Aripiprazole Improves Spinal Cord Injury in Rats: Involvement of Inflammatory Pathways

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Abstract- Macrophages and glial activation contribute to the pathophysiology of spinal cord injury (SCI). Some preclinical studies have shown the anti-inflammatory effects of aripiprazole (ARP). In the current study, we evaluated the anti-inflammatory effects of ARP in a rat SCI model. Forty male Wistar rats underwent either T9 vertebra laminectomy or were used as a sham-operated group without laminectomy. There were four major groups in this study: a sham-operated, three treatments (normal saline [vehicle] control versus ARP 10 and 20 mg/kg/day for three days after surgery, first dose 30 minutes post-surgery) SCI groups. We evaluated locomotor scaling and neuropathic pain behavioral tests over 28 days. On Day 28, tissue samples were investigated for neuroinflammatory and histopathology changes through flow cytometry and ELISA. ARP (10 and 20 mg/kg/day, 3 days) treatment significantly reduced locomotors disability (P<0.01) and mechanical (P<0.01) and thermal allodynia (P<0.001) scores. Additionally, Levels of tumor necrosis factor (TNF)-α level and interleukin (IL)-10 were significantly altered in ARP-treated spinal cord tissues 28 days after SCI (P<0.01). Moreover, spinal cord tissue expression of M1 and M2 macrophages, as well as M1/M2 ratio, were reduced in ARP-treated SCI animals, concurrent with decreased M1 and increased M2 and M1/M2 in dorsal root ganglion (P<0.001). Our study indicates that ARP has therapeutic effects on SCI via the reduction of neuroinflammation and SCI sensory and locomotor abnormalities.

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Keywords: Aripiprazole; Spinal cord injury; Neuropathy; Neuroinflammation; Microglia; Macrophages

Introduction

Spinal cord injury (SCI) is still considered important and complicated with regard to its pathophysiological pathway and its outcomes, impacting patients' quality and expectancy of life (1). According to an epidemiological study 2, SCI prevalence has varied from 236 to 1298 per million inhabitants from 1950 to 2012, making SCI an important neurological condition, and any effort to improve the quality of life of patients with SCI is crucial (2,3).

There are two injury phases in SCI: (i) primary SCI, related to mechanical damage that occurs at the time of spinal trauma, and (ii) secondary SCI, which is biochemical and pathological alterations after the injury that can deteriorate by the passage of time (4,5). Various mechanisms have been proposed as underlying the secondary SCI, which include edema and destruction of spinal micro-vascularization, ischemia-reperfusion injury, and immune reaction via microglia, macrophages, neutrophils and related inflammatory mediators like interleukin (IL)-1β and tumor necrosis factor (TNF)-α (6-9). This inflammation can cause macrophage and microglial imbalance (9). Infiltrating monocyte-derived macrophages (M1) play a role as a maintainer of inflammation; meanwhile, resident microglial (M2) leads to neuronal regeneration and anti-inflammation (10-14). A high ratio of M1/M2 macrophage/resident microglia can cause neural autophagy, apoptosis, and central nervous system (CNS) neurotoxicity (11,15). Neural

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demyelination and notable impairment of the patient's mobility and sensation rehabilitation after primary SCI are then seen (15). Therefore, exploring target inflammatory pathways and testing related drug candidates that can inhibit these pathways would lead us to perceive a possible improvement in SCI patients.

Aripiprazole (ARP) is a partial agonist for the dopamine D2 receptor that is commonly prescribed as an atypical antipsychotic medication for the treatment of bipolar disorder and schizophrenia (16,17). Aripiprazole also has an agonistic effect on 5-HT1A receptors, while it antagonizes 5HT2A receptors (18). Recent studies have indicated an anti-inflammatory property for ARP (19-24). ARP was shown to dose-dependently inhibit the mRNA expression of inflammatory enzymes and cytokines, including inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor (TNF)-α; thereby reducing the production of prostaglandins and NO (19). ARP is capable of reducing various inflammatory cytokines (e.g., interleukin [IL]-1β, IL-6, TNF-α, TNF-R1, IL-12, IL-23, IL-1Ra, TGF-β1, and IL-4) and increasing IL-10 levels in schizophrenic patients (24). It also targets activator protein-1 (AP-1) and nuclear factor (NF)-κB (19). This anti-inflammatory property of ARP is reported to be related to its effects on macrophages and microglial cells in the CNS (19,25,23), as it easily crosses the brain-blood barrier (23,26). Several mechanisms may underly the anti-inflammatory property of ARP, which include (i) inhibition of Toll-like receptor (TLR)2-mediated microglial activation and related brain inflammation (19,27); (ii) inhibition of c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase kinase 4 (MKK4), MKK7, and transforming growth factor (TGF) β-activated kinase 1 (TAK1) that are necessary for the activator protein-1 signaling pathway (19,28), (iii) inhibition of autophosphorylation and kinase activities of Src and Syk, two crucial contributors to the NF-κB signaling (29,19,30); (iv) increase in the expression of kinase II α-dependent Ca2+/calmodulin expression in microglial cells (31). Accordingly, ARP has been shown to prevent form renal ischemia/reperfusion injury (32).

