

The Association Between Alpha-7 Nicotinic Acetylcholine Receptor and Microglial Polarization in Spinal Cord Injury: Nicotine as an Alternative Therapy for Neuroinflammation

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Research

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Abstract

Background: Nicotine is an agonist of alpha-7 nicotinic acetylcholine receptor ($\alpha 7$ nAChR). The association between the expression of $\alpha 7$ nAChR and neuroinflammation has been extensively reported. Herein, we assessed the efficacy of Nicotine in the management of spinal cord injury (SCI) complications and mediating mechanisms.

Methods: In this study, 64 male rats were randomly allocated to 7 SCI and a sham-operated groups. SCI was induced through an aneurysmal clip at the T9/T10 level. The group list consists of a non-treated group as the control, four Nicotine-treated groups receiving 0.5, 1, 1.5, and 3 mg/kg of the drug, a Methyllycaconitine (MLA, 1.5 mg/kg)-treated group, a group of rodents receiving MLA plus the most effective dosage of Nicotine, and a sham one. Locomotion and mechanical allodynia were assessed during 28 days using the Basso, Beattie, Bresnahan (BBB) and von Frey methods, respectively. In the end, spinal cord samples were taken to assess cavity formation, the expression levels of M1 and M2 macrophages, pro-inflammatory and anti-inflammatory factors, as well as $\alpha 7$ nAChR and NF- κ B gene levels.

Results: Repeated measures analysis revealed significant effect of time-treatment interaction on locomotion [$F(42, 336) = 120.2, p < 0.001$] and mechanical sensitivity [$F(35, 280) = 45.47, p < 0.001$]. Behavioral response to Nicotine was dose-dependent, and 1 mg/kg of this reagent was the most efficient dosage. H&E staining represented lesser histopathological disruptions in Nicotine-treated animals. SCI increased the M1/M2 ratio ($p < 0.001$) via shifting macrophages polarization towards M1 subset and 1 mg/kg of Nicotine could attenuate this ratio ($p < 0.001$) through reversing the shift. Meanwhile, Nicotine administration resulted in a significant elevation of $\alpha 7$ nAChR and a reduction of NF- κ B genes. Finally, in the Nicotine group, there were declines in the levels pro-inflammatory biomarkers, including TNF- α , IL-1 β , and IL-6, while IL-10 was found higher than the control group (p values < 0.05). MLA-treated groups showed almost none of the aforementioned alterations.

Conclusion: Single-dose therapy with Nicotine could improve locomotor and sensory complications of SCI. Nicotine possible mechanism of action is through increasing the $\alpha 7$ nAChR level, which alleviates neuro-inflammation by changing microglial phenotyping.

Introduction

Spinal cord injury (SCI) is still considered as a significant and life-long condition after a broad spectrum of studies because of its complex pathogenesis and the following probable motor and sensory complications that may have negative effects on patient's quality and expectancy of life. These adverse effects are mostly continuous due to the incomplete rehabilitation of the central nervous system (CNS). SCI has an estimated annual incidence of 12.1–57.8 cases per million worldwide [1]. SCI has two different phases of injury. The primary phase of SCI occurs at the moment of spinal trauma and is described as mechanical damage while the second phase is defined as the following biochemical and

pathological changes after the injury that worsens by the passage of time. Neuroinflammation plays a fundamental role in the latter phase where dysregulation in the activity of several immune cells such as neutrophils, monocyte-derived macrophages, and resident microglia leads to an imbalance between pro-inflammatory and anti-inflammatory mediators [2].

The macrophages polarization paradigm refers to the phenotyping of macrophages in response to various exogenous and endogenous stimulants and has two main subsets. M1 macrophages are responsible for the release of pro-inflammatory factors that bring about neurodegenerative effects, while M2 macrophages have neuroprotective characteristics due to its stimulatory impact on anti-inflammatory responses. Therefore, it is not surprising that a higher ratio of M1/M2 macrophages is accompanied by the dysfunction in the CNS repair, and neurotoxicity [3]. This phenomenon occurs in the second phase of SCI and notably impairs the patient's locomotor and sensory rehabilitation. In this manner, several previous studies of SCI have pharmacologically targeted the relative proportions of M1 and M2 markers to manage the neuroinflammation [4].

The alpha-7 nicotinic acetylcholine receptor ($\alpha 7$ nAChR) is a member of the nicotinic acetylcholine receptors family, recognized for its diverse roles within the nervous system [5, 6]. It has been shown that $\alpha 7$ nAChR participates in the modulation of neuroinflammatory processes and therefore, has been of great interest. Nicotine, which is a potent agonist of $\alpha 7$ nAChR, has shown significant effectiveness in terms of SCI behavioral (i.e., locomotor dysfunction) and molecular deficits (i.e., neuroinflammation). It is hypothesized that the $\alpha 7$ nAChR level affects the microglial polarization within the CNS via the nuclear factor-kappa b (NF- κ B) signaling pathway. This issue has been confirmed by several studies of cerebral brain injury [6–8]. Nevertheless, as far as we know, this is the first study that evaluates the association between $\alpha 7$ nAChR, NF- κ B, microglial polarization, neuroinflammation, and behavioral recovery in an SCI setting.

The discovery of novel therapeutic reagents for the treatment of SCI motor and sensory complications seems necessary due to limited available medications. Up to now, the beneficial effects of a few numbers of drugs have been reported on adverse events of SCI, acting via changing the microglial polarization at the lesion region [9, 10]. The therapeutic effects of Nicotine have also been mentioned in SCI; however, its exact mechanism of action warrants further investigation [11]. In the present study, we determined to present a novel pathway in SCI by apprehending the role of $\alpha 7$ nAChR in neuroinflammation and behavioral recovery after SCI.

Materials And Methods

Study population

Sixty-four male Wistar rats, weighing 240–300 g, were provided from Tehran Pasteur Institute and were housed in separate cages that were equipped with free access to a sufficient amount of tap water and chow pellets. The room temperature was 23 ± 2 °C with $50 \pm 5\%$ humidity and a 12-hours light/dark cycle.

The study protocol was executed in agreement with the National Institutes of Health (NIH) Guide for the Care and Use of laboratory animals (NIH publication No. 86 – 23, Eighth Ed.) and institutional and governmental concerns for animal care and use (Approval ID: IR.TUMS.MEDICINE.REC.1398.922). All possible efforts were made to minimize the study population and their suffering.

Drugs

Nicotine and Methyllycaconitine (MLA) were obtained from Sigma, St Louis, Missouri. Ketamine HCl (Gedeon Richter Ltd, Budapest, Hungary) and Xylazine HCl (Bayer, Leverkusen, Germany) were also purchased. Drugs were dissolved in physiologic saline and used through intraperitoneal injection.

Study groups

In a randomized setting, animals were assigned 8 to equal groups. Four groups received Nicotine (Sigma-Aldrich, St. Louis, Missouri, United States) in the doses of 0.5, 1, 1.5, and 3 mg/kg in the attempt of identifying the amount with optimal behavioral outcomes, including locomotion and neuropathic pain recovery. 1.5 mg/kg of MLA was administered to one group while another group received the optimum dosage of Nicotine in addition to 1.5 mg/kg of MLA. The control and sham-operated groups merely received vehicle (saline 0.9%). All groups were followed for 28 days after surgery. All data, including lab tests, locomotor, and sensory evaluations were collected by different observers, blinded to groups. All drugs were intraperitoneally administered 30 min prior to the surgery.

Surgical procedure

A combination of 86 mg/kg Ketamine HCL and 13 mg/kg Xylazine HCL was injected intraperitoneally to anesthetize each animal. Dorsal hairs of the rodents were shaved with an electric razor, and the surgical site was cleaned with povidone-iodine. A prophylaxis injection of 30 mg/kg cefazolin was administered to all rodents before the beginning of the surgery. In the next step, each rat was located on a sterile, heating operating board in a prone position. After posterior midline incision and muscle dissection, complete laminectomy was operated on the T9/T10 vertebrae. The injury was induced on the spinal cord by compression with 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) for 60 seconds. Finally, the wound sites were sutured, and animals were kept in a 35 °C incubator until recovery. The post-operative care was performed administrating normal saline (2 ml), cefazolin (20 mg/kg) and buprenorphine (0.1 mg/kg) for seven successive days. Voiding was performed twice a day manually until complete functional rehabilitation. Of note, the sham group underwent posterior midline incision without the induction of SCI.

