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Magnesium yields opposite effects on the nuclear and cytosolic cascades of apoptosis in different rat brain regions

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Abstract. – OBJECTIVE: Magnesium is considered as potential neuroprotective and therapeutic agent, but certain studies have provided evidence of its apoptotic effectiveness in neurons. We aimed to evaluate the possible apoptotic effects of long-term magnesium use in healthy adult rat brains.

MATERIALS AND METHODS: Magnesium citrate and magnesium glycinate compounds were administered orally to rats for 8 weeks (36 mg/kg). Expression levels of Bcl-2, Bax and Cyt-C genes were analyzed by real-time polymerase chain reactions (RT-PCR) in the prefrontal cortex, hippocampus and striatum regions. Bcl-2, Bax and CytC protein levels were measured using ELISA kits. Tissue sections were evaluated histopathologically with hematoxylin-eosin staining.

RESULTS: Compared to the control group, the magnesium-administered groups indicated gene expression reductions in almost all brain regions; pro-apoptotic Bax, anti-apoptotic Bcl-2 and Cyt-C gene expression levels were reduced. With magnesium, the Bcl-2 and Bax protein levels were increased. Bax/Bcl-2 gene and protein ratio were also increased in the striatum and hippocampus, whereas Cyt-C protein levels were decreased or did not change in the magnesium treated groups. There was no pathological finding in histological evaluation.

CONCLUSIONS: Long-term magnesium usage can promote apoptotic cascade in brain tissue by increasing Bax/Bcl-2 ratio. Cyt-C, a prominent factor processing caspase pathway, was decreased or unchanged. In addition, taking into account the histological evaluation, we supposed that the absence of Cyt-C in the cytosol can prevent the subsequent apoptotic pathway. Consequently, we obtained the findings of apoptotic initiation with magnesium in brain, but this cascade seems to be arrested at later stages.

Key Words:
Magnesium citrate, Magnesium glycinate, Apoptosis, Bcl-2, Bax, Cyt-C.

Introduction

Following sodium, magnesium (Mg) is the most abundant second cation in cells. In a holistic approach, Mg is an essential mineral with pleiotropic impacts (e.g., in activity of various enzymes, ion balance) in both cytosols and organelles. In the neurological system, in addition to being a protective element against neuronal-directed excitotoxicity, Mg is an organizer in neuronal and neuromuscular synaptic conductance. The neuroprotective and neuromodulator effects of Mg supplementation have garnered attention[1]. Research has demonstrated that agricultural processes, processed foods, dietary habits, diuretics and proton pump inhibitors can lead to magnesium deficiency[2-5], and consequently, the importance of daily Mg supplementation has been increased.

Approximately 30% of the enzymes and proteins that maintain cell function and vitality depend on Mg[6]. Mg and Mg-dependent kinases are required in the maintenance of vital cell functions (such as cell proliferation and maturation, signal transduction, the production and function of second messengers, the activation of ATP/ATPase pumps associated with electrolyte homeostasis, and bio-energetic functions) as well as regulating cell death and apoptosis[7-10]. Low Mg levels contribute to glutamatergic neurotransmission, leading to excitotoxicity and neuronal cell death.

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Abnormal glutamatergic neurotransmission has been associated with many neurological and psychiatric disorders (migraine, chronic pain, epilepsy, Alzheimer's, Parkinson's, stroke, depression, and anxiety)[11,12].

As Mg yields such a diverse and broad spectrum of functions, its effects on various cell types differ. The role of magnesium in the nervous system has been extensively investigated in clinical and experimental research on different pathological processes (chronic pain, fibromyalgia, anxiety-depression, epilepsy, dementia, Parkinson's disease). In some of these diseases, Mg levels in the brain or cerebrospinal fluid were reduced or normal. In others, magnesium therapy was preventive, ineffective or even curative. Since there is insufficient evidence on the efficacy of Mg supplementation in neurological disorders, no consensus is reached on magnesium usage in these pathologies[1,13]. Furthermore, the apoptotic effectiveness of Mg in the nervous system is unclear[13-15].

From a physiological point of view, cells must contain a certain amount of magnesium in order to prevent deterioration of the cell functions. Mg can be found in organelles, and is especially bonded to nucleic acids, chromatin, phospholipids, and proteins. Mg is scarce in the cytosol. While the magnesium concentration in eukaryotic cells is 17-20 mM, it is 15-18 mM in organelles (nucleus, mitochondria, endoplasmic reticulum) and below 1 mM in free active form in the cytosol and organelle lumens[16]. The entry of Mg into the brain is largely blocked by the blood-brain barrier. Due to the concentration gradient, the transition of Mg from blood to brain tissue requires active transport[13]. Addressing these difficulties, *in vivo* studies test the effect of Mg on the passage of the blood-brain barrier, its entry into the brain tissue and its cleavage to the active form. In our previous study, we reported the passage of Mg glycinate and Mg citrate into mice brain tissue at mice dose corresponding to human dose (405 mg/70 kg bodyweight); total Mg levels in the brain were higher than in subjects who did not receive Mg supplementation[3]. Since these Mg compounds are well absorbed into the brain tissue, hence they can affect cellular functions in the brain.

Mg supplementation has been suggested in the Dietary Reference Intake (DRIs) and Recommended Dietary Allowance (RDAs) guidelines as 320-420 mg/day (men and women, respectively) aged 30-50 years[17,18]. An increase in Mg levels can cause toxic effects such as diarrhea, muscle weakness, arrhythmia, and deterioration in kidney functions, hypokalemia, and metabolic alkalosis[19]. However, there is no evidence whether long-term Mg supplementation has negative effects on brain cells. Healthy individuals without Mg deficiency often receive Mg supplements as a result of reports of positive effects on the nervous system. The intake of Mg may not create a change in the free-active form and may be ineffective or cause harmful results such as pathological apoptosis. In our present study, by supplementing Mg glycinate and Mg citrate for 8 weeks, we investigated the effect of on the apoptotic processes in rat brains.

Materials and Methods

Animals

The tissue materials used in this study were obtained from another protocol: the Institutional Review Board/Ethical Review Board No. 02.10.2018/36/2018 titled "Investigation of the effects of chronic Mg use on brain, skeletal and smooth muscle function and biochemistry in rats". The study had previously been approved by the institutional ethics committee.

All female adult outbred Sprague Dawley rats (250-300 gr) were obtained from the Dokuz Eylül University Faculty of Medicine, Experimental Animals Laboratory (Izmir, Turkey). All rats were placed in individual cages at constant room temperature (22 ± 1°C) and humidity (60%), 12-hour light-12 hour dark cycles and free access to standard feed. All animal experiments were conducted in accordance with the policies of the NIH Guide for the Care and Use of Laboratory Animals.

