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Protective effects of Rosemary extract and/or Fluoxetine on Monosodium Glutamate-induced hippocampal neurotoxicity in rat

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Abstract

The use of Monosodium Glutamate (MSG) as a food flavor enhancer is increasing worldwide despite its neurotoxic effects. Fluoxetine (FLX) and Rosemary extract (RE) are known to have beneficial neuroprotective properties. Rats were divided into five groups: control group; MSG group, rats received 2 g/kg/day intraperitoneal (i.p.) injections of MSG for seven days; RE/MSG group, rats received 50 mg/kg/day of oral RE for 28 days starting prior to MSG; FLX/MSG group, rats received 10 mg/kg/day of oral FLX for 28 days beginning before MSG; and RE/FLX/MSG group, received combined treatments as mentioned above. Rats underwent the Barnes maze test, in addition to histopathological, immunohistochemical, morphometric and ultrastructural evaluations for their hippocampi. MSG increased the number of errors and escaped latency in the Barnes maze test that was significantly minimized in the three treatment groups. The MSG group exhibited pyramidal cell (PC) degeneration, shrunken glial cells and massive vascular dilatation that were improved with RE and/or FLX treatment. The number of glial fibrillary acidic protein (GFAP)-immunopositive cells were increased, and the number of PCs was decreased in the MSG group, while these values were significantly reversed with the three treatment groups with the most significant improvement at RE/FLX/MSG one. Ultrastructurally, PCs were shrunken with degenerated nuclei, dilated endoplasmic reticulum, swollen mitochondria, and vacuolations in the MSG group that were improved with RE and/or FLX. In conclusion, the combined RE and FLX treatment can ameliorate the toxic effect of MSG on rat hippocampus probably through its antioxidant and anti-inflammatory effects.

Keywords: Monosodium Glutamate, Rosemary extract, Fluoxetine, neurodegeneration, rat hippocampus.

Introduction

Exposure to certain chemicals can produce neurotoxicity resulting in neuronal cell degeneration [1]. One of these chemicals is Monosodium Glutamate (MSG), which is used as a flavor enhancer in various food industries [2, 3]. MSG consumption between 0.3 g and 1 g is safe; however, this daily consumption is growing worldwide to be estimated as an average of 3–4 g [4]. Since Glutamate is considered a pivotal central nervous system (CNS) excitatory neurotransmitter, its excess leads to massive neuronal damage and other complications [5].

Swamy *et al.* (2014) [6] reported that MSG is responsible for alteration in antioxidant defense homeostasis and increased nonspecific permeability to several ions due to lost neuronal membrane integrity, and pathological intracellular metabolic processes changes. A lower cyclic adenosine monophosphate (cAMP)-activated protein kinase activity and increased levels of apoptosis mediator may be implicated for such sequences [7].

Hippocampus is one of the major structures forming the limbic system, which is included in its posterior part. It is the earliest and most severely damaged structure at various neuropsychiatric illnesses, such as Alzheimer's disease (AD) and epilepsy [8].

Fluoxetine (FLX) is a selective serotonin reuptake inhibitor (SSRI) antidepressant and is one of the most excessively consumed medications [9]. Hippocampus is involved in antidepressant and anxiolytic actions of SSRIs [10]. FLX treatment in rodents promotes neuroplasticity, especially in the hippocampus, by inducing neuronal proliferation, increasing survival of the newly formed cell, accelerating neuronal maturation, and increasing both dendritic arborization and length in the dentate gyrus (DG) [11]. Nevertheless, it has a wide range of common side effects, such as headache, agitation, anorexia, insomnia, sexual dysfunctions, bleeding, and hyperprolactinemia [12].

Rosmarinus officinalis (RO) is one of the popular herbs. Its extract has antioxidant properties attributed to its Carnosic Acid, phenolic diterpenes, and Carnosol content [13]. It has antiviral, anti-inflammatory, cytoprotective, antithrombotic, antidiabetic, and neuroprotective effects partly due to decreasing neuronal oxidative damage. As a natural food preservative, RO could potentially replace the artificial additives to avoid their harmful properties [14].

Aim

The present study aimed to evaluate and compare the preventive effects of Rosemary extract (RE) and FLX alone or in combination on the MSG-induced pathological changes of the hippocampus in adult rats.

Materials and Methods

Animals

Sixty male Sprague–Dawley albino rats (10–12 weeks

old, weighing 225±25 g) were obtained from Animal House of Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. They were kept in temperature-controlled rooms, housed in separate spacious wire mesh cages, and provided with water and food *ad libitum*, with a 12-hour light/dark cycles.

Experimental design

After two weeks of acclimatization, the rats were randomly divided into five equal groups (n=12), as following:

▪ Group I (control group):

– Subgroup A: as a negative control subgroup that had no intervention.

– Subgroup B: as a positive control, one that received 1 mL of distilled water, daily for 28 days by oral gavage; in addition to intraperitoneal (i.p.) injection of 1 mL/kg of saline, daily for seven days starting from day 22 after the first oral gavage.

▪ Group II (MSG group): rats received daily MSG 2 g/kg/day *via* i.p. injection for seven days [15].

▪ Group III (RE/MSG group): rats received 50 mg/kg/day of RE for 28 days by oral gavage [16]. In addition, on day 22, MSG was given daily by i.p. injection for seven days.

▪ Group IV (FLX/MSG group): rats received 10 mg/kg/day of FLX for 28 days by oral gavage [17]. In addition, on day 22, MSG was administrated daily by i.p. injection for seven days.

▪ Group V (RE/FLX/MSG group): rats received RE in combination with FLX daily for 28 days by oral gavage, and on day 22, MSG was given daily by i.p. for seven days.