Locomotion

The hindlimb locomotor function was assessed using the Basso, Beattie, Bresnahan (BBB) rating scale in an open field at baseline and on days 1, 3, 7, 14, 21, and 28 after the surgery [12]. For each rat, the BBB score of hindlimbs were averaged and the result was documented. The assessor was blinded to the study groups. Score 21 indicates normal locomotion of hind limbs while 0 accounts for no locomotor function.

Von Frey

For each rodent, the withdrawal thresholds of both left and right hind paws were assessed using the simplified up-down (SUDO) method of von Frey testing, which is valid and induces minimal stress to the rodents [13]. The assessments were conducted prior to SCI induction and on days 7, 14, 21, and 28 of the experiment. First, the rats were habituated to the test platform, which is an elevated box with a netting floor. Then, a series of von Frey hairs (a logarithmic series of 20 calibrated Semmes-Weinstein monofilaments; Stoelting Co., Wood Dale, IL) was smacked from below the platform to the central region of the plantar surface of the rat hind paws and were held for a maximum of 5 seconds. Prominent paw withdrawal or flinch were considered as positive responses. The start point of the test was with filament 10 (4.31, 2.0 gr).

Sample acquisition

At day 28 post-operation, half of the rats in each group were anesthetized with a mixture of 86 mg/kg Ketamine HCL and 13 mg/kg Xylazine HCL and then, 150 ml of phosphate buffered saline (PBS, pH 7.4) was perfused to their heart, followed by 250 ml of formalin-acid picric mixture (4% paraformaldehyde, 0.4% picric acid in 0.16 M phosphate buffer, pH 7.4) for fixation of the tissues. Three cm of the spinal cord was selected from the center of the injury site. Specimens were prepared and embedded in paraffin. The paraffinized specimens were sliced into samples with a thickness of 5 micrometers by a Leica 2135 microtome (Deerfield, IL, USA) for Hematoxylin and Eosin (H&E) and immunohistochemistry (IHC) assays. The other half of the rats underwent spinal cord dissection and the extracted samples were kept inside liquid nitrogen in a - 70 °C freezer for Enzyme-Linked Immunosorbent Assay (ELISA) and RT-PCR.

Histopathological analysis

As mentioned above, half of the specimens were stained with Hematoxylin and Eosin (H&E) dye for histopathological assessment. The staining protocol is completely described in our previous publication [10]. Briefly, after deparaffinization and rehydration of the specimens, the nuclei were stained by rinsing the slides in hematoxylin solution. In the next step, bluing was performed in 0.2% ammonia water. Then, the slides were counterstained in eosin-phloxine for 1 min and afterward samples were rinsed in 90%, 96% and 100% ethanol for 2 min. Finally, slides were embedded in xylene and mounting medium. The final products were examined using an optical microscope (resolution: 40×) to determine tissue and cavity percentages in the lesion sites.

IHC

IHC method was performed on half of the spinal cord specimens to evaluate the expression of the surface receptors of M1 and M2 macrophages, which are CD86 and CD206, respectively. The samples were rinsed by 0.1 M PBS in 4 steps. Afterward, in the attempt of retrieving the antigens, sections were placed in 2 normal hydrochloric acid (HCL) solutions for 30 min. Then, the effect of HCL was neutralized by Borate buffer. In the next step, a 3% solution of Triton X-100 was used for 30 min to increase the cell

membranes permeability. Goat serum (10%) was added as an extra color to the background during a period of 30 min to block nonspecific reaction of the antibodies. Next, a primary antibody (ab220188, Abcam) diluted with PBS (1:100) was added and the combination was transferred to a 2–8 °C refrigerator for 24 hours. Next, a secondary antibody (ab 182422, Abcam) diluted with PBS (1:150) was added and the processed samples were incubated for 1.5 hours in a 37 °C incubator. Afterward, 4',6-diamidino-2-phenylindole (DAPI) was added in a dim place to counterstain the nuclei. Notably, the samples were washed by PBS between the steps. Finally, the macrophages were calculated, dividing prepared samples into 5 separate areas via an Olympus fluorescent microscope (× 400) and images were taken from each area. The captured images were analyzed using the ImageJ software (Fiji version) [14]. The result was reported as the percentage of positive immunolabeled cells over the total cells in each selected region (the ratio number of positively stained cells/total number of cells × 100).

Real-Time RT-PCR

Four main phases were conducted for qRT-PCR analysis of $\alpha 7$ nAChR and NF- κ B genes expression. First, total RNA was extracted from the specimens using Qiazol reagent (Qiagen, Germany). Second, one microgram of mRNA was reverse transcribed to cDNA according to manufacturer's instructions (Fermentas, USA). Third, an Applied Biosystems 7300 Fast Real-Time PCR System with SYBR green PCR master mix (Applied Biosystems, CA, USA) was employed for real time RT-PCR analysis. The primer sequences are listed in Table 1. The thermocycler conditions were as follows: 95°C for 15 min for activation of DNA polymerase, followed by 45 cycles of amplification at 94°C for 15 sec, 60°C for 15 secs and 72° for 30 sec. At last, melting curve analysis was conducted to confirm whether all primers provided a single PCR product. Sequences were normalized with GAPDH as a control. The $2^{-\Delta\Delta C_t}$ method was used to measure the relative expression of $\alpha 7$ nAChR.

Table 1
Primers for RT-PCR

Gene name	Primers (Forward and reverse)
$\alpha 7$ nAChR	F: AAAATGCCTAAGTGGACCAGAA
	R: TCACTGCAGATCACCTCACTCT
NF- κ B	F: GTGCAGAAAGAAGACATTGAGGTG
	R: AGGCTAGGGTCAGCGTATGG
GAPDH	F: TCAGAGCAAGAGAGGCATCC
	R: GGT CATCTTCTCACGGTTGG
Abbreviations: Alpha-7 nicotinic acetylcholine receptor, $\alpha 7$ nAChR; Glyceraldehyde 3-phosphate dehydrogenase, GAPDH; Nuclear factor kappa b, NF-Kb; Reverse transcription polymerase chain reaction (RT-PCR).	

ELISA

Three pro-inflammatory factors, tumor necrosis factor-alpha (TNF- α), interleukin 1 beta (IL-1 β), and interleukin 6 (IL-6) and an anti-inflammatory mediator, interleukin 10 (IL-10) were measured. The specimens were first homogenized in lysis buffer and then centrifuged for 20 min at 13,000 rpm at 4 °C. Finally, the ELISA (Abcam, Cambridge, UK) method was employed to quantify the level of aforementioned mediators.

Statistical analysis

All statistical analyses were performed using the SPSS software, version 25 (IBM Corp., Armonk, NY). The graphs were drawn using the GraphPad Prism software, version 7 (San Diego, CA, USA). The behavioral data were analyzed by the general linear model (GLM) repeated measures analysis followed by Tukey's post hoc test. Besides, the one-way analysis of variance (ANOVA) test followed by Tukey's post hoc test was employed to evaluate differences of histopathological scorings, pro-inflammatory and anti-inflammatory mediators, as well as $\alpha 7$ nAChR and NF- κ B genes expression between the groups. The p values $< 0.05^*$, $< 0.01^{**}$ and $< 0.001^{***}$ were accepted as statistically significant. Comparisons between two groups are reported as mean difference (95% confidence interval), which is abbreviated to MD (95% CI).

Results

Locomotion

According to locomotor assessment using the BBB scale, pre-operation scores were similar between the groups (p values > 0.05). One day after SCI induction, locomotor scores declined significantly in all groups except for the sham-operated (Fig. 1A). Repeated measure ANOVA analysis indicated significant effect of time [$F(6, 336) = 4865, p < 0.001$] and effect of the interaction between treatments and time [$F(42, 336) = 120.2, p < 0.001$] on locomotor scores during 28 days. Moreover, multiple comparisons using the Tukey as a post-hoc test exhibited the highest locomotor scores in rodents which received 1 mg/kg of Nicotine [Nicotine (1 mg/kg) vs control: MD (95% CI) = 3.321 (2.45 to 4.19), $p < 0.001$]. Evidently, the BBB scores of all groups endured significantly lower comparing to sham (p values < 0.001). However, there was no significant difference between Nicotine 1 mg/kg and Nicotine 1.5 mg/kg treated groups ($p = 0.736$). Further comparisons between the groups are illustrated in Fig. 1A.

