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Spinal cord regeneration by modulating bone marrow with neurotransmitters and Citicholine: Analysis at micromolecular level

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ABSTRACT

Background: Spinal cord injury results in disruption of brain-spinal cord fibre connectivity, leading to progressive tissue damage at the site of injury and resultant paralysis of varying degrees. The current study investigated the role of autologous bone marrow modulated with neurotransmitters and neurotransmitter stimulating agent, Citicholine, in spinal cord of spinal cord injured rats.

Methods: Radioreceptor assay using [3H] ligand was carried out to quantify muscarinic receptor. Gene expression studies were done using Real Time PCR analysis.

Results: Scatchard analysis of muscarinic M1 receptor showed significantly decreased B_{max} ($p < 0.001$) and K_d ($p < 0.01$) compared to control and significant reversal ($p < 0.001$) in both the treatment groups (spinal cord injury treated with 5HT and GABA, and spinal cord injury treated with Citicholine). Muscarinic M1 receptor gene expression in spinal cord injured group showed significant down regulation ($p < 0.001$) compared to control, and both the treatment groups significantly reversed ($p < 0.001$) these changes to near control when compared to spinal cord injured group. The confocal microscopic study using specific antibody of muscarinic M1 confirmed the gene expression studies.

Conclusion: Thus our results suggest that the neurotransmitters combination along with bone marrow or Citicholine with bone marrow can reverse the muscarinic receptor alterations in the spinal cord of spinal cord injured rats, which is a promising step towards a better therapeutic intervention for spinal cord injury because of the positive role of cholinergic system in regulation of both locomotor activity and synaptic plasticity.

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At a glance commentary

Scientific background on the subject

Spinal cord injury (SCI) affects many pathways in brain and spinal cord. A proper understanding of alterations in central signalling will help in devising better treatment options. The present study investigated the role of autologous bone marrow modulated with neurotransmitters and Citicholine in spinal cord injured rats for functional recovery.

What this study adds to the field

Promising results in our animal study showed that treatment with 5HT and GABA with bone marrow aspirate, and Citicholine with bone marrow aspirate can facilitate functional recovery of altered muscarinic receptor function. The modulation of bone marrow aspirate by neurotransmitters or citicholine have opened a new treatment strategy in SCI.

Spinal cord injury (SCI) has permanent and devastating neurological deficits and disability. SCI is a progressively debilitating condition with poor prognosis and remote possibility of repair of the damaged neurons in the Central Nervous System [1]. The spinal cord acts as the primary information pathway between the brain and peripheral nervous systems of the body. SCI causes tissue damage through both primary and secondary mechanisms. The primary mechanical injury results in damage to neuronal and vascular tissue. Neurological dysfunction results more from the secondary changes than the primary neuronal damage [2]. Axons are damaged beyond repair and neural cell membranes are broken in spinal cord injury. During spinal shock, even undamaged portions of the spinal cord become temporarily disabled and cannot communicate normally with the brain. Therefore, effort has been undertaken to develop *in vivo* models of SCI and to study the cellular and molecular mechanisms of synaptic connections and information processing in the spinal cord.

Cholinergic motor neurons stimulate muscle contraction. The imbalance in the excitation and inhibition within the motor circuit disrupts coordinated body muscle contraction [3]. Acetylcholinesterase, the enzyme that catalyses a reaction of hydrolysis of acetylcholine to choline and acetate [4], is regarded as a specific marker for cholinergic function [5] and is used as a differentiation marker. Receptors activate a multitude of signalling pathways important for modulating neuronal excitability, synaptic plasticity and feedback regulation of acetylcholine (ACh) release [5].

