EXPERIMENTAL STUDY

Evaluation of rat major celluler prion protein for early diagnosis in experimental rat brain trauma model

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ABSTRACT

BACKGROUND: Although traumatic brain injury (TBI) is an important problem, there has been no widespread utilization of neurobiomarkers to aid the diagnosis of TBI. This study was conducted to evaluate serum S100B and prion protein (PrPC) levels in rats with TBI.

METHODS: In this study, 15 albino rats were categorized into three groups as follows: sham-operated (1), control (6) and trauma (8) groups. The TBI model was based on the modified free falling model. S100B, PrPC levels were measured using ELISA. Brain specimens were obtained for the pathological examination.

RESULTS: Serum S100B and PrPC levels were found to increase in T group at both 2h and 24h after trauma ($p<0.002$, $p<0.002$, respectively). We also found higher histopathological injury scores of brain tissues in the T group. Only a positive correlation was found between serum PrPC levels and the extent of brain injury ($p=0.039$, $r=0.731$). Using ROC analysis, among the two serum markers investigated, both of them revealed the same sensitivity and specificity for diagnosing TBI.

CONCLUSION: The changes in serum S100B and PrPC levels showed good sensitivity in our experimental model. Therefore, PrPC could be helpful in the early prognostic prediction in patients with TBI. Further studies are needed to test our findings in humans following TBI (penetrating bodies, blunt trauma) to definitively acknowledge it as a reliable biomarker and its subsequent diagnostic utility.

Keywords: Major cellular protein prion (PrPC); S100B; traumatic brain injury.

INTRODUCTION

Traumatic brain injuries may cause longterm neurological morbidities by primary and secondary mechanisms. Primary damage occurs at the time of the incident due to mechanical injury and cannot be prevented, although secondary injury is induced by the biochemical and physiological process can be avoided.[1] TBI is a very important public health issue because of its effects on extensive psychological, physical and social impacts with a high economic concern.[2] Moreover, according to the WHO, many diseases will be overshot by TBI as the major cause of death and disability by the year 2020. Neurological examination and neuroimagining tools are the main implements of the diagnosis of TBI in the acute period. One of them, computed tomography (CT) scanning, is prone to radiation exposure and has low sensitivity in some cases. The other tool, magnetic resonance imaging (MRI), can induce useful information, but it is restricted by cost, limited availability, and concerns to use in unstable patients.[3,4] There is limited knowledge in the early diagnosis and diagnostic and prognostic tools for risk stratification of TBI patients. Therefore, to validate and introduce rapid diagnosis employing biomarkers from blood tests into the clinical setting is the key for this population. Although several studies exploring many promising

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biomarkers,[5,6] there is a lack of any Food and Drug Administration–approved biomarkers for clinical use.[6,7] A new confirmed blood neuro biomarkers may decline unnecessary radiation exposure and provide an opportunity for early diagnosis and by this way, care of patients with TBI may be improved.

S100B is the major low-affinity calcium-binding protein in astrocytes[8] that contributes to regulating intracellular levels of calcium; it is considered as a marker of astrocyte injury or death. S100B is one of the most largely studied biomarkers in brain injury.[9–14] Elevated levels of S100B in serum have been associated with an increased incidence of the postconcussive syndrome and problems with cognition.[15,16] Moreover, studies have reported that serum levels of S-100B are related to MRI abnormalities and with neuropsychological examination disturbances after TBI.[17,18] Notable interactions between elevated serum levels of S100B and CT abnormalities were spotted by several studies.[19,20] It has been suggested that assaying S100B concentration to clinical decision tools for mild TBI patients could potentially diminish the number of CT scans by 30%.[19] We should note that these results have not been consistently reproduced and no relations between S100B and CT abnormalities have been spotted by many other investigators.[21–23]

Accumulating evidence has shown that cellular prion protein (PrPC) host-coded membrane-bound glycoprotein plays pivotal roles in processes, such as copper binding, the regulation of cell death and the modulation of several signal transduction pathways.[24–26] However, the actual physiological function of this stays is unknown. *In vitro* and *in vivo* studies have reported that interaction between PrPC and amyloid-β oligomers could be a useful pathology of Alzheimer's disease because of the high receptor affinity of PrPC for these oligomers.[27] The main objective of this study was to determine the feasibility of using circulating S100B and PrPC levels at 2h and 24 h in rats following TBI.

