

TiO₂-Nanowired cerebrolysin attenuated hyperthermia induced ubiquitin overexpression and brain pathology

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ABSTRACT

Our military personnel are often engaged in combat operations in desert environments during summer months across the World. In such situations, apart from their exposure to missiles, roadside bomb or other explosive related nanoparticles exposure, they are also exposed to silica dust (SiO₂) nanoparticles. Previous experiments from our laboratory showed that SiO₂ exposure exacerbates heat stress induced brain pathology. Thus, this is likely that our soldiers that are exposed to SiO₂ nanoparticles under heat stress are prone to central nervous system (CNS) dysfunction. Since ubiquitin is present in almost all cells and responsible for intracellular protein degradation, in present innovation we examined ubiquitin expression in heat stress and its modification with SiO₂ nanoparticles in a rat model. Our observations show that SiO₂ enhances ubiquitin overexpression in heat stress and that could be one of the key mechanisms responsible for brain pathology.

Keywords: ubiquitin, silica dust, brain pathology, heat stress, military, brain dysfunction

1 INTRODUCTION

Exposure to military personnel to high environment heat in Middle East or other desert environment leads to silica dust (SiO₂) intoxication [1,2]. This could lead to abnormal brain function and brain pathology [3]. Thus, efforts should be made to understand the basic cellular and molecular mechanism of brain dysfunction caused by a combination of SiO₂ and heat stress for development of effective therapeutic measures [4]. Ubiquitin is a small molecular weight protein found in almost all cells and tissues in all

organisms [5]. The most well known function of ubiquitin is to regulate protein interaction or degradation to maintain homeostasis [see 5]. However, upregulation of ubiquitin in brain or spinal cord is seen following various neurodegenerative diseases or following traumatic insults. The functional significance of such an enhanced ubiquitin expression is still unknown [4,5]. Interestingly, expression of ubiquitin in heat stroke or following nanoparticles exposure is still unknown. Keeping these views in mind, in this innovation we examined ubiquitin expression in a rat model of heat stress in which animals are also intoxicated with SiO₂ nanoparticles [3,6].

Previous experiments from our laboratory demonstrated an exacerbation of brain pathology in heat stress following exposure to SiO₂ nanoparticles [3]. It seems quite likely that under such situations, ubiquitin protease system may also be affected. We have shown that Cerebrolysin (Ever NeuroPharma, Austria), a balanced composition of several neurotrophic factors and active peptide fragments in high doses was able to thwart SiO₂ induced exacerbation of brain pathology in heat stress [3,4,6]. This suggests that enhanced delivery of Cerebrolysin either using high doses of the compound or following nanodelivery one could achieve better therapeutic effects in SiO₂ induced brain dysfunction following heat stress [6]. Thus, it would be interesting to see whether nanodelivery of Cerebrolysin could affect ubiquitin expression, if any in the brain following the combination of SiO₂ exposure and heat stress in our rat model. We examined ubiquitin expression in the brain using immunohistochemistry following heat stress in normal and in SiO₂ treated animals using standard protocol.

In addition, effects of titanium oxide (TiO₂) nanowired Cerebrolysin on ubiquitin expression and brain pathology was also examined in this model.

2 MATERIALS & METHODS

Experiments were carried out on Male Sprague-Dawley Rats (200-300 g) that housed at controlled room temperature ($21 \pm 1^\circ \text{C}$) with 12 h light and 12 h dark schedule. Food and water were supplied ad libitum before 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

Normal or SiO₂ intoxicated rats were exposed to 4 h heat stress in a Biological Oxygen Demand Incubator (BOD) maintained at 38°C with relative humidity (45-47%) and wind velocity (20-25 cm/sec) were kept constant [3,4]. A group of saline treated or diabetic rats were exposed in identical conditions at 21°C for comparison.

2.2 SiO₂ Nanoparticles exposure

Rats were treated with commercial SiO₂ nanoparticles (IoLiTec Ionic Liquids Technologies GmbH, D-74076 Heilbronn, Deutschland) in a dose of 50 mg/kg, i.p. (Size 50-60 nm) daily once for 1 week. This dose and schedule of treatment results in accumulation of SiO₂ in blood plasma comparable to that seen in human exposure for long periods [3].

