

# TiO<sub>2</sub> Nanowired Cerebrolysin reduces neuron-specific ubiquitin carboxyl-terminal esterase-L1 (UCHL1) in Alzheimer's Disease and brain pathology

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## ABSTRACT

This innovation deals with involvement of neuron-specific ubiquitin carboxyl-terminal esterase-L1 (UCHL1) in Alzheimer's Disease (AD) induced brain pathology and neuroprotection by modulation of the enzyme with multimodal drug Cerebrolysin administered using TiO<sub>2</sub>-nanowired biotechnology in model experiments. AD like brain pathology was induced by intracerebroventricular (i.c.v.) administration of amyloid beta peptide (A $\beta$ P 1-40, 250 ng/10  $\mu$ l) daily for 4 weeks. This treatment resulted in significant elevation of UCHL1 in various brain regions associated with breakdown of the blood-brain barrier (BBB) and brain pathology. Administration of TiO<sub>2</sub> nanowired Cerebrolysin (25  $\mu$ l, NWCBL, i.c.v.) starting from 1 week after the onset of A $\beta$ P infusion thwarted UCHL1 elevation and brain pathology. These observations are the first to demonstrate that nanodelivery of cerebrolysin is capable to attenuate AD pathology by modulating UCHL1 enzyme, not reported earlier.

**Keywords:** Alzheimer's Disease, brain pathology, ubiquitin carboxyl-terminal esterase-L1, Cerebrolysin

## 1 INTRODUCTION

Our military personnel during combat operations are vulnerable to various kinds of traumatic brain injuries. These soldiers are prone to develop neurodegenerative diseases e.g., Alzheimer's Disease (AD) over time [1,2]. Thus, efforts should be made to reduce the consequences of brain injury or development of AD pathology. Recent studies show that brain injury alone or followed by AD

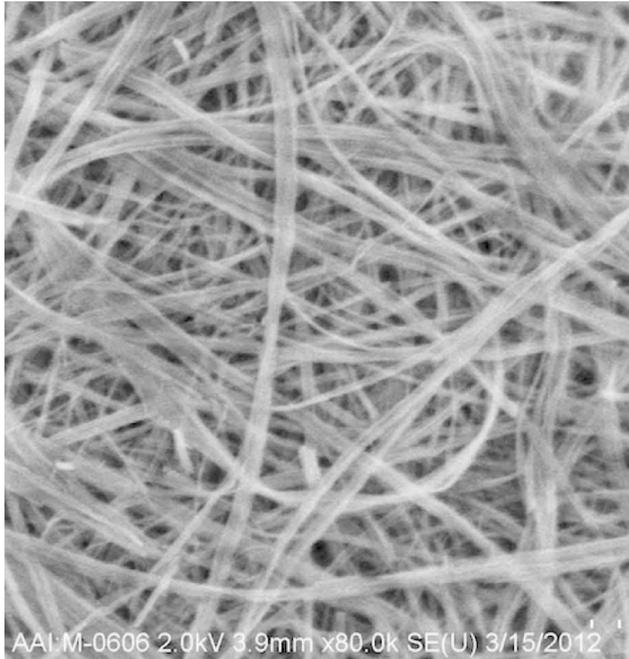
leads to enhanced levels of plasma and cerebrospinal fluid (CSF) concentrations of neuron-specific ubiquitin carboxyl-terminal esterase-L1 (UCHL1) [1,4]. The level of UCHL1 correlates well with neurological symptoms of AD as well as brain pathologies [4]. The UCHL1 is a protein selectively expressed in neurons due to alterations in ubiquitin proteasome pathways [1]. An accumulation, overload or malfunction of UCHL1 results in protein aggregation in the AD brain. This would result in structural changes in the protein substrates thereby preventing the recognition and degradation of Amyloid beta protein (A $\beta$ P) by the UCHL1 [1,4]. Altered UCHL1 levels indicate defective proteolysis that could cause synaptic dysfunction as well as clearance of A $\beta$ P from the brain [2,3]. Thus, regulation of UCHL1 for therapeutic purposes may affect the pathogenesis of AD. There are also reports that UCHL1 is facilitating lysosomal degradation of Amyloid precursor protein (APP) by increasing free ubiquitin levels [1,4]. Thus, it remains to be seen whether enhanced levels of UCHL1 is reducing the AD pathology in the brain or multimodal drugs e.g., cerebrolysin that is known neuroprotective agent [2,3,5] and may affect UCHL1 regulation in AD.