Therefore, considering the above-outlined ARP potentials in neuroprotection and anti-inflammation as well as important roles of inflammatory signaling, especially in the secondary SCI, in the current study, we evaluated the effects of treatment with ARP on functional outcomes (i.e., motor function as well as neuropathic pain) as well as alterations in spinal cord histopathology and inflammatory markers post SCI to determine whether (i) ARP has any therapeutic value post-SCI and (ii) inflammatory signaling is involved in such effects of ARP. To address this, we utilized an animal model of SCI in rats.

Materials and Methods

Animals and ethical considerations

We used a total of 40 adult male Wistar rats (weight between 240 and 300 g) from Tehran Pasteur Institute. All rats were kept at a standard room temperature of 23±2º C and a 12-hour light/dark cycle with free access to tap water and food (chow pellets). All the animal experimentations were conducted according to the Declaration of Helsinki principles and the institutional Association for the Study of Pain guidelines for American experiments and after ethical approval by the Tehran University of Medical Sciences ethics committee (Code# 983081, Date 06/14/2019).

Drugs

We used the following drugs: aripiprazole (ARP), xylazine, buprenorphine, and cefazolin (Sigma-Aldrich, St. Louis, Missouri, USA) as well as ketamine hydrochloride (Gedeon Richter Ltd, Budapest, Hungary). Physiologic saline (90% NaCl solution) was used as a solvent for all drugs, which were administered via intraperitoneal (i.p.) injection.

Spinal cord injury (SCI) model

After induction of anesthesia by intraperitoneal administration of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), the dorsal hair of rats was cut off and sterilized with povidone-iodine. Then, the animals were placed in a prone position on a sterile, heating operating board. Then, animals underwent a complete laminectomy on T_9 vertebral level. Induction of SCI was made by a 60-second compression using an aneurysmal clip (YASARGIL Aneurysm clip system, Titanium mini clips FT712T; closing force, 110 g [1.08 N]; 4.7 mm Blade length; 3.8 mm maximum opening diameter) (33,34). After suturing the wound sites, animals were given buprenorphine (0.1 mg/kg) and cefazolin (20 mg/kg) and maintained in an incubator (35° C) till they gained full recovery. Animals received cefazolin and buprenorphine daily for 6 days post-operation (33).

Study groups

There were four major experimental groups in this study (total of 40 rats): two ARP-treated groups which separately received 10 and 20 mg/kg/day for three days after surgery (first dose 30 minutes post-surgery), one

control group that only received vehicle (normal saline 0.9%), and one sham-operated group (operation without SCI). All groups were monitored over 28 days post-surgery. The collection of all experimental data was made by separate, blinded observers. Each group of behavioral studies (*i.e.*, locomotor function test and neuropathic pain evaluation) consisted of 6-8 rats. For pathological assessment of spinal tissues, ELISA analysis, and flow cytometry, each group consisted of 3 rats.

Locomotor function test

As well-documented in our previous studies (33,34), we assessed locomotor function based on BBB (Basso, Beattie, Bresnahan) locomotor rating scale in an open field on day 0 prior to the surgery as well as days 1, 3, 7, 14, 21, 28 after surgery. Two blinded investigators were observed. The platform and recorded scoring, and the lesser number was confirmed. Their range of scoring was between 0 and 21 according to weight bearing, lean movements, coordination, and individual joint movements (33). Score 21 demonstrates normal locomotion of hind limbs; however, score 0 means no locomotor function. Screenings were given for every hind limb, and the mean score was recorded at each session (33,35).

Neuropathic pain assessment

Hot plate test

This test is a common method of investigating nociception in rats and mice (36). This test (36) was conducted by placing animals on a hot plate (50° C) on day 0 prior to the surgery and 7, 14, 21, and 28 days after the surgery. Then, the response time for behavioral changes (*i.e.*, stomping, jumping, paw licking, and escaping from the hot plate) was observed and recorded (36).

