

Intraspinal administration of TiO₂ nanowired cerebrolysin with mesenchymal stem cells has superior neuroprotective effects on cord pathology and functional outcome following a focal spinal cord trauma

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ABSTRACT

Military personnel are quite vulnerable to heat stroke in hot environment leading to mental dysfunction. Due to severe work stress and irregular food habits they could develop hypertension and diabetes. In this innovation, we demonstrate that a combination of heat stress with diabetes (DB) and hypertension (HY) adversely affect brain function resulting in mental abnormalities and exacerbation of brain pathology. Our observations in a rat model show that a combination of diabetes (DB) and hypertension (HY) exacerbates blood-brain barrier (BBB) breakdown, edema formation and brain injury. It appears that excessive upregulation of nitric oxide synthase (NOS) and heme oxygenase-2 (HO-2) following heat stroke in DBHY rats resulting in excessive brain pathology. In such situation, TiO₂ nanowired delivery of cerebrolysin has superior effects in reducing BBB breakdown, brain edema, NOS and HO-2 expression and brain pathology in DBHY rats after heat stroke as compared to cerebrolysin alone, not reported earlier.

Keywords: spinal cord injury, mesenchymal stem cells, edema, blood-spinal cord barrier, intraspinal delivery, TiO₂ nanowired cerebrolysin, neuroprotection

1 INTRODUCTION

Military personnel are often victims of spinal cord injury (SCI) for which there is no suitable treatment available. Thus, exploring new therapeutic avenues using select combination of novel neuroprotective agents is the need of the hour to improve the quality of life of SCI patients. Previous experiments from our laboratory show that a focal SCI inflicted in rat by making an incision of the right dorsal horn of the T10-11 segment resulted in pronounced functional disability on Tarlov Scale, inclined plane angle test and walking on a mesh grid in a progressive manner after 12 and 24 h trauma. This behavioral dysfunction correlated well with breakdown of the blood-spinal cord barrier (BSCB), edema formation and cell injuries seen in both the rostral and caudal segments after SCI in a progressive manner. Recording of spinal cord evoked potentials using epidural electrodes at rostral and caudal to lesion segments exhibited significant increase in latency and amplitude changes indicating loss of spinal cord conduction.

Several lines of evidences suggest that intraspinal administration of mesenchymal stem cells (MSCs) improves functional outcome and enhance spinal cord conduction. However, MSCs induced restoration of

BSCB and reduction in edema formation is not well known. In present innovation, we examined the role of MSCs alone or in combination with cerebrolysin (CBL)- a multimodal drug on SCI induced restoration of cellular and behavioural functions in a rat model.

2 MATERIALS & METHODS

Experiments were carried out on Male Wistar rats (200-250 g body weight) housed at controlled room temperature (21±1°C) with 12 h light and 12 h dark schedule. Food and tap water were supplied *ad libitum* before the experiment. All the experiments were carried out according to the Guidelines & Care for Laboratory Animals as described by National Institute of Health and approved by Local Institutional Ethics Committee.

2.1 Spinal cord injury

SCI was inflicted in Equithesin anesthetized rats over the right dorsal horn of the T10-11 segments (2 mm deep and 4 mm long) and the animals were allowed to survive 12 or 24 h after trauma (Fig. 1). Uninjured rats were served as controls [2-4].

2.2 Treatment with cerebrolysin and mesenchymal stem cells

In separate groups of SCI rats MSCs (10⁶ cells) and CBL (50 µl) were administered into the rostral and caudal spinal cord around the lesion site after 3 h injury using a 100 µl Hamilton syringe connected to a constant infusion pump (10 µl/min). Since nanodelivery of MSCs or CBL has superior neuroprotective effects in CNS injury, we also examined TiO₂ nanodelivery of MSCs and CBL in SCI [1,3].

2.3 Blood-spinal cord barrier and edema formation

The BSCB breakdown was examined in T9 and T12 segments using Evans blue (EB) and [¹³¹I]-Iodine leakage across the brain microvessels after intravenous administration of these tracers (EBA 2 % solution 3 ml/kg, and radioiodine 100 µCi/kg) 5 min before the end of the experiment. Spinal cord edema was determined using regional water content by wet and dry weights of the T9 and T12 segments [2-4].

2.4 Spinal cord pathology

At the end of the experiments, the animal were perfused with 4 % buffered paraformaldehyde through heart preceded by a brief physiological saline rinse under deep

anesthesia. After perfusion, T9 and T12 segments were dissected out and tissue samples were placed in same fixative at 4°C for 24 h. After that the tissue samples were embedded in paraffin using standard protocol. About 3-µm thick paraffin sections were cut and stained with Nissl or H&E stain according to commercial protocol [1-6].

