Role of nitric oxide in neurotoxicity (Immunohistochemical study)

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دور أكسيد النيتريك في السمية العصبية (دراسة نسيجية مناعية)

الملخص:

المقدمة: الإجهاد التأكسدي هو عامل رئيسي في الاضطرابات والتلف العصبي، وتلعب الجذور الحرة دورًا مهمًا، وهناك احتمال أن يكون أكسيد النيتريك هو المسبب للسمية العصبية عن طريق التفاعل مع السوبر أكسيد الذي يشكل جذر بيروكسي نيتريت عالي السمية.

الهدف: يهدف هذا البحث إلى معرفة دور أكسيد النيتريك في تلف الخلايا العصبية من خلال الدراسة المناعية النسيجية.

الطريقة: تم تقسيم التجربة إلى 9 مجموعات، كل مجموعة تحتوي على 6 فئران تم اختيارها عشوائياً. المجموعة 1: مجموعة المقارنة الطبيعية. المجموعة 2: Aβ(1−42). المجموعة 3: L−أرجينين. المجموعة 4: L-NAME. المجموعة 5: 7−نيتروبندازول. المجموعة 6: دونيبيزيل. المجموعة 7: دونيبيزيل + L−أرجينين. المجموعة 8: دونيبيزيل+ L-NAME. المجموعة 9: دونيبيزيل+7−نيتروبندازول. تم إحداث السمية العصبية عن طريق حقن Aβ (1−42) في القشرة المخية الأنفية الداخلية للفئران لجميع الحيوانات باستثناء مجموعة المقارنة الطبيعية. بعد انتهاء التجربة تمت إزالة الدماغ بسرعة لدراسة الأنسجة .

النتائج: Aβ (1−42) أدى إلى ارتفاع مستوى COX-2 والتغير النسيجي بشكل ملحوظ بالمقارنة مع مجموعة المقارنة الطبيعية. بالمقارنة مع الفئران المعالجة بـ Aβ، فإن L-arginine وL-NAME و7−nitroindazole وdonepezil بشكل منفصل لم يحسن التغيرات في مستوى COX−2 والتغير النسيجي. يعود مزيج دونيبيزيل مع NI−7 إلى طبيعته.

الخلاصة: أكسيد النيتريك يلعب دورًا مركزيًا في السمية العصبية وأن تنظيم مستوى أكسيد النيتريك هو مفتاح الاستقرار والوقاية من الاضطراب والتلف العصبي.

الكلمات المفتاحية: *أكسيد النيتريك، الإجهاد التأكسدي، الالتهاب العصبي، بيتا أميلويد، L − NAME، دونيبيزيل، L− أرجينين، 7−نيتروبندازول.*

Abstract:

Background: Oxidative stress is a key factor in the neurodegenerative disorder. Free radicals may play an important role. Nitric oxide may be implicated in neurotoxicity by a reaction with superoxide, which forms a highly toxic peroxynitrite radical.

Aim: This research aimed to investigate the role of nitric oxide in neuronal damage through Immunohistochemical study.

Material and method: The experiment was divided into 9 groups, each group containing

6 rats selected randomly. Group 1: control normal saline. Group 2: $A\beta_{(1-42)}$. Group 3: L-arginine. Group 4: L-NAME. Group 5: 7-nitroindazole Group 6: donepezil. Group 7: donepezil+ L-arginine. Group 8: donepezil+ L-NAME. Group 9: donepezil+7-nitroindazole. Neurotoxicity was induced by injection of $A\beta_{(1-42)}$ into the rat entorhinal cortex of all animals except the control normal saline group. After the end of the experiment brain was removed quickly for histopathological study.

Results: $A\beta_{(1-42)}$ significantly elevated COX-2 level when compared with control. when compared with Aβ treated rats, L-arginine, L-NAME, 7-nitroindazole and donepezil separately did not improve the changes in COX-2 level. The combination of donepezil with 7-NI is back to normal.

