Original article DOI: 10.1515/aiht-2017-68-2878

Neuroprotective effects of eugenol against aluminium-induced toxicity in the rat brain

Mahmoud M. Said[1] and Marwa M. Abd Rabo[2]

Biochemistry Department, Faculty of Science, Ain Shams University, Cairo[1], Hormone Evaluation Department, National Organization for Drug Control and Research (NODCAR), Giza[2], Egypt

[Received in August 2016; Similarity Check in August 2016; Accepted in February 2017]

Aluminium (Al) is a neurotoxic metal that contributes to the progression of several neurodegenerative diseases. The aim of the present study was to evaluate the protective effect of dietary eugenol supplementation against aluminium (Al)-induced cerebral damage in rats. Male Wistar rats were divided into four groups: normal controls, rats fed a diet containing 6,000 μg g^{-1} eugenol, rats intoxicated daily with aluminium chloride (84 mg kg^{-1} body weight) *p. o.* and fed either a basal diet or a eugenol-containing diet. Daily oral administration of Al for four consecutive weeks to rats significantly reduced brain total antioxidant status (TAS) (11.42±0.31 μmol g^{-1} tissue, $p<0.001$) with a subsequent significant enhancement of lipid peroxidation (MDA) (32.55±1.68 nmol g^{-1} tissue, $p<0.002$). In addition, Al enhanced brain acetylcholinesterase activity (AChE) (46.22±4.90 U mg^{-1} protein, $p<0.001$), tumour necrosis factor alpha (TNF-α) (118.72±11.32 pg mg^{-1} protein, $p<0.001$), and caspase 3 (Casp-3) (8.77±1.26 ng mg^{-1} protein, $p<0.001$) levels, and in contrast significantly suppressed brain-derived neurotrophic factor (BDNF) (82.74±14.53 pg mg^{-1} protein, $p<0.002$) and serotonin (5-HT) (1.54±0.12 ng mg^{-1} tissue, $p<0.01$) levels. Furthermore, decreased glial fibrillary acidic protein (GFAP) immunostaining was noticed in the striatum of Al-intoxicated rats, compared with untreated controls. On the other hand, co-administration of dietary eugenol with Al intoxication restored brain BDNF (108.76±2.64 pg mg^{-1} protein) and 5-HT (2.13±0.27 ng mg^{-1} tissue) to normal levels, enhanced brain TAS (13.43±0.24 μmol g^{-1} tissue, $p<0.05$), with a concomitant significant reduction in TNF-α (69.98±4.74 pg mg^{-1} protein) and Casp-3 (3.80±0.37 ng mg^{-1} protein) levels ($p<0.001$), as well as AChE activity (24.50±3.25 U mg^{-1} protein, $p<0.001$), and increased striatal GFAP immunoreactivity, compared with Al-treated rats. Histological findings of brain tissues verified biochemical data. In conclusion, eugenol holds potential as a neuroprotective agent through its hydrophobic, antioxidant, and anti-apoptotic properties, as well as its neurotrophic ability against Al-induced brain toxicity in rats.

KEY WORDS: *acetylcholinesterase; apoptosis; brain-derived neurotrophic factor; oxidative stress; serotonin*

Aluminium (Al), the most abundant metal on earth that constitutes 8.13 % of the crust, may enter the human body through food, drinking water, and Al-containing drugs (1, 2). As a result of the wide usage of such a well-established neurotoxin (3-5), Al has become a global public health problem. Reports have also defined it as an important etiological factor in the progression of several neurodegenerative disorders as it influences more than 200 biologically important reactions (6, 7). Aluminium-induced brain toxicity was reported in rats by many authors, including administration of aluminium chloride hexahydrate (25 mg kg^{-1} day^{-1}) for one month by oral gavage (8), daily oral treatment with aluminium chloride (100 mg kg^{-1}) for 42 days (2), or administration of aluminium chloride (17 mg kg^{-1} day^{-1}) orally for four consecutive weeks (9, 10). The neurotoxicity of Al is likely to be the result of a combination of several mechanisms, including oxidative brain injury, induction of apoptosis and neuronal damage, neuroglia inflammatory reaction, and reduced neurotransmitter biosynthesis (6, 11).

The pharmacological and therapeutic properties of medicinal plants have been attributed to different chemical constituents isolated from their crude extracts. Many naturally-occurring compounds were reported to prevent neural damage and become, therefore, important therapeutic candidates for brain disorders. Eugenol (4-allyl-1-hydroxy-2-methoxybenzene), an effective antioxidant phenolic compound extracted from clove (*Syzygium aromaticum*), is one of these compounds (12). It is approved for use as a food additive by the U.S. Food and Drug Administration and is on the list of substances "generally recognised as safe"(13).

A wide range of pharmacological properties for eugenol in nephrotoxicity, chronic inflammation, cancer, as well as metastasis were demonstrated (14-17). In addition, eugenol was previously reported to protect neuronal cells from excitotoxic and oxidative injury in mouse cortical cultures (18). Furthermore, it prevented from 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in mouse striatum (19) and provided a neuroprotective effect against hippocampal CA1 neuronal ischemia-induced injury in gerbils (20). It

Correspondence to: Dr Mahmoud M. Said, Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo, Egypt
E-mail: *mahmoudmsaid@sci.asu.edu.eg*

also proved to protect against acrylamide-induced neuropathy (21) and chlorpyrifos-induced brain toxicity (12) in rats, which may at least in part be attributed to its antioxidative stress activity.

Considering the beneficial *in vitro* and *in vivo* effects of eugenol, including its antioxidant, cytotoxic, antiproliferative, and anti-inflammatory properties, the current study was undertaken to investigate the neuroprotective potential of eugenol against Al-induced cerebral damage in rats and to shed light on the underlying mechanisms involved in such protection.

MATERIALS AND METHODS

Chemicals

Eugenol was obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan) and aluminium chloride ($AlCl_3.6H_2O$; CAS No. 7784-13-6) was purchased from Loba Chemie (Mumbai, India). All other chemicals used were of analytical grade.

Animals

Adult male Wistar rats (eight weeks old) were obtained from the breeding unit of the Egyptian Stock Holding Company for Biological Products, Vaccines, Sera and Drugs (Helwan, Egypt). The animals were housed in plastic cages on wood-chip bedding and had free access to commercial pelleted diet and distilled water before the start of the experiment. They were acclimatised for one week. Animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (22) and all applicable institutional guidelines were followed.

Experimental protocol

In total, 40 rats were randomly allocated to four equally sized groups (10 rats per group). Rats in the first group were assigned to a powdered basal AIN-93M diet (23) serving as the control group (Control), whereas the animals in the second group (EUG) were administered a basal diet containing eugenol (6,000 μg g^{-1}) (24). Rats in the third ($AlCl_3$) and fourth (EUG+$AlCl_3$) groups were administered $AlCl_3$ (84 mg kg^{-1} body weight equivalent to 17 mg ions of Al) *p.o*. daily using an intragastric tube for four consecutive weeks (25) and were assigned to the basal and eugenol-containing (6,000 μg g^{-1}) basal diets, respectively. Being used as a food additive in many products (ice cream, baked goods, gelatines, puddings, chewing gums, and meat products) (13), we thought it was better to administer eugenol through diet rather than a single bolus dose, especially as eugenol is rapidly absorbed and metabolised in the liver (26). Eugenol-containing diets were prepared once weekly by mixing eugenol with the powdered basal diet in a blender for 15 min, which was then stored at 4 °C in the dark (24). The diets were available *ad libitum* and were given to animals by freshly replenishing feed trays twice weekly, where food consumption was recorded. No mortalities were previously recorded in rats fed with diets containing eugenol (6,000-50,000 μg g^{-1}) in a fourteen-day study (13). The lowest dose (6,000 μg g^{-1}) was, therefore, previously selected (24) and herein adopted. As for aluminium, three doses were previously evaluated in rats (6, 17, and 50 mg kg^{-1}) and the minimum effective dose of aluminium that induced a biological toxic effect was set at the level of 17 mg kg^{-1} (25), which was selected in the current study.

