Original Article

The Potential Therapeutic Effects of Agmatine, Methylprednisolone, and Rapamycin on Experimental Spinal Cord Injury

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Abstract

Objective: In spinal cord injury (SCI), the primary mechanical damage leads to a neuroinflammatory response and the secondary neuronal injury occurs in response to the release of reactive oxygen species (ROS). In addition to the suppression of inflammation, autophagy plays a significant role in the survival of neurons during secondary SCI. The present study aimed to examine the anti-inflammatory and autophagic effects of agmatine and rapamycin in SCI and to compare the results with methylprednisolone (MP) used in the clinic.

Materials and Methods: In this animal-based experimental study, thirty adult male Sprague-Dawley rats were randomly divided into five groups as sham-control, injury, injury+MP, injury+rapamycin, injury+agmatine groups. SCI was induced by compressing the T7-8-9 segments of the spinal cord, using an aneurysm clip for one minute, and then rats were treated daily for 7 days. Seven days post-treatment, damaged spinal cord tissues of sacrificed rats were collected for microscopic and biochemical examinations using histopathologic and transmission electron microscope (TEM) scores. Malondialdehyde (MDA) and glutathione peroxidase (GPx) levels were spectrophotometrically measured.

Results: The results of this study showed that the damaged area was smaller in the rapamycin group when compared to the MP group. Many autophagic vacuoles and macrophages were observed in the rapamycin group. Degeneration of axon, myelin, and wide edema was observed in SCI by electron microscopic observations. Fragmented myelin lamellae and contracted axons were also noted. While MDA and GPx levels were increased in the injury group, MDA levels were significantly decreased in the agmatine and MP groups, and GPx levels were decreased in the rapamycin group.

Conclusion: The results of our study confirmed that rapamycin and agmatine can be an effective treatment for secondary injury of SCI.

Keywords: Agmatine, Methylprednisolone, Rapamycin, Spinal Cord Injury

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Introduction

Acute traumatic spinal cord injury (SCI) is a serious condition that occurs unexpectedly, usually with lifetime consequences that affect the patient and their family (1). There are ongoing studies to treat these patients, who are usually dependent on others, by undergoing intense rehabilitation programs (2, 3). SCI is classified into primary and secondary injuries. The primary injury is directly caused by the trauma. Activation of a cascade of cellular, vascular, and biochemical events such as inflammation, autophagy, and oxidative stress results in secondary injury. For all mechanisms of secondary injury, inflammation plays a significant role in removing deteriorating and damaged tissue. The goal of pharmacological therapy is to avoid or reduce secondary injury by inhibiting the inflammatory process, lipid peroxidation, and immune response (4). Autophagy is a well-known cellular pathway characterized by the deterioration of cytoplasmic proteins and organelles under stress conditions such as injury (5). Autophagy minimizes spinal cord Ischemia/Reperfusion (I/R) injury by suppressing apoptosis and inflammation when activated early (6, 7). Previous studies have shown that the mammalian target of rapamycin (mTOR) signaling pathway is crucial in the regulation of autophagy (8, 9) by inhibiting the signals and alleviates neural tissue injury (10). In addition, neurotrophic and neuroprotective effects of Rapamycin have been demonstrated (11, 12).

Currently, the most commonly used drug in treating SCI in clinical practice is methylprednisolone (MP) with a long-acting anti-inflammatory effect. MP

inhibits inflammatory cytokines and lipid peroxidation which provides maintenance of calcium balance, modulation of immune-inflammatory cells, and restores blood flow of the spinal cord (13). MP has been suggested as a treatment for SCI, but its utilization has been limited due to its adverse effects (14). The use of MP after acute SCI has been controversial for more than 20 years (15). MP therapy at a high dose is not recommended for the treatment of pediatric acute SCI (16, 17). A high-dose 24-hour administration of MP applied within 8 hours of injury, provides a minor improvement on long-term motor recovery and should be considered a treatment option for patients with SCI (18-22). Agmatine provides neuroprotective effects against inflammation, apoptosis, oxidative stress, mitochondrial dysfunction, and excitotoxicity. Agmatine contributes positively to recovery after SCI through modulating the macrophage phenotype (19).

