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Role of Donpezil alone and in combination with L. argenine, L. NAME and 7-nitroindazole against $AlCl_3$ induced neurotoxicity.

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Alzheimer's disease (AD) is a progressive age-related neurodegenerative disorder characterized by progressive impairment of memory and cognitive functions. Oxidative stress, neuroinflammation, and neurotransmitters disturbance may play an important role. This study aimed to evaluate the effect of donepezil alone and in combination with nitric oxide synthase (NOS) substrate L-arginine, and nitric oxide synthase inhibitor (NOSi) N^g-nitro-l-arginine methyl ester hydrochloride (L-NAME) and 7-nitroindazole against aluminum chloride ($AlCl_3$) induced neurotoxicity. A total of 36 male albino rats were divided to six groups: control normal saline, $AlCl_3$. ,Donepezil, Donepezil+ L-arginine, Donepezil+ L-NAME, and Donepezil+7-nitroindazole. Neurotoxicity was induced by $AlCl_3$ in a dose of 10 mg/Kg subcutaneously for 30 days. After the experimental period, brain was removed for determination of acetylcholinesterase activity (AChE), levels of fatty acids fractions by high performance liquid chromatography (HPLC), interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF-α). $AlCl_3$ significantly increase brain levels of AChE, IL-1β, and TNF-α, and arachidonic acid (AA) and linoleic acid (LA). Treatment with Donepezil alone and in combination with L.NAME and 7-nitroindazole significantly attenuates these changes. In conclusion, regulation of nitric oxide (NO) level is the key for stability and prevention of neurodegenerative diseases. Combination of Donepezil with nitric oxide synthase inhibitors especially 7-nitroindazole attenuates the oxidant and inflammatory induced by $AlCl_3$.

Keywords: $AlCl_3$, L. NAME, Donpezil, fatty acids, HPLC

INTRODUCTION

Alzheimer's disease (AD) is a highly complex neurodegenerative disorder that has multiple factors which contribute to its etiology in terms of initiation and progression, which is of unknown etiology in more than 90% of the cases (Rosales-Corral et al., 2012). AD is associated with initial memory loss, followed by language impairment (aphasia), motor function is intact (*apraxia*), inability to recognize object, sensory function is intact (*agnosia*), and impairment in judgment, executive functioning, and visuospatial functioning (Sosso et al., 2017).

Cholinergic system had an important role in cognitive functions, developing memory, concentration, attention, and psychiatric symptoms of AD (Grabowski and Damasio 2004). AD brains characterized by severe degeneration of cholinergic neurons, reduced ACh synthesis and levels, decreased choline levels as well as

down-regulated activity of choline acetyltransferase (ChAT), and the deregulations of ACh receptors (Melancon et al., 2013). Oxidative stress is defined as the imbalance between the generation of reactive oxygen/nitrogen species (ROS/RNS) and the cells ability to neutralize them by the antioxidant defense (Koopman et al., 2010)Oxidative stress has been recognized as a contributing factor in aging and in the progression of multiple neurodegenerative diseases including AD (Ramassamy et al., 2000; Onnies and Trushina 2017) Free radicals include superoxide anions (O_2·−), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH·), and hydroxyl ions (OH−) (Abdel Moneim 2015). Brain phospholipids contain a high percentage of polyunsaturated fatty acids (PUFA), including arachidonic acid as a result of free radical overproduction, there is a reduced content of PUFA in the brain in AD (Skoumalova and Hort 2012). Nitric oxides (NO) have various physiological roles including vascular regulation (vasodilation), neuronal transmission, and host defense against microbe invasion (Yoshitake et al., 2016). NO can also be neurotoxic primarily due to its free radical properties, and it has been implicated in neurodegenerative diseases (Law, 2001). Nitric oxide has been implicated in the pathogenesis of a variety of neurodegenerative diseases including Alzheimer's disease (Smith 1997). NO react with superoxide to form the strong oxidant peroxynitrite radical (Szabó et al, 2007). Neurons synthesize NO as a response to the activation of NMDA receptors by glutamate. NO is synthesized in the brain upon demand as in cognitive condition for which NO activity is required. (Garthwaite et al., 1995). Inflammation have been strongly linked with pathogenesis of AD (Blasko et al., 2003) Inflammatory cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF- α), and interleukin 6 (IL-6), located close to amyloid plaques (Cacquevel et al., 2004). Aluminum is considered to be one of the environmental factors causing neurodegenerative disorders such as AD (Kawahara and Kato-Negishi 2011). It has been established as a neurotoxic agent (Walton 2006). Aluminum is characterized by relatively low redox activity; however, it can bind to brain phospholipids which carry negative charges and contain polyunsaturated fatty acids that are readily attacked by free radicals (Exley 2004).