2.3 Nanodelivery of Cerebrolysin

We used normal or TiO₂ nanowired Cerebrolysin (2.5 ml/kg) in heat stress rats with or without SiO₂ exposure administered intravenously once daily for 3 days before subjection of rats to 4 h heat stress [4,6].

2.3 Parameters Measured

The following parameters to assess brain pathology were measured and normal or SiO₂ intoxicated rats after heat stress and following treatment with Cerebrolysin.

2.3.1 Blood-brain barrier

The blood-brain barrier (BBB) leakage was measured using Evans blue albumin (EBA) and radioiodine (¹³¹Iodine] extravasation in the brain. For this purpose the EBA (2 % of 0.3 ml/100g body weight) was administered intravenously 5 min before termination of the experiment. After washing out if intravascular tracer with 0.9 % saline perfused through heart at 90 Torr, the brain were dissected out and examined for blue staining. The tissue pieces from

selected brain areas were then dissected out weighed and radioactivity determined in a Gamma Counter (Packard, USA). Before saline perfusion about 1 ml whole blood was withdrawn from cardiac puncture to determine radioactivity or EBA concentration in the whole blood. Leakage of these tracers was expressed as percentage increase in the brain over blood concentration [3,4,6].

2.3.2 Brain Edema formation

The brain edema formation was determined using measurement of water content in the brain. For this purpose, small tissue pieces of brain were dissected out and weighed immediately to determine their wet weight. After that these tissue pieces were kept in an oven maintained at 90°C for 72 h to obtain their dry weight. The percentage water content was calculated from the differences between wet and dry weight of the samples [4,6].

2.3.3 Neuronal injury

Neuronal injury was evaluated using Nissl or Haematoxylin & Eosin (HE) staining on paraffin sections using standard histopathological techniques [4,6]. For this purpose, animals were perfused in situ with 4 % buffered paraformaldehyde preceded with a brief saline rinse through cardiac puncture. After in situ fixation, the brain were removed and kept in the same fixative for 24 h. On the 2nd day coronals sections of the brain were cut passing through the hippocampus and the blocks were embedded in paraffin using standard procedures. About 3 μm thick sections were cut and stained with HE or Nissl using commercial protocol [3].

The sections were examined under an Inverted Carl-Zeiss Microscope and the images were recorded using a digital Olympus camera [6]. The number of damaged or distorted neurons in designated anatomical areas were counted manually and compared between controls; heat stressed healthy or diabetic rats with or without MSCs treatment.

2.3.3 Ubiquitin Immunohistochemistry

Ubiquitin immunohistochemistry was done using standard protocol on 3- μm thick paraffin sections using Ubiquitin antibody (ab7780; Dilution 1:100). The immunostaining was developed using Avidin-biotin complex (ABC) techniques [4,6].

2.4 Statistical analyses

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

3 RESULTS

3.1 Ubiquitin expression in heat stress

Heat stress resulted in marked upregulation of ubiquitin expression within the neurons and also in glial cells like structures in the cerebral cortex. This overexpression of ubiquitin was further enhanced in animals intoxicated with SiO₂ nanoparticles after heat stress (Table 1). In these animals, breakdown of the BBB to EBA and radioiodine was also most marked as compared to saline treated heat stressed group. The neuronal damages were also much

more intense in SiO₂ treated heat stressed animals as compared to normal rats subjected to identical hyperthermia (Table 1).

Brain edema formation as seen using water content measurement closely followed ubiquitin overexpression and BBB leakage as well as neuronal injuries. Thus, brain edema was most marked in SiO₂ intoxicated heat stressed group as compared to saline treated heat stressed animals (Table 1). The magnitude and intensity of neural injuries were most marked in the edematous areas of the cerebral cortex (results not shown).

