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Research Article

AGMATINE ADMINISTRATION ALLEVIATES NERVE DAMAGE AND IMPROVES NERVE FUNCTION IN METHOTREXATE-INDUCED PERIPHERAL NEUROPATHY IN RATS

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ABSTRACT

Objective: Chemotherapeutic agents can produce neurodegenerative changes. This study was conducted to assess the therapeutic potential of agmatine, a neuromodulator, on methotrexate induced neurodegeneration in sciatic nerve.

Materials and Methods: 40 male Wistar albino rats were assigned into four groups at random as control, methotrexate, agmatine and methotrexate-agmatine. Methotrexate was injected intraperitoneally at a 37.5 mg/kg/week dose for 3 weeks. Afterwards, agmatine was administered intraperitoneally twice a day at a 40 mg/kg dose for 7 days. Sciatic functional index, nociceptive pain perception and behavioral changes were analyzed every week. Nerve conduction velocity was evaluated. Apoptotic activity and mitophagy, histopathological changes in sciatic nerves were examined.

Results: Methotrexate administration resulted in a prolonged escape time to the platform and decreased the time spent in the quadrant in the water maze test; elevated nociceptive latencies; decreased the number of frames passed in the open field test; reduced sciatic NCV and SFI score. Besides, methotrexate administration caused a reduction in myelin thickness and axon diameter in sciatic nerve and a more intense glial fibrillary acidic protein immunoreactivity. Methotrexate administration triggered an increase in the Bax/Bcl2 protein expression ratio without changing the expression level of Parkin, indicating a slight apoptotic activation. agmatine administration improved methotrexate induced changes in behavioral performances, nociceptive pain perception, nerve conduction, SFI scores and histopathological changes.

Conclusion: Agmatine has been demonstrated to possess a therapeutic potential in methotrexate induced degeneration and peripheral neuropathy in the rat sciatic nerve.

Keywords: Methotrexate, Agmatine, Sciatic nerve, Peripheral neuropathy

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INTRODUCTION

Methotrexate (MTX) is a folic acid analog and a first-line synthetic antimetabolite commonly used in the treatment of cancer types affecting the nervous system and certain chronic autoimmune inflammatory diseases (1). Serious side effects associated with MTX use include progressive hepatotoxicity and nephrotoxicity, peripheral neuropathy, axonopathy, and varying degrees of demyelination. Due to these severe consequences, it may be necessary to adjust the treatment dose or even discontinue the medication, which can lead to incomplete treatment and serious health issues for many patients (2). Some of these conditions, especially neuropathy and demyelination induced by MTX usage, may last for several years adversely affecting patient welfare. Although the adversative effects of MTX administration on central nervous system has been of great concern, little is known on the effects of MTX administration on peripheral nervous system. Whilst, when MTX use is unavoidable, it is crucial to develop methods to prevent and/or treat probable peripheral nervous system degeneration. The long-term use of chemotherapeutic agents at high therapeutic levels was stated to affect the plasticity of the nervous system leading to irreversible neuronal damage (3) and neuropathic pain.

Agmatine (AgM) is an endogenous neuromodulator known for modulating various functions both in central and peripheral nervous system. As it possesses N-methyl-D-aspartate receptor (NMDAR) antagonist and nitric oxide synthase (NOS) inhibitor activities (4), the studies on the effect of AgM on peripheral nervous system have been focused on its role in the modulation of neuropathic pain and its antinociceptive function. AgM was reported to play an antiplasticity and antinociceptive role to reverse pain induced by cisplatin-induced neuropathy (5) peripheral nerve injury (6, 7), inflammation and spinal cord injury (7). Additionally, AgM has been reported to enhance cognitive functions through its analgesic, anti-inflammatory, and neuroprotective effects, and it has been suggested to be beneficial in the treatment of neurodegenerative diseases (4, 7). Consequently, this study was addressed to evaluate the remedial potential of AgM on peripheral nervous system neuropathy or degeneration induced by MTX.

MATERIALS AND METHODS

Animals

All experimental procedures to be applied were firstly approved by the Adnan Menderes University Experimental Animal Ethics Committee with an approval number 64583101/2020/074 and the experiments were accomplished in compliance with ethical guidelines. 11-week old adult male Wistar-albino rats (n = 40) (250-300 g) were maintained in a controlled ambient environment and were assigned randomly into four experimental groups, each consisting of 10 animals, namely a control group (C), a MTX administered group (MTX), an only AgM administered group (AgM) and a group where the rats recieved AgM after MTX

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administration. Figure 1 demonstrates the time schedule of drug administrations and the measurements performed by the use of the animals belonging to experimental groups.

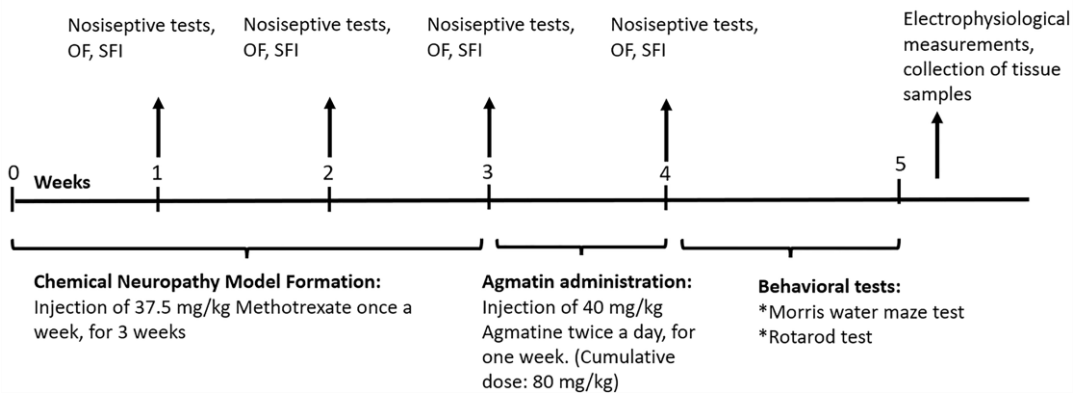

Figure 1. Schematic diagram demonstrating the timing of experimental procedures applied on rats.

Drugs and their administration

In order to achieve a rat model of methotrexate-induced neuropathy, MTX (item no: BD43028, BLD pharm, China) was administered to MTX and MTX-AgM groups at a dose of 37.5 mg/kg, dissolved in physiological saline and injected (i.p.) once a week for 21 days (8). Following the neuropathy induced by methotrexate injection over a period of three weeks, the treatment groups (AgM and MTX-AgM) were administered with a 40 mg/kg dose of AgM (item no: BD112829, BLD phram, China), dissolved in physiological saline and injected (i.p.) twice a day (at a 12-hour interval) for seven days (9).