Aim of the study

This study aimed to evaluate the role of nitric oxide in Alzheimer's disease through studding the effect of nitric oxide synthase (L-arginine), and nitric oxide synthase inhibitors (L-NAME) and 7-nitroindazole on the acetylcholiesterase inhibitor (donepezil) against $AlCl_3$ induced neuronal damage and cognitive impairments.

MATERIALS AND METHODS

Chemicals

Aluminum chloride (AlCl3) was purchased from Merck, Darmstadt, Germany. L.NAME, L-arginine, 7-nitroindazole and fatty acids standards were purchased from Sigma Chemicals Co. (Munich, Germany). Acetonitrile, methanol, ethanol, N-hexane, 2-propanol and phosphoric acid HPLC grade were purchased from Sigma ALDRICH, Germany.

Donepezil tablets (10mg) were purchased from Pfizer Egypt.

Experimental animals

Male albino rats weighting 140-180g were used for induction of brain injury. Animal obtained from the Animal house of National Research Center, Giza, Egypt. Animals were housed in individual suspended stainless cages in controlled environment (22-25°c) and 12 hours light, 12 hours dark with food and water *ad libtium* freely available. The guidelines of the ethical care and treatment of the animals underwent the regulations of the ethical committee of National Research Centre (NRC), Egypt.

Induction of brain injury

$AlCl_3$ was dissolved in normal saline; pH was adjusted at 7.4 and subcutaneously injected in rats in a dose of 10 mg/Kg body weight daily for 30 days (Lakshmi et al., 2015).

Induction of drugs

L-arginine (750mg/kg body weight), L-NAME L-NAME (50 mg/kg body weight), 7-nitroindazole and Donpezil (25 mg/kg body weight) were administered subcutaneously daily for 15 days after 30 days from beginning of $AlCl_3$ injection. All drugs were dissolved in saline and freshly prepared immediately before use.

Experimental design

36 rats were divided to six groups each one containing 6 rats. Group I: control group: healthy rats injected by normal saline. Group II: healthy rats were injected with $AlCl_3$ for 30 days. Group III ($AlCl_3$+Donpezil) healthy rats were injected with

$AlCl_3$ for 30 days followed by injection of Donpezil .Group IV: ($AlCl_3$+Donpezil+ L-arginine): healthy rats were injected with $AlCl_3$ for 30 days followed by injection of Donpezil in combination with L-arginine. Group V: ($AlCl_3$+Donpezil+L-NAME): healthy rats were injected with $AlCl_3$ for 30 days followed by injection of Donpezil in combination with L-NAME. Group VI: ($AlCl_3$+Donpezil+7-nitroindazole): healthy rats were injected with $AlCl_3$ for 30 days followed by injection of Donpezil in combination with 7-nitroindazole.

At the end of the experimental period brain was removed quickly and placed in iced normal saline, perfused with the same solution to remove blood cells, blotted on filter paper and frozen at -80°C.

Preparation of tissue homogenate

The frozen tissue was cut into small pieces and homogenized in 5 ml cold buffer (0.5 g of Na_2HPO_4 and 0.7 g of NaH_2PO_4 per 500 ml deionized water (pH 7.4) then centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant was removed for biochemical parameters estimation (Manna et al., 2005).

