Monosodium Glutamate-Induced Cerebellar Toxicity; Possible Role of Nitric Oxide in Adult Albino Rats

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Abstract: Background: Several studies indicated that monosodium glutamate (MSG) disrupts the metabolism, the development, and the functions of various organs, such as liver, thymus, ovaries, kidney, and many parts of brain, including cerebellum. Nitric Oxide (NO) is known to be responsible for the organization of many biological events in the mammalian body as a second messenger and a neural messenger. Some studies found that NO is a neuroprotective substance while others qualify it as a neurotoxic. Aim: This study was designed to investigate the effect of non-selective inhibition of nitric oxide synthase enzyme isoforms on cerebellar structure and function in normal rats and in rats with MSG-induced cerebellar toxicity. **Material and methods**: The study groups included thirty two male adult albino Sprague-Dawley rats which were divided into four groups; group I (control), group II (LNAME-treated), group III (MSG- treated) and group IV (combined LNAME and MSG-treated). Motor coordination was assessed by rotarod test. Cerebellar nitrite concentration was measured. Histopathological evaluation of cerebellar structure and immunohistochemical examination for caspase-3 were done. **Results:** Both LNAME and MSG significantly impaired cerebellar function and resulted in marked cerebellar injury and an increase in apoptosis. This effect was most prominent in group III. **Conclusion**: Inhibition of nitric oxide synthase enzyme disturbed cerebellar structure and function in normal rats and potentiated MSG-induced cerebellar toxicity. Further studies are required for better understanding of the role of the NO pathway and developing therapeutic strategies that are based on manipulation of NO synthesis.
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1. Introduction

Monosodium glutamate (MSG) is a salt form of L -glutamic acid and is commonly used as a flavor enhancer worldwide. The Chinese restaurant syndrome is induced by ingestion of food containing monosodium glutamate salts. Individuals present with burning sensation on the back, neck, shoulders and abdomen. There is also nausea, vomiting, headache, flushing, chest pain and rarely life-threatening bronchospasm. [1]. the safety of MSG's usage has generated much controversy [2]. Glutamate has a very low acute toxicity under normal circumstances; the oral dose that is lethal to 50% of subjects (LD_{50}) in rats and mice is 15,000–18,000 mg/kg body weight [3]. On the other hand, several studies indicated that MSG with its glutamate excitatory characteristics disrupts the metabolism, the development, and the functions of various organs, such as liver, thymus, ovaries, kidney, and many parts of brain, including cerebellum [1]. In addition MSG- induced cerebellar toxicity was confirmed in previous studies [3, 4].

Nitric oxide (NO) is produced by three distinct isoforms of nitric oxide synthase (NOS): endothelial (eNOS), neuronal (nNOS) and inducible nitric oxide synthase (iNOS) [5]. NO is known to be responsible for the organization of many biological events in the mammalian body as a second messenger and a neural messenger [6].

Among the three enzymatic isoforms producing NO; nNOS and eNOS are expressed in the cerebellum while iNOS is not appreciably expressed in normal animals but starts to appear in pathologic conditions. The nNOS isoform has the distinctively highest level of expression, and its protein content in the cerebellum is comparatively higher than in other brain regions [7]. Free nitric oxide is highly reactive and unstable therefore it is difficult to measure nitric oxide. Nitric oxide reacts with oxygen to form nitrite and nitrate which are measurable forms of nitric oxide and are used as indirect measures of nitric oxide levels [8]. Some studies found that NO is a neuroprotective substance while others qualify it as a neurotoxic [6]. Disruption to NO synthesis and metabolism underlies many of the pathophysiological processes that occur after brain injury, and appears to occur regardless of the mode of injury. The available evidence suggests that NO derived from eNOS is neuroprotective after acquired brain injury whereas NO synthesized by

iNOS contributes to further damage, and that this difference is due to differences in timing, spatial location and concentration of NO generated by each isoform [9].

Also it was observed that exposing culture of cerebellar granular neurons to pharmacologic inhibition of NOS activity through the broad spectrum inhibitor N-nitro- L-arginine methylester (L-NAME) caused a progressive apoptotic death of cells, reaching 60% after 4 days of pharmacologic treatment [7].

Taken together, this study was designed to investigate the effect of non-selective inhibition of nitric oxide synthase enzyme isoforms on cerebellar structure and function in normal rats and in rats with MSG-induced cerebellar toxicity.

2 - Material and methods:

This study was performed at Physiology and Histology departments, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

2.1 *Animals:*

Thirty two male adult albino Sprague-Dawley rats, body weight 185 - 210 gm purchased from center for experimental animals, Faculty of Veterinarian Medicine, Zagazig University were used in the study.

