

# Co-administration of TiO<sub>2</sub> nanowired cerebrolysin and alpha-melanocyte stimulating hormone has superior neuroprotective effects on brain pathology following concussive head injury after sleep deprivation

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## ABSTRACT

Sleep deprivation (SD) is a serious problem in military personnel during combat operations. Previous observations from our laboratory showed marked brain pathology following SD in rats from 12 h to 72 h in a progressive manner. In this innovation we demonstrate that an additional concussive head injury (CHI) that is very common in soldiers during combat operation could exacerbate SD induced brain damage and behavioral dysfunction. We found a several fold increase in BBB permeability to Evans blue albumin (EBA) and radioiodine (<sup>131</sup>I), brain edema in several brain regions associated with neuronal injuries after CHI in SD at 48 h. Interestingly, the brain derived neurotrophic factor (BDNF) levels and alpha-melanocyte stimulating hormone ( $\alpha$ -MSH) showed greater decline in CHI animals after SD. Thus, TiO<sub>2</sub> nanowired delivery of cerebrolysin (2.5 ml/kg, i.v.) together with  $\alpha$ -MSH 4 to 6 h after CHI in SD significantly increased BDNF and  $\alpha$ -MSH levels and reduced brain pathology seen at 48 h. This treatment also improved behavioral functions significantly in CHI rats after SD. These observations are the first to show that nanodelivery of cerebrolysin with  $\alpha$ -MSH has superior neuroprotective effects in SD following CHI, not reported earlier.

**Keywords:** Sleep deprivation in Military, brain pathology, Brain derived neurotrophic factor,  $\alpha$ -MSH, Cerebrolysin, TiO<sub>2</sub> nanowired delivery, Concussive head injury

## 1 INTRODUCTION

Sleep deprivation (SD) is a serious problem in military personnel during combat operations. Normally they have only a few hours of sleep under severe stressful conditions [1]. This leads to profound mental and cognitive dysfunctions. Previous studies from our laboratory show that 12 to 48 h of SD results in widespread breakdown of the blood-brain barrier (BBB) associated with brain edema and cellular injuries. Since concussive head injury (CHI) is often associated with SD in military, in this investigation we examined a combination of CHI and SD on brain pathology and behavioral dysfunction in a rat model [1,2]. To understand the role of neurotrophic factor and alpha-melanocyte stimulating factor ( $\alpha$ -MSH) associated with SD, we measured regional distribution of brain derived neurotrophic factor (BDNF) and  $\alpha$ -MSH in SD with CHI. In addition, we used exogenous supplement of Cerebrolysin (a balanced composition of several neurotrophic factors and active peptide fragments) alone or together with  $\alpha$ -MSH both tagged with TiO<sub>2</sub> nanowires) [4-6] to reduce SD induced brain pathology after CHI and behavioral dysfunctions.

## 2 MATERIALS & METHODS

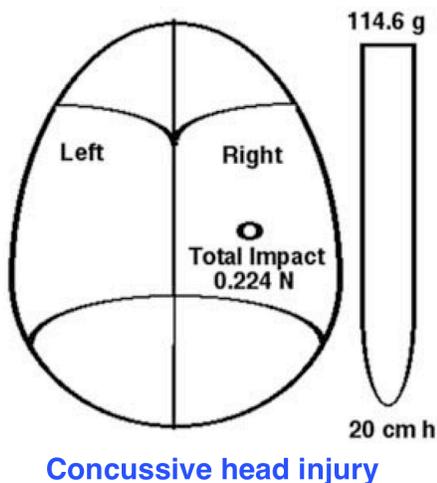
Experiments were carried out on Male Wistar rats (200-250 g body weight) housed at controlled room temperature ( $21\pm 1^\circ\text{C}$ ) with 12 h light and 12 h dark schedule. Food and tap water were supplied *ad libitum* before the experiment. All the experiments were carried out according to the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

### 2.1 Sleep deprivation

Animals were subjected to SD using the well-established inverted flowerpot model that selectively deprives them from paradoxical sleep (PS) [1,2]. In this model each rat is placed on an inverted flower pot (diameter 6.5 cm) surrounded by a water-filled Plexiglas chamber up to the 1 cm below the surface of the flowerpot with free access to food and water. The water temperature was maintained at  $30\pm 1^\circ\text{C}$  [1,2]. SD was induced in rats up to 48 h as described earlier [2]. Rats placed at room temperature were used as controls.