Experimental Design

The rats were divided into three groups. (1) Control group (n=6), (2) magnesium citrate (MgC, n=6), magnesium glycinate (MgG, n=6). For eight weeks both Mg compounds were administered orally. The daily elementary Mg dose was 36 mg per kg bodyweight dissolved in 1 milliliter tap water. Based on the calculations suggested in one of our previous studies, the prescribed physiological oral Mg supplement dose currently applied for humans (5.78 mg/kg) was adapted to rats. Control animals were given the same volume of tap water orally. Finally, all rats were anesthetized by CO2 inhalation, which has a rapid and short-term anesthetic effect.

The prefrontal cortex, hippocampal and striatum tissues were removed and randomly divided into two sections: right and left; one part was placed in a 1 ml RNa stabilization solution for RNA isolation and the other was also stored at −80°C for biochemical analysis.

Total RNA Extraction and cDNA Synthesis

The total RNAs were extracted from the brain tissues of rats in each group. Tissue samples were immediately placed in 2 ml sterile centrifuge tubes containing RNA stabilization solution (RiboSaver, GeneAll Biotechnology Co., LTD). Total RNA was isolated using an RNA isolation kit (Hybrid-RTM, GeneAll Biotechnology Co., LTD) in accordance with the manufacturer's protocol. RNA purity and concentration were determined by absorbance readings at 260 nm and 280 nm (NanoDrop™1000 Spectrophotometer, Thermo Scientific, Waltham, MA, USA). 1.5 μg of total RNA was reverse transcribed into cDNA using a High-Capacity cDNA Synthesis Kit with RNase Inhibitor according to the manufacturer's protocol (A.B.T.™ Laboratories, Turkey). Each cDNA sample (100 ng/μl) was used as template for qRT-PCR using SYBR Green (2X One-Step qRT-PCR MasterMix, A.B.T.™ Laboratories, Turkey).

Real Time Relative Quantitative RT-PCR

Quantitative real time PCR (qRT-PCR) was performed using the Rotor-Gene Q PCR system (Qiagen, Hilden, Germany) with SYBR-Green MasterMix to determine the expression levels of apoptosis-related genes. Based on the user manual of 2X qPCR SYBR-Green MasterMix (without ROX) (A.B.T.™ Laboratories, Turkey), the qPCR assay was performed with a 20 μl final reaction volume consisting of 10 μl of 2X SYBR-Green, 0.6 μl of forward and reverse primers, 2 μl of cDNA template and 6.8 μl ddH_2O. PCR conditions for mRNAs as follows; pre-denaturation at 94°C for 10 min, denaturation at 95°C for 10 s, annealing at 58°C for 15 s and extension at 72°C for 20 s, for 40 cycles. The hypoxanthine phosphoribosyl-transferase (HPRT) housekeeping gene was used as an endogenous control for mRNA expression. The sequences of the primers are provided in Table I. All the primers were designed and synthesized by Triogen Biotechnology Co., LTD (Turkey) and all real-time PCR reactions were conducted using a Rotor-Gene Q PCR detection system (Qiagen). The relative expression of each mRNAs (BCL-2, BAX, and CYC) were calculated to the normalizer gene HPRT and calculated using the $2^{-\Delta\Delta Ct}$ method. Calculations were performed using Light Cycler 2.0 software.

Biochemical Measurements

Frozen samples of the prefrontal cortex, hippocampal and striatum tissues were weighed and homogenized with steel beads using BioSpec Mini-Beadbeater-16 (BioSpec Products Inc., USA) in 10 volumes of PBS, pH:7.4 and centrifuged at 5000 g for 15 min at 4°C. The supernatants were used for all biochemical analyses.

Tissue ELISA measurements for Bcl-2, Bax and CytC were performed according to the kit protocol. Bcl-2 levels (Rat B-cell leukemia/lymphoma 2 ELISA kit, Catalog Number E0037Ra,Bioassay Technology Laboratory, Wuhan, China with assay sensitivity 0.026 ng/ml and range 0.05 ng/ml – 10 ng/ml); Bax levels (Rat Bcl-2 associated X protein, Bax ELISA Kit, Catalog Number E0034Ra, Bioassay Technology Laboratory, Wuhan, China with assay sensitivity 0.12 ng/ml and range 0.2 ng/ml – 60 ng/ml) and CytC levels (Rat Cytochrome C, CYCS ELISA kit, Catalog Number E1939Ra, Bioassay Technology Laboratory , Wuhan, China with assay sensitivity 14.35 ng/L and range 30 ng/L – 9000 ng/L) were measured using commercially available ELISA kits specific for rats. All results were calculated in mg protein per tissue. Protein analysis was performed according to the manufacturer guide of the BCA protein Assay kit (Catalog Number 23227, Pierce™ BCA Protein Assay Kit, USA). Absorbency changes were measured using a microplate reader (ELx800, BioTek Instruments, Inc., Winooski, VT, USA) at 450 nm for ELISA kits and at 560 nm for protein assay kit.

Histopathological Analyses

Brain tissues were fixed with formalin and blocked with paraffin. Coronal sections comprised from the prefrontal cortex, hippocampus and striatum regions of the brain were prepared and sliced 4 μm thick. Apoptosis findings were

Table I. The sequences of primers.

Gene	Forward primer	Reverse primer
Bax	5′-AAGAAGCTGAGCGAGT-3′	5′-GCCCATGATGGTTCTG-3′
Bcl	5′-ACCTGACGCCCTTCAC-3′	5′-AGGTACTCAGTCATCCAC-3′
CytC	5′-TGGGTGATGTTGAGAAAG-3′	5′-TTTGTTCCAGGGATGTACT-3′
HPRT	5′-CCGGGTGGACCATTACTTAGG-3′	5′-TTCCAGAGGCCGTCCAAAG-3′

examined in H&E (hematoxylin and eosin) stained sections. The presence of apoptotic body, nuclear fragmentation, and pycnotic nuclei were examined for the identification of apoptotic cells. Apoptosis was evaluated in the BX51 Olympus microscope (Olympus, Tokyo, Japan). Camera system with DP Controller and DP Manager Software (Olympus America, Melville, NJ, USA) was used to record the images.

Statistical Analysis

All statistical procedures were performed by SPSS 24.0 (IBM Corp., Armonk, NY, USA). The differences among the groups were analyzed using the one-way ANOVA test. Bonferroni and Tamhane multiple-comparison post hoc tests were performed when a statistically significant difference was found between groups. The results are presented as mean ± S.E.M, where $p < 0.05$ was considered statistically significant.