Chemicals

MSG was purchased from Fisher Scientific Co. LLC (Pittsburgh, PA, USA), in crystals. FLX was purchased under the trade name of Prozac® 20 mg oral capsules (Eli Lilly & Co., Ltd., USA).

RE preparation

A quantity of 50 g of dried RO leaves was extracted with 250 mL of 75% Methanol in Soxhlet condenser for six hours, then filtered through Schleicher & Schuell (S & S) filter paper (No. 604), and the solvent was vacuum distilled at 40°C in a rotary evaporator. The remaining extract was finally dried in a vacuum oven at 30°C for two hours. Finally, the extract was kept in the dark bottles at 4°C in the refrigerator until use [16].

Barnes maze test

Cognitive functions were assessed using the Barnes maze test five days after the last MSG injection, preceded by four days of daily training of the rats. A bright light was used to motivate the rats to escape. Only one hole leads to a target escape box. The test was performed in a quiet room and monitored using a Sony® video camera for detection of the number of errors (total number of incorrect trials before reaching the target hole) and escape latency (time elapsed to reach the target hole in seconds) [18].

Histopathological (HP) study

At the end of the experiment, rats were sacrificed by cervical decapitation under Ketamine anesthesia. Brains were collected immediately and then cut through the median sagittal plane into two halves. The left cerebral hemispheres were fixed in 10% neutral buffered formalin for 24 hours, dehydrated in graded concentrations of Ethanol, and embedded in paraffin blocks, then 5 µm-thick sections were cut to be stained using Hematoxylin–Eosin (HE) (Sigma, St. Louis, MO, USA). Finally, sections were examined using Olympus DP70 light microscope (Tokyo, Japan) [19].

Immunohistochemistry

For glial fibrillary acidic protein (GFAP) immunohistochemical (IHC) staining of the astrocytes, the specimens were fixed in Picric Acid–Paraformaldehyde–Glutaraldehyde fixative for 10 days and paraffin blocks were prepared. Paraffin sections of 5 µm thickness were mounted onto glass slides coated with poly-L-Lysine. Treatment in Xylene then Acetone for 10 minutes each was done to remove the paraffin wax. After a brief incubation (1–5 minutes) with two changes of phosphate-buffered saline (PBS, pH 7.4), hydrogen peroxide in Methanol was used to block the endogenous peroxidase activity. Sections were then incubated in a wet chamber with normal goat serum for 20 minutes at room temperature to suppress background staining. Sections were incubated with polyclonal rabbit anti-GFAP antiserum (Sigma, Aldrich, UK) (1:500 dilution in PBS) at 4°C for 24 hours. Biotinylated mouse anti-rabbit immunoglobulin incubation for 30 minutes, as a secondary antibody, and then with the Avidin–Biotin complex for 45 minutes, as peroxidase conjugate, were performed. Visualization of the immunocomplex was achieved using 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich, UK). Sections were counterstained with Hematoxylin, coverslipped and finally examined under the light microscope using a magnification of ×400 [20].

Morphometric analysis

A morphometric study was conducted in HE-stained and GFAP-immunostained sections using the image analyzer Leica Q image system (Leica Microsystem Corp., Cambridge, UK). From each animal, five sections were investigated, and from each section, five different fields were observed at a magnification of ×400. All data regarding the numerical cell density were expressed as cell/mm^2. At HE-stained sections, the number of pyramidal cells (PCs) and granular cells (GCs) were counted in each group. Counting of PCs and GCs was regarding the areas of their cellular layers in the *Cornu Ammonis* (CA1) and DG respectively with the exclusion of the neuropil areas of each field during the assessment of cellular density. Furthermore, the numbers of positive GFAP-immunoreactive astrocytes also were estimated in the CA1 and DG. These cells were qualified as GFAP-immunopositive when a cell body was observed with extended processes. GFAP expressing fibers without a clear cell body were left out of the quantification [21].

Transmission electron microscopy

Hippocampi extracted from the right cerebral hemisphere were fixed in a buffered 2.5% Glutaraldehyde solution and then in 1% Osmium Tetroxide and dehydrated in ascending graded Ethanol concentrations. Semithin (0.3 µm-thick) sections were cut and stained with Toluidine Blue. Ultrathin sections were cut using MT 600-XL RMC ultratome and were stained with Uranyl Acetate and Lead Citrate. The sections were examined with a JEOL-1010 (Japan) transmission electron microscope (TEM) at the Regional

Center for Mycology and Biotechnology Transmitting Electron Unit (Al-Azhar University, Cairo, Egypt) and photographed under different magnifications.

Statistical analysis

Data were collected, revised, verified, and then edited on a personal computer using a Statistical Package for the Social Sciences (SPSS®) software (version 24). Data were expressed as mean ± standard deviation (SD). The analysis of variance (ANOVA) with Tukey's *post hoc* test was used for the comparison between groups according to their quantitative data. Pearson's correlation coefficient was used to evaluate the connection between different variables of the study. Finally, results were considered significant when the probability value (p) was less than 0.05.

Results

Barnes maze test results

Regarding the Barnes maze test, there was a statistically significant increase in the escape latency and the number of errors in the MSG group than the control one. Furthermore, the RE/MSG, FLX/MSG and RE/FLX/MSG groups showed a statistically significant decrease in the escape latency and the number of errors compared to the MSG group. Otherwise, there was no statistical difference between the three treatment groups according to their numbers of errors. In contrast, escape latency was significantly decreased in the RE/FLX/MSG group compared to the RE/MSG and FLX/MSG ones (Table 1).