Stem/progenitor cells derived from the ependymal region of the spinal cord have the ability to self-renew and are multipotential for neurons and glia. These cells have the ability to regenerate the injured mammalian spinal cord [6]. Treatments using human embryonic stem cells have shown improved mobility in rats with spinal cord injuries, providing the first physical evidence that the therapeutic use of these cells can help restore motor skills lost from acute spinal cord

tissue damage. Haematopoietic system is used as a source of progenitor cells for the CNS and it also has the property to differentiate into both microglia and macrophage when injected directly to the brain of adult mice. Although bone marrow cells (BMC) normally give rise to mesenchymal derivatives, such as osteoblasts, adipocytes, myoblasts and chondrocytes, recent studies indicated that these cells were capable of remarkable phenotypic plasticity. BMC is induced to differentiate into cells with surface markers characteristic of neurons [7].

The bulbospinal monoamine transmitters, released from serotonergic, noradrenergic, and dopaminergic systems, exert modulatory control over spinal sensory systems as acetylcholine, an intrinsic spinal cord biogenic amine transmitter. Generally, the monoamines facilitate motor activity [8]. The administration of Serotonin (5-HT) and Gamma aminobutyric acid (GABA) as therapeutic agents for cell proliferation and differentiation is a novel approach. Our earlier studies showed that 5-HT and GABA acting through specific receptor subtypes 5HT₂ (+) and GABA [9] respectively, control cell proliferation and act as co-mitogens. Serotonin and GABA along with bone marrow cells in combination showed reversal of glutamate receptors and motor abnormality shown in the Parkinson's rat model [9].

Citicholine is an exogenous source for acetylcholine synthesis, a key neurotransmitter [10]. Supplementation with Citicholine can increase the amount of choline available for acetylcholine synthesis and aid in rebuilding membrane phospholipid stores after depletion [11].

The main objective of the present study was to investigate role of autologous bone marrow modulated with neurotransmitters and neurotransmitter stimulating agent, Citicholine, in spinal cord of spinal cord injured rats for the functional recovery.

Materials and method

Animals

Male adult Wistar rats of 200–250 g body weight were used for the experiments. Each group consisted of 8–10 rats. They were housed in separate cages under 12-h light and 12-h dark periods and were maintained on standard food pellets and water *ad libitum*. All animal care and procedures were in accordance with Institutional, CPCSEA and National Institute of Health guidelines.

Experimental design

Under all aseptic precautions and ether anaesthesia, monoplegia was induced by shearing between the T9 and T12 vertebra of the experimental rats. A specially designed rubber chamber with silastic catheter [12] was inserted subcutaneously and the tip of silastic tube inserted to the injury site and fixed with sutures. Spinal cord injury was confirmed by monoplegia. Those rats that developed monoplegia after 3 h of the surgery were selected for further experiments. These rats were randomly divided into the following groups as (i) Control (C), (ii) Spinal cord injured (SCI), (iii) Spinal cord injured + Bone

Marrow + Serotonin + GABA (SCI + BM + 5HT + G) and (iv) Spinal cord injured + Bone Marrow + Citicholine (SCI + BM + CT). The spinal cord-injured group was given physiological saline solution through the chamber daily for 21 days. SCI + BM + 5HT + G group was given BM (10^5 cells) on day one followed by a combination of 5HT and GABA (7 mg/kg body weight, Sigma chemicals) through the chamber for 21 days. SCI + BM + CT group was given BM (10^5 cells) on day one followed by CT (7 mg/kg body weight, Lupin pharmaceuticals) through the chamber for 21 days. BM was aspirated using a needle inserted through the top of the femoral intercondylar groove.

Tissue preparation

Rats were sacrificed by decapitation on the 22nd day of the experiment. The injured spinal cord T9–T12 region was dissected out quickly over ice. The tissues collected were stored at -80°C until assayed.