MATERIALS AND METHODS

Fifteen female Wistar albino rats, weighing 220–230 g, from the Animal Laboratory of YeditepeUniversity (İstanbul Turkey), were randomly divided into three groups. The first group was sham-operated (S, n=1) group in which only a craniotomy was performed, the others control (C, n=6) and trauma (T, n=8) groups. All the rats were put under environmentally controlled conditions in a 12/12 h light/dark and granted free access to food and water. This study was approved by the Animal Care and Ethics Committee of Yeditepe University School of Medicine (date: 01.03.2016, no: 522).

Experimental Procedure of TBI

A special weight-drop device developed by Marklund et al.[28] was used to deliver a standard diffuse traumatic injury. Under ketamine hydrochloride anesthesia (80 mg/kg) and xyslazine (10 mg/kg) intraperitoneally (i.p.), rats were placed in a prone position. A craniotomy (6X9 mm^2), centered over the right parietal cortex at bregma -3.5 and 3.5 mm lateral to the midline, was done using a dental drill. The weight-drop device, a 5 g weight, was allowed to fall freely from a height of 50 cm to induce TBI. The device's aim is to prevent bouncing of the weight by allowing a single compression.

Animals that survived after 24 h were the ones to be used in this study and animals that died during trauma and observation were eliminated. Blood samples were collected before and after trauma (after 2^{nd} and 24^{th} hrs, 1 mL sample every time). All serum samples were centrifuged and stored at -80°C using an ultracold freezer.

We noted physiological parameters of the rats after 24 h and the rats were anaesthetized with an i.p. injection of ketamine hydrochloride. After intracardiac blood drawing for analysis, all rats were sacrificed, and the brains were carefully taken out for pathological examination. Blood serums were held at -80 °C till analysis. Both serum S100B (Bioassay Technology Laboratory E0075Ra, Rat S100 Calcium Binding Protein B) and PrPC (Bioassay Technology Laboratory E1368Ra, Rat Majör Prion Protein (PRNP)) levels were measured with ELISA method.

Histopathological Examination

The intact brain was finally placed into 10% formaldehyde. The extent of brain damage was detected by morphological findings. Tissue samples were obtained from the hippocampus, pons and cerebellum. Axial sections were stained with hematoxylin and eosin (H&E). Stained specimens were examined blindly under an Olympus BX40 light microscope by a pathologist. In the histopathological examination of brain tissues, edema, hemorrhage, neuronal damage, vascular congestion, retraction ball-diffuse axonal damage and their extensity were estimated. The semi-quantitative scores reflect the approximate percentage of axonal, vascular and neuronal changes observed in the section. Results were scored as grade 0 (no changes), grade 1 (dilatation), grade 2 (dilatation and stasis), grade 3 (dilatation, statis and parenchymal hemorrhage).

Statistical Analyses

Statistical analyses were performed using MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013). Descriptive analysis was used to determine continuous variables (mean, Standard deviation, minimum and maximum). Nonparametric statistical analyses were used: some differences between two distinct groups were regulated using the Mann-Whitney U test; differences between more than two groups were determined using the Kruskal-Wallis test. Differences between two dependent groups were determined using the Wilcoxon test; differences between more than two dependent groups were determined using the Friedman test. A p-value of <0.05 was considered statistically significant.

Table 1. Serum biochemical parameter levels in groups

	0h	2h	24h	p*	p¹	p²	p³
	Mean±SD Median (Min-Max)	Mean±SD Median (Min-Max)	Mean±SD Median (Min-Max)				
S100B (ng/L)							
Control	5.09±0.7	11.6+1.1	11.8±5.2	0.042	0.028	0.058	0.753
	5.1 (4.2–6.2)	11.4 (10.2–13.5)	13.1 (5.2–17.2)				
Trauma	4.9±0.6	81.6±21.9	411.8±141.6	0.001	0.012	0.012	0.012
	4.6 (4.2–6.2)	81.7 (48.6–118.6)	387.7 (235.7–585.9)				
PRNP (ng/mL)							
Control	1.4±0.3	1.1±0.09	2.4±0.3	0.006	0.173	0.028	0.028
	1.3 (1–1.8)	1.09 (1.02–1.3)	2.4 (2.1–2.7)				
Trauma	1.3±0.2	7.7±0.7	24.6±5.7	0.001	0.012	0.012	0.012
	1.2 (1–1.8)	7.7 (6.8–9.3)	25.7 (16.1–31.7)				

