Neuroendocrinology Letters Volume 37 No. 2 2016
ISSN: 0172-780X; ISSN-L: 0172-780X; Electronic/Online ISSN: 2354-4716
Web of Knowledge / Web of Science: Neuroendocrinol Lett
Pub Med / Medline: Neuro Endocrinol Lett

ORIGINAL ARTICLE

Biochemical manifestations of the nervous tissue degradation after the blood-brain barrier opening or water intoxication in rats

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Submitted: 2015-10-29 Accepted: 2015-11-20 Published online: 2016-04-29

Key words: **Osmotic BBB opening; Water intoxication; Cerebrospinal fluid; Myelin basic protein; Neuron specific enolase; Tau protein**

Neuroendocrinol Lett 2016; **37**(2):114–120 **PMID:** 27179573 NEL370216A03

Abstract

OBJECTIVES: The aim of the study was to determine changes of biomarkers of nervous tissue degradation in experimental model of osmotic blood-brain barrier opening or water intoxication and to find whether they correspond to changes in well defined clinical entities.

METHODS: In the cerebro-spinal fluid taken via the suboccipital puncture, myelin basic protein (MBP ng/ml), neuron-specific enolase (NSE ng/ml) and TAU-protein (Tau pg/ml) were determined by ELISA in 19 controls and 29 experimental rats several hours or one week after the experimental intervention.

RESULTS: Significant difference between the control and experimental groups was revealed only for the concentration of myelin basic protein. After the BBB opening, its level dramatically increased within hours and dropped back to control values within one week. Water intoxication induced only dilutional hypoproteinorachia. No significant changes were found in NSE and levels of TAU-protein were not detectable.

CONCLUSION: 1. Increased permeability of cytoplasmic membranes induced by water intoxication does not alter any of monitored CSF biomarkers. 2. Osmotic opening of the BBB *in vivo* experiment without the presence of other pathological conditions leads to a damage of myelin, without impairment of neurons or their axons.

Abbreviations:

ACC	- arteria carotis communis
ACE	- arteria carotis externa
ACI	- arteria carotis interna
BBB	- Blood-Brain Barrier
CG	- control group
CSF	- cerebrospinal fluid
DW	- distil water
EG	- oedema group
GOS	- Glasgow Outcome Scale
HH	- hyperhydrated rats
MBP	- myelin basic protein
MV	- mean value
NSE	- Neuron-Specific Enolase
WI	- water intoxication

INTRODUCTION

Our previous work has confirmed the essential role of homeostatic mechanisms for all physiological processes in the CNS. Experimental violation of homeostasis by means of osmotic opening of the BBB, or water intoxication (WI) can induce structural changes in the CNS, including cell damage (Pokorny *et al.* 2002; Kozler & Pokorny 2003; Kozler *et al.* 2013). Effect of these experimental procedures can be assessed by analysis of specific biomarkers in the cerebro-spinal fluid (CSF).

Impairment of myelin structure can be recognised by elevated level of myelin basic protein (MBP), neuronal damage by neuron specific enolase (NSE) and axonal impairment by changes in tau protein (Tau) in CSF. The dynamic of the response can be studied by sampling the CSF at various time intervals after the BBB opening and / or after the completion of water intoxication. The aim of the study was to correlate our experimental data (concentration of biomarkers in CSF) with similar data in clinical studies.

MATERIAL AND METHODS

Adult male Wistar strain laboratory rats (weight 270–540 g) were used in our experiments. All experiments were approved by the Ethical Committee of the First Faculty of Medicine (Charles University in Prague) and were in agreement with the Guidelines of the Animal Protection Law of the Czech Republic and Guidelines for the treatment of laboratory animals EU Guidelines 86/609/EEC.

Microsurgical exposure of the internal carotid (ACI)

Animals were put into the state of general anaesthesia using intraperitoneal application of thiopental in the dose of 4 mg/100 g and allowed to ventilate spontaneously throughout the procedure. Starting from a skin incision along the midline between the upper end of the sternum and the mandible, the whole common carotid artery (ACC, arteria carotis communis) was exposed with a standard microsurgical technique and, beyond its bifurcation, also the proximal portions of the ACI and external carotid (ACE, arteria carotis externa), which was ligated close beyond the bifurcation. An intraluminal catheter was introduced into the ACC trunk from the arteriotomy for selective application of mannitol. With the application over and the catheter removed, the ACC was ligated distal to and proximal to the arteriotomy. The operation concluded with a single-layer suture (Kozler & Pokorný 2003).

