Spinal cord regeneration and functional recovery: neurotransmitter's combination and bone marrow cells supplementation

C. S. Paulose[1,*], P. S. John[2], R. Sreekanth[2], Mathew Philip[2], C. Padmarag Mohan[2], Jobin Mathew[1], T. Peeyush Kumar[1], Jes Paul[1], Pretty Mary Abraham[1], Sherin Antony[1], Binoy Joseph[1], Anu Joseph[1], Amee Krishnakumar[1], T. R. Anju[1], S. Reas Khan[1], K. Santhosh Thomas[1] and M. S. Nandhu[1]

[1]Molecular Neurobiology and Cell Biology Unit,
Centre for Neuroscience, Department of Biotechnology,
Cochin University of Science and Technology, Cochin 682 022, India
[2]Department of Orthopedics, Medical College,
Kottayam 686 004, India

We report here the regenerative cell proliferation and functional recovery of spinal cord injury after supplementation with neurotransmitter's combination and bone marrow cells. Monoplegia was induced in male Wistar rats by producing a wound between 12th and 13th vertebra and they were treated with neurotransmitter's combination and bone marrow cells for 21 days. GABA and 5HT receptors were down-regulated in the spinal cord of the injured rats and the treatment reversed the parameters to near control. Nuclear staining and reward seeking locomotor test support spinal cord regeneration. Our results suggest the neurotransmitter's combination with the bone marrow cells as a successful treatment for re-establishing the connections and functional recovery of spinal cord injury.

Keywords: Bone marrow cells, Gamma aminobutyric acid, neurotransmitters, serotonin, spinal cord.

SPINAL cord injuries are often caused by road accidents, whereas fall from height is another significant cause of injury. The spinal cord contains nerve fibers which carry messages between the brain and different parts of the body. If it is damaged by different levels of shearing, one or several of the body functions are impaired and even total paraplegia occurs. In the present scenario, there are no treatments for restoring locomotor function in the victims.

It has been demonstrated that nerve cells outside the brain and spinal cord can regenerate[1]. These experiments encouraged the idea that adult nerve cells in spinal cord proliferate and re-establish the connections in appropriate growth environment[2,3]. Neurotransmitters relay, amplify and modulate signals between neurons. Serotonin (5HT) and Gamma aminobutyric acid (GABA) acting through specific receptor subtypes $5HT_2$ (ref. 4) and $GABA_{A,B}$ (ref. 5) respectively, control cell proliferation as comitogens. Neurotransmitters' integration into biodegradable polymers results in a biomaterial that successfully promotes nerve growth, which is necessary for victims of central nervous system (CNS) injury, stroke or certain neurodegenerative diseases to recover sensory, motor, cognitive or autonomic functions. 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 re-myelination following white matter injury[6]. Metabotropic glutamate receptors modulate neuronal development and survival. They have been identified in oligodendrocyte progenitor cells. Findings showed the cell-specific effect of serotonin on regenerating neurons within the adult central nervous system by increasing the calcium concentration of the cells[7].

One approach for repairing spinal cord injuries in animals is by transplanting cells or pieces of peripheral nerves that produce substances that create an environment for axons to grow. It is suggested that implanting cells from the peripheral nervous system (PNS) into the area of a CNS injury helps neuronal regeneration. Because the environment of the PNS supports axon regeneration, it is believed that re-creating this environment in the spinal cord allows CNS axons to regenerate after an injury. Ideally, this environment would also direct the growing nerves to the correct targets[8]. Research supporting the pre-clinical studies to assess the safety, feasibility and efficacy of implanting autologous bone marrow stem cells into spinal cord injury was done in patients[9].

Regeneration is also carried out by foetal tissue implantation containing stem cells, progenitor cells and growth factors which promote axonal growth. Stem cells can differentiate into various cell types, depending on the signals they receive. Foetal tissue transplantation into spinal cord with the right chemical signals helps them to develop into neurons and supporting cells, re-establishing lost circuits. Bone marrow cells have been shown to actively re-myelinate spinal cord once administered directly or intravenously[10].