Neuropathic pain

In order to assess mechanical allodynia, the von Frey method was performed weekly after induction of SCI and the withdrawal threshold was compared between the groups. Mechanical allodynia in sham-operated group remained intact while there was significant decline in the control group within one week after the surgery [MD (95% CI) = 39.3 (31.10 to 47.50), $p < 0.001$]. The decreasing trend of resistance to mechanical pressure is evident from day 7 of the study (Fig. 1B). Repeated measure analysis revealed significant effect of time [$F(5, 280) = 1956, p < 0.001$] and also time \times treatment interaction [$F(35, 280) = 45.47, p < 0.001$], which represents different effectiveness of the administered treatments over time.

Particularly, Nicotine in doses of 1 mg/kg and 1.5 mg/kg could significantly rise the final mechanical threshold compared to the control group (p values = 0.04 and 0.01, respectively). Nevertheless, none of the groups achieved a mechanical resistance similar to the sham. Of note, 1 mg/kg of Nicotine was selected as the optimal dosage for molecular assessments due to the most locomotor and sensory alleviating response to the administration at this dose.

Spinal cord histopathology

Figure 2 represents the mean cavity percentage in the spinal cord tissue 28 days after the induction of SCI. The one-way ANOVA test revealed significant difference between the groups [$F(4, 25) = 101.1, p < 0.001$]. According to the Tukey post-hoc test, SCI induction with no treatment significantly increased the mean cavity percentage in the spinal cord tissue comparing to sham-operated group [MD (95%CI) = 28.52 (23.74 to 33.29), p value < 0.001]. However, treating spinal cord injured rats with 1 mg/kg of Nicotine significantly reduced the cavity percentage compared to the control group during the experiment period [MD (95% CI) = 8.47 (3.70 to 13.24), $p < 0.001$]. Additionally, there was no significant difference between control group and both MLA + 1 mg/kg Nicotine-treated ($p = 0.187$) and MLA-treated groups ($p = 0.628$).

M1/M2 expression ratio

One-way ANOVA analysis followed by the Tukey post-hoc test demonstrated a significant elevation in the expression level of CD86 marker post-SCI ($p < 0.001$), while a significant decline was observed in the expression level of CD206 at 28 days' post-injury (dpi) ($p < 0.001$) (Fig. 3). Interestingly, activation of $\alpha 7$ nAChR by administration of 1 mg/kg Nicotine to the injured rats significantly attenuated the expression of M1 marker and in parallel, increased the expression of M2 marker (p values < 0.001). However, administration of 1.5 mg/kg of $\alpha 7$ nAChR selective antagonist, MLA, could alter the expression of neither CD86 ($p = 0.217$) nor CD206 ($p = 0.266$) at 28 dpi. In summary, Nicotine alleviated the elevated M1/M2 ratio induced by SCI ($p < 0.001$), whereas MLA did not exert any significant effect on classical and alternative macrophage polarization ($p = 0.058$).

M1 downstream markers

TNF- α , IL-1 β , and IL-6 are inflammatory, neurodegenerative biomarkers that were measured to evaluate the downstream pathways of M1 macrophages post-SCI and the consecutive treatments. Here, it was shown that administration of Nicotine with dosage of 1 mg/kg could significantly subside the level of TNF- α , IL-1 β , and IL-6 in the spinal cord tissue comparing to the control group (p values < 0.001) (Fig. 4). However, single dose of MLA altered none of the aforementioned inflammatory factors (TNF- α : $p = 0.071$; IL-1 β : $p = 0.413$; IL-6: $p = 0.366$). Interestingly, meaningful decline in the expression of IL-1 β was observed in the MLA + Nicotine group ($p = 0.036$).

M2 downstream marker

IL-10 is an anti-inflammatory, neuroprotective biomarker that was measured to assess the downstream pathways of M2 macrophages in response to SCI and the following treatments. The one-way ANOVA analysis resulted in different IL-10 level among the groups [$F(4,15) = 28.18, p < 0.001$] (Fig. 5). The Tukey post hoc analysis suggested the positive effect of Nicotine on IL-10 level since rodents which received 1 mg/kg of Nicotine represented higher IL-10 level compared to the control ones ($p = 0.010$).

NF- κ B gene expression

According to Fig. 6, RT-PCR analysis of NF- κ B gene was statistically different among the experiment groups [$F(4,15) = 23.24, p < 0.001$]. It was shown that rodents with SCI exhibit considerably higher expression level of NF- κ B gene in comparison to the sham rats ($p < 0.001$). Compared to the control group, the NF- κ B gene level in the Nicotine group was found to be lower ($p = 0.009$), which suggests the direct role of Nicotine in the expression of this gene. Neither MLA ($p = 0.753$) nor MLA + Nicotine ($p = 0.772$) injection was significantly different with the vehicle administration to the control rodents.

α 7 nAChR gene expression

As Fig. 7 represents, RT-PCR analysis of α 7 nAChR gene expression demonstrated significant difference between the groups [$F(4,15) = 19.07, p < 0.001$]. Interestingly, the Tukey post-hoc test indicated that SCI reduces the expression level of α 7 nAChR gene at 28 dpi in the spinal cord ($p < 0.001$). Surprisingly, pre-treatment with either 1 mg/kg of Nicotine ($p = 0.033$) or 1.5 mg/kg of MLA ($p = 0.001$) could relatively reverse the reduced gene expression. In contrast, there was no significant difference in the expression of α 7 nAChR between the control group and MLA + Nicotine group ($p = 0.993$).

Discussion

Our behavioral assessments indicated that pre-treatment with Nicotine could improve secondary complications of SCI such as locomotor dysfunction and neuropathic pain. The Nicotine alleviating effects appeared to be dose-dependent since 1 mg/kg of Nicotine had the most notable impact on behavioral recovery, while 3 mg/kg of the reagent apparently had no significant effect on locomotion and mechanical sensation. Therefore, the optimal dosage (1 mg/kg) was chosen for molecular assay. Spinal cord injured rats exhibited reduced levels of α 7 nAChR gene compared to the sham ones, which was partially reversed by administration of Nicotine. Moreover, Nicotine attenuated the elevated levels of NF- κ B gene, cavity formation, M1 macrophages, and inflammatory factors while it simultaneously increased M2 macrophages and an anti-inflammatory marker (IL-10) at the injury site. Notably, these alterations were absent in the MLA-treated rodents. Our proposed model regarding the association between α 7 nAChR and neuroinflammation in SCI is summarized in Fig. 8.

Neuro-inflammatory responses are integrated with secondary SCI, which is initiated in the first 24 hours after primary SCI and would last chronically. Neuroinflammation plays a critical role in the development of neuropathic pain and locomotor exasperation, induced by SCI [15]. Although various mechanisms

have been suggested to explain deficiencies after SCI, macrophages activity has been recently regarded as one of the main factors influencing neuroinflammation and axonal survival [16]. In general, it seems that nervous-immune interactions take a remarkable part in the process of secondary SCI, which potentially could lead to significant positive and negative effects on neural survival and neural regeneration. In detail, M1/M2 macrophage polarization paradigm is acting as a seesaw in post-SCI neuroinflammation. The M1 phenotype, recognized as “classic macrophage polarization”, produce inflammatory markers (e.g., TNF- α , IL-1 β , IL-6) that bring about neurotoxic and inflammatory interactions, whereas the M2 phenotype, known as “alternative macrophage polarization”, tends to mediate neuro-regenerative responses through secretion of anti-inflammatory factors (e.g., IL-10). However, it appears that one-sided shift to M1 phenotype during the secondary SCI may facilitate undesirable outcomes varying from excessive inflammatory responses to fibrosis scarring [17]. Previous studies have confirmed this issue, showing that transplantation of M2 macrophages or manipulation of the existing macrophages towards M2 phenotype could lessen neuroinflammation and histopathological abnormalities [4, 18–21]. In this study, we aimed to elucidate the effects of $\alpha 7$ nAChR modification on neuroinflammation and histopathology in an animal model of SCI considering the role of M1/M2 macrophage polarization paradigm. To the best of our knowledge, this is the first study that examines this relationship in SCI setting.