Conclusion: we concluded that NO plays a central role in neurotoxicity and the regulation of NO level is the key to stability and prevention of neurodegenerative disorder.

Keywords: *Oxidative stress, Neuroinflammation, Beta amyloid, L- NAME, Donepezil, L-arginine, Nitric oxide, 7-nitroindazole.*

Introduction

Neurodegenerative disorders are among the deterioration in neuronal function, leading to brain diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (Volkman, R.; Offen, D. Concise Review: Mesenchymal Stem Cells in Neurodegenerative Diseases. *Stem Cells* 2017, *35*, 1867–1880.).

Oxidative stress has been recognized as a contributing factor in ageing and in the progression of many neurodegenerative diseases including AD. There is a clear relationship between oxidative stress and Aβ (Ramassamy et al., 2000; Onnies and Trushina, 2017).

Free radicals are produced in the matrix of mitochondria by leakage of electrons from the inner membrane and react with oxygen to form superoxide anions (O_2 ·−). This can further react to generate other forms of ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH·), and hydroxyl ions (OH−). The RNS is generated when O2 ·− reacts with nitric oxide (NO) to form peroxynitrite (ONOO−). These can then in consecutive reactions forms other types of RNS, like nitrogen dioxide (·NO_2) and nitrosoperoxycarbonate ($ONOOCO_2$ −). Astrocytes and microglia studied as other sources of ROS and RNS in brain that produce these species when activated (Persson et al., 2014; Abdel Moneim, 2015).

Nitric oxide (NO) is an endogenous gaseous mediator and has different physiological roles such as vascular regulation (vasodilation), neuronal transmission, and host defense against microbial invasion (Alam et al., 2002; Hardingham et al., 2013).

Beta-amyloid (Aβ): Aβ plays a central role in the cause of neuronal damage. it is a peptide with high resistance to proteolytic degradation. It contains 37–43 amino acids, but the 1–40 and 1–42 isoforms are the most common. The 1–42 amyloid peptide isoform has the greatest toxicity (Reale et al., 2012; Sanabria-Castro et al., 2017).

Cyclooxygenase (COX): Two distinct isoforms of cyclooxygenase have been characterized, a constitutive form, cyclooxygenase-1 (COX-1), and a mitogen-inducible form, cyclooxygenase-2 (COX-2). COX-1 and COX-2 are coded by 2 distinct genes

located on human chromosome 9 and 1, respectively. COX-2 plays a special role in normal neuronal function such as synaptic activity and memory consolidation and in neurotoxicity. (Pasinetti and Aisen, 1998; Luisa Minghetti, 2004) Cyclooxygenase (COX), also known as prostaglandin (PG) H synthase. PGH_2 is the precursor of the prostaglandins PGE_2, PGD_2, PGI_2, and $PGF_2\alpha$, and the thromboxane TXA_2 (Vlad et al., 2008; Breitner et al., 2011). In neuronal damage COX-2 accumulates in neurons (Pasinetti and Aisen, 1998; Ho et al., 2001; Hoozemans et al., 2001).

Donepezil: is a piperidine derivative that reversibly inhibits acetylcholinesterase. Donepezil is approved for use in all stages of AD, mild, moderate, and severe (Ravikumar et al., 2006; Lee et al., 2015).

L-Arginine: is an essential amino acid, included in diverse physiological and pathological processes, such as noradrenergic, noncholinergic neuro-transmitters in learning and memory, synaptic plasticity, and neuroprotection. It may play an important role in age-related degenerative diseases.

L-arginine -NO- cGMP pathways: L-arginine is the precursor of NO. Soluble guanylyl cyclase (sGC) is the major physiologically relevant receptor for NO, which mediates the production of cGMP from GTP (Archer et al., 1994; Wang, 2012).