*Friedman test, ¹0h-2h, ²0h-24h, ³2h-24h (Wilcoxon test).
S100B: Bioassay Technology Laboratory E0075Ra, Rat S100 Calcium Binding Protein B; PRNP: Rat majör prion protein; SD: Standard deviation; Min: Minimum; Max: Maximum.

ROC curves were constructed using both trauma and control subject serum S100B and PrPC levels in an attempt to form a specificity- sensitivity relationship; areas under the ROC curve were calculated according to standard methods.[18] The diagnostic accuracy of the serum levels determined at study entry was expressed as the area under the receiver operating characteristic curve (AUC), which was derived from logistic regression analysis.[19] These values were calculated for the cut-off from the AUCs.

RESULTS

Physiological Measurements

No rats died throughout all the experimental experience. There was no difference between the physiological measurements of rats before trauma and at the end of the experiment apropos weight, respiration, heart rate and rectal temperature.

Biochemical Analyses

Serum S100B and PrPC levels and histopathological scores for each group were shown in (Table 1). There were no significant differences in using serum S100B and PrPC levels at the beginning of this experiment in both groups. We found statistically significant differences in these markers at both 2nd and 24th hrs of the experiment when compared to C and T groups ($p<0.001$) (Fig. 1, 2).

In control and trauma groups, serum S100B and PrPC levels were statistically significantly different when compared 0, 2 and 24hrs (Friedman $p<0.05$). In the trauma group, there were significant differences at both S100B and PrPC levels at dual comparisons using all sampling time (0h-2h, 0h-24h, 2h-24h) (Wilcoxon $p<0.016$, after Bonferroni correction). However, these differences were not observed in the control group.

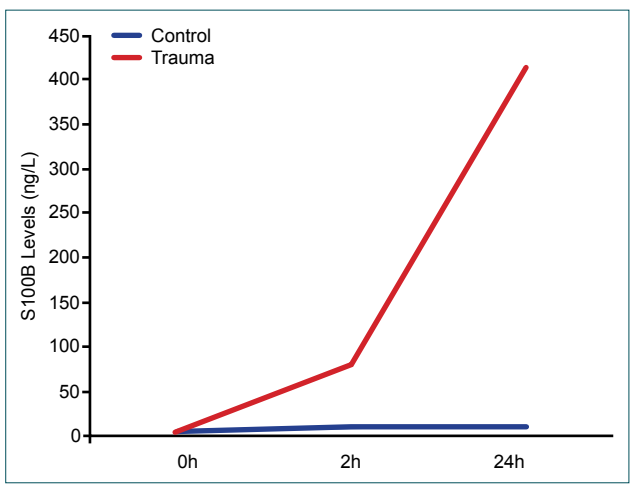

Figure 1. Serum S100B levels at the all time points in both gropus.

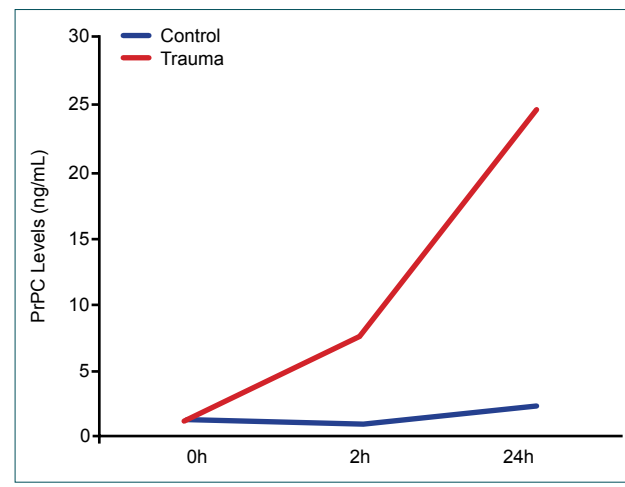

Figure 2. Serum PrPC levels at the all time points in both gropus.