$\alpha 7$ nAChR is a member of nicotinic acetylcholine receptors family and is expressed on both neuronal and non-neuronal cells, including endothelial cells, dendritic cells, macrophages, B cells and T cells. It has been well established that $\alpha 7$ nAChRs are involved in various inflammatory contexts such as sepsis, hemorrhage, rheumatoid arthritis, brain ischemia, myocardial infarction, Alzheimer’s disease, schizophrenia, pain and SCI [22–25]. In addition, a remarkable number of studies have explored the effect of $\alpha 7$ in the extent of traumatic brain injury (TBI), which could be considered a close model to SCI. It has been shown that TBI could result in reduced levels of $\alpha 7$ nAChR in the injured region at both acute and chronic phases after the damage [26]. Besides, Nicotine has been found effective in the improvement of neural loss and behavioral outcomes of subjects with TBI [27, 28]. The effects of $\alpha 7$ nAChR on regulation of inflammation in TBI models have been supported by observing that vagal nerve stimulation leads to the reduction of pro-inflammatory factors (TNF- α , IL-1 β , IL-6) through activation of the receptor [29, 30].

Various studies have revealed neuroprotective influences of $\alpha 7$ nAChR on the spinal cord tissue. There is solid evidence concerning the stimulatory effects of $\alpha 7$ nAChR agonists on the level and activity of the receptor [23, 31–34]. Rong et al. has indicated that pre-treatment with $\alpha 2$ -adrenoreceptor selective agonist could enhance locomotor recovery after SCI among rats via amplification of $\alpha 7$ nAChR pathway [32]. Furthermore, it has been reported that $\alpha 7$ nAChR participates in determining the severity of neuropathic pain and mechanical allodynia. In this respect, Loram et al. demonstrated that both systemic and local administrations of $\alpha 7$ nAChR agonist could improve mechanical resistance to the forces, probably via reducing the spinal TNF- α level in a rat model of neuropathic pain [35, 36]. In two distinct studies, Ravikumar et al. showed that both single and multiple administrations of Nicotine, a potent $\alpha 7$ nAChR agonist, attenuate oxidative stress, pro-inflammatory markers, and NF- κ B activity while enhance sparing of spinal cord tissue and therefore, the recovery of SCI-induced locomotor dysfunction [11, 37]. Moreover,

Richardson et al. indicated that nonsmokers with SCI experience lower grades of neuropathic pain after Nicotine therapy [38]. Furthermore, Lee et al. suggested that Nicotine may reduce inducible nitric oxide synthase (iNOS) protein and mRNA levels, probably due to the activation of $\alpha 7$ nAChR on microglial cells [39]. Herein, we also detected significant improvement in functional and molecular status of the spinal cord injured rodents following administration of 1 mg/kg of Nicotine, whereas 3 mg/kg of Nicotine appeared less beneficial. Interestingly, paradoxical effects of Nicotine have been an issue in previous relevant studies and have been related to several features, including dosage, timing and selectivity [25, 40].

Previous studies have addressed the role of $\alpha 7$ nAChR in the orchestration of cholinergic anti-inflammatory pathway (CAP), which is a key element in modulation of neuroinflammation through restoring tissue redox homeostasis [41]. In detail, in the process of secondary SCI, neuroinflammation is facilitated when injured cells within the CNS release DAMPs (damage associated molecular patterns) like high mobility group box 1 protein (HMGB1) that bind to toll-like receptors (TLRs) and successively over-activate the NF- κ B, which is a crucial modulator in the initiation of inflammatory cascades and macrophages polarization. One of the main reasons for Nicotine therapeutic effects is that there is an opposite interaction between $\alpha 7$ nAChR and NF- κ B, presumably via Jak2/STAT3 signaling pathway [41]. In another word, activation of $\alpha 7$ nAChR could reduce neuroinflammation via inhibiting the expression of NF- κ B and therefore, reducing inflammatory cytokines production [42, 43]. Interestingly, it has been demonstrated that this pathway is reversible in case of MLA administration. Our results confirm this association as activation of $\alpha 7$ nAChR was accompanied by lower level of NF- κ B and subsequently higher TNF- α , IL-1 β , and IL-6 levels.

There is mounting evidence that $\alpha 7$ nAChR is associated with microglial activity. For instance, it was shown that pre-conditioning with Nicotine could prevent LPS-induced activated microglial cells from releasing pro-inflammatory cytokines and this effect was faded following the administration of MLA [44]. Several *in vivo* studies have also pointed to the anti-inflammatory effects of $\alpha 7$ nAChR on microglial cells. In a rat model of focal brain ischemia, Guan et al. discovered that Nicotine regulates microglial proliferation and correspondingly the levels of TNF- α , IL-1 β and neural loss even in the acute phase of post-injury and these effects were reversed by pre-treatment with α -bungarotoxin [45]. Several studies of CNS have exclusively discussed the role of $\alpha 7$ nAChR in the polarization status of M1/M2 macrophages. In two recent studies of brain ischemia, it was noticed that activation of $\alpha 7$ nAChR is related to the inhibition of NF- κ B, reduction of M1 markers (CD68, IL-1 β , TNF- α , IL-6) and lesion volume, elevation of M2 markers (CD206, Arg-1, IL-10), as well as both short-term and long-term behavioral recoveries [6, 7]. Meanwhile, Ma et al. demonstrated that electroacupuncture therapy could enhance axonal survival via activation of $\alpha 7$ nAChR that leads to reduction of M1 markers and simultaneous elevation of M2 markers in a rat model of brain stroke [8].

There has been increasing interest regarding the role of M1/M2 macrophages polarization paradigm in mediation of secondary SCI, as well. A few experiments have targeted this theory to find novel therapeutic options for SCI. The shift of macrophages to the M1 phenotype has been attributed to the activation of

NF- κ B in response to the binding of HMGB1 to TLRs. Results obtained from related investigations are consistent, suggesting higher levels of M1 markers (e.g., CD86) and lower levels of M2 markers (e.g., CD163, CD206) at the injury region of spinal cord. Observing higher concentrations of pro-inflammatory (e.g., TNF- α , IL-1 β , IL-6) and lower concentrations anti-inflammatory (e.g., IL-10) downstream markers confirms this alteration. Therefore, it is not surprising to detect neurodegenerative activities due to the established neuroinflammation [3, 17, 46]. Here, we consistently detected similar results in non-treated SCI rodents. Additionally, we discovered that elevation of α 7 nAChR level due to Nicotine therapy is correlated with shift reversion to M2 phenotype, probably through inhibition of NF- κ B. To the best of our knowledge, this is the first study that addresses the association between α 7 nAChR and macrophages polarization, indicating congruence with previous CNS models.

This study has several limitations. Promotion in SCI outcomes could be related to either suppression of excessive inflammation through inhibiting M1 polarization or enhancement of axonal repair through stimulating M2 polarization; these two seem undistinguishable *in vivo* models. However, recent innovative points of view highlight the importance of M1/M2 plasticity, suggesting that there is a dynamic time-dependent interaction between the two mechanisms after SCI [47, 48]. After all, in this study, we concluded that Nicotine could improve SCI outcomes and neuroinflammation possibly via activation of α 7 nAChR; however, further clarification of the binding lines between α 7 nAChR and macrophages polarization pattern is warranted in SCI setting [49]. On a side note, studies containing larger sample size are recommended to provide better insight into the dose-dependent effects of Nicotine.

Conclusion

In summary, we indicated that Nicotine could play a promising role in the treatment of SCI motor and sensory adverse events due to its anti-inflammatory and neuroprotective effects, possibly via increasing the expression of α 7 nAChR. However, these effects might vary depending on the content, timing and selectivity. Additionally, it should be noted that NF- κ B seems to link neuroinflammation transpiring within secondary SCI to α 7 nAChR as a key modulator of CAP in microglial polarization. Precise blueprints of these effects require further attention.