Results

Bax, Bcl-2 and Cyt-C gene and protein levels, Bax/Bcl-2 ratios were determined, and statistical analyses were performed in the MgC, MgG and control groups. The gene and the protein expression data and statistical results in the brain regions of the three groups are provided in Tables II and III. The presentations of the data as a bar graph are shown in Figures 1, 2 and 3.

Prefrontal Cortex Measurements

Bax protein did not change in the prefrontal cortex of MgG compared to controls (Figure 1B). In this group, all other gene and protein expressions (Bax, Bcl-2, Cyt-C) in the prefrontal cortex decreased (Figure 1A, 1C-F). In the prefrontal cortex of the MgC group, all gene expressions had decreased as well, and except for the increase in Bax protein, no change was observed in other protein expressions (Figure 1). In the prefrontal cortex for both Mg groups, the Bax/Bcl-2 ratio decreased at the gene level but did not change at the protein level (Figure 1G,1H).

Hippocampus Measurements

When compared to the controls, Bcl-2 and Cyt-C gene levels decreased in the hippocampus of the MgG group (Figure 2C, 2E). In the MgG group, the Bax/Bcl-2 ratio of the hippocampus increased at both gene and protein levels (Figure 2G, 2H). Other gene and protein levels did not change (Figure 2A, 2B, 2D, 2F). Compared to the control group, the Cyt-C gene level decreased and the Bax protein level increased in the hippocampus of the MgC Group.

Striatum Measurements

In comparison to the control group, the Bax gene and protein levels increased in the striatum samples of the MgG group (Figure 3A, 3B). Bcl-2 gene levels decreased (Figure 3C) and protein levels did not change (Figure 3D). The CYT-C gene level increased but the protein level did not vary in the striatum samples of the MgG group (Figure 3E, 3F). The Bax/Bcl-2 ratio increased in the MgG group at both gene and protein levels (Figure 3G, 3H). In the striatum samples of MgC, the Bcl-2 gene level decreased (Figure 3C). Bax and Bcl-2 protein levels increased (Figure 3B, 3D); however, the Bax/Bcl-2 protein levels did not alter (Figure 3H). Cyt-C protein levels decreased (Figure 3F).

Histological Assessment

In the control group, there were no cells displaying Caspase-3 positive staining in the sections of the brain prefrontal cortex, striatum and hippocampus regions. Likewise, no cells showing Caspase-3 positive staining were detected in the Mg-administered groups. There were no findings of apoptotic body, nuclear fragmentation and pycnotic nuclei (Figure 4A, 4B, 4C).

Discussion

Various experimental and clinical studies have reported that Mg has beneficial effects on cognitive brain functions[20-22]. We researched apoptosis in the rat brain regions (the prefrontal cortex, hippocampus and striatum areas) which process cognitive function. The rats were administered Mg for 8 weeks which translates to an approximate five year duration of human life[23].

Up-regulation in the gene expression was detected only in the Bax and Cyt-C genes in the striatum of the MgG group. We did not observe an up-regulatory effect on the gene expressions in the MgC groups. Most of the genes we studied were downregulated in the prefrontal cortex, striatum and hippocampus regions. MgC showed suppressive effects on the Cyt-C gene in the hippocampus, on Bcl-2 in the striatum, and on all three genes in the prefrontal cortex. MgG demonstrated similar impacts on gene expressions. In addition, MgG down-regulated the Bcl-2 gene in the hip-

Table II. The comparison of Bax, Bcl-2 ve Cyt-C protein levels (ng protein / per mg tissue proteins) in rat brain regions.

Relative Expression Unit Mean±SE		Prefrontal Cortex			Hippocampus			Striatum		
		ANOVA	post hoc (compared to control)	Relative Expression Unit Mean ± SE	ANOVA	post hoc (compared to control)	Relative Expression Unit Mean ± SE	ANOVA	post hoc (compared to control)	
Bcl2	Mg citrate group	0.19± 0.04	$p<0.0001$ F(2.15)=287.43	$p<0.0001$	0.73± 0.18	$p<0.0001$ F(2.15)=17.68	$p>0.05$	0.34± 0.03	$p<0.0001$ F(2.15)=45.78	$p<0.0001$
	Mg glycinate group	0.15± 0.04		$p<0.0001$	0.12±0.01		$p<0.0001$	0.79± 0.07		$p<0.03$
	control	1			1			1		
Bax	Mg citrate group	0.01± 0.004	$p<0.0001$ F(2.15)=250.92	$p<0.0001$	0.58±0.12	$p=0.009$ F(2.15)=6.62	$p=0.07$	0.35± 0.03	$p<0.0001$ F(2.15)=28.17	$p>0.05$
	Mg glycinate group	0.04± 0.03		$p<0.0001$	0.77± 0.06		$p>0.05$	2.30± 0.32		$p=0.01$
	control	1			1			1		
CytC	Mg citrate group	0.01± 0.003	$p<0.0001$ F(2.15)=107.38	$p<0.0001$	0.59±0.14	$p=0.001$ F(2.15)=35.55	$p>0.05$	0.35± 0.03	$p=0.011$ F(2.15)=11.89	$p>0.05$
	Mg glycinate group	0.05±0.008		$p<0.0001$	0.03±0.007		$p<0.0001$	2.86±0,65		$p=0.01$
	control	1			1			1		
Bax/Bcl2 ratio	Mg citrate group	0.18±0.06	$p<0.0001$ F(2.15)=110.92	$p<0.0001$	0.95±0.14	$p<0.0001$ F(2.15)=47.81	$p>0.05$	1.12± 0.23	$p<0.001$ F(2.15)=17.76	$p>0.05$
	Mg glycinate group	0.25± 0.02		$p<0.0001$	6.60±0.80		$p<0.0001$	2.90± 0.37		$p<0.0001$
	control	1			1			1		

Table III. The comparison of Bax, Bcl-2 ve Cyt-C gene expression levels in rat brain regions.