Nociceptive tests

Hot plate and tail flick tests were applied as nociceptive tests at the end of the first and third weeks of MTX administration and at the end of the week of Agmatine administration (on the fourth week). In the tail-flick test, a beam of radiant heat was focused on the lower one-third portion of the tail via an automated device (May Tic., Ankara, Türkiye), and the latency for a tail withdrawal upon thermal stimulation was recorded in seconds. In the hot-plate test, the rats were placed onto a preheated (55 ± 0.3°C) plate (May Tic., Ankara, Türkiye) and the time needed for a response, for instance licking the hind legs or jumping, to the thermal stimulation was documented. With the aim of avoiding irreversible injury, the cut-off time was set to 10 s in both tests.

Sciatic functional index (SFI)

Sciatic Function Index (SFI) was measured once a week for five weeks. The hind paws of the animals were coated with blue ink and the animal was let to move freely on a blotting paper. The paw-prints were then used to calculate the SFI where at least three paw prints were evaluated per animal in a single

measurement. Before the drug administrations started, paw-prints collected from all animals were considered as the healthy prints for calculations, and the paw-prints of every animal was compared to this first collected healthy paw-print of the same animal throughout the drug administration period. The paw-prints were loaded to ImageJ-Win64 program (NIH, USA) and the following distances were measured: the 3rd toe-to-heel distance (NPL) for the healthy foot, the 1st to 5th toe distance (NTS) for the healthy foot, the 2nd to 4th toe distance (NITS) for the healthy foot, the 3rd toe-to-heel distance (EPL) for the damaged foot, the 1st to 5th toe distance (ETS) for the damaged foot, and the 2nd to 4th toe distance (EITS) for the damaged foot. The 'multiple linear regression formula' was used in the SFI calculation as previously reported (10,11).

Behavioral analyses

Open Field Test: All experimental groups were tested with the open field test once a week throughout the duration of the experiment. The platform for the open field test is a 20 x 20 cm area with a white floor divided into a total of 16 squares by black lines. Each rat was placed on the open field platform from the same corner square of the platform. For each rat, a 2-minute observation period was recorded with a camera. During this period, the total number of squares the rat crossed, as well as the frequency of rearing and grooming, were recorded and scored (12). The locomotor activities of the rats were assessed using the data obtained from the open field test.

Rotarod test: The test was executed at the fifth week of experiments, after the cessation of AgM administration. The rats were separately placed on the rotating rod of the platform (at a speed of 5 rpm), and their latencies to fall from the rotarod platform were recorded in a 1-minute period, and their latencies to fall from the rotarod platform were recorded and analyzed with a video camera (13). Data obtained from the rotarod test were used to evaluate the balance, coordination, and locomotor activities of the rats.

***Morris's water maze (MWM) test*:** A circular swimming pool (150 cm in diameter with 60 cm depth) was allocated into four equal quadrants, where different symbols were assigned to each quadrant and a transparent hidden platform was placed in the pool, submerged 2 cm below the surface in one quadrant. The rats were placed in the pool facing different quadrants each time, and a total of 4 trials were conducted. The escape latencies of the rats to the hidden platform in the designated quadrant were recorded with a stopwatch, and this phase of the experiment was repeated for four days. On the fifth day, the hidden platform was removed, and the time spent by the rats in the previously designated quadrant was recorded. All MWM test trials were recorded with a video camera to confirm the measured latencies. The escape latencies to the

platform during the first four days and the time spent in the designated quadrant after the removal of the platform on the fifth day were evaluated to determine the learning and memory abilities of the rats (14).

Electrophysiological measurements

Electrophysiological measurements were performed using a four-channel clinical electromyography (EMG) device (4-channel Nicolet Viking Quest VIASYS, Natus Medical Inc., CA, USA) at the Faculty of Veterinary Medicine, Aydın Adnan Menderes University. For the electrophysiological measurements, the rats were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). Then the hind limbs were shaved starting from the point where the femur meets the hip joint to the point corresponding to the back of the femoral knee joint, and cleaned. The measurements were performed using reusable surface electrodes. The active recording electrode was placed in the center of the plantar surface of the paw, the reference electrode was placed on the heel, and the ground electrode was positioned at the base of the tail. Two electrical stimulations were applied by a small bipolar surface electrode (Natus Medical Incorporated, Pleasanton, CA, USA) from a proximal and a distal stimulation point. Proximal stimulation was applied from the point aligned with the hip end of the femur, and distal stimulation was applied from the point aligned with the back of the knee joint of the femur. The EMG device settings and amplifier filter settings were adjusted to 10 Hz-10 kHz, sweep speed to 1 ms, stimulation duration to 0.5 ms, and gain to 2 mV. Electrical stimulations were manually applied at right angles with the respective electrode at random intervals. For each rat, a total of three compound muscle action potential (CMAP) recordings were obtained in response to the proximal and distal stimulations, and the averages of the calculations were considered in data analysis. From the CMAP recordings, proximal and distal latencies (ms), total CMAP duration (ms) and peak-to-peak amplitude (mV) were analyzed using the VIASYS Nicolet Viking Quest software program. The sciatic nerve motor nerve conduction velocity was calculated by taking the ratio of the distance between the stimulating electrodes to the difference between the proximal and distal latencies.

Afterwards, the animals were sacrificed and sciatic tissue samples were dissected from both limbs. The right sciatic nerves to be used in histological analysis were placed into 10% formalin for fixation. The left sciatic nerves were stored at -80°C until further usage in Western blot analysis.

Western blot analysis

For the determination of protein expression levels, sciatic nerves were homogenized by sonication in a 5X (w:v) RIPA lysis buffer (Catalog # 20188, Merck Millipore, Germany) containing a 1:10 (v:v) protease inhibitor cocktail (Catalog # K297, BioVision, USA) and sonicated (HD2200, Bandelin SONOPULS, Germany) on ice until the tissue was homogenized. Then homogenates were centrifuged at 16.000 g for 10 minutes at 4ºC to collect the supernatants. The protein concentration in lysates was determined by using Bradford assay kit (Catalog # P010A, ABY Bioscience, USA). Tissue lysates were then mixed with 6 µl loading dye (Catalog # 751-

00, Pagesta, 5X SDS-PAGE sample buffer, Korea) and boiled at 95°C for 5 min. Each sample containing an equal amount of protein (30 μg) was loaded onto the polyacrylamide gel (4 and 12% stacking and resolving gels, respectively), underwent gel electrophoresis, transferred onto polyvinylidene fluoride membranes (Catalog # D9160172A, Bio-Rad, USA) and blocked. The membranes were then incubated with primary antibodies against Bcl-2 (1:1000, Catalog # bsm-3304M, Bioss, USA), Bax (1:1000, Catalog # bsm-33283M, Bioss, USA), Parkin (1:1000, Catalog # 4211S, Cell signaling, Netherlands), and β-Actin (1:1000, Catalog # sc-47778, Santa Cruz, USA), at 4°C overnight. After washing, the membranes were incubated with the secondary antibody (1:5000, anti-mouse IgG HRP, Catalog # 7076S, Cell Signaling, Netherlands) at room temperature for 2 h. Afterwards the membrane was washed, added with the ECL substrate (Catalog #1705060, BIO-RAD, California, USA) to be visualized using the UVP system (G:Box Syngene, DR4V/1309, UK) with antibody-specific varying exposure times. The protein band intensities were measured by the usage of ImageJ (MD, USA, 64-bit version) software. The expression level of each protein was compared to that of β-actin (reference protein).