Neurotransmitter's combination as therapeutic agents for cell proliferation and differentiation is important in spinal cord regeneration. Upregulation of GABA and 5HT receptor subtypes were reported in accelerated fracture healing[11]. In the present study, we investigated the structural and molecular changes during recovery of spinal cord injury by re-establishing the connections using combinations of neurotransmitters along with bone marrow cells.

Male Wistar rats were selected for the experiments. All the animals were housed in separate cages under 12 h light and 12 h dark periods and were maintained on stan-

*For correspondence. (e-mail: cspaulose@cusat.ac.in)

dard food pellet and water *ad libitum*. All animal care and procedures were in accordance with the institutional CPSCEA guidelines. Under all aseptic precautions and ether anesthesia, monoplegia was induced by producing a wound between the 12th and 13th vertebra of the experimental rats using a sterilized needle. A specially designed rubber chamber with silastic tube[12] was inserted subcutaneously, with 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 (Figure 1). These rats were randomly divided into spinal cord-injured and treatment groups. The spinal cord-injured group was given physiological saline solution through the chamber daily for 21 days. Treatment group was given bone marrow cells for one time and a combination of GABA and serotonin (1 μg/kg body weight) through the chamber for 21 days. Bone marrow was aspirated using a needle inserted through the top of the femoral intercondylar groove.

Spinal cord regeneration and re-establishment of connections were assessed by the motor recovery – reward seeking locomotor test. Rats were deprived of food for 24 h continuously after which a source of food was kept at a distance of 1 m in a special unidirectional pathway. The time taken by the rat to reach the food source from this 1 m distance was taken as an indirect measurement of motor recovery. Molecular studies of GABA and $5HT_{2A}$ receptor kinetics, gene expression studies of $GABA_{A\alpha 1}$ and $5HT_{1A}$, and confocal imaging of nuclei using TOPRO-3 stain were also done to confirm recovery from the spinal cord injury.

The reward seeking locomotor test showed that locomotor ability decreased significantly ($P < 0.001$) in the spinal cord-injured rats. The treatment with the neurotransmitter's combination and bone marrow cells reversed these changes significantly ($P < 0.001$) to near control (Table 1).

Scatchard analysis of [^{3}H]GABA against GABA in the spinal cord showed a significant decrease ($P < 0.001$) in the B_{max} of the spinal cord-injured group compared to the control. K_d showed a significant decrease ($P < 0.01$) in the spinal cord-injured group compared to the control. The treatment with neurotransmitter's combination and bone marrow cells significantly ($P < 0.001$) reversed the B_{max} to near control with no significant change in the affinity (Table 2). Scatchard analysis of [^{3}H] ketanserin against ketanserin showed a significant decrease ($P < 0.001$) in the B_{max} of the spinal cord-injured rats compared to the control. K_d showed no significant change. The treatment with neurotransmitter's combination and bone marrow cells significantly ($P < 0.001$) reversed the B_{max} to near control (Table 2).

$GABA_{A\alpha 1}$ gene expression in the spinal cord decreased significantly ($P < 0.001$) in the spinal cord-injured rats. The treatment with neurotransmitter's combination and bone marrow cells reversed this change significantly ($P < 0.001$) to near control (Table 3). $5HT_{1A}$ gene expression in the spinal cord decreased significantly ($P < 0.001$) in the spinal cord-injured rats. Treatment using neurotransmitter's combination and bone marrow cells reversed this change significantly ($P < 0.001$) to near control (Table 3).

Confocal imaging of the treated group showed more cell nuclei with TOPRO-3 staining, indicating active regeneration of cells at spinal cord injury site compared to the control and spinal cord-injured rats (Table 4 and Figure 2).

Table 1. Reward seeking locomotory test of control, spinal cord-injured and treated rats

Animals	Control (s)	Spinal cord-injured (s)	Treatment (s)
Day 10	11 ± 0.3	45.3 ± 0.3	43.4 ± 0.5
Day 20	12 ± 0.5	44.9 ± 0.2	31.9 ± 0.7*
Day 30	10 ± 0.9	46.5 ± 0.8	24.6 ± 0.9*
Day 40	9 ± 0.4	45.5 ± 0.6	14.9 ± 0.2*

Values are mean ± SEM of 4–6 separate experiments. Each group consists of 6–8 rats.
*$P < 0.001$ when compared to control.

