

Original Research Article

Implication of cholinergic transmission in rat model of spinal cord injury: A potential therapeutic target

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Abstract

Purpose: To assess the involvement of cholinergic transmission in the etiology of spinal cord injury (SCI) in a rat model.

Methods: Male adult rats (Wistar) with body weight ranging from 200 to 250 g were equally allocated into 2 groups: test (SCI) and control (non-SCI). Clipping method was used to induce SCI. Thereafter, motor function was measured using rotarod. Each rat was sacrificed by decapitation, and the cortex was excised for use in the study of the involvement of cholinergic transmission in SCI using real time quantitative polymerase chain reaction (RT-PCR) and western blot analysis (WBA).

Results: Significant upregulation in acetylcholine esterase (AChE) was observed in the cortex of SCI rats, relative to non-SCI rats ($p < 0.005$). Results from cholinergic receptor binding studies revealed significantly decreased maximum binding (B_{max}) and dissociation constant (K_d) values for muscarinic receptors in SCI rats, when compared to non-SCI rats. Moreover, the reduction in intensity of cholinergic receptors was significantly greater in the cerebral cortex of SCI group compared to non-SCI group.

Conclusion: The results of this study suggested that the reduction in cortical cholinergic transmission impairs motor functions in SCI, and plays a major role in motor deficits in SCI.

Keywords: Spinal cord injury, Cholinergic receptor, Acetylcholine esterase, Nicotinic receptor, Muscarinic receptor

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INTRODUCTION

Spinal cord injury (SCI) is one of the leading causes of severe incapacitation associated with high health care expenses [1,2]. Globally, SCI affects more than 2 million individuals [2]. Understanding CNS pathways after SCI is key to establishing an accurate treatment for the disease [1]. During SCI, changes at neurotransmitter levels in motor cortex are

helpful for the accurate understanding of brain-spinal cortex synchronization. Studies have reported the participation of several cortical areas in the control of motor function in individuals with stroke during the recovery period [1-4]. However, majority of these studies have failed to show accurate mechanism of recovery in the stroke patients. Thus, understanding the effect of neurotransmitter adaptations in cortex could help in recognizing its role in controlling

motor functions [5-8].

The acetylcholine released from cortex has several functional roles, one of which is regulation of motor function. The roles of cholinergic receptors in regulating spinal cord functions are well-known [9-12]. The muscarinic receptors in CNS regulate learning and memory, and are also involved in controlling several sensory, motor, and autonomic routes. Muscarinic acetylcholine receptor plays a vital role in the functioning of sensory and motor structures [3]. Acetylcholine is produced through acetyltransferase. In neurons, acetylcholine is transferred into synaptic vesicles using acetylcholine transporter. It is then hydrolyzed by acetylcholinesterase, resulting in execution of specific pharmacological actions. Several studies have reported the use of choline acetyltransferase as potential bio-marker for assessing the status of cholinergic conduction, where it serves as indicator for the functional activity of cholinergic innervations [13-15]. Currently, very little is known about the involvement of cholinergic transmission in rat model of motor deficits. Thus, the current study was intended to evaluate the position of the cholinergic pathway in SCI.

EXPERIMENTAL

Animals

Male adult rats (Wistar) having body weight from 200 to 250 gram were kept in isolated cages with 12-h day/12-h night cycle, with *ad libitum* access to rat feed and drinking water. The study protocol of this experiment was approved by Animal Ethics Committee of the Hunan Academy of Medical Sciences and Hunan Provincial People's Hospital, China (approval no. AEC-HPPH/238/2018), and followed the principle laid down in the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and National Research Council's Guide for the Care and Use of Laboratory Animals [16]. The rats were equally allocated in 2 groups: test (SCI) and control (non-SCI). Chronic SCI was induced using the clipping method. Each rat was sacrificed by decapitation, and the cortex was dissected and stored at -80°C prior to assays.

Motor function assessment using rotarod test

Following the induction of SCI, the motor function of the rats was tested using rotarod test. In this test, each rat was trained 5 times before taking actual reading to assess its motor function. The actual reading was recorded for each rat at

different speeds (rpm): low (10 rpm), medium 15 rpm, and high/fast (25). In addition, retention time was measured at these rpm values in both groups.