Histological analysis

The formalin was removed from the fixated right sciatic nerves under running water and the tissues were dehydrated through passages of ethanol (Catalog # UN1170, Isolab, Germany). Subsequently, the tissues were cleared in xylene at 60°C (Catalog # 108297, Merck, Germany) and infiltrated with paraffin (Catalog # 8002-74-2, Tekkim, Türkiye) and embedded in paraffin blocks. 5 μm thick serial sections were obtained from the blocks via a rotary microtome (RM 2265, Leica, Germany) with a microtome blade (Catalog # 3051835, Thermo Fisher MX35 Premier, Singapore). Paraffin sections obtained from the sciatic nerve tissue were stained using histochemical methods with Hematoxylin (Catalog # RRSP67-E, Atom Scientific, United Kingdom) & Eosin (Catalog #RRSP35-E, Atom Scientific, United Kingdom), Luxol Fast Blue (Catalog # RRSK345-100, Atom Scientific, United Kingdom), and Masson Trichrome (Catalog # RRSK20-100, Atom Scientific, United Kingdom). Additionally, immunohistochemical staining was performed using GFAP Rabbit monoclonal primary antibody (Catalog # bsm-52254R, Bioss, USA). The stained sections were visualized using an Olympus CX21 microscope (Olympus, Japan). The LFB stained images were further loaded to ImageJ (NIH, USA, 64-bit version) software program and myelin sheath thickness, axon and myelinated fiber diameter in the sections were calculated.

Statistical analysis

The data obtained in the study were expressed mean ± standard error of mean (SEM) and analyzed via SPSS Statistics software (version 26.0, IBM, USA). Kolmogorov-Smirnov test was used to confirm that the data exhibited a normal distribution. Intergroup comparisons of normally distributed data were accomplished via Student's t-test or One-way ANOVA and Tukey's test was performed as a post-hoc test, while non-normally

distributed data were compared using non-parametric Kruskal-Wallis test. A p value equal to or less than 0.05 was accepted as statistically significant. For the denotation of the degree of significance, an asterisk (*) was used for the comparisons of all groups with regard to the control (C); a dagger (†) was used for comparisons with regard to the MTX administered group; and a hashtag (#) was used for comparisons with respect to the AgM treatment group. (*p<0.05, **p<0.01, ***p<0.001).

RESULTS

Nociceptive pain perception

The results from the nociceptive tests showed that a 3-week administration of MTX led to significantly prolonged latencies of responses to thermal painful stimuli when compared to that of control (Table 1). This result indicates a decrease in thermal pain perception due to MTX administration. It was observed that AgM treatment after MTX administration (MTX-AgM group) resulted in a reduction in the latencies of hot plate and tail flick responses to thermal painful stimuli compared to the MTX-treated groups (p<0.05) (Table 2).

Table 1. Results of the nociceptive tests recorded as latency (s) of a reaction given in response to thermal painful stimuli during the 3-week period of chemical neuropathy induction induced by MTX.

		Latency (s)	
Nociceptive Test	Groups	**1st week**	**3rd week**
Hot Plate	C	5.85 ± 0.28	5.75 ± 0.25
	MTX	7.65 ± 0.39***	7.94 ± 0.28***
Tail Flick	C	6.49 ± 0.15	6.54 ± 0.12
	MTX	6.79 ± 0.13	7.49 ± 0.12***

The data were represented as mean ± standard error of mean. Differences in means were compared by student t-test. P-values equal or less than 0.05 were considered as statistically significant (*p<0.05, **p<0.01, ***p<0.001). The degree of significance was symbolized with asterisks (*) for the comparisons to that of control group (C).

Sciatic functional index (SFI)

Figure 2a-2d demonstrates the paw-prints obtained from the experimental groups and Figure 2e shows the results of the 5-week SFI analysis. In the measurements taken over five weeks, no treatment was applied to the experimental groups during the first week. In the SFI measurements taken at the end of the 2nd and 3rd weeks, where only MTX was administered to relevant groups, as well as those taken in the 4th week, a statistically significant reduction in SFI values was observed in the MTX and MTX-AgM groups compared to that of control (p<0.001).

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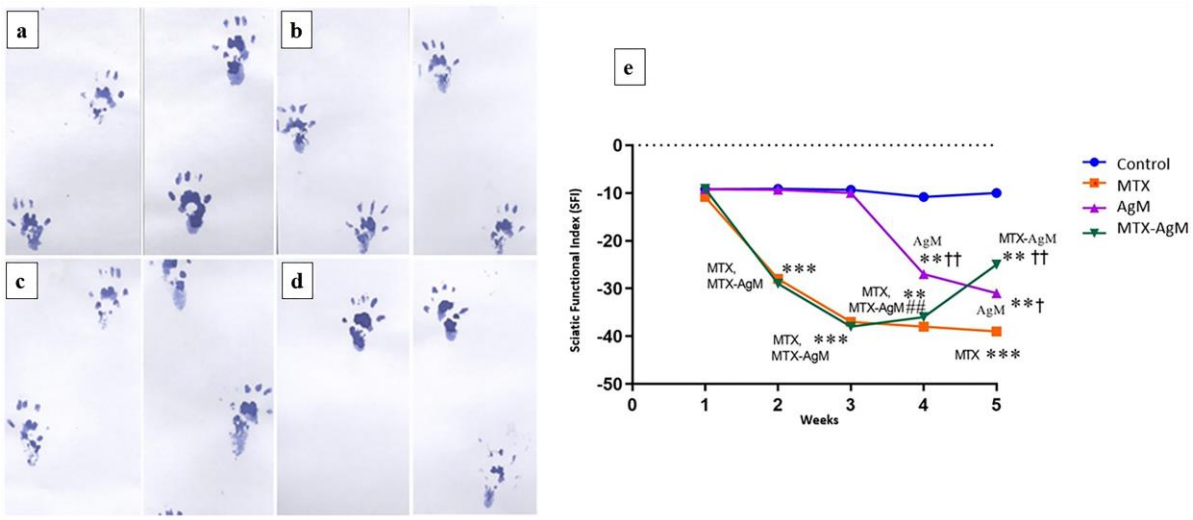

Figure 2. Representative photographs of the paw-prints of rats in a) Control group, b) MTX group, c) AgM group, d) MTX-AgM group; and e) the alterations in the SFI values of the experimental groups throughout the study. Data were represented as mean ± standard error of mean. Differences in between variances were compared by One-way ANOVA test with Tukey's test applied as a post-hoc test. A p value equal to or less than 0.05 was accepted as statistically significant (*p<0.05, **p<0.01, ***p<0.001). The degree of significance was indicated by an asterisk (*) for comparisons of all groups with respect to the control (C) group; by a dagger (†) for comparisons with respect to the MTX administered group; and by a hashtag (#) for comparisons with respect to the AgM treatment group.