Tissue sampling

At the end of the four-week treatment period, rats were killed by cervical dislocation and the fore-brains (including the cerebral areas with hippocampus and striatum) were isolated on ice and then divided sagittally into two parts using a sharp blade. One part was stored in ice-cold sterile physiologic saline at -20 ˚C (seven hemispheres were kept for tissue homogenisation and three for trace metal analysis), while the other one was fixed in 10 % phosphate-buffered formalin for 48 h for histological examination.

Preparation of brain homogenate

Fore-brains were made into 10 % homogenates with ice-cold physiologic saline using a glass homogeniser (27), and the homogenates were centrifuged at 1,800 g for 20 min at 4 ˚C (Sigma 2-16PK, SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany). The supernatant was immediately separated on ice, aliquoted, and stored at -20 ˚C until further biochemical analysis.

Biochemical assays

Brain lipid peroxidation was measured in terms of malondialdehyde (MDA) using thiobarbituric acid (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) (28). Total antioxidant status (TAS) was evaluated by a colorimetric method based on the ability of brain antioxidants to suppress the production of thiobarbituric acid reactive substances (TBARS). TBARS are released following benzoate degradation by means of hydroxyl radicals (•OH) that form in a Fenton-type reaction (29). Brain acetylcholinesterase activity (AChE) was determined colourimetrically (30) using a kit provided by Sigma-Aldrich (St Louis, MO, USA). Tumour necrosis factor alpha (TNF-α) concentration was measured in the brain homogenate using a rat TNF-α ELISA kit provided by R&D Systems (Minneapolis, MN, USA), whereas brain caspase 3 (Casp-3) level and brain-derived neurotrophic factor (BDNF) concentrations were analysed using rat ELISA kits supplied by MyBioSource (MyBioSource, Inc., San Diego, CA, USA). The total protein level was determined in brain homogenates (31).

Determination of brain serotonin (5-hydroxytryptamine, 5-HT)

Equal volumes of brain homogenate and ice-cold HPLC-grade methanol were mixed, followed by centrifugation at 4,000 g at 4 ˚C for 10 min (Z 36 HK high-speed centrifuge, HERMLE Labortechnik GmbH, Germany). The supernatant was filtered through a 45 µm filter and then analysed by high performance liquid chromatography (Agilent 1100, Agilent Technologies, Waldbronn, Germany) (32). Samples (20 µl) were injected into the loop injector (Rheodyne 210A, USA). AQUA C_{18} 5 µm 150 mm x 4.6 mm column (Phenomenex, USA) was used. The mobile phase consisted of 20 mmol L^{-1} potassium phosphate (pH 3) and methanol (97:3 v v^{-1}) and the elution flow rate was 1.5 mL min^{-1}. Serotonin was detected using a UV variable wavelength detector (G1314A-1100 LC VWD, Agilent Technologies, Waldbronn, Germany) and its concentration (ng mg^{-1} tissue) was calculated using a calibration curve of standard serotonin (Sigma Aldrich. St Louis, MO, USA).

Determination of dietary aluminium level and brain trace metals concentration

The wet digestion method was used for the analysis of dietary aluminium and trace metals [Iron (Fe), Zinc (Zn), and Copper (Cu)] in brain tissues (33). Briefly, one cerebral hemisphere of a rat brain (or 0.2 g diet) was incubated overnight at 40 ˚C in a screw-capped polypropylene tube with 0.8 mL concentrated nitric acid (RIEDEL-DE HAËN, Germany) and 0.2 mL concentrated sulfuric acid (Merck, Germany). The mixture was then heated at 70 ˚C for 2 h and 105 ˚C for 1 h, yielding a clear yellow solution, and the digest was allowed to cool and then diluted to 3 mL with deionised water. Multi-element analysis was done using the Inductively Coupled Argon Plasma Spectrometer (ICAP 6500 Duo, Thermo Scientific, England).

Histological examination

Fixed brain specimens were processed and embedded in paraffin. Serial four µm-thick sections were made, stained with haematoxylin and eosin (H&E) and examined by light microscopy.

Immunohistochemistry

Four micrometre-thick sections of fore-brains were incubated with mouse monoclonal antibody to glial fibrillary acidic protein Ab-1(GFAP; 1:500) (Thermo Scientific, CA, USA). Sections were then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000) (Thermo Scientific, CA, USA) and counterstained with haematoxylin for microscopic examination.

Statistical analysis

Equal variance between the groups was first checked using the Levene's test for homogeneity of variances and statistical analysis of the difference between the mean values was carried out using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test for multiple comparisons. SPSS statistical software 22.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used for all analyses. A value of $p<0.05$ was considered statistically significant for all tests.

RESULTS

Food consumption and average intake data

No mortality was recorded during the experimental study. As for eugenol, its acute toxicity is low when administered orally, with LD_{50} values ranging from 1,930 to 2,680 mg kg^{-1} day^{-1} in rats (34, 35). Its sub-chronic toxicity is also low, with no observed adverse effect level (NOAEL) ranging from 900->2,000 mg kg^{-1} day^{-1}. Furthermore, up to 1.0 % eugenol in the diet (10,000 µg g^{-1}) did not cause adverse effects (26). On the other hand, the LD_{50} of aluminium chloride is reported to be 3,800 mg kg^{-1} (36) in rats. A non-significant change was recorded in food consumption among different groups (Table 1). Aluminium content in the basal diet of rats was found to be 16 µg g^{-1} and based on food consumption data, the average intake of dietary Al in different groups ranged from 1.27 to 1.56 mg kg^{-1} day^{-1}. Rats fed eugenol-containing diets, either alone or intoxicated with Al, consumed 480 and 580 mg kg^{-1} day^{-1} eugenol, respectively (Table 1).

Table 1 *Average intake data of rats treated with aluminium with or without eugenol mixed in the diet*

Groups	Average intake			
	Food consumption (g per rat per day)	Dietary Al consumption (mg kg^{-1} b.w. per day)	Al administered orally (mg kg^{-1} b.w. per day)	Eugenol (mg kg^{-1} b.w. per day)
Control	15.72±3.56	1.40	--	--
EUG	12.42±1.90	1.27	--	480
$AlCl_3$	15.61±1.83	1.56	17	--
EUG+$AlCl_3$	14.22±0.80	1.55	17	580

Values are mean±SEM (n=10)