Table 2. The comparison of biochemical parameters differences at time of sampling according to groups

	Control	Trauma	P*
	Mean±SD Median (Min-Max)	Mean±SD Median (Min-Max)	
S100B (ng/L)			
0–2	-6.5±1.1	-76.7±22.3	0.001
	-6.4 (-8.3–-5.0)	-77.3 (-114.4–-43.3)	
0–24	-6.7±5.8	-406.9±142.2	0.001
	-8.2 (-13–0.9)	382.8 (-581.7–-230.4)	
2–24	-0.2±6.2	-330.2±122.6	0.001
	-1.8 (-6.1–7.4)	-304.6 (-471.9–-187.1)	
PrPC (ng/mL)			
0–2	0.2±0.3	-6.4±0.9	0.001
	0.2 (-0.3–0.7)	-6.3 (-8.3–-5)	
0–24	-1.01±0.5	-23.3±5.7	0.001
	-1 (-1.7–-0.5)	-24.6 (-30.1–-14.8)	
2–24	-1.3±0.2	-16.9±5.9	0.001
	-1.3 (-1.5–-0.9)	-17.1 (-24.9–-8.9)	

*Mann-Whitney U test. S100B: Bioassay Technology Laboratory E0075Ra, Rat S100 Calcium Binding Protein B; PrPC: Cellular prion protein; SD: Standard deviation; Min: Minimum; Max: Maximum.

We found significant variations of serum S100B and PrPC levels between 0h-2h, 0h-24h and 2h-24h in both control and trauma groups (Table 2). The only significant correlation was found between PrPc levels and extent of injury in the trauma group (p=0.039, r=0.731).

To compare S100B and PrPC levels for the prediction of TBI, we performed ROC curves. The two serum markers investigated revealed similar sensitivity and specificity for diagnosing TBI (Table 3, Fig. 3).

Histopathological Results

Pathological examination results were significantly higher in the trauma group after 24 h as expected. Light microscope images of stasis and bleeding are shown in Figure 4. We found grade 1 features in group C and S rats. In the trauma group, 6 of 8 rats have grade 2, and 2 of 8 rats have grade 3 features.

DISCUSSION

There are several models for mimicking the brain injury in humans, such as lateral and central fluid impact, wounding with hard objects, acceleration (weight drop from a height), injection, cold injury, local stress, and penetrating injury models. The acceleration model has been thought to be idle for diffuse brain injury.[29] Thus, it was chosen in our study. Our results showed that both serum S100B and PrPC levels were found significantly increased after trauma at both 2 and 24 hrs. We also found a significant positive correlation between serum PrPC levels and the extent of brain injury at all time points. We also measured the same sensitivity and specificity for the diagnosis of TBI.

Traumatic brain injury that arises from a sudden external force is a set of secondary functional and/or pathological amendments within the brain. It represents an important problem in public health.[30] Research on mild TBI has examined the role of biomarkers as prognostic and diagnostic tools for brain damage, such as glial fibrillary acidic protein, neuron-specific enolase, ubiquitin C-terminal hydrolase-L1, Tau protein and the S100B protein.[31] They could have potentially predictive effects about damage to reduce CT use in TBI diagnosis.[32] S100B is one of the most studied biomarkers for a brain injury to date and could be useful for the diagnosis of TBI.[33] S100B is a calcium-binding protein with a biological half-life of 30–120 min. It is synthesized Schwann cells primarily, and in astroglial cells.[34] After blood–brain barrier injury or neuronal damage, this protein is released into the bloodstream.[35] Although S100B is still a reassuring connected marker, its utility in the setting of multiple traumas remains questionable because it is also elevated in trauma patients without head injuries.[36–38] In addition to these findings, there are studies on serum and the cerebrospinal fluid. There is a clear positive interaction with the degree of brain damage.[39,40] The exact role of S100B has protein in the pathogenesis of TBI remains unclear. Excessive production of S100B from astrocytes in brain injury may generate toxic milieu. S100B have potential effects on both stimulation of the production of proinflammatory cytokines and direct neurotoxicity.[41] Several findings indicated that S100B protein is important in the inflammatory reaction in the earlier period and associated with the secondary brain injury.[39,40]