At the end of the 4th week, where AgM was administered, the SFI values of the MTX and MTX-AgM groups were observed to be close to each other, where the SFI values of both groups were significantly lower compared to that of control (p<0.01) and to that of only AgM administered group (p<0.01). At the end of the 5th week, the SFI values of the MTX group was the lowest among the other experimental groups with a statistically significant difference with respect to control (p<0.001).

Table 2. Results of the nociceptive tests recorded as latency (s) of a reaction given in response to thermal painful stimuli during the 4th week of AgM treatment.

Nociceptive Test	Latency (s)			
	Groups	1st week	3rd week	4th week
Hot Plate	C	5.85 ± 0.28	5.75 ± 0.25	5.9 ± 0.26
	MTX	7.65 ± 0.39	7.94 ± 0.28	$7.77 \pm 0.2^{***}$
	AgM	5.96 ± 0.3	5.99 ± 0.28	$5.88 \pm 0.31^{†††}$
	MTX-AgM	7.53 ± 0.39	7.76 ± 0.16	$7.13 \pm 0.14^{†}$
Tail Flick	C	6.49 ± 0.15	6.54 ± 0.12	6.48 ± 0.11
	MTX	6.79 ± 0.13	$7.49 \pm 0.12^{***}$	$7.64 \pm 0.16^{***}$
	AgM	6.34 ± 0.05	6.58 ± 0.16	$6.43 \pm 0.09^{†††}$
	MTX-AgM	6.71 ± 0.13	7.03 ± 0.11	$6.91 \pm 0.21^{†}$

The data were represented as mean ± standard error of mean. Differences in between variances were compared by One-way ANOVA test with Tukey's test applied as a post-hoc test. A p value equal to or less than 0.05 was accepted as statistically significant (*p<0.05, **p<0.01, ***p<0.001). The degree of significance was indicated by an asterisk (*) for comparisons of all groups with respect to the control (C) group; by a dagger (†) for comparisons with respect to the MTX administered group (*p<0.05, **p<0.01, ***p<0.001).

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However, the SFI values of the MTX-AgM group was observed to be significantly increased at the end of the 5th week with respect to that of MTX ($p<0.01$) and only AgM administered groups, and the SFI values of the MTX-AgM group were observed to be approaching to that of control. Taking these results into consideration, MTX administration in the first three weeks led to a decrease in sciatic functioning and SFI values, and the treatment of AgM to MTX administered rats led to an increase in sciatic functioning and SFI values at the end of the 5th week. Additionally, AgM administration alone also causes to a decrease in SFI values, even one week after the cessation of the AgM administration.

Electrophysiological recordings

The results of the measurements obtained from CMAP recording are presented in Table 3. As can be seen from the table, there was a significant decrease in nerve conduction velocity in the MTX group ($p<0.001$) and the MTX-AgM group ($p<0.01$) compared to that of control, where the NCV of the AgM treated MTX group was slightly higher to that of MTX administered group. It was also observed that the only AgM administered group had a significantly higher nerve conduction velocity compared to the MTX group ($p<0.05$). The decrease in nerve conduction velocity in the MTX and MTX-AgM groups indicates the presence of myelin damage. The total CMAP duration was observed to be significantly prolonged in the MTX-AgM group compared to the MTX group ($p<0.05$), with no significant differences observed among other groups. In addition, there was a slight decrease in distal amplitude (peak-to-peak (P-P)) values in the MTX, AgM, and MTX-AgM groups compared to the control group. However, this decrease is not statistically significant. This suggests that axonal damage may have remained at a cellular level.

Table 3. The results of the alterations in motor nerve conduction velocities, peak-to-peak amplitude and duration of the compound muscle action potential recordings (CMAP) of the sciatic nerves in the experimental study groups.

Electrophysiological measurements	Control	MTX	AgM	MTX-AgM
Nerve conduction velocity (m/s)	64.02 ± 0.96	52.46 ± 1.15***	62.48 ± 1.39†††	55.46 ±1.07***
Amplitude (peak-to-peak) (mV)	3.88 ± 0.61	3.33 ± 0.47	2.72 ± 0.36	3.51 ± 0.9
Total CMAP duration (ms)	2.71 ± 0.12	2.68 ± 0.08	3.31 ± 0.22	3.48 ± 0.20†

The data were represented as mean ± standard error of mean. Differences in between variances were compared by One-way ANOVA test with Tukey's test applied as a post-hoc test. A p value equal to or less than 0.05 was accepted as statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). The degree of significance was indicated by an asterisk (*) for comparisons of all groups with respect to the control (C) group; by a dagger (†) for comparisons with respect to the MTX administered group.

According to the electrophysiological measurement results obtained from the sciatic nerve, MTX treatment led to a reduced nerve conduction velocity and a decreased CMAP duration, while the treatment of AgM following MTX administration increased nerve conduction velocity and CMAP duration, bringing them closer to normal values.

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Behavioral analyses

According to the results of the open field test, MTX treatment was found to significantly decrease the line crossing frequency compared to that of control ($p<0.001$), while AgM treatment after MTX administration significantly increased the line crossing frequency compared to the MTX group ($p<0.001$), bringing it closer to the control group.

Table 4. Results of the behavioral tests for all experimental groups.

Measurements	Control	MTX	AgM	MTX-AgM
Open Field (OF)				
Line crossing frequency	16.2 ± 0.95	6.5 ± 0.87***	13.5 ± 0.81†††	13.2 ± 0.91†††
Grooming frequency	1.7 ± 0.47	0.7 ± 0.42	2.2 ± 0.38	1.11 ± 0.34
Rearing Frequency	2.7 ± 0.78	2.6 ± 0.54	2.5 ± 0.31	2.1 ± 0.57
Rotarod				
Latency to fall (s)	60.00 (55.00-62.50)	50.00 (45.00-55.00)	60.00 (55.00 - 62.50)	62.50 (50.00 - 57.50)
Morris Water Maze (MWM)				
Platform escape latency (s)	16.7 ± 2.15	26.7 ± 2.84*	17.1 ± 2.09†	19.5 ± 2.63
Time in the quadrant (s)	25.6 ± 1.75	15.22 ± 1.99**	24.6 ± 2.16†	22.8 ± 1.05†

In the open field and rotarod tests, the data were represented as mean ± standard error of the mean. Differences in between variances were compared by One-way ANOVA test with Tukey's test applied as a post-hoc test. In the rotarod test, fall latency data were analyzed using the independent-samples Kruskal-Wallis test and data was expressed as median and interquartile ranges. A p value equal to or less than 0.05 was accepted as statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). The degree of significance was indicated by an asterisk (*) for comparisons of all groups with respect to the control (C) group; by a dagger (†) for comparisons with respect to the MTX administered group.