Tail-flick latency

We used a Tail-Flick Analgesia Meter (IITC life science model 33t; Los Angeles, California, United States) to measure Tail-flick latency (TFL) at the same above-outlined time point. After a 45-minute adjustment time, TFL was assessed by recording the time for each animal to move its tail from the painful thermal incitement while its dorsum of tails was exposed to a beaming heat source (33). Each rat was subjected to five tests. The mean withdrawal time was then calculated in each rat (33).

Mechanical allodynia

Von Frey test (Bioseb, USA) was utilized to measure the mechanical allodynia at the same above-outlined time point. Overall, each rat is located in a small cage with a penetrable bottom (33). A monofilament is applied perpendicularly to the plantar surface of the hind paw until it buckles, delivering a constant pre-determined force for 2 to 5 s. The test is positive if rats exhibit any nocifensive behaviors, including brisk paw withdrawal, licking, or shaking of the paw, either during the application of the stimulus or immediately after the filament is removed (33). In our experiments, to assess hyperalgesia, cutaneous sensitivity to Von Frey hairs was recorded as a 50% withdrawal threshold for each paw, and the average scores of paws (grams) for each rat were calculated and recorded (33).

Histopathology assessments

To assess spinal tissues assessment, we had 3 animals in each group. On day 28, post-surgery, anesthesia was induced by ketamine (80 mg/kg) and xylazine (10 mg/kg). Then, phosphate buffer saline (PBS, 150 ml, pH=7.4) was transcardially perfused to the right atrium in order to exsanguinate the rodents. This was followed by 4% paraformaldehyde (PFA, 250 ml) perfusion. The extracted spinal cords were post-fixed in 4% PFA, kept overnight in PBS, cryoprotected in 30% sucrose, and blocks were prepared by freezing the samples in optimal cutting temperature (OCT) compound. These sections were from the spinal cord tissue that was excised at the site of injury. Specimens respectively were put in alcohol 50% to 96% for a total of eight hours for dehydration. Then, the specimens were put in xylenol solution (10 minutes, 2 times). Finally, the specimens were placed in melted paraffin (50º C, 1 hour for 2 times). The paraffinization specimens were put in a cast full of paraffin. Glasses were then put in the oven (60 C, for 30 minutes) and were put in xylenol again (15 minutes for two times) to completely remove paraffin. The prepared specimens were stained with hematoxylin and then washed in distilled water for 1 minute and were put in lithium carbonate for a second. At last, the glasses were put in eosin dye for 3 minutes. At the end of staining, rehydration was made by putting the glasses in 90% and then Absolute alcohol and was immersed in xylenol (2 times, 15 minutes) for clarification. An optical microscope was utilized to assess tissue damage (evidenced by observing vacuolization, cyst formation, hemorrhage, inflammation, and apoptosis) (30).

ELISA analysis

To measure the inflammatory mediators IL-10 and TNF-α, the spinal cord specimens were processed and

analyzed with enzyme-linked immunosorbent assay (ELISA; Abcam, Cambridge, UK). Each group consisted of 3 animals. After perfusing PBS into the heart of sacrificed rats, the spinal cord samples were harvested and homogenized in lysis buffer. Samples were centrifuged (4000 r.p.m for 15 minutes at 4º C) and used for TNF-α and IL-10 level measurement by ELISA method (33).

Flow cytometry

We utilized the previous standard method for flow cytometry (37). Each group consisted of 3 animals. In these experiments, we assessed the levels of M1 and M2 and their ratio (M1/M2) in both the dorsal root ganglion (DRG) and spinal cord. The excised spinal cord specimens were digested with 600 U/ml collagenase II (Sigma-Aldrich, St. Louis, Missouri, United States) and 60 U/ml DNase I solutions diluted in Hanks buffered saline and filtered (30-µm separation filter) to develop single-cell suspensions. A goat serum-blocking reagent (Gibco, UK) was added to block non-specific interactions. Afterward, an antibody (10 µl) incubation was applied for a total of 5×10^5 ml of cells for 60 minutes at room temperature. Then, we performed a flow cytometry analysis. To assess macrophage phenotype M1 and M2 over the total cell population, the CD86+ cells (Code number CD86 [105007]: PE anti-mouse CD86 antibody [BioLegend, USA]) over the total CD80+ cell population were investigated for macrophages with M1 phenotype as the first initial gating strategy. Then, the CD206+ cells (Code number CD206 [141703]: FITC anti-mouse CD206 Antibody [BioLegend, USA]) over the total CD80+ cell population were assessed for macrophages with M1 phenotype as the second gating strategy. Cells positive for the M2 phenotype marker (CD206) and M1 phenotype marker (CD86) were then evaluated to identify the mixed M1/M2 phenotype. We used a Navios Flow Cytometer and the Kaluza analysis software (Beckman Coulter, Milan, Italy) analysis (37).