ng protein/per mg tissue proteins Mean±SE		Prefrontal Cortex			Hippocampus			Striatum		
		ANOVA	post hoc (compared to control)	ng protein/ per mg tissue proteins Mean±SE	ANOVA	post hoc (compared to control)	ng protein/ per mg tissue proteisn Mean±SE	ANOVA	post hoc (compared to control)	
Bcl2	Mg citrate group	0.63±0.09	p=0.003 F(2.15)=8.85	p>0.05	0.90±0.12	p=0.002 F(2.15)=10.07	p>0.05	1.56±0.10	p<0.0001 F(2.15)=16.81	p=0.04
	Mg glycinate group	0.28±0.02		p=0.03	0.37±0.03		p>0.05	0.94±0.72		p>0.05
	control	0.52±0.05			0.65±0.06			1.13±0.04		
Bax	Mg citrate group	0.53±0.09	p=0.006 F(2.15)=7.37	p=0.04	0.62±0.05	p=0.006 F(2.15)=7.29	p=0.06	0.94±0.01	p=0.001 F(2.15)=10.60	p=0.01
	Mg glycinate group	0.21±0.04		p>0.05	0.40±0.08		p>0.05	1.03±0.08		p=0.001
	control	0.30±0.03			0.30±0.02			0.66±0.04		
CytC	Mg citrate group	0.02±0.007	p=0.016 F(2.15)=5.55	p>0.05	0.06±0.003	p>0.05		0.05±0.02	p=0.001 F(2.15)=10.36	p=0.03
	Mg glycinate group	0.01±0.003		p=0.01	0.05±0.008			0.16±0.01		p>0.05
	control	0.03±0.001			0.06±0.003			0.12±0.007		
Bax/Bcl2 ratio	Mg citrate group	0.82±0.03	p>0.05		0.70±0.05	p=0.025 F(2.14)=4.85	p>0.05	0.61±0.03	p<0.0001 F(2.15)=38.35	p>0.05
	Mg glycinate group	0.78±0.15			1.15±0.24		p=0.028	1.09±0.06		p<0.0001
	control	0.57±0.04			0.48±0.07			0.58±0.02		

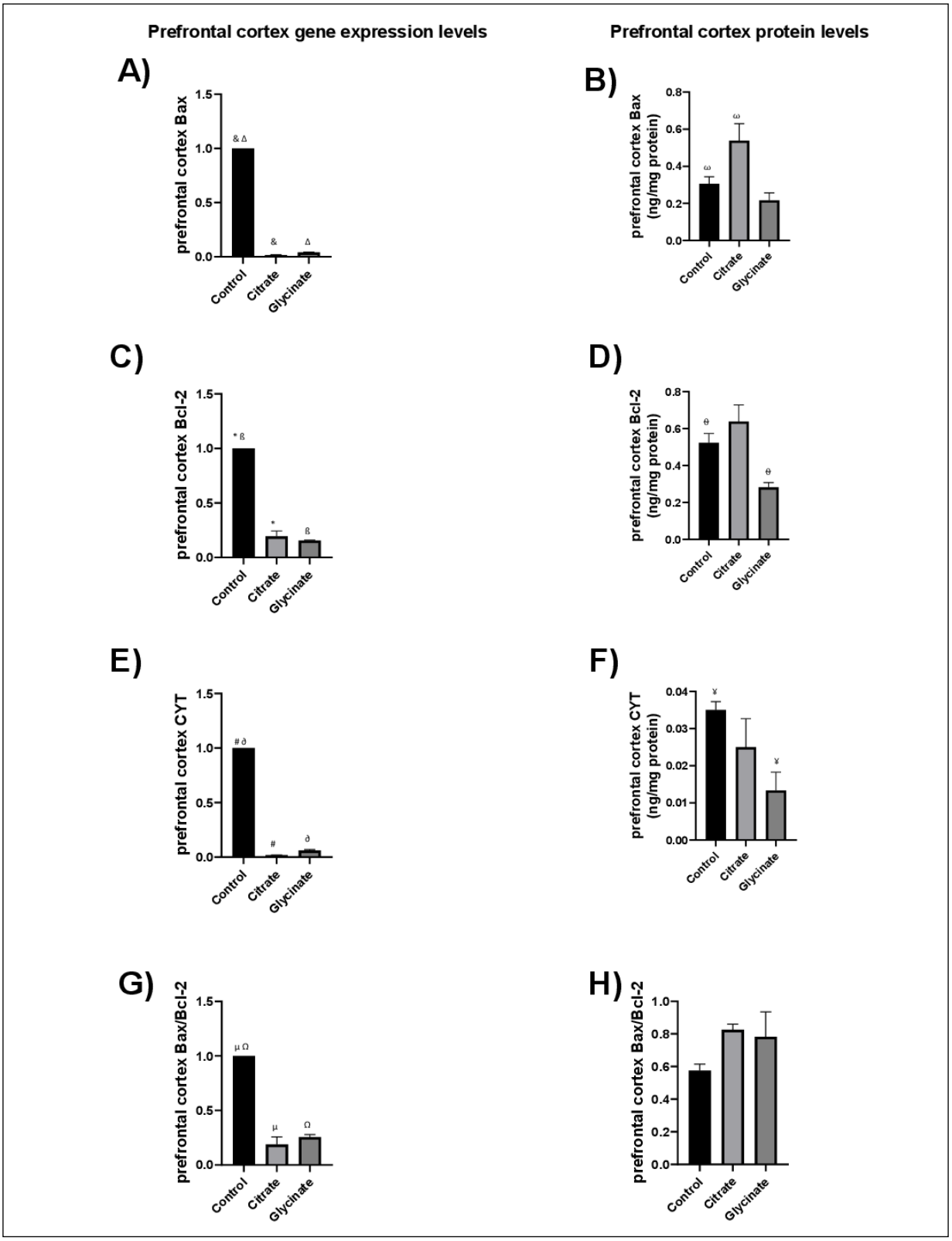

Figure 1. Gene and protein expression levels in the rat prefrontal cortex. Bax gene decreased in the MgC and in the MgG groups compared to the control group, and the Bax protein increased significantly in the MgC group (**A**, **B**). Bcl-2 gene and CYT-C gene levels decreased in both Mg groups, while protein levels decreased in the MgG group (**C**-**F**). The Bax/Bcl-2 gene ratio decreased in both Mg groups compared to the control group, but protein levels did not change (**G**, **H**).

