Effect of acetylcholine in rat brain in promoting early recovery of spinal cord injury by inhibiting monoamine oxidase enzyme: Pre-clinical evidence

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Abstract: Spinal cord injury (SCI) is one of the most common causes of severe incapacity and has been associated with high health care expenditures. The present study designed to assess the effect of acetylcholine in rat brain (cortex) promotes early recovery of SCI by inhibiting monoamine oxidase enzyme. Male adult wistar rats (200–250 gram in body weight) were kept in isolated cages with 12-day and dark cycle, and were offered regular food and water (as and when required) during the day. Protocol was approved by ethics committees of Soochow University. Rats were distributed into two groups: 1) Test group: SCI group, treated with acetylcholine; 2) Control group (SCI group, not treated with acetylcholine). SCI was induced by clipping between T12 and T13 vertebra. Rats were surrendered by decapitation, and the cortex was removed and was stored at −80°C for investigation. Acetylcholine inhibited acetylcholinesterase enzyme and MAO isoform (MAO-A or -B) in the cerebral cortex. Motor function of rats after inducing SCI was tested using rotarod test, and retention time was measured at different RPM speed (low [10rpm], medium [15] and high/fast [25]) in both the groups. In the rotarod test, the rats with treated with acetylcholine had significantly more retention time as compared to control rats SCI at all rotations (10, 15 and 25 rpm). Whereas, the rats of the control group had significantly lesser retention time as compared to rats with treated with acetylcholine at all rotations (10, 15 and 25 rpm) [p=0.003]. Our study results showed significant improvement in activity of cholinergic and monoaminergic transmission, which thereby improve motor function in rats with SCI after treatment with acetylcholine. This study results suggested, that there is need to target MAO enzyme in brain for developing effective treatment for SCI. The finding of this study gives the new idea to researchers to develop therapy for treatment of SCI based on MAO enzyme target.

Keywords: Spinal cord injury, acetylcholine, monoamine oxidase enzyme, acetylcholinesterase enzyme.

INTRODUCTION

Spinal cord injury (SCI) is one of the most common reasons of severe incapacity and has been linked with high health care expenditures. Generally, SCI affects 2 to 5 million of individuals, thus understanding its etiology pathways in SCI is a key to begin an accurate treatment for SCI (Dong, 2006; Erickson, 1994; James, 1981; Jiang, 2007). Majority of these studies have failed to conclude accurate mechanism of recovery among SCI patients. Thus, understanding the effect of neurotransmitter variations in cortex could help to recognize its role in controlling motor functions (Kuhar, 1978; Kus, 2003; Levine, 1997; Lotze, 2006). Acetylcholine releases from hippocampus and cortex, has several functional role including motor function. The role of cholinergic receptors in controlling spinal locomotor system is previously identified (Messi, 1997; Mooradian, 1988; Myers, 2005). Role of cholinergic receptors in CNS is well known for the regulation of learning and memory, and 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 (James, 1981). Acetylcholine is produced mainly through acetyltransferase enzyme. In neuron, acetylcholine is supplied into synaptic vesicles using Acetylcholine transporter. Acetylcholine is then fragmented using acetyl cholinesterase enzyme, which is distributed in several tissue to execute specific pharmacological action (Marc, 1991; Mess, 1997; Saltarelli, 1987; Ward, 2000).

Also, it has been reported that monoamine oxidases (MAO) is present in various part of CNS and spinal cord area, and has been associated with oxidative stress which generate hydrogen peroxide, results in disruption of key biochemical parameters such as noradrenaline, adrenaline and dopamine (Osman, 2008; Ozaita, 1997). With the good understanding of monoamine oxidase involvement in SCI, one can establish an accurate treatment for SCI based on monoamine oxidase inhibition. Reduction in noradrenaline (NA) and serotonin (5-HT) after SCI has been well established in pre-clinical studies. It has been observed that the delay in SCI recovery possibly due to reduction in levels of monoamines such as NA and 5 HT. The possible reason of decreased in monoamines levels could be over activity of monoamine oxidase enzyme, which is not yet established in either in pre-clinical or in

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clinical studies. Reduction in NA and 5 HT results in delayed SCI recovery as shown in few animal studies. We hypothesize that inhibition of monoamine oxidase enzyme (both types: MAO-A and MAO-B) may improve the SCI recovery by increasing level of NA and 5 HT in brain or spinal cord area. Moreover, reduction in cholinergic transmission in cortex impairs the motor function in SCI, and it plays a major role in motor deficits in SCI. Thus, there is a need of treatment which improves cholinergic transmission by activating cholinergic system, and also which inhibits monoamine oxidase enzyme for effective treatment of SCI. Effect of acetylcholine in inhibition of MAO enzyme is well established (Osman, 2008; Ozaita, 1997). The present study designed to assess the effect of acetylcholine in rat brain (cortex) promotes early recovery of SCI by inhibiting monoamine oxidase enzyme.

