

# TiO<sub>2</sub>-nanowired delivery of cerebrolysin thwarts exacerbation of sleep deprivation induced decline in regional brain derived neurotrophic factor, brain pathology and behavioral dysfunctions following emotional stress

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## ABSTRACT

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. In this innovation we demonstrated that a combination of emotional stress and sleep deprivation (SD) exacerbates brain pathology and behavioral dysfunction in a rat model. Thus, in these animals several fold increase in BBB permeability to Evans blue albumin (EBA) and radioiodine (<sup>125</sup>I) was observed in several brain regions associated with neuronal injuries as compared to normal animals after SD. Behavioral disturbances were also exacerbated in this model. Interestingly, the brain derived neurotrophic factor (BDNF) levels showed greater decline in stressed animals after SD. Treatment with cerebrolysin (2.5 ml/kg, i.v.) in normal rats 4 to 6 h after SD significantly increased BDNF levels and reduced brain pathology. However, in stressed rats after SD TiO<sub>2</sub> nanowired cerebrolysin (2.5 ml/kg, i.v.) is needed to enhance BDNF levels and attenuate brain pathology. TiO<sub>2</sub> nanowired cerebrolysin also improved behavioral functions significantly in stressed rats after SD. These observations are the first to show that nanodelivery of cerebrolysin has profound neuroprotective effects in SD following emotions stress, not reported earlier.

**Keywords:** Sleep deprivation in Military, brain pathology, Brain derived neurotrophic factor, Cerebrolysin, nanodelivery, Immobilization stress

## 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 emotional stress is always associated with SD in military, in this investigation we examined a combination of emotional stress and SD on brain pathology and behavioral dysfunction in a rat model [1,2]. To understand the role of neurotrophic factor in SD and emotional stress we measured regional distribution of brain derived neurotrophic factor (BDNF) and Insulin like growth factor-1 (IGF-1) in SD. In addition, we used exogenous supplement of Cerebrolysin alone or tagged with nanowires (TiO<sub>2</sub>-nanowired cerebrolysin) that is a balanced composition of several neurotrophic factors and active peptide fragments [4-6] to reduce SD induced brain pathology and behavioral dysfunction.

## 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 Immobilization Stress

Rats were subjected to partial immobilization in a plastic tube with holes for free air circulation. Rats were immobilized for 1 h daily for 2 weeks.

### 2.3 Brain derived neurotrophic factor

Brain derived neurotrophic factor (BDNF) was measured in control or SD rats using Rad BDNF ELISA Kit (ERIGF1, Thermo Scientific, Frederick, MD, USA). The IGF-1 levels (ng/g) was measured in parietal cerebral cortex, hippocampus and cerebellum using commercial protocol.

### 2.4 Insulin like Growth Factor-1

Insulin like growth factor-1 (IGF-1) was measured in control or SD rats using Rat IGF-1 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

Cerebrolysin (CBL, Ever NeuroPharma, Austria) was 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) was administered 4 to 6 h after SD. For comparison, normal CBL (2.5 or 5 ml/kg, i.v.) was also given in separate groups of SD [5,6]. The animals were allowed to survive 48 h.

### 2.6 Blood-Brain Barrier and brain edema

In control and SD animals blood-brain barrier (BBB) breakdown to Evans blue albumin (EBA) and radioiodine (<sup>131</sup>Iodine) was examined after intravenous administration of these tracers (EBA 2 % solution 3 ml/kg, and radioiodine 100  $\mu\text{Ci/kg}$ ) 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 studied 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

After 48 h of SD, normal rats 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 (Table 1). The brain edema formation and neuronal damages were also exacerbated by several folds SD group as compared to normal animals. Neuronal distortion and damages are more frequent in the brain areas showing edema formation or sponginess of the neuropil. In general hippocampus showed greater neuronal damages in the CA-3 and 4 areas along with dentate gyrus as compared to CA-1 and CA-2 areas of the hippocampus. Cerebellar granule cells and Purkinje cells both showed cellular swelling, distortion and damage in the vermis as well as the lateral cerebellar cortices in a selective and specific manner.

At transmission electron microscopy (TEM), membrane vacuolation, synaptic damage and edema are frequent in neuropil from the above brain regions (Fig. 1).