Biochemical assays

Determination of brain cholinesterase activity

Cholinesterase activity was determined using quantitative colorimetric kinetic assay (BEN Biochemical Enterprise, Milano, Italy) according to Young (2000)

Determination of brain fatty acids

Brain fatty acids (FA) were fractionated and analyzed according to (El-Khayat et al., 2013).

Determination of brain Interleukin – 1β (IL- 1β)

IL- 1β ELISA kit purchased from assaypro was used for quantitative detection of brain IL- 1β according to the method described by Barland et al., (2004).

Determination of brain tumor necrosis factor-α (TNF- α)

TNF- α ELISA kit was purchased from Assaypro was used for quantitative detection of brain TNF-α as described by Taylor (2001).

Statistical Analysis

All data was expressed as mean ± SE. Distribution of the data was verified to be normal using Tests of Normality (SPSS package). Statistical significance was tested by one way analysis of variance (ANOVA).

RESULTS

Effects of $AlCl_3$, L.argenine, L. NAME, 7-nitroindazole and Donpezil on AChE in brain tissue.

Figure (1) summarized the recorded mean values in different studied groups. $AlCl_3$ significantly elevate level of AChE by 459% when compared with control group. Donpezil alone significantly increased AChE level when compared with control by 114%, and decreased AChE level when compared with $AlCl_3$ group by 62%. Combination of Donpezil with L.argenine, L. NAME and 7-nitroindazole significantly decreased AChE level when compared with $AlCl_3$ by 65%, 73% and 80%, respectively. Donpezil with L.argenine group significantly increased AChE level when compared with control by 96%, but there is no significant difference between Donpezil with L. NAME and 7-nitroindazole when compared with control table (1).

Effects of $AlCl_3$, L.argenine, L. NAME, 7-nitroindazole and Donpezil on ALA, AA and LA level in brain tissue.

Figure (2) summarized the recorded mean values in different studied groups.

$AlCl_3$ significantly decrease ALA level by 90% when compared with control group. Donpezil alone significantly decreased ALA level when compared to control 31%, and significantly increased ALA when compared with $AlCl_3$ by 550%. Combination of Donpezil with L.argenine, L. NAME and 7-nitroindazole were significantly increased ALA level when compared with $AlCl_3$ by 86%, 371% and 679%, respectively. Donpezil with L.argenine, and L. NAME significantly decreased ALA level when compared with control by, 81% and 50% respectively, there is no significant difference between Donpezil with 7-nitroindazole and control. In addition, in the table (2) and figure (3), $AlCl_3$ significantly increase AA level by 60% when compared with control group. Donpezil alone significantly decreased AA level when compared with control by 23%, and significantly decreased AA when compared with $AlCl_3$ by 52%. Combination of Donpezil with L.argenine, L. NAME and 7-nitroindazole were significantly decreased AA level when compared with $AlCl_3$ by 19%, 29% and 46% respectively. Donpezil with L.argenine, significantly increased AA level when compared with control by, 29%. There is no significant difference between Donpezil with L. NAME and 7-nitroindazole and control.

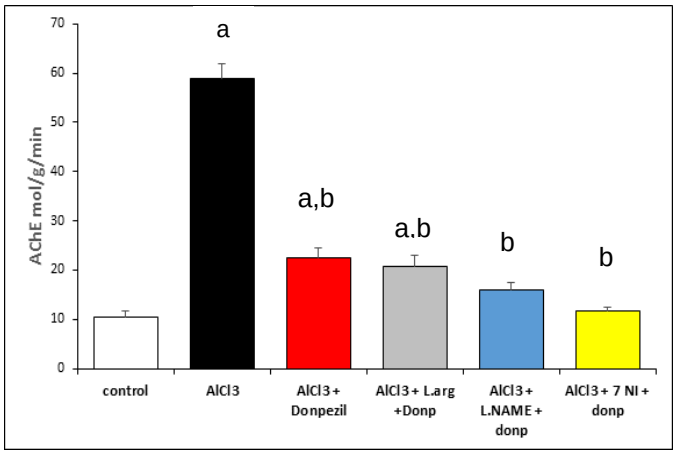

Figure (1): Effects of Donpezil alone and in combination with L.argenine, L. NAME and 7-nitroindazole on AChE in brain tissue against $AlCl_3$ induced neurotoxicity.