Muscarinic M1 receptor binding studies in the spinal cord

Muscarinic M1 receptor binding assays were done using specific antagonist [^3H] QNB in the T9–T12 spinal cord region of control, injured and treatment groups. The tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50 mM Tris buffer, pH 7.4 containing 1 mM EDTA. The supernatant was then centrifuged at $30,000\times g$ for 30 min and the pellets were re-suspended in appropriate volume of Tris EDTA buffer, pH 7.4. Muscarinic M1 binding assay was done using different concentrations i.e., 0.1–2.5 nM of [^3H] QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250 μl containing appropriate protein concentrations (200–250 μg). Nonspecific binding for muscarinic M1 receptor was determined using 100 μM of pirenzepine (Sigma Chemical Co.). Tubes were incubated at 22°C for 60 min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50 mM Tris–HCl buffer pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

Analysis of the receptor-binding data

Linear regression analysis of scatchard plots

The data were analysed according to Scatchard. The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis.

Analysis of gene expression by real-time pcr

RNA was isolated from the spinal cord using Tri reagent. Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit. Real-Time PCR assays were performed in 96-well plates in ABI 7300 Real-Time PCR instrument (Applied Biosystems). PCR analysis were conducted with gene-specific primers and fluorescently labelled Tag for muscarinic M1 (designed by Applied Biosystems). Endogenous control, β -actin was labelled with a report dye, VIC. All reagents were purchased from Applied Biosystems. The thermocycling

profile conditions were as follows: 50°C for 2 min – Activation, 95°C for 10 min – Initial Denaturation, 95°C for 15 s – Denaturation 40 cycles, 50°C for 30 s – Annealing, 60°C for 1 min – Final Extension. The $\Delta\Delta\text{CT}$ method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) of the target mRNAs to the CT-values of the internal control β -actin in the same samples ($\Delta\text{CT} = \text{CT Target} - \text{CT } \beta\text{-actin}$). It was further normalized with the control ($\Delta\Delta\text{CT} = \Delta\text{CT} - \text{CT Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta\text{CT}}$).

Protein determination

Protein was measured by the method of using bovine serum albumin as standard [13].

Muscarinic M1 receptor expression studies in the spinal cord of control and experimental rats using confocal microscope

Control and experimental rats were deeply anesthetized with ether. The rat was transcardially perfused with PBS, pH- 7.4, followed by 4% paraformaldehyde in PBS. After perfusion the spinal cord T9–T12 region were dissected and immersion fixed in 4% paraformaldehyde for 1 h and then equilibrated with 30% sucrose solution in 0.1 M PBS, pH-7.0. 10 μm sections were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBST (PBS in 0.01% Triton X-100) for 20 min. Spinal cord slices were incubated overnight at 4°C with rat primary antibody for muscarinic M1 (Sigma Aldrich, diluted in PBST at 1: 1000 dilution). After overnight incubation, the spinal cord slices were rinsed with PBST and then incubated with secondary antibody of FITC (Chemicon, diluted in PBS at 1: 1000 dilution).

Histochemical analysis using TOPRO-3

Experimental rats were anesthetized with urethane (0.75 mg/kg body weight intraperitoneal) and transcardially perfused with Phosphate Buffer Saline (PBS) (pH-7.4) followed by 4% paraformaldehyde in PBS. After perfusion spinal cord was dissected out and fixed in 4% paraformaldehyde for 1 h and then equilibrated with 30% sucrose solution in PBS (0.1 M). 10 μm sagittal sections of spinal cord were taken using Cryostat (Leica, CM1510 S). Nuclear stain TOPRO-3 (diluted 1: 1000 in PBS) was added and kept for 10 min at room temperature. The sections were observed and photographed using confocal imaging system (Leica TCS SP 5). Quantification was done by using Leica Application Suit Advanced Fluorescence (LASAF) software by considering the mean pixel intensity was directly related to the fluorescence emitted from the sections. The nuclear densities of the experimental groups are correlated with mean pixel intensity patterns. The fluorescence obtained depends on the number of receptors specific to the added nuclear stain or antibody.

Statistical analysis

Statistical evaluations were done with analysis of variance (ANOVA), using GraphPad Instat (version 2.04a, San Diego, USA).