Osmotic opening of the BBB

Mannitol 20% (1 098 mosmol/l) in a dose of 5 ml/kg was selectively applied in the ACI at a rate of 0.12 ml/sec (Rapoport 2000). After the surgical intervention, animals were placed in boxes offering standard access to food and drink.

Water intoxication

For hyperhydration the standard model of water intoxication was used. Animals received distilled water in the amount corresponding to 20% of their body weight. The volume was divided into three parts and administered intraperitoneally in 8 hours interval during 24 hours (Olson *et al.* 1990).

CSF sampling

Modified method of CSF sampling via suboccipital puncture as described elsewhere was used (Nirogi *et al.* 2009; Lai *et al.* 1983; van den Berg *et al.* 2002; Rosenling 2008).

CSF in amount of 0.15 to 0.2 ml was extracted in 19 intact rats (control group) and in 29 rats divided into two experimental groups with animals exposed to osmotic BBB opening or water intoxication. CSF was sampled in the interval up to 4 hours after the BBB opening and after WI completing (group 1, 15 animals) or one week after the BBB opening and after WI completing (group 2, 14 animals) (see Table 1).

CSF analysis

In all animals, concentration of myelin basic protein in CSF was determined (MBP ng/ml) by MBP Elisa BECKMAN together with the neuron specific enolase (NSE ng / ml Method: Elecsys NSE Roche) and the protein Tau (Tau pg/ml, Elisa Total Tau EUROIMMUN).

Statistics

Values of MBP, NSE and Tau protein in all groups of animals were statistically evaluated using Kruskal-Wallis and Mann-Whitney non-parametric tests.

Tab. 1. Characteristics of experimental and control groups.

	No	Body weight (gr)
Control group	19	278-540 (Ø 382.8)
BBB – Hours (Ø 2.6 H)	6	270-295 (Ø 286.3)
WI – Hours (Ø 2.5 H)	9	294-425 (Ø 335.6)
BBB – 1 week	6	384-468 (Ø 422.8)
WI – 1 week	8	412-455 (Ø 441.7)

RESULTS

Concentration of studied markers (MBP, NSE and Tau protein) in the cerebrospinal fluid in the control and two experimental groups is given in Table 2.

In samples of CSF from 19 control animals, average concentrations of MBP were 9.11 ng/ml (normal values up to 4 ng/ml, NSE 1.55 ng/ml (normal values up to 23 ng/ml), and tau protein was below the positive range (levels under 614 pg/ml are taken as negative and/or at not detectable level). The given normal concentrations (MBP 4 ng/ml, NSE 23 ng/ml) apply for humans. In 6

experimental animals, samples of CSF were taken in the time interval from 95 to 200 minutes (within 3 hours, in average within 157 minutes) after the opening of the BBB. MBP concentration was 14.8 ng/ml, which is the upper limit of normal values, and significantly higher concentration than in control rats. Average NSE level was 0.94 ng/ml, which is less than normal values.

In 9 experimental animals, samples of CSF were taken in the time interval 120 to 230 minutes (within 4 hours, in average within 177.5 minutes) after the last i.p. DW dose. MBP concentration was 4.04 ng/ml, which is within the standard range and NSE concentrations was 0.74 ng/ml, which is less than the normal value.

In 6 animals samples of CSF were taken one week after the BBB opening. MBP concentration was 8.23 ng/ml, which is higher than normal value and significantly less than the value obtained in the interval of 3 hours after the BBB opening. NSE concentration was 3.16 ng/ml, which is less than the normal value.

In 8 animals samples of CSF were taken one week after the water intoxication (after the last DW dose). MBP concentration was 4.89 ng/ml which is within the normal range; NSE concentration was 0.93 ng/ml, which is less than the normal values.

Tab. 2. CSF composition (average values ± standard deviation).