Statistical analysis

SPSS version 25 (Chicago, Illinois, United States) was utilized to conduct statistical analyses. The behavioral and tissue outcome measures were analyzed by either two-way or one-way repeated measure analysis of variance (ANOVA) followed by Tuckey's post hoc test for multiple comparisons. A *P* less than 0.05 was considered statistically significant.

Results

Locomotor scaling

Pre-operation locomotor scores were not statistically different among the three groups ($P>0.05$). As shown in figure 1, at day 1 post-SCI, locomotor scores decreased in both control and ARP (10 and 20 mg/kg/day)-treated groups ($P<0.001$), but there was no significant change in the sham-operated group without SCI induction ($P>0.05$). A two-way ANOVA analysis showed a remarkable effect of time ($F_{6,129}=1324$, $P<0.001$) and significant interaction between groups and time ($F_{18,129}=193.1$, $P<0.01$) on locomotor scores over 28 days. Multiple comparisons using Tukey's post hoc test showed locomotor scores' increase in both ARP-treated groups (both 10 and 20 mg/kg/day for 3 days) compared to the saline-treated control group (respective mean difference [95% CI]=3.10 [2.24-3.96], 2.65 [1.82-3.49], $P<0.01$). The BBB scores of both ARP and saline-treated groups were significantly less than the sham-operated group ($P<0.01$).

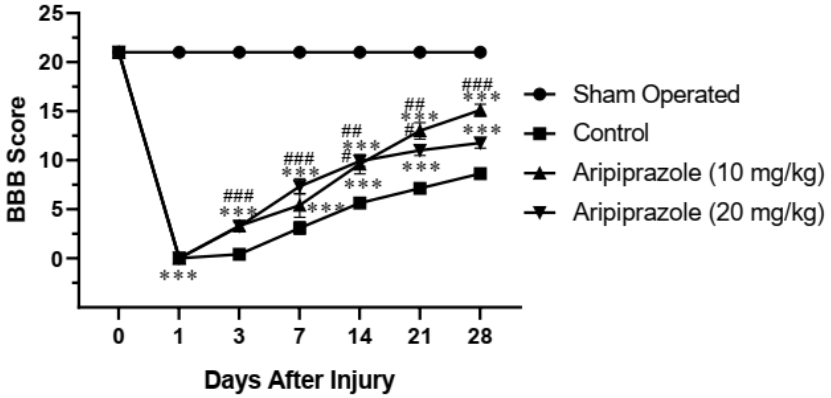

Figure 1. Line chart of BBB locomotor scores of rats in 4 groups of sham-operated, aripiprazole (10 and 20 mg/kg/day) and saline (as control) groups. Aripiprazole at both doses improved hind limb locomotor recovery through 28 days. The data are presented as mean±SD (6 rats per each group). *** $P<0.001$ compared to the sham-operated group; ### $P<0.001$ in comparison with the saline-treated control group

Hot plate test

This was performed weekly post-surgery to assess the medullary and brain reflexes. As depicted in figure 2A, the hot-plate scores markedly dropped after SCI in control and ARP-treated animals in comparison to the sham-operated group (P<0.001). We observed that time was an independently effective factor in hot plate score changes; however, ARP at both doses (10 and 20 mg/kg/day) did not exert any significant (P>0.05) effects on the hot plate scores at day 28 compared to control. Moreover, after 28 days, Scores were still significantly different between sham-operated and ARP-treated groups (P<0.001).

Tail-flick latency

Although at day 7, there was a decline in response to thermal incitements, this was statistically significant on the 14th day (P<0.01) in comparison to the sham-operated group (figure 2B). Assessment of weekly alterations in latency with repeated measure ANOVA showed a significant improvement in the TFL changes with ARP treatment as evidenced by increased latency and inhibited the nociceptive phenomena induced by SCI beginning at the 14th day over 4-week monitoring in comparison with the control group (P<0.001); the effect that was more significant for ARP at 20 than 10 mg/kg/day at 21st day compared to control group (P<0.01).

Mechanical allodynia

As demonstrated in figure 2C, there was no significant difference in mechanical allodynia between groups before the SCI induction (P>0.05). Two-way ANOVA revealed a remarkable upshot of time ($F_{4,76}$=8.29, P<0.01) and meaningful interaction between groups and time ($F_{12,76}$=4.59, P<0.01). Multiple comparisons using Tukey's post hoc test showed a markedly elevated mechanical allodynia threshold in ARP-treated groups compared to the control group mean difference [95% CI]=90.8 [39.32-142.3], 80.33 [30.80- − 129.9], P<0.01 for the 10 mg/kg and 20 mg/kg doses, respectively). Notably, the ARP-treated group and sham-operated group were similar 28 days following the SCI induction (P>0.05).