Abbreviations

SCI

Spinal cord injury; MLA:Methyllycaconitine; BBB:Basso, Beattie, Bresnahan; α 7 nAChR:Alpha-7 nicotinic acetylcholine receptor; NF- κ B:Nuclear factor-kappa B; IL:Interleukin; H&E:Haematoxylin and Eosin; CNS:Central nervous system; NIH:National institutes of health; PBS:Phosphate buffered saline; IHC:Immunohistochemistry; ELISA:Enzyme-linked immunosorbent assay; DAPI:4',6-Diamidino-2-phenylindole; CD:Cluster of differentiation; TNF- α :Tumor necrosis factor-alpha; RT-PCR:Reverse transcription polymerase chain reaction; RNA:Ribonucleic acid; DNA:Deoxyribonucleic acid; GLM:General linear model; GAPDH:Glyceraldehyde-3-phosphate dehydrogenase; MD:Mean difference; CI:Confidence interval; DPI:Days post injury; TBI:Traumatic brain injury; DAMPS:Damage associated molecular patterns;

HMGB1:High mobility group box 1; TLR:Toll like receptor; Jak2/STAT3:Janus kinas/ Signal transducer and activator of transcription; CAP:Cholinergic anti-inflammatory pathway.

Declarations

Conflicts of interest

Authors have no conflict of interest to disclose.

Acknowledgments

None

Authors contribution

AB, KM, ZES, SB, PRSA, SH, MMK, and MK participated in data acquisition, molecular assessments, and preparation of the manuscript. ARD and SMA designed the manuscript, provided the outlines for the presentation of the study, supervised the study process and edited the final manuscript. All authors have reviewed the process of data analysis, writing of the manuscript and approved the final article.

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Availability of data and materials

The datasets used and/or analyzed during this study are available on reasonable request from the corresponding author.

Ethics approval and consent to participate

The study protocol was executed in agreement with the National Institutes of Health (NIH) Guide for the Care and Use of laboratory animals (NIH publication No. 86-23, Eighth Ed.) and institutional and governmental concerns for animal care and use (Approval ID: IR.TUMS.MEDICINE.REC.1398.922).

Consent for Publication

Not applicable

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Figures

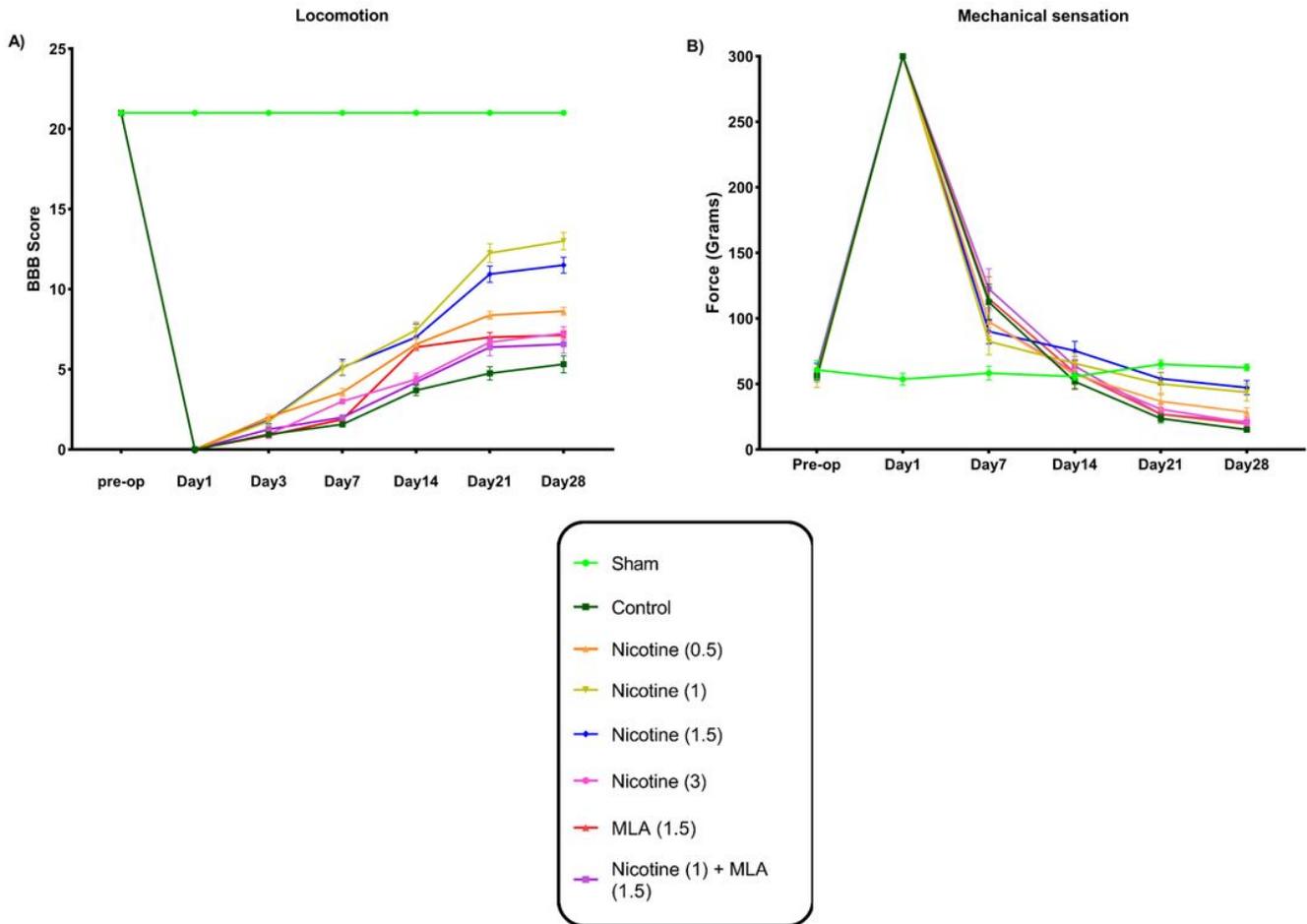


Figure 1

Repeated measure for comparison of the effects of the treatment groups on motor and sensory complications of SCI during the study period. A) BBB locomotor scores of the groups during 28-days period. Values are represented as the mean \pm standard error mean (SEM). B) Von Frey scores for the assessment of the mean resistance of the study groups to mechanical pressure. Values are represented as the mean \pm SEM.