Table 1 – ***No. of errors and escape latency in Barnes maze test of the study groups***

Group \ Parameter	No. of errors	Escape latency
Control	0.67±0.65	8.50±2.32
MSG	3.42±0.9[a]	69.33±3.98[a]
RE/MSG	1.08±0.79[b]	42±4.05[a,b]
FLX/MSG	1.5±0.8[b]	49.17±3.04[a–c]
RE/FLX/MSG	0.67±0.78[b]	11.83±4.02[b–d]

Values are mean ± SD. [a]p<0.05 *vs* control group, [b]p<0.05 *vs* MSG group, [c]p<0.05 *vs* RE/MSG group, and [d]p<0.05 *vs* FLX/MSG group. Statistical analysis was performed by ANOVA, followed by Tukey's *post hoc* test. ANOVA: Analysis of variance; FLX: Fluoxetine; MSG: Monosodium Glutamate; RE: Rosemary extract; SD: Standard deviation.

HP findings

In the control group HE-stained sections, CA1 was formed of three characteristic layers: *stratum oriens*, *stratum pyramidale* and *stratum radiatum*. The *stratum pyramidale* consisted of 4–5 rows of closely packed PCs with large vesicular nuclei and triangular basophilic cytoplasm. *Stratum oriens* and *stratum radiatum* showed scattered glial cells and blood capillaries (Figure 1A). DG had three layers: polymorphic, granular, and molecular layers. The granular layer was formed of 8–10 rows of closely packed small rounded GCs with large vesicular nuclei and thin basophilic cytoplasm (Figure 2A).

CA1 and DG of the MSG group showed massively scattered disorganized degenerated PCs and GCs; in addition, many PCs appeared to be ghost-like cells. Glial cells were numerous, shrunken, deeply stained, and surrounded with pericellular halos. Furthermore, the blood capillaries were dilated and congested (Figures 1B and 2B).

The RE/MSG and FLX/MSG groups showed partial preservation of their cellular organization and integrity, as the PCs and GCs mostly had large vesicular nuclei and preserved cytoplasm. However, few of them showed signs of degeneration and the presence of few ghost-like PCs. Furthermore, CA1 and DG layers had few deeply stained glial cells with relatively wide pericellular halos. Moreover, blood capillaries normally appeared or minimally dilated (Figure 1, C and D; Figure 2, C and D).

The RE/FLX/MSG group showed that almost all the PCs and GCs were with vesicular nuclei and scanty cytoplasm. In addition, glial cells had dense nuclei and were surrounded with thin clear halos, and most of the blood capillaries were not congested or dilated (Figures 1E and 2E).

IHC results

GFAP-immunostained sections showed star-shaped astrocytes within the different layers of CA1 and DG in the control group (Figure 3, A and B). Moreover, the MSG group revealed a marked increase in the number of astrocytes and their cytoplasmic processes exhibiting massive immunoreactivity (Figure 3, C and D).

The RE/MSG and FLX/MSG groups showed that GFAP immunoexpression was minimal in the astrocytes with fewer branching cell processes within the layers of CA1 and DG. However, the DG of the FLX/MSG group revealed more intense astrocytic immunoreactivity than in CA1 (Figure 3, E–H). On the other hand, the RE/FLX/MSG group revealed mild GFAP-immunoexpression in all layers of CA1 and DG; however, this immunoexpression was also higher in the DG than in CA1 (Figure 3, I and J).

Morphometric assessment

The number of GFAP-immunopositive cells was significantly increased in the MSG group *versus* the control one. On the other hand, only the RE/MSG and RE/FLX/MSG groups showed a significant decrease in the astrocytic number compared to the MSG group (Figure 4). Furthermore, Pearson's correlation between the number of GFAP-immunopositive cells and Barnes maze test results demonstrated a significant positive correlation (n=60, r=0.772, p<0.001).

The number of the PCs significantly decreased in the MSG group *versus* the control one. On the other hand, the RE/MSG, FLX/MSG, and RE/FLX/MSG groups showed a significant increase in the PCs compared to the MSG group. Furthermore, the RE/FLX/MSG demonstrated a significant increase in the PC count compared to the other groups (Figure 5). Moreover, PC density had a significant negative correlation with Barnes maze test results (n=60, r=−0.933, p<0.001) and also a significant negative correlation with the number of GFAP-immunopositive cells (n=60, r=−0.744, p<0.001).

The number of the GCs showed a significant decrease in the MSG group *versus* the control one. On the other hand, the RE/MSG, FLX/MSG, and RE/FLX/MSG groups showed a significant increase in the GCs compared to the MSG one (Figure 6). Furthermore, the GC density correlation with Barnes maze test results was significantly negative (n=60, r=−0.922, p<0.001), and also was significantly negative with the number of GFAP-immunopositive cells (n=60, r=−0.705, p<0.001). Nonetheless, there was a significant positive correlation between both PC and GC densities (n=60, r=0.961, p<0.001).