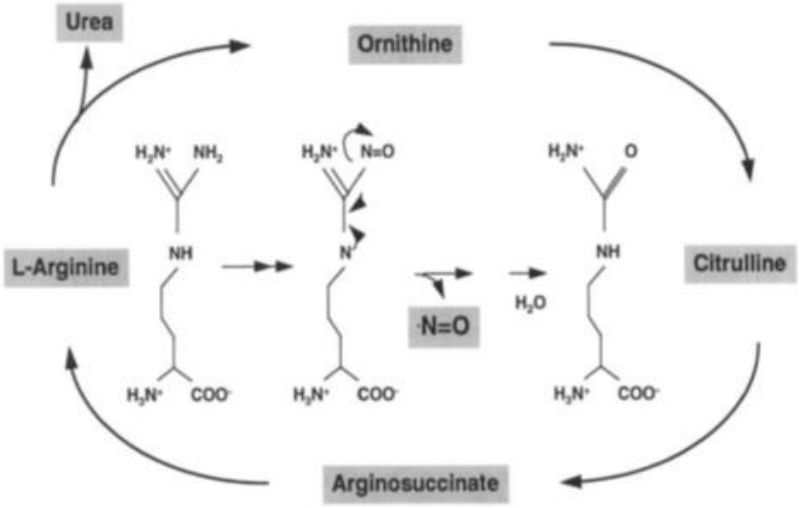

Figure 1: Biosynthetic pathway of NO. (Asiimwe et al., 2016).

L-NAME: N-nitro-L-arginine methyl ester (L-NAME) is a non-specific NOS inhibitor. It has an antioxidant action and is suggested as a strong neuroprotective effect (Stevanović et al., 2009).

7-Nitroindazole (7-NI): acts as a selective inhibitor for neuronal NOS. It may act by reducing oxidative stress or by decreasing the amount of peroxynitrite formed in these tissues (Yildiz Akar et al., 2007; Mutlu et al., 2011).

This paper aimed to investigate the role of nitric oxide in neuronal damage through Immunohistochemical study.

Materials and Methods:

Experimental animals:

Male albino rats weighing 140-180g were used for induction of brain injury. Animals were obtained from the Animal House of the National Research Center, Giza, Egypt.

Animals were housed in individual suspended stainless cages in a controlled environment (22-25°c) and 12 hours light, 12 hours dark with food and water ad libtium freely available.

Methods:

54 rats were divided into nine groups each one containing 6 rats. Group 1: control injected by normal saline. Group 2. Aβ1-42. Group 3. Aβ1-42 + L-arginine (Sigma-Aldrich, Germany) (750 mg/kg s.c). Group 4. Aβ1-42 + L-NAME (50 mg/kg s.c). Group 5. Aβ1-42 + 7-nitroindazole (25 mg/kg s.c). Group 6. Aβ1-42 + Donepezil (10 mg/kg s.c). Group 7. Aβ1-42 + Donepezil + l-arginine (750 mg/kg s.c). Group 8. Aβ1-42 + Donepezil + L-NAME (50 mg/kg s.c). Group 9. Aβ1-42 + Donepezil + 7-nitroindazole (25 mg/kg s.c)

Duration of experiment two weeks

Induction of A beta 1-42

Rats were anaesthetized with ethyl ether. An incision was made in scalp and hole was drilled in the skull over the injection site. The 30-gauge needle was lowered into the dorsal hippocampus. Coordinates for the anterior–posterior (from bregma), medial–lateral (from midline), and dorsal-ventral (from the surface of the skull) axes were −2.3, ±2.5, and −1.5 mm, respectively. The bilateral intrahippocampal infusion was administrated via a 10.0 µl Hamilton microsyringe with a 30-gauge needle fitted to the arm of the stereotaxic instrument. Double-distilled water as vehicle for peptides was used in the study as a control infusion. A 0.6 µl volume of oligomer $A\beta_{1-42}$, freshly made $A\beta_{1-42}$ peptide solution, or double-distilled water alone (as a vehicle control) was slowly infused at a rate of 0.2 µl/min. After an additional 5 min, to assure adequate diffusion, the needle was slowly retracted. (Huang *et al*., 2007). Injection of human Aβ1-42 into the rat entorhinal cortex.