## 2 MATERIALS & METHODS

Experiments were carried out on Male Wistar rats (200-250 g body weight) housed at controlled room temperature (21 $\pm$ 1 $^{\circ}$ 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 Alzheimer's Disease Pathology

AD like brain pathology was induced by A $\beta$ P (1-40) administration intraventricularly (i.c.v.) in the left lateral ventricle 250 ng/10  $\mu$ l once daily for 4 weeks using standard procedures [3]. After 30 days of infusion, various biochemical and pathological parameters were measured as described earlier [2,3,6].



**Fig. 1. TiO<sub>2</sub> Nanowires are used for Cerebrolysin tagging for nanodelivery [see Ref. 2].**

## 2.2 Brain Pathology

After 30 days of the 1<sup>st</sup> A $\beta$ P infusion, the rats exhibited breakdown of the blood-brain barrier (BBB) extravasation of endogenous/exogenous protein tracers, brain edema formation, A $\beta$ P deposits in several parts of the brain. The BBB breakdown was examined using Evans blue (EB) and [<sup>131</sup>I]iodine leakage across the brain microvessels after intravenous administration of these tracers (EB 2 % solution 3 ml/kg, and radioiodine 100  $\mu$ Ci/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]. In separate groups of animals, immunohistochemistry of albumin, glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) was examined. Neuronal changes were studied using histopathological examination of Nissl or Haematoxylin & Eosin (HE) staining [3,6].

## 2.3 UCHL1 ELISA

UCHL1 levels in the cerebral cortex, hippocampus and cerebellum were measured using a UCHL1 sandwich enzyme-linked immunosorbent assay (ELISA). Mouse monoclonal anti-human UCHL1 antibody was used for this purpose using standard procedures [1].

### 2.3.1 UCHL1 Immunohistochemistry

UCHL1 immunohistochemistry was performed on 3- $\mu$ m thick paraffin sections using Mouse monoclonal anti-human UCHL1 antibody (WH0007345M1 SIGMA, St. Louis, USA) with dilution 1:1600 in PBS with 1.5% normal goat serum, 0.2% Triton X-100, 0.2% gelatin and 1  $\mu$ l/ml sodium azide and incubated for 48 h at 4°C. The immunoreaction was visualized using the avidin-biotin-peroxidase complex (Vectastain ABC-Elite Kit, Vector Laboratories, Burlingame, CA, USA).

### 2.3.2 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 [2,3]. The TiO<sub>2</sub> nanowired Cerebrolysin (25  $\mu$ l, NWCBL) was infused into the left cerebral ventricles daily starting from 1 week after the onset of A $\beta$ P infusion and terminated 1 week before the last infusion. For comparison, normal CBL was administered in identical doses instead of NWCBL.

### 2.3.3 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 Brain Pathology

After 30 days of the 1<sup>st</sup> A $\beta$ P infusion, the rats exhibited profound breakdown of the BBB as evident with extravasation of endogenous serum albumin as well as exogenous protein tracers, e.g., EB and radioiodine in the cerebral cortex, hippocampus and the cerebellum (Table 1). In these brain areas, significant increase in brain water content was also observed indicating brain edema formation (Table 1).