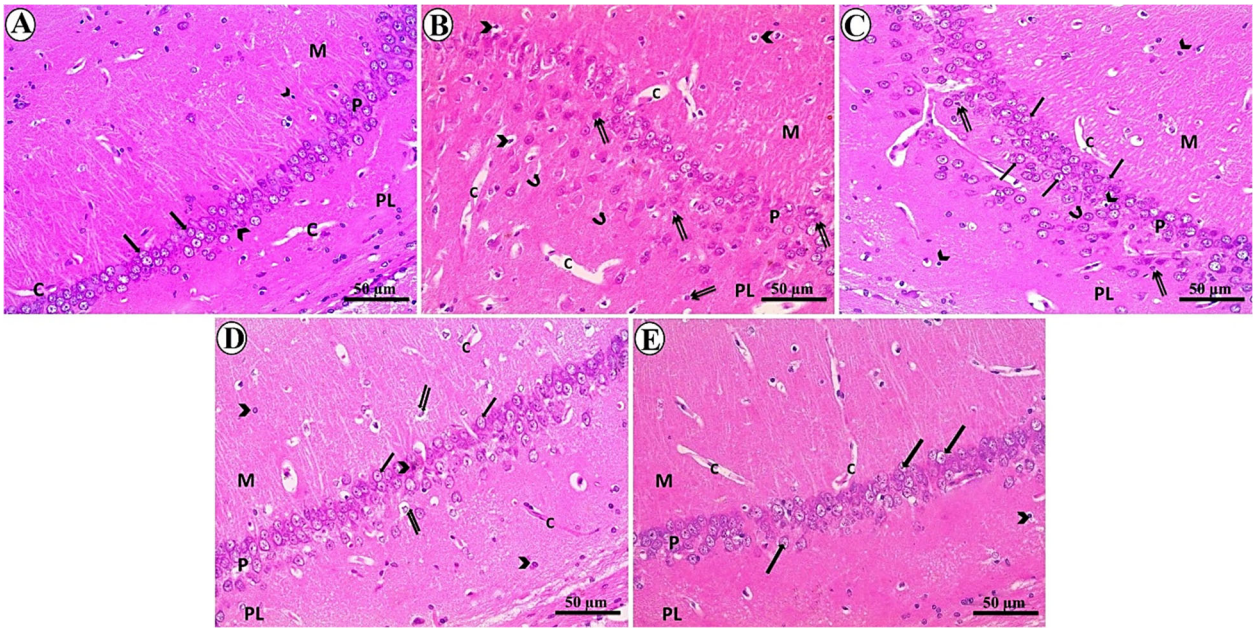

Figure 1 – ***Photomicrographs of CA1 sections in rat hippocampus: (A) Control group showing the*** **stratum pyramidale** ***{P} with PCs (arrows), and glial cells (arrowheads). The*** **stratum oriens** ***{O} and*** **stratum radiatum** ***{R} with normal neuronal processes and capillaries {C}; (B) MSG group showing many shrunken and scattered PCs with darkly stained nuclei (double arrows) and others as ghost-like cells (curved arrows). Dark shrunken deeply stained glial cells with pericellular halos (arrowheads) and dilated capillaries {C}; (C) RE/MSG group showing disorganized PCs (arrows), few degenerated PCs (double arrow), ghost-like cells (curved arrow), darkly-stained glial cells with pericellular halos, while others are normal (arrowheads)and minimally dilated capillaries {C}; (D) FLX/MSG group showing disorganized PCs (arrows), few cells with shrunken nuclei and vacuolated cytoplasm (double arrows), normal glial cells (arrowheads), and non-dilated capillaries {C}; (E) RE/FLX/MSG group showing normal PCs (arrows), glial cells (arrowheads) and blood capillaries {C}. HE staining: (A–E) ×400. Scale bar: (A–E) 50 µm. CA1: Cornu Ammonis 1; FLX: Fluoxetine; HE: Hematoxylin–Eosin; MSG: Monosodium Glutamate; PCs: Pyramidal cells; RE: Rosemary extract.***