After the end of the experiment brain was removed quickly from each rat and washed with ice-cold saline, fixed in 10% buffered formalin, dehydrated in graded ethanol and embedded in paraffin using standard procedures. Sections of 4 µm thickness were stained for cyclooxygenase-2 (COX-2) to examine under the light microscope. sections were deparaffinized and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature. The specimens were then incubated with anti COX-2 antibodies as the primer antibody at a 1:200 dilution. The specimens were counter stained with H&E.

Results:

Examination of sections of the cerebral cortex of the control group showed a negative reaction of COX-2 as indicated by the absence of the brown color (Figure 2), while the brain of Aβ (Figure 2) treated rats showed a positive reaction of COX-2 represented by brown color.

When compared with Aβ, the combination of donepezil with 7-NI (Figure 10) back to normal. Other drug-treated groups did not show improvement indicated by positively stained neurons.

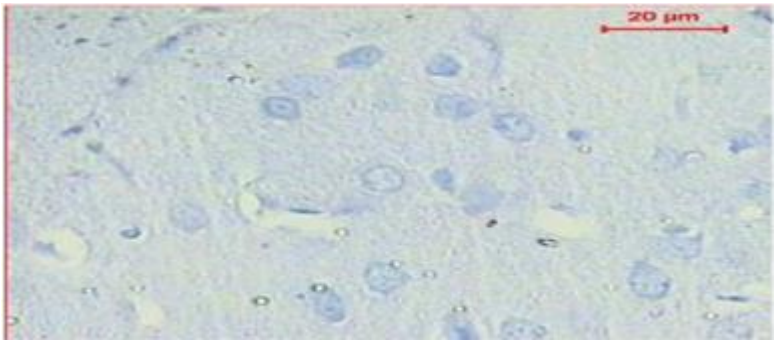

Figure (2): A micrograph of sections of cerebral cortex of control rat showing negative stained neurons (Immunohistochemical stain of COX-2; Scale bar: 20 µm).

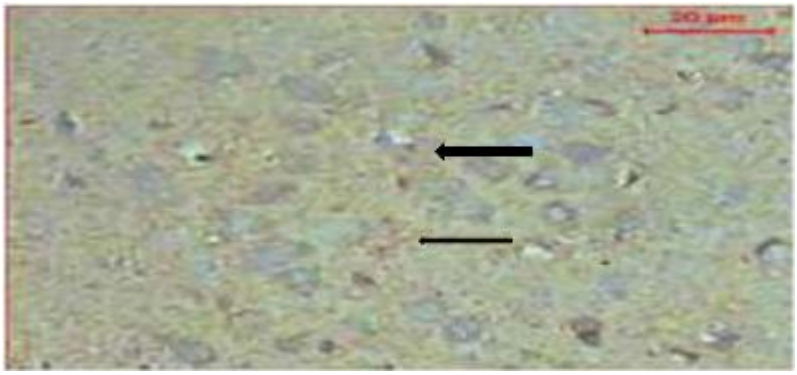

Figure (3): A micrograph of sections of cerebral cortex of rat given Aβ 1-42 showing positive stained neuron (Immunohistochemical stain of COX-2; Scale bar: 20 µm).