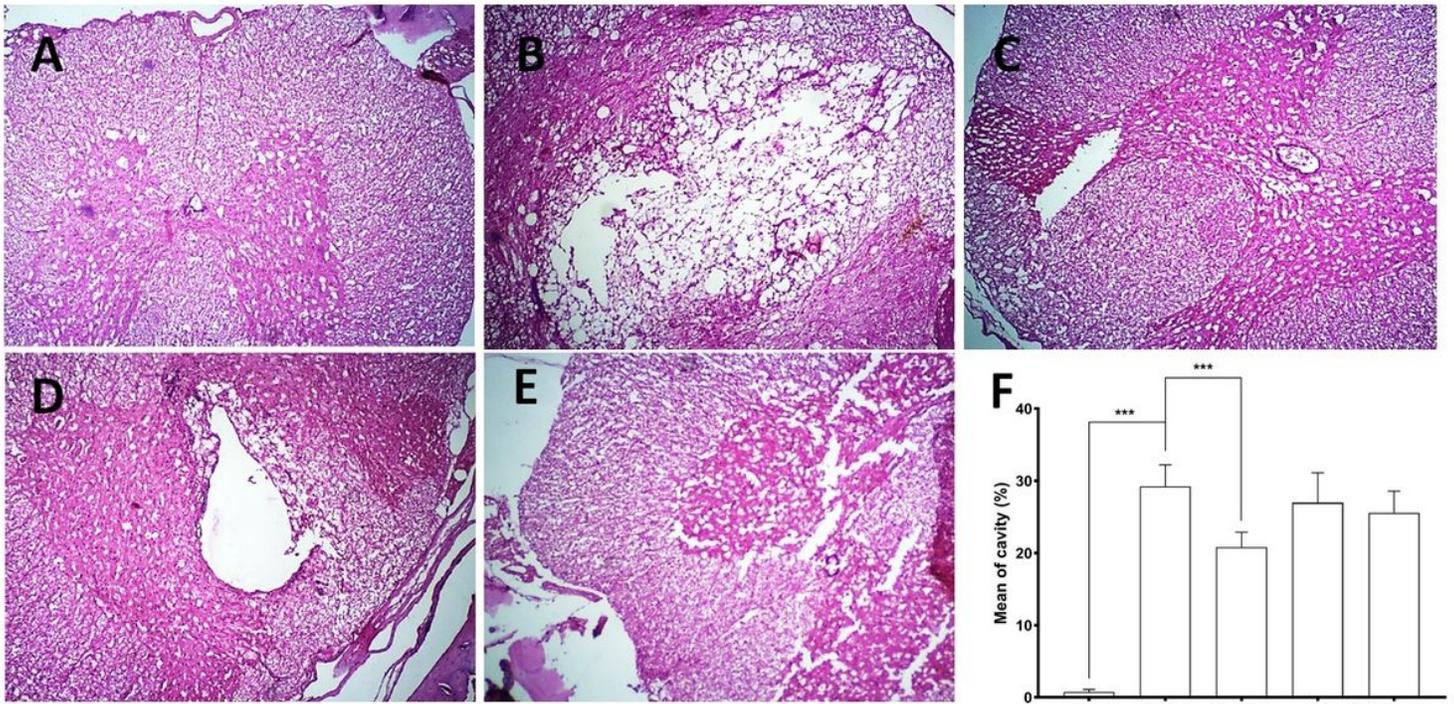


Figure 2

Histopathological pictures of the groups, produced by H&E staining of spinal cord sections at the injury region after a period of 28 days: (A) sham, (B) control, (C) Nicotine (1 mg/kg), (D) MLA (1.5 mg/kg), (E) Nicotine (1 mg/kg) + MLA (1.5 mg/kg). (F) analyzes the differences of cavity formation between the groups (mean \pm standard deviation). The cavity percentage was significantly lower in the sham group compared to the others. Significant differences with the control group were shown in the graph. ***: $p < 0.001$.

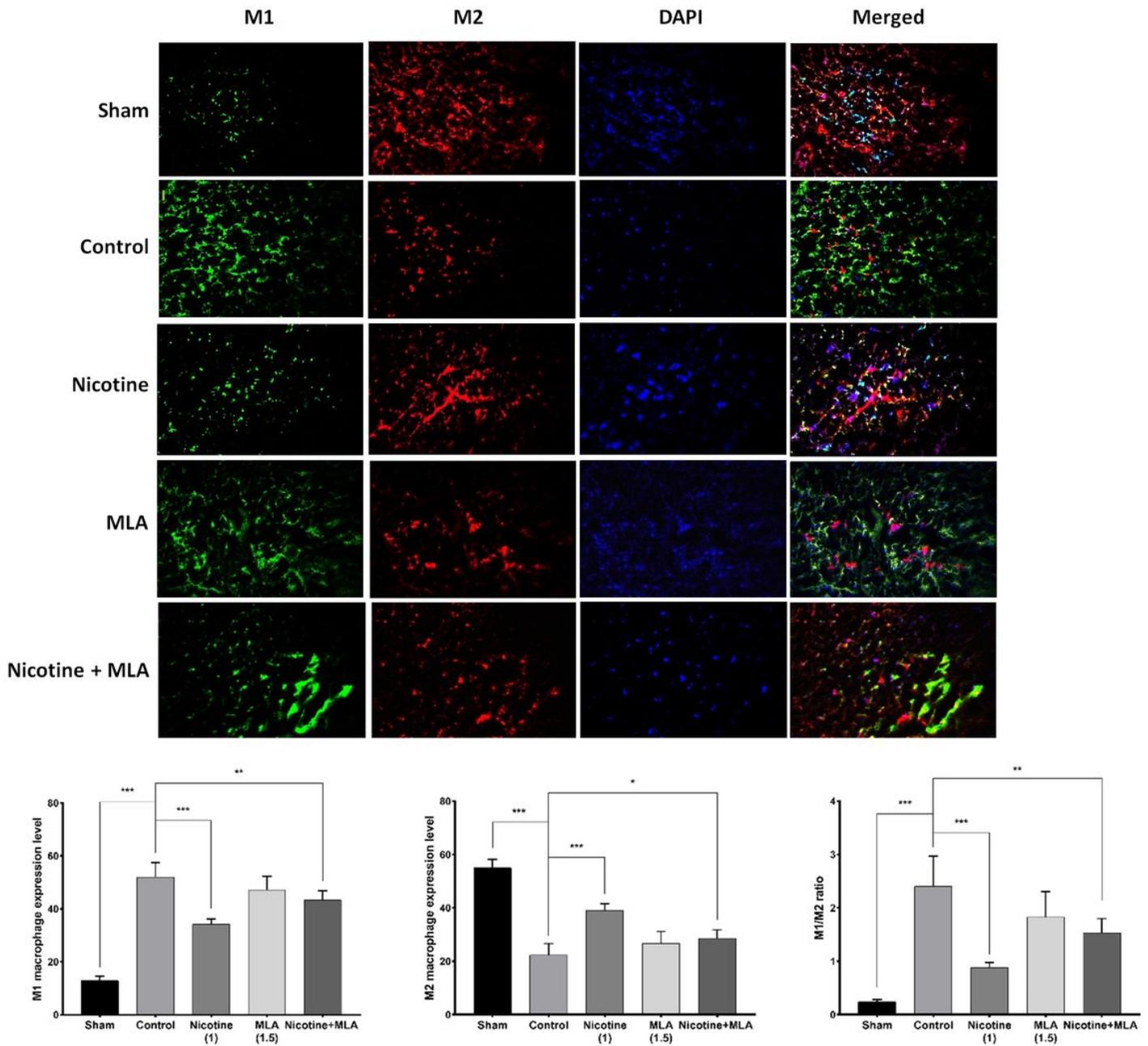


Figure 3

Immunohistochemical staining of M1 and M2 macrophages in the injury site of spinal cord. Bar charts depict the expression of M1 and M2, as well as the relative M1/M2 ratio in the study groups (mean \pm standard deviation). *: $0.01 < p < 0.05$; **: $0.01 < p < 0.001$; ***: $p < 0.001$.

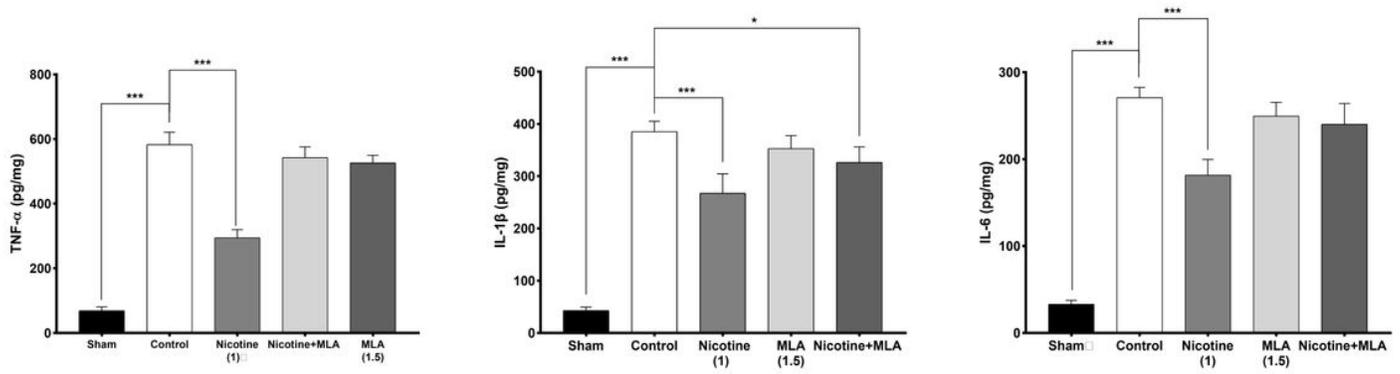


Figure 4

Comparison of pro-inflammatory factors, including TNF- α , IL-1 β , and IL-6 between the groups 28 days after SCI. Values are represented as the mean \pm standard deviation. Significant differences with the control group are shown. *: $0.01 < p < 0.05$; ***: $p < 0.001$.