2.2 ***Ethical Considerations***: This study was performed in accordance with the Guide for the ethical care and use of laboratory animals 2011(10). All rats were left to acclimatize for one week prior to the experiment and were housed in plastic cages maintained at controlled room temperature (22-24 C) with 12 hour diurnal (day and night change) with free access to standard pellet animal diet and tap water. Rats were equally divided into four weight-matched groups: group I: (control), group II: (LNAME-treated), group III: (MSG-treated) and group IV: (combined administration of LNAME and MSG).

2.3 Methods:

2.3.1. Groups II and IV: LNAME was administered by intraperitoneal (IP) injection at a concentration of 100 mg/kg dissolved in saline in group II [6] LNAME was given one hour before MSG administration in group IV [8].

2.3.2. Groups III and IV: animals in these groups were subjected to chemically-induced cerebellar injury by daily (IP) injection of 6 mg/Kg of MSG for10 days in group III [4]. LNAME was given one hour before MSG administration in group IV [8].

2.3.3. Rotarod test:

Is a test used to evaluate the cerebellar functions of rats via assessment of balance and motor coordination. Each rat was placed on a rotating rod or rotarod (10 cm long and 4 cm in diameter). The rod rotation speed was adjusted to be 20 rpm. Each rat was left on the rotating rod and the time elapsed from putting the animal on the shaft of the rotarod, till it falls to the ground (latency) was recorded. The maximal physiological time that was allowed for each rat was 2-4 minutes [11].

2.3.4. Measurements of cerebellar nitrite concentration: After motor coordination and balance assessment, the anaesthetized rats were decapitated and brains were quickly dissected and washed with ice-cold saline. Cerebellar hemispheres were separated and one of them was processed for histological and immunohistochemical evaluation. The second cerebellar hemisphere was kept in – 80°C degree for subsequent homogenization and measuring the concentration of cerebellar nitrite. Cerebellar nitrite was measured by spectrophotometric method [12] using its specific kits (Biodiagnostic, Egypt).

2.3.5. Histological, immunohistochemical examination: 6µm cerebellar paraffin sections were stained by Haematoxylin and Eosin (H, E), Mallory's phosphotungestic acid haematoxylin (PTAH), cresyl fast violet and immunohistochemical stain using polyclonal antibody to caspase-3 (Santa Cruz, USA, at dilution 1:150) for evaluation of apoptosis was done. Qualitative and quantitative assessments were done for histological and immunohistochemical changes in the cerebellum. Quantitative assessment included measurement of mean dendrite length. Optical density was done for Nissl's granules and caspase-3 expression [4].

2.3.6. Statistical analysis: All the data was expressed as mean ± standard error of mean (SEM) and analyzed using Statistical Package for Social Sciences (SPSS) program version 20. All the comparisons among groups were carried out using one way Analysis of Variance (ANOVA) followed by Bonferroni post hoc test. Data were considered statistically significant with $P \leq 0.05$.

3- Results:

3.1. Rotarod test:

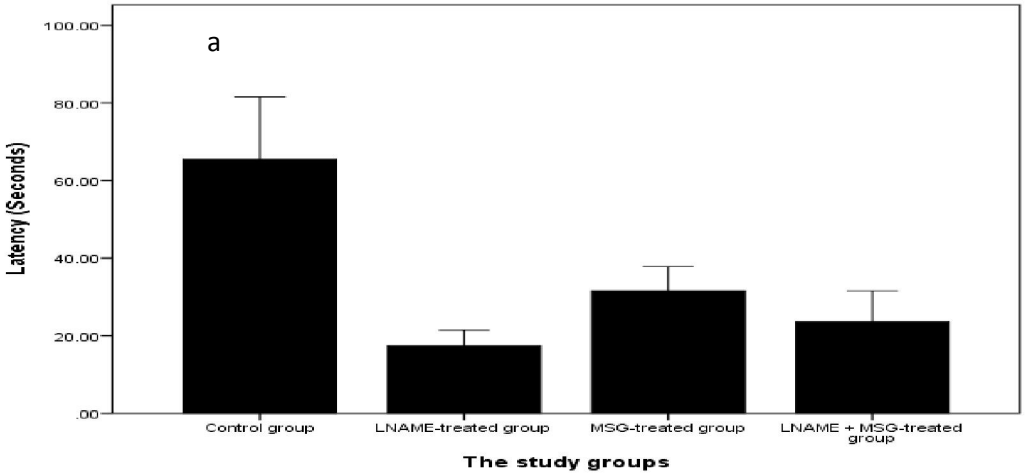
All treated groups (II,III and IV) had significant decrease in the latency time in rotarod test versus the control group I ($p<0.05$) Figure (1).

3.2. Cerebellar nitrite levels: Cerebellar nitrite levels were significantly decreased in groups II, III, and IV versus the control group ($p<0.05$) as shown in table (1).