MATERIALS AND METHODS

In this study, male adult Wistar rats (200–250 gram in body weight) were kept in isolated cages with 12-day and dark cycle, and were offered regular food and water (as and when required) during the day. Protocol was approved by ethics committee of Soochow University, China. All the animal maintenance procedure was in promise with Institutional ethics committee. Male adult Wistar rats were equally distributed in two groups: 1) Rats with SCI (test group); 2) Rats without SCI (control group). Spinal cord injury was caused by clipping vertebra (T12 and T13). Rats were sacrificed by beheading, and cerebral cortex was separated and was kept at −80°C for analysis.

Motor function of rats after inducing SCI was tested using rotarod test. In rotarod test, each rat was trained for 5 times before taking actual reading to assess its motor function. The actual reading was recorded for each rat at different RPM speed (low [10rpm], medium [15] and high/fast [25]). Retention time was measured at different RPM speed (low [10rpm], medium [15] and high/fast [25]) in both the groups. Acetylcholinesterase enzyme activities in the prefrontal cortex, hippocampus and hypothalamus were measured using the validated method. Dissected brain regions were crushed in chilled phosphate buffer (alkaline pH) with the help of Mixer/homogenizer. The mixture was centrifuged at one thousand G for 10 minutes at 4 0C temperature. Each sample was mixed with chilled phosphate buffer (alkaline pH), and then small amount of ACT (acetyl thiocholine iodide) and DTNB were added to the mixture. Acetyl cholinesterase degrades ACT into two compounds: 1) thiocholine (TC) and acetate. Thereafter, TC reacted with DTNB results in yellow color, which measure acetyl cholinesterase activity. Intensity of yellow color is directly proportional to acetyl cholinesterase activity. Higher intensity of yellow color results in greater acetyl cholinesterase activity, which was measured using spectrophotometer at the absorbance of four hundred twelve nm. Absorbance after analysis of test and control sample was noted, and then enzyme activity in test and control group was assessed. Acetyl cholinesterase activity between test and control group was compared using appropriate statistical test. Also, in both groups (SCI group and non-SCI group), MAO was measured in brain tissue, using amended technique of Naois and his co-workers. Brain tissue was homogenized in ice cold solution containing sucrose and phosphate buffer, then sample was centrifuged (at 1.2 k G for 10 min). After centrifugation, supernatant solution was collected and subjected to centrifugation at 16k G for 10 min at 5 degree Celsius. Then, it is washed with phosphate buffer and put it in the same buffer for measurement of MAO enzyme (MAO-A and B). Serotonin has been used as substrate for MAO-A enzyme, whereas benzylamine has been used as substrate for MAO-B enzyme. Analysis was performed using spectrophotometer and absorbance was recorded at 242 nm. Moclobimide was used as MAO-A inhibitor, and has been used as +Ve control. For MAO-B, selegeline was used as MAO-B inhibitor, and has been used as +Ve control. Effect of Acetylcholine on expression of MAO enzymes was studied using by Western blot analysis. Sample was dispersed in formaldehyde solution (4%), and was stained with eosin/hematoxylin. Then it was freeze in N2O (liquid) and then kept at -20°C for subsequent investigation. Western blot analysis was performed using protein sample, which was removed from tissues/cells, and then fixed with gel electrophoresis and stimulated with cellulose sheath. Then, the cellulose sheath was dwindling at 4°C during whole night along with antibodies with MAO.

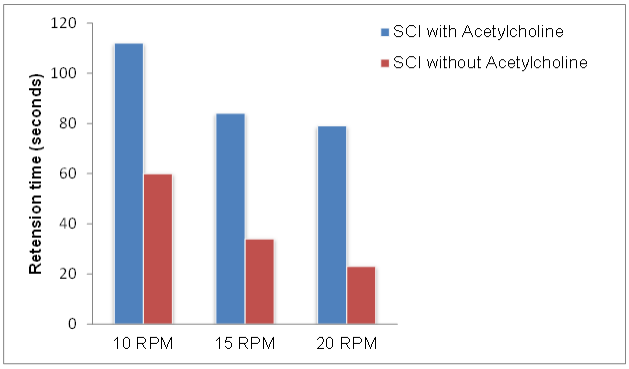
Since this study was designed as a preliminary, thus there is no formal sample size calculation was executed for this study. We have planned to include at least 20 male rats in each group (so total 40 rats in both groups). Data/variables which fall under numerical data were analyzed using appropriate statistical test, namely t test (Gaussian distribution) or Mann Whitney test (non-Gaussian distribution). All the statistical analysis was performed using SPSS statistical analysis software.