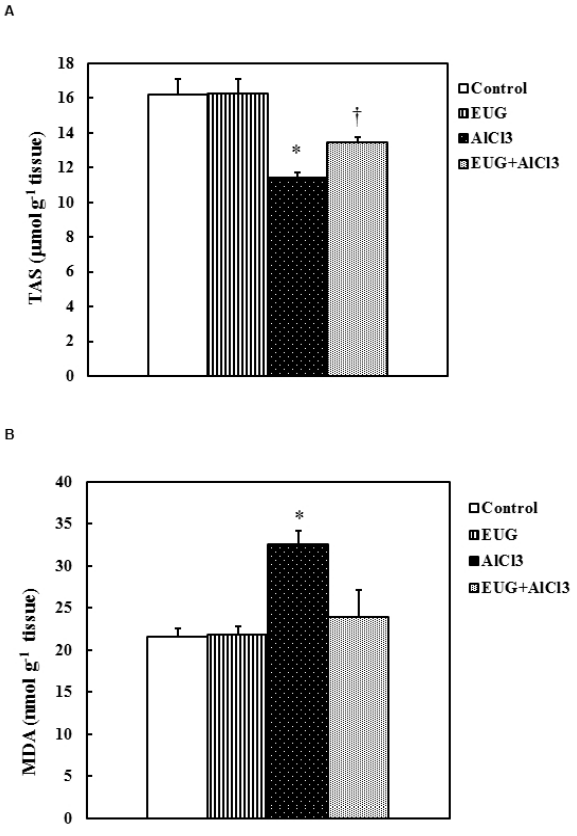

Figure 1 *Effect of eugenol and/or aluminium on brain total antioxidant status (**A**) and lipid peroxidation (**B**) in rats. Values are mean ± SEM (n=7). Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. Significance: * p<0.05* versus *Control, EUG and EUG+AlCl$_3$ groups; † p<0.05* versus *Control, EUG, and AlCl$_3$ groups*

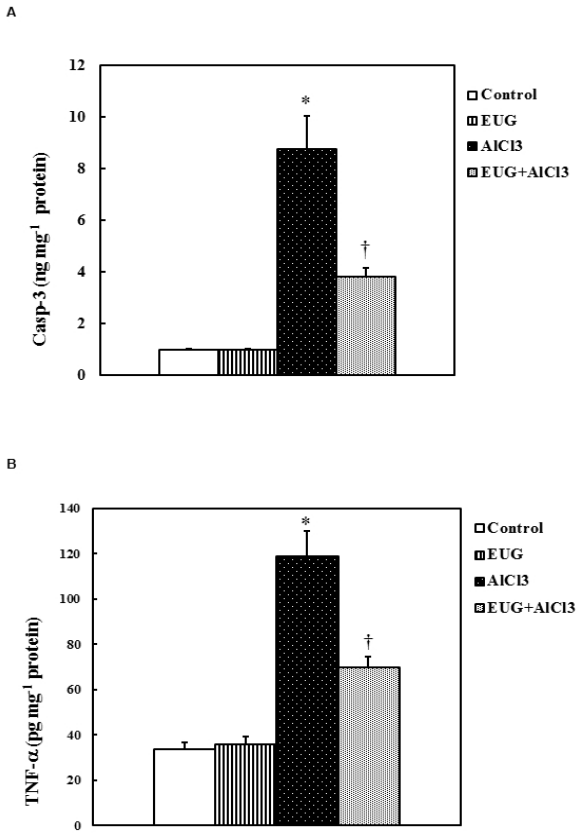

Figure 2 *Effect of eugenol and/or aluminium on brain caspase 3 (**A**) and tumour necrosis factor alpha (**B**) levels in rats. Values are mean ± SEM (n=7). Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. Significance: * p<0.05* versus *Control, EUG and EUG+AlCl$_3$ groups; † p<0.05* versus *Control, EUG, and AlCl$_3$ groups*

Effect of eugenol and/or aluminium on brain TAS and LPO

Treatment with $AlCl_3$ alone resulted in a significant reduction in brain TAS (29.42 %, p<0.001), along with a significant rise in lipid peroxidation expressed as MDA (50.42 %, p<0.002) compared with controls. In contrast, rats treated with $AlCl_3$ and fed with eugenol-supplemented diets displayed a significant enhancement in brain endogenous TAS compared with $AlCl_3$-treated rats (17.60 %, p<0.05) with a concomitant return to control MDA levels (Figure 1).

Effect of eugenol and/or aluminium on brain Casp-3 and TNF-α concentrations

Apoptosis was severely induced in the brain of $AlCl_3$-treated rats as manifested by sharp parallel significant elevations in Casp-3 (777 %, p<0.001) and TNF-α (250.62 %, p<0.001) levels, compared with controls. On the other hand, co-administration of eugenol with $AlCl_3$ produced a marked significant reduction in Casp-3 (56.67 %, p<0.001) and TNF-α (41.05%, p<0.001) levels, compared with $AlCl_3$-only-treated rats (Figure 2).

Effect of eugenol and/or aluminium on brain AChE activity and BDNF level

Rats intoxicated with $AlCl_3$ displayed a sharp significant increase in the AChE activity (204.48 %, p<0.001) accompanied with a significant depletion in BDNF concentration (33.72 %, p<0.002), compared with controls. On the contrary, eugenol supplementation with $AlCl_3$ normalised brain BDNF level and significantly reduced the AChE activity (46.99 %, p<0.001), with respect to $AlCl_3$ treatment (Figure 3).

Effect of eugenol and/or aluminium on brain serotonin level

Subchronic toxic administration of $AlCl_3$ to male rats significantly suppressed brain serotonin level (37.65 %, p<0.01) compared with controls, whereas co-administration of eugenol along with $AlCl_3$ intoxication normalised brain serotonin concentration (Figure 4).

Effect of eugenol and/or aluminium on brain trace metal level

A significant elevation in Cu level was recorded in the brain of Al-intoxicated rats (21.02 %, p<0.05) compared

with controls, whereas rats treated with $AlCl_3$ and fed eugenol-supplemented diets manifested a return to normal Cu levels (Figure 5).

Histological findings

Microscopic examination of the brain cerebral cortex and hippocampus of control rats showed normal histological structure of the meninges, neurons of the cerebral cortex, and the small pyramidal cells of the pyramidal layer of the hippocampus (Figures 6 a and 6 b). Similar normal structures were revealed in the brain sections of eugenol-supplemented rats. On the other hand, daily oral treatment of rats with $AlCl_3$ for four successive weeks caused severe congestion in the blood vessels of the meninges, associated with marked karyopyknosis and degeneration of cerebral cortex neurons and the small pyramidal cells of the hippocampus (Figures 6 c and 6 d). Animals fed with eugenol-supplemented diets along with $AlCl_3$ intoxication showed minimum damage to the brain tissue as demonstrated by reducing neuronal cell damage in the cerebral cortex and preservation of the small pyramidal cells of the hippocampus (Figures 6 e and 6 f).