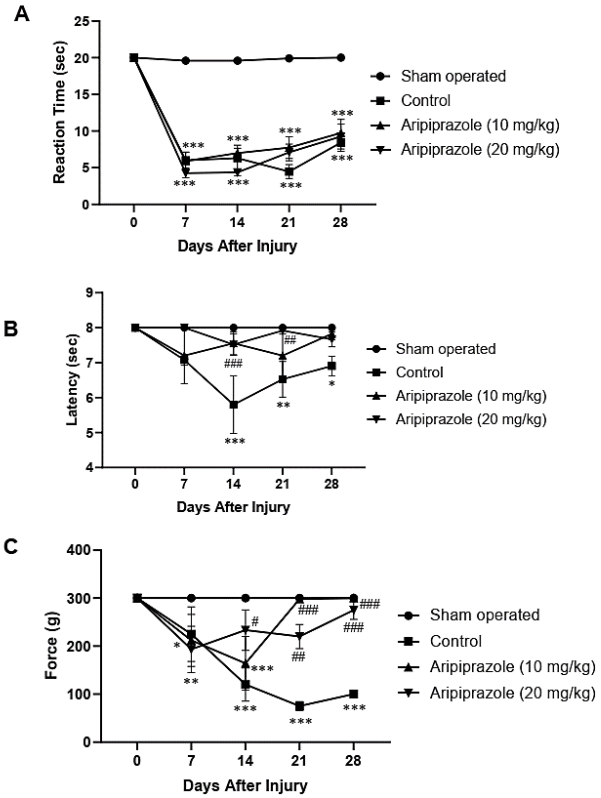

Figure 2. Line chart of (A) hot plate test scores, (B) mean tail-flick latencies (TFL), and (C) von Frey filaments test scores of rats in 3 groups of sham-operated, aripiprazole (10 and 20 mg/kg/day) and saline (as control) groups. The data are presented as mean±S.D. (6-8 rats per group). *P<0.05, ** P<0.01, and *** P<0.001 compared to the sham-operated group on the same day; $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, and $^{\#\#\#}$ P<0.001 compared to the saline-treated control group

ELISA of inflammatory markers

One-way ANOVA test revealed a significant difference in the levels of TNF-α and IL-10 between all groups ($F_{3,8}$=31.60, P<0.01, $F_{3,8}$=175.2, P<0.01 respectively) at 28 days post-SCI (figure 3). Tukey's post-hoc test also showed a significant reduction in TNF-α levels in ARP-treated groups (10 and 20 mg/kg) compared to the control group (for both doses, mean difference [95% CI]= -73.89 [-115.6 − -32.2], -67.78 [-109.5 − -26.09], P<0.01, respectively) and the significant increase in IL-10 level (mean difference [95% CI]=25 [11.6-38.84], 21.67 [7.83-35.5], P<0.01, respectively).

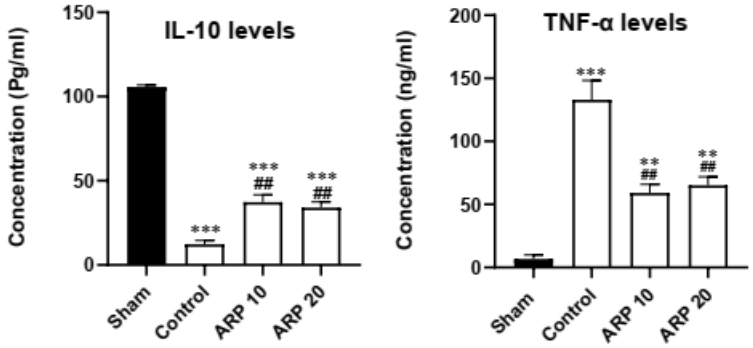

Figure 3. Cytokines levels, (A) TNF-α and (B) IL-10, in spine tissues at day 28 in either sham-operated or SCI groups of control rats (*i.e.*, saline) and aripiprazole (ARP, 10 and 20 mg/kg/day)-treated rats. The data are presented as mean±S.D. (3 rats per group). ** P<0.05 and *** P<0.001 in comparison with the sham-operated group on the same day; $^{\#\#}P$<0.01 in comparison to the saline-treated control group

Histopathological assessment

Histopathological scoring using Luxol fast blue staining was utilized in order to investigate any differences in myelination among groups (figure 4). One-way ANOVA test revealed a statistically significant difference between groups ($F_{3,8}$=89.7, P<0.01). There was significant myelination (pix/μm2) difference between ARP-treated groups (10 and 20 mg/kg) and the control group following the Tukey post-hoc analysis (mean difference [95% CI]=26 [17.33-34.67], 20 [11.33-28.67], P<0.01, respectively).