### 2.2 Concussive head injury

After 48 h of SD, rats were subjected to CHI by dropping a weight of 114.6 g over the right parietal skull causing an impact of 0.224 N on the brain without skull fracture (Fig. 1).



### Concussive head injury

Fig. 1. Concussive head injury (CHI) model in the rat.

### 2.3 Alpha-melanocyte stimulating hormone

Alpha-melanocyte stimulating hormone ( $\alpha$ -MSH) was measured (pg/g) in control, SD with or without CHI in rats using commercial MSH ELISA Kit (Lifespan Bioscience Corporation, Seattle, Wa, USA) in parietal cerebral cortex,

hippocampus and cerebellum according to standard protocol.

### 2.4 Brain derived neurotrophic factor

Brain derived neurotrophic factor (BDNF) was measured in control, SD rats with or without CHI using Rat BDNF ELISA Kit, Thermo Scientific, Frederick, MD, USA). The BDNF levels (ng/g) were measured in parietal cerebral cortex, hippocampus and cerebellum using commercial protocol.

### 2.5 TiO<sub>2</sub>-nanowired delivery of Cerebrolysin and $\alpha$ -MSH

Cerebrolysin (CBL, Ever NeuroPharma, Austria) and  $\alpha$ -MSH were tagged with TiO<sub>2</sub> nanowires according to standard protocol [4-6]. The TiO<sub>2</sub> nanowired Cerebrolysin (2.5 ml/kg, i.v., NWCBL) with or without  $\alpha$ -MSH (100  $\mu\text{g}/\text{kg}$ ) was administered intravenously 4 to 6 h after SD. For comparison, normal CBL (2.5 or 5 ml/kg, i.v.) and  $\alpha$ -MSH (100  $\mu\text{g}/\text{kg}$ ) was also given in separate groups of SD with or without CHI [5,6]. The animals were allowed to survive 48 h after CHI.

### 2.6 Blood-Brain Barrier and brain edema

In control, SD animals with or without CHI the blood-brain barrier (BBB) breakdown to Evans blue albumin (EBA) and radioiodine ( $^{131}\text{I}$ -Iodine) was examined after intravenous administration of these tracers (EBA 2 % solution 3 ml/kg, and radioiodine 100  $\mu\text{Ci}/\text{kg}$ ) 5 min before the end of the experiment [1,4]. Brain edema was determined using regional water content by wet and dry weights of the brain samples [5].

### 2.7 Brain Pathology

In separate groups of animals, neuronal changes were examined using histopathological examination of Nissl or Haematoxylin & Eosin (HE) staining on 3- $\mu\text{m}$  thick paraffin sections [4,5].

### 2.8 Behavioral parameters

Rota-rod performance, inclined plane angle test and walking on a mesh grid was used to evaluate behavioural functions in SD rats with or without stress as described earlier [4,5].

### 2.9 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 SD and Brain Pathology in CHI

CHI inflicted after 48 h of SD exhibited profound breakdown of the BBB as evident with extravasation of endogenous Evans blue albumin (EBA) and radioiodine in the cerebral cortex, hippocampus and the cerebellum as compared to SD alone. The brain edema formation and neuronal damages were also exacerbated by several folds in SD group after CHI as compared to normal animals. Neuronal distortion and damages were also exacerbated after CHI in SD group in the brain areas showing edema formation or sponginess of the neuropil.

At transmission electron microscopy (TEM), membrane vacuolation, synaptic damage and edema were exacerbated in CHI following SD in the above brain regions (results not shown).