Table 1 Scatchard analysis of muscarinic M1 receptor using [³H] qnb binding against pirenzepine in the T9–T12 region of spinal cord of control and experimental groups of rats.

| Animal status | Bmax (fmol/mg protein) | Kd (nM) |
|-----------------------|------------------------------|----------------------------|
| C | 585.3 ± 56.76 | 2.81 ± 0.16 |
| SCI | 328.8 ± 29.88 ^a | 2.16 ± 0.14 ^b |
| SCI + BM | 354.8 ± 25.80 ^a | 2.05 ± 0.10 ^b |
| SCI + BM + 5HT + GABA | 474.5 ± 39.74 ^{a,d} | 2.59 ± 0.15 ^{b,e} |
| SCI + BM + CT | 465.9 ± 32.97 ^{a,d} | 2.55 ± 0.14 ^{b,e} |

Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 8–10 rats. Abbreviations: C: Control; SCI: Spinal Cord Injury; SCI + BM: Spinal Cord injury + Bone Marrow; SCI + BM + 5HT + G: Spinal Cord Injury + Bone Marrow + Serotonin + GABA; SCI + BM + CT: Spinal Cord injury + Bone Marrow + Citicholine; a: $p < 0.001$; b: $p < 0.01$ when compared to control; d: $p < 0.001$; e: $p < 0.01$ when compared to spinal cord injured.

Results

Muscarinic M1 receptor binding in the spinal cord of control and experimental group of rats

The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on the X-axis and bound/free on the Y-axis. The B_{max} is a measure of the total number of receptors present in the tissue and the K_d is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity. Scatchard analysis of [³H] QNB binding against

pirenzepine to study muscarinic M1 receptor binding parameters showed a significant decrease ($p < 0.001$) in B_{max} and K_d in the spinal cord of spinal cord injured rats when compared to control. SCI + BM + 5HT + GABA and SCI + BM + CT groups showed a significant reversal ($p < 0.01$) in B_{max} and K_d to near control when compared to spinal cord injured rat [Table 1].

Muscarinic M1 gene expression in spinal cord of control and experimental group of rats

Relative quantification method was used to analyse real time PCR data. The $\Delta\Delta CT$ method of relative quantification was used to determine the fold change in expression. Real-time PCR analysis of muscarinic M1 receptor mRNA showed a significant down regulation ($p < 0.001$) in spinal cord injured rats when compared to control. SCI + BM + 5HT + GABA and SCI + BM + CT groups showed a significant reversal ($p < 0.001$) to near control when compared to spinal cord injured rat [Fig. 1].

Muscarinic M1 receptor antibody staining in spinal cord of control and experimental group of rats using confocal microscopy

The sections were observed and photographed using confocal imaging system (Leica SP 5). Expression of muscarinic M1 receptor was analysed using pixel intensity method. The given pixel value is the net value which is deducted from the negative control pixel value.

The muscarinic M1 receptor antibody staining in the spinal cord showed significant down regulation ($p < 0.001$) in spinal cord injured rats when compared to control. SCI + BM + 5HT + GABA and SCI + BM + CT groups showed a