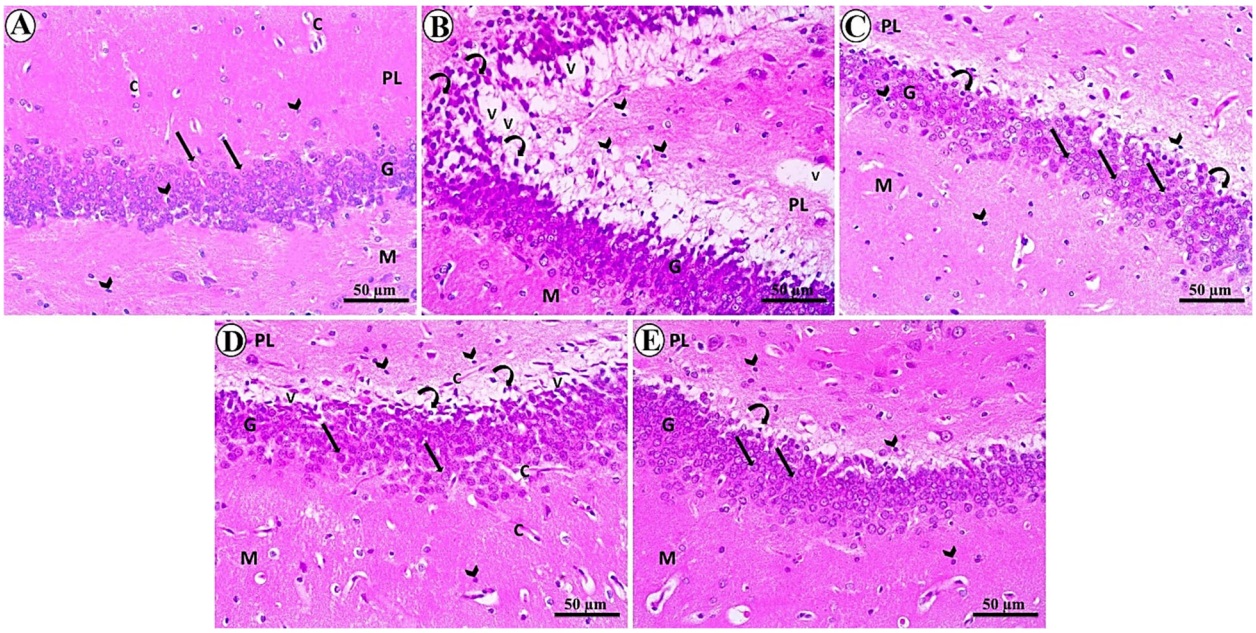

Figure 2 – ***Photomicrographs of DG sections in rat hippocampus: (A) Control group showing the granular layer {G} with closely packed granular cells (GCs) (arrows) and glial cells (arrowheads). The polymorphic {PL} and molecular {M} layers with normal capillaries {C}; (B) MSG group showing disorganized deeply stained GCs (curved arrows) with cytoplasmic vacuolations {V} and glial cells with surrounding wide clear halos (arrowheads); (C) RE/MSG showing GCs (arrows), few shrunken deeply stained cells with vacuolations (curved arrows). Few glial cells are surrounded by clear halos (arrowheads); (D) FLX/MSG group showing few shrunken GCs (curved arrows), minimal vacuolations {V}, and GCs (arrows) and glial cells with shrunken deeply stained nuclei (arrowheads); (E) RE/FLX/MSG group showing normal GCs (arrows), few shrunken GCs with vacuolations (curved arrow), and normal glial cells (arrowheads) and blood capillaries {C}. HE staining: (A–E) ×400. Scale bar: (A–E) 50 µm. DG: Dentate gyrus; FLX: Fluoxetine; GCs: Granular cells; HE: Hematoxylin–Eosin; MSG: Monosodium Glutamate; RE: Rosemary extract.***

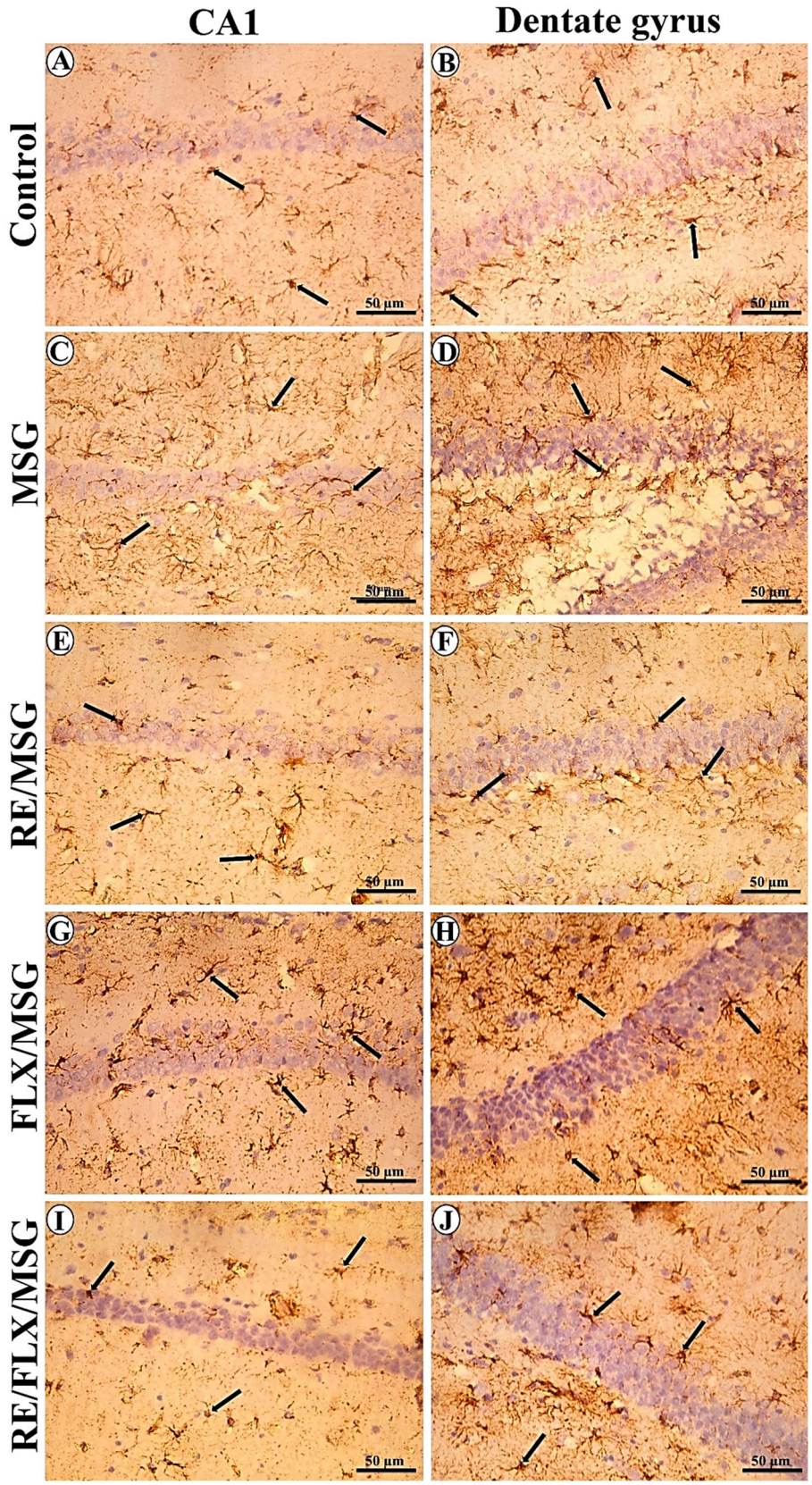

Figure 3 – ***Photomicrographs of GFAP-immunostained sections of CA1 and DG of rat hippocampus of the study groups: (A) CA1 of the control group showing mild immunoreactivity (arrows); (B) DG of the control group showing slight immunoreactivity (arrows); (C) CA1 of the MSG group with extensive immunoreaction; (D) DG of the MSG group showing massive immunoreaction; (E) CA1 of the RE/MSG group with minimal immunoreactivity; (F) DG of the RE/MSG group showing mild immunoreaction; (G) CA1 of the FLX/MSG group with slight immunoreactivity; (H) DG of the FLX/MSG group with moderate immunoreactivity; (I) CA1 of the RE/FLX/MSG group showing mild immunoreactivity; (J) DG of the RE/FLX/MSG group with mild immunoreactivity. Anti-GFAP antibody immunostaining: (A–J) ×400. Scale bar: (A–J) 50 µm. CA1: Cornu Ammonis 1; DG: Dentate gyrus; FLX: Fluoxetine; GFAP: Glial fibrillary acidic protein; MSG: Monosodium Glutamate; RE: Rosemary extract.***