#### 3.2 SD and regional brain BDNF and $\alpha$ -MSH Levels in CHI

BDNF and  $\alpha$ -MSH measurement using ELISA showed a significant decrease in this neurotrophic factor content following 48 h SD in all brain regions examined. Plasma  $\alpha$ -MSH and BDNF level shows significant reduction ( $\alpha$ -MSH  $8.34 \pm 0.23$  vs. Control  $20.34 \pm 0.12$  pg/ml; BDNF  $8.23 \pm 0.11$  vs. control  $22.34 \pm 0.21$  pg/ml) in SD group after CHI as compared to SD group alone ( $\alpha$ -MSH  $15.13 \pm 0.12$  pg/ml; BDNF  $14.23 \pm 0.08$  pg/ml).

#### 3.3 TiO<sub>2</sub> Cerebrolysin with $\alpha$ -MSH and Brain pathology

Intravenous administration of TiO<sub>2</sub> nanowired Cerebrolysin with  $\alpha$ -MSH 4 to 6 h after the onset of CHI in 48 h SD resulted in profound neuroprotection in terms of restoration of the BBB function and reduction in brain edema and volume swelling along with neuronal damages caused by a combination of CHI and SD (Figs. 2 & 3).

#### 3.4 TiO<sub>2</sub> Cerebrolysin with $\alpha$ -MSH and BDNF level

Intravenous administration of  $\alpha$ -MSH (100  $\mu$ g/kg) together with cerebrolysin significantly induced neuroprotection in CHI or SD groups alone. However, TiO<sub>2</sub> nanowired delivery of  $\alpha$ -MSH and cerebrolysin is needed to induce neuroprotection in SD rats after CHI (Figs. 2 & 3). The levels of  $\alpha$ -MSH and BDNF were also restored by this treatment in SD rats after CHI ( $\alpha$ -MSH  $22.34 \pm 0.12$  pg/ml; BDNF  $23.34 \pm 0.17$  pg/ml).

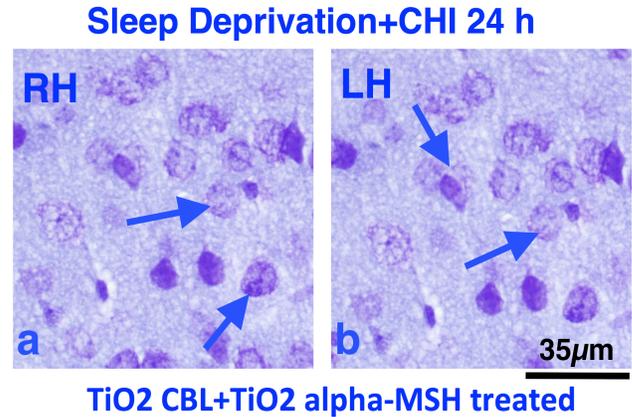


Fig. 2. Shows neuroprotection by Treatment with TiO<sub>2</sub>-nanowired cerebrolysin and nanowired alpha melanocyte stimulating hormone (MSH) in right hemisphere (RH, a) and left hemisphere (LH, b) after concussive head injury (CHI) following sleep deprivation (SD) and 24 h survival. Neuronal damages were significantly reduced by the combined treatment (arrows) in both RH and LH (a,b). Nissl stain on 3- $\mu$ m thick paraffin sections. Bar: 35  $\mu$ m.

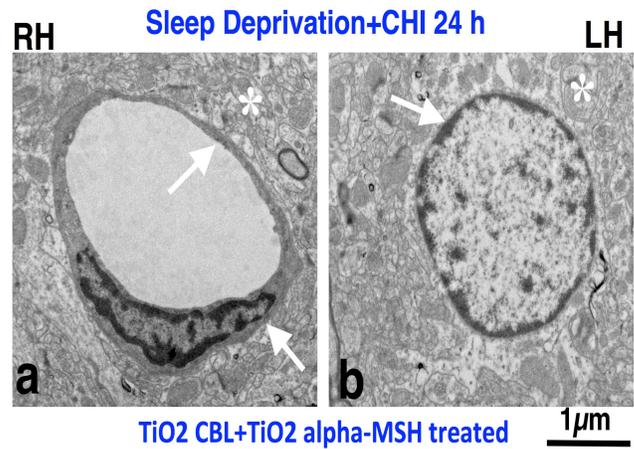


Fig. 3. Low power Transmission electron micrograph showing neuroprotection following treatment with TiO<sub>2</sub>-nanowired cerebrolysin with  $\alpha$ -MSH significantly reduced the neuropil damages, edema and sponginess in SD with CHI in right injured half (RH, a) and left uninjured half (LH, b). Bar: 1  $\mu$ m.