### 3.2 SD and regional brain BDNF Levels

BDNF measurement using ELISA showed a significant decrease in this neurotrophic factor content following 48 h SD in all brain regions examined. Thus, there was a significant decrease of BDNF content by 75 % in hippocampus followed by 50 % in parietal cerebral cortex and about 40 % in the cerebellum following SD 48 h as compared to control group.

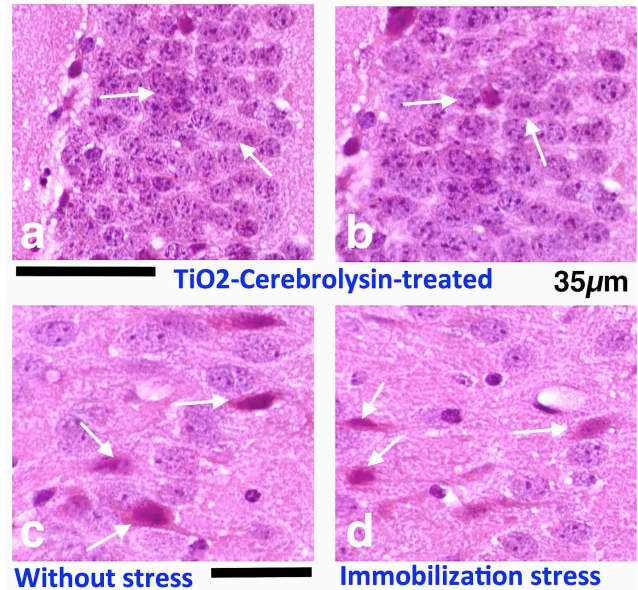
### 3.3 SD and regional IGF-1 Levels

IGF-1 measurement using ELISA showed a significant decrease in SD following 48 h in all brain regions examined. A significant decrease in IGF-1 content by 80 % in hippocampus was seen followed by 55 % decrease in the parietal cerebral cortex and about 35 % reduction in the cerebellum following 48 h SD as compared to control group.

### 3.4 TiO<sub>2</sub> Cerebrolysin and Brain pathology

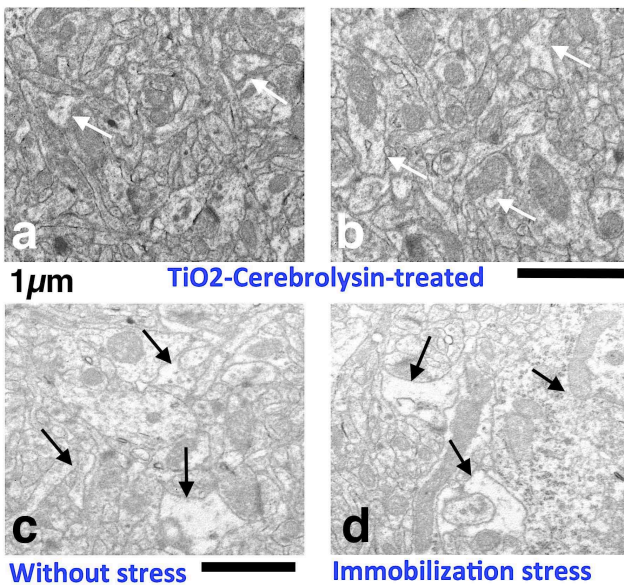
Treatment with TiO<sub>2</sub> nanowired Cerebrolysin (NWCBL) 4 to 6 h after the onset of SD resulted in profound neuroprotection in terms of restoration of the BBB function and reduction in brain edema and volume swelling along with protection of nerve cells against damage caused by SD (Figs. 1 & 2).

#### Sleep Deprivation 48 h Hippocampus CA1



**Fig. 1.** Shows loss of neurons and sponginess with edema in the hippocampus CA1 area in SD (c) and its exacerbation of neuronal damages by emotional stress (d). Treatment with TiO<sub>2</sub>-nanowired cerebrolysin significantly reduced the neuronal damages, edema and sponginess in SD (a) or in SD combined with emotional stress (b). H&E stain on 3-µm thick paraffin sections. Bar: 35 µm.