**Table 1. Neuron-specific ubiquitin carboxyl-terminal esterase-L1 (UCHL1) levels in the brain in Alzheimer's Disease and its modification with TiO<sub>2</sub>-nanowired cerebrolysin (NWCBL).** AβP 1-40 infused into lateral ventricle 250 ng/10 μl once daily for 4 weeks. Cerebrolysin (CBL) or TiO<sub>2</sub> nanowired CBL(NWCBL) was infused identically 25 μl 1 week after AbP for 2 weeks.

Expt. Group	Brain Pathology Cerebral Cortex		UCHL1 level				Neural Injury	
	<sup>131</sup> I %	Brain Water %	CSF ng/ml	Cerebral cortex ng/g	Hippo-campus ng/g	Cerebellum ng/g	Cerebral cortex nr	Hippo-campus nr
A. Saline Control	0.25±0.07	74.76±0.21	9.5±2.0	3.2±0.56	4.8±0.21	3.6±0.32	2±3	3±2
B. AβP infusion	3.28±0.12**	78.34±1.02**	67.7±7.23**	11.78±2.34**	23.32±0.89**	34.32±2.32**	218±23**	189±12**
C. AβP infusion+CBL	1.89±0.34*#	76.09±0.21**#	34.23±0.21**#	7.03±0.21**#	13.21±0.23**#	18.23±0.18**#	78±12**#	56±12**#
D. AβP infusion+NWCBL	0.76±0.09**#^	75.13±0.10*#^	15.34±0.08**#^	5.45±0.09**#^	6.78±0.08**#^	6.36±0.21**#^	12±4**#^	16±8**#^

Values are Mean±SD of 5 to 6 rats at each point. \* P <0.05, \*\* P <0.01 from control A; # P <0.05 from B; ^ P <0.05 from C. ANOVA followed by Dunnett's test for multiple group comparison with one control. For details see text.

### 3.2 UCHL1 Level in CSF and AD Brain

Our ELISA studies showed a significant increase in the UCHL1 level in the CSF and in key brain areas e.g., parietal cerebral cortex, hippocampus and cerebellum [see Table 1]. The increase in CSF UCHL1 was much more pronounced (7-fold) than in cortical brain tissues. Interestingly hippocampus and cerebellum also showed more than 6- to 8-fold increase after AβP infusion [Table 1]. This increase in UCHL1 level in the cortex and in hippocampus closely corresponded with cellular injuries in these brain areas [Table 1]. In these AD brains AβP deposits, albumin leakage, gliosis and axonal damages (Fig. 3) were also most prominent.

### 3.3 UCHL1 Immunohistochemistry in AD

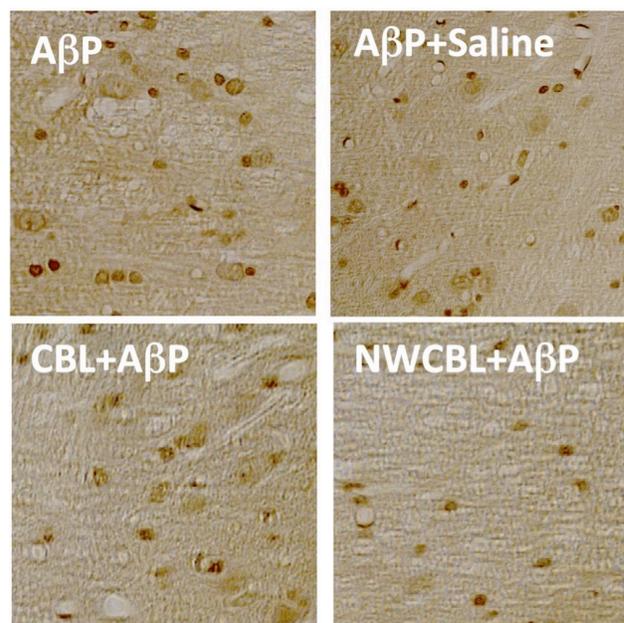
AβP infusion also upregulated UCHL1 immunoreactivity in several parts of the brain e.g., cerebral cortex, hippocampus and cerebellum. The UCHL1 activity was largely seen in damaged and distorted nerve cells located within the edematous neuropil. An example of UCHL1 immunoreactivity following AβP infusion in the parietal cerebral cortex is shown in Fig. 2.