In the histopathological study (figure 5), compared to a sham-operated group, significant damage in the control group was seen post-SCI associated with cell apoptosis and histopathological damage in anterior horn regions. Moreover, there were long interval spaces between neurons in the control group, mainly resulting from neuronal death. On the other hand, there were less severe spinal cord and anterior horn damages in ARP (10 and 20 mg/kg)-treated groups compared with the control group.

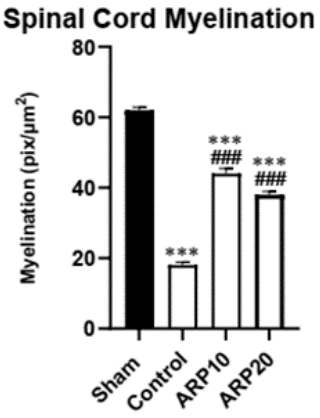

Figure 4. Spinal cord myelinization in the spinal cord tissue sections at the injury site 28 days post SCI. *** P<0.001 in comparison to the sham-operated group and ### P<0.001 in comparison with the saline-treated control group

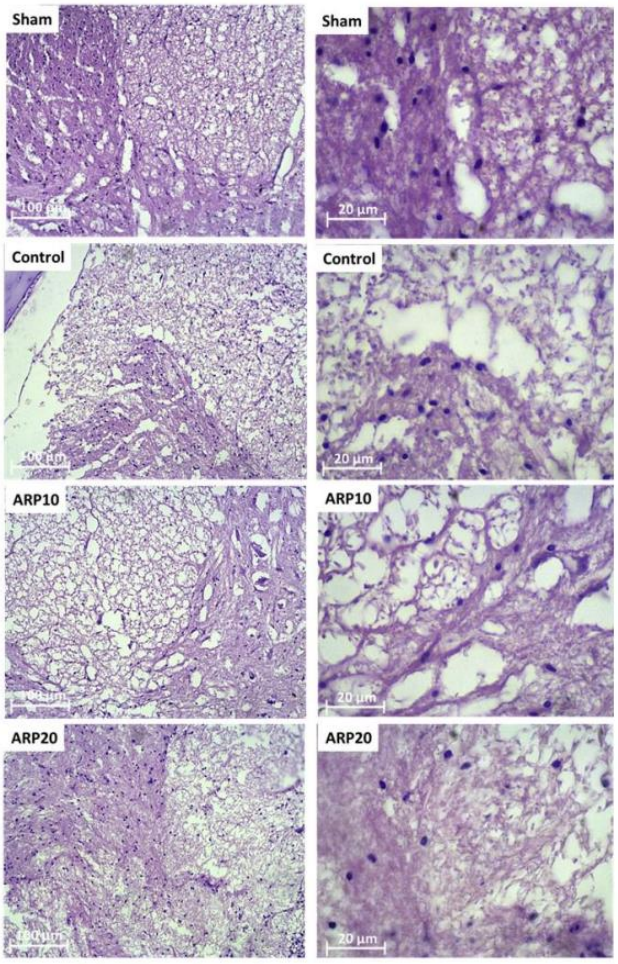

Figure 5. Spinal cord sections histology at the injury site 28 days post-SCI. Sections are stained by Luxol fast blue in order to investigate differentiation in myelination between all groups of sham-operated (Sham), saline control (Control), and aripiprazole (ARP10 and ARP20, 10 and 20 mg/kg/day, respectively)-treated groups

M1/M2 macrophages assessment

Eventually, M1 and M2 levels and their ratio (M1/M2) were assessed in the dorsal root ganglion (DRG) and spinal cord. One-way ANOVA analysis revealed that all procedures could change the spinal cord M1 level by using immunohistochemistry (figure 6A-C, ($F_{3,8}$=303.6, P<0.01). Tukey's post hoc analysis demonstrated a significant elevation of the expression of M1 after inducing the SCI (P<0.001); on the other hand, ARP treatment at both doses significantly decreased the M1 expression in the spinal cord compared to the control group (P<0.001). Similar results were found in the evaluation of spinal cord M2 level ($F_{3,8}$=257.8, P<0.01).