3.3. Histopathological results: The histological changes that were detected in the cerebellar tissue in group II included wide displacement and distortion of the Purkinje cells as shown in (figure 2), significant decrease in purple Nissl's granules within the Perikarya of Purkinje cells (figure 3), and non-significant decrease in the length of Purkinje cell dendrites (figure 4). Group III showed distorted Purkinje cells. Some Purkinje cells were displaced, distorted and shrunken, leaving vacuoles in the

intercellular spaces (figure 2). Also there was a significant decrease in purple Nissl's granules within the perikarya of Purkinje cells (figure 3) and non-significant decrease in the length of Purkinje cell dendrites (figure 4). In group IV cerebellar tissue showed shrunken irregular Purkinje cells with various shapes. Some Purkinje cells had dark stained cytoplasm, deeply stained nuclei and surrounded with vacuolated neutrophil and other cells had karyolytic nuclei (figure 2). Also a significant decrease in purple Nissl's granules within the perikarya of Purkinje cells (figure 3) and non-significant decrease in the length of Purkinje cell dendrites (figure 4).

3.4. Immunohistochemical results: The optical density of caspase-3 immunoreactivity was significantly increased in groups II, III and IV versus group I. In addition group II showed significant increase in caspase-3 immunoreactivity versus group III. In group IV the optical density of caspase-3 immunoreactivity was significantly increased vs. groups II and III (figure 5).



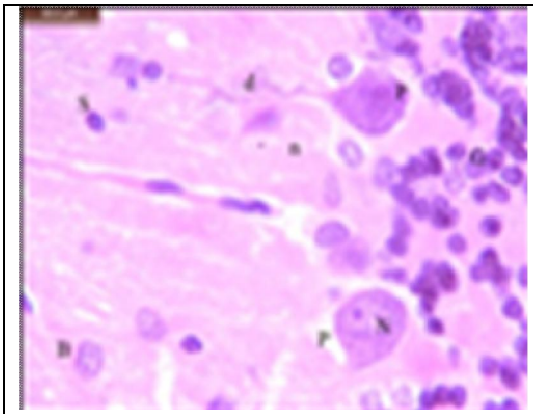
(Figure 1): Rotarod test revealed a significant difference in latency time (seconds) in group I (control group) versus groups II, III and IV (P<0.05).

Table 1 shows a significant decrease in cerebellar nitrite concentrations in groups II, III and IV versus control group I (P<0.05).

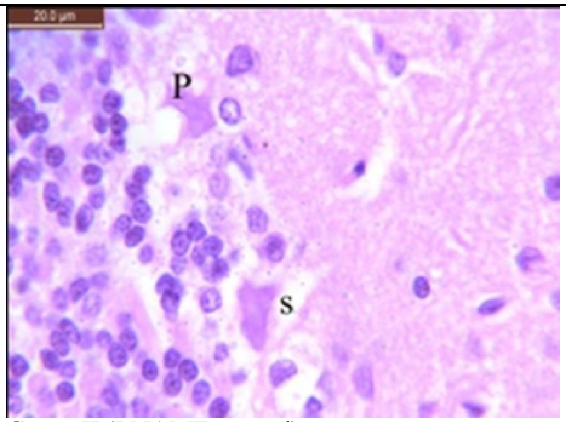
Table (1) Concentrations of cerebellar nitrite in the studied groups

Cerebellar nitrite Concentrations in (µmol/L)	Group I Mean ± SEM	Group II Mean ± SEM)	Group III Mean ± SEM)	Group IV Mean ± SEM)
	[a]9 ± 4.04	2.33 ± 0.62	1.33 ± 0.21	2.2 ± 0.49

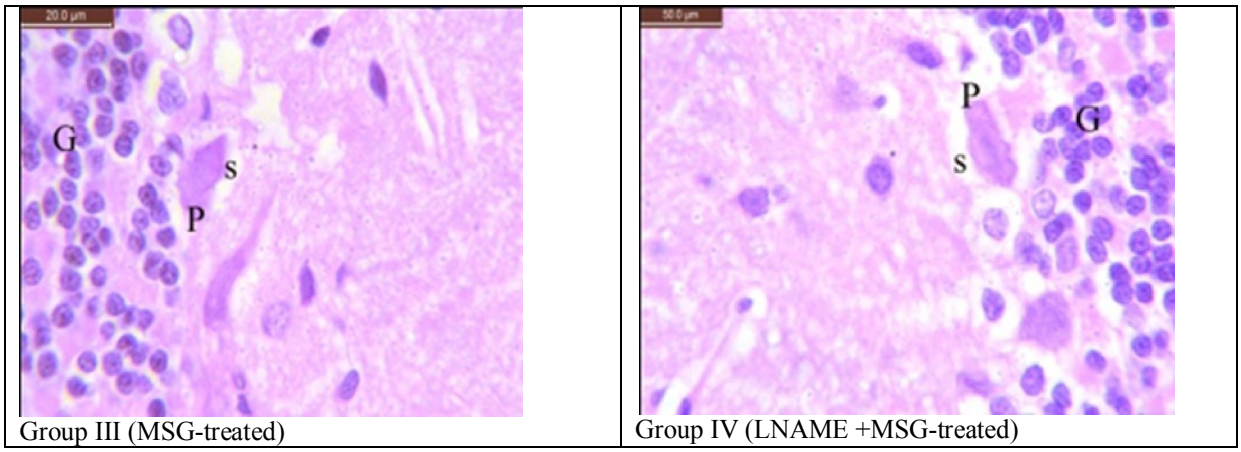
[a]***Significant difference in cerebellar nitrite concentrations (p<0.05)***