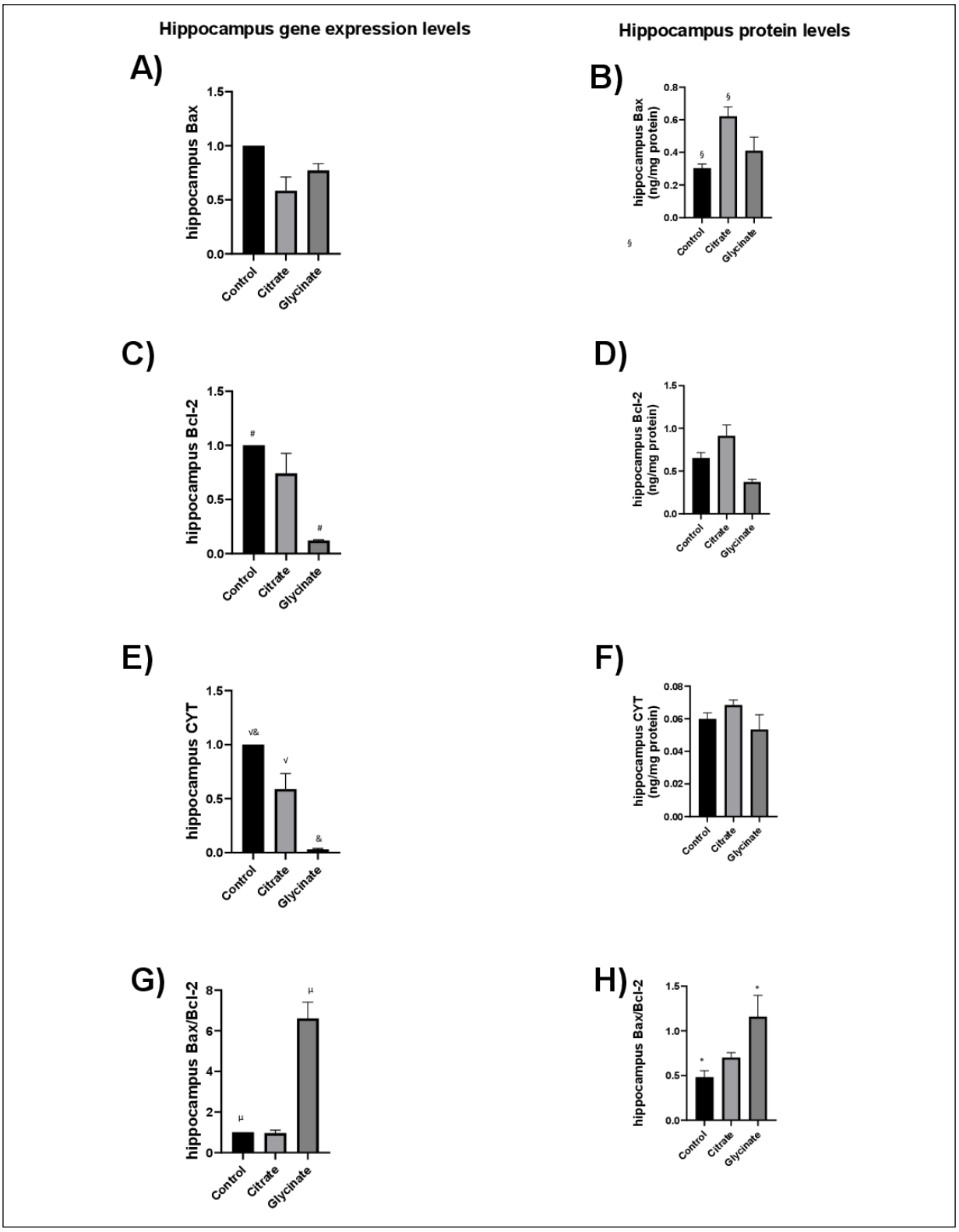

Figure 2. Gene and protein expression levels in the rat hippocampus. The Bax gene levels show differences among the groups; the Bax protein level increased in the MgC group (**A**, **B**). In comparison to the control group, the Bcl-2 gene level decreased in the MgG group and protein levels did not change in both groups (**C**, **D**). The Cyt-C gene level decreased significantly in both groups, but protein levels did not change (**E**, **F**). The Bax/Bcl-2 ratio increased in the MgG group in both gene and protein levels (**G**, **H**).

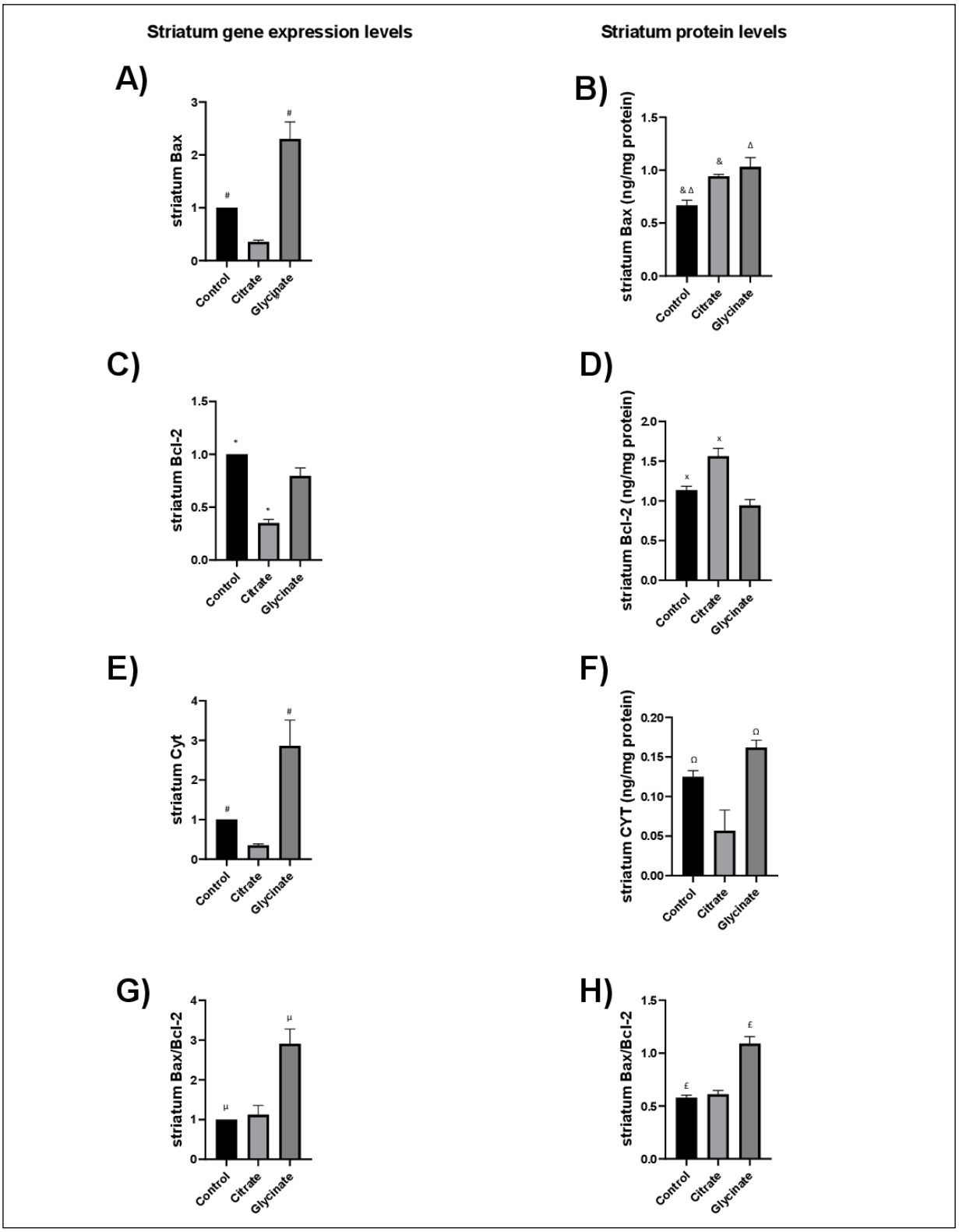

Figure 3. Gene and protein expression levels in the rat striatum. Bax and Bcl-2 gene levels decreased in the MgC group, while protein levels increased (***A***-***D***). The Bax gene and protein levels increased in the MgG group (***A***, ***B***). The Bcl-2 gene level decreased in the MgG group compared to the control group, but protein levels did not change (***C***, ***D***). Compared to the control group, the CytC level increased in the MgG group, and protein levels decreased in the MgC group (***E***, ***F***). Bax/Bcl-2 levels increased in both gene and protein levels in the MgG group (***G***, ***H***).