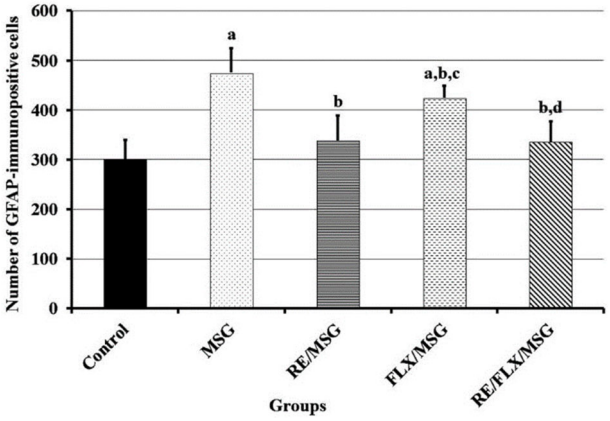

Figure 4 – ***Number of GFAP-immunopositive cells of the study groups. Values are mean ± SD. Statistical analysis was performed by ANOVA, followed by Tukey's post hoc test. ANOVA: Analysis of variance; FLX: Fluoxetine; GFAP: Glial fibrillary acidic protein; MSG: Monosodium Glutamate; RE: Rosemary extract; SD: Standard deviation.***

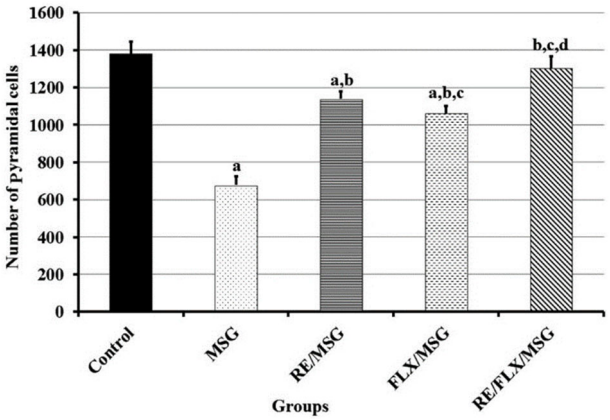

Figure 5 – ***Number of PCs of the study groups. Values are mean ± SD. Statistical analysis was performed by ANOVA, followed by Tukey's post hoc test. ANOVA: Analysis of variance; FLX: Fluoxetine; MSG: Monosodium Glutamate; PCs: Pyramidal cells; RE: Rosemary extract; SD: Standard deviation.***

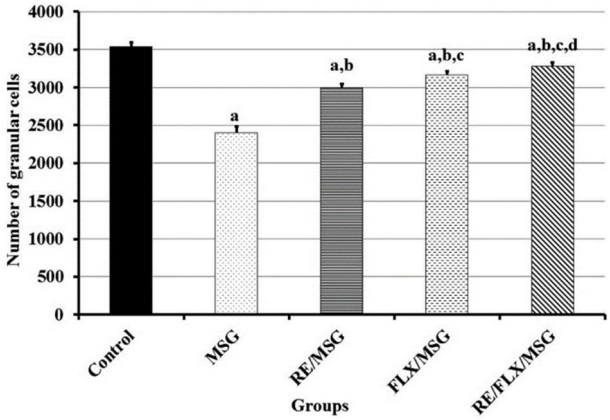

Figure 6 – ***Number of GCs of the study groups. Values are mean ± SD. Statistical analysis was performed by ANOVA, followed by Tukey's post hoc test. ANOVA: Analysis of variance; FLX: Fluoxetine; GCs: Granular cells; MSG: Monosodium Glutamate; RE: Rosemary extract; SD: Standard deviation.***

Electron microscopic findings

With TEM, the control group PCs had distinct cytoplasmic membrane and well-defined euchromatic nuclei with prominent nucleoli. Furthermore, the cytoplasm contained different normal sizes of mitochondria, free ribosomes, and rough endoplasmic reticulum (rER) (Figure 7A). In the MSG group, the PCs were with shrunken nuclei, irregular nuclear membranes, and dense clumping nuclear chromatin. In addition, there were dilated cisternae of rER, swollen mitochondria and large cytoplasmic vacuolations (Figure 7, B and C).

The PCs of the RE/MSG group had euchromatic nuclei with the most regular nuclear membrane and prominent nucleoli. Furthermore, there were non-dilated rER, minimally swollen mitochondria and absent cytoplasmic vacuolations (Figure 7D).

By TEM assessment of the FLX/MSG group, the PCs mainly were with euchromatic nuclei, prominent nucleoli, and slightly irregular well-defined nuclear membrane. Moreover, there were small vacuolations, slightly dilated rER and edematous mitochondria (Figure 7E). The PCs of the RE/FLX/MSG group showed well-defined round to oval nuclei, prominent nucleoli, distinct regular nuclear membrane, and intact rER and mitochondria (Figure 7F).

Discussions

Glutamate is one of the most abundant excitatory neurotransmitters of the brain, playing an important role in learning and memory. MSG is sodium salt of L-Glutamate and used worldwide as a flavor enhancer, increasing the palatability of food [22]. However, it is toxic to the hippocampus of humans and laboratory animals, especially at high doses through increasing the excitability of neuronal cells, increasing influx of Ca^{2+} ions *via* Glutamate-gated channels, triggering catabolic processes, and releasing reactive oxygen species (ROS) that finally lead to neuronal injury [7].

Neuroprotection against Glutamate-induced neurotoxicity is, therefore, a therapeutic strategy for preventing and treating both acute and chronic neurodegeneration [23].