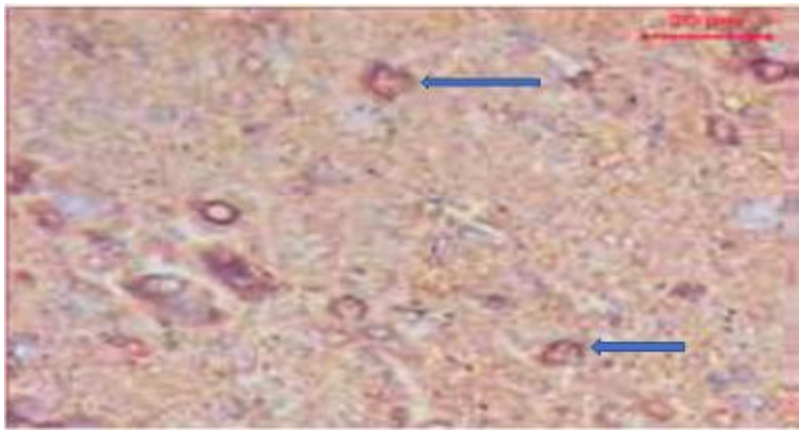

Figure (4): A micrograph of sections of cerebral cortex of rat given Aβ 1-42+L-arginine showing positive stained neuron (Immunohistochemical stain of COX-2; Scale bar: 20 µm).

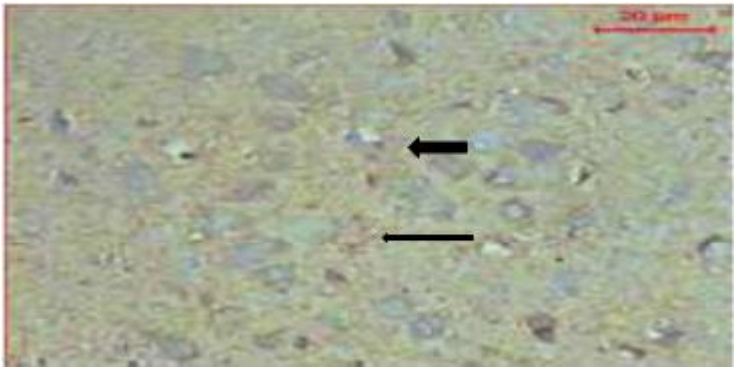

Figure (5): A micrograph of sections of cerebral cortex of rat given Aβ 1-42+L-NAME showing positive stained neuron (Immunohistochemical stain of COX-2; Scale bar: 20 µm).

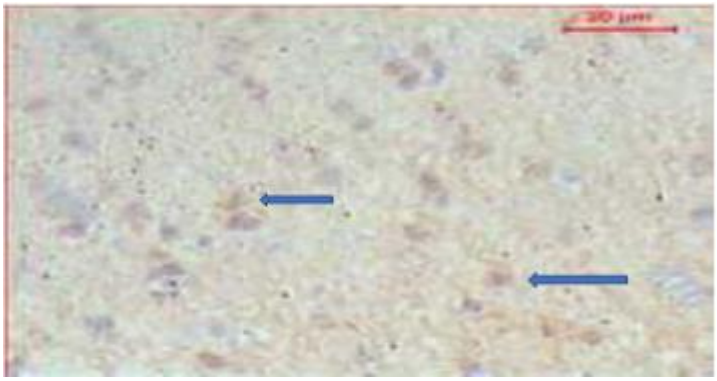

Figure (6): A micrograph of sections of cerebral cortex of rat given Aβ 1-42+7- NI showing positive stained neuron (Immunohistochemical stain of COX-2; Scale bar: 20 µm).

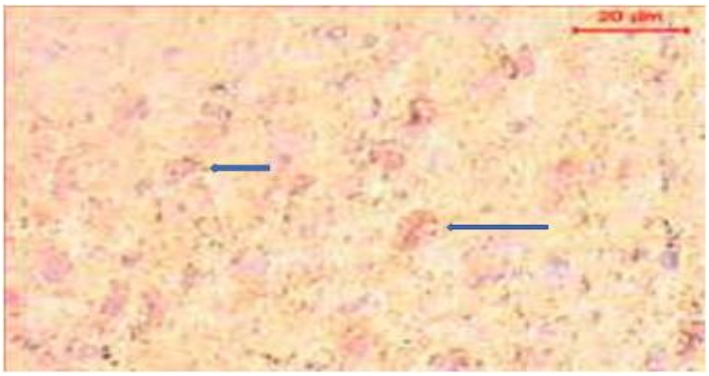

Figure (7): A micrograph of sections of cerebral cortex of rat given Aβ 1-42 +Donepezil showing positive stained neuron (Immunohistochemical stain of COX-2; Scale bar: 20 µm).