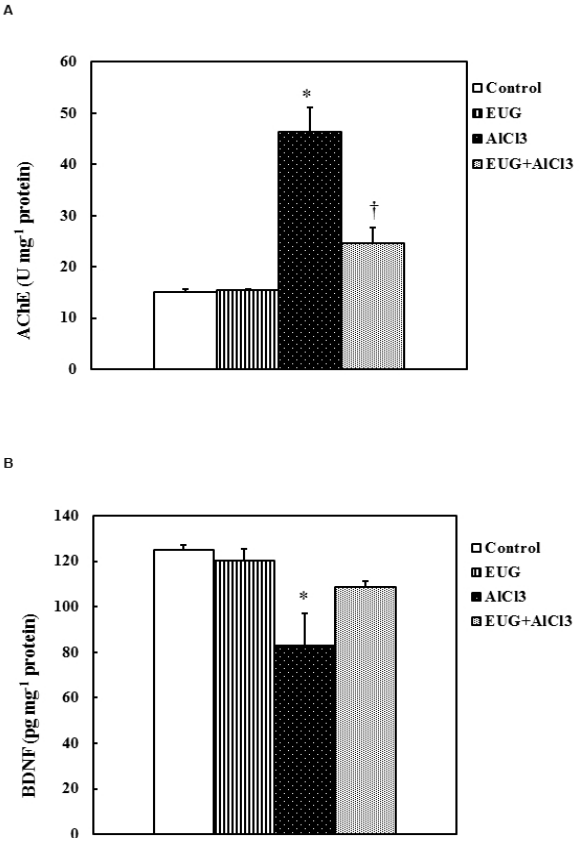

Figure 3 *Effect of eugenol and/or aluminium on brain acetylcholinesterase activity (**A**) and brain-derived neurotrophic factor level (**B**) in rats. Values are mean ± SEM (n=7). Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. Significance: *p<0.05* versus *Control, EUG and EUG+AlCl$_3$ groups; †p<0.05* versus *Control, EUG, and AlCl$_3$ groups*

Immunostaining of GFAP

Normal GFAP immunoreactivity was seen in astrocytes and glial fibres in the striatum of control (Figure 7 a) and eugenol-treated animals (Figure 7 b). On the other hand, decreased GFAP immunoreactivity was noticed in the striatum of $AlCl_3$-intoxicated rats (Figure 7 c), whereas an apparent restoration of GFAP immunoreactivity was observed in rats treated with $AlCl_3$ and fed with eugenol-supplemented diets (Figure 7 d). Scoring of neuronal damage is illustrated in Table 2.

DISCUSSION

The brain is especially vulnerable to oxidative stress, more than any other organ due to its large oxygen consumption, high lipid content, and low mitotic rate and antioxidant levels. The participation of free radical-induced oxidative brain cell injury in aluminium toxicity has been previously proposed (37-39). Our data demonstrated that sub-chronic aluminium administration to rats significantly reduced the brain total antioxidant status (TAS) with concomitant enhancement of lipid peroxidation (LPO), verifying the oxidative stress-related changes that contribute to Al neurotoxicity.

Although Al cannot change its redox state, it exacerbates free radical damage and lipid peroxidation initiated by iron. This can be attributed to the fact that, like iron, Al binds to the iron regulatory protein (IRP), which influences the expression of iron-binding proteins. This causes further stabilisation of iron in its ferrous (Fe^{+2}) state that promotes Fe^{+2}-induced LPO by the Fenton reaction, modifying therefore the biophysical properties of membranes. Oxidative damage resulting from aluminium and deregulated iron is indissociable in aluminium-loaded neurons, where their combined activity provides an oxidative environment (6, 11, 37).

In the present study, amelioration of the brain TAS with the depletion of LPO in Al-intoxicated rats simultaneously treated with eugenol suggests a lowered oxidative stress and protection of neuronal cell integrity. Eugenol has a broad range of biological actions in spite of its relatively simple molecular structure. Because of its hydrophobic property, orally administered eugenol seems to cross the blood brain barrier (BBB) and acts *in situ* (40) to inhibit lipid peroxidation (as a chain-breaking antioxidant) and to enhance endogenous antioxidant mechanisms (12, 14, 24). Eugenol possesses antioxidant property by virtue of its phenolic hydroxyl group in its structure that donates electrons to quench free radicals. It also prevents the oxidation of Fe^{2+} by H_2O_2 in the Fenton reaction, which generates hydroxyl radicals ($^{\bullet}OH$) involved in the initiation of lipid peroxidation (14).

Al disrupts the pro-inflammatory cytokine/neurotrophic balance that plays an important role in neurodegenerative disorders (38). Al intoxication in the current study induced

Table 2 *Histological and immunohistochemical alterations in the brain of rats treated with aluminium with or without eugenol mixed in the diet*

		Control	EUG	$AlCl_3$	EUG+$AlCl_3$
Cerebral Cortex	Karyopyknosis of neuronal cells	-	-	+++	+
Hippocampus	Karyopyknosis of pyramidal cells	-	-	+++	-
Striatum	GFAP expression	+++	+++	+	++

Scoring key: Nil (-), mild (+), moderate (++), and severe (+++)

brain apoptosis as demonstrated by increased brain Casp-3 and TNF-α levels. This is verified histologically, as severe degenerations of cerebral cortex neurons and small pyramidal cells of the hippocampus were noticed. Induction of apoptosis and necrosis, as well as increased expression of inflammatory cytokines by Al, were previously reported in neuronal cells both *in vitro* (41) and *in vivo* (42). Owing to its affinity for negatively charged ligands, Al binds to the phosphate groups of nucleoside di- and triphosphates, DNA, RNA, and amino acids, influencing therefore energy metabolism, the functions of various protein kinases and phosphatases, the expression of various genes essential for brain functions, as well as the accumulation of highly phosphorylated cytoskeleton proteins. In addition, Al binds to various metal-binding proteins and influences homeostasis of other metals (such as Cu in the present study). These ultimate macromolecular alterations cause apoptotic death of neurons and glial cells (6).

The brain-derived neurotrophic factor (BDNF) plays an important role in regulating the survival and differentiation of selective populations of neurons during development. It is synthesised predominantly in the CNS by neurons, and is highly expressed in the hippocampus and cortex, two brain regions which are known to be important for learning and memory (43). BDNF has trophic effects on serotonergic (5-HT) neurons in the central nervous system. It has been reported that decreased expression of BDNF, which maintains normal neuronal circuits in the brain, appears to play an important role in the development of several neurodegenerative diseases. The reduction of BDNF following Al intoxication in the current study was previously reported (2, 9, 38). In addition, Al impairs various enzymes involved in neurotransmitters biosynthesis and one of its potential toxic mechanisms of action may include disruption in serotonergic neurotransmissions in the brain hippocampus (6, 44). Therefore, it is reasonable to suggest that Al suppressed brain serotonin concentration either directly, by reducing its biosynthesis, or indirectly, by downregulating BDNF level, which has trophic effects on serotonergic neurons.

The ability of eugenol to inhibit apoptosis and pro-inflammatory cytokine secretion and subsequent protection of neuronal cells in the present study is related to its effect to counterbalance Al-induced oxidative stress and regulate the generation of the inflammatory cytokines. Eugenol was previously reported to inhibit the pro-inflammatory mediators (nitric oxide production and cyclooxygenase 2 expression) in lipopolysaccharide-induced RAW264.7

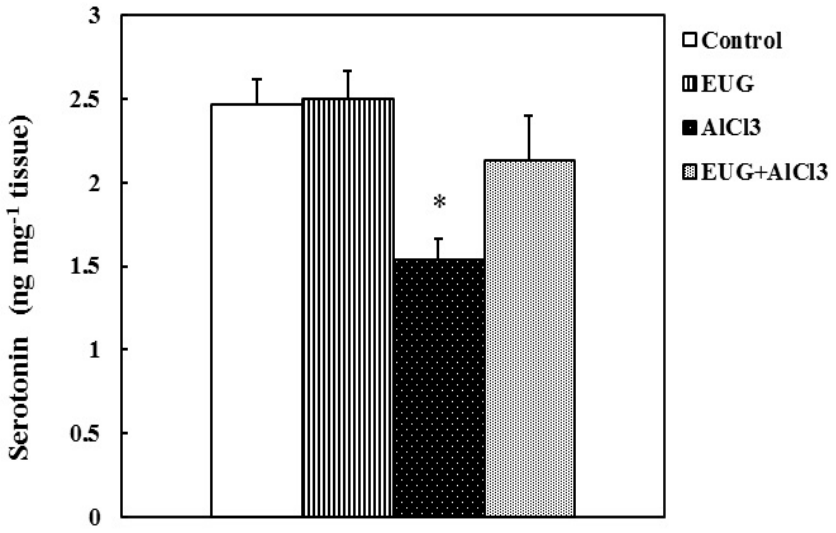

Figure 4 *Effect of eugenol and/or aluminium on brain serotonin concentration in rats. Values are mean ± SEM (n=5). Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. Significance: *p<0.05* versus *Control, EUG, and EUG+$AlCl_3$ groups*