#### Sleep Deprivation 48 h Parietal Cortex layer III



**Fig. 2.** Low power Transmission electron micrograph shows vacuolation and sponginess in the parietal cerebral cortical neuropil (arrows) in SD (c) and its exacerbation by emotional stress (d). Treatment with TiO<sub>2</sub>-nanowired cerebrolysin significantly reduced the neuropil damages, edema and sponginess in SD (a) or in SD combined with emotional stress (b). Bar: 1 µm.

### 3.5 TiO<sub>2</sub> Cerebrolysin and BDNF level

Our results further show that NWCBL treatment also partially but significantly restored the BDNF levels in all the brain areas examined after 48 h SD. Thus, in NWCBL treated animals BDNF level was significantly elevated after 48 h SD than the untreated group. This restoration of BDNF level by NWCBL in SD rats was almost 80 to 90 % complete as compared to the control group.

### 3.6 TiO<sub>2</sub> Cerebrolysin and IGF-1 level

NWCBL treatment also restored IGF-1 levels in the cortex, hippocampus and cerebellum near normal level in both normal SD as well as in stressed animals subjected to 48 h SD. The magnitude of IGF-1 restoration was significantly higher than the BDNF restoration after TiO<sub>2</sub>-nanowired cerebrolysin treatment.

### 3.7 TiO<sub>2</sub> Cerebrolysin and Neuronal Injury

The NWCBL was able to reduce neuronal injuries in all brain areas examined as seen either at light (Fig. 1) or electron microscopy (Fig. 2). Thus, in NWCBL treated SD rats neuronal damages are much less evident in TiO<sub>2</sub>-nanowired cerebrolysin treated animals after SD in normal

or stressed rats (Figs. 1 & 2). This effect was also seen at the ultrastructural level (Fig. 2). Thus, TEM studies showed less vacuolation, edematous expansion or synaptic damage as compared to untreated SD rats (Fig. 2). At light microscopy also, several neurons were healthy with a distinct nucleus and clear nucleolus in NWCBL treated SD rats in different brain areas as compared to untreated rats that exhibited marked neuronal damages, distortion and loss of nucleus and nucleolus in many brain areas after 48 h SD (Fig. 1).

### 3.8 TiO<sub>2</sub> Cerebrolysin and behavioural functions

SD 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 additional stress. TiO<sub>2</sub> nanowired cerebrolysin significantly attenuated these behavioural dysfunctions in SD in both normal and stressed animals.

## 4 DISCUSSION

The salient findings in this investigation clearly show that SD is capable to induce brain pathology at 48 h. Furthermore, our study showed that prior emotional stress e.g., immobilization aggravates SD induced brain pathology. This suggests that stress potentiates SD induced brain damage [5-6]. Our observations further showed that SD was able to reduce BDNF and IGF-1 levels in the brain that correlates well with the brain pathology. This indicates that a decrease in BDNF and IGF-1 levels is instrumental in brain damage in SD. This idea is further supported by TiO<sub>2</sub> nanowired cerebrolysin therapy. Cerebrolysin is a balanced composition of several neurotrophic factors including BDNF, IGF-1 and other active peptide fragments [3]. Nanodelivery of cerebrolysin is thus able to restore the BDNF and IGF-1 contents in rats following SD. This could be one of the mechanisms by which NWCBL reduced the brain pathology in SD

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The reasons for NWCBL effectiveness could be due to the fact that TiO<sub>2</sub> tagged Cerebrolysin effectively penetrates deeper into the brain or establish cellular communication better than other forms of cerebrolysin delivery [4-6]. Obviously, restoration of BBB function and reduction in brain edema are instrumental in neuronal survival [4]. A slow degradation or metabolism of NWCBL within the brain may also be responsible for maintaining high level of BDNF in the brain of SD rats resulting in neuroprotection.

## 5 CONCLUSION

Our observations are the first to show that SD pathology is related with a decrease in BDNF and IGF-1 levels in the brain and NWCBL restores the level of these neuroprotective agents in SD in the most efficient way. This suggests that NWCBL is the most efficient in inducing neuroprotection in SD, not reported earlier.

It remains to be seen whether NWCBL given after longer duration of SD i.e., 12 h after could still be able to restore BDNF or IGF-1 levels, a subject that is currently being examined in our laboratory.

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