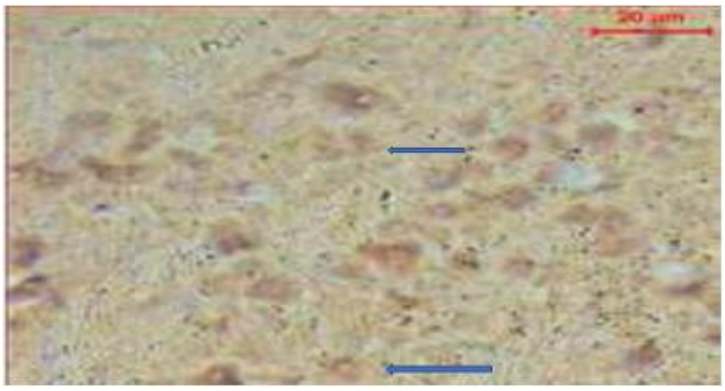

Figure (8): A micrograph of sections of cerebral cortex of rat given Aβ 1-42 +Donepezil + L-arginine. showing positive stained neuron (Immunohistochemical stain of COX-2; Scale bar: 20 µm).

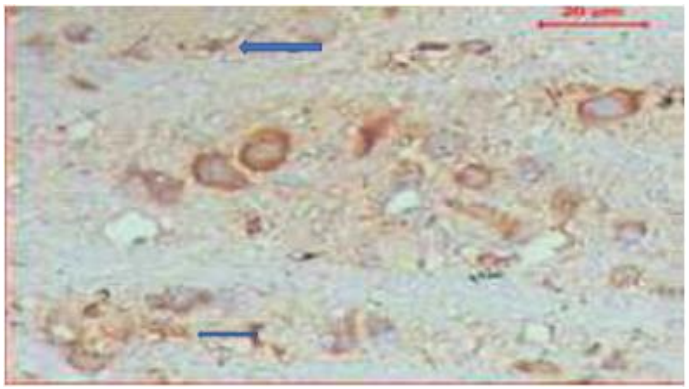

Figure (9): A micrograph of sections of cerebral cortex of rat given Aβ 1-42 +Donepezil + L-NAME showing positive stained neuron (Immunohistochemical stain of COX-2; Scale bar: 20 µm).

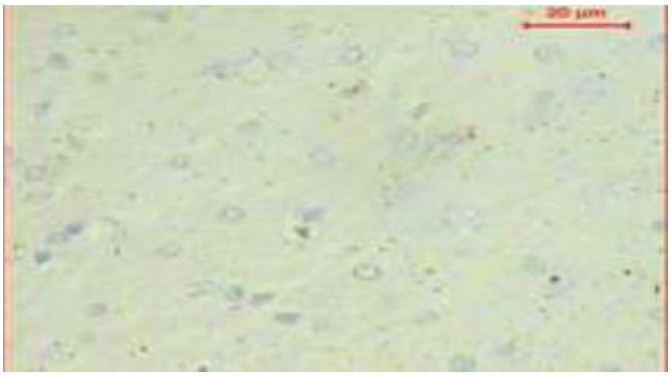

Figure (10): A micrograph of sections of cerebral cortex of rat given Aβ 1-42 +Donepezil + 7-NI showing negative stained neuron (Immunohistochemical stain of COX-2; Scale bar: 20 µm).

Discussion and Conclusion:

The role of nitric oxide in neuronal damage and cognitive impairments was studied by using L-arginine as a substrate for NOS and L-NAME, 7-nitroindazole as NOS separately and in combination with donepezil as acetylcholiesterase inhibitor. Brain oxidative stress and inflammation biomarkers were determined.