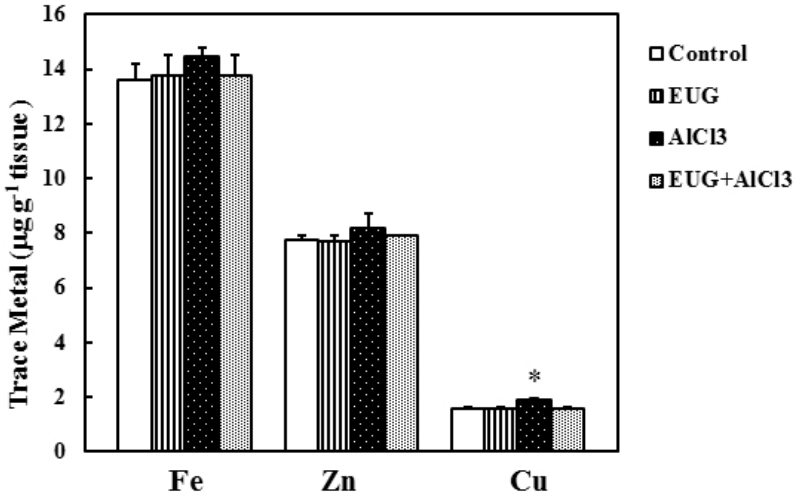

Figure 5 *Effect of eugenol and/or aluminium on brain Fe, Zn, and Cu concentrations in rats. Values are mean ± SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. Significance: *p<0.05* versus *Control, EUG, and EUG+AlCl$_3$ groups*

macrophages (45) and TNF-induced NF-kB activation in ML1-a cells (46). In addition, a direct neurotrophic effect of eugenol on BDNF was previously reported since the administration of eugenol in drinking water for two consecutive weeks to male mice induced BDNF gene expression in the hippocampus (47). Similarly, eugenol was able to reverse the stress-induced changes of serotonin level in all rat brain regions and such modulation in brain functions is attributed to the ability of eugenol to regulate voltage-gated cation channels and the release of neurotransmitters (48). Taken altogether, eugenol exerts a multitude of actions, either directly or indirectly, to restore a normal neurotrophic and serotonergic balance in the brain of Al-intoxicated rats. In accordance with the biochemical finding, histological examination revealed a neuroprotective potential of eugenol against brain-induced toxicity as demonstrated by reducing neuronal cell damage of the cerebral cortex and the preservation of small pyramidal cells of the hippocampus.

Acetylcholinesterase (AChE, EC 3.1.1.7) hydrolyses acetylcholine in cholinergic brain synapses and at neuromuscular junctions into choline and acetate. AChE is a significant biological component of the membrane that contributes to its integrity. Induction of the AChE activity causes faster acetylcholine degradation and consequent lowered stimulation of acetylcholine receptors, which produces a reduction of diverse cholinergic (learning and memory), as well as non-cholinergic (cell proliferation and neurite outgrowth) functions (12, 21). In the current study, sub-chronic administration of Al induced significantly the brain AChE activity. Comparable results were previously reported (2, 3). Al exerts its cholinotoxic effects by blocking the provision of acetyl-CoA or by impairing the choline acetyltransferase activity (49). Also, Al can interact with plasma membrane lipids and affect the activity of membrane associated enzymes, including AChE. The increase in the

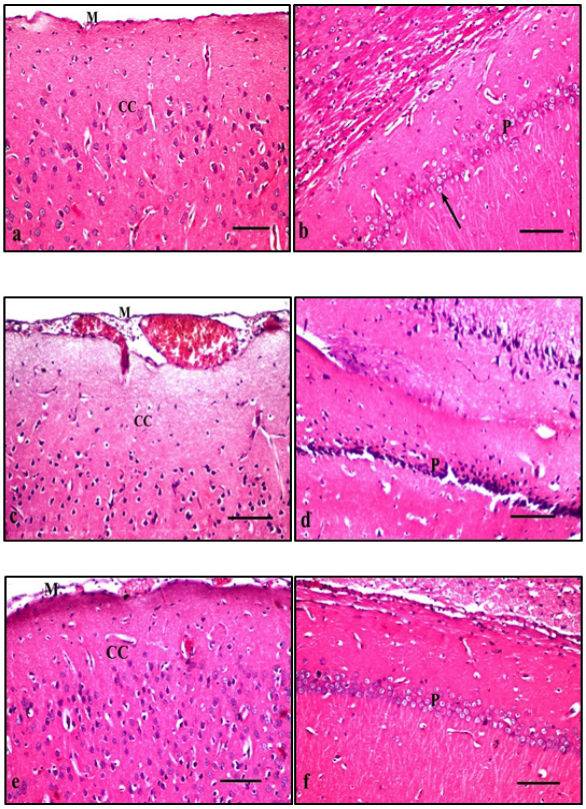

Figure 6 *Photomicrographs of rat brain sections stained with haematoxylin and eosin (bar=25 µm). The normal histological structure of the meninges (M) and cerebral cortex (CC) with intact neurons (**a**) and the pyramidal layer of the hippocampus (P), composed of 4-5 compact layers of small pyramidal cells (arrow) most with vesicular nuclei (**b**), were seen in control animals. AlCl$_3$ intoxication resulted in severe congestion in the blood vessels of the meninges, karyopyknosis of neuronal cells of the cerebral cortex (**c**) and pyramidal cells of the hippocampus, which showed darkened nuclei (**d**). Protection against AlCl$_3$ brain toxicity with eugenol was evident by reducing karyopyknotic neuronal cells of the cerebral cortex (**e**) and the preservation of small pyramidal cells of the hippocampus (**f**)*

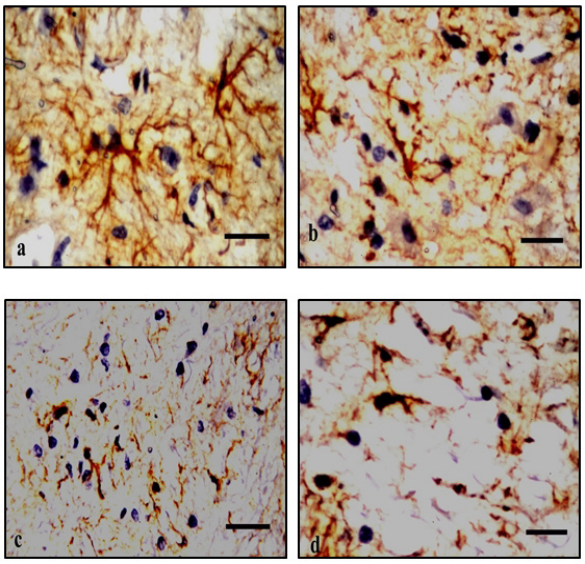

Figure 7 *Normal morphology and pattern of GFAP immunoreactivity were seen in astrocytes and glial fibres in the striatum of control (**a**) and eugenol-treated animals (**b**). A considerable decrease in GFAP immunoreactivity was observed in the striatum of rats intoxicated with $AlCl_3$ (**c**), whereas an apparent increase in GFAP immunoreactivity was noticed in the striatum of rats treated with $AlCl_3$ and fed with eugenol-supplemented diets (**d**) (bar=20 μm)*

brain AChE activity following aluminium exposure of rats was attributed to an allosteric interaction between Al with the peripheral anionic site of the enzyme molecule (50). The ability of eugenol to directly suppress the enhanced AChE activity following Al intoxication in the current study could be explained with the finding of an *in vitro* study that demonstrated a potential anti-cholinesterase property of eugenol (51). Similarly, eugenol was found to normalise the cortical AChE level in acrylamide-induced brain neuropathy in rats (21).

