuroprotective influence on histopathology in an AlCl_3 -triggered AD model.

Histopathological changes in hippocampus in different rat groups may be explained by the biochemical and transcriptional gene expression changes which are previously discussed.

Conclusion

The current study highlighted that clopidogrel improves behavioral and biochemical function in AlCl_3 -treated rat brain, an effect that could be explained mainly by its anti-inflammatory properties. Clinical studies are required to evaluate the potential neuroprotective effect of clopidogrel in AD patients.

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الملخص العربى

تأثير الحماية العصبية المحتمل لعقار الكلوبيدوجريل على مرض الزهايمر المستحث بكلوريد الألومنيوم فى الجرذان

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نبذة مختصرة :

الخلفية والهدف: يعد مرض الزهايمر احد الأمراض الشائعة سريعة التطور التى تتميز بالضمور العصبى. تساهم آليات جزيئية متعددة مثل تكوين الأميلويد، زيادة فسفرة بروتين التاو ، انخفاض النقل العصبى الكولينىرجى ، الإجهاد التأكسدي ، والإلتهاب العصبى فى كيفية حدوث المرض. الغرض من هذه الدراسة هو تقييم تأثير الحماية العصبية المحتمل لعقار الكلوبيدوجريل فى نموذج الزهايمر المستحث بكلوريد الألومنيوم فى الجرذان.

الطرق: تم تقسيم أربعين من جرذان سبراج داوى من الذكور البالغين عشوائياً إلى أربع مجموعات (عشرة جرذان لكل منها): المجموعة الضابطة، مجموعة كلوريد الألومنيوم (100مجم / كجم عن طريق الفم)، مجموعة كلوريد الألومنيوم والميمانتين (10مجم / كجم عن طريق الفم)، و مجموعة كلوريد الألومنيوم والكلوبيدوجريل (00مجم / كجم عن طريق الفم). تم تناول كلوريد الألومنيوم والأدوية مرة واحدة يومياً لمدة 20 يوماً. تم تقييم التعلم المكاني والذاكرة المكاني عن طريق اختبار متاهة موريس المائية. بعد القتل الرحيم ، تم تقييم نشاط انزيم أستيل كولين استيراز وقياس تركيزات TNF- α . IL-1 β كيميائياً فى الهيپوكامباس فى جميع المجموعات. وعلاوة على ذلك ، تم تحليل التعبير الجيني لجين APP فى الهيپوكامباس لكل المجموعات من خلال تفاعل كمي وحقيقي لسلسلة البوليميراز. تم فحص نسيج الهيپوكامباس باثولوجيا فى جميع المجموعات للتحقق من وجود لويحات الأميلويد باستخدام صبغة الهيماتوكسيلين والإيوسين وصبغة الكونغو.

النتائج: أدى تناول الجرذان للكلوبيدوجريل مع كلوريد الألومنيوم لمدة 20 يوماً إلى تحسن ذو دلالة إحصائية في العجز المعرفي الناجم عن كلوريد الألومنيوم . علاوة على ذلك ، أدى تناول الجرذان للكلوبيدوجريل مع كلوريد الألومنيوم إلى انخفاض ذو دلالة إحصائية في نشاط انزيم أستيل كولين استيراز وتركيزات $TNF-\alpha$, $IL-1\beta$ والتعبير الجيني لجين APP في الهيبوكامباس مقارنةً بالجرذان التي تناولت كلوريد الألومنيوم فقط. أدى تناول الجرذان للكلوبيدوجريل مع كلوريد الألومنيوم إلى انخفاض ذو دلالة إحصائية في تركيزات $TNF-\alpha$, $IL-1\beta$ في الهيبوكامباس مقارنةً بالجرذان التي تناولت الميمانتين مع كلوريد الألومنيوم. بالإضافة إلى ذلك ، قلل تناول الجرذان للكلوبيدوجريل مع كلوريد الألومنيوم من ترسب لويحات الأميلويد في أنسجة الهيبوكامباس المصبوغة بالهيماتوكسيلين والايوسين و الكونغو مقارنةً بالجرذان التي تناولت كلوريد الألومنيوم فقط.

الخلاصة: أظهرت هذه النتائج أن عقار كلوبيدوجريل تمكن من تحسين العجز المعرفي الناجم عن كلوريد الألومنيوم في الجرذان . ومن المحتمل ان تاثير الحماية العصبية لعقار الكلوبيدوجريل في الزهايمر المستحث بكلوريد الألومنيوم حدث من خلال تاثيره المضاد للإلتهاب كما يتضح من قدرته على تقليل تركيزات $TNF-\alpha$, $IL-1\beta$ في الهيبوكامباس او من خلال تاثيره المثبط لنشاط انزيم أستيل كولين استيراز و/ أو تقليل التعبير الجيني لجين APP في الهيبوكامباس.