### 3.4 Effect of Cerebrolysin Treatment

When TiO<sub>2</sub> nanowired Cerebrolysin (25 μl, NWCBL) was infused into the left cerebral ventricles daily starting from 1 week after the onset of AβP infusion and terminated 1 week before the last infusion, there was a significant reduction in the UCHL1 levels in various parts of the brain as well as brain pathology was significantly reduced. Neuronal loss, gliosis and AβP deposits were significantly reduced indicating that UCHL1 is modulating AD pathology, not reported earlier. On the other hand normal cerebrolysin when administered in higher doses (100 μl) under identical conditions was able to achieve comparable

reduction in UCHL1 level and pathological changes in the above brain areas [Table 1, Fig. 2].

At the ultrastructural level, TiO<sub>2</sub>-nanowired cerebrolysin was also able to reduce myelin vesiculation following AβP infusion most effectively as compared to normal cerebrolysin administration (Fig. 3).



**UCHL1 Immunoreactivity in AD**

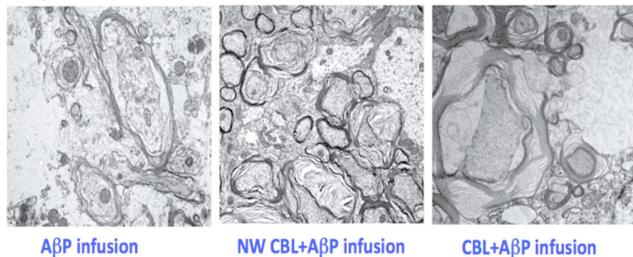
**Fig. 2. NWCBL reduces UCHL1 upregulation in the cortex following AβP infusion induced AD like symptoms x 40.**

## 4 DISCUSSION

Our novel findings clearly show TiO<sub>2</sub> nanowired cerebrolysin is capable to attenuate AD induced brain pathology. Furthermore our observations are the first to point out that TiO<sub>2</sub>-nanowired cerebrolysin also resulted insignificant reduction in CSF and brain UCHL1 levels after AβP infusion induced AD like symptoms. These

observations are further confirmed using immunohistochemistry of UCHL1 that showed marked downregulation in TiO<sub>2</sub>-nanowired cerebrolysin treated groups.

#### Myelin vesiculation in cortex



**Fig. 3. TiO<sub>2</sub> Nanowired cerebrolysin (NWCBL) significantly reduced AD pathology as seen using myelin vesiculation in the cortex by low power Transmission electron Microscope (x 600).**

The basic mechanisms by which cerebrolysin is able to thwart brain pathology and UCHL1 levels in AD brain is still unclear [1,4]. However, it appears that strengthening of the BBB function by cerebrolysin is instrumental in reducing brain edema formation and neuronal restoration in AD [2]. If neurons are less damaged by cerebrolysin, it could be that release or expression of UCHL1 is also reduced [4]. Alternatively, a direct effect of cerebrolysin on UCHL1 activity may also be responsible for neuroprotection, a feature that requires additional investigation.

Potential of cerebrolysin induced neuroprotection caused by TiO<sub>2</sub> 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 [2,3,5,6]. Higher doses of normal cerebrolysin having better effects in AD on brain pathology and UCHL1 activity are in line with this hypothesis.

## 5 CONCLUSION

In conclusion, our observations indicate that TiO<sub>2</sub> nanodelivery of cerebrolysin has superior effects on downregulation of UCHL1 activity resulting in reduction in AD pathology following AβP infusion. These results suggest that the neuroprotective effects of cerebrolysin in AD are mediated through modulation of UCHL1 activity, not reported earlier.

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 UCHL1 activity in AD models. This is a feature currently being investigated in our laboratory.

## 6 ACKNOWLEDGEMENTS

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