The present study aimed to examine the protective effects of MP, agmatine, and rapamycin on the prevention and reduction of secondary injury following SCI in rats and to compare the findings among the experimental groups both microscopically and biochemically.

Material and Methods

Animals

All experimental procedures were approved by Bolu Abant Izzet Baysal University Animal Experiments Ethics Board (No: 300-59) and were in accomplice with the Care and Use of Laboratory Animals published by the US National Institutes of Health. Thirty male Sprague-Dawley rats (120-160 g, 3 months old) were housed at room temperature (22 ± 2°C) under a 12 hours light/dark cycle. They were fed standard rat chow (210 kcal/100 g/day) and drank tap water ad libitum.

Experimental procedure

Rats were anesthetized by intramuscular injection of 100 mg/kg Ketamine (Alfamine 10%, 100 mg/ml, 10 ml, Alfasan, Holland) and 10 mg/kg Xylazine (Alfazyne 2%, 20 mg/ml, 30 ml, Alfasan, Holland). The dorsum was shaved while the rat was in the prone position and the site of surgery was cleaned with povidone-iodine. Using the guidance of the spinous processes, an incision was made at the level of the T_{7-9} vertebrae. The paravertebral muscles were dissected and the spinal cord was exposed by laminectomy. Leaving the dura intact, the pressure was exerted for 60 seconds with an aneurysm clip (10). When the clip was released contusion was observed at the site of pressure. The wound was closed in anatomical planes. Paraplegia was observed when the rats were awake. In subsequent days, daily wound care was performed. Micturition was induced twice a day by applying pressure on the bladder.

Five experimental groups were constructed:

Group 1: Sham-control (n=6): The paravertebral muscles of the rats in this group were sectioned, the spinal cord was opened at T_{7-8-9} levels and the wound was closed after laminectomy, without exerting pressure.

Group 2: Injury (n=6): The paravertebral muscles of the rats in this group were sectioned and laminectomy was carried out at T_{7-8-9} levels of the spinal cord. In line with Rivlin and Tator's clip compression method, the pressure was exerted on the spinal cord segments for one minute using an aneurysm clip, leaving the dura intact. The clip was removed at the end and the planes were closed anatomically.

Group 3: Injury+MP (n=6): 30 mg/kg MP (Prednol L, Mustafa Nevzat, Turkey) was administered intraperitoneally (IP) within 5 minutes after the procedure and for 7 days to rats in which pressure was exerted with aneurysm clip.

Group 4: Injury+Rapamycin (n=6): 1 mg/kg Rapamycin (Rapamune®, Wyeth, USA) was given via oral gavage for 7 days to rats in which pressure was exerted.

Group 5: Injury+Agmatine (n=6): 50 mg/kg Agmatine (Sigma, USA) was administered IP for 7 days to rats in which pressure was exerted.

Daily monitored rats were weighed on day 7 under anesthesia, rats were sacrificed by taking intracardiac blood. The region of the spinal cord, where the lesion was observed, is termed as "core area". Five mm long "core area" was removed with five mm proximal and distal ends. Tissue samples were separated under a stereomicroscope for microscopic and biochemical evaluation.

Light microscopy

Tissues were fixed in 10% formaldehyde for 24 hours and processed for paraffin embedding. 5 µm sections were stained with Hematoxylin Eosin and Luxol Fast Blue. Neuronal degeneration, cellular edema, hemorrhage/congestion, and inflammation were analyzed with Nikon Eclipse 80i light microscope (Nikon Instruments Europe BV, Amsterdam, Nederland) for the histological score. A semiquantitative scoring system, ranging from 0 to 3, was used to grade histopathological changes as follows: 0=normal, 1=mild, 2=moderate, and 3=severe (23).