RT-PCR and Western blot assay (WBA)

Real-time PCR assay was conducted in 96-well kits in a PCR instrument. The PCR assay was performed with the primers for AChE, ChAT, M1 receptor, M3 and nicotinic receptor, with RT- β -actin as internal control. Total protein (approx. 50 μg) was extracted from cells or tissues of cortex and fractionated using 10 % SDS-polyacrylamide gel electrophoresis. The bands were then transferred to nitrocellulose membrane, and images were captured using Odyssey Infrared Imaging System. The loading control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Immunohistochemistry

Dissected cortex was immersed in phosphate buffer (pH 7.4) and 4 % paraformaldehyde solution for 60 min and then put in sucrose solution (30 %). After 1 h, the cortex was sliced into different sections using cryostat. Each section of cerebral cortex was treated with phosphate buffer at pH 7.4 for half an hour, and then incubated with muscarinic or nicotinic acetylcholine receptor antibody. Images of cerebral cortex sections were taken using confocal imaging method. The expressions of muscarinic and nicotinic acetylcholine receptors were evaluated using pixel intensity technique.

Receptor-binding assays

Receptor-binding was determined using Scatchard method for assessment of receptor binding variables such as B_{max} (maximum binding), and k_d (dissociation constant). Usually, B_{max} is used to measure expression of receptors available in cortex sample, while k_d is an index of the affinity of the muscarinic and nicotinic receptors for ligands.

Statistical analysis

Statistical analysis of data was performed using SPSS statistical analysis software. Comparison of retention times and the expressions of muscarinic and nicotinic receptors in cortical region between both groups (SCI and non-SCI) were analyzed by *t*-test. Data related to muscarinic receptor binding analysis in cortex for both groups were analyzed using non-parametric test (Mann Whitney test). Pixel intensities in the

cortex between both groups were analyzed by non-parametric test.

RESULTS

Rotarod results

In the rotarod test, significantly lower retention time was observed in SCI rats than in the non-SCI rats at all rotations (10, 15 and 25 rpm) ($p = 0.003$; Figure 1).

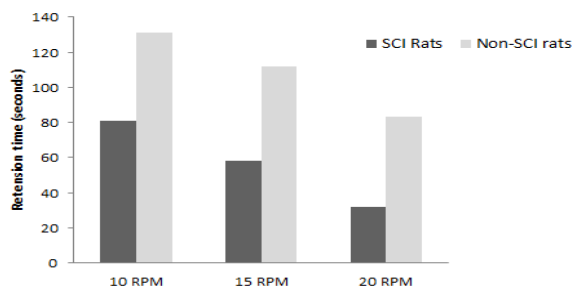


Figure 1: Comparison of retention times (seconds) after rotarod test in rats with and without SCI; * $p < 0.001$, compared to rat without SCI

Expressions of AChE and choline AChE

Results from RT-PCR assay revealed that the expressions of AChE and choline AChE were significantly greater in SCI group compared to non-SCI group. Western blot analysis showed significantly higher upregulation of AChE in the cortex region of SCI group as compared to non-SCI group (Figure 2).

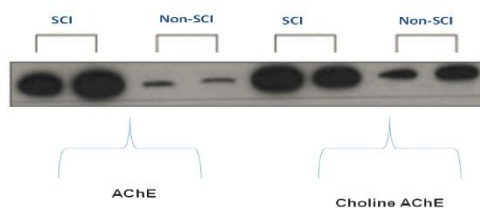


Figure 2: Western blot analysis showing the expressions of AChE and choline AChE in cortex regions of SCI and non-SCI rats

Expressions of muscarinic and nicotinic acetylcholine receptors

Receptor binding analysis using pixel intensity showed that the expressions of muscarinic and nicotinic acetylcholine receptors were significantly decreased in SCI rats, when compared to non-SCI rats (Figure 3).

Muscarinic receptor binding

Muscarinic receptor binding analysis in the cortex regions of SCI and non-SCI rats showed that

B_{max} and k_d values for muscarinic receptor type 1 (MRT1), MRT3 and total MR were significantly lower in SCI group as compared to non-SCI group. Results are shown in Table 1.