However, grooming frequency and rearing frequency remained unchanged among the groups. These results indicated that MTX treatment causes a reduction in locomotor activity, whereas AgM administered after MTX increases locomotor activity (Table 4). MTX treatment adversely affected the rats' ability to learn and retain the location of the hidden platform ($p<0.01$); however, AgM treatment following MTX administration improved these abilities compared to the MTX group and brought the values closer to those of the control group ($p<0.05$) in the MWM test (Table 4). Rotarod test results revealed that MTX administration led to a slight decrease in the latency to fall from the apparatus and AgM treatment following MTX administration improved this parameter, however these changes were not statistically significant (Table 4).

Western blot analysis

The expression levels of Bcl-2, Bax and Parkin proteins, in comparison to those of β-actin, were evaluated to obtain information on the apoptotic activation and induction of mitophagy in the sciatic nerves (Figure 3). As seen in the figure, Bcl-2 protein expression was significantly reduced ($p<0.05$) in the only AgM administered group. The absence of statistically significant differences in Bcl-2 protein expression levels, which plays a role in the anti-apoptotic pathway and suppresses apoptotic pathway activation, in the MTX-treated groups suggests that applied MTX dose or duration was not able to suppress apoptosis.

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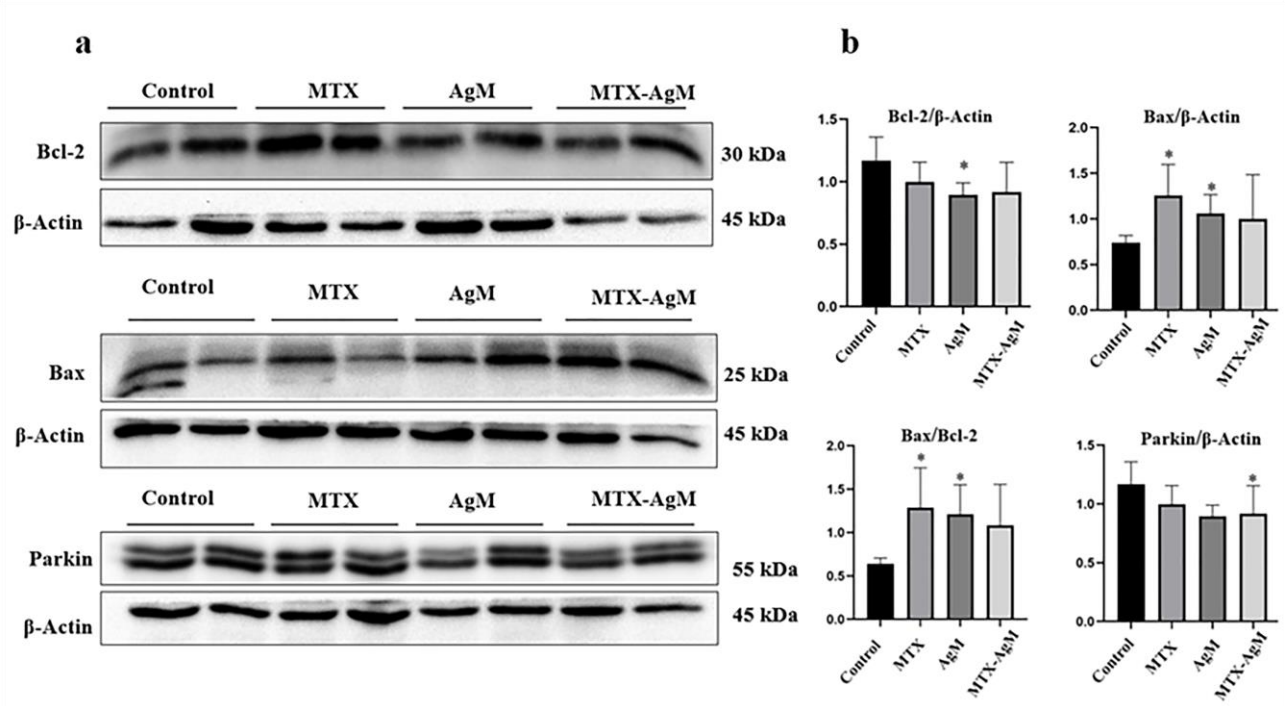

Figure 2. Results representing the alterations in protein expression levels. a) Representative Western blot images of anti-apoptotic Bcl-2, pro-apoptotic Bax, marker of mitophagy Parkin proteins and β-actin protein; b) the calculated protein band intensity results of the expression levels Bcl-2, Bax and Parkin proteins compared to that of β-actin and the Bax/Bcl-2 expression ratio. The represented Western blot images for each protein investigated were cropped from different blots, where same exposure was used for visualization. The samples were derived from the same experiment and the blots were processed in parallel. The data were represented as mean ± standard error of mean. Differences in between variances were compared by One-way ANOVA test with Tukey's test applied as a post-hoc test. A p value equal to or less than 0.05 was accepted as statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). The degree of significance was indicated by an asterisk (*) for comparisons of all groups with respect to the control (C) group; by a dagger (†) for comparisons with respect to the MTX administered group.

Moreover, Bax/β-actin protein expression levels were observed to be significantly increased ($p<0.05$) in MTX and only AgM administration groups compared to the control. As Bax is involved in the pro-apoptotic pathway and its levels indicates the degree of apoptotic pathway activation, the observed increase in Bax expression may suggest that the dose and duration of MTX treatment used in this study was able to initiate the activation of the apoptotic pathway. The same effect is also seen in the AgM-only group ($p<0.05$). However, AgM treatment following MTX administration (MTX-AgM) only led to a slight decrease Bax protein expression levels. Similarly, Bax/Bcl-2 expression ratio was significantly increased in the MTX and AgM groups, while the Bax/Bcl-2 expression ratio was slightly decreased in the MTX-AgM group. When this ratio is evaluated, it may be suggested that MTX administration activates apoptosis and that AgM treatment following MTX administration might be effective in reducing the induced apoptosis. MTX administration resulted in a slight decrease in Parkin/β-actin expression levels, whereas AgM treatment following MTX

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administration (MTX-AgM) led to a statistically significant decrease ($p<0.05$) in Parkin/β-actin expression compared to the control.

Histological analysis

Figure 4 demonstrates the representative images of luxol fast blue (LFB) (Figure 4a-4d), Masson's trichrome (MTC) (Figure 4e-4h) and hematoxylin-eosin (H&E) (Figure 4i-4l) histochemically stained, and GFAP immunohistochemically stained (Figure 4m-4p) sciatic nerve sections.

