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Serum pNF-H levels in the first six hours after experimental mild traumatic brain injury in rats

E. KARESIOGLU, H.I. CIKRIKLAR, V.A. DURAK, B. AYDIN, A. ARDIC, E. ARMAGAN

Emergency Medicine Department, Bursa Uludağ University Medical Faculty, Bursa, Turkey

Abstract. – OBJECTIVE: Mild traumatic brain injury (mTBI) cases with a normal CT scanning account for the vast majority of all TBI patients. The aim of this study was to investigate the course of serum Phosphorylated Neurofilament Heavy Chain (pNF-H) levels in the first six hours after trauma in rats in experimental mTBI.

MATERIALS AND METHODS: In this experimental animal study, 32 female Sprague-Dawley rats were enrolled equally (n=8) into 3 experimental groups and 1 control group. In experimental groups, animals were exposed to a mTBI with a free fall of 50-gram metal disc from a height of 80 cm. We compared serum pNF-H levels at the 2nd, 4th, and 6th hours after traumatic brain injury in the experimental groups with the control group.

RESULTS: Serum pNF-H levels at the 2nd and 4th hours after traumatic brain injury were statistically significantly higher than the control group. Serum pNF-H levels gradually decreased at the 4th and 6th hours compared to the 2nd hour and decreased to a similar level to the control group at the 6th hour after injury.

CONCLUSIONS: A high serum pNF-H value, could be used in the diagnosis and management of mTBI patients.

Key Words:
Emergency department, Mild traumatic brain injury, Phosphorylated neurofilament heavy chain.

Introduction

The annual global incidence of traumatic brain injury (TBI) is estimated at approximately 27 million, and it causes a significant socioeconomic burden due to its high morbidity and mortality rates[1]. TBI cases were classified as mild, moderate, and severe according to the Glasgow Coma Scale (GCS) score[2]. However, GCS is generally not reliable enough to differentiate between mild and moderate TBI cases. Therefore, magnetic resonance imaging (MRI) or computed tomography (CT) scans are used to evaluate the level of TBI according to current guidelines[3]. Even though MRI is more sensitive than CT to detect changes in almost all post-traumatic lesions aside from bleeding and acute fractures, routine MRI usage is limited in clinical practice by resource availability and operating costs[4].

The short-term complications are rare in the patients who suffered mild TBI (mTBI)[5]. Nevertheless, these patients are at further risk for progressive neurocognitive dysfunction, neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and psychiatric disorders including depression, mixed affective disorder and bipolar disorder[6]. However, CT findings are normal in up to 30% of mTBI cases[7] and mTBI cases with a normal CT scan account for the vast majority (80-90%) of all TBI patients[8]. Therefore, a valid and reliable biomarker, which is detectable in the acute period of TBI, is critical for the diagnosis of mTBI with normal CT[7].

Following a TBI, various biomarkers are released in the cerebrospinal fluid (CSF) or serum, based on the severity of the injury[9]. These biomarkers can be used for many purposes including estimating the presence of mTBI, and predicting the severity, treatment success and prognosis of the disease[9,10]. Neurofilaments (NF), which are located in the axonal region and regulate the structure, stability and diameter of neurons, can be detected in blood by enzyme-linked immunosorbent assay, electrochemiluminescence assay ultra-sensitive single molecule array methods method[11]. NFs are composed of five filament families divided according to their molecular mass. The largest of these is neurofilament heavy chain (NF-H), followed by medium chain (NF-M), light chain (NF-L), α-internexin and peripheralin in descending order of molecular weight[12]. Among NFs, NF-H is seen to be superior to others in axonal injury[13,14]. The phosphorylated form of NF-H (pNF-H) has been found to be secreted in CSF

Corresponding Author: Halil Ibrahim Cikriklar, MD; e-mail: halilcikriklar@uludag.edu.tr

and blood both after TBI in recent animal and human studies[14-16].

From the aspect of admission time, the majority of TBI patients were admitted to the emergency department (ED) within the first 6 hours[17,18]. All procedures performed in patients with mTBI in ED took approximately 6 hours[18-20]. For the reason of the need to make rapid and accurate decisions for TBI patients in EDs, miscellaneous diagnostic and management protocols were proposed[5,21]. However, the use of biomarkers in these protocols is not common. Although there are various studies[22,23] on the duration of pNF-H release, to the best of our knowledge, there is no study investigating the increase in pNF-H blood levels in the first six hours after mTBI.