a=Significantly different from the control value at p< 0.05, $AlCl_3$ = Aluminum chloride, 7-NI = 7-nitroindazole. b =Significantly different from the $AlCl_3$ value at p< 0.05, AChE= acetylcholinesterase.

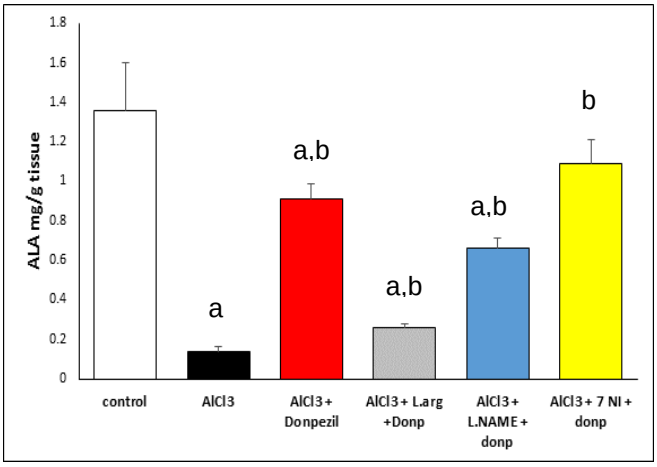

Figure (2): Effects of Donpezil alone and in combination withL.argenine, L. NAME 7-nitroindazole on ALA in brain tissue against $AlCl_3$ induced neurotoxicity.

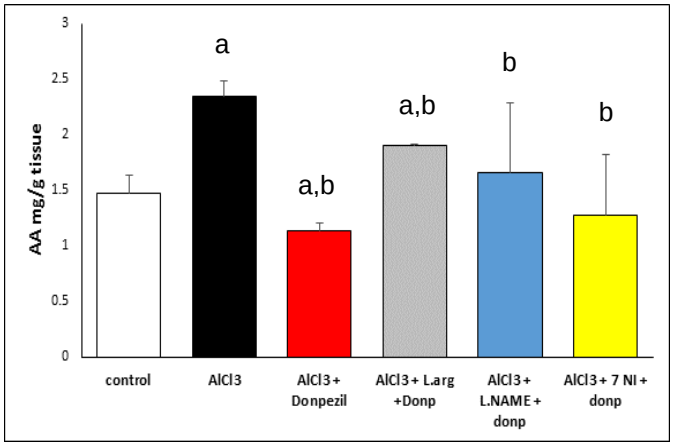

Figure (3): Effects of Donpezil alone and in combination withL.argenine, L. NAME and 7-nitroindazole on AA in brain tissue against $AlCl_3$ induced neurotoxicity.

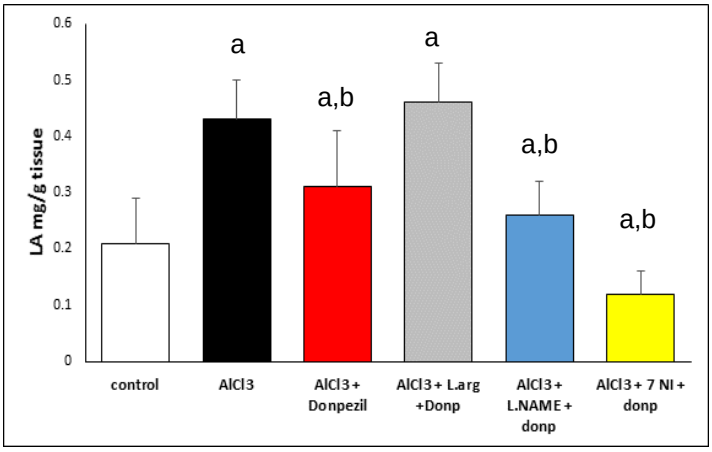

Figure (4): Effects of Donpezil alone and in combination withL.argenine, L. NAME and 7-nitroindazole on LA in brain tissue against $AlCl_3$ induced neurotoxicity.