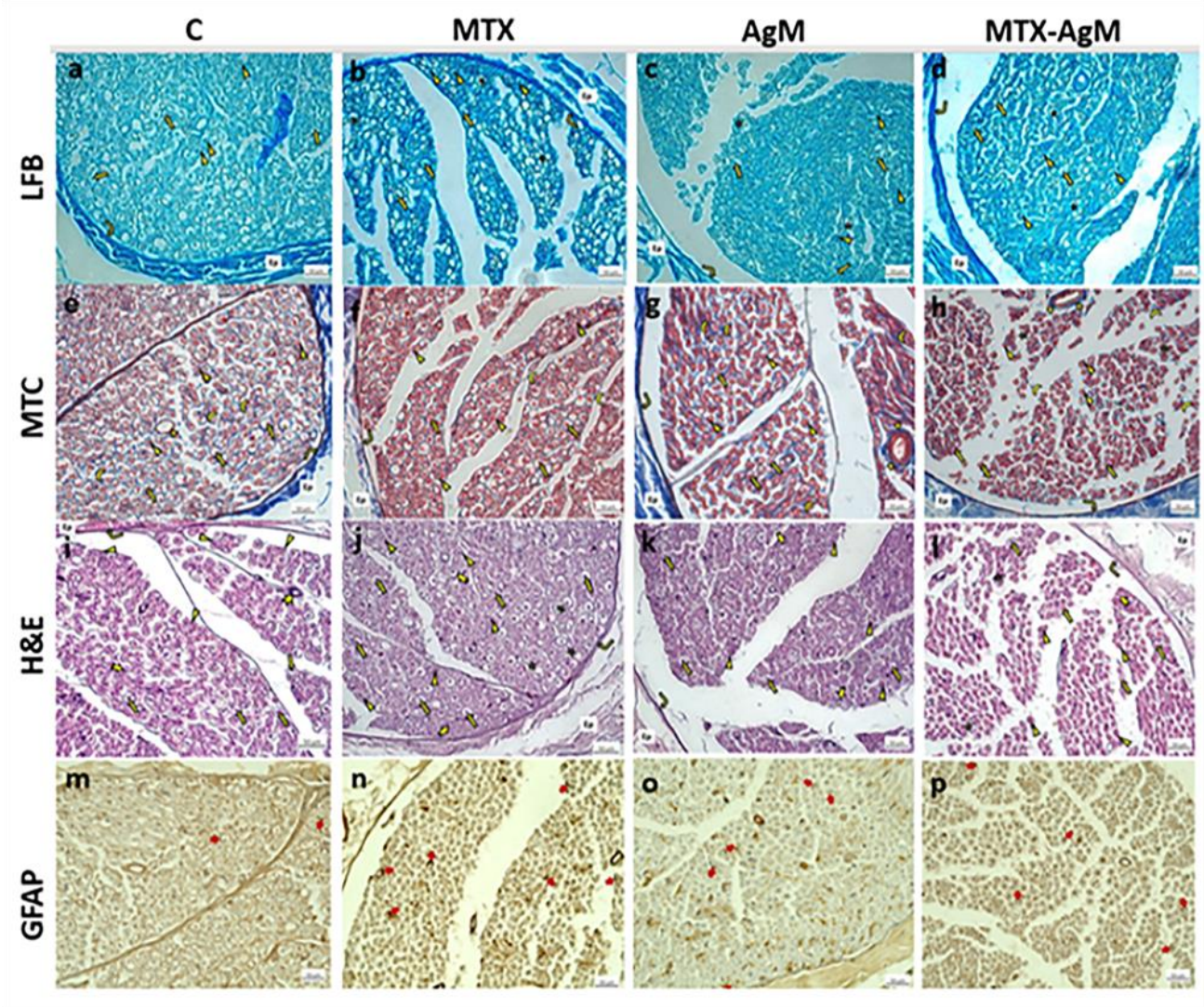

Figure 3. Representative images of the results of Luxol fast blue (LFB) (a, b, c, d), Masson's trichrome (MTC) (e, f, g, h), Hematoxylin–eosin (H&E) (i, j, k, l) and GFAP (m, n, o, p) stained sciatic nerve tissue sections belonging to the experimental groups. Notable features are indicated on the figure as follows: In the LFB staining, the following were identified: Epineurium (Ep), Perineurium ( ), Axon ( ), Myelin ( ), Axonal Swelling ( ); In the MTC staining, the following were identified: Epineurium (Ep), Perineurium ( ), Axon ( ), Myelin ( ), Blood Vessels ( ), Collagen Fibers ( ). In the H&E staining, the following were identified: Epineurium (Ep), Perineurium ( ), Axon ( ), Myelin ( ), Blood Vessels ( ), Schwann Cells ( ), Axonal Swelling ( ). In the GFAP immunostaining, the following were identified: GFAP positive schwann cells ( ). Magnification: 40X, Scale bar: 50 μm.

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LFB staining enables the visualization of myelin and myelinated axons in nerve sections. Hematoxylin-eosin staining was used to visualize the general morphological structure of the sciatic nerve tissue and to distinguish between the nucleus and cytoplasm. Masson trichrome staining was used to visualize the structure of the connective tissue surrounding the nerve tissue as a sheath; and immunohistochemical staining with GFAP primary antibody was used to visualize the immunoreactivity of glial cells in the sciatic nerve tissue. Using the ImageJ-Win64 program, measurements of myelin sheath thickness, axon diameter and myelinated fiber diameter from LFB stained sections, and scoring of GFAP immunoreactivity in sciatic nerve Schwann cells were performed (Figure 5). According to the data obtained from histopathological analyses of the sciatic nerve, MTX treatment was found to decrease myelin thickness, axon and myelinated fiber diameter ($p<0.001$). Instead, AgM treatment following MTX administration was observed to increase axon diameter, myelin sheath thickness ($p<0.001$), and myelinated nerve fiber diameter ($p<0.05$) compared to the MTX group. In addition, the significant rise in the number of GFAP-positive Schwann cells ($p<0.001$) in the MTX group indicates nerve degeneration. AgM treatment following MTX administration (MTX-AgM) was found to reduce GFAP immunoreactivity compared to the MTX group ($p<0.001$). Interestingly, in the group with only AgM treatment, an increase in GFAP immunoreactivity was observed compared to the control group ($p<0.01$). Still, the reduction of GFAP immunoreactivity upon AgM treatment following MTX administration suggests the potential of AgM in the recovery from MTX-induced neurodegeneration (Figure 5).