FLX is a widely used antidepressant acting through inhibition of serotonin reuptake into the synaptic terminals of the CNS and also has blocking effects on various ion channels, including Ca^{2+} channels [24].

RO is native to the Mediterranean region, also cultivated in several European countries and in the USA, and recently has been used for medical purposes because of its antioxidant properties [25].

In this study, the toxic effects of MSG were investigated on the rat hippocampus, and, in addition, the possibility of RE and/or FLX to modulate this toxic effect was evaluated.

In the present study, MSG induced a considerable degree of hippocampal neurodegeneration, leading to a significantly impaired spatial memory, as proved by the Barnes maze test. The defective memory parameters may be due to increased activity of acetylcholinesterase enzyme resulting in the reduction of acetylcholine and decrease in cognition. The most consistent losses of acetylcholine were seen in cholinergic neurons of the basal forebrain that provide a major source of cholinergic innervation to the cerebral cortex and hippocampus, playing a key role in memory and attention [26].

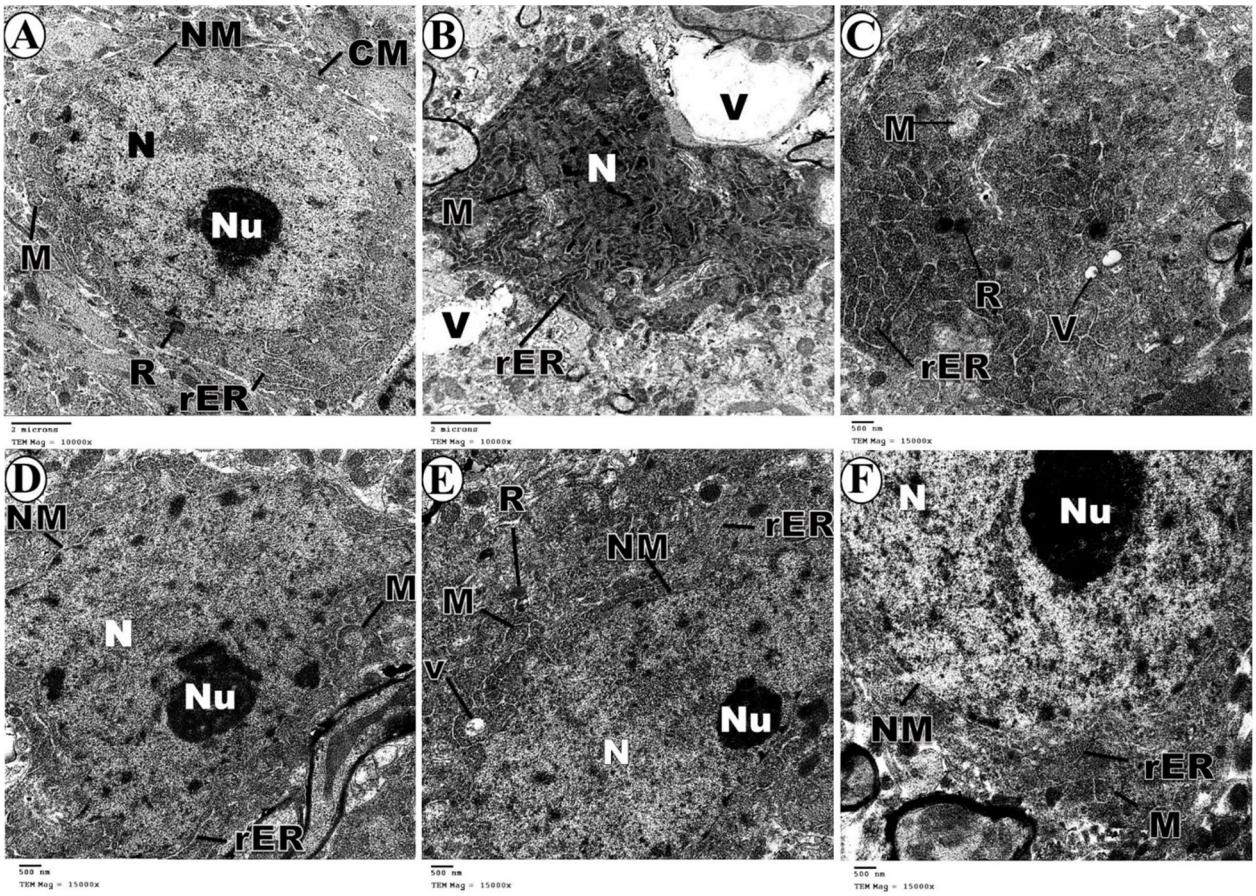

Figure 7 – ***Electron micrographs of PCs: (A) Control group showing a PC with intact cell membrane {CM}, euchromatic nucleus {N}, prominent nucleolus {Nu}, intact nuclear membrane {NM}, and normal mitochondria {M}, ribosomes {R} and rough endoplasmic reticulum {rER}; (B and C) MSG group showing shrunken irregular PC having a nucleus {N} with indistinct nuclear membrane, and cytoplasm with dilated fragmented rough endoplasmic reticulum {rER}, swollen mitochondria with loss of cristae {M}, vacuolations {V} and free ribosomes {R}; (D) RE/MSG group showing a PC with euchromatic nucleus {N}, regular nuclear membrane {NM}, distinct nucleolus {Nu}, normal rough endoplasmic reticulum {rER} and minimally dilated mitochondria {M}; (E) FLX/MSG group showing well-defined nucleus {N}, slightly irregular intact nuclear membrane {NM} and prominent nucleolus {Nu}. The cytoplasm is with small vacuolations {V}, minimally dilated cisternae of rough endoplasmic reticulum {rER}, slightly swollen mitochondria {M} and ribosomes {R}; (F) The RE/FLX/MSG group shows normal nucleus {N} and nucleolus {Nu}, intact nuclear membrane {NM}, and normal rough endoplasmic reticulum {rER} and non-swollen mitochondria {M}. Transmission electron microscopy (TEM): (A and B) ×10 000; (C–F) ×15 000. Scale bars: (A and B) 2 μm; (C–F) 500 nm. PCs: Pyramidal cells.***

The administration of RE and FLX in the present work resulted in a significant decrease in the escape latency and the number of errors indicating an improvement in cognition defects induced by MSG. These RE effects may be due to the induction of neurogenesis in the hippocampus through its antioxidant properties [13, 27]. Chronic FLX administration had a significant recovery of the cognitive functions in rats with DG lesions [28].