Table 2. [^{3}H] GABA and [^{3}H] ketanserin binding parameters in the spinal cord of control, spinal cord-injured and treated rats

	[^{3}H]GABA against GABA		[^{3}H]Ketanserin against ketanserin	
Group	B_{max} (fmoles/mg protein)	K_d (nM)	B_{max} (fmoles/mg protein)	K_d (nM)
Control	84 ± 2.4	14.2 ± 0.97	55 ± 6.0	0.2 ± 0.04
Spinal cord-injured	37 ± 2.9**	13.2 ± 1.3*	13 ± 0.23**	0.2 ± 0.03
Treated	100 ± 3.2***	10.5 ± 1.2	50 ± 4.2***	0.2 ± 0.04

Values are mean ± SEM of 4–6 separate experiments. Each group consists of 6–8 rats.
*$P < 0.01$; **$P < 0.001$ when compared to control; ***$P < 0.001$ when compared to spinal cord-injured group.

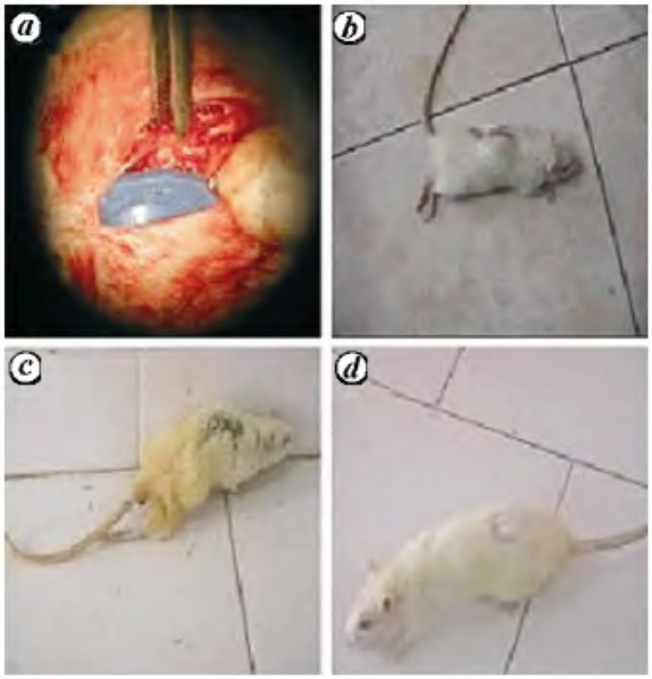

Figure 1. ***a***, Subcutaneous implantation of chamber and silastic tubes into the site of injury; ***b***, Rat immediately after surgery; ***c***, Rat after 10 days of treatment, and ***d***, Rat completely recovered.

Table 3. Real-time PCR amplification of $GABA_{A\alpha1}$ and $5HT_{1A}$ receptor mRNA in spinal cord of the control, spinal cord-injured and treatment rat groups

	$GABA_{A\alpha1}$	$5HT_{1A}$
Experimental group	RQ value	RQ value
Control	0	0
Spinal cord-injured	–0.66 ± 0.05*	–0.72 ± 0.06*
Treatment	–0.16 ± 0.07**	–0.13 ± 0.05**

Values are mean ± SD of 4–6 separate experiments. Each group consists of 6–8 rats.
*$P < 0.001$ when compared to control, **$P < 0.001$ when compared to spinal cord-injured group.

Table 4. Pixel intensity of TOPRO-3 stained spinal cord of control, spinal cord-injured and bone marrow, and neurotransmitter treated rats

Condition	Pixel intensity
Control	212380
Spinal cord-injured	309860*
Bone marrow and neurotransmitter treated	427837**

Values are mean ± SD of 4–6 separate experiments. Each group consists of 6–8 rats.
*$P < 0.001$ when compared to control, **$P < 0.001$ when compared to spinal cord-injured group.

Spontaneous recovery after spinal cord injury is delayed by the limited ability of mammalian central nervous system to re-establish functional neural connections; re-myelinate, spread nerve fibers and replace lost cells[13]. Spinal cord injury leads to sensory motor loss and disruption of autonomic nervous system. Most spinal cord injury victims also develop chronic pain conditions that severely reduce quality of life.

