

Neuroprotective effects of cerebrolysin in exacerbation of blood-brain barrier breakdown, neuropathology and upregulation of nitric oxide synthase and hemeoxygenase in diabetes and hypertension after heat stroke

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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: Heat stroke, diabetes, hypertension, brain pathology, blood-brain barrier, brain edema, Nitric oxide, heme oxygenase, TiO₂ nanowired cerebrolysin

1 INTRODUCTION

Military personnel are often exposed to heat stress in desert areas either during peacekeeping or combat operations [1,2]. In such situations, they are quite vulnerable to heat stroke and brain pathology. Due to severe work stress and irregular food habits they often develop hypertension and diabetes. A combination of heat stress with diabetes and hypertension could adversely affect their brain function resulting in mental abnormalities [1,2]. Thus, efforts are needed to find out suitable therapeutic strategies to reduce mortality or morbidity caused by heat stroke alone. It appears that heat stroke induced formation of free radicals could play important roles.

Nitric oxide (NO) and carbon monoxide (CO) are free radical gases that are synthesized within the neurons, glial and endothelial cells by the enzymes nitric oxide synthase (NOS) and hemeoxygenase (HO) respectively [1]. Massive expression of NOS and HO could contribute to breakdown of the blood-brain barrier (BBB), edema formation and neuronal injuries.

Previous studies in our laboratory show that upregulation of NOS and HO occurs following whole body hyperthermia (WBH) at 38° for 4 h that is similar to clinical symptoms of brain pathology and heat stroke [1-3].

However, upregulation of NOS and HO in DBHY rats after WBH is not well known [4,5]. Thus, a possibility exists the DBHY rats after WBH induce exacerbation of NOS and HO upregulation that could exacerbate brain pathology. Since cerebrolysin is a balanced composition of several neurotrophic factors and active peptide fragments, in this investigation we examined the influence of TiO₂ nanowired delivery of cerebrolysin on NOS and HO upregulation in DBHY rats after WBH in relation to brain pathology 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 Exposure to heat stress

Animals were exposed to heat stress in a biological oxygen demand (BOD) incubator at 38°C for 4 h. The relative humidity (45-57 %) and wind velocity (20-25 cm/sec) was kept constant. The animals develop heat stroke symptoms similar to clinical situations [1-4].

2.2 Hypertension and diabetes

Rats were made hypotensive using 2 kidney 1 clip (2K1C) method. For this purpose, a silver clip with an opening of 0.5 mm was placed over left renal artery to induce partial ischemia. This treatment results in development of clinical grade hypertension in these rats after 4 to 6 weeks (mean arterial blood pressure, MABP 150 to 170 Torr). These hypertensive rats then made diabetic by injecting streptozotocine (50 mg/kg, i.p.) daily for 3 days. This treatment results in blood sugar elevation within a week by 20 to 40 mM/L [2-5].

Control group show blood sugar level 5-6 mM/L and MABP of 90-105 Torr.

2.3 Blood-brain barrier and brain edema

The BBB breakdown was examined 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. Brain edema was determined using regional water content by wet and dry weights of the brain samples [2-4].

2.4 Nitric oxide synthase and heme-oxygenase immunohistochemistry

Nitric oxide synthase (NOS) neuronal isoform (nNOS) and heme-oxygenase (HO) constitutive isoform (HO-2) was examined using immunohistochemistry on paraffin sections (3 µm thick) employing Anti-nNOS (neuronal) antibody (ab1376) and Anti-Heme oxygenase 2 antibody (ab90515) respectively according to commercial protocol.

2.5 Histopathology

In separate groups of animals, neuronal changes were examined using histopathological techniques for Nissl or Haematoxylin & Eosin (HE) staining [2,6].

2.6 TiO₂-nanowired Cerebrolysin

Cerebrolysin (CBL, Ever NeuroPharma, Austria) was tagged with TiO₂ nanowires according to standard protocol [2,3]. The TiO₂ nanowired Cerebrolysin (2.5 ml/kg, i.v. NWCBL) was administered either 1 or 2 h after the onset of a 4 h heat stress session. For comparison, normal cerebrolysin was administered in identical doses instead of NWCBL [2,4,6,7].