		Mean±SD		
	No	**MBP norm**	**NSE norm**	**Tau positivity**
controls	19	9.11±5.23	1.55±1.54	not present
BBB - Hours	6	14.847±0.258	0.942±0.425	not present
WI – Hours	9	4.04±4.14	0.74±0.83	not present
BBB – 1week	6	8.23±5.02	3.16±3.71	not present
WI – 1 week	8	4.89±4.43	0.93±0.39	not present

As stated above the given normal concentrations (MBP 4 ng/ml, NSE 23 ng/ml) apply for humans. The elevated concentration of MBP to 4–8 ng/ml shows a chronic lesion of myelin, while all concentrations above 9 ng/ml indicate acute myelin damage (Greene *et al.* 2012). In rats, the concentration of both markers in the CSF is notably higher. Therefore, evaluation of experimental results has to consider the trends of changes and not the absolute values of the observed concentrations (Rosenling 2008). This explains why concentration of MBP in our experimental material was higher than in humans and why changes resulting from experiments were significant.

Statistically significant results were found only for MBP. After osmotic opening of the BBB in the first experimental group the MBP values were found greater than 14 ng/ml, which corresponds to the acute myelin damage in humans. In the second experimental group MBP values were about 8 ng/ml, which corresponds to a chronic myelin lesion in humans. The difference between these values is significant, though the absolute value of the MBP concentration in the second experimental group was almost the same as in the control group. Considering the trends rather than absolute values of concentration we can interpret our results with conclusion, that in the acute phase (H) MBP concentration significantly increased while in the chronic phase (W) MBP concentration was significantly reduced – see Figure 1.

After the water intoxication, MBP concentrations in both experimental groups were lower than that in the control group. However, only the difference between the control and first experimental group (H) was significant. It is possible to speculate that the lower MBP concentration in CSF resulted from the dilution of proteins after hyperhydration – see Figure 2.

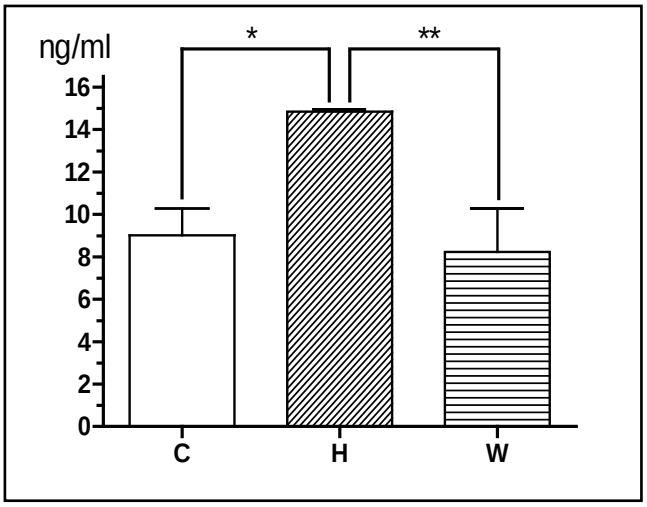

Fig. 1. Myelin basic protein concentration in CSF after osmotic BBB opening (mannitol administered (MA) rats, concentration of MBP is given in ng/l, **=p<0.001, *=p<0.01), control (C), Hours (H), Week (W).

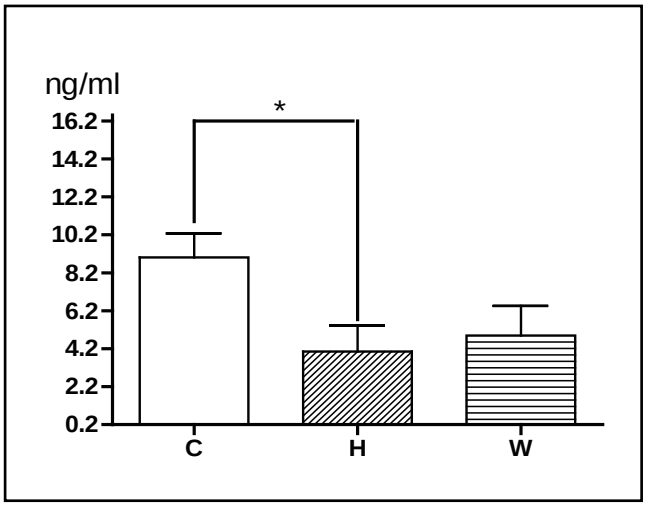

Fig. 2. Myelin basic protein concentration in CSF after water intoxication (hyperhydratation). Concentration of MBP is given in ng/l, *=p<0.01), control (C), hours (H), week (W).