a=Significantly different from the control value at p< 0.05, AlCl3 = Aluminum chloride, 7-NI = 7-nitroindazole. b =Significantly different from the AlCl3 value at *p*< 0.05, ALA= Alpha linolenic acid
AA= Arachedonic acid, LA= Linolenic acid.

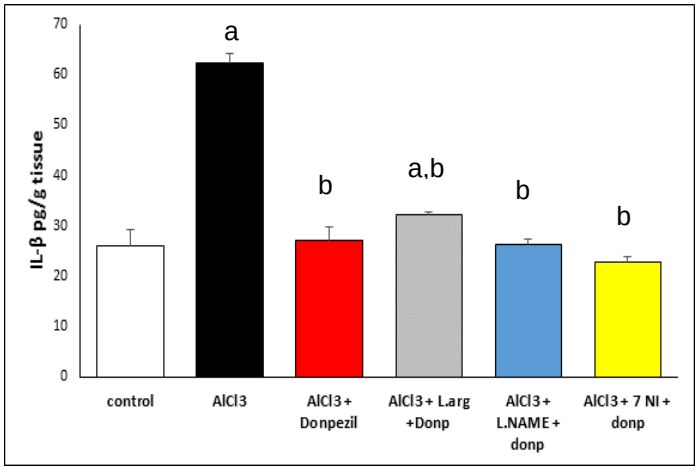

Figure (5): Effects of Donpezil alone and in combination with L.argenine, L. NAME and 7-nitroindazole on IL-1β level in brain tissue against $AlCl_3$ induced neurotoxicity.

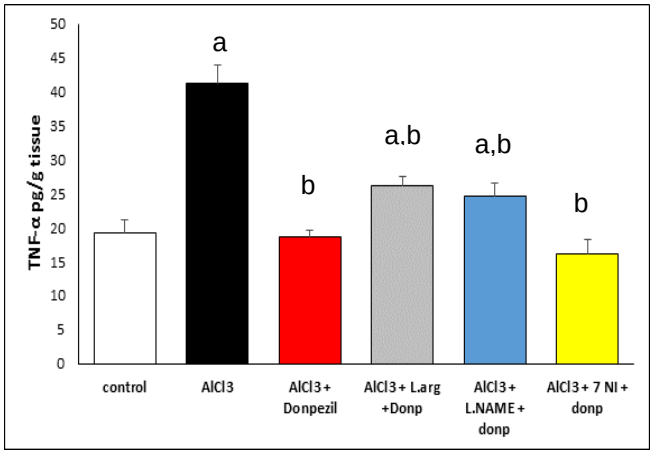

Figure (6): Effects of Donpezil alone and in combination withL.argenine, L. NAME and 7-nitroindazole on TNF-α level in brain tissue against $AlCl_3$ induced neurotoxicity.

a=Significantly different from the control value at *p*< 0.05, $AlCl_3$ = Aluminum chloride, 7-NI = 7-nitroindazole. b=Significantly different from the $AlCl_3$ value at *p*< 0.05, IL-1 β =interleukin, TNF_ α = tumor necrosis factor

Furthermore, in table (2) and figure (4), $AlCl_3$ significantly increase LA level by 105% when compared with control group. Donpezil alone significantly increased LA when compared with control by 48%, and significantly decreased LA level when compared with $AlCl_3$by 28%. Combination of Donpezil with L.argenine, L. NAME and 7-nitroindazole were Donpezil with L. NAME and 7-nitroindazole significantly decreased LA level when compared with $AlCl_3$ by 40% and 72% respectively, but there is no significant difference between Donpezil with L.argenine and $AlCl_3$ when compared with control, Donpezil with L.argenine significantly increased LA level by 119%, Donpezil with 7-nitroindazole, significantly decreased LA level by 43%, but there is no significant difference between Donpezil with L. NAME and control.