2.7 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-brain barrier and brain edema

Heat exposure in DBHY rats resulted in marked exacerbation of the BBB breakdown as evident with greater leakage of exogenous protein tracers, e.g., EBA and radioiodine in the cerebral cortex, hippocampus, thalamus, hypothalamus and cerebellum. In these brain areas, significant increase in brain water content was also observed indicating brain edema formation and volume swelling. In general these pathological changes in DBHY rats after stress was 2- to 4-fold higher than the normal healthy rats subjected to identical heat stress.

3.2 NOS and HO immunoreactivity

Heat stress in DBHY animals resulted in profound exacerbation of nNOS and HO-2 upregulation in several brain areas exhibiting BBB leakage and brain edema. This

upregulation of NOS and HO immunoreactivity was 2- to 4-fold higher in some brain areas as compared to immunoreaction seen in normal animals after an identical heat exposure (Fig. 1). The most marked upregulation in DBHY animals was evident in cerebral cortex, hippocampus and cerebellum.

3.3 Brain pathology

Heat exposure in DBHY rats exacerbated neuronal injuries in several parts of the brain exhibiting BBB breakdown and edema formation. Thus, loss of neurons in the cerebral cortex, hippocampus and cerebellum was the most prominent as compared to identical heat stress in normal healthy rats. The number of damaged and distorted neurons increased significantly by 2- to 4-fold in after heat stress in DBHY rats as compared to normal animals after identical heat exposure. The occurrences of damaged cells are seen in the areas showing marked upregulation of nNOS or HO-2 immunoreactivity.

3.4 Effect of Cerebrolysin Treatment

Treatment with TiO₂ nanowired Cerebrolysin (2.5 ml/kg, i.v., NWCBL) 1 or 2 h after the onset of heat exposure in DBHY animals resulted in a significant reduction in the nNOS and HO-2 immunoreaction in several parts of the brain.

In these brain areas, brain pathology was also significantly reduced. Neuronal loss, neuronal damages and distortion was markedly reduced by NWCBL in DBHY rats after heat stress. On the other hand normal cerebrolysin when administered in higher doses (10 mg/kg, i.v.) under identical conditions was able to achieve comparable reduction in NOS and HO immunoreaction and pathological changes in the above brain areas [Fig. 2].

At the ultrastructural level also, TiO₂-nanowired cerebrolysin was also able to reduce edema, membrane disruption and sponginess in DBHY animals after heat exposure most effectively as compared to normal cerebrolysin administration (Fig. 3).

TiO₂ nanowired cerebrolysin resulted in significant reduction in the BBB breakdown to EBA and radioiodine tracers in several brain areas in DBHY rats after heat stress. A close parallelism was observed between the magnitude of reduction in the BBB breakdown and the intensity of edema formation in following NWCBL treatment of DBHY rats after heat stress.

4 DISCUSSION

Our novel findings clearly show that TiO₂ nanowired cerebrolysin is capable to attenuate heat stroke induced brain pathology in DBHY rats. Furthermore our observations are the first to point out that TiO₂-nanowired cerebrolysin has superior effects in inducing

neuroprotection in DBHY rats after heat stroke as compared to cerebrolysin in lower doses. These observations suggest that TiO₂-nanowired cerebrolysin could be used in clinics in heat stroke cases complicated with co-morbidity factors, not reported earlier.

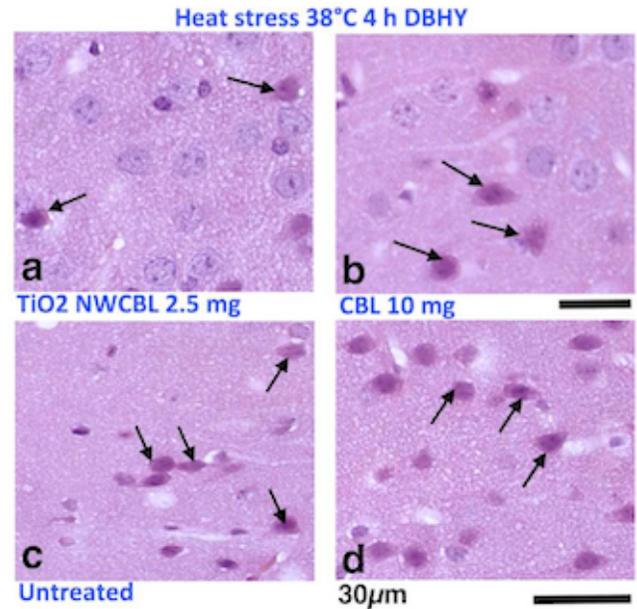


Fig. 1. TiO₂ NWCBL reduces neuronal damages in the cortex following 4 h heat exposure in diabetic and hypertensive rats.