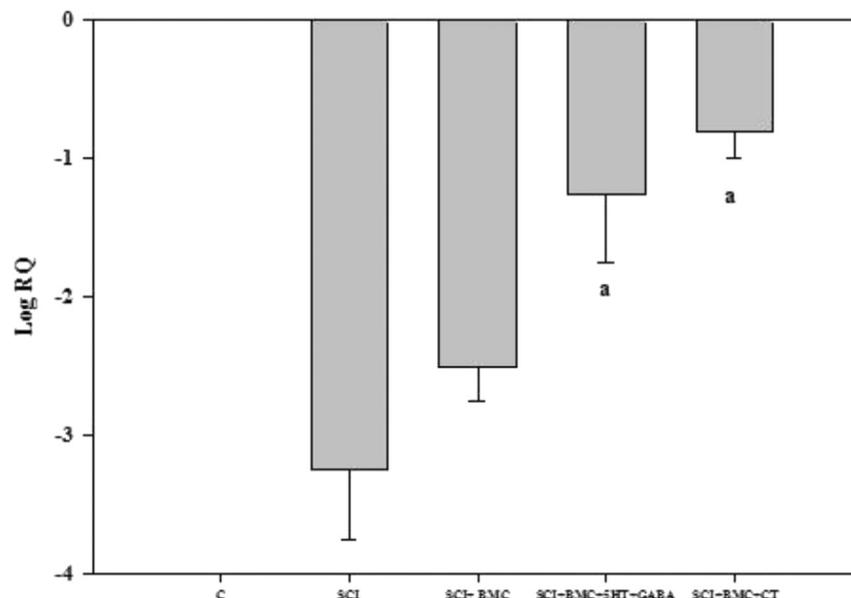


Fig. 1 Real Time PCR Amplification of Muscarinic M1 Receptor mRNA in the T9–T12 Region of Spinal Cord of Control and Experimental Groups of Rats. Values are Mean ± S.E.M. of 4–6 separate experiments. Each group consists of 8–10 rats. a $p < 0.001$ when compared to control. Abbreviations used: C: Control; SCI: Spinal Cord Injury; SCI + BMC: Spinal Cord injury + Bone Marrow; SCI + BMC + 5HT + GABA: Spinal Cord Injury + Bone Marrow + Serotonin + GABA; SCI + BMC + CT: Spinal Cord injury + Bone Marrow + Citicholine; a: $p < 0.001$; b: $p < 0.01$ when compared to control; d: $p < 0.001$; e: $p < 0.01$ when compared to spinal cord injured.

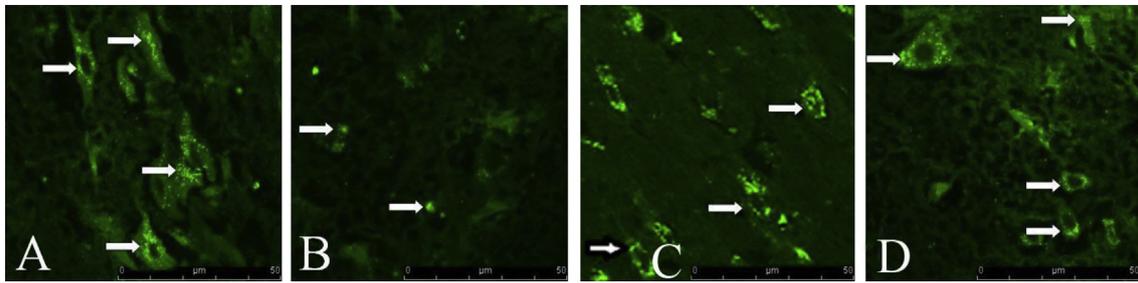


Fig. 2 Confocal image of muscarinic M1 receptors of Control (A), Spinal Cord Injury (B), Spinal Cord Injury + Bone Marrow + 5HT + GABA (C) and Spinal Cord injury + Bone Marrow Cell + Citicholine (D) rats using immunofluorescent microscope with muscarinic M1 receptor specific primary antibody and FITC labeled as secondary antibody. Arrows in white shows muscarinic M1 receptors. Scale bar = 50 μm . The arrows depict neuronal visualisation in treatment groups similar to the control which is not seen in injured group.

significant reversal ($p < 0.001$) to near control when compared to spinal cord injured rats. The arrows depict neuronal visualisation in treatment group similar to the control which is not seen in injured groups [Fig. 2].