Effects of $AlCl_3$, L.argenine, L. NAME, 7-nitroindazole and Donpezil on IL-1β and TNF-α level in brain tissue.

In table (3) and figure (5), $AlCl_3$ significantly increase IL-1β level by 140% when compared with control group. Donpezil alone significantly decreased IL-1β level when compared with $AlCl_3$ by 57%, and there is no significant difference and control. Combination of Donpezil with L.argenine, L. NAME and 7-nitroindazole were significantly decreased IL-1β level when compared with $AlCl_3$ by 48%, 58% and 63% respectively. Donpezil with L.argenine, significantly increased IL-1β level when compared with control by, 22%, but there is no significant difference between Donpezil with L. NAME, and 7-nitroindazole when compared with control. In addition, in table (3) and figure (6), $AlCl_3$ significantly increase TNF-α level by 114% when compared with control group. Donpezil alone significantly decreased TNF-α when compared with $AlCl_3$ by 55%, but there is no significant difference with control. Combination of Donpezil with L.argenine, L. NAME and 7-nitroindazole were significantly decreased TNF-α level when compared with $AlCl_3$ by 37%, 40% and 61% respectively. Donpezil with L.argenine, L. NAME significantly increased TNF-α level when compared with control by, 36% and 2 9% respectively, but there is no significant difference between Donpezil with 7-nitroindazole and control.

DISCUSSION

In the present study, AlCl3 significantly increase the AChE activity when compared with control. This finding supported by previous studies (Yellamma et al., 2010; John et al, 2015) who concluded that acute, subacute, and chronic exposure to aluminum leads to its accumulation in different brain regions with subsequent elevation in cholinergic neurotransmitter and its associated enzymes. In addition Silva et al., (2007) concluded that a substantial increase in AChE activity was observed in AlCl3 exposure rats, which denotes impaired cholinergic function. The present study show that, Donpezilcould protect AD rats against AChE activity elevation induced in AlCl3-intoxicated rats. Combination of Donpezil with L. NAME and 7-nitroindazole, but not L.argenine, showed good improvement in the AChE activity. The obtained data in accordance with Sugimoto et al. (2002) and Wilkinson et al. (2004) who found that, boosting cholinergic activity through increasing available acetylcholine (ACh) with the use of centrally acting cholinesterase inhibitors like donepezil is a cornerstone of therapy and results in clinically measurable benefit in cognitive function, and behavior. Other finding shows that, Donepezil hydrochloride is a hexaoxypyridine oxide and the generation-II specific reversible central AchE inhibitor. It can increase the concentration of Ach by inhibiting the hydrolysis of Ach to improve the cognitive function of AD patients (Jing Li et al., 2006)

Neurons when compared with other cells, it has low level of anti-oxidant defense molecules such as glutathione (GSH). (Chaitanya et al., 2012; Bourogaa et al., 2013)The brain is subjected to free radical induced lipid peroxidation because high level of lipid content and tissue oxygen consumption. Lipid peroxidation is a measure of tissue destruction (Juranekand Bezek 2005; Attrey et al., 2012; Balu et al., 2005; Kumar and Gill, 2014)