Group I (Control)



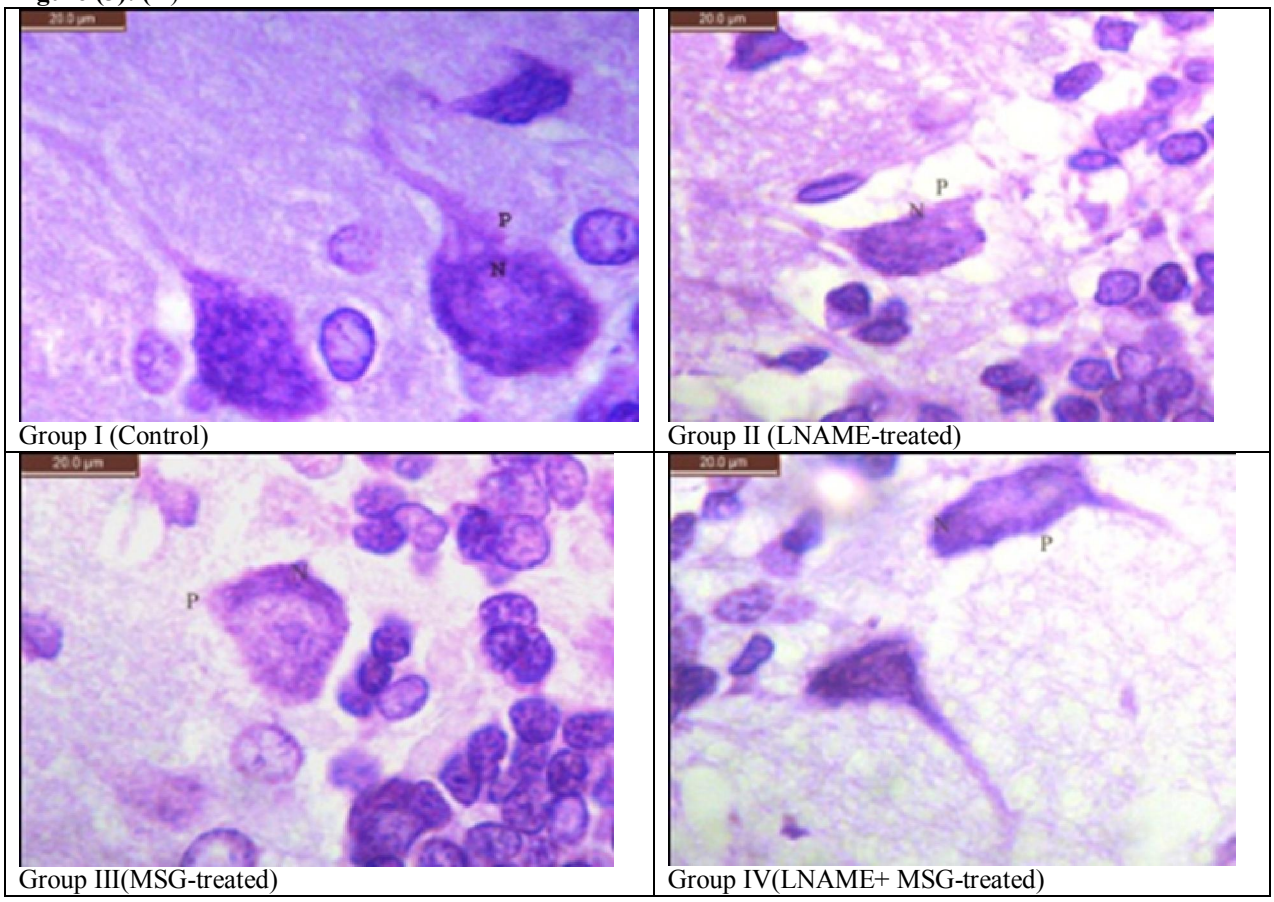
Group II (LNAME-treated)