Tukey's post hoc test demonstrated a significant elevation in SCI-induced M2 level in control animals in comparison with the sham-operated group (P<0.001); however, ARP markedly reduced the M2 expression compared to the control group (P<0.001). At last, the M1/M2 phenotype macrophages were significantly different among all experimental groups ($F_{3,8}$=571.3, P<0.01). Post-hoc analysis indicated that the M1/M2 cells also significantly decreased in the ARP-treated animals compared to control animals (P<0.01).

In the next step, by using flow cytometry, the M1 and M2 levels, as well as their ratio in DRG, were examined (figure 6D-F). One-way ANOVA analysis revealed that ARP (20 mg/kg/day for 3 days) markedly changed the M1 level in DRG ($F_{2,6}$=40.99, P<0.01). Tukey's post hoc analysis showed M1 level in DRG increased (P<0.001) in the SCI group, whereas ARP markedly decreased compared to control (P<0.001). One-way ANOVA was performed to assess the M2 level ($F_{2,6}$=35.80, P<0.01). Tukey's analysis showed a significantly decreased M2 level in the SCI control animals compared to the sham-operated group (P<0.001). Also, ARP significantly reversed M2 levels in DRG compared to control groups

(P<0.001). In the DRG, M1/M2 ratio was significantly different among all groups ($F_{2,6}$=580.9, P<0.01). Post hoc tests showed that this ratio significantly enhanced after SCI induction in comparison to sham-operated animals (P<0.001). On the other hand, the M1/M2 ratio was significantly elevated in the ARP group compared to the control group (P<0.001).

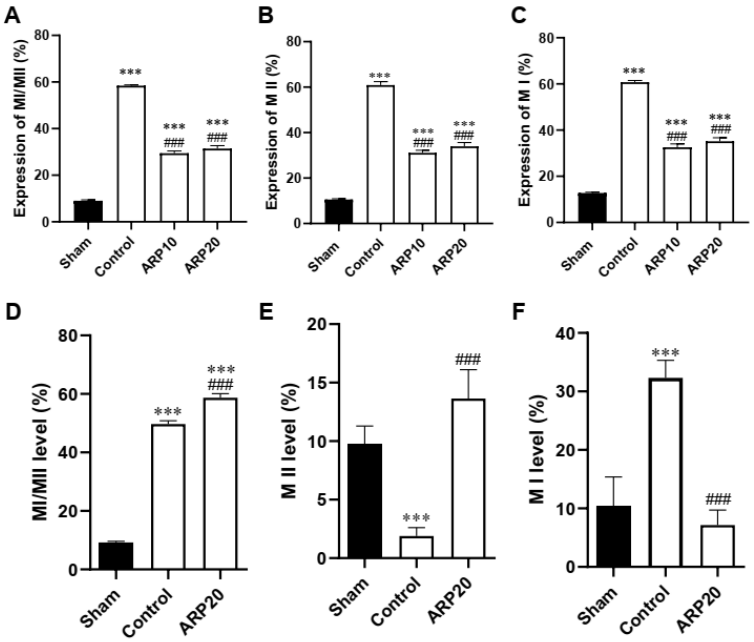

Figure 6. Levels of M1 and M2 and their ratio (M1/M2) in both spinal cord (A-C) and dorsal root ganglion (DRG, D-F) of sham-operated, saline control, and aripiprazole (ARP, 10 and 20 mg/kg/day)-treated groups. The data are presented as mean±S.D. (3 rats per group). *** P<0.001 compared to the sham-operated group on the same day; ### P<0.001 in comparison with the saline-treated control group

Discussion

The current study, for the first time, revealed that administration of ARP (10 and 20 mg/kg/day for 3 days) post-SCI induction robustly improved the spinal SCI outcomes because it exerted considerable improvement in both locomotion and neuropathic tests as well as in histopathologic and myelination assessment. We also found a decrease in TNF-α and an increase in IL-10 in spinal cord tissue in the ARP-treated group 28 days post-SCI induction in comparison with saline-treated control rats. Spinal cord injury would cause acute and chronic neuro-inflammation, resulting in more demyelination and more deterioration in locomotion and neuropathy evaluation (38,33). Our data revealed that ARP significantly reduced demyelination and improved locomotion and neuropathy scores in SCI rats.