Histochemical analysis using TOPRO-3 staining

TOPRO-3 staining showed a significant increase ($p < 0.001$) in the nuclear staining in spinal cord injured rats when compared to control. SCI + BM + 5HT + GABA and SCI + BM + CT groups showed a significant reversal ($p < 0.001$) to near control when compared to spinal cord injured rats. This indicates the marked increase in cell density in the spinal cord of treatment groups [Fig. 3].

Discussion

The pathophysiological changes caused by spinal cord injury affect multiple systems, the extent of which is related to the severity and level of injury. After the primary mechanical injury to spinal cord, there is a complex secondary injury cascade that leads to the progressive death of otherwise potentially viable axons and cells, which in turn impairs endogenous recovery processes [14]. Neuroprotective strategies aimed at preventing damage arising from secondary injury processes provide some hope for tissue sparing and improved functional outcome. This, in conjunction with the

fact that current treatment options are limited, hastens the need to find novel therapeutic agents.

The present study has investigated the role of bone marrow aspirate in combination with neurotransmitters like GABA and Serotonin, and bone marrow aspirate with Citicholine, for the treatment spinal cord injured rats. Transplantation approaches using cellular bridges [15], foetal central nervous system cells [16,17], fibroblasts expressing neurotrophin-3 [18], hybridoma cells expressing inhibitory protein-blocking antibodies, or olfactory nerves ensheathing glial cells transplanted into the acutely injured spinal cord have produced axonal regrowth or functional benefits [19]. Pittenger et al., reported that mesenchymal stem cells derived from the bone marrow, differentiated into osteocytes, chondrocytes and adipocytes [7]. The multipotent adult progenitor cells derived from the bone marrow [20] which comprise approximately 0.125% of the total bone marrow cells [21,22], are multipotent stem cells with the capacity to differentiate, under specific experimental conditions [23], into several different types of cells, including osteoblasts, adipocytes, chondrocytes, skeletal muscle fibres, cardiomyocytes, hepatocytes, neural cells and epithelial cells of the lung and intestinal tract. It has recently been reported that the bone marrow-derived cells also have the potential to develop into neural lineages, such as neurons and astrocytes, both *in vivo* [24] and *in vitro* [25]. Bone marrow cells that are adherent in the culture of bone marrow aspirates have already been used for the treatment of the injured spinal

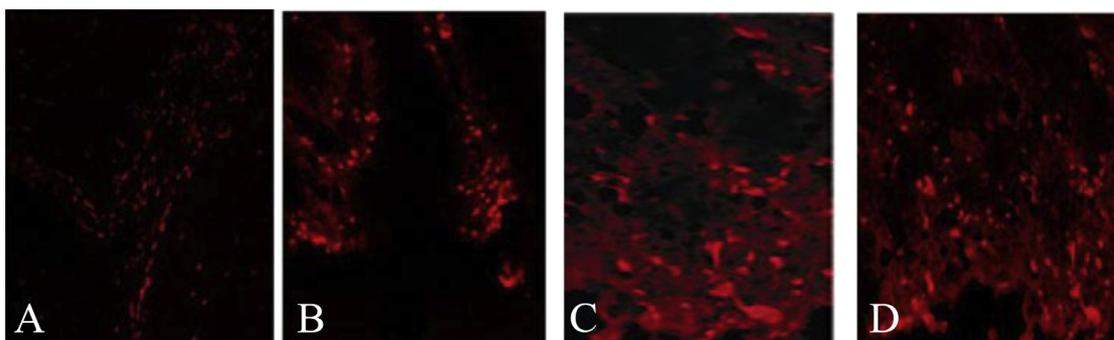


Fig. 3 Confocal image of TOPRO 3 staining of Control (A), Spinal Cord Injury (B), Spinal cord injury + Bone marrow + 5HT + GABA, (C) and Spinal Cord injury + Bone Marrow l + Citicholine, (D) Scale bar = 50 μm .

cord and brain [26]. The transplantation of bone marrow cells by direct injection into the lesion might promote tissue repair in the injured spinal cord. The effects of transplanted bone marrow cells on tissue repair, as described above, suggest that some trophic factors might be released from bone marrow cells to promote the tissue repair [27].