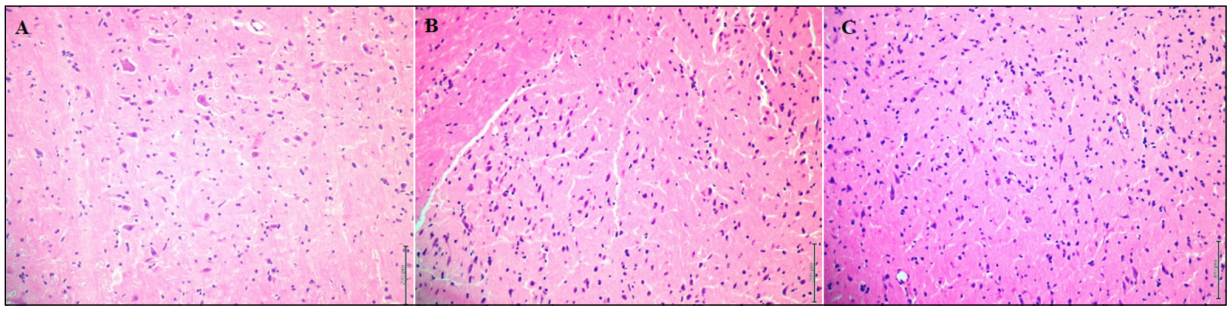

Figure 4. The histopathological image of the striatum region of rats. There was no finding supporting apoptosis in neurons and glial cells of the control, MgC and MgG groups (**A**, **B**, **C**, respectively). (H&E staining, magnification 10X, the scale bar indicates 200 ηm).

pocampus. The suppressive effect of Mg on gene transcription may be due to many different mechanisms. With Nuclear Magnetic Resonance (NMR) spectroscopy, it was noted that the opening base pairs were impeded by Mg while transcription continued[24]. This outcome was explained based on the tenet that Mg increases DNA stability through attachment without changing its chemical structure. Another study reported that Mg binds to the PO4 skeleton thus preventing the degradation of DNA[25]. The down-regulatory effect of Mg on genes may occur during mRNA chain elongation. Mg may interfere during mRNA strand formation; long oligomer strands cannot be produced if Mg is present in the medium[26]. In other words, Mg in the nucleus may yield negative effects on mRNA formation but the regulatory effects of Mg on apoptotic genes are not clear.

Mg has many effects on cell nucleus. Mg can activate calcium/magnesium-dependent endonucleases (to cut DNA), exonucleases (to repair DNA), ligases, and topoisomerases[15,27,28]. Mg-dependent DNase1L1 endonuclease enzyme cleaves to chromatin during apoptosis[29]. Apoptotic endonucleases produce DNA degradation, an important marker of apoptosis. Mg increases DNA fragmentation by activating endonucleases in the cell nucleus undergoing apoptosis[30]. The transcription of the genome can be inhibited because of endonuclease activity.

The Bax/Bcl-2 gene ratio in the MgG increased in exception of the prefrontal cortex. The ratio we focused on in the MgC group was unchanged in the striatum and hippocampus. The Rheostat Model, explained after the discovery of heterodimerization between Bax and Bcl-2, determines cell destiny. The Bcl-2 concentration must be high according to the Bax concentration in living cells to enable them to attach and sequestrate Bax. When the Bax concentration is higher than Bcl-2, Bax molecules compose dimers, and this leads to cell death [31]. The results on the nuclear stage of apoptosis demonstrated that MgG activates the apoptotic process in the hippocampus and striatum; the Bax/Bcl-2 gen ratio increased in these regions. This may be associated with the more pronounced suppressive effect of Mg on the Bcl-2 gene transcription compared to the Bax gene transcription.

The outflow of mRNA from the nucleus does not always result in cytosolic protein synthesis. Therefore, the translation of apoptosis-related proteins in the cytosol was also examined. Interestingly, protein synthesis levels were inversely correlated to mRNA levels. It has been reported that approximately 40% of changes in protein expression correlate with transcription [32]. In other words, about 60% of the change in protein expression is associated with other regulations (posttranscriptional, translational, or protein degradation regulation). In a study conducted to examine the mismatch of protein and mRNA expression, it was reported that the discordance mainly occurs in non-differential gene expressions, and there is a high correlation between mRNA and protein levels in differential gene expressions[33]. In our study, transcriptional changes in non-differential genes may have been canceled by biological regulation mechanisms.

In the prefrontal cortex, hippocampus and striatum regions, Bax protein expressions increased significantly in the MgC group. In the MgG group, Bax protein was higher than in the control only in the striatum and did not change in the other regions. Bcl-2 protein levels were lower in the prefrontal cortex of MgG group and higher in the striatum of the MgC group. The ratio of Bax/Bcl-2 protein expression was also observed to be raised

in the hippocampus and striatum regions of the MgG group and remained unaltered in the MgC group.

Bax and Bcl-2 proteins are predominantly found in the cytosol. These are proteins that regulate mitochondrial cell death-intrinsic apoptosis. However, during apoptosis in order to degrade proteins and DNA through caspase activity, Cyt-C must pass outwards through the mitochondrial permeability transition pores (MPTP) into the cytosol[34]. In this process, Bcl-2 mediates the inhibition of Cyt-C secretion from the mitochondrial pore[35]. Although Bax/Bcl-2 ratios generally increased in both gen and protein levels in our study, Cyt-C levels decreased or did not change. Our results suggest that Mg generates apoptosis-initiating signals but fails to raise Cyt-C. The stable or decreased levels of Cyt-C may be associated with the increasing cytosolic degradation of Cyt-C (by E3 ligases) or the blocking of Cyt-C secretion.