Electron microscopy

Tissues were taken and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer for 24 hours. After rinsing with phosphate buffer, tissues were post-fixed with 2% osmium tetroxide in sodium phosphate buffer. Dehydration was accomplished by gradual ethanol series and embedded in epoxy resin. The sections were viewed under a Carl Zeiss EM900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany). Transmission electron microscope (TEM) scores of each animal were evaluated in 5 different categories. Axonal myelin scoring; 0: normal myelin layers, 1: vesiculated myelin, 2: cracked myelin layers, 3: honeycombed and extruded vesicles, General axonal score; 0: normal, 1: light edema, 2: mild edema, 3: severe edema and loss of

structure, Intracytoplasmic edema scoring; 0: absent, 1: light, 2: mild, 3: severe (cell membrane defect), Nucleus scoring; 0: normal, 1: clumping, 2: sparse chromatin, 3: severe damage, Mitochondrion score; was evaluated as 0: normal, 1: light edema, 2: mild edema, 3: severe edema and loss of structure.

Biochemical examination

Malondialdehyde (MDA, Cayman, lipid hydroperoxide Assay Kit, Catalog no:705003) and Glutathione peroxidase (GPx, Cayman Kit, Catalog no: 703102) were assayed in samples according to the manufacturer guidelines. The absorbance of each well at 500 nm wavelength was determined by BioRad Benchmark Plus Microplate Spectrophotometer (BioRad Laboratories, London, England) and the activity was calculated according to a formula. The results were expressed as nmol/min/ml for GPx, where nmol was for MDA and U/mL for superoxide dismutase (SOD).

Statistical analyses

Animal weight and all biochemical parameters were analyzed by One-Way ANOVA and Post hoc Tukey test using GraphPad Prism, USA 3.0 statistical software. $P<0.001$ was considered statistically significant. The histological variables were summarized as mean ± standard deviation (SD). The results of the experimental groups were compared using Kruskal-Wallis, and pairwise post hoc comparisons were performed using Mann Whitney U tests with Bonferroni correction. Statistical Package for Social Sciences 25.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for conducting the analysis. Statistical significance was set at $P<0.05$.

Results

Biochemical findings

The MDA and GPx levels in the experimental groups are shown in Table 1.

Malondialdehyde

MDA has been used as a primary indicator of lipid peroxidation of membranes. There were significant differences among the sham control,injury and Agmatine groups. MP and Agmatine significantly decreased the MDA levels when compared to the injury group ($P<0.001$). The increased MDA measurements in the injury groups were expected and the results indicated that SCI damaged lipid membranes. Rapamycin did not have any beneficial effect on lipid peroxidation. The most effective drug in reducing the level of MDA was MP, with MDA levels comparable to the sham control group. Rapamycin significantly decreased the MDA levels.

Table 1: MDA and GPx levels in the experimental groups

Variables	Sham (n=6)	Injury (n=6)	Injury+MP (n=6)	Injury+Rapamycin (n=6)	Injury+Agmatine (n=6)	P value[a]
GPx	91.29 ± 23.02^{a}	309.7 ± 22.51^{b}	262.6 ± 39.78^{c}	132.0 ± 25.02^{d}	202.5 ± 30.31^{e}	<0.001
MDA	2.395 ± 0.374^{f}	3.720 ± 0.188^{g}	2.422 ± 0.151^{h}	3.942 ± 0.143^{i}	3.117 ± 0.240^{j}	<0.001

Data expressed as mean ± SD. P values of One-Way Anova and Post hoc Tukey test. $^{a-e}$; <0.001, $^{b-d}$; <0.001, $^{b-e}$; <0.001, $^{c-d}$; <0.001, $^{f-g}$; <0.001, $^{a-d}$; <0.001, $^{f-j}$; <0.001, $^{g-h}$; <0.001, $^{g-j}$; <0.01, $^{h-l}$; <0.001, $^{h-j}$; <0.001, $^{i-j}$; <0.001, MDA; Malondialdehyde, and GPx; Glutathione peroxidase.