#### 3.5 TiO<sub>2</sub> Cerebrolysin $\alpha$ -MSH and Neuronal Injury

TiO<sub>2</sub> cerebrolysin together with  $\alpha$ -MSH induced marked neuroprotection as seen either at light (Fig. 2) or electron microscopy (Fig. 2) in CHI rats after SD. Thus, rats with CHI after SD neuronal damages are much less evident in TiO<sub>2</sub>-nanowired cerebrolysin and  $\alpha$ -MSH treated animals (Fig. 3). This effect was also seen at the ultrastructural level (Fig. 3).

### 3.6 TiO<sub>2</sub> Cerebrolysin $\alpha$ -MSH and behavioural functions

SD alone induced marked deterioration on Rota-rod, inclined plane angle tests and walking on a mesh grid. These behavioral functions in SD were further aggravated by CHI. TiO<sub>2</sub> nanowired cerebrolysin together with  $\alpha$ -MSH significantly attenuated these behavioural dysfunctions in SD in both normal and CHI inflicted animals (results not shown).

## 4 DISCUSSION

The main findings of this investigation show that CHI inflicts on SD exacerbates brain pathology seen at 48 h. This suggests that soldiers who are deprived of regular sleep may have serious consequences if they get additional brain injury. It appears that alterations in neurotransmitters and decrease in BDNF and  $\alpha$ -MSH levels in the brain caused by SD could result in greater damage following an additional CHI [5-6]. This idea is further strengthened by the fact that SD alone was able to reduce BDNF and  $\alpha$ -MSH levels in the brain that correlates well with the brain pathology. This indicates that a decrease in BDNF and  $\alpha$ -MSH levels is instrumental in brain damage in SD following CHI. This idea is further supported by co-administration of TiO<sub>2</sub> nanowired cerebrolysin with  $\alpha$ -MSH. Cerebrolysin is a balanced composition of several neurotrophic factors including BDNF, IGF-1 and other active peptide fragments [3]. Thus, nanodelivery of cerebrolysin is able to restore the BDNF contents in SD rats following CHI. Likewise  $\alpha$ -MSH nanodelivery also enhanced the level of this hormone that was depleted by SD and CHI combination. This could be one of the main mechanisms by which cerebrolysin and  $\alpha$ -MSH together significantly reduced the brain pathology following CHI in SD.

The reasons for superior effectiveness of cerebrolysin and  $\alpha$ -MSH could be due to the fact that their nanowired delivery may effectively penetrate deeper into the brain or establish cellular communication better than other normal administration [4-6]. Obviously, restoration of BBB function and reduction in brain edema are instrumental in neuronal survival [4]. A slow degradation or metabolism of nanowired cerebrolysin and  $\alpha$ -MSH within the brain may further enhance their high level in the brain of SD rats after CHI causing superior neuroprotection.

## 5 CONCLUSION

Our observations are the first to show that SD pathology enhanced by CHI is related with a decrease in BDNF and  $\alpha$ -MSH levels in the brain. Thus, nanodelivery of these agents restores the level of these neuroprotective components following CHI in SD in the most efficient manner. This suggests that nanowired delivery of agents is

the most efficient in inducing neuroprotection following CHI in SD, not reported earlier.

It remains to be seen whether nanodelivery of other neuroprotective drugs given with  $\alpha$ -MSH or cerebrolysin after longer duration of CHI in SD i.e., after 12 or 24 h insult could still be able to restore BDNF or  $\alpha$ -MSH levels, inducing neuroprotection. This a subject that is currently being examined in our laboratory.

## 6 ACKNOWLEDGEMENTS

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