Neurotransmitters relay, amplify and modulate signals between neurons and are the key regulators of movement. 5-HT and GABA, acting through specific receptor subtypes 5HT₂ and GABA_{A,B}, control cell proliferation as co-mitogens [9]. GABA receptors are involved in early events during neuronal development. The presence of GABA receptors in developing oligodendrocytes provides a new mechanism for neuronal–glial interactions during development and offers a novel target for promoting remyelination following white matter injury. Findings showed the cell-specific effect of Serotonin on regenerating neurons within the adult CNS of the pond snail by increasing the calcium concentration of the cells. The combination of neurotransmitters as therapeutic agents for cell proliferation and differentiation has importance in spinal cord regeneration. Up regulation of GABA and 5-HT receptor subtypes were reported in accelerated bone fracture healing [28]. 5-HT, GABA and bone marrow in combination potentiates a restorative effect by reversing the alterations in glutamate receptor binding, dopamine receptor binding and gene expression that occur during Parkinson's disease. Thus, it is evident that 5-HT and GABA along with bone marrow aspirate, renders protection against oxidative, related motor and cognitive deficits which makes them clinically significant for cell-based therapy. Earlier studies from our lab has also shown that anti-oxidant and co-mitogenic property of 5-HT functionally reversed the dopamine receptors alterations in rotenone-induced hemi-Parkinson's rat [29].

Our results showed a decreased muscarinic M1 receptor binding in the spinal cord injured rats. These experiments also demonstrated a decreased expression of muscarinic M1 receptor gene which further highlights the receptor data at the molecular level. It is evident that the decrease in acetylcholine is reflected as decrease in muscarinic receptors, which play a major role in locomotor function [30]. Citicholine can increase the availability of acetylcholine by increasing the endogenous production. The treatment groups showed a reversal in the muscarinic M1 receptor binding and gene expression which implicates the increase in the muscarinic receptors and hence an increase in acetylcholine which is a major neurotransmitter in peripheral movement. Earlier studies have reported changes in the cholinergic transmission in spinal cord injured rats [31,32]. Changes in acetylcholine receptor have been implicated in the pathophysiology of many major diseases of the central nervous system. The muscarinic acetylcholine receptors are widely distributed throughout the body, but are predominantly expressed within the parasympathetic nervous system and exert both excitatory and inhibitory control over central and peripheral tissues. Muscarinic stimulation is a potent and complete inducer of neuritogenesis acting on both ends, directly on neurons by stimulating an intracellular signalling pathway and on astrocytes by affecting the secretion of guiding factors. In addition, acetylcholine induces expression of genes associated with neuronal differentiation.

The various phases of the usual or normal reaction to the experienced loss which follows a spinal cord injury include: denial, withdrawal, internalized hostility, externalized hostility and reaction against dependence [33]. About one in five people with spinal cord injury (SCI) report problems with sleep, poor energy, low mood, loss of interest in things they used to enjoy, or changes in appetite. Some of these changes in basic biological functions are attributed to the direct effects of SCI on life. The stress of experiencing and living with SCI cause changes in brain chemicals that regulate these bodily functions, magnifying the negative effects of SCI.

Promising results in our animal study showed that treatment with 5HT and GABA with bone marrow aspirate, and Citicholine with bone marrow aspirate can facilitate functional recovery of altered muscarinic receptor function. Thus, autologous transplantation of bone marrow along with neurotransmitter combination or bone marrow with Citicholine produced significant functional improvement after spinal cord injury in the two treatment groups of rats, suggesting its role as a novel treatment for spinal cord injury in humans. The modulation and differentiation of bone marrow aspirate by neurotransmitters or neurotransmitter producing agents like Citicholine, have opened a new treatment strategy in SCI.

Conflicts of interest

The authors has no conflicts of interest.

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