NSE concentrations in experimental groups did not differ from the control group – see Figures 3 and 4.

Tau protein was not detectable in either group.

DISCUSSION

A common feature of clinical conditions which are accompanied with changes in the concentration of biomarkers in cerebrospinal fluid is the increased permeability of BBB and cytoplasmic membranes. However, neither of these processes has been described as an independent and the sole cause of the CNS structural damage. Changes in the concentration of biomarkers in CSF are associated with a specific pathological process that impairs the structure.

Damage of myelin was revealed using myelin basic protein (MBP), for impairment of neurons – neuron specific enolase (NSE), and for impaired axons we used protein Tau (Tau). The literary data proved that those clinical pathological conditions which are accompanied with increased concentrations of biomarkers in cerebrospinal fluid include: sclerosis multiplex (Barry *et al.* 1991; Davies *et al.* 1987; Mukherjee *et al.* 1985), stroke (Barry *et al.* 1991; Yan *et al.* 2014; Hay *et al.* 1984; Lima *et al.* 1987; Giovannoni *et al.* 2006; Mciver *et al.* 2010), head injury (Barry *et al.* 1991; Yan *et al.* 2014; Lima *et al.* 2004; Noseworthy *et al.* 1985; Liu *et al.* 2006; Mukherjee *et al.* 1985), Alzheimer's disease (Goedert *et al.* 1989; Zilka *et al.* 2010; Rosén *et al.* 2013; Tsai *et al.* 2014; Roder *et al.* 2007), some brain tumors (Golfinos *et al.* 1997; Kalwy & Smith 1994; Tapia *et al.* 1981) and stress (Li *et al.* 2014). At the same time, it is clear that the literary sources indicate the coexistence of the afore mentioned clinical conditions with increased permeability of the BBB and cytoplasmic membranes (Engelhardt & Liebner 2014; Alvarez 2011). Our previous experimental work demonstrated that osmotic insult and water intoxication increase permeability of the BBB and the cytoplasmic membranes (Pokorný *et al.* 2002; Kozler & Pokorný 2003; Kozler *et al.* 2013) with parallel functional consequences (Maresova *et al.* 2014). We can therefore conclude that we have an experimental model for testing the dynamic of changes of biomarker in the cerebrospinal fluid (CSF), without the necessity to impair nervous tissue by pathological process related to the precisely-defined clinical entities.

Although myelin basic protein (MBP) also occurs in the peripheral nervous system, it is present mainly in the CNS. MBP determines adaptive processes of internal environment to CNS disorders, it is responsible for myelin integrity and it has also other functions. It participates in the transmission of extracellular signals to oligodendrocytes via membrane proteins (e.g. acetin, tubulin), to which it binds. If MBP function in these physiological processes fails, a structural lesion of myelin can develop. Such lesions are accompanied by changes of MBP concentration in CSF (Davies *et al.* 1987; Boggs 2006; Kalwa & Smith 1994; Deber & Reynolds 1991; Simons & Trotter 2007). Also in several other clinical pathologies MBP is a useful marker of impaired internal environment of the brain. In the clinic and in appropriate experimental models *in vivo* the determination of the concentration of MBP in CSF enables to estimate the degree of impairment of physiological functions. MBP level is estimated primarily in such disorders as multiple sclerosis, cerebral ischemia (stroke), traumatic brain injury and certain brain tumors. All of these entities are associated with different degrees of increased permeability of the blood brain barrier (BBB). (Engelhardt & Liebner 2014). In patients one week after the brain injury, MBP concentration in CSF correlated with the degree of brain damage, patients with better results in TBI according to the GOS scale (Glasgow Outcome Scale) had lower concentrations of MBP (Noseworthy *et al.* 1985). Davies studied the CSF MBP levels in 129 patients with various types of CNS lesions, of which 26 patients suffered from multiple sclerosis. In these patients, he found the high concentration of MBP