Group III (MSG-treated) Group IV (LNAME +MSG-treated)

Figure 2: A photomicrograph of a section in the cerebellar cortex from **group I** showing scattered small stellate (**S**) and basket cells (**B**) in the molecular layer. Large pyriform cells with vesicular nuclei (**P**) are shown in the Purkinje cell layer. Crowded small cells with deeply stained nuclei (**G**) in the granular layer**.** A photomicrograph of a section in the cerebellum of a rat from **group II** showing Purkinje cells (P) are widely displaced and distorted. A photomicrograph of **group III** showing distorted Purkinje cells (P), some Purkinje cells are displaced, distorted and shrunken, leaving vacuoles in intercellular spaces. A photomicrograph of a section in the cerebellar cortex of **group IV** showing shrunken irregular Purkinje cells with various shapes. One Purkinje cell has dark stained cytoplasm and deeply stained nucleus and surrounded with vacuolated neutrophil. Another cell has karyolytic nucleus [H&E x 400].

Figure (3): (A)



Group I (Control) Group II (LNAME-treated)

Group III(MSG-treated) Group IV(LNAME+ MSG-treated)

Figure (3): (B)

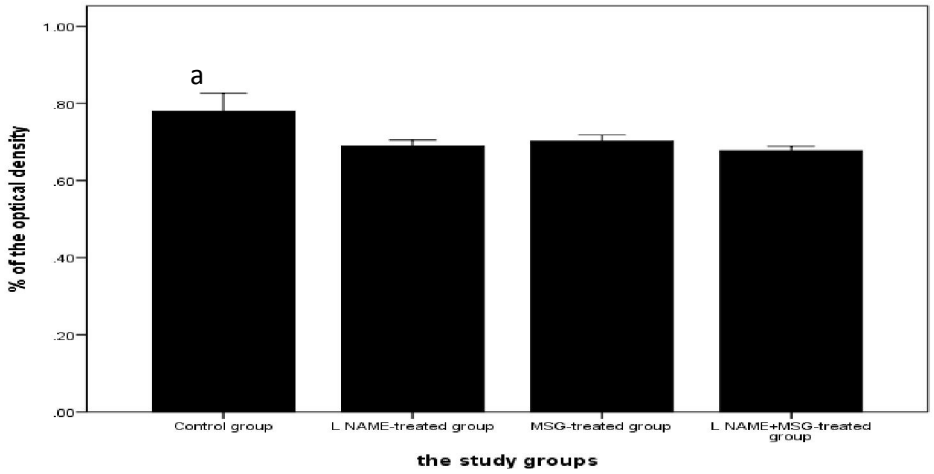


Figure (3): (A): A photomicrograph of a section in the cerebellum of a rat from group I showing purple Nissl's granules in the perikarya of Purkinje cells with deeply stained nuclei. A photomicrograph of a section in the cerebellum of group II showing decreased purple Purkinje granules (N) in the perikarya of Purkinje cells (P). A photomicrograph of a section in the cerebellum of group III showing decreased purple Nissl's granules (N) in the perikarya of Purkinje cells (P). A photomicrograph of a section in the cerebellum of group IV showing decreased purple Nissl's granules (N) in the perikarya of Purkinje cells (P) [Cresyl fast violet x 1000]. (B): A Significant increase in the percentage of optical density of Nissl's granules in group I versus groups II, III and IV (P<0.05).

Figure (4 A):