Table 3. Diagnostic performance of S100B and PrPC at 24h

	AUC	p-value	Cut-off*	Sensitivity	95% Lower CI	95% Upper CI	Specificity	95% Lower CI	95% Upper CI
S100B	0.816	0.018	235.667	85.71	42.1	99.6	85.71	42.1	99.6
PRNP	0.878	<0.001	16.136	85.71	42.1	99.6	85.71	42.1	99.6

*Sensitivity, specificity, and predictive values were calculated for the cutoff, which represented the best discrimination as derived from the receiver operating characteristic curves. The area under the receiver operating characteristic curves (AUC), confidence interval (CI). S100B: Bioassay Technology Laboratory E0075Ra, Rat S100 Calcium Binding Protein B; PRNP: Rat majör prion protein; PrPC: Cellular prion protein.

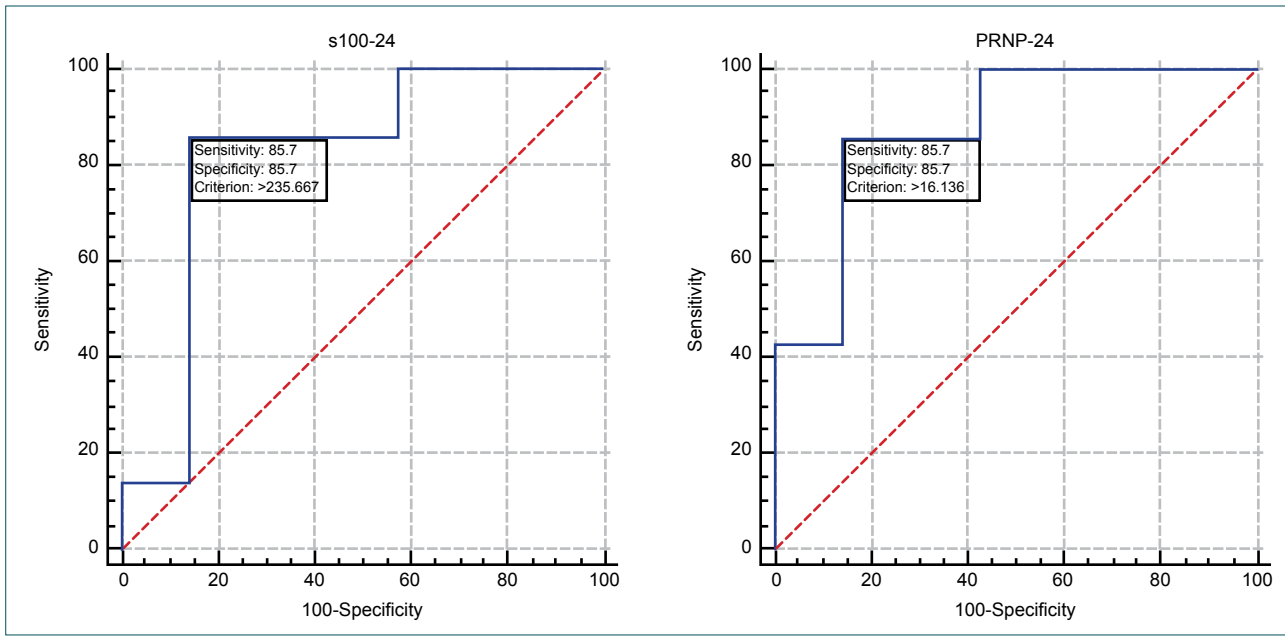

Figure 3. Evaluation of serum S100B and PrPC for the diagnosis of TBI. Receiver operating characteristic curves were drawn with the data of these markers from all rats. AUC, the area under curve.