2.5 TiO₂-nanowired Cerebrolysin and MSCs

Cerebrolysin (CBL, Ever NeuroPharma, Austria) or MSCs (Santa Clara, CA 95050, USA; Cat # RASMX-01001) was tagged with TiO₂ nanowires according to standard protocol [2,3]. The TiO₂ nanowired Cerebrolysin (50 µl NWCBL) together with MSCs (10⁶) was administered 3 h after SCI. For comparison, normal cerebrolysin was administered in identical doses instead of NWCBL [2,4,6,7] with normal MSCs (10⁶) 3 h SCI. The animals were allowed to survive either 12 h or 24 h after injury.

2.6 Statistical Analyses

ANOVA followed by Dunnett's test for multiple group comparison with one control was used to analyze statistical significance of the data obtained. A p-value less than 0.05 was considered significant.

3 RESULTS

3.1 Blood-spinal cord barrier and edema formation

Focal SCI resulted in marked iprogressive ncrease in the BSCB breakdown to Evans blue and radioiodine in the T9 and T12 segments associated with edema formation at 12 and 24 h after injury. The magnitude and intensity of BSCB disruption and edema formation was further exacerbated when the identical SCI was performed at HE (results not shown).

3.2 Spinal cord pathology

The cellular changes following SCI correlated well with the edema formation and leakage of Evans blue in the T9 and T12 cord segments. Thus, neuronal damages, perineural edema, sponginess and expansion of the neuropil in the T9 and T12 segments were more pronounced when the SCI was inflicted at HE as compared to normal room temperature (Fig. 2). These neuronal damages were also associated with gliosis and myelin degeneration in the areas exhibiting BSCB disruption (results not shown). It appears that the rostral segment (T9) showed a more marked cellular damage as compared to the caudal segment (T12) after SCI at both normal room temperature or at HE (unpublished observation).

3.3 Effect of Cerebrolysin and MSCs treatment

Our observations showed that co-administration of MSCs and CBL significantly reduced BSCB breakdown, edema formation and cell injuries at 12 h but not at 24 h after SCI performed at normal room temperature. However, when the SCI was inflicted at HE this combination was not that much effective in inducing neuroprotection in the cord (results nor shown).

3.4 Effect of TiO₂ Cerebrolysin with nanowired MSCs Treatment

On the other hand, treatment with TiO₂ nanowired Cerebrolysin (50 μ l, NWCBL) together with nanowired MSCs (10⁶) at 3 h afre SCI resulted in significant neuroprotection in the cord at both 12 and 24 h after the insult at HE (see Fig. 2).

Nanodelivery of CBL or MSCs alone was also able to induce mild neuroprotection in the cord after SCI at HE. However, the combination of TiO₂ nanowired delivery of CBL and MSCs has the most superior neuroproetctive effects inSCI at HE (Fig. 2).

At the ultratructural level also, TiO₂-nanowired cerebrolysin with MSCs reduced myelin vesiculation, membrane disruption and synaptic damages in the cord in both the T9 and T12 segments afre SCI at HE (results nor shown).

3.5 Behavioral dysfunction in SCI

A focal SCI inflicted in rat by making an incision of the right dorsal horn of the T10-11 segment resulted in pronounced functional disability on Tarlov Scale, inclined plane angle test and walking on a mesh grid in a progressive manner after 12 and 24 h trauma (results not shown). When the TiO₂-nanodelivery of MSCs and CBL was done in identical conditions, this treatment strategy significantly attenuated cord pathology and improved behavioral dysfunctions after 24 SCI (results not shown).

4 DISCUSSION

Our novel findings clearly show that TiO₂ nanowired cerebrolysin together with nanowired MSCs is capable to attenuate SCI induced cord pathology effectively after 24 h trauma. Furthermore our observations are the first to point out that TiO₂-nanowired cerebrolysin with MSCs has superior effects in inducing neuroprotection rats after SCI inflicted at hot environment. These observations suggest that nanowired MSCs with TiO₂-nanowired cerebrolysin has an added value for superior neuroprotection. This

means that this combined treatment could be used in military clinics instantly to protect spinal cord damage in soldiers up to 24 h afre injury. This time window will allow to transprt injured soldiers to a specialized clinic from the war zone, not reported earlier.

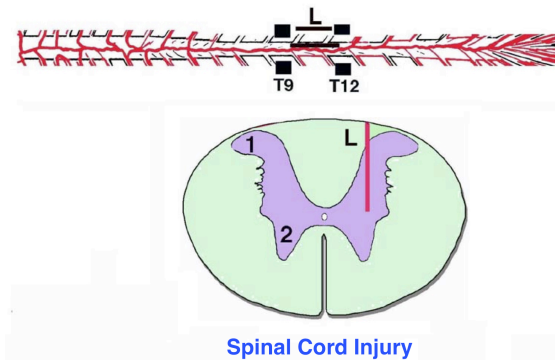


Fig. 1. Rat model of spinal cord injury. A longitudinal incision (L) was made on the right dorsal horn of the T10-11 segments and morphological analysis was done on the rostral T9 and caudal T12 segments. The dept of the lesion (L) is extended ti lamina VIII in the right dorsal horn. Left dorsal (1) and ventral (2) horns were examined at light microscopy for morphological alterations and subsequent neuroprotection.