Astrocytes, the most abundant cell type in the brain, are responsible for providing nutrients to neurons. They are indispensable for the formation and stabilisation of synapses, as well as for the regulation of the concentration of various molecules supporting energy metabolism of neurons and are therefore critical for their survival (9, 52). Glial fibrillary acidic protein (GFAP), a structural protein composed of intermediate filaments synthesised in astrocytes, is a classical astrocyte marker. Decreased GFAP immunostaining in the brain of Al-intoxicated rats in the current study demonstrates the vulnerability of astrocytes to Al neurotoxicity, which contributes to neuronal loss. It has also been suggested that Al treatment impairs astrocytes to protect neurons from glutamate-induced excitotoxicity. The progressive accumulation of Al in astrocytes may protect neurons in early times after intoxication. Nevertheless, the pathological accumulation of Al may damage astrocytes at later time points (53, 54). A favourable effect of eugenol on basal glycogen synthesis in primary mouse astrocytes was previously demonstrated (52). Furthermore, a probable direct or indirect effect of eugenol to preserve brain astrocytes against Al-induced neurotoxicity is not excluded, which warrants further investigations.

In conclusion, the present study confirms the susceptibility of the brain to the toxic effect of increased oral exposure to aluminium in rats. The neurotoxicity of Al is likely to be the result of a combination of several mechanisms including oxidative brain injury and enhanced lipid peroxidation, disruption of neurotrophic, cholinergic, and serotonergic functions, and induction of apoptosis with ultimate neuronal and astrocyte damages. A neuroprotective role of eugenol against Al intoxication is verified through its antioxidant and antiapoptotic potential, as well as its neurotrophic property (Figure 8). Unfortunately, we were not successful in obtaining clear results concerning the measurement of Al concentration in the brain of rats by means of ICAP spectrometry. Also, eugenol's ability to prevent Al bioaccumulation in the brain tissue may need further histochemical analysis (55). Since eugenol causes a significant delay in the neurodegenerative markers of the current Al-induced toxicity model, it represents a promising therapeutic adjuvant for brain disorders and related neuropathic conditions in further clinical investigations.

Acknowledgement

The authors would like to acknowledge the valuable histological and immunohistochemical comments provided by Dr Adel M. Bakeer, Professor of Pathology, Faculty of Veterinary Medicine, Cairo University, Egypt.

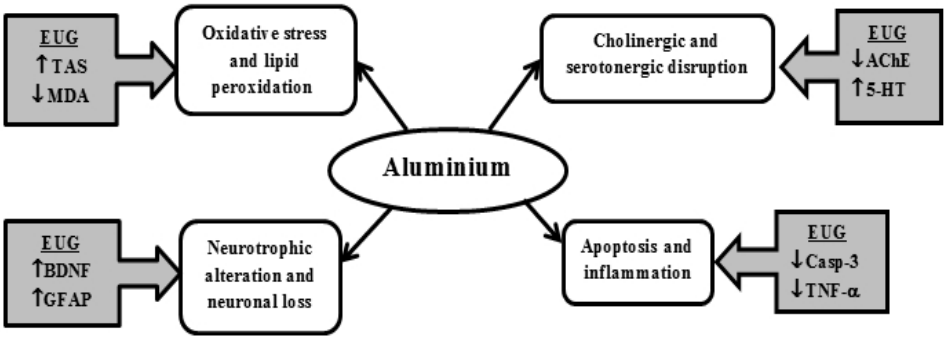

Figure 8 *A graphical scheme illustrating some of the proposed mechanisms involved in aluminium-induced brain toxicity and the probable actions by means of which eugenol exerts its neuroprotective effect*

Conflict of interest

The authors declare that there are no conflicts of interest.