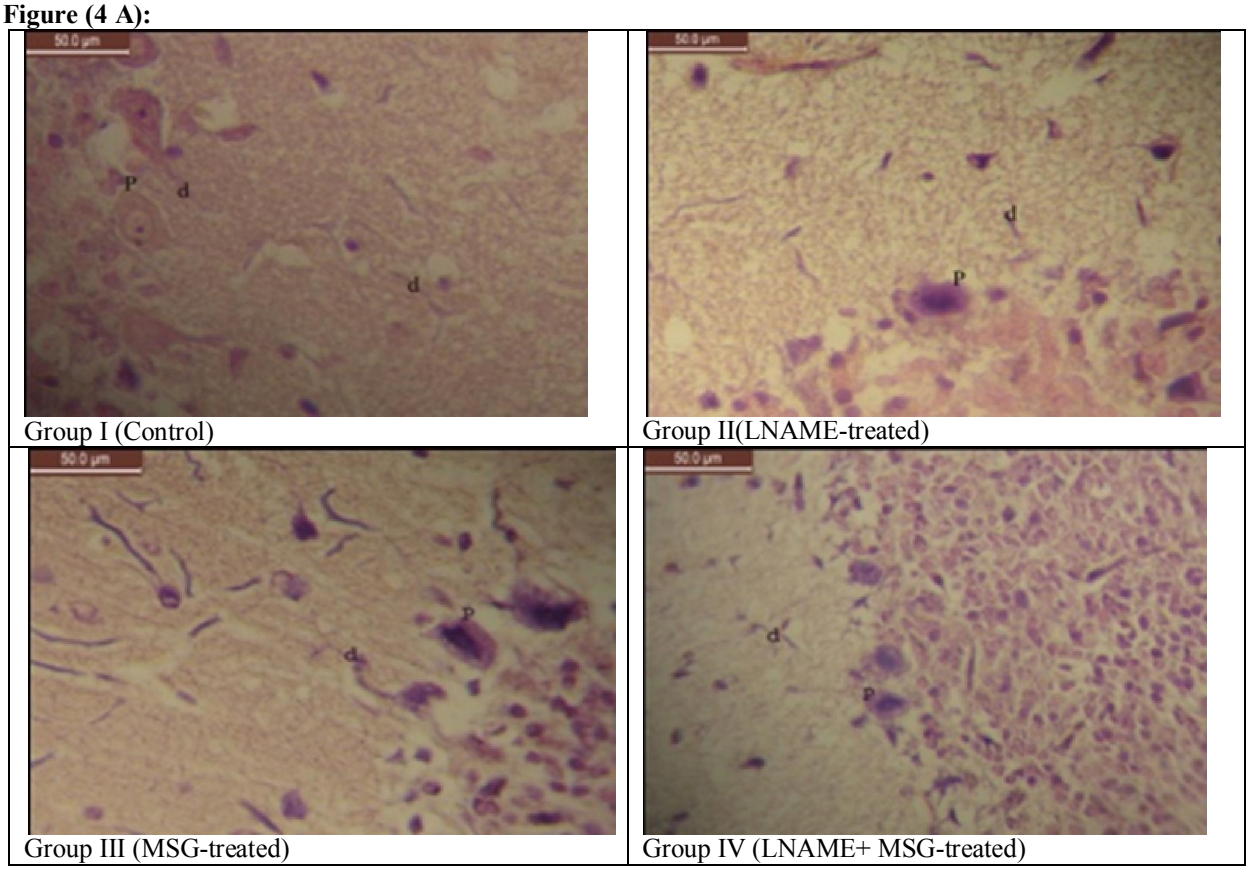


Figure (4):(B)

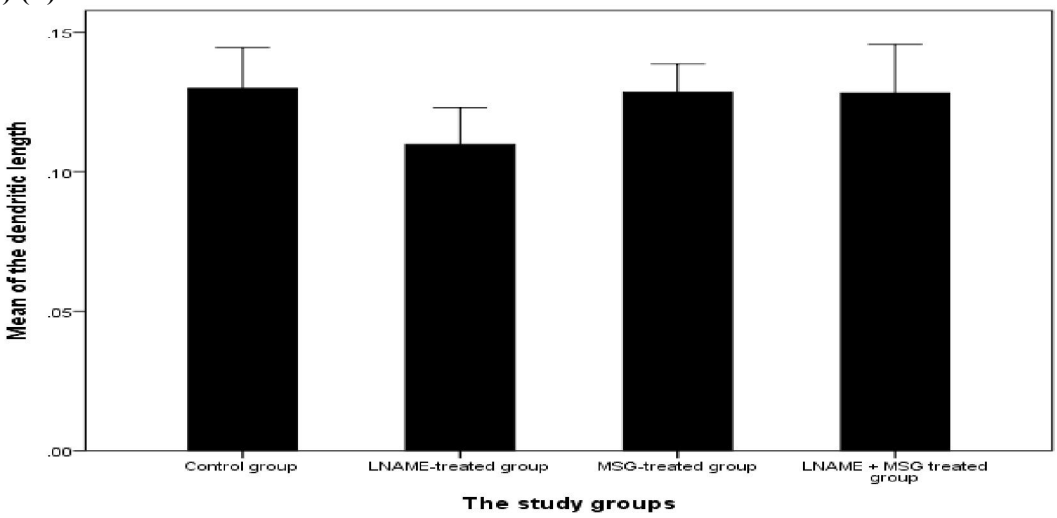
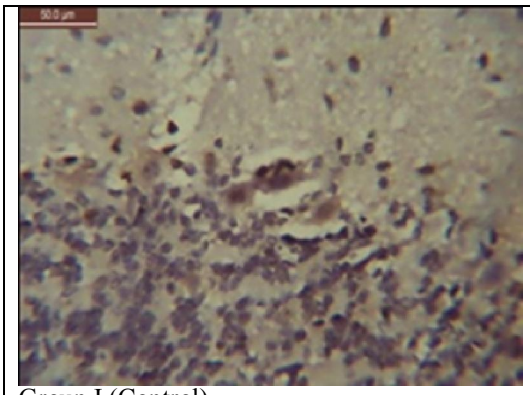
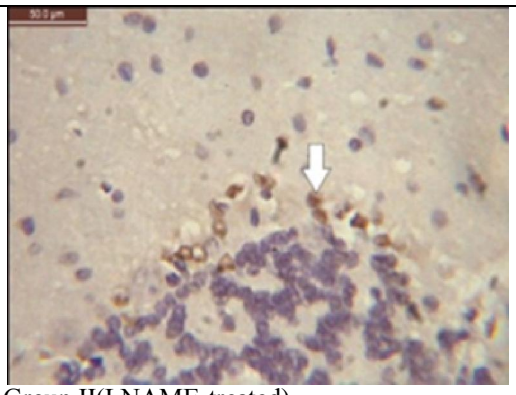
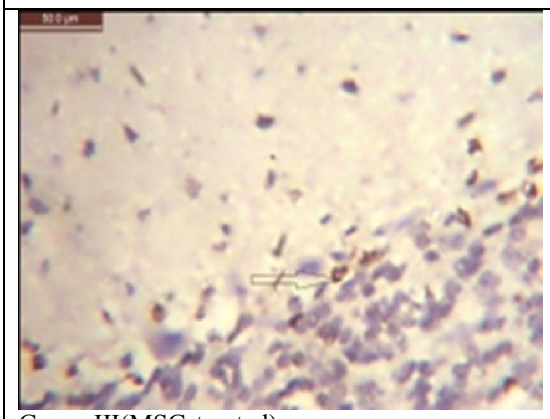