Histologically, disorganization of PC and GC layers of CA1 area and DG respectively was noticed with degenerative changes of their cells, as well as decreased cellular density of these layers. Such neuronal degeneration is due to the excitotoxicity phenomenon, which is responsible for neuronal degeneration by overstimulation of glutamatergic post-synaptic receptors as an effect of high doses of L-Glutamate. Several abnormalities such as glial reaction and increased expression of the N-methyl-D-aspartate (NMDA) receptor subunits have been observed in the rat hippocampus after MSG exposure, leading to the occurrence of cell death in CA1 and some cytoarchitectural modifications in the surviving neurons, which finally produce functional alterations in the hippocampal integrative activity [29].

Ultrastructural changes of hippocampal neurons in the present work due to MSG toxicity appeared in the form of shrunken cells, irregular nuclear membranes, dense chromatin, dilated cisternae of rER, swollen mitochondria, and large cytoplasmic vacuolations, which may be due to that MSG administration causes cellular degeneration in the hippocampus and increased concentration of cholinesterase in the brain tissues and plasma [30]. Glutamate-induced neuronal death mediated by both apoptotic and necrotic mechanisms leads to excitotoxic and oxidative injury. Excitotoxicity involves the overactivation of Glutamate receptors by increasing doses of MSG leads to rapid cytotoxic events [31]. Glutamate causes over-activation of NMDA and α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors leading to prolonged depolarization and increased intracellular Ca^{2+} ions influx that causes activation of mitochondria, and release of enzymes like phospholipase and protein kinases, causing the degradation of proteins and cell death [32].

The study results revealed an increase in both glial cell density and GFAP immunoreaction in MSG-treated rats. MSG exposure induced a significant increase in the number

of astrocytes in GFAP-immunostained sections that resulted in a permanent consequence of MSG neuroexcitation effects and could represent reactive gliosis [18]. Excitotoxicity phenomenon is accompanied by astrocytes reactivity in the form of hypertrophy of glial processes, and an increase of the astrocytic GFAP immunoreactivity (astrogliosis) and proliferation (astrocytosis) [33].

In the present study, RE oral administration prior to the injection of MSG mostly preserved PC integrity and organization in the CA1 region and DG, respectively. This protective effect of RE against the neuronal degeneration of the hippocampus is induced by kainic acid in rats [11]. The Carnosic Acid, an effective component of RE, improves the PC survival in the CA1 region in the rat model with AD and protects neuronal cells from ischemic brain injury by scavenging ROS [34, 35].

In this study, astrocytic GFAP immunoexpression was massive within all layers of CA1 area and DG after administrated MSG, whereas significantly decreased with the RE and FLX treatments. This decrease after RE administration may be due to decreased ROS generation in response to the increased activity of superoxide dismutase, which is a primary antioxidant enzyme [36]. FLX treatment reverses the damage in hippocampal neurons, which was most comparable with the control group [17]. Also, it produces an increase in hippocampal neurogenesis and a decrease in neuronal degeneration [37]. Pearson's correlations resulted in significant negative correlations between GFAP immunopositive cell number and neuronal cell densities suggesting that reactive astrocytes may have a specific role in neuronal degeneration by losing their supportive role.

In the present study, astrocytic GFAP immunoexpression was mild in the CA1 area and DG in the FLX/MSG group. Although the number of the astrocytes was lower in the MSG group than in the FLX/MSG one, there was no statistical difference between both groups, as kainic acid-induced gliosis in rodents was suppressed by FLX administration because of its anti-inflammatory effect [38]. FLX prevents the infiltration of macrophages and inhibits the messenger ribonucleic acid (mRNA) expression of inflammatory mediators after an injury resulting in a decrease in activation of microglia and astrocyte in hippocampal regions [12, 39].

The present study results revealed that RE was more effective than FLX in preventing of pathological changes of the hippocampus induced by MSG, which proved by the HP, IHC assessments.

The upper hand of the combined RE and FLX treatment may be due to their synergic effects, including inhibition of voltage gated Ca^{2+} channels, leading to decreased Ca^{2+} influx into the cells [40], and this might decrease the Ca^{2+} intracellular overload produced by MSG. In addition, this may also be due to the combined antioxidant effect of RE and the neurogenic effect of FLX [41].

Conclusions

MSG causes significant cognitive, HP, morphometric, and ultrastructural changes in the hippocampus cells and increases the astrocytic GFAP immunoexpression. Furthermore, RE or FLX treatment before MSG administration has a preventive effect; however, combined RE and FLX treatment has a better preventive effect than a single agent treatment, probably through its antioxidant and anti-inflammatory effects.

Ethical consideration

All experimental procedures performed in this study were done in accordance with the ethical standards of the Institutional Animals Ethics Committee of Suez Canal University, Ismailia, Egypt.

Conflict of interest

The authors declare that they have no conflict of interests.

Authors' contribution

All authors have been personally, equally, and actively involved in substantive work leading to the manuscript and will hold themselves jointly and individually responsible for its content.

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