Cyt-C enters the mitochondria after being synthesized in the cytosol. It is a hemeprotein that aids the electron transport chain activity and inhibition of reactive oxygen species in the inner membrane of mitochondria. Cyt-C can also dissociate from mitochondria in various physiological processes such as cell differentiation. In addition, its transition to the cytosol does not always initiate the apoptotic process[36]. The secretion of Cyt-C from mitochondria against apoptotic stimuli is followed by an increase in calcium within the mitochondria, a decrease in mitochondrial membrane potential, and the formation of MPTPs. By reducing mitochondrial permeability, Mg can inhibit the secretion of Cyt-C, which is bound with acidic phospholipids in the inner membrane of mitochondria[37]. Therefore, in our study, although Bax/Bcl ratios increased as a result of Mg, Cyt-C may not have been able to pass into the cytosol due to decreased mitochondrial permeability. Various studies have reported that Mg ions added to the medium inhibit Cyt-C and enzyme secretion from mitochondria[10,38]. The authors hypothesized that positively charged Mg ions bind negatively charged phospholipids and proteins to form membrane resistance. A recent *in vivo* study regarding the neurotoxin effects on substantia nigra exhibited a decrease in Mg entry into cells and an increase in Caspase-3 levels. Consequently, decreased Caspase-3 levels and increased cell viability were noted when Mg was added[39]. This finding also supports the suppressive effect of Mg on Cyt-C and Caspase-3 activities. The suppression of Caspase-3 activity by Mg may have also been associated with its P2X7 nonselective cation channel blocking effect[40]. This receptor channel is present in the central nervous system and an increase in Caspase-3 positive cells has been reported with its activation[41]. Mg may have terminated the cytosolic apoptosis cascade by blocking P2X7.

Unlike most other cells, neurons are programmed to remain alive throughout life. Therefore, they have to be more resistant to apoptotic signals than other cells. E3 ligases can degrade Cyt-C. PARC/CUL9, one of the E3 ligases, is higher in neuronal cells than in other cells. It was observed that Cyt-C was increased in cells lacking this ligase, and Cyt-C was rapidly degraded in cells with high ligase[42]. It is likely that neurons use ligase and various Cyt-C-inactivating pathways to escape apoptosis.

Conclusions

According to the Bax/Bcl-2 ratio we recorded in the hippocampus and striatum, we could claim that MgG triggers the apoptosis cascade at the initial stage. Additionally, we could not determine any enhancement in the Cyt-C, which is required to execute apoptosis. The Cyt-C level was unaltered in the hippocampus and decreased in the prefrontal cortex and striatum. Apoptosis can be completed if Cyt-C exists in cytosol. The outcome related with detected Cyt-C leads us to surmise Mg is stimulatory in the initial phase of the apoptotic cascade, but later steps of the cascade are suppressed during Cyt-C release. Inhibition of Cyt-C may be related to the direct action of Mg or the ability of nerve cells to protect against apoptosis. Our chemical findings were supported by histological findings. There was not apoptosis in the healthy brain cells in the control group; the brain cells of the subjects that had received Mg displayed the same appearance.

The existing literature indicates that Mg increases neuronal apoptosis. Long-term use of magnesium triggers the apoptotic cascade in the brain, but our findings suggest that this cascade is probably not complete. Further studies of Cyt-C's expression and activity in the cytosol during apoptosis may clarify the course of the apoptotic cascade.

Conflict of Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' Contribution

Servet Kızıldağ: Conceptualization, Methodology, Investigation, Writing original-draft. Basar Koç: Conceptualization, Methodology, Investigation, Formal Analysis. Serap Kurt: Investigation. Ferda Hosgorler: Validation, Formal analysis, Writing- Reviewing and Editing. Asuman Argon: Investigation. Mehmet Ateş: Conceptualization, Supervision. Sefa Kızıldağ: Methodology, Validation, Supervision. Nazan Uysal: Conceptualization, Methodology, Supervision All authors have reviewed and approved the article.

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Ethics Statement

The present animal study was approved by Institutional Medicine Animal Care Committee (Dokuz Eylul University, Number 15.03.2018-36/2018).

References

1) Kirkland AE, Sarlo GL, Holton KF. The Role of Magnesium in Neurological Disorders. Nutrients 2018; 10: 730.
2) Cakmak I, Yazici A, Tutus Y, Ozturk L. Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. Eur J Agron 2009; 31: 114-119.
3) Ates M, Kizildag S, Yuksel O, Hosgorler F, Yuce Z, Guvendi G, Kandis S, Karakilic A, Koc B, Uysal N. Dose-Dependent Absorption Profile of Different Magnesium Compounds. Biol Trace Elem Res 2019; 192: 244-251.
4) Verhas M, de la Guéronnière V, Grognet JM, Paternot J, Hermanne A, Van den Winkel P, Gheldof R, Martin P, Fantino M, Rayssiguier Y. Magnesium bioavailability from mineral water. A study in adult men. Eur J Clin Nutr 2002; 56: 442-447.
5) Musso CG. Magnesium metabolism in health and disease. Int Urol Nephrol 2009; 41: 357-362.
6) Buchachenko AL, Kouznetsov DA, Orlova MA, Markarian AA. Magnetic isotope effect of magnesium in phosphoglycerate kinase phosphorylation. PNAS 2005; 102: 10793-10796.
7) de Baaij JH, Hoenderop JG, Bindels RJ. Magnesium in man: implications for health and disease. Physiol Rev 2015; 95:1-46.
8) Romani AM, Scarpa A. Regulation of cellular magnesium. Front Biosci 2000; 5: D720-D734.
9) Jahnen-Dechent W, Ketteler M. Magnesium basics. Clin Kidney J 2012; 5: i3-i14.
10) Pilchova I, Klacanova K, Tatarkova Z, Kaplan P, Racay P. The Involvement of Mg(2+) in Regulation of Cellular and Mitochondrial Functions. Oxid Med Cell Longev 2017; 2017: 6797460
11) Mathew AA, Panonnummal R. 'Magnesium'-the master cation-as a drug—possibilities and evidences. BioMetals 2021; 34: 955-986.
12) Wolf G, Keilhoff G, Fischer S, Hass P. Subcutaneously applied magnesium protects reliably against quinolinate-induced N-methyl-d-aspartate (NMDA)-mediated neurodegeneration and convulsions in rats: Are there therapeutical implications? Neurosci Lett 1990; 117: 207-211.
13) Yamanaka R, Shindo Y, Oka K. Magnesium Is a Key Player in Neuronal Maturation and Neuropathology. Int J Mol Sci 2019; 20: 3439.
14) Dribben WH, Eisenman LN, Mennerick S. Magnesium induces neuronal apoptosis by suppressing excitability. Cell Death Dis 2010; 1: e63.
15) Trapani V, Mastrototaro L, Wolf F. Magnesium and the yin-yang interplay in apoptosis. Magnesium in the Central Nervous System. Adelaide (AU): University of Adelaide Press, 2011.
16) Maret W, Wedd AG, editors. Binding, transport and storage of metal ions in biological cells. 2014.
17) Institute of Medicine Standing Committee on the Scientific Evaluation of Dietary Reference I. The National Academies Collection: Reports funded by National Institutes of Health. Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. Washington (DC): National Academies Press (US). National Academy of Sciences, 1997.
18) Fiorentini D, Cappadone C, Farruggia G, Prata C. Magnesium: Biochemistry, Nutrition, Detection, and Social Impact of Diseases Linked to Its Deficiency. Nutrients 2021; 13: 1136.
19) Ajib FA, Childress JM. Magnesium Toxicity. StatPearls Publishing, 2021.
20) Famitafreshi H, Karimian M. Deficit in Memory Is Associated with Paradoxical Regulation of Magnesium and Manganese in Isolated Male Rats. Ann Neurosci 2018; 25: 241-246.
21) Daher I, Le Dieu-Lugon B, Dourmap N, Lecuyer M, Ramet L, Gomila C, Ausseil J, Marret S, Leroux P, Roy V, Mestikawy SE, Daumas S, Gonzalez B, Leroux-Nicollet I, Cleren C. Magnesium Sulfate Prevents Neurochemical and Long-Term Behavioral