Table 2: Comparison of histopathological scores among experimental groups

Variables	Sham (n=6)	Injury (n=6)	Injury+MP (n=6)	Injury+Rapamycin (n=6)	Injury+Agmatine (n=6)	P value[a]
Neuronal degeneration	0 ± 0^{a}	3 ± 0^{b}	3 ± 0^{c}	2 ± 0^{d}	2.5 ± 0.55^{e}	<0.05
Cellular edema	0 ± 0^{f}	2.5 ± 0.55^{g}	2.5 ± 0.55^{h}	1 ± 0^{i}	1.5 ± 0.55^{j}	<0.05
Hemorrhage/ congestion	0 ± 0^{k}	1 ± 0^{l}	2.5 ± 0.55^{m}	1 ± 0^{n}	1.5 ± 0.55^{o}	<0.05
Inflammation	0 ± 0^{r}	1 ± 0^{s}	2.5 ± 0.55^{t}	1 ± 0^{u}	1 ± 0^{v}	<0.05

Data expressed as mean ± SD and median (interquartile range). P value[a]; P values of Kruskal Wallis H test. P values obtained from Bonferroni adjusted Mann Whitney U tests for pairwise comparisons: a-b; 0.022, a-c; 0.022, a-d; 0.022, a-e; 0.022, b-d; 0.022, c-d; 0.022, f-g; 0.022, f-h; 0.022, f-i; 0.022, f-j; 0.022, g-i; 0.022, h-i; 0.022, k-l; 0.022, k-m; 0.022, k-n; 0.022, k-o: 0.022, l-m; 0.022, m-n; 0.022, r-s; 0.022, r-t; 0.022, r-u; 0.022, r-v; 0.022, s-t; 0.022, t-u; 0.022, t-v; 0.022, and MP; Methylprednisolone.

Glutathione peroxidase

A significant difference was found between all groups in the Kruskal-Wallis test (P<0.001). When compared to the sham group, GPx levels were significantly elevated in the injury group. Rapamycin and Agmatine decreased GPx levels significantly but the levels were still higher than the sham group. Rapamycin was more effective, with values close to the sham group. MP decreased the GPx levels less than Rapamycin and Agmatine.

Microscopic findings

Histopathologic scores are summarised in Table 2. Spinal injury significantly induced neuronal degeneration, cellular edema, congestion, and inflammation when compared with the sham group. Among all treatments, only Rapamycin was able to prevent neuronal degeneration. Rapamycin and Agmatine treatment both decreased cellular edema. Interestingly MP treatment significantly increased inflammation scores (Table 2, P<0.022).

In the injury group, the integrity of the spinal cord was compromised, with the boundaries between the grey and white matter obscured. It was found that there was diffused edema, an increase in inflammatory cells (Fig.1B, C), axonal degenerations, and disruption of the myelin sheath (Fig.2B).

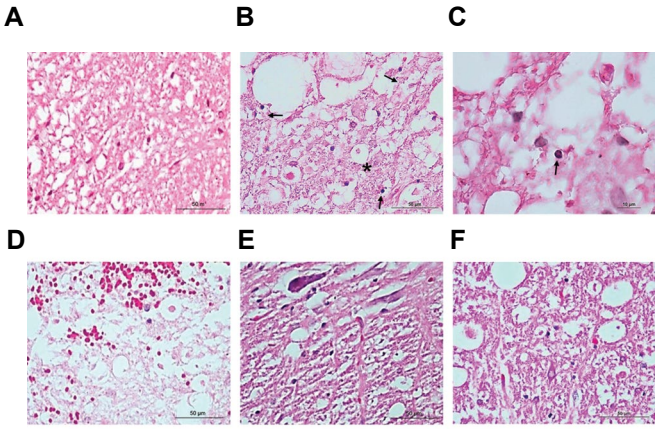

Fig.1: Histopathological assessment of Hematoxylin Eosin stained sections. **A.** Sham group (scale bar: 50 µm), **B.** Increased inflammatory cell (→), degeneration axons, and myelin sheaths (*) were seen in injury group (scale bar: 50 µm), **C.** Injury group (scale bar: 10 µm), **D.** MP group (scale bar: 50 µm), **E.** Rapamycin group (scale bar: 50 µm), and **F.** Agmatine group (scale bar: 50 µm).