Figure (4): (A): A photomicrograph of group I showing blue discoloration of myelinated nerve fibers in the white matter. A photomicrograph of a section in the cerebellar cortex of group II revealed decreased in length of Purkinje cell dendrites. A photomicrograph of a section in the cerebellar cortex of group III demonstrating decreased in length of Purkinje cell dendrites. A photomicrograph of group IV showing decreased in length of Purkinje cell dendrites [PTAH x1000]. (B): The mean of dendritic length in the different experimental group (P>0.05).


Group I (Control)


Group II(LNAME-treated)


Group III(MSG-treated)

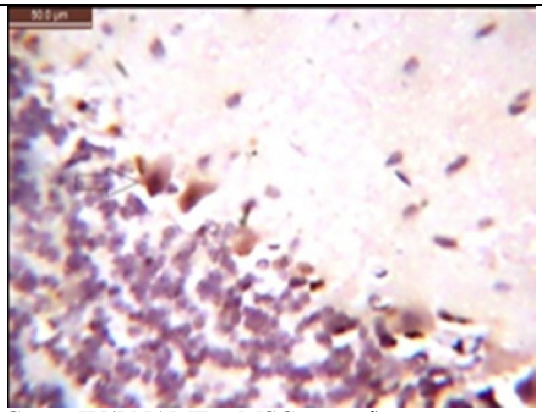

Group IV(LNAME + MSG treated)

Figure (5): (B)

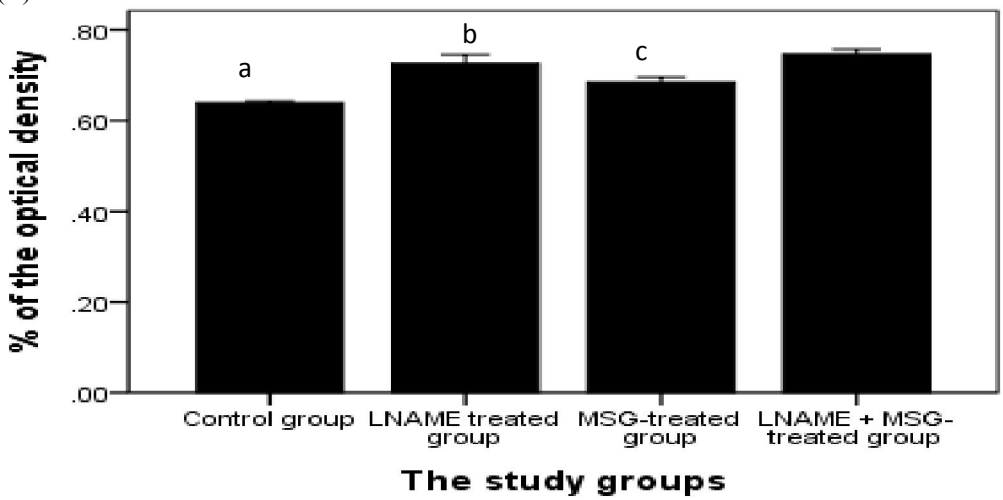


Figure (5): (A): Photomicrographs in the cerebellum of studied groups showing increased caspase-3 immunoreactive cells (white arrow) in groups II, III and IV [Caspase 3 x 1000]. (B): The percentage of optical density of caspase-3 immunoreactive cells in the study groups show significant decrease in optical density of caspase-3 immunoreactive cells in group I ($p<0.05$)[a] versus groups II, III and IV. Significant increase in optical density of immunoreactive cells in group II ($p<0.05$)[b] versus group III ($p<0.05$)[c]. Significant increase optical density of immunoreactive cells in group IV versus, group II and IV ($p<0.05$).