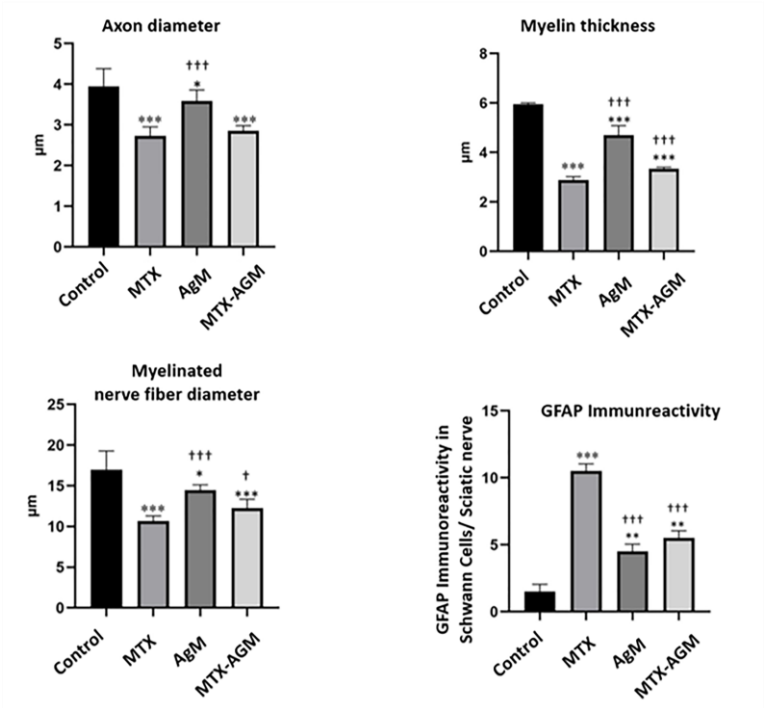

Figure 4. The results of the alterations in axon diameter, myelin sheath thickness, myelin fiber diameter, and GFAP positive immunoreactivity in Schwann cells in the experimental study groups. The data were represented as mean ± standard error of the mean. Differences in between variances were compared by One-way ANOVA test with Tukey's test applied as a post-hoc test. A p value equal to or less than 0.05 was accepted as statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). The degree of significance was indicated by an asterisk (*) for comparisons of all groups with respect to the control (C) group; by a dagger (†) for comparisons with respect to the MTX administered group.

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DISCUSSION

This study was conducted to evaluate the therapeutic potential of AgM in a MTX-induced peripheral neuropathy rat model. MTX, a chemotherapeutic agent, is being extensively utilized in the treatment of solid tumors and cancers observed in the nervous system at high acute doses, as well as in the treatment of some chronic autoimmune inflammatory diseases, for instance rheumatoid arthritis, at chronic low doses (15). The extensive utilization of MTX in the treatment of various diseases brings with it a wide range of side effects, for instance progressive hepatotoxicity and nephrotoxicity, cognitive impairments, peripheral neuropathy, varying degrees of axonopathy, and myelin damage, which also limits its use in therapy (16). Peripheral neuropathy, one of the frequent side effects of chemotherapeutic agents, significantly reduces the life quality of patients. As the dose and duration of the administered chemotherapeutic agent increase, the severity of the resulting neuropathy can vary.

AgM, an endogenous neuropeptide, is a well-known precursor polyamine in the synthesis of polyamines in plants and bacteria (17). AgM, which is known to possess a regulatory role in the nervous system, has a broad pharmacological effect spectrum, including anti-nociceptive and neuroprotective properties. It has been suggested that AgM could be a candidate for new therapeutic targets through better understanding of the underlying molecular mechanisms in relevant physiological and pathological processes (18). and has been used in numerous studies. However, this study is first to report the efficacy of AgM on MTX-induced peripheral neuropathy.

AgM was reported to produce antihyperalgesic and anti-allodynic outcomes in animal models of chronic neuropathic and inflammatory pain (19). In a rat model of neuropathy induced by intravenous cisplatin injection, a single dose of 100 mg/kg AgM was reported to not to lead to a significant effect on tail flick latencies whereas resulted in an improvement of nerve conduction velocities (20). In light of this information, it can be concluded that AgM provides effective treatment in nerve injuries, while the anti-nociceptive effect of AgM occurs only under chronic neuropathic conditions. Consistent with the findings in literature, in our study AgM treatment demonstrated a greater anti-nociceptive effect following MTX-administration, increased nerve conduction velocity, and AgM administration alone to healthy rats was found to have a lower allodynia perception. Future studies should further explore whether AgM induces degenerative changes in healthy subjects or shows therapeutic efficacy only in response to a damage mechanism. The results of this study demonstrated the effectiveness of a total 80 mg/kg AgM (i.p.) treatment in repairing nerve damage caused by MTX-induced neuropathy in rats, and our findings emphasize the importance of clarifying the mechanisms of action of AgM.

In addition, SFI was calculated to examine the effects of MTX and AgM administration on sciatic nerve function and to support the findings with electrophysiological results. MTX administration caused a decrease in SFI values, while AgM treatment following MTX administration resulted in an increase in SFI values, one

week after the cessation of AgM treatment. Additionally, AgM administration alone in healthy rats also caused a decrease in SFI values. Consistent with these findings, studies in the literature have shown that oral administration of 0.05 mg/kg MTX for 25 days in a rat rheumatoid arthritis model significantly decreased SFI values (20). The results of this study reveals that MTX administration at a dose of 37.5 mg/kg resulted in a significant reduction in sciatic nerve conduction and function, suggesting that MTX administration leads to peripheral nervous system degeneration. AgM treatment following MTX administration was observed to mitigate these adverse effects, leading to an improvement in sciatic nerve function and conduction.

One possible reason for the decreased nerve conduction and function is the loss of myelin sheath upon MTX administration. Histochemical staining of the sciatic nerve tissue with LFB revealed that MTX administration led to a significant reduction in axon diameter, myelin thickness, and the diameter of myelinated nerve fibers compared to the control group. The decrease in axon, myelin, and myelinated fiber diameters in the MTX group can be considered evidence of chemical neuropathy induced by MTX in the experimental animals. This reduction in axonal diameter in the MTX group explains the decrease in amplitude values in the electrophysiological measurement results of this study. Similarly, the reduction in myelin sheath thickness in the MTX group explains the slower nerve conduction velocity observed in the electrophysiological measurements of this study; additionally, the axonal and myelin damage in the MTX group supports the observed decline in nociceptive pain perception in this study. In addition, AgM treatment following MTX administration led to a slight increase in nerve conduction, together with a significant increase in myelin sheath thickness and myelinated fiber diameters. These findings are consistent with studies reporting that AgM application increases nerve conduction velocities and CMAP amplitudes. According to the GFAP immunohistochemical staining results in sciatic nerve tissue, an increase in GFAP expression was observed in the MTX group compared to the control group and compared to the AgM and MTX-AgM groups. This significant increase in GFAP expression in the MTX group is known to originate from Schwann glial cells in the peripheral nervous system (PSS), which become reactive as a result of systemic damage (21). GFAP expression was also increased in the only AgM administered group compared to the control group. However, the reduction in GFAP expression in the MTX-AgM group compared to the MTX group suggests that AgM application has a regenerative effect on MTX-induced nerve damage in the sciatic nerve, bringing it closer to the control group. The significant increase in GFAP expression in the AgM group compared to the control group and the significant decrease in the MTX-AgM group suggest that AgM may substantially improve the damage mechanism and may not show the same efficacy when administered to healthy rats, similar to the findings of others (22).