In the MP treatment group, axonal shortening, disruption of the myelin sheath, and various small axons (Fig.2C) were seen. Large macrophages containing phagocytic material (Fig.2D) were seen in the Rapamycin group and this observation was not found in the MP and Agmatine treated groups. The thin myelin sheath, prominent lysosomal granule of macrophages (Fig.2E) were observed in the Agmatine treatment group.

Electron microscopic examination demonstrated degeneration of the myelin sheath and axons in the injury group (Fig.3B). In Methylprednisolone, Agmatine, and Rapamycin groups, the injury of myelin sheath was less than the injury group (Fig.3B-D). Rapamycin group contained granular material between the myelin sheath and in axons.

All scores of the injury groups were higher than the sham group by TEM evaluation (Table 3). Axonal Myelin scores were significantly decreased in MP and Rapamycin treated groups compared to the injury group (P=0.011 and P=0.007, respectively). Axonal myelin was preserved with MP and Rapamycin treatment. Intracytoplasmic edema score, nucleus score, and mitochondrion score were significantly decreased in the rapamycin group compared to the injury group (P=0.006, P=0.026, P=0.007, respectively). These results suggest that methylprednisolone ultrastructurally protected the axonal myelin and mitochondrion but cellular structures were found to be best preserved by rapamycin.

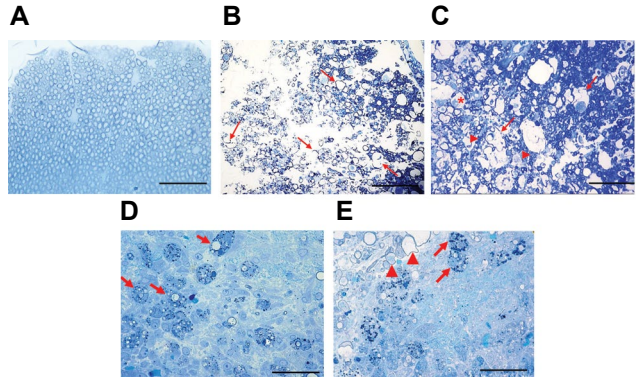

Fig.2: Histopathological assessment of semithin sections stained with Toluidin Blue-Pyronin. **A.** Sham group, **B.** Injury group, degeneration axons and myelin sheaths (→), **C.** Methylprednisolone group, axonal shortening (*), disruption of the myelin sheath (→) and various small axons (►) were seen, **D.** Rapamycin group, increased large macrophages (→) containing phagocytic material are seen in the Rapamycin group compared with other treatment groups, and **E.** Agmatine group, thin myelin sheath (►), prominent lysosomal granule of macrophages (→) (scale bar: 50 µm).

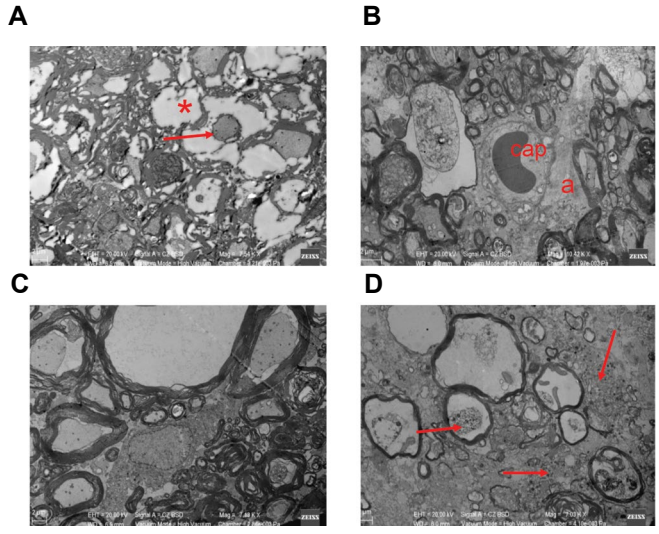

Fig.3: TEM sections. **A.** Injury group, edema areas (*), degenerated myelin and axon structures (→). **B.** Methylprednisolone group, injury of myelin sheaths less than injury group. **C.** Agmatine group, axon and myelin structure. **D.** Rapamycin group, granular material between myelin sheaths and in axons (→). Lead citrate-uranyl acetate. TEM; Transmission electron microscope.