The aim of our study was to investigate the course of pNF-H, which might be a promising biomarker in distinguishing the injury, in serum samples of the rats with an experimental mTBI in the first six hours after trauma.

Materials and Methods

Study Design and Experimental Animals

This study, which was designed as an animal experiment, was carried out in Bursa Uludag University Neurovascular Research Laboratory. 32 female Sprague-Dawley rats weighed between 250-300 g were randomly allocated (n=8) into 4 study groups.

After obtaining the rats from Bursa Uludag University Experimental Animal Breeding Center, they were kept in cages with free access to food and water at a room temperature of 18-22°C for 12 hours in dark and 12 hours in light cycles. The experimental protocol applied on rats is in accordance with national and international animal experimentation legislation and guidelines, and we conducted this study in adherence to the Animal Research [Reporting of *In Vivo* Experiments (ARRIVE) guidelines][24].

Study Groups

The 32 rats were randomly allocated into 3 experimental groups and 1 control group, and all animals reached the planned sacrifice time. The animals in the control group (Group 1) were placed in the experimental setup under anesthesia, and blood samples were taken without developing TBI. In the three experimental groups, animals were exposed to an experimental mTBI under anesthesia, and the blood samples for pNF-H were taken at 2nd, 4th, or 6th hours after injury (experimental groups were defined as Group 2, Group 3 and Group 4 according to three different blood sample collection time, respectively).

Anesthesia

All rats were placed in a box and exposed to 3% sevoflurane for anesthesia. Sevoflurane concentration was calculated by confirming the loss of the deafness reflex in rats. After the administration of anesthesia, mTBI was established in the animals in experimental groups, and intracardiac blood samples were collected from all animals, and then they were euthanized.

Traumatic Brain Injury

The weight drop model of Marmarou et al[25] was used when inducing TBI in rats under anesthesia. In this model, 50-gram metallic disc was dropped by free fall from a height of 80 cm to induce mTBI. First, anesthetized rats were placed on the foam mattress in the prone position, and then, to distribute the weight evenly over the skull, a stainless-steel metal disc with a length of 10 mm and a thickness of 3 mm was fixed to the midline of the skull using toothpaste. Rats were fixed to coincide with the lower end of the experimental trauma mechanism. Neither skull fracture nor death was occurred after exposure to trauma.

Measurement of pNF-H Levels

Blood samples were taken in tubes including no anticoagulant or preservative, and serums were separated using centrifugation at 5,000 rpm for 10 minutes at +4 degrees. The pNF-H levels in serum samples were analyzed with rat ELISA Kits (Biossay Technology Laboratory, Shanghai, China) at a wavelength of 450 nanometers using a spectrophotometer (Biossay Technology Laboratory, Shanghai, China) in accordance with the manufacturers' instructions. Serum pNF-H levels are expressed as "pg/mL".

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). The mean and standard deviation (SD) values were used to present the descriptive statistics of continuous numeric variables. One-Way ANOVA test was used to compare serum pNF-H levels among the study groups. Post-hoc comparisons were conducted by using the Holm-Sidak test. A p-value <0.05 was accepted as the statistical significance level.

Table I. Comparison of serum pNF-H levels between study groups.

Study Group	Serum pNF-H Level (pg/mL)		*p*-value
	Mean	SD	
Group 1 (Control)	575.36	28.22	
Group 2 (Post-traumatic 2nd hour)	665.23	20.02	<0.001
Group 3 (Post-traumatic 4th hour)	639.78	27.97	
Group 4 (Post-traumatic 6th hour)	593.40	33.5	

SD: Standard deviation. *One-Way ANOVA test was used.

Results

The comparison of pNF-H serum levels between study groups is presented in Table I. The mean serum pNF-H level was 575.36±28.22 pg/mL in the control group, 665.23±20.02 pg/mL in Group 2, 639.78±27.97 pg/mL in Group 3, and 593.40±33.55 pg/mL in Group 4. A statistically significant difference in the distribution of serum p-PN-H levels was found between the study groups (p<0.001, Table I).