Nitric oxide may play a dual role (neuroprotective or neurotoxic) in CNS. (Siles, 2002) NO, in addition to its vasoactive and immunological properties, it has important neurophysiological functions. However, its protective and regulatory effects may be related to either stimulation of cGMP synthesis by the soluble guanylate cyclase or S-nitrosylation of the NMDA receptor and probably other cellular targets. (Lipton, 1993; Garthwaite and Boulton, 1995) The major signalling pathway activated by nitric oxide is the activation of the soluble guanylate cyclase. (Ignarro, 1989) Because its free radical properties, NO can also be neurotoxic primarily, and it has been involved in many neurodegenerative diseases including AD. (Smith, 1997) Other mechanism of NO toxicity related to its ability to react with many other free radicals involves its reaction with superoxide to form the strong oxidant peroxynitrite radical.

NO also binds to COX and it may act as an inhibitor of this enzyme at physiological concentrations leading to mitochondrial damage occurring in the ischemic brain and at inflammatory sites. (Talas, 2002; Muza, 2005; Toiber, 2005) The cyclic nucleotide cGMP is presented as a sensitive marker of intracellular NO formation. These findings confirm the role of the NO/cGMP pathway and point specifically to cGMP as a causative mediator in antioxidant protection. (Nina Grosser and Henning Schröder, 2003)

The free radical production induced damage to the cell by damaging the DNA, cytosolic and membrane-bound macromolecules. (Filipcik et al., 2006; Hardas et al., 2013)

Inflammation clearly occurs in the brain of neuro disease and the classical mediators of inflammation. Neurons can also express enzymes such as cyclooxygenases (COXs). (Vasto et al., 2007; Hoozemans et al., 2008; Vasto et al., 2008)

Inflammatory cytokines located close to amyloid plaques might be cytotoxic when chronically produced. (Cacquevel et al., 2004) The innate immune response that occurs in the brain induce the accumulation of inflammatory mediators, free radicals, complement components and microglia activation. These neuroinflammation makers are typically shown in association with the neuropathology of AD. (Weiner and Selkoe, 2002) The TNFα is an O-glycosylated, homotrimeric cytokine included in the maintenance of a wide spectrum of biological processes involving cell proliferation, differentiation, apoptosis, lipid metabolism, coagulation, insulin resistance and cancer. (Dong et al., 2015; Holmstrup et al., 2017)

Neurons, as well as other brain cells, can express enzymes such as COXs which are considered important in inflammatory cells, it has been demonstrated that COX-2 enzymes play a considerable role in the pathophysiology of neurodiseases. (Florinda List et al., 2010) In the present study, expression of neuronal COX-2 elevated in Aβ treated group. This results in harmony with Ho et al., 2001 who found that expression of neuronal COX-2 elevated in AD brain. Other study showed that, the expression of the constitutively expressed COX-1 and the inflammatory induced COX-2 has been intensively investigated in neuro diseases. (Hoozemans et al., 2008) In addition, Luisa Minghetti, 2004 presented that**,** COX-2 is rapidly expressed in several cell types in response to growth factors, cytokines, and pro-inflammatory molecules and has emerged

as the isoform primarily responsible for prostanoid production in acute and chronic inflammatory conditions. Furthermore, several studies reported increased neuronal COX-2 immunoreactivity compared to control brain tissues. (Yasojima et al., 1999; Yermakova et al., 2001)

The present finding is in agreement with Jenny Johansson, 2015 who reported that targeting toxic inflammatory prostaglandin signalling downstream of COX may potentially slow or prevent progression to neurodegeneration.

Combining donepezil with 7-nitroindazole significantly enhanced the protection against neurotoxicity of Aβ when compared with each drug alone. The best results were obtained by the combination of Donepezil with 7-nitroindazole, This effect may be related to the prevention of NO overproduction, stimulation of anticholinesterase activity, reducing oxidative damage in the brain, and protection against neuroinflammation.

Conclusion:

We concluded that NO plays a central role in neurotoxicity and the regulation of NO level is the key to stability and prevention of neurodegenerative disorder.

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