REFERENCES

1. Hirata-Koizumi M, Fujii S, Ono A, Hirose A, Imai T, Ogawa K, Ema M, Nishikawa A. Evaluation of the reproductive and developmental toxicity of aluminium ammonium sulfate in a two-generation study in rats. Food Chem Toxicol 2011;49:1948-59. doi: 10.1016/j.fct.2011.04.035
2. Lin WT, Chen RC, Lu WW, Liu SH, Yang FY. Protective effects of low-intensity pulsed ultrasound on aluminum-induced cerebral damage in Alzheimer's disease rat model. Sci Rep 2015;5:9671. doi: 10.1038/srep09671
3. Bilkei-Gorzo A. Neurotoxic effect of enteral aluminium. Food Chem Toxicol 1993;31:357-61. doi: 10.1016/0278-6915(93)90191-Z
4. Hirata-Koizumi M, Fujii S, Ono A, Hirose A, Imai T, Ogawa K, Ema M, Nishikawa A. Two-generation reproductive toxicity study of aluminium sulfate in rats. Reprod Toxicol 2011;31:219-30. doi: 10.1016/j.reprotox.2010.11.004
5. Petit TL, Biederman GB, McMullen PA. Neurofibrillary degeneration, dendritic dying back, and learning-memory deficits after aluminum administration: implications for brain aging. Exp Neurol 1980;67:152-62. PMID: 7349982
6. Kawahara M, Kato-Negishi M. Link between aluminum and the pathogenesis of Alzheimer's disease: the integration of the aluminum and amyloid cascade hypotheses. Int J Alzheimers Dis 2011;2011:276393. doi:10.4061/2011/ 276393
7. Zhang L, Jin C, Liu Q, Lu X, Wu S, Yang J, Du Y, Zheng L, Cai Y. Effects of subchronic aluminum exposure on spatial memory, ultrastructure and L-LTP of hippocampus in rats. J Toxicol Sci 2013;38:255-68. doi: 10.2131/jts.38.255
8. Zaky A, Mohammad B, Moftah M, Kandeel KM, Ahmad R, Bassiouny AR. Apurinic/apyrimidinic endonuclease 1 is a key modulator of aluminum-induced neuroinflammation. BMC Neurosci 2013;14:26. doi: 10.1186/1471-2202-14-26
9. Ghoneim FM, Khalaf HA, Elsamanoudy AZ, Abo El-khair SM, Helaly AMN, Mahmoud EM, Elshafey SH. Protective effect of chronic caffeine intake on gene expression of brain derived neurotrophic factor signaling and the immunoreactivity of glial fibrillary acidic protein and Ki-67 in Alzheimer's disease. Int J Clin Exp Pathol 2015;8:7710-28. PMCID: PMC4555665
10. Mahdy KA, Gouda NAM, Marrie AH, Yassin NAZ, El-Shenawy SMA, Farrag ARH, Ibrahim BMM. Protective effect of ginger (*Zingiber officinale*) on Alzheimer's disease induced in rats. J Neuroinfect Dis 2014;5:159. doi: 10.4172/2314-7326.1000159
11. Zatta P, Lucchini R, van Rensburg SJ, Taylor A. The role of metals in neurodegenerative processes: aluminum, manganese, and zinc. Brain Res Bull 2003;62:15-28. PMID: 14596888
12. Singh V, Panwar R. *In vivo* antioxidative and neuroprotective effect of 4-allyl-2-methoxyphenol against chlorpyrifos-induced neurotoxicity in rat brain. Mol Cell Biochem 2014;388:61-74. doi: 10.1007/s11010-013-1899-9
13. National Toxicology Program. Carcinogenesis Studies of Eugenol (CAS No. 97-53-0) in F344/N Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser 1983;223:1-159. PMID: 12778213
14. Said MM. The protective role of eugenol against gentamicin-induced nephrotoxicity and oxidative damage in rat kidney. Fundam Clin Pharmacol 2011;25:708-16. doi: 10.1111/j.1472-8206.2010.00900.x
15. Manikandan P, Vinothini G, Vidya Priyadarsini R, Prathiba D, Nagini S. Eugenol inhibits cell proliferation via NF-κB suppression in a rat model of gastric carcinogenesis induced by MNNG. Invest New Drugs 2011;29:110-17. doi: 10.1007/s10637-009-9345-2
16. Júnior PL, Câmara DA, Costa AS, Ruiz JL, Levy D, Azevedo RA, Pasqualoto KF, de Oliveira CF, de Melo TC, Pessoa ND, Fonseca PM, Pereira A, Araldi RP, Ferreira AK. Apoptotic effect of eugenol envolves G2/M phase abrogation accompanied by mitochondrial damage and clastogenic effect on cancer cell *in vitro*. Phytomedicine 2016;23:725-35. doi: 10.1016/j.phymed.2016.03.014
17. Nam H, Kim MM. Eugenol with antioxidant activity inhibits MMP-9 related to metastasis in human fibrosarcoma cells. Food Chem Toxicol 2013;55:106-12. doi: 10.1016/j.fct.2012.12.050
18. Wie MB, Won MH, Lee KH, Shin JH, Lee JC, Suh HW, Song DK, Kim YH. Eugenol protects neuronal cells from excitotoxic and oxidative injury in primary cortical cultures. Neurosci Lett 1997;225:93-6. PMID: 9147382
19. Kabuto H, Tada M, Masahiro Kohno M. Eugenol [2-methoxy-4-(2-propenyl)phenol] prevents 6-hydroxydopamine-induced dopamine depression and lipid peroxidation inductivity in mouse striatum. Biol Pharm Bull 2007;30:423-7. doi: 10.1248/bpb.30.423
20. Won MH, Lee JC, Kim YH, Song DK, Suh HW, Oh YS, Kim JH, Shin TK, Lee YJ, Wie MB. Postischemic hypothermia induced by eugenol protects hippocampal neurons from global ischemia in gerbils. Neurosci Lett 1998;254:101-4. doi: 10.1016/S0304-3940(98)00664-8
21. Prasad SN, Muralidhara. Neuroprotective efficacy of eugenol and isoeugenol in acrylamide-induced neuropathy in rats: behavioral and biochemical evidence. Neurochem Res 2013;38:330-45. doi: 10.1007/s11064-012-0924-9
22. National Research Council (US) Committee for the Update of the Guide. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington (DC): National Academies Press; 2011.
23. Reeves PG, Nielsen FH, Fahey GC, Jr. AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123:1939-51. PMID: 8229312
24. Said MM, Ogawa K, Pitchakarn P, Takahashi S, Asamoto M, Shirai T. Cyclooxygenase 2 and prostaglandin E2 are not involved in N-nitrosodiethylamine-initiated early rat hepatocarcinogenesis. J Toxicol Pathol 2009;22:263-71. doi: 10.1293/tox.22.263
25. Krasovskiĭ GN, Vasukovich LY, Chariev OG. Experimental study of biological effects of lead and aluminum following oral administration. Environ Health Perspect 1979;30:47-51. doi: 10.1289/ehp.793047
26. Marin Municipal Water District Vegetation Management Plan DRAFT-1/1/2010, Herbicide Risk Assessment. Chapter 6 - Clove oil (Eugenol). Available at http://www.marinwater.org/documentcenter/view/253