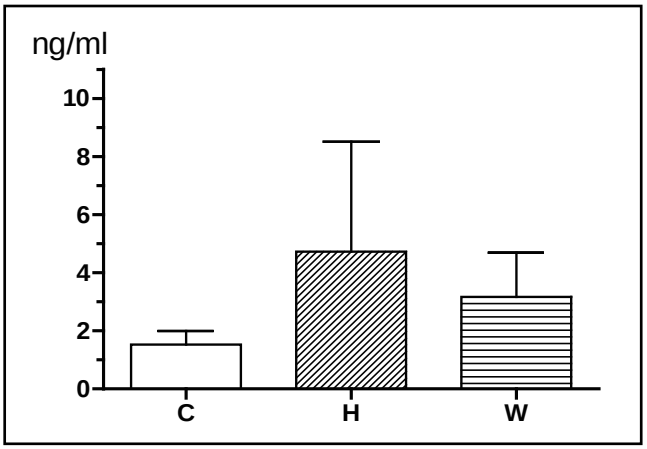

Fig. 3. Neuron specific enolase concentration in CSF after osmotic BBB opening (mannitol administration). Concentration of NSE is given in ng/l, control (C), hours (H), week (W).

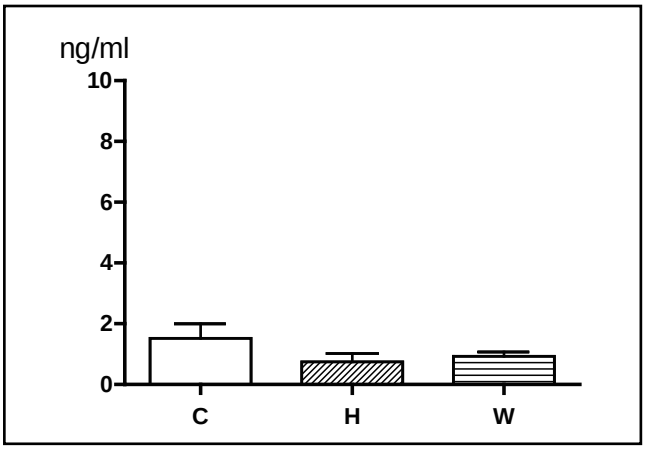

Fig. 4. Neuron specific enolase concentration in CSF after water intoxication (hyperhydratation). Concentration of NSE is given in ng/l, control (C), hours (H), week (W).

in CSF during the ongoing attack of disease in 81%; in patients with multiple sclerosis in remission he never found elevated levels of CSF MBP (Davies *et al.* 1987).

Enolase (2-phospho-glycerate D hydrolysis) is a protein with a heterodimeric structure consisting of subunits α, β, and γ. The αα and γγ isoenzymes are known as neuron-specific enolase (NSE), because it was found almost exclusively in neurons (Rider *et al.* 1975). Furthermore NSE is stable in biological fluids and as a freely soluble cytoplasmic protein it easily penetrates into CSF during damage/injury of the neuronal membrane. For this reason, determination of the concentration of CSF NSE serves as a suitable marker of neuronal damage (Marangos *et al.* 1987). Some studies have shown that NSE concentration in CSF is a reliable indicator of the severity of neuronal damage (Hay *et al.* 1984; Lima *et al.* 1987). For example, Lima (Lima *et al.* 2004) studied NSE concentration in CSF after brain injury in humans. Already the fifth hour after the traumatic brain injury higher concentration of NSE in comparison with the controls were found in the cerebrospinal fluid, with a difference in concentration according to the severity of injury. For minor injury with a good prognosis the increase was small, while in severe injuries with poor prognosis, concentrations of NSE in CSF was up to 6 times higher than in controls. Similar experience was published by Yan (Yan *et al.* 2014). He demonstrated significant correlation between the concentration of biomarker (MBP and NSE) in the CSF and the state of consciousness, CT pathology and the final state of brain injury (GOS) in humans. These findings contrast with observations of other authors. Marchi *et al.* studied NSE concentrations in cerebrospinal fluid during the treatment of patients with brain lymphoma using methotrexate administered intraarterially after previous osmotic opening of the BBB. NSE concentrations during treatment remained constant without any increase. On the basis of these results he concluded that the concentration of NSE would not increase, because the opening of the BBB does not lead to neuronal damage (Marchi *et al.* 2003).