RESULTS

A total of 40 rats included in this study (n=20 in each group), all 40 male adult Wistar rats were subjected into statistical analysis. Before bio-chemical analysis, all rats were subject to rotarod test to assess motor function. In rotarod test, the SCI rats treated with acetylcholine had significantly more retention time as compared to SCI rats without acetylcholine treatment at all rotations (10, 15 and 25 rpm). Fig. 1 comparing the retention time in SCI rats with and without treatment of acetylcholine.

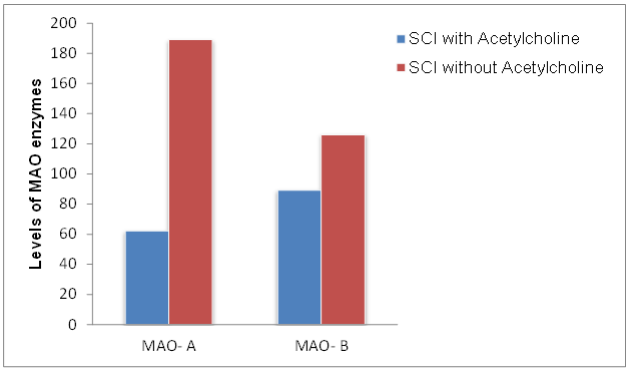
Western blot analysis showed a statistical significant downregulation of MAO enzymes activity was observed

in the cerebral cortex of rats treated with acetylcholine as compared to rats with without acetylcholine treatment [p<0.0023]. Also, a statistical significant down regulation of MAO-A/B enzymes activity was observed in the cerebral cortex of rats treated with acetylcholine as compared to rats treated without acetylcholine (fig. 2).



Abbreviation: SCI= Spinal Cord Injury; RPM: Rotation per minutes

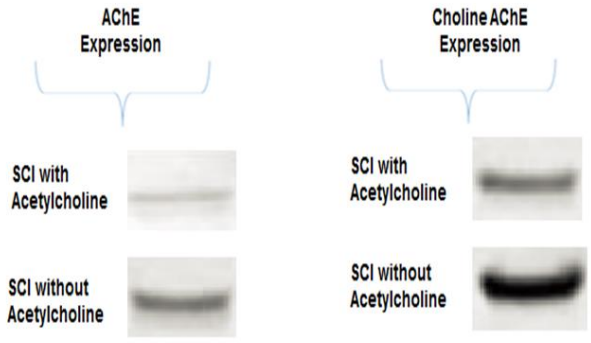
Fig. 1: Comparison of retention time (seconds) after rotarod test in rats with and without treatment of Acetylcholine



Abbreviation: SCI= Spinal Cord Injury; MAO=Monoamine oxidase

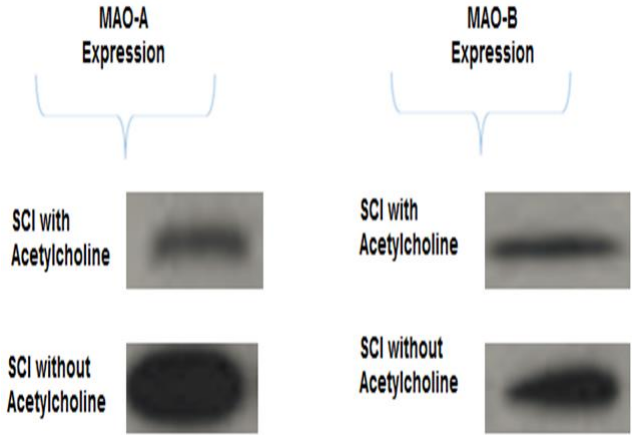
Fig. 2: Level of MAO-A and MAO-B enzyme in cortex region of SCI rats with and without treatment of Acetylcholine

In addition, we have observed improve cholinergic transmission in rats treated with acetylcholine as compared to rats treated without acetylcholine [p<0.001]. Fig. 2 comparing the expression of MAO-A and MAO-B in rats treated with acetylcholine as compared to rats treated without acetylcholine. Fig. 3 comparing the expression of AChE and choline AChE in rats treated with acetylcholine as compared to rats treated without acetylcholine. Fig. 4 comparing the expression MAO enzymes activity (MAO-A and MAO-B) in rats treated with acetylcholine as compared to rats treated without acetylcholine. There was significant greater reduction of pixel strength for cholinergic and monoaminergic activity in cortex region of rats that were not treated with acetylcholine as compared to the rats that were treated with acetylcholine (table 1). Pixel strength for cholinergic and monoaminergic activity in cortex region was greater in rats who were treated with acetylcholine as compared to the rats that were not treated with acetylcholine This indicates greater intensity of cholinergic and monoaminergic transmission in cortex region of in rats that were treated with acetylcholine as compared to the rats who were not treated with acetylcholine.



