

Research Article

Bone Marrow Mesenchymal Stromal Cells and Dantrolene Promote Functional Locomotor Improvement in Wistar Rats after Spinal Cord Injury

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Abstract

This study aimed to evaluate the effects of bone marrow mesenchymal stromal cells (BMSC) and dantrolene (DAN) in spinal cord injury (SCI). Twenty-five male Wistar rats were divided into five groups: BMSC; BMSC+ DAN; DAN; positive control (PC) - trauma and placebo; and negative control (NC)-no trauma+placebo. Laminectomy was performed at T12 level in all animals, followed by a weight-drop model of SCI, except for the NC group. One hour later, the BMSC+DAN and DAN groups received 10mg/kg of DAN and after seven days the BMSC and BMSC+DAN groups received 1×10^6 BMSC intravenously. At those times, other groups received the same volume of placebo. Basso, Beattie, Bresnahan (BBB) locomotor scale was performed for 28 days to access neurological status. Traumatized animals showed severe paraplegia. There was a significant neurological improvement in groups BMSC, BMSC+ DAN and DAN from the 22th, 25th and 28th days, respectively ($p < 0.05$) compared to PC group. It was concluded that bone marrow mesenchymal stromal cells and dantrolene, alone or combined, for the treatment of SCI in rats promote functional neurological improvement.

ABBREVIATIONS

SCI: Spinal Cord Injury; BMSC: Bone Marrow Mesenchymal Stromal Cells; BBB: Basso, Beattie and Bresnahan Locomotor Rating Scale; DAN: Dantrolene; NIH: National Institutes of Health; UFMG: Federal University of Minas Gerais; T6: Sixth Thoracic Vertebrae; L1: First Lumbar Vertebrae; T12: Twelfth Thoracic Vertebrae; PC: Positive Control; NC: Negative Control; PBS: Phosphate-Buffered Saline

INTRODUCTION

After a spinal cord injury (SCI) it is well known that there is a low recovery capacity of injured tissue due to many factors that inhibit neural cell regeneration and axonal regrowth [1]. Following the SCI, there is an acute inflammation, caused by hemorrhage, edema, tissue or cell necrosis and inflammatory cytokine release, increased levels of oxygen free radicals, and

proteases. The secondary injury is caused subsequently by tardive apoptosis, demyelination, chronic inflammation, and the formation of glial scar [2,3].

The target for therapeutic intervention is an attempt to stop the progression of neuro degeneration and reverse the loss of neurons and glia induced by trauma [4-6]. The development of any form of pharmacological therapy that can reduce or alleviate even some of those adverse outcomes and promote motor, sensory and/or autonomic functional benefits has proven difficult due to the complexity of the injury [7]. Moreover, it is unlikely that a single line therapy promotes full functional return [4,8]. The real need for combined therapies has been widely accepted, and agents that may act in different signaling pathways directly involved in secondary injury are of particular interest.

Bone marrow mesenchymal stromal cells (BMSC) have shown promising results improving locomotor recovery after

SCI in many clinical conditions alone or as adjuvant therapy. After systemic injection they are able to migrate to the lesion site and promote marked anti-apoptotic and anti-inflammatory action, induce repair of nerve cells, and restore nerve function by secreting neurotrophic factors and anti-inflammatory cytokines [3,9-11]. However, the low cell viability after transplantation to an injured area, due to an ischemic microenvironment with little supply of essential nutrients and oxygen, obviously restricts the efficacy of this promising therapy [1,5,12].

On the other hand, dantrolene (DAN) is a drug that inhibits cytosolic calcium overload [13-16], a key element in apoptotic signaling pathway after SCI [2]. Its neuroprotective effects have been shown on *in vitro* [17,18] and *in vivo* models of SCI [19,20]. Thus, it was hypothesized that DAN could provide a better microenvironment to support the transplanted cells that would result in a more prominent clinical improvement.

However, the effects of BMSC combined with DAN on SCI remain uncharacterized. Therefore, the aim of the current study was to investigate the potential clinical effects of BMSC, DAN and their combination on the functional improvement in Wistar rats submitted to SCI using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale.

MATERIALS AND METHODS

All procedures were performed according to the principles adopted by the NIH Guide for the Care and Use of Laboratory Animals and by the Ethics Committee on Animal Use (protocol number UFMG46/2012). Twenty-five male Wistar rats aged 12 weeks and weighing 320–350 g were used in this study. Rats were kept under a 12/12 h light-dark cycle for 14 days of acclimation with commercial rodent food and water *ad libitum*.