Tau-protein (τ-protein, Tau) is a protein component that occurs in neurons and stabilizes microtubular structures of their axons. In Alzheimer's disease abnormal phosphorylation of this protein occurs, accompanied with increased transfer into the cerebrospinal fluid. Elevated Tau concentrations in the cerebrospinal fluid became a reliable marker of axonal damage in various clinical conditions, but especially in Alzheimer's disease (Goedert *et al.* 1989). Zilka demonstrated a direct correlation between the progressive deterioration of cognitive function and increased concentrations of Tau in cerebrospinal fluid (Zilka *et al.* 2010). A similar correlation between Tau concentrations in cerebrospinal fluid and the degree of brain deterioration in Alzheimer's disease was proved by imaging methods also by other authors (Rosén *et al.* 2013). The fact that elevated levels of Tau in CSF are a selective marker of axonal injury was confirmed in the study of Tsai who demonstrated that in other dementias (in the study a case of normal-pressure hydrocephalus is shown) Tau levels in CSF are not increased (Tsai *et al.* 2014).

Our work shows that statistically significant results were found only for MBP changes. After the osmotic opening of the BBB, in the first experimental group (sampling of cerebrospinal fluid within several hours), a significant increase of the concentration of MBP was observed, corresponding to our earlier finding of acute myelin damage. In the second experimental group (CSF sampling after one week) concentration of MBP was significantly reduced which again corresponds to chronic myelin lesion (see Figure 1). It is possible to conclude that our results show certain similarity with the findings of a clinical study by Davies *et al.* Those authors demonstrated high concentrations of MBP in patients with multiple sclerosis in the stage of acute attacks of the disease, while at the stage of remission the high MBP levels did not occur (Davies *et al.* 1987). Similarly in another clinical trial Noseworthy reported higher concentrations of MBP levels in CSF in patients with severe brain injury and worse clinical course than in the group of patients with better outcomes (Noseworthy *et al.* 1985). Another current and very detailed paper on the role of the BBB in the pathophysiology of multiple sclerosis and TBI (Engelhardt & Liebner 2014) has shown correlation between the degree of the BBB permeability increase and the clinical course. In the light of these facts, we can conclude that also our results show a biphasic course of changes in the levels of CSF MBP within one week after the osmotic opening of the BBB in rats in the model which is not based on a pathological insult. It can be considered as evidence that the increased permeability of the BBB itself induces structural damage to the myelin.

After water intoxication, we found in both experimental groups low concentrations of MBP which corresponded to normal values (see Figure 2). The finding can be explained by the fact that our model represents a dilution hypoproteinorachii with elevated water content which is similar to dilutional hyponatremia. Our results indicate that it is the increased permeability of the BBB and not the increased permeability of cytoplasmic membranes that leads to the myelin impairment which brings elevated levels of MBP in CSF.

Our next results showed that the concentrations of NSE in neither of the experimental groups was significantly different from the control group (see Figures 3 and 4). These findings are consistent with the statement of Marchi, who, based on the above study on the therapeutic opening of the BBB in the treatment of brain lymphoma, concluded that NSE concentrations can be increased, since the opening of the BBB leads to neuronal damage (Marchi *et al.* 2003). On the other hand, Lima have demonstrated that soon after the severe TBI, concentration of NSE in CSF was increased but as a

result of the injury, not due to the opening of the BBB (Lima *et al.* 2004).

For Tau protein indetectable values were observed in all groups which confirmed the findings of the above authors, who related detectable and especially elevated levels of CSF tau protein only with the damage to axons.

CONCLUSIONS

Our results indicate that the increased permeability of cytoplasmic membranes, induced in our study by water intoxication, does not impair cell structure in the CNS and the concentration of biomarkers in CSF is not therefore increased. Osmotic opening of BBB with the consequent increase of water content in the nervous tissue and unbalanced CNS homeostasis can impair structure of myelin, which is reflected in our results by a significant increase of MBP concentration in CSF during the acute phase (CSF was sampled within hours) and also during the chronic phase (CSF was sampled within a week). Osmotic opening of the BBB does not affect the integrity of neurons and axons and therefore the levels of NSE or Tau protein in CSF were not elevated.

Our experiments proved that osmotic opening of the BBB *in vivo* experiment without the presence of other pathological conditions leads to a damage of myelin, without impairment of neurons or their axons.

ACKNOWLEDGEMENTS

Supported by grant P – 34/LF 1/7

Conflict of Interest: There is no conflict of interest

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