4- Discussion

Recently MSG is commonly sold in Egyptian supermarkets and many Egyptians consume it almost daily [13]. In the current study, it was found that MSG treatment in rats resulted in cerebellar damage and dysfunction. This result was in accordance to other studies [1,3 and 4]. The currently observed increase in apoptosis of cerebellar neurons with MSG treatment may explain that damage and dysfunction. Several mechanisms were suggested to be involved in MSG-induced cerebellar toxicity. One potential mechanism is glutamate excitotoxicity which based on that with an accumulation of glutamate in the synapses overstimulation of both A-amino- 3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and metabotropic glutamate receptors may be triggered. This results in the increase of intracellular calcium levels which in turn activates calcium-dependent degradative enzymes and apoptotic pathways. In addition, the massive influx of calcium ions may disrupt normal synaptic functions [1]. Also the increased oxidative stress may mediate the MSG-induced cerebellar injury through the depletion of glutathione which resulted in a form of cell injury called oxidative glutamate toxicity [14].

MSG treatment caused a significant decrease in cerebellar nitrite. In accordance with this result Da Cunha et al found that NO production is decreased in rats with MSG-induced obesity [14]. This finding may be attributed to the inhibitory effect of MSG on inducible nitric oxide synthase i(NOS) as suggested by Amagase *et al.* [16].

In the current study, it was observed that inhibition of NO synthase enzyme isoforms by LNAME significantly disturbed cerebellar structure and function in normal rats and potentiated the MSG-induced cerebellar injury. Also immunohistochemical evaluation of caspase-3 expression in cerebellar tissues showed significant apoptotic activity with LNAME treatment in normal rats and in rats with MSG-induced cerebellar toxicity. In line with these observations, Ciani *et al.* demonstrated that NO is essential for the survival of differentiating neurons and concluded that blocking NO production results in apoptotic death of differentiated neurons [17].

In addition the neuroprotective role of NO was documented against ethanol- induced cerebellar ataxia and in models of excitotoxicity [7].

Also the observed negative effect of NOS inhibitor on cerebellar function may be attributed to impairment in glutamate/NO/cGMP pathway in the cerebellum. In this pathwayglutamate activation of NMDA receptors increases Ca^{2+} in the post-synaptic neuron. Ca^{2+} binds to calmodulin and activates different enzymes including neuronal nitric oxide synthase n(NOS), increasing the formation of NO. This NO, in turn, activates soluble guanylatecyclase, increasing the formation of c GMP. This pathway modulates important cerebellar processes [18]. An impairment of glutamate/nitric oxide/c GMP signaling pathway was suggested as one of the molecular mechanisms that involved in rats with chronic hyperammonemia without or with liver failure [16] and in rats with ethanol induced cerebellar ataxia [19].

In contrast to previously mentioned findings it was suggested that NO may play a neurotoxic role and inhibitors of nitric oxide synthase enzymes may have a neuroprotective role in iron- induced cerebellar purkinje cell loss in rats [20] and in zinc induced cerebellar cells loss [6]. These controversial observations may be explained by that "NO can either function as a beneficial physiological agent utilized for essential functions such as differentiation or neurotransmission, or as a pathological agent that causes or exacerbates central nervous system (CNS) disease and injury. Whether NO is helpful or harmful depends on a variety of factors, such as the cellular environment in which NO is released, the rate of NO flux, as determined by which NOS isozyme is activated, and what array of second messenger cascades are available for utilization by NO for beneficial or toxic cell signaling" [21]. For example physiological amounts of NO are neuroprotective, whereas higher concentrations are clearly neurotoxic [22]. In addition inflammatory conditions lead glial cells to assume a neurodestructive phenotype and that induction of i(NOS) and overproduction of NO play a central role in the neurodegenerative mechanism [7]. So manipulation of NO synthesis and metabolism may be a future neurotherapeutic option by giving NO donors in cases lack NO and by inhibiting NO synthesis in cases with NO neurotoxicity.

Conclusion:

Inhibition of nitric oxide synthase enzyme disturbed cerebellar structure and function in normal rats and potentiated MSG-induced cerebellar toxicity in rats. Further studies are required for better understanding of the role of the NO -pathway and developing therapeutic strategies that are based on manipulation of NO- synthesis.

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