Table 3: Comparison of TEM score among experimental groups

Variables	Sham (n=6)	Injury (n=6)	Injury+MP (n=6)	Injury+Rapamycin (n=6)	Injury+Agmatine (n=6)	P value[a]
1. Axonal myelin score	0.16[a]	2.66[b]	1.5[c]	1.33[d]	2.16[e]	<0.05
2. General axonal score	0.33[a]	1.66[b]	1.33[c]	0.83[d]	1.83[e]	<0.05
3. Intracytoplasmic edema score	0[a]	1.83[b]	1.33[c]	1.16[d]	1.83[e]	<0.05
4. Nucleus score	0[a]	2[b]	2[c]	1.16[d]	1.83[e]	<0.05
5. Mitochondrion score	0.16[a]	2[b]	1.16[c]	0.83[d]	1.66[e]	<0.05

P value[a]; P values of Kruskal Wallis H test. P values obtained from Bonferroni adjusted Mann Whitney U tests for pairwise comparisons: 1: a-b; 0.002, a-c; 0.005, a-d; 0.006, a-e; 0.002, b-c; 0.011, b-d; 0.007, c-e; 0,043, d-e; 0.018, 2: a-b; 0.007, a-c; 0.014, a-e; 0.004, d-e; 0.023, 3: a-b; 0.001, a-c; 0.002, a-d; 0.019, a-e; 0.002, b-d; 0.006, d-e; 0.030, 4: a-b; 0.002, a-c; 0.001, a-d; 0.001, a-e; 0.002, b-d; 0.026, c-d; 0.005, 5: a-b; 0.003, a-c; 0.006, a-d; 0.0027, a-e; 0.004, b-c; 0.026, b-d; 0.007, and d-e; 0.018

Discussion

SCI always leads to cell death. Programmed cell death (PCD) is a critical cascade after SCI, and various forms of PCD including apoptosis, autophagy have been discovered in recent years (20).

Modulation of autophagy is a possible therapeutic approach in SCI management (5). Previous studies have well described the activation of the macrophage in the pathogenesis of SCI (21, 22). M1 macrophages are induced by toll-like receptor ligands or pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ and oxidative metabolites. The M1: M2 ratio is very important in SCI repair (23, 24). Agmatine modulates the macrophage phenotype and contributes to healing after SCI (19).

Phagocytic neutrophils and macrophages also produce excessive ROS and myeloperoxidase after SCI. Oxidative damage plays an important role in neuronal damage after SCI. MP treatment at SCI was determined by National Acute SCI Study III (25). Celik et al. (26) found that MP reduced the MDA levels after SCI. Ozturk et al. (27) found that antioxidant enzymes, such as SOD and GPx, were increased by MP treatment in SCI. Our results showed that at day 7 of the spinal injury, tissue MDA and GPx levels in the injury group were elevated compared to the injury group. Likewise, MP decreased the level of MDA significantly, with levels close to the sham control group. MP prevents lipid peroxidation, one of the mechanisms implicated in SCI. There have been a very limited number of studies on the use of Rapamycin in central or peripheral nervous system injuries and we did not come across any biochemical studies. The levels of MDA in the Rapamycin group were increased, close to the level of the injury group. Compared to the injury group, GPx level was significantly decreased. Agmatine has a protective effect against apoptotic cell damage due to its ability to act as a free radical scavenger (28). Agmatine leads to alleviation in neuroinflammation, oxidative damage, and proapoptotic signaling (29). Kotil et al. (30) reported that Agmatine decreased MDA level significantly but not as much as the level of the control group. Our results showed that in the Agmatine group, MDA and GPx levels decreased significantly in comparison to the injury group, but the levels were still higher than the sham control group. Agmatine was shown to reduce the levels of MDA indicating that free radical-mediated neuronal damage was prevented.