27. Liu Z, Niu W, Yang X, Wang Y. Effects of combined acupuncture and eugenol on learning-memory ability and antioxidation system of hippocampus in Alzheimer disease rats via olfactory system stimulation. J Tradit Chin Med 2013;33:399-402. doi: 10.1016/S0254-6272(13)60186-7
28. Wills ED. Lipid peroxide formation in microsomes. General considerations. Biochem J 1969;113:315-24. doi: 10.1042/bj1130315
29. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V. Method for the measurement of antioxidant activity in human fluids. J Clin Pathol 2001;54:356-61. doi: 10.1136/jcp.54.5.356
30. Ellman GL, Courtney KD, Andersen V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95. doi: 10.1016/0006-2952(61)90145-9
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75. PMID: 14907713
32. Pagel P, Blome J, Wolf HU. High-performance liquid chromatographic separation and measurement of various biogenic compounds possibly involved in the pathomechanism of Parkinson's disease. J Chromatogr B Biomed Sci Appl 2000;746:297-304. doi: 10.1016/S0378-4347(00)00348-0
33. van Ginkel MF, van der Voet GB, de Wolff FA. Improved method of analysis for aluminum in brain tissue. Clin Chem 1990;36:658-61. PMID: 2323045
34. Sober HA, Hollander F, Sober EK. Toxicity of eugenol; determination of LD50 on rats. Proc Soc Exp Biol Med 1950;73:148-51. doi: 10.3181/00379727-73-17608
35. Taylor JM, Jenner PM, Jones WI. A comparison of the toxicity of some allyl, propenyl and propyl compounds in the rat. Toxicol Appl Pharmacol 1964;6:378-87. doi: 10.1016/S0041-008X(64)80002-8
36. Buraimoh AA, Ojo SA, Hambolu JO, Adebisi SS. Behavioural endpoints of adult wistar rats, following aluminium chloride exposure. Br J Pharmacol Toxicol 2011;2:273-6.
37. Parekh KD, Dash RP, Pandya AN, Vasu KK, Nivsarkar M. Implication of novel bis-imidazopyridines for management of Alzheimer's disease and establishment of its role on protein phosphatase 2A activity in brain. J Pharm Pharmacol 2013;65:1785-95. doi: 10.1111/jphp.12149
38. Ahmed HH, Estefan SF, Mohamd EM, Farrag AH, Salah RS. Does melatonin ameliorate neurological changes associated with Alzheimer's disease in ovariectomized rat model? Ind J Clin Biochem 2013;28:381-9. doi: 10.1007/s12291-012-0284-x
39. Stevanović ID, Jovanović MD, Jelenković A, Ninković M, Đukić M, Stojanović I, Čolić M. The effect of inhibition of nitric oxide synthase on aluminium-induced toxicity in the rat brain. Gen Physiol Biophys 2009;28:235-42. PMID: 19893106
40. Irie Y. Effects of eugenol on the central nervous system: Its possible application to treatment of Alzheimer's disease, depression, and Parkinson's disease. Curr Bioact Comp 2006;2:57-66.
41. Johnson VJ, Kim S, Sharma RP. Aluminum maltolate induces apoptosis and necrosis in neuro-2a cells: potential role for p53 signaling. Toxicol Sci 2005;83:329-39. doi: 10.1093/toxsci/kfi028
42. Savory J, Herman MM, Ghribi O. Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain. J Inorg Biochem 2003;97:151-4. doi: 10.1016/S0162-0134(03)00258-7
43. Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME. Ca^{2+} influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 1998;20:709-26. doi: 10.1016/S0896-6273(00)81010-7
44. Abu-Taweel GM, Ajarem JS, Ahmad M. Neurobehavioral toxic effects of perinatal oral exposure to aluminum on the developmental motor reflexes, learning, memory and brain neurotransmitters of mice offspring. Pharmacol Biochem Behav 2012;101:49-56. doi: 10.1016/j.pbb.2011.11.003
45. Li W, Tsubouchi R, Qiao S, Haneda M, Murakami K, Yoshino M. Inhibitory action of eugenol compounds on the production of nitric oxide in RAW264.7 macrophages. Biomed Res 2006;27:69-74. PMID: 16707845
46. Chainy GB, Manna SK, Chaturvedi MM, Aggarwal BB. Anethole blocks both early and late cellular responses transduced by tumor necrosis factor: effect on NF-kappaB, AP-1, JNK, MAPKK and apoptosis. Oncogene 2000;19:2943-50. doi: 10.1038/sj.onc.1203614
47. Irie Y, Itokazu N, Anjiki N, Ishige A, Watanabe K, Keung WM. Eugenol exhibits antidepressant-like activity in mice and induces expression of metallothionein-III in the hippocampus. Brain Res 2004;1011:243-6. doi: 10.1016/j.brainres.2004.03.040
48. Garabadu D, Shah A, Ahmad A, Joshi VB, Saxena B, Palit G, Krishnamurthy S. Eugenol as an anti-stress agent: modulation of hypothalamic-pituitary-adrenal axis and brain monoaminergic systems in a rat model of stress. Stress 2011;14:145-55. doi: 10.3109/10253890.2010.521602
49. Alleva E, Rankin J, Santucci D. Neurobehavioural alteration in rodents following developmental exposure to aluminum. Toxicol Ind Health 1998;14:209-21. PMID: 9460176
50. Kaizer RR, Corrêa MC, Gris LR, da Rosa CS, Bohrer D, Morsch VM, Schetinger C. Effect of long-term exposure to aluminum on the acetylcholinesterase activity in the central nervous system and erythrocytes. Neurochem Res 2008;33:2294-301. doi: 10.1007/s11064-008-9725-6
51. Dalai MK, Bhadra S, Chaudhary SK, Bandyopadhyay A, Mukherjee PK. Anti-cholinesterase activity of the standardized extract of *Syzygium aromaticum* L. Pharmacogn Mag 2014;10(Suppl 2):S276-82. doi: 10.4103/0973-1296.133275
52. Sartorius T, Peter A, Schulz N, Drescher A, Bergheim I, Machann J, Schick F, Siegel-Axel D, Shurmann A, Weigert C, Haring HU, Hennige AM. Cinnamon extract improves insulin sensitivity in the brain and lowers liver fat in mouse models of obesity. PLoS One 2014;9:e92358. doi: 10.1371/journal.pone.0092358
53. Silva Junior AF, Aguiar MSS, Carvalho Junior OS, Santana LdNS, Franco ECS, Lima RR, de Siqueira NVM, Feio RA, Faro LRF, Gomes-Leal WG. Hippocampal neuronal loss, decreased GFAP immunoreactivity and cognitive impairment following experimental intoxication of rats with aluminum citrate. Brain Res 2013;1491:23-33. doi: 10.1016/j.brainres. 2012.10.063
54. Guo-Ross SX, Yang EY, Walsh TJ, Bondy SC. Decrease of glial fibrillary acidic protein in rat frontal cortex following aluminum treatment. J Neurochem 1999;73:1609-14. doi: 10.1046/j.1471-4159.1999.0731609.x
55. Walton JR. A bright field/fluorescent stain for aluminum: its specificity, validation, and staining characteristics. Biotech Histochem 2004;79:169-75. doi: 10.1080/10520290400015423

Neuroprotektivni učinci eugenola protiv aluminijem izazvane toksičnosti u mozgu štakora

Aluminij (Al) je neutrotoksični metal koji pridonosi razvoju nekoliko neurodegenerativnih bolesti. Cilj ovog istraživanja bio je procijeniti zaštitni učinak eugenola protiv aluminijem izazvanog moždanog oštećenja u štakora. Mužjaci Wistar soja štakora bili su podijeljeni u četiri skupne: kontrolna skupina, štakori čija je hrana sadržavala 6,000 μg g^{-1} eugenola, štakori kojima je oralnim putem svakodnevno davan aluminijev klorid (84 mg kg^{-1} tjelesne težine) uz uobičajen režim prehrane ili uz prehranu obogaćenu eugenolom. Dnevna doza aluminija tijekom četiriju uzastopnih tjedana značajno je smanjila ukupni antioksidacijski status (TAS)(11,42 ± 0,31 μmol g^{-1} tkiva, $p < 0,001$) te je povećala lipidnu peroksidaciju (MDA)(32,55 ± 1,68 nmol g^{-1} tkiva, $p < 0,002$). Nadalje, tretman s Al potaknuo je aktivnost acetilkolinesteraze u mozgu (AChE)(46,22 ± 4,90 U mg^{-1} protein, $p < 0,001$) te povisio razine tumornoga nekroznog faktora alfa (TNF-α)(118,72 ± 11,32 pg mg^{-1} protein, $p < 0,001$) i kaspaze 3 (Casp-3)(8,77 ± 1,26 ng mg^{-1} protein, $p < 0,001$), a značajno je smanjio razine neurotrofnoga moždanog faktora (BDNF)(82,74 ± 14,53 pg mg^{-1} protein, $p < 0,002$) i serotonina (5-HT)(1,54 ± 0,12 ng mg^{-1} tkivo, $p < 0,01$). Isto tako, u usporedbi s kontrolnom skupinom koja nije bila tretirana, u strijatumu štakora tretiranih aluminijem primijećeno je imunobojenje glijalnih fibrilarnih kiselih proteina (GFAP). S druge strane, istovremenim uzimanjem eugenola i aluminija ponovo su uspostavljene normalne razine BDNF-a (108,76 ± 2,64 pg mg^{-1} protein) i 5-HT-a (2,13 ± 0,27 ng mg^{-1} tkiva), a moždani je TAS povećan (13,43 ± 0,24 μmol g^{-1} tkiva, $p < 0,05$). Nadalje, istodobno su se razine TNF-α (69,98 ± 4,74 pg mg^{-1} protein) i Casp-3 (3,80 ± 0,37 ng mg^{-1} protein) značajno smanjile ($p < 0,001$), kao i aktivnost AChE (24,50 ± 3,25 mg^{-1} protein, $p < 0,001$), te je imunoreaktivnost GFAP-a u strijatumu povećana u usporedbi sa štakorima koji su tretirani aluminijem. Histološki nalazi moždanog tkiva potvrđuju biokemijske podatke. Zaključno, eugenol ima potencijal kao neuroprotektivna tvar ne samo zbog svojih hidrofobnih, antioksidacijskih i antiapoptotskih svojstava nego i zbog svoje neurotrofne sposobnosti djelovanja protiv aluminijem izazvane toksičnosti u mozgu štakora.

KLJUČNE RIJEČI: *acetilkolinesteraza; apoptoza; neurotrofni moždani faktor; oksidacijski stres; serotonin*