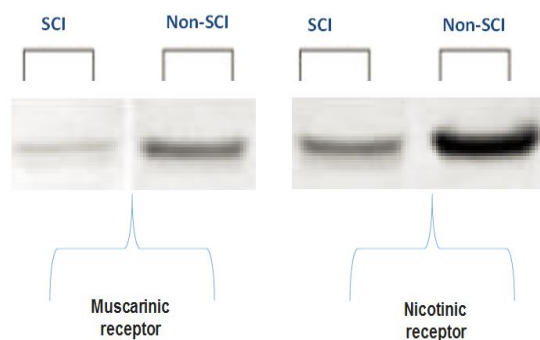


Figure 3: Western blot analysis showing the expressions of muscarinic and nicotinic receptors in cortical regions of SCI and non-SCI rats

Table 1: Muscarinic receptor binding analysis showing B_{max} and k_d for MRT1, MRT3 and total MR in the cortex region

Variable	SCI rats (n=20)	Non-SCI rats (n=20)
Muscarinic receptor (total)*		
B_{max} (fmol per mg protein)	113.1±3.10	221.1±13.10
k_d (nM)	0.41 ± 0.21	2.41 ± 0.01
Muscarinic receptor type 1 (MRT1)*		
B_{max}	123.4± 11.70	253.2 ± 18.5
k_d	3.17 ± 0.33	8.87 ± 0.13
Muscarinic receptor type 3 (MRT3)*		
B_{max}	124.1± 15.70	241.1± 15.7
k_d	1.31 ± 0.92	4.61 ± 0.81

Data are presented as mean ± SD. n: total number of subjects; * $p < 0.001$

Pixel intensity

There was significantly greater reduction of pixel intensity (mean) in cortex region of SCI rats, relative to non-SCI rats (Table 2). This indicates reduced intensity of cholinergic receptors in cortex region of SCI rats. A similar trend in low pixel intensity was observed in the assessment of nicotinic receptors in the cortex region of SCI rats. Pixel intensity was significantly reduced in SCI rats, when compared to non-SCI rats. The cumulative results of both receptors indicated decreased activities of nicotinic and muscarinic receptors in cortex region of SCI rats when compared to non-SCI group.

Table 2: Pixel intensity in cortex of SCI and non-SCI rats

Variable	Rats with SCI (n=20)	Non-SCI rats (n=20)
Muscarinic receptor (MRT1)*	28.13 ± 3.14	67.18 ± 4.14
Nicotinic receptor (alpha 7)*	32.19 ± 2.15	84.43 ± 8.35

Data are presented as mean ± SD. n: total number of subjects; * $p < 0.001$

DISCUSSION

The results of current study showed that there was significant decrease in activity of cholinergic receptors, which led to reduction in motor function associated with the cholinergic receptor or neurotransmitter in SCI. Cholinergic receptor function was impaired in the SCI rats. In addition, it was observed that the activity of AChE, the enzyme activity which regulates cholinergic transmission, was significantly impaired in SCI rats, when compared with non-SCI rats. This indicates reduced intensity of cholinergic receptors in cortex region of SCI rats, relative to non-SCI rats. Low pixel intensities were observed for cholinergic receptors in cortex region of SCI rats.

The results of the present study suggest a metabolic impairment in cholinergic system of CNS. In addition, a gene expression of acetylcholine esterase was significantly higher in the cortex region of SCI rats. This resulted in accentuated degradation of acetylcholine which also controls motor functions, and hence impaired cholinergic transmission in SCI. In SCI, increased expression of acetylcholine esterase results in low levels of acetylcholine, which further reduces motor functions. Increased expression of acetylcholine esterase has been observed in Alzheimer disease, a neurodegenerative disorder [17-19]. Defects in motor function in SCI are possibly due to impaired transmission of cholinergic pathways.

The role of cholinergic receptors (nicotinic and muscarinic receptors) in regulating spinal cord functions have been previously reported [13-15,17,18]. Muscarinic receptors in CNS are associated with the regulation of learning and memory, and are also involved in controlling several sensory, motor, and autonomic routes. Muscarinic receptor of acetylcholine plays a vital role in functioning of sensory and motor structures [3]. The expressions of several receptors in nociceptive pathway are changed in the dorsal horn region of spinal cord after peripheral nerve injury. In this situation, the

stimulation of nicotinic receptor of acetylcholine stimulates survival of spinal motor neurons [19]. These results indicate that impairment of cholinergic transmission acts as one of key contributors to motor deficits in SCI. Thus, cholinergic system may be a useful target for an effective treatment option for motor deficits associated with SCI.

CONCLUSION

This study demonstrates that impairment of cholinergic transmission via cholinergic receptors (muscarinic and nicotinic) may result in development of SCI in rats. The reduction in cholinergic transmission in the cortex impairs motor function and plays a major role in motor deficits in SCI. Thus, the targeting of the cholinergic system may help to find effective treatment for motor deficits in SCI.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors involved in design of study, data acquisition, analysis and writing including revising the paper. All authors have read and approved the manuscript for publication.

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