Abbreviation: SCI= Spinal Cord Injury; AChE = Acetyl cholinesterase

Fig. 3: Western blot analysis showed expression of AChE and choline AChE in cortex region of SCI rats with and without treatment of Acetylcholine



Abbreviation: SCI= Spinal Cord Injury; MAO=Monoamine oxidase

Fig. 4: Western blot analysis showed expression of monoamine oxidase A and B in cortex region of SCI rats with and without treatment of Acetylcholine

DISCUSSION

Spinal cord injury is a one of common cause of disability, responsible for morbidity and mortality worldwide (Messi, 1997; Mooradian, 1988; Myers, 2005; James, 1981; Jiang, 2007; Osman, 2008; Ozaita, 1997). It has been reported that the cholinergic and monoaminergic transmission in brain plays an important role in governing

Table 1: Pixel intensity in cortex in SCI rats with and without treatment acetylcholine

Variables	SCI rats with Acetylcholine (N=20)	SCI rats without Acetylcholine (N=20)
Cholinergic Activity		
Muscarinic receptor	89.23 (2.44)*	45.18 (3.14)
Nicotinic receptor	83.32 (1.34)*	49.13(2.15)
Monoaminergic Activity		
MAO-A	83.13 (1.24)*	36.18 (1.14)
MAO-B	79.12 (3.14)*	29.36 (1.15)

Data are presented as mean (SD). *P value less than 0.001 compared to SCI rats without Acetylcholine
Abbreviation: N: total number of subjects; SCI= Spinal Cord Injury; MAO=Monoamine oxidase

motor function (James, 1981; Jiang, 2007; Osman, 2008; Ozaita, 1997). To establish an accurate treatment for Spinal cord injury, understanding of cholinergic and monoaminergic transmission involvement in spinal cord injury is critical.

This study results showed there was significant improvement in activity of cholinergic and monoaminergic transmission, which thereby improve motor function in rats with SCI after treatment with acetylcholine. In this study, we found that there was impaired functioning of cholinergic and monoaminergic transmission in SCI rats. In addition, we noted that the enzyme activity which regulates cholinergic and monoaminergic transmission was significantly impaired in SCI rats that were not treated with acetylcholine. Whereas the enzyme activity which regulates cholinergic and monoaminergic transmission was significantly improved in SCI rats that were treated with acetylcholine. Our finding suggested that there was metabolic impairment in cholinergic and monoaminergic system of CNS as we saw that there was significantly higher expression of ACh esterase and MAO enzyme in rats, which was reduced after treatment with acetylcholine. Reduced expression of ACh esterase and MAO enzyme in cortex results after acetylcholine treatment improves motor functions in rats with SCI (Ward, 2003; Osman, 2008; Ozaita, 1997; James, 1981; Yamamura, 1974). In SCI, over activation of acetylcholine esterase and MAO enzyme results in low levels of acetylcholine, which further reduces motor functions. Increased expression of acetylcholine esterase and MAO enzyme has seen in several neurodegenerative disorders such as Alzheimer disease (Wessler, 2008; Yamamura, 1974; Zaninetti, 1999; Osman, 2008; Ozaita, 1997). Deficits in motor function in SCI condition is possibly due to impaired transmission of cholinergic and monoaminergic pathways. Acetylcholine releases from hippocampus and cortex, has several functional role including motor function. The role of cholinergic system in controlling spinal locomotor system is previously identified (Saltarelli, 1987; Ward, 2003; Wessler, 2008; Yamamura, 1974; Zaninetti, 1999). In summary, the present study suggest new therapeutic target in the managing of Spinal cord injury. This study results suggested, that there is need to target MAO enzyme in brain for developing effective treatment for Spinal cord injury. The finding of this study gives the new idea to researchers to develop therapy for treatment of SCI based on MAO enzyme target. We inspire conducting well designed clinical study to confirm the finding of this study in patients with SCI.

CONCLUSION

Our study results showed significant improvement in activity of cholinergic and monoaminergic transmission, which thereby improve motor function in rats with SCI after treatment with acetylcholine. This study results suggested, that there is need to target MAO enzyme in brain for developing effective treatment for Spinal cord injury. The finding of this study gives the new idea to researchers to develop therapy for treatment of SCI based on MAO enzyme target. We inspire conducting well designed clinical study to confirm the finding of this study in patients with SCI.

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