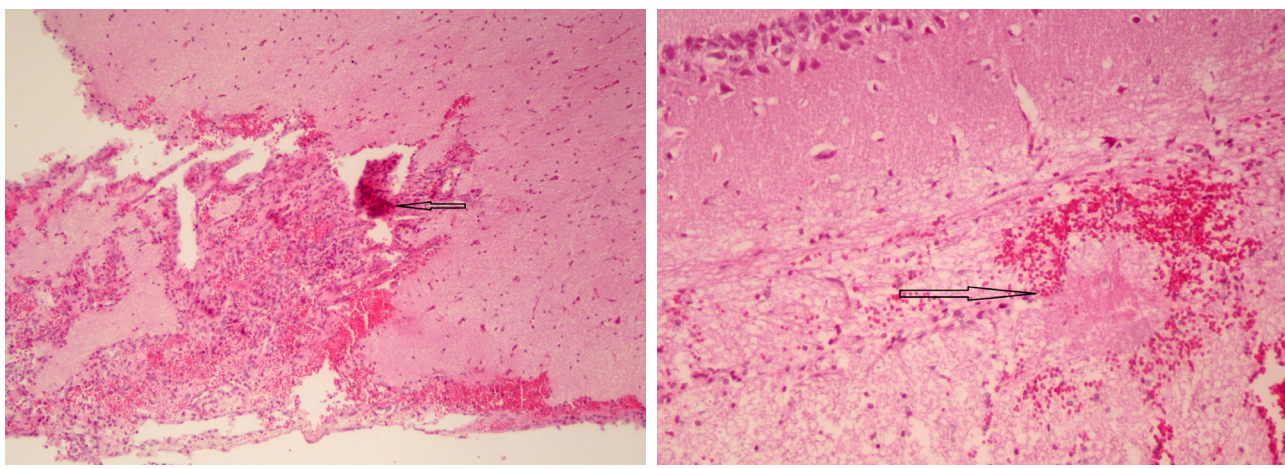

Figure 4. Axial sections were stained with hematoxylin and eosin (H&E). Light microscope (BX40) images of stasis and bleeding respectively.

It was commented on the potential of S100B to reduce CT scanning in mild TBI.[42] S100B is a useful biomarker not only in adults but also in children. Even though S100B is quite sensitive, it has poor specificity in this population. S100B could be a marker to prevent an intracranial injury but cannot be used as the sole marker owing to its specificity.[43] However, only Scandinavian guidelines include its early measurement in the initial clinical management of minimal, mild and moderate TBI to rule out the presence of intracranial lesion.[44] In addition to this, the American College of Emergency Physicians recommends the use of the CT scan on patients with mild TBI only when levels of S100B in serum are >0.10 µg/L.[45] Interestingly, post-mortem cerebrospinal fluids S100B levels are significantly elevated, after a fatal TBI.[46]

PrPC is a ubiquitous glycoprotein distributed throughout many cell types, especially within the central nervous system (CNS).[47] PrPC may play a decisive but unclear role in T-cell function because of T cell activation. However, interestingly, PrPC knockout mice have only minor alterations in immune function.[48] Physiological levels of PrPC exhibit a neuroprotective effect following hypoxic brain damage *in vivo*.[49] The mechanism underlying neuroprotection involves a copper-binding activity that protects cells from oxidative damage.[25] Moreover, membrane-associated PrPC with an extracellular glycosyl-phosphatidylinositol anchor may release into the systemic circulation under shear forces. Therefore PrPC may serve as a potential biomarker for TBI.[50] However, it was demonstrated in an animal model that plasma PrPC is a potential biomarker for determining primary Blast-induced TBI.[50] The applied force on the brain tissue may dislocate PrPC from its neuronal lipid rafts and allow the protein to collect within the systemic circulation. Therefore, the circulating levels could be used as a potential biomarker for mild TBI diag-

nosis.[51] The authors also reported that a mild positive interaction correlation between plasma PrPC levels and increasing blast intensity.[50] Moreover, neurodegenerative changes after TBI is also associated with elevated plasma PrPC levels.[52] Recently, it was suggested that PrPC is important in mediating TBI related pathology.[53] On the other hand, PrPC is expressed on endothelial cells and could change at the other pathological conditions, such as cerebrovascular disease, vascular endothelial damage, or hypertension.[54]