Although previous clinical studies have reported antinociceptive effects of ARP monotherapy in psychiatric patients with comorbid nociceptive painful syndromes (39), to the best of our knowledge, no preclinical study has evaluated such effects of ARP on an SCI model in rodents. For the first time, our data revealed that ARP administration could improve functional outcomes post-SCI in rats, including locomotor activity and neuropathic pain. Consistent with our results, there is also evidence that either systemic or local ARP treatment in mice could inhibit formalin-induced paw licking and prostaglandin E_2-induced hyperalgesia in the paw pressure test (40,41). Our data suggest a potential benefit of ARP for neuropathic pain and locomotor activity after SCI.

ARP is a partial dopamine $D_2/D_3/D_4$ receptors agonist with an agonistic effect on 5-HT_{1A} and an antagonistic effect on $5HT_{2A}$ receptors (18) and is prescribed as an atypical antipsychotic medication for the treatment of a variety of neuropsychiatric disorders (16,17). In addition to its antipsychotic effects, ARP can modulate macrophage and microglial functions (42,27,19). Our present data also revealed that ARP administration leads to a switch from activated macrophages with the pro-inflammatory role to an anti-inflammatory phenotype; in fact, ARP has been shown to protect against the neurotoxicity of pro-inflammatory macrophages (27,19).

Previous studies have also reported that decreasing M1 and increasing M2 macrophages have therapeutic properties in the SCI (43-45). M2 cells produce the anti-inflammatory cytokines IL-10 and transforming growth factor (TGF)-β and decrease iNOS, caspase-3, and pro-inflammatory cytokine generation (45-47). M2 cells also hinder inflammation through NOS inhibition and arginase-1 up-regulation (47). On the other hand, TNF-α could affect M1 expression in tissue-activated macrophages (48). M1-activated macrophages cause more TNF-α expression (48,45). Altogether, one possible and important mechanism of ARP action is its capability to reduce pro-inflammatory cytokines (*e.g.*, IL-12, IL-6, IL-1β, and TNF-α) and increase anti-inflammatory cytokines (*e.g.*, IL-10) (22,49,24,21). Accordingly, ARP has been shown to have protective effects against renal ischemia/reperfusion injury in rats through the reduction of IL-1β and TNF-α levels (32). Consistently, our present data revealed that ARP-treated SCI rats had markedly reduced pro-inflammatory TNF-α and elevated anti-inflammatory IL-10 levels compared to control SCI groups. This is consistent with the prior study in which ARP could inhibit the expression of iNOS, COX-2, IL-6, IFN-β, and TNF-α in peptidoglycan-stimulated macrophages (RAW264.7 cells) (19). Several mechanisms may underly such anti-inflammatory effects of ARP, which include inhibition of TLR2-mediated microglial activation as well as inhibition of JNK, MKK4, MKK7, and TAK1 that are essential for the activator protein-1 signaling pathway (19,28). Another mechanism is the inhibition of autophosphorylation and kinase activities of Src and Syk, which are crucial contributors to the NF-κB signaling (29,19,30). Elevated expression of kinase II α-dependent Ca^{2+}/calmodulin expression in microglial cells is also another suggested mechanism underlying the anti-inflammatory effects of ARP (31). Additionally, ARP treatment post-SCI led to improvement in both locomotor and neuropathic pain scores. We also observed that post-SCI administration of ARP altered the macrophage/microglia polarization, which is associated with better rehabilitation in spinal cord-injured rats.

Ample preclinical evidence suggests an essential role for macrophage phenotypes in the pathophysiology of different stages of SCI (50). Specifically, interferon (IFN)-γ plus lipopolysaccharides (LPS)-stimulated M1 macrophages are toxic to neurons and lead to axonal dieback, whereas IL-4-stimulated M2 macrophages exert promoting effects on axon growth, re-myelination, resolution of inflammation and tissue repair (51,52,44). Accordingly, markers of M1 or M2 macrophages are commonly considered for assessment of the anti-inflammatory property of various agents in the treatment of SCI. On the other hand, finding the relative ratio of M1 or M2 markers and transcription factors may be more informative in identifying the macrophage phenotypes (e.g., M1 and M2) related to various conditions (52-55). Our present data also revealed that ARP-treated groups had improvement in the spinal cord histopathology as well as M1/M2 macrophage ratio compared to control rats. These data provide novel evidence that the improving effects of ARP on functional outcomes post SCI in our study could be related to its modulatory effects on spinal cord histopathology, M1/M2 macrophage balance, as well as pro/anti-inflammatory cytokines.

In conclusion, the present study, for the first time, revealed that post-SCI treatment with ARP (10 and 20 mg/kg) markedly improved functional outcomes of SCI as evidenced by improving locomotor activity as well as neuropathic pain in rats. We also found that ARP treatment improved neuronal tissue damage and neuroinflammation in the spinal cord tissue in SCI groups.

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