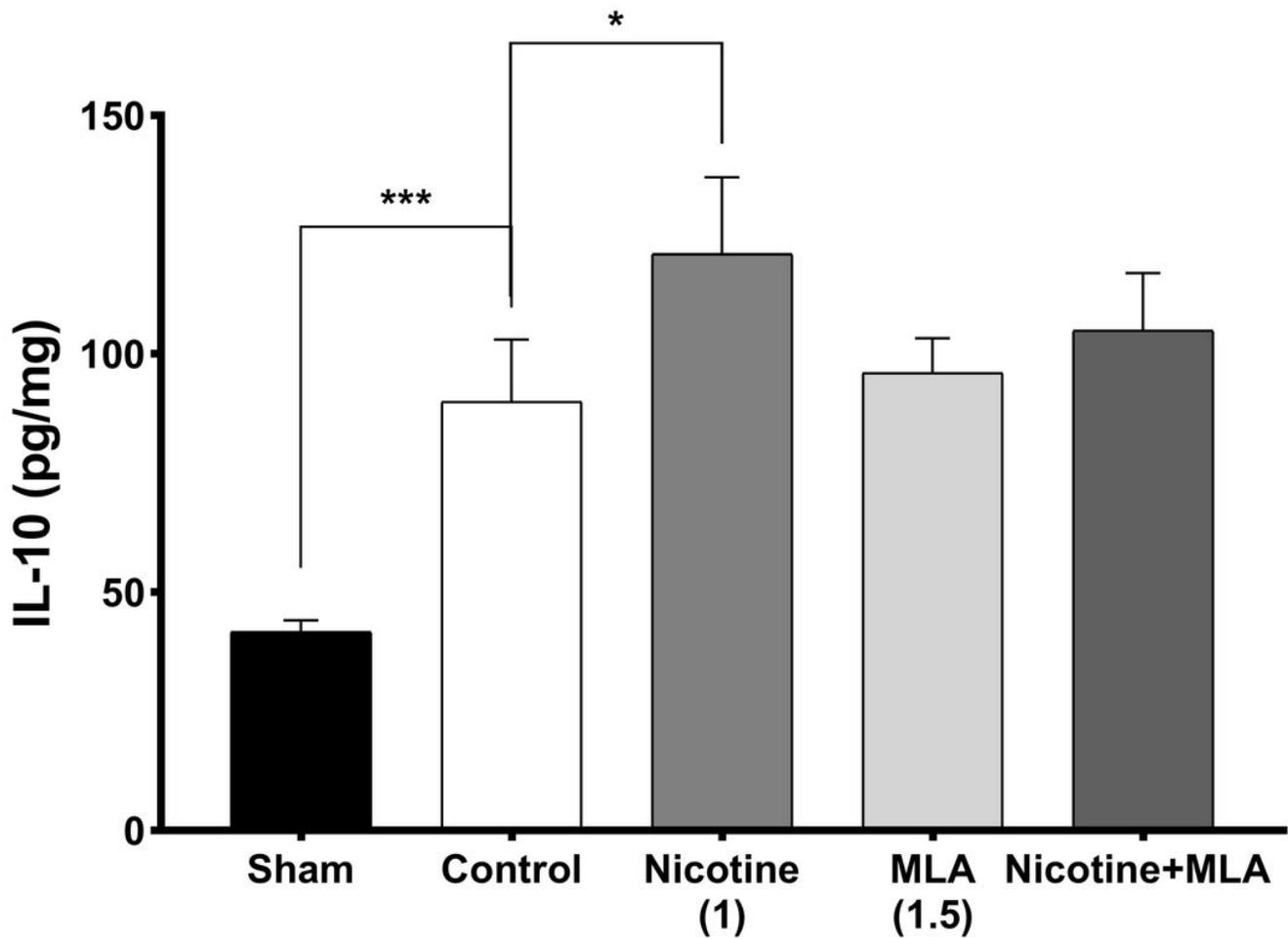


Figure 5

Comparison of IL-10 level between the groups. Values are reported as the mean \pm standard deviation. Significant differences with the control group are indicated. *: $0.01 < p < 0.05$; ***: $p < 0.001$.

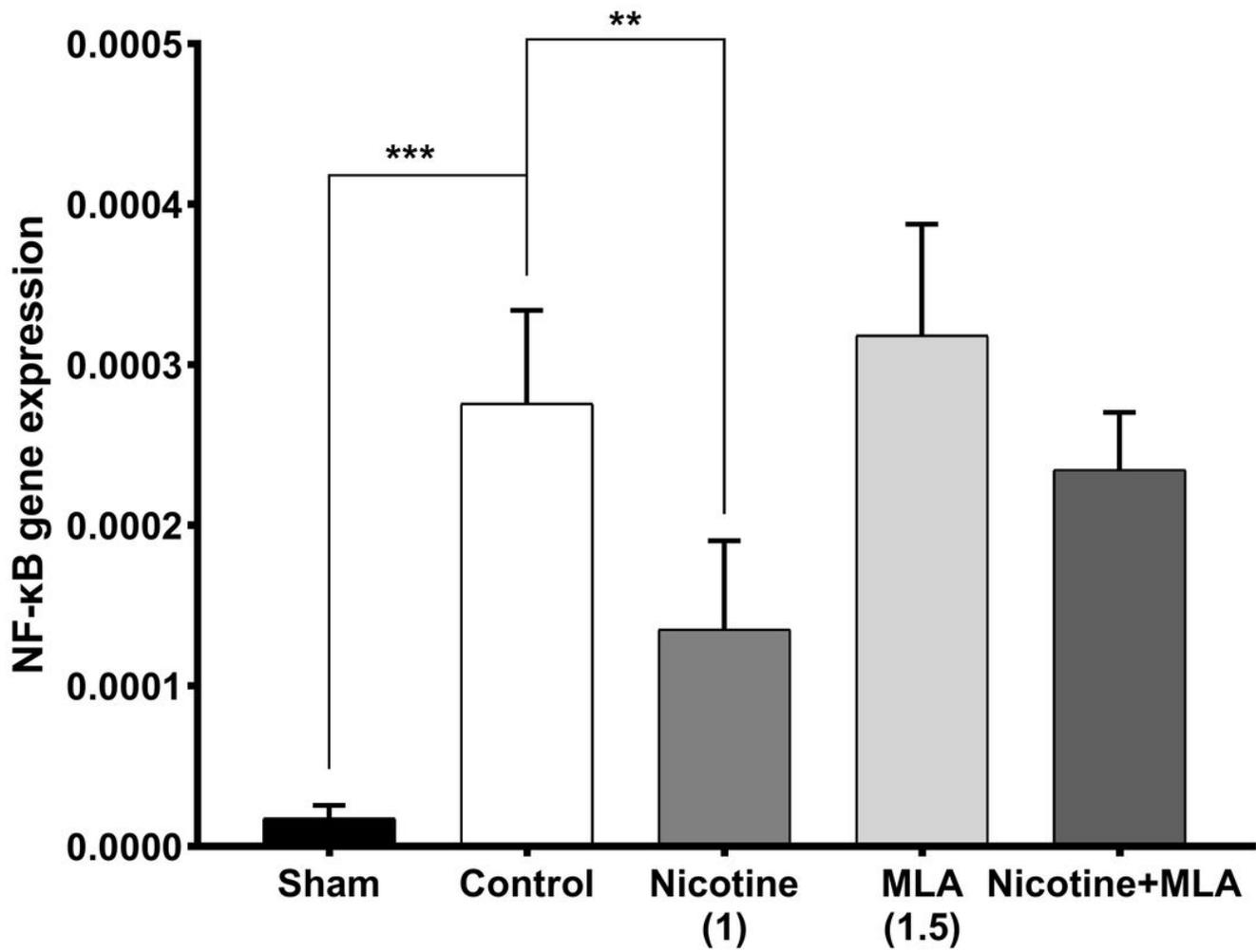


Figure 6

Nuclear factor kappa b (NF- κ B) gene expression in the study groups. Significant differences with the control animals are manifested. **: $0.01 < p < 0.001$; ***: $p < 0.001$.