Consequences of Neonatal Excitotoxic Lesions: Comparison Between Male and Female Mice. J Neuropathol Exp Neurol 2017; 76: 883-897.

22) Al-Ghazali K, Eltayeb S, Musleh A, Al-Abdi T, Ganji V, Shi Z. Serum Magnesium and Cognitive Function Among Qatari Adults. Front Aging Neurosci 2020; 12: 101.

23) Sengupta P. The Laboratory Rat: Relating Its Age With Human's. Int J Prev Med 2013; 4: 624-630.

24) Every AE, Russu IM. Influence of magnesium ions on spontaneous opening of DNA base pairs. J Phys Chem B 2008; 112: 7689-7695.

25) Kohll X, Antkowiak P, Chen W, Nguyen B, Stark W, Ceze L. Stabilizing synthetic DNA for long-Term data storage with earth alkaline salts. Chem Commun 2020; 56: 3613-3616.

26) Motsch S, Tremmel P, Richert C. Regioselective formation of RNA strands in the absence of magnesium ions. Nucleic Acids Res 2019; 48: 1097-1107.

27) Fei L, Tian S, Moysey R, Misca M, Barker JJ, Smith MA, McEwan PA, Pilka ES, Crawley L, Evans T, Sun D. Structural and Biochemical Studies of a Moderately Thermophilic Exonuclease I from Methylocaldum szegediense. PLos One 2015; 10: e0117470.

28) Hwang W, Yoo J, Lee Y, Park S, Hoang PL, Cho H, Yu J, Hoa Vo TM, Shin M, Jin SM, Park D, Hyeon C, Lee G. Dynamic coordination of two-metal-ions orchestrates λ-exonuclease catalysis. Nat Commun 2018; 9: 4404.

29) Lauková L, Konečná B, Janovičová Ľ, Vlková B, Celec P. Deoxyribonucleases and Their Applications in Biomedicine. Biomolecules 2020; 10: 1036.

30) Fang M, Zhang H, Xue S. Differential effects of ca2+ and Mg2+ on endonuclease activation in isolated promyelocytic HL-60 cell nuclei. Science in China Series C. Life Sci 1998; 41: 351.

31) Maes ME, Schlamp CL, Nickells RW. BAX to basics: How the BCL2 gene family controls the death of retinal ganglion cells. Prog Retin Eye Res 2017; 57: 1-25.

32) de Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C. Global signatures of protein and mRNA expression levels. Mol Biosyst 2009; 5: 1512-1526.

33) Koussounadis A, Langdon SP, Um IH, Harrison DJ, Smith VA. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. Sci Rep 2015; 5: 10775.

34) Luis-García ER, Becerril C, Salgado-Aguayo A, Aparicio-Trejo OE, Romero Y, Flores-Soto E, Mendoza-Milla C, Montaño M, Chagoya V, Pedraza-Chaverri J, Hafidi ME, Orozco-Ibarra M, Pardo A, Selman M. Mitochondrial Dysfunction and Alterations in Mitochondrial Permeability Transition Pore (mPTP) Contribute to Apoptosis Resistance in Idiopathic Pulmonary Fibrosis Fibroblasts. Int J Mol Sci 2021; 22: 7870.

35) Bagci EZ, Vodovotz Y, Billiar TR, Ermentrout GB, Bahar I. Bistability in apoptosis: roles of bax, bcl-2, and mitochondrial permeability transition pores. Biophys J 2006; 90:1546-1559.

36) Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G. Mechanisms of cytochrome c release from mitochondria. Cell Death Differ 2006; 13:1423-1433.

37) Bonora M, Patergnani S, Ramaccini D, Morciano G, Pedriali G, Kahsay AE, Bouhamida E, Giorgi C, Wieckowski MR, Pinton P. Physiopathology of the Permeability Transition Pore: Molecular Mechanisms in Human Pathology. Biomolecules 2020; 10: 998.

38) La Piana G, Gorgoglione V, Laraspata D, Marzulli D, Lofrumento NE. Effect of magnesium ions on the activity of the cytosolic NADH/cytochrome c electron transport system. FEBS J 2008; 275: 6168-6179.

39) Sun Y, Sukumaran P, Singh BB. Magnesium-Induced Cell Survival Is Dependent on TRPM7 Expression and Function. Mol Neurobiol 2020; 57: 528-538.

40) Ozen M, Xie H, Shin N, Al Yousif G, Clemens J, McLane MW, Lei J, Burd I. Magnesium sulfate inhibits inflammation through P2X7 receptors in human umbilical vein endothelial cells. Pediatr Res 2020; 87: 463-471.

41) Turner CM, Tam FW, Lai PC, Tarzi RM, Burnstock G, Pusey CD, Cook HT, Unwin RJ. Increased expression of the pro-apoptotic ATP-sensitive P2X7 receptor in experimental and human glomerulonephritis. Nephrol Dial Transplant 2007; 22: 386-395.

42) Gama V, Swahari V, Schafer J, Kole AJ, Evans A, Huang Y, Cliffe A, Golitz B, Sciaky N, Pei XH, Xiong Y, Deshmukh M. The E3 ligase PARC mediates the degradation of cytosolic cytochrome c to promote survival in neurons and cancer cells. Sci Signal 2014; 7: ra67.