Bone Mesenchymal Stromal Cells Culture

Bone mesenchymal stromal cells (BMSC) were isolated from the tibias and femurs of five 8-week-old clean male Wistar rats. BMSC were isolated and expanded as previously described [21]. BMSC were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The culture medium was changed once every 3 days, and the non-adherent cells were discarded. The identity of the BMSC was confirmed by cell morphology, adherence to plastic and specific surface antigen expression. Morphological characterization of the BMSC was observed under an inverted phase contrast microscope (IX70; Olympus, Tokyo, Japan). BMSC were harvested at 90% confluence. Passages 8 BMSC were used in this experiment. Anti-rat CD45, CD54 and CD90 (BD Pharmingen, Franklin Lakes, NJ, USA) were used [22]. BMSC were trypsinized and stained with fluorescein in labeled antibodies for flow cytometric analysis.

Spinal Cord Injury

Pre-anesthetic medication was performed with tramadol (2 mg/kg, orally) and induction and maintenance was carried out with isoflurane administered by mask in a semi-opened system. The animals were positioned in prone position, prepared for aseptic surgery and received prophylactic antibiotic therapy with cephalothin (30 mg/kg, intravenous). Skin and subcutaneous tissue were incised in the dorsal midline extending from T6 to

L1, the paravertebral muscles dissected and laminectomy of T12 was performed with the employment of a pneumatic drill. After visualization of the spinal cord covered by the intact dura, a compressive model of SCI was performed, as previously described [23-25], using a weight of 70 g/cm loading to the dorsal surface of the spinal cord. Afterwards, the site was irrigated with saline, the muscles approximated, and the reduced dead space and skin sutured using an unabsorbed suture. During anesthetic recovery, the animals were kept warm in a box heated approximately to 37°C. They received tramadol (2 mg/kg, orally), every 8 h for three days. Abdominal massage was performed three times a day in all animals to assist with urination and defecation.

Dantrolene treatment

One hour after SCI, the BMSC+DAN and DAN groups received 10mg/kg of dantrolene (Cristália Lab. Itapira, SP, and Brazil) diluted in water for injection, in single dose intraperitoneally. The BMSC, PC and NC groups received the equivalent volume of water for injection alone as placebo.

Cell Transplantation

Seven days after SCI, the injured rats from BMSC and BMSC+DAN groups received 200 µL phosphate-buffered saline (PBS) containing 1×10^6 injected into the lateral vein of the tail. The DAN, PC and NC groups received 200 µL PBS as placebo.

Behavioral assessment

Functional tests were scored using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale at pre-surgery (0) and on the 1st, 4th, 7th, 10th, 13th, 16th, 19th, 22th, 25th and 28th days post-surgery. Two independent blinded examiners observed and video-recorded the hind limb movements and assessed the animal's locomotor function [26].

Statistical analysis

Measurement data are expressed as the mean scores. Statistical analyses were performed with Prism 7 for Windows (Graph Pad Software, La Jolla, CA, USA). BBB locomotor rating scores at different time points were carried out using repeated measures analysis of variance and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Characteristics of BMSC

Rat BMSC from allogeneic bone marrow grew in a monolayer of large, flat cells at low plating densities. As the cells approached confluency, they assumed a more spindle-shaped, fibroblastic morphology. Using flow cytometry analysis, undifferentiated rat BMSC were demonstrated as negative for CD45 (<2,45%) which surface marker associated with lympho-hematopoietic cells (Ishii *et al.*, 2005). Using both flow cytometry analysis and immunocytochemistry, cells were positive for CD54 and CD90 (82,27% and 87,89%, respectively), which are reported to be surface markers of BMSC [22].

Behavioral assessment

To evaluate the locomotor recovery with animal models of

spinal cord injury, BBB scale which is a sensitive and reliability of locomotor rating scale and set up by Basso, Beattie and Bresnahan is widely used [26]. BBB scale is estimated by observing the movements of lower limbs and joints of rats in open field. The full scores of BBB rating scale are 21 points which means normal function. The fewer score the rats get, the worse function they have [26]. BBB score is a generally accepted method of evaluation for the degree of SCI and treatment effect. It is useful for clinical application in the future with meta-analysis of random animal trials [27].

In this study, twenty-four hours after SCI (1st day), all traumatized rats had severe paraplegia showing score 0, meaning no observable hind limb movements. Subsequently, the animals were evaluated every three days until the 28th day. None of the animals showed complete recovery of functions in BBB locomotor scale after 28 days of follow-up. However, in all groups except the PC, rats gradually recovered some motor function varying over the observation period.