Our results showed that the treatment using neurotransmitter's combination and bone marrow cells of the same individual significantly improved the recovery from spinal cord injury. Pittenger *et al.*[14] reported that mesenchymal stem cells derived from the bone marrow differentiated into osteocytes, chondrocytes and adipocytes. It is also reported that multipotent adult progenitor cells derived from the bone marrow[15], which comprise approximately 0.125% of the total marrow cells[16], are multipotent stem cells with the capacity to differentiate, under specific experimental conditions[17] 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*[18] and *in vitro*[19]. Bone marrow cells which are adherent in the culture of bone marrow aspirates, have already been used for the treatment of the injured spinal cord[20] and brain. Recent studies indicated that transplantation of bone marrow cells by direct injection into the lesion might promote tissue repair in the injured spinal cord by reducing the size of the cavity at the lesion. 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[21].

Our study showed that GABA and 5HT receptors were downregulated in the spinal cord of the spinal cord-injured rats. The treatment using neurotransmitter's combination and bone marrow cells reversed the changes near to control. GABA receptors play a crucial role in modulating motor neuron excitability and firing rates in spinal cord injury. Repeated pulses of 5HT initiate a cascade of gene activation that leads ultimately to the growth of new synaptic connections in *Aplysia*[22]. 5HT and GABA have been recognized to cause proliferation of a variety of cells in culture[23,24]. Cellular cyclic nucleotides and protein phosphorylation pathways play an important role in the intracellular signalling process for growth regulation by serotonin. Scatchard analysis of GABA and $5HT_{2A}$ receptors and gene expression of $GABA_{A\alpha1}$ and $5HT_{1A}$ receptor subunit in the spinal cord, showed significant downregulation in the spinal cord-injured group compared to control. All these changes were reversed significantly by the treatment using neurotransmitter's combination and bone marrow cells. Confocal imaging of spinal cord sections of treated group showed active cell proliferation compared to the control and spinal cord-injured group. Recovered rats performed functionally

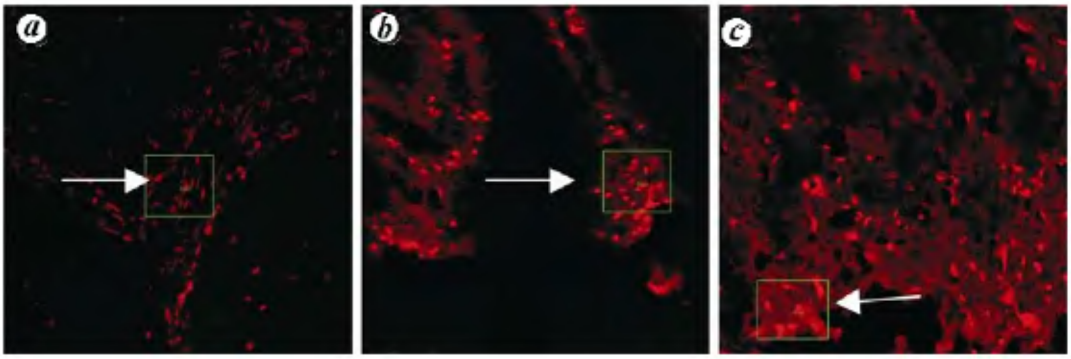

Figure 2. TOPRO-3 stained spinal cord (*a*) Control, (*b*) Spinal cord-injured, (*c*) Bone marrow and neurotransmitter treated. Images were taken using Leica TCS SP5, confocal microscope. Arrows indicate stained nuclei.

similar to control rats in the reward seeking locomotor test and in the molecular studies of GABA and 5HT indicating the role of the combination and the individual's bone marrow cells in regeneration of the spinal cord. Thus, our results showed that GABA and 5HT acting through their specific receptors play a crucial role in the spinal cord regeneration in combination with bone marrow cells. There is proliferation and differentiation of cells re-establishing the connections in the injured spinal cord resulting in the functional recovery of the individual rat. This is suggested to have clinical benefit for spinal cord-injured paraplegics.

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