TBI may affect anyone and can enhance the risk of certain brain diseases. TBI may alter the brain, producing pathologies, such as energy depletion, ionic imbalances, toxic aggregates, inflammation, and cell death. Thus, brain trauma may result in disease-causing and disease-accelerating capabilities, ultimately being the main reason for these affected individuals to develop neurodegenerative disorder.[55]

PrPC has been reported to be upregulated following focal cerebral ischemia; thus, it is possible that the rise in plasma PrPC content may be partially attributed to damaged ischemic regions in the brain as a result of blast exposure.[56] Because the extent of blast-induced damage in the brain is unclear, it is possible that PrPC mRNA and protein expression may also be affected. It is certain, however, that the rise in PrPC concentration is yet another part of the unique pathology complex associated with primary bTBI.[56–64] Although there are many studies in literature for s100B after TBI, there are limited studies about S100B and prion protein C together. Detecting cellular prion protein as a biomarker could be thought important for TBI. As prion protein levels lead clinicians to diagnose TBI earlier and to forecast neurodegenerative disease. In addition, we know that S100 B has poor specificity. Therefore, using several biomarkers simultaneously is useful for early diagnose and other clinical aspects.

Further studies are needed to test our findings in humans following TBI (penetrating bodies, blunt trauma) to definitively acknowledge it as a reliable biomarker and its subsequent diagnostic utility.

To date, several biomarkers have been investigated, but none of them has been definitively established as having clinically practical screening qualities.[33] We report that mean PrPC concentration is significantly increased compared with controls at both 2nd and 24th hrs after insult. Higher PrPC levels found after TBI may arise from its neuroprotective role in brain tissue. In the literature, it was shown that raised PrPC levels in blast TBI at 24th h. To our knowledge, this is the first study investigating PrPC levels at the early period following injury. In this study, we demonstrated circulating PrPC as a potential biomarker for early diagnosis of TBI. We also reported a positive correlation between PrPC and the extent of the injury.

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DENEYSEL ÇALIŞMA - *ÖZET*

Deneysel beyin travması oluşturulan sıçanlarda erken tanı için sellüler prion protein(PrPC)'nin değerlendirilmesi

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AMAÇ: Travmatik beyin yaralanması önemli bir problem olmasına rağmen, tanıda yardımcı olan yaygın kullanımlı bir nöro-biyobelirteç yoktur. Bu çalışma, S100B ve prion protein (PrPC) düzeylerini travmatik beyin yaralanması oluşturulan sıçanlarda değerlendirmeyi amaçlamaktadır.
GEREÇ VE YÖNTEM: On beş albino cinsi sıçan; sham (1), kontrol (6) ve travma (8) olarak üç gruba ayrıldı. Travmatik beyin yaralanması modifiye serbest düşme modeli ile oluşturuldu. S100B, PrPC düzeyleri ELISA yöntemi ile ölçüldü. Beyin örnekleri patolojik inceleme için çıkarıldı.
BULGULAR: Serum S100B ve PrPc düzeyleri travma grubunda, travmadan sonraki 2. ve 24. saatlerde yüksek bulundu (sırasıyla, $p<0.002$, $p<0.002$). Ayrıca travma grubundan alınan beyin örneklerinde histopatolojik yaralanma skoru yüksek bulundu. Sadece serum PrPc düzeyleri ile çıkarılan beyin dokusundaki yaralanma skoru arasında pozitif korelasyon vardı ($p=0.039$, $r= 0.731$). ROC analizi ile iki parametre incelendiğinde travmatik beyin yaralanmasının tanısında sensitivite ve spesifitesi aynı bulundu.
TARTIŞMA: S100B ve PrPc düzeyleri bizim deneysel modelimizde tanıda iyi sensitiviteye sahiplerdi. PrPc travmatik beyin yaralanması ile gelen hastalarda güvenilir bir belirteç olarak kullanılabilir. Bu biyo-belirteçin tanısal değerini gösterecek klinik çalışmalara ihtiyaç vardır.
Anahtar sözcükler: S100B; sellüler prion protein (PrPC); travmatik beyin hasarı.

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