Table 1. Superior neuroprotective effects of nanodelivered Cerebrolysin on Ubiquitin and brain pathology in hyperthermia

Expt. Type	Control	Heat Stress 4h	SiO ₂ +HS	CBL+HS+ SiO ₂	TiO ₂ -CBL+HS+ SiO ₂
EBA mg%	0.24±0.08	1.84±0.12**	2.47±0.12**#	1.08±0.18*a	0.58±0.10*#b
[¹³¹ I]Iodine %	0.34±0.08	2.03±0.10**	3.03±0.16**#	1.76±0.21*a	0.68±0.11*#b
Brain Water %	75.36±0.12	80.14±0.14**	82.68±0.38**#	77.44±0.37*a	76.08±0.13*#b
Neuron Injury Nr	1±2	234±45**	445±61**#	132±13*a	34±8*#b
Ubiquitin +cells Nr	2±2	43±8**	87±6**#	23±8*a	12±6*#b

Values are Mean±SD of 5 to 6 rats. CBL = Cerebrolysin, * P <0.05, ** P <0.01 from control, # P <0.05 from Heat stress, a P <0.05 from SiO₂, b P <0.05 from CBL+HS. TiO₂-CBL = nanodelivered Cerebrolysin. For details see text.

3.2 Nanowired delivery of MSCs is superior

Interestingly, in Cerebrolysin (2.5 ml/kg, i.v.) treatment resulted in marked neuroprotection in saline treated heat stressed rats after 4 h (results not shown), however, this dose has much less beneficial effects in SiO₂ intoxicated rats after heat stress (Fig. 1, Table 1). Thus, only a mild but significant reduction in ubiquitin expression, BBB disruption, brain edema formation and neuronal injuries was evident (Table 1). On the other hand, when SiO₂ intoxicated rats were exposed to heat stress in TiO₂-nanowired cerebrolysin (2.5 ml) treated animals, significant reduction in ubiquitin expression, BBB breakdown, neuronal injuries and edema formation was observed (Fig. 1, Table1).

Nanodelivery of Cerebrolysin also significantly reduced behavioral symptoms and stress response of heat stress in SiO₂ intoxicated group (results not shown).

Cerebrolysin could be used to influence ubiquitin expression in several neurodegenerative diseases e.g., Parkinson's, Alzheimer's, Huntington's as well as in multiple sclerosis and traumatic CNS injuries for effective therapy in future [5].

Another important finding from this innovation show that TiO₂-nanowired Cerebrolysin is needed to effectively contain brain pathology and ubiquitin expression in SiO₂ intoxicated heat stressed rats. This suggests that our soldiers who are chronically exposed to SiO₂ nanoparticles in desert and engaged in combat stress at high environmental heat may get effective treatment using nanowired cerebrolysin for their mental anomalies, if needed. However, further study in this area is needed to explore the long-term strategies of ubiquitin expression in relation to heat stress and SiO₂ intoxication.

How ubiquitin overexpression leads to brain pathology is still speculative [see 5]. There are reasons to believe that abnormal cellular proteins expression occurring in CNS disease of diverse conditions necessitates upregulation of ubiquitin expression in different brain areas [3-6]. Alternatively, cellular and molecular stress could be also responsible for ubiquitin overexpression leading to a cascade of events causing brain damage [3-6]. Thus, downregulation of ubiquitin in Cerebrolysin treated group

4 DISCUSSION

Our novel findings clearly show that Cerebrolysin has the capability to thwart ubiquitin expression in the brain following heat stress. This innovation supports the idea that

may represent diminished stress level at the cellular and molecular levels or suppression of neurodestructive protein expression in the brain that normally occur in untreated CNS insults [3,5].

The possible mechanisms of the superior effects of nanodelivered Cerebrolysin in heat stress are unclear. Rapid distribution of the drug within the brain as well as slow degradation of the compound due to its bonding with nanowires could partially be responsible for such a superior action of Cerebrolysin [3,4,6]. Obviously, long-lasting effects of Cerebrolysin in high concentration could lead to cell membrane stability resulting in reduction in the BBB dysfunction and neuronal injuries. A reduction cellular stress and injury signals may also be responsible for downregulation of ubiquitin expression after heat stress. However, further studies are needed to clarify these points.