Table II and Figure 1 show the post-hoc pairwise comparison of serum pNF-H levels between study groups. Firstly, the pNF-H levels increased statistically significantly at the 2nd hour after injury (p<0.001), then although it started to decrease by the 4th hour, the levels were still statistically significantly higher than the control group (p=0.001). The decrease in serum pNF-H levels continued at the 6th hour after injury, and finally, it declined to a statistically similar level to the control group (p=0.257). The decrease in pNF-H level at the 4th hour after TBI compared to the 2nd-hour level was not statistically significant (p=0.217). However, pNF-H level at the 6th hour was statistically significantly lower than at the 2nd hour (p<0.001). The decrease in pNF-H level at the 6th hour after TBI compared to the 4th-hour level was statistically significant (p=0.020, Table II and Figure 1).

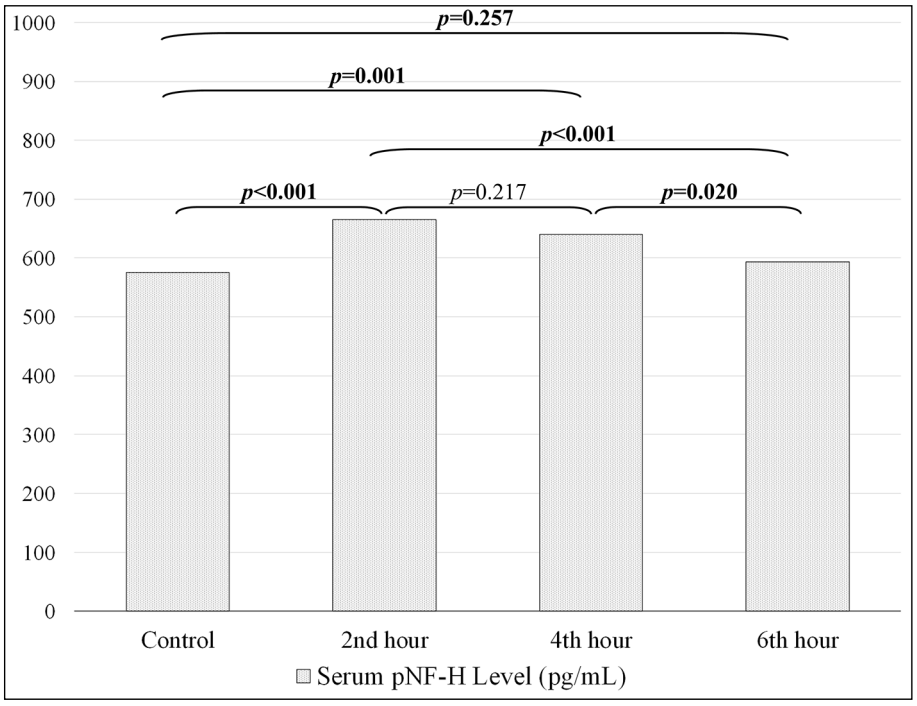

Figure 1. Course of pNF-H level after injury.

Table II. Post-hoc pairwise comparison of serum pNF-H levels between study groups.

Pairwise Comparison	Difference in Mean (pg/mL)	*p*-value*
Group 2 - Group 1	89.87	<0.001
Group 3 - Group 1	64.42	0.001
Group 4 - Group 1	18.04	0.257
Group 2 - Group 3	25.45	0.217
Group 2 - Group 4	71.83	<0.001
Group 3 - Group 4	46.39	0.020

*Holm-Sidak test was used.

Discussion

Despite all the advances in diagnosis and treatment procedures, TBI is still a clinical entity in EDs with high morbidity and mortality, and mTBI accounts for approximately 80-90% of all TBI admissions[3]. Most patients with mTBI have normal imaging and clinical features at presentation, and for this reason, the brain injury can be missed[26]. Due to the long-term effects of mTBI cases and missed diagnoses, the usage of biomarkers in this area come into prominence[7].

In our study, we set up an experimental mTBI rat model to examine the course of the serum levels of pNF-H, a possible candidate biomarker for TBI, in the first 6 hours after trauma, which is the most common presentation period of TBI cases[8,18]. According to our findings, while the serum pNF-H levels measured at the 2nd hour and 4th hour after TBI were statistically significantly higher than the control group's values, the levels gradually decreased by the 4th and 6th hours compared to the 2nd hour, and finally reached the approximately normal values at the 6th hour.

The focus in mTBI cases should be on the problems that may develop when the patients are considered normal and discharged after the observation period. Nearly 5% of adult patients who are admitted to the ED for mTBI have a subsequent admission, which is related to the age, CT features and symptomology of the patients, within 72 hours after discharge[27]. Late admissions to the hospital with chronic neurological degeneration due to mild TBI or clinical worsening after discharge may cause neurological damage[8,18,19]. Therefore, there is a need to distinguish the patients with mTBI, who might present with complex clinical presentations or complications after discharge, from the patients without injury.