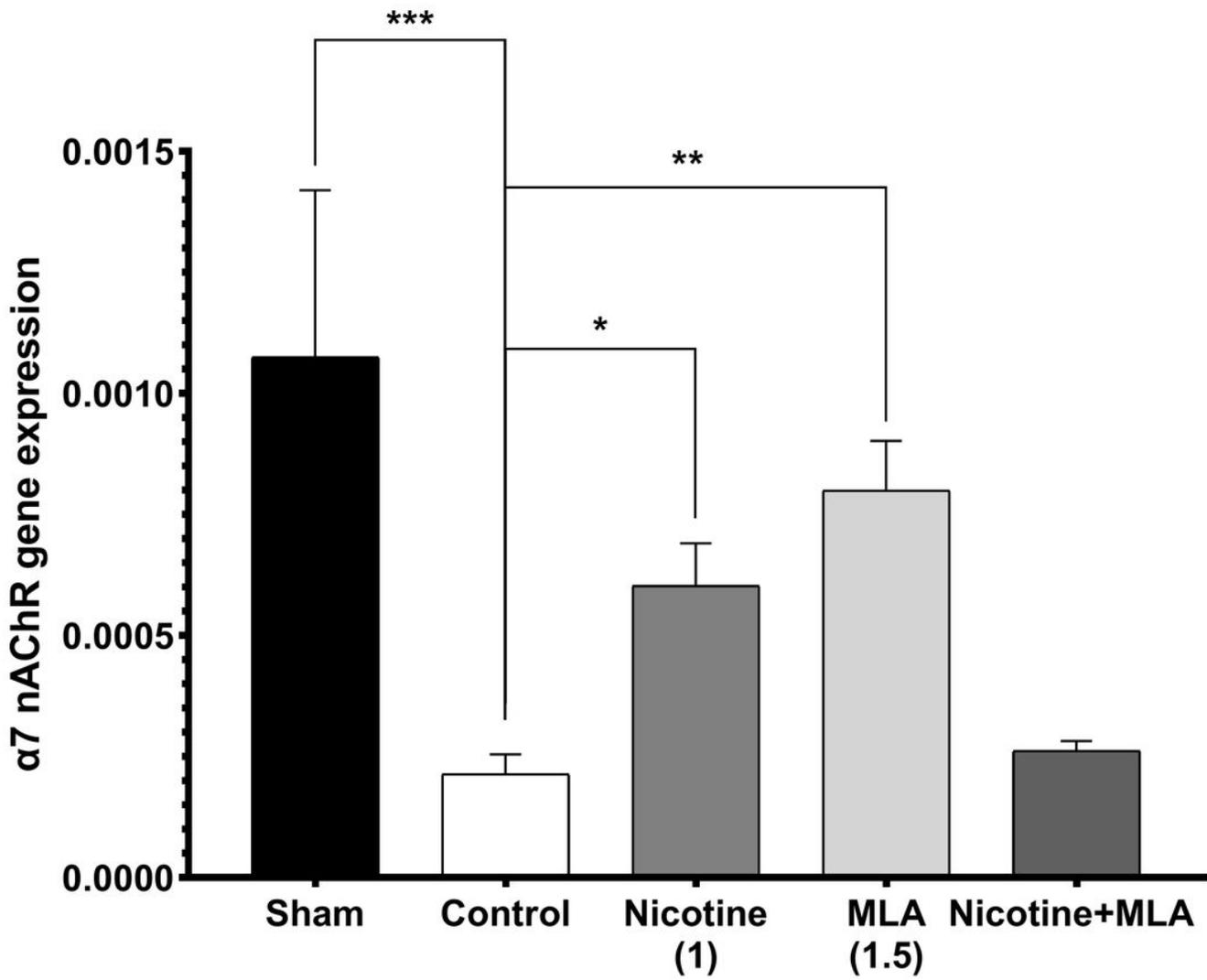


Figure 7

Alpha-7 nicotinic acetylcholine receptor ($\alpha 7$ nAChR) gene expression in the study groups at 28 dpi. Significant differences with the control group are marked. *: $p < 0.05$; **: $0.01 < p < 0.001$; ***: $p < 0.001$.

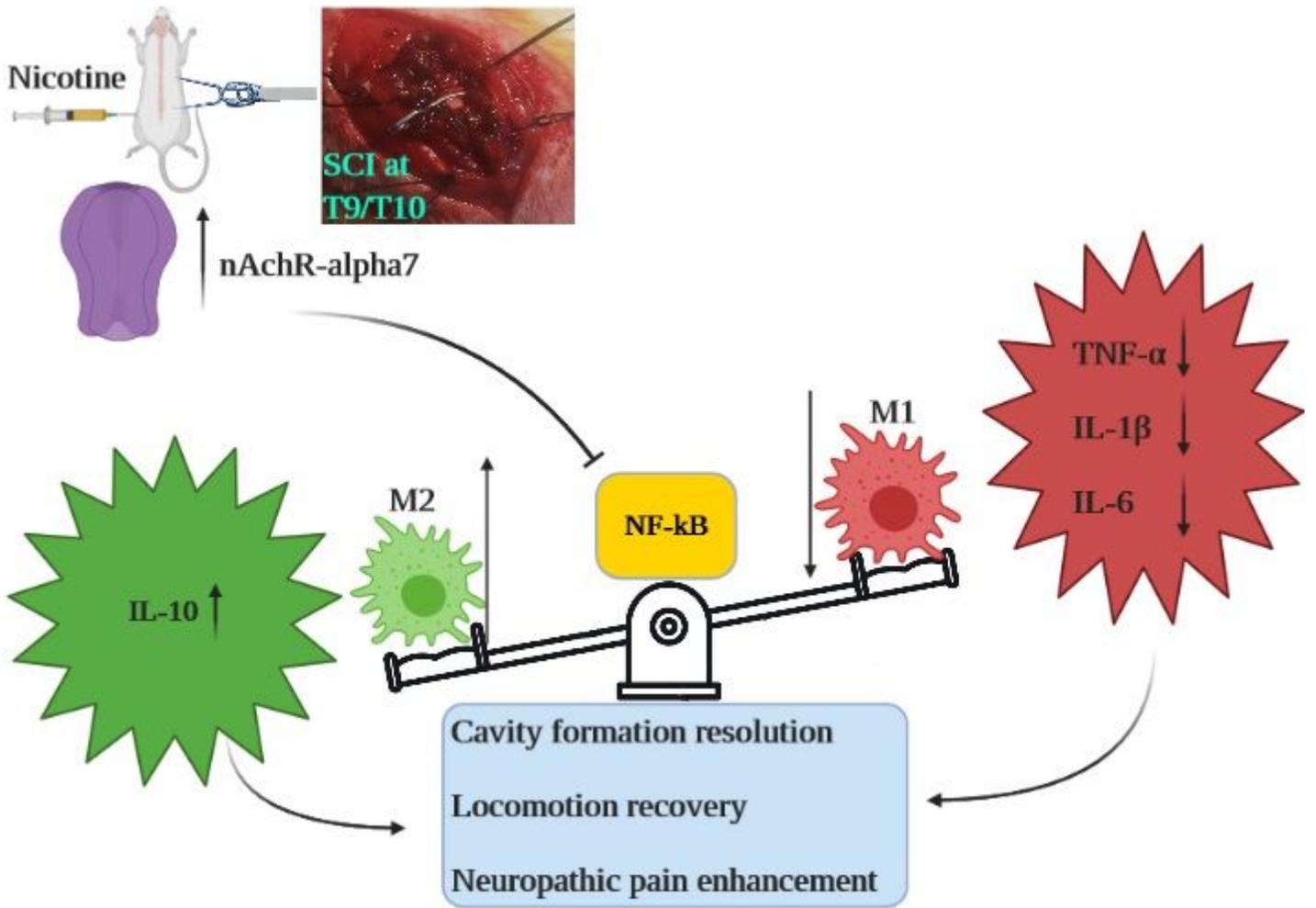


Figure 8

Cavity formation, M1 macrophages, and inflammatory factors