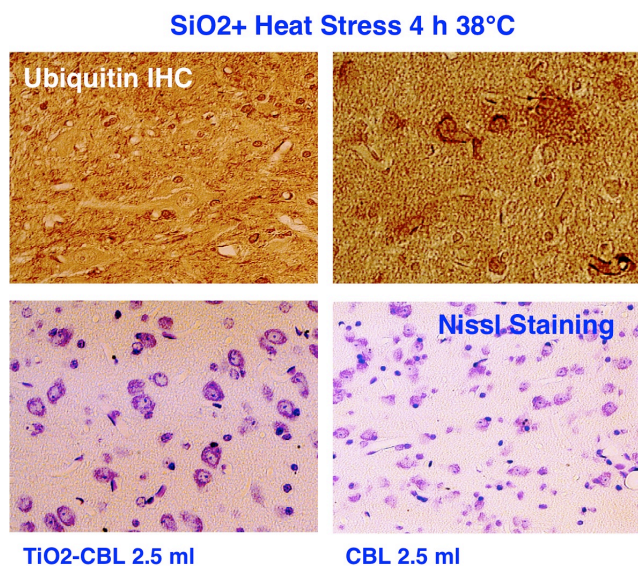


Fig. 1. TiO₂-Nanowired Cerebrolysin induces superior neuroprotection in SiO₂ intoxicated heat stressed rats. Bar = 35 μm.

5 CONCLUSION

Taken together our innovation demonstrated novel features of Cerebrolysin in attenuating ubiquitin expression in the brain following heat stress leading to reduction in brain pathology. TiO₂-nanowired Cerebrolysin appears to be more effective in inducing downregulation of ubiquitin and neuronal injuries that are exacerbated following SiO₂ intoxication in hyperthermia. These observations open up new avenues for Cerebrolysin research in hyperthermia-induced neurodegeneration. In addition, our research further suggests that novel drug discovery targeting ubiquitin-protease system is needed to effectively treat neurological disease in future clinical settings.

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7 REFERENCES

- [1] Stacey M, Woods D, Ross D, Wilson D. Heat illness in military populations: asking the right questions for research. *J R Army Med Corps.* 2014 Jan 3. doi: 10.1136/jramc-2013-000204.
- [2] Nindl BC, Castellani JW, Warr BJ, Sharp MA, Henning PC, Spiering BA, Scofield DE. Physiological Employment Standards III: physiological challenges and consequences encountered during international military deployments. *Eur J Appl Physiol.* 2013 Nov;113(11):2655-72. doi: 10.1007/s00421-013-2591-1. Epub 2013 Feb 22.
- [3] Ciechanover A. Intracellular protein degradation: from a vague idea through the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. *Bioorg Med Chem.* 2013 Jun 15;21(12):3400-10. doi: 10.1016/j.bmc.2013.01.056. Epub 2013 Feb 8. Review.
- [4] Sharma HS, Sharma A, Mössler H, Muresanu DF. Neuroprotective effects of cerebrolysin, a combination of different active fragments of neurotrophic factors and peptides on the whole body hyperthermia-induced neurotoxicity: modulatory roles of co-morbidity factors and nanoparticle intoxication. *Int Rev Neurobiol.* 2012;102:249-76. doi: 10.1016/B978-0-12-386986-9.00010-7. Review.
- [5] Sharma HS, Muresanu DF, Patnaik R, Sharma A. Exacerbation of brain pathology after partial restraint in hypertensive rats following SiO₂ nanoparticles exposure at high ambient temperature. *Mol Neurobiol.* 2013 Oct;48(2):368-79. doi: 10.1007/s12035-013-8502-y. Epub 2013 Jul 6.
- [6] Sharma HS, Sharma A. Nanowired drug delivery for neuroprotection in central nervous system injuries: modulation by environmental temperature, intoxication of nanoparticles, and comorbidity factors. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2012 Mar-Apr;4(2):184-203. doi: 10.1002/wnan.172. Epub 2011 Dec 8. Review.

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