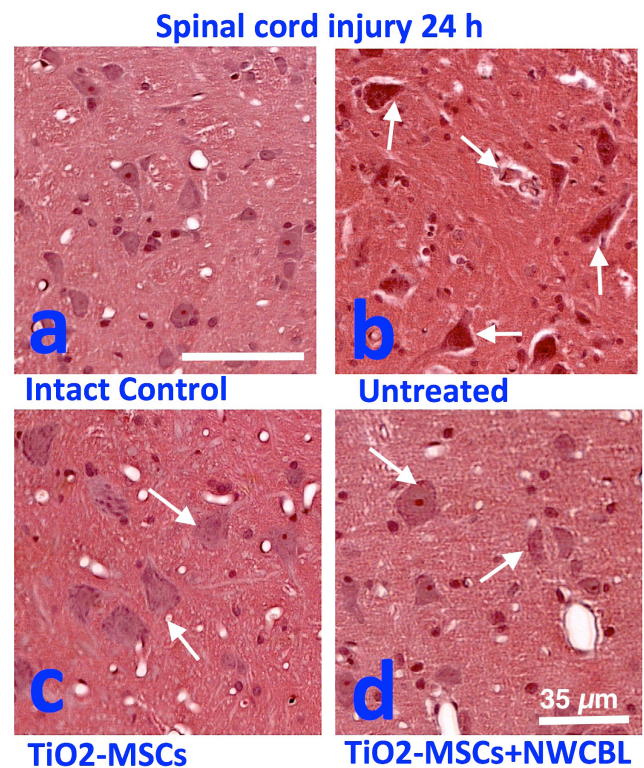


Fig. 2. TiO₂ Nanowired cerebrolysin (NWCBL) together wih TiO₂ mesenchymal stem cells (MSCs, d) induced superior neuroprotection in spinal cord injury (SCI, b) as compared to TiO₂-MSCs alone (c). Intact control (a). H&E stain on 3- μ m thick paraffin sections. Bar = 35 μ m.

The possible mechanisms by which TiO₂ nanowired cerebrolysin and MSCs combine are capable to attenuate cord pathology in SCI at HE is unclear. However, it appears that a reduction in oxidative stress and nitric oxide synthase (NOS) could play important roles [1-4,6,7]. Both cerebrolysin and MSCs are capable of strengthening the cell membrane of endothelial cells due to their neurotrophic and antioxidant properties together with the cell regeneration capabilities [1-3]. This could be another important factor for reducing brain pathology by the combination of cerebrolysin and MSCs in SCI [2-5,7]. A significant reduction in BSCB breakdown and cord edema formation in TiO₂-nanowired cerebrolysin and MSCs treated groups further support the idea.

HE exposure alone could induce oxidative stress and free radical formation [2-5]. This effect could be exacerbated by additional exposure to SCI. Since HE and SCI alone induces NOS upregulation in the cord, it is quite likely that oxidative stress and lipid peroxidation could alter membrane permeability resulting in greater BSCB leakage to proteins. Obviously, extravasation of proteins into the spinal cord fluid compartment will lead to vasogenic edema formation. Volume swelling of the cord and exposure of blood components to neurons, glial and myelin due to leaky BSCB will induce further cell and tissue injury [4-7]. Accordingly, greater neuronal, glial and axonal injuries seen in this study at HE is in line with this idea.

Potentiation of cerebrolysin and MSCs induced neuroprotection caused by TiO₂ nanowired delivery could either be due to their enhanced penetration within the cord or to a slow degradation or metabolism of these agents within the cord tissue [2-6]. It would be interesting to see whether this combination when applied intraspinally at different time intervals after SCI could still be able to induce remarkable neuroprotection e.g., 6 or 8 h after injury over a period of 24 h post trauma period.

5 CONCLUSION

In conclusion, These observations are the first to show that intraspinal administration of nanowired MSCs and CBL have superior neuroprotective effects in SCI and reduces exacerbation of cord pathology at HE, not reported earlier.

It remains to be seen whether nanodelivery of cerebrolysin and MSCs using other technology e.g., Poly (L-lactide-co-glycolide) could also be equally effective in containing spinal cord pathology at HE. This is a feature that is currently being investigated in our laboratory.

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