The first change that appears following SCI is the edema of the white matter. Edema is more prominent around the vessels and neurons and in astrocytes. Areas of hemorrhage are frequent, with numerous neutrophils in the initial phase and lymphocytes and macrophages in later phases. Damaged axons and myelin, swelling, and vacuolization of the myelin sheath are present. Degeneration, pyknotic nucleus, hemorrhage, congestion, edema, necrosis, inflammatory cell infiltration were determined in post-SCI neurons (26, 31, 32). Macrophages release proinflammatory cytokines, nitric oxide, and proteases (21). In the present study, a large area of injury, with disruption of the grey and white matter elements, edema, hemorrhage, and abundant inflammatory cells was observed under the light microscope. Even though the damage persisted in the treatment groups, the areas of damage at the same levels of sections were smaller than in the injury group. Numerous large macrophages with intracytoplasmic phagocytic material were found in the area of injury, especially in the Rapamycin treated group. Thus, the expansion of the secondary injury area was prevented. Large macrophages were less in number in the Agmatine and MP administered groups. MP inhibited edema in rats with acute SCI (31, 33). In our study, it was observed that edema was less in Rapamycin treated group.

Changes observed by a light microscope were also defined at the fine structure level by electron microscopy. EM findings were consistent with the previously published data. Degeneration, edema, fragmented myelin sheaths, and disruption of axon structures were observed in the injury group. It was determined that the edema in the

treatment groups decreased but the separation of myelin sheaths did not completely recover. In experimental studies, separation of myelin sheaths, swelling of mitochondria, axon withdrawal, and intraneural vacuoles were observed in SCI (31, 32). Wang et al. (34) observed the formation of autophagic vesicles with engulfed lipid droplets and swelled mitochondria by electron microscopy analysis on the first day after SCI. The abundance of macrophages is a sign of induction of autophagia by Rapamycin via inhibiting mTOR and Rapamycin decreases inflammation at the lesion site (10, 35). Moreover, it increased autophagic activity, blocked apoptotic signals (36), and decreased mitochondrial apoptosis in SCI (37).

The damaged site was smaller in the treatment groups compared to the injury groups. Rapamycin and Agmatine administrated for 7 days after induction of spinal injury helped to alleviate secondary injury to some extent. Biochemically, lipid peroxidation, as manifested by MDA, was reduced by MP and Agmatine, with MP being more effective than Agmatine. In the experimental SCI, there were no reports of rapamycin changes in MDA and GPx. It was observed that while rapamycin did not decrease the MDA level, it was able to reduce the GPx level. It was evaluated that Rapamycin did not have any effects on the antioxidant system. Although the side effects of MP are defined, MP was not effective in reducing edema and inflammation in the acute injury model used in our study. Rapamycin increased the number of macrophages, which plays a significant role in the healing of spinal injury.

Conclusion

In the present study, we compared the possible therapeutic effects of rapamycin, agmatine, and MP on experimental SCI in rats by microscopic and biochemical parameters. We found that rapamycin has the best laboratory results in all treatment groups according to the parameters that were used for investigation. Partially increasing autophagy and reducing inflammation in the tissue makes Rapamycin a promising therapeutic agent for SCI. However, laboratory-only data presentation is the weakness of our study. Further research and functional tests are required to demonstrate clinical effects.

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Authors' Contributions

T.F., A.K., F.T., N.A.; Participated in study design, data collection and evaluation, drafting, and statistical analysis. T.F., A.R.G.; Performed the surgical procedure. T.F., A.K.; Contributed extensively to the interpretation of the data and the conclusion. C.O., E.S.; Conducted molecular experiments and biochemical analysis. All authors performed editing and approving the final version of this manuscript for submission, also participated in the finalization of the manuscript and approved the final draft.

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