Arachidonic acid is the second PUFA in the brain it corresponds to around 20% of the total amount of the neuronal fatty acids and is mainly esterified in membrane phospholipids. AA released by phospholipases A, and converted to numerous eicosanoids which are key mediators in neuroinflammation.(Smith and Song 2002; Matsumura and Kobayashi 2004; Pecchi et al., 2009; Vasilache et al., 2015) The level of intracellular free AA and the balance between the releasing enzymes and its incorporation in membrane phospholipids can be critical for neuroinflammation and synaptic dysfunctions associated AD.(Funk 2001; Latham et al, 2007)The present study demonstrated that, injection of AlCl3 significantly elevate the

Arachedonic acid (AA) and Linolenic acid (LA), while its significantly decrease Alpha linolenic acid (ALA) when compared with control. This finding in accordance of Stanley I. Rapoport 2008 who reported that, in conditions such as neuroinflammation and excitotoxicity, additional AA is released at cytokine and glutamatergic N-methyl-D-aspartate receptors, and AA cascade enzymes are overexpressed. In addition, high levels of AA have been found in neurodegenerative disorders such as Alzheimer's disease.(Hoozemans et al., 2001)

Inflammation have been strongly linked with pathogenesis of AD (Blasko et al, 2003) Inflammatory cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF- α), and interleukin 6 (IL-6), located close to amyloid plaques, which might be cytotoxic when chronically produced.(Jeohn et al., 1998; Cacquevel et al, 2004) The innate immune response that occurs in the brain leads to the accumulation of inflammatory mediators such as TNF-α, IL-1, IL-6, free radicals, complement components and microglia activation. These neuro inflammation makers are typically observed in association with AD neuropathology.(Weiner and Selkoe 2002) The present findings reported a significant increase in IL-1β and TNF-α level in AlCl3 treated rats when compared with control. This results in agreement with Mohamed et al, 2018 who reported that, a significant increase in TNF-α level in the cortex and hippocampus of AlCl3-intoxicated rats. Passos et al., 2010reported that inflammation might contribute to the neurodegenerative effects of AlCl3, through the increased expression of cytokines such as TNF-α in activated microglia around amyloid plaque. The present data showed that, Donpezil significantly decreased the AA, and LA level when compared with AlCl3-treated rats. Combination of Donpezil with L. NAME and 7-nitroindazole enhancement this protection. On the other hand, in the present study, Donpezil significantly increased the ALA level when compared with AlCl3-treated group. Combination of Donpezil with 7-nitroindazole enhancement this protection. The present study also reported that, Donpezil significantly decreased the IL-1β level when compared with AlCl3-treated rats. Combination of Donpezil with L. NAME and 7-nitroindazole enhancement this protection. In addition, the present study demonstrated that, Donpezil significantly decreased the TNF-α level when compared with AlCl3 group. Combination of Donpezil with L. NAME and 7-nitroindazole enhancement this protection. This finding is in harmony with Yoshiyama et al., 2010 who found that, neuroprotective mechanisms of donepezil against neuroinflammation and tau pathology. This data in line of Olivenza et al., 2000who reported that A sustained overproduction of NO via NOS expression may be responsible, at least in part, for some of the neurodegenerative changes caused by stress and support a possible neuroprotective role for NOS inhibitors in this context. Accordingly, suppression/ inhibition of NOx synthesis by NG-nitro-L-arginine methyl ester (L-NAME) might act protectively. (Talas et al., 2002)

In this study, L.argenine as substrate for NOS, when combined with Donpezil usually not significantly improves the changes in most tested parameter when compared with AlCl3-treated rats. That's effects may be due to overproduction of NO could be involved in the etiology of a broad spectrum of neuropathologic disorders. (Miranda et al, 2000) Combination of Donpezil with L. NAME and 7-nitroindazole enhancement the protection against neurotoxicity of AlCl3 when compared with Donpezil alone. This effect may be due to stimulation of anticholinesterase activity, antioxidant, and anti-inflammatory.

CONCLUSION

Regulation of nitric oxide level is a critical factor in stability and prevention of neurodegenerative effects. Using of NOS inhibitors, L. NAME and 7-nitroindazole enhancement the protective effects of Donepezil against $AlCl_3$ neurotoxicity.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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