During the 28 days of evaluation there could not be observed any significant difference in neurological recovery among the groups BMSC, BMSC+DAN and DAN ($p > 0.05$). On the other hand, compared to PC group there was a significant neurological recovery in groups BMSC from the 22th day, BMSC+DAN from the 25th day and DAN at 28th day after SCI (Figure 1). After 22 days, the animals treated with BMSC had a slight movement of two joints and extended movement of the third ($p < 0.05$). After 25 days, they

showed extensive movement of all three joints ($p < 0.01$) and at day 28 sweeping with no weight support or plantar support with no weight support ($p < 0.001$). Rats treated with BMSC+DAN at the 25th and 28th days had extensive movement of two joints and slight movement of the third ($p < 0.05$). Rats treated with DAN at the 28th day had a slight movement of two joints and extended movement of the third ($p < 0.05$).

Few studies have been performed to investigate the clinical effects of DAN after SCI. Dantrolene afforded neurologic functional improvement by anti-oxidative status in a model of spinal cord ischemia /reperfusion injury induced by abdominal aortic occlusion in rabbits [18] and in a traumatic balloon model, also in rabbits that showed decreased TUNEL-positive cells counting after DAN injection [19]. Most recently, our research team demonstrated the first direct evidence connecting DAN with caspase-3 activity inhibition and neuronal viability preservation in an *in vivo* model of traumatic SCI, strengthening evidence of its neuroprotective effect via anti-apoptotic mechanisms [20]. Now, to the best of our knowledge, this is the first time that DAN has been reported to improve clinical status in a weight-drop model of SCI.

Furthermore, several studies have shown the ability of BMSC on the functional neurologic recovery in rats submitted to SCI via different mechanisms of action [11]. In agreement with other authors [8,28,29], in the present study the BBB scores of animals treated with cells (BMSC and BMSC +DAN), showed a significant improvement on the motor capacity ($p < 0.05$) starting two weeks after its application.

Numerous studies have been shown that BMSC may mediate transient paracrine mechanisms rather than long-term cell engraftment and cell replacement [3,11]. Moreover, it is well known that when injected intravenously BMSC are able to migrate to the lesion site and secrete anti-inflammatory, immunomodulatory, anti-apoptotic, trophic and angiogenic factors, thus playing a neuro protective role [27,30-32].

On the other hand, the hypothesis that DAN associated with BMSC could anticipate some beneficial clinical outcome was not significantly proven, although it is clearly possible to note a trend of better improvement in this group from the 4th day till the 19th day compared to the other groups (Figure 1).

Finally, it is speculated that the significant functional improvement reported in the present study should be related to the preservation of neural cells due to the DAN action enabling continuity to stimulating the production and action of endogenous neurotrophins associated with the possibility of endogenous repair due to neurotrophic factors released by the grafted BMSC.

These findings suggest that bone marrow mesenchymal stromal cells and dantrolene may provide a promising therapeutic strategy for the management of SCI and future investigation, such as concerning long-term clinical evaluation, must be carried out to elucidate the broad potential of these combinations on SCI.

CONCLUSION

Our results provide experimental evidence for the potential

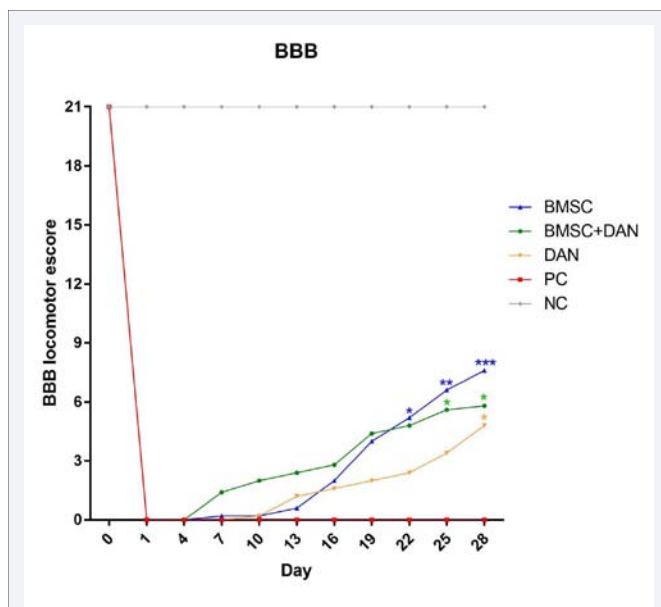


Figure 1 Dispersion graph of mean locomotor scores showing a clear and progressive motor improvement in spinal cord injured Wistar rats followed-up for 28 days, according to the Basso–Beattie–Bresnahan (BBB) scale, in the groups treated with bone marrow mesenchymal stromal cells (BMSC), dantrolene (DAN) and both (BMSC + DAN) when compared to positive control (PC) group that received placebo. Negative control (NC) group received laminectomy only and had no locomotor deficits.

(* = $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$)

therapeutic significance of bone marrow mesenchymal stromal cells, dantrolene and both combined in clinical treatment of SCI. Further research is required to study the mechanisms of the protective effects of bone marrow mesenchymal stromal cells and dantrolene on spinal cord injury.

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