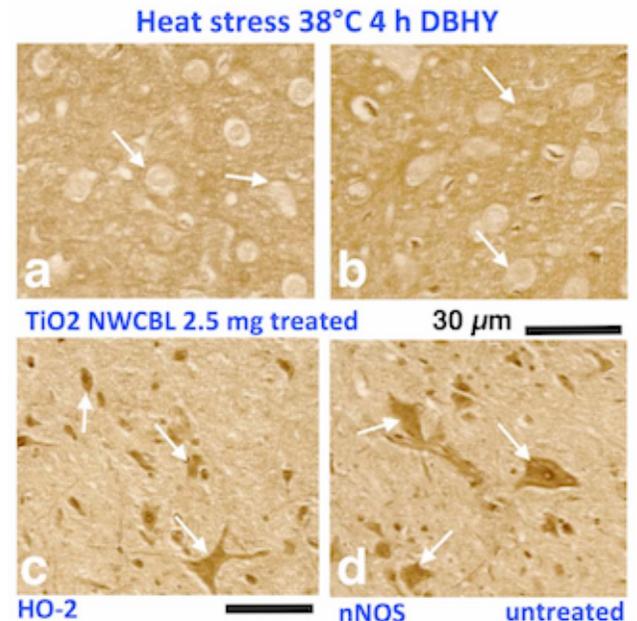


Fig. 2. TiO₂ Nanowired cerebrolysin (NWCBL) significantly reduced heme-oxygenase-2 and neuronal nitric oxide synthase immunoreactivity in the cerebral cortex after heat stress in diabetic and hypertensive rats.

The possible mechanisms by which TiO₂ nanowired cerebrolysin is capable to attenuate brain pathology in heat stroke complicated with DBHY syndrome is unclear. However, it appears that a reduction in nNOS and HO-2 immunoreactivity by TiO₂-nanowired cerebrolysin could play important roles [1-4]. In addition, strengthening the cell membrane of endothelial cells due to neurotrophic and antioxidant effects of cerebrolysin could be another important factor for reducing brain pathology [2-5,7]. A significant reduction in BBB breakdown and brain edema formation in TiO₂-nanowired cerebrolysin treated groups in DBHY rats further support the idea.

DBHY alone could induce oxidative stress and free radical formation [2-5]. This effect could be exacerbated by additional exposure to heat stress. This could be one of the main reasons for upregulation of nNOS and HO-2 expression. It is quite likely that oxidative stress and lipid peroxidation could alter membrane permeability resulting in greater BBB leakage to proteins. Obviously, extravasation of proteins into the brain fluid compartment will lead to vasogenic edema formation. Volume swelling of the brain in a close cranial compartment could compress the brain cells and also expose unwanted substances from the vascular compartment causing brain injury [4-7]. Accordingly, neuronal, glial and axonal injuries could occur in DBHY rats and also following heat stress. Alternatively, a direct effect of cerebrolysin on endothelial membrane stability may also be responsible for neuroprotection, a feature that requires additional investigation. Potentiation of cerebrolysin induced neuroprotection caused by TiO₂ nanowired delivery could be due to either an enhanced penetration of the drug within the brain or due to a slow degradation or metabolism of the compound within the CNS [2- 6]. Higher doses of normal cerebrolysin having quite remarkable effects on neuroprotection in DBHY rats after heat exposure are in line with this hypothesis.

5 CONCLUSION

In conclusion, our observations indicate that TiO₂ nanodelivery of cerebrolysin has superior effects on DBHY induced brain pathology after heat stress. These results suggest that military personnel who are often exposed to heat stress with or without additional co-morbidity factors i.e. diabetes and/or hypertension may get better therapeutic strategies using cerebrolysin in the future.

It remains to be seen whether nanodelivery of cerebrolysin using other technology e.g., Poly (L-lactide-co-glycolide) could also be equally effective to contain brain pathology in heat stress with DBHY conditions. This is a feature that is currently being investigated in our laboratory.

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