Although, cranial MRI and CT are currently frequently used in EDs, both MRI and CT can be normal in mTBI cases[18,19]. Covino et al[28] evaluated the risk of delayed ICH after mTBI in patients using anticoagulants. 15 (2.2%) out of 685 patients with negative CT at presentation developed ICH on control CT. Gordon[11] reported that neurofilaments increase in TBI cases as well as diseases with neuronal damage such as ischemic stroke, Creutzfeldt-Jakob Disease, Parkinson's Disease, Alzheimer's Disease, frontotemporal dementia and multiple sclerosis.

Gatson et al[16] examined 34 people with mild TBI and reported that pNF-H levels were higher in CT-positive cases than in CT-negative cases. In another study[10], Tau protein, GFAP, and NFL were compared as biomarkers between 277 mTBI patients and 49 healthy controls, and both three biomarkers were found to be higher in mTBI patients than in healthy controls. Additionally, the authors concluded that the combination of these three biomarkers had a valuable discriminatory success for detecting MRI abnormalities, even in CT normal mTBI patients.

Shibahashi et al[29] examined the pNF-H levels 24 and 72 hours after trauma in 32 moderate and severe TBI cases, and they found a significant increase, especially 24 hours after trauma. An optimal cut-off value of 240 μg/ml for the 24th hour and 80 μg/ml for the 72nd hour was accepted by the authors to predict the long-term unfavorable outcome of TBI, and these values were found to be significant in terms of management and follow-up in TBI cases. Otani et al[22] evaluated the pNF-H levels at the 24th and 72nd hours, 1st and 2nd weeks in 15 male and 5 female patients with TBI and found that the serum pNF-H level increased and peaked at two weeks after trauma in TBI cases. The authors reported that the increase of serum pNF-H was correlated to the clinical outcome of patients and therefore serum pNF-H measurements in TBI cases could be beneficial for clinical follow-up.

Shaw et al[23] investigated the serum level of pNF-H after trauma in rats and reported that serum pNF-H levels increased both in animals with spinal cord injury and TBI. Anderson et al[15] examined post-traumatic pNF-H levels in an experimental rat model and found that the pNF-H levels had the first peak at the 24th hour and the second peak at 48th hour. Yang et al[30] studied pNF-H in serum and cerebrospinal fluid across three rat TBI models (controlled cortical impact, parasagittal fluid percussion and penetrating ballistics-like brain injury models) and reported that CSF and serum pNF-H levels are elevated by 24 hours after injury in all models. The differences in the course and temporality of the elevation in serum pNF-H levels between studies might be due to the variations of trauma models and the severity of the injury. In our study, we examined serum levels in the first 6 hours after TBI in rats from an emergency medicine perspective and found an increase in the first 4 hours and a decrease thereafter.

Limitations

There are limitations in our study. First, this is an animal study and the probable limitations due to the nature of animal experiments might be valid in our study. For instance, stress factors of the laboratory environment might have effects on animals. We tried to reduce such factors by providing them an adequate amount of light, heat, food and water.

Conclusions

In our experimental rat mTBI model, serum pNF-H levels at the 2nd hour and 4th hour after injury were statistically significantly higher than the control group; this value gradually decreased at the 4th and 6th hours compared to the 2nd hour and reached a similar level to the control group at the 6th hour.

In line with these results and the evidence obtained from the literature, we concluded that a high serum pNF-H value, especially in the first 2 or at most in the first 4 hours after trauma, could be used in the diagnosis and management of mTBI patients who have a possibility of negative imaging features. However, large-scale diagnostic studies in humans are needed to support our findings.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Authors' Contributions

HIC, EK, VAD, AA substantially contributed to conception and design, acquisition of data, analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and provided final approval of the version to be published. BA, EA contributed to acquisition of data, interpretation of data, drafting the article or revising it critically for important intellectual content, and provided final approval of the version to be published.

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Availability of Data and Material

The datasets generated and/or analyzed during the current study are available from corresponding author.

Informed Consent

Not required.

Ethics Approval

Ethical approval for this study was obtained from Uludağ University Faculty of Medicine Animal Experiments Local Ethics Committee (Approval date and number: 01.06.2021 and 2021-07/01).

ORCID ID

Halil Ibrahim Cikriklar: 0000-0002-8073-6207.

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