We have also examined some protein expression levels associated with apoptotic/anti-apoptotic and mitophagy mechanisms to explain how AgM demonstrates its effectiveness in reversing myelin damage and functional loss caused by MTX. The regulation of apoptosis mechanisms is managed by the Bcl-2/Bax gene family. The removal of damaged mitochondria through a process called mitophagy, which was reported to be

facilitated through PINK1 and Parkin related pathways, is critical for maintaining proper cellular function. Downregulation of the mitophagy leads to increased toxicity and ultimately neuronal death. Therefore, the regulation of both apoptotic and mitophagy pathways plays a crucial role in controlling neuronal dysfunction. According to our findings, MTX administration activated the pro-apoptotic pathway, resulting in an increase in Bax expression and a non-significant decrease in anti-apoptotic Bcl2 expression, together with a significant increase in Bax/Bcl-2 ratio. The increase in Bax expression may indicate that the MTX treatment dose and duration have triggered the activation of the apoptotic pathway. In the MTX-AgM group, however, AgM treatment was found to slightly reduce Bax protein expression levels and the Bax/Bcl-2 ratio. In the literature, AgM was reported to reduce the increased Bax protein expression induced by 15 μM cisplatin addition in the HEI-OC1 cell line, where only 8mM AgM addition onto HEI-OC1 cell line also resulted in a slight increase in the proportion of late apoptotic cells (23). Similarly, in the current study, AgM administration to healthy rats also resulted in an increase in the Bax/β-actin protein expression levels and Bax/Bcl-2 expression ratio and decreased Bcl-2 expression compared to the control group, suggesting that AgM alone might trigger the apoptotic pathway in healthy subjects, which should be explored in future studies. The Bax/Bcl-2 expression ratio is considered an important factor in determining the degree of apoptotic activation in the literature (24,25). Based on this ratio, it can be suggested that MTX treatment may have activated apoptosis, and AgM administered following MTX may be effective in reducing the induced apoptosis. Regarding Parkin protein expression levels, MTX administration led to a non-significant decrease compared to the control group, and the MTX-AgM treatment did not alter this situation but caused a statistically significant decrease compared to the control group. The Bax/Bcl-2 ratio showed a significant increase in both MTX and AgM groups, while Parkin levels exhibited a non-significant decrease compared to the control group. This finding indicates that the 37.5 mg/kg dose of MTX used in the experiment may tend to suppress the mitophagy pathway and that the administered dose of AgM may not be sufficient to reverse this effect. These results should be validated by future studies, and the effects of different doses of MTX or AgM should be investigated.

In literature, the administration of a combination of MTX (5 mg/kg) with other chemotherapeutic agents to a breast cancer mouse model was observed to prolong the escape latency to the platform in the water maze test but did not create a significant difference in the total number of squares traveled in the open field test (26,27). In another study, the combined administration of cyclophosphamide (40 mg/kg) and MTX (37.5 mg/kg) did not affect the fall latencies in the rotarod balance test (2). Our data indicate that MTX administration adversely affects the rats' memory and learning abilities, does not create a significant difference in grooming and rearing frequency, but causes a decrease in the total number of squares traveled, indicating a negative impact on locomotor activity. In this context, similar to findings in the literature, it can be said that the 37.5 mg/kg MTX used in our study induces nerve degeneration in the subjects. However, AgM treatment following MTX administration demonstrated a positive effect on learning and memory abilities and increased locomotor

activity. Additionally, the positive effect of AgM on cognitive abilities in behavioral tests is noteworthy and warrants future investigations.

AgM is a polyamine that is highly conserved in nature being found in bacteria, plants, invertebrates and vertebrates. It is present endogenously in brain and various organs of the body, such as the stomach, intestine, aorta and spleen, and generally found in a heterogeneous distribution throughout the tissues (28). Therefore; in experimental studies, the observed outcomes may vary depending on the organ studied and the dose of exogenous AgM used, where short and long-term administration of exogenous AgM is generally regarded as safe and non-toxic (29). Apart from its role to play in the excretion of Na+ ions, insulin and glucose metabolism in the periphery, AgM has been reported to be involved in a number of neurobiological processes exhibiting antinociceptive, anticonvulsant and antidepressant activity and is especially important in central neurotransmission (30). However; mechanistic studies have been focused on the effects of AgM through its NMDA antagonism, NOS inhibition and imidazoline or α2-adrenergic receptor interactions (28) and any other potential receptors or pathways of AgM action is yet to be resolved. AgM was reported to exert no significant alterations in locomotion, behavior and motor performance in naïve animals (28, 31), as supported by the findings of this study, where AgM administration to healthy rats did not result in a significant alteration in nociceptive latencies, locomotor activity and behavior of the rats. On the contrary, AgM administration following MTX resulted in an improvement in nociceptive latencies, locomotor activity and behavior of the rats. However, to the best of our knowledge, this study is the first to report the AgM related alterations in sciatic functional index, sciatic motor conduction and histopathological alterations, such as myelin thickness, axon and myelinated fiber diameter or GFAP immunoreactivity. Our results indicated that AgM administration following MTX improved MTX-induced alterations in SFI and increased myelin thickness and myelinated fiber diameter and decreased GFAP immunoreactivity. However, AgM administration to healthy rats resulted in a significant decrease in SFI, myelin thickness, axon and myelinated fiber diameter together with an increase in GFAP immunoreactivity. This discrepancy needs to be confirmed together with the elucidation of other possible targets of action of AgM in future studies and the outcomes of different doses of AgM or MTX administration should also be explored.

CONCLUSION

MTX induces neuropathy in rats, as indicated by the evidence of axonal damage associated with a reduction in axon diameter and a decrease in amplitude of CMAPs, myelin damage linked to a reduction in myelin sheath thickness and a decrease in nerve conduction velocity; an increase in apoptotic pathway activation and a decrease in the activation of the pathway for the elimination of damaged mitochondria, increased reactivity in glial cells, delayed responses to thermal stimuli, deterioration in learning and memory abilities, decreased locomotor activity, and a reduction in sciatic functional index values. Additionally, AgM

treatment following MTX administration has the potential to ameliorate the damage caused by MTX in sciatic nerves. However, AgM administration to naive rats showed a similar damage profile to the MTX-induced damage in some of the parameters studied compared to the control group, suggesting that AgM may has a therapeutic potential for ameliorating damage in existing degenerative conditions. The mechanisms and pathways underlying this property of AgM should be examined in more detail in future studies. Since this study used a single dose of AgM and MTX, the data obtained are somewhat limited, and different dosage studies should be conducted. This study only focused on the expression levels of one apoptotic/anti-apoptotic and mitophagy pathways, and the sample size of the study was rather small to point out the molecular mechanisms of AgM and MTX. Future studies should be conducted to confirm the mechanism of action of the drugs with larger sample sizes and explore protein expression levels in other pathways to better elucidate the mechanism of action. Additionally, the link between AgM treatment as well as MTX administration with inflammation should be assessed in detail in future studies.

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Authorship contributions

Conception – HFY, OBG; Design – HFY, OBG; Supervision – OBG; Surgical and Medical Practices – HFY; Data collection and/or processing – HFY; Analysis and/or interpretation – HFY, OBG; Literature search – HFY, OBG; Writing – HFY, OBG; Critical review – OBG. All authors have read and approved the final version of the manuscript.

Data availibity statement

The datasets generated in the current study are available from the corresponding author on request.

Declaration of competing interest

The authors declare no competing interests.

Ethics

The Adnan Menderes University Experimental Animal Ethics Committee granted approval for this study (approval number: 64583101/2020/074).

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