Serum biomarkers based neurotrauma severity scale: a study in the mice model of fluid percussion injury

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The study aimed to investigate the significance of serum biomarkers in the severity grading of traumatic brain injury (TBI). For this purpose, mice underwent fluid percussion injury (FPI) at three discrete severity levels, mild, moderate, and severe. The severity of trauma was verified by the qualitative and quantitative histopathology of the brain. The serum samples were analyzed for the potential changes in ubiquitin C-terminal hydrolase-1 (UCHL-1), S100β, interleukin-6 (IL-6), corticosterone, and β-endorphin at 24 and 72 h post injury. A multifold increase in the values of UCHL-1 was reported at all severity extents of FPI. However, TBI severity-dependent increase in UCHL-1 was reported on 72 h following FPI but not at 24 h. S100β values were significantly augmented in the mild and moderate group at both the time point but not in the severe group. Serum level of IL-6 was significantly increased in the mild injury group at 24 h but not in the moderate and severe. At 72 h, IL-6 showed a reverse trend. β-endorphin and corticosterone were sensitive at an early stage only. Such unique dynamics of each biomarker enable us to propose TBI severity scale in the term of biomarkers codes to predict the extent of neurotrauma. Our preclinical study presents a predictive model for further clinical validation.

Key words: traumatic brain injury, biomarkers, fluid percussion injury, severity scale

INTRODUCTION

Traumatic brain injury (TBI) is a major global burden of epidemic proportions leading to acute and chronic disability. The common etiological factors for TBI are highway traffic accidents, combat operations, and sports activities. Accurate and timely diagnosis is one of the therapeutic challenges in the clinical management of TBI. With the advent of modern neuroimaging techniques and methods, it is now possible to detect trauma insults at the sub-millimeter level if not matching histological precision. However, pre-hospital decision-making in the treatment of TBI is challenging in an austere setting such as the operational environment and remote suburban settlements, especially in the developing world.

TBI represents two complex spectra of damage, the primary and secondary injuries. The primary injury is irreversible and occurs immediately following the head trauma. It may lead to the deformation or damage to the vasculature, axons, glia, and neurons to a varying extent (Johnson et al., 2013; Hemphill et al., 2015; McKee and Daneshvar, 2015; Kaur and Sharma, 2018). Depending on the therapeutic regimes and strategies, the secondary injury can be reversible and occurs for hours and days following the initial assault. The secondary injury may involve a rather more complex cascade of neurophysiological and biochemical responses to the primary insult sufficient to modulate the functional outcomes of the brain. The major measurable functional dysfunctions involve cognitive, affective, and sensorimotor changes (Zhao et al., 2012; Johnstone et al., 2015). Therefore, the only available field-based assessment method employed currently is the Glasgow Coma Scale (GCS). Though GSC is a valuable method for the point of care diagnosis, it requires a skilled practitioner and results may be affected by...
concomitant factors like alcohol, drug, or psychological trauma (Teasdale and Jennett, 1974; Shahin et al., 2010; Mood et al., 2011). Moreover, mild TBI is difficult to diagnose by GCS and sometimes even by modern imaging techniques. Hence, such forms of TBI that remain undiagnosed may result in severe neurobehavioral dysfunctions at a later stage.

A suitable biomarker or a set of biomarkers for the TBI diagnosis should be convenient enough to use at the point of care, require minimal training and expertise, readily accessible, and easily sampled in the field. The biofluid-based reliable biomarkers for rapid triage in the prognosis of TBI are of utmost importance for treatment strategies. The suitable markers for TBI necessitate two fundamental requisites, a reliable animal model, and selection of potential biomarkers. Human is regarded as the ultimate animal models for human diseases and disorders. However, severity grading in human TBI patients may not be accurate due to unknown and varying degrees of impact, time after injury, age, gender, and concomitant factors like disease, drug, lifestyle, and nutritional status. Therefore, the rodent model may prove to be superior to the human patient to cause discrete grades of traumatic brain injury. TBI can be induced to experimental animals by impact, rapid acceleration or deceleration, blast waves, crush, and penetrating projectile. The most commonly employed models are cortical impact, weight drop, blast waves, and fluid percussion injury (FPI) (Xiong et al., 2013; Galliano et al., 2015). Although every model represents a unique feature in causing the specific pattern of trauma, the FPI model is popular due to its accuracy and reproducibility. Moreover, the observable effect of FPI, the transient apnea, and concomitant seizures represent the clinical symptoms of TBI (Kharatishvili et al., 2006). The selection of TBI biomarkers is challenging because it represents a complex spectrum of pathophysiological changes consisting of several interacting, and interdependent biological reactions at the organ, tissue, cellular, and sub-cellular levels. Major targets of TBI are neuronal bodies, dendrites, and axons along with the astroglial cells and the myelin-forming oligodendrocytes. Therefore, recent findings are mostly centered on the biomarkers for neuronal cell body injuries like ubiquitin C-terminal hydrolase-L1 (UCH-L1) and glial markers like S100β protein.

Therefore, a comprehensive study using a reproducible TBI model is required to evaluate potential changes in the biomarker/s of a multiparametric panel. For this purpose, mice underwent FPI at three discrete severity levels, mild, moderate, and severe. The serum samples were analyzed for the potential changes in UCH-L1, S100β, interleukin-6 (IL-6), corticosterone, and β-endorphin at 24 and 72 h post-injury.

**METHODS**

**Animals**

Male adult (11 weeks-old) C57BL/6J mice weighting 26±2g were obtained from in-house inbred colonies of the institute. The mice were kept in an experimental room having ambient temperature 22±1°C, 12 h light, and 12 h dark cycle, with ad libitum food and water availability. All the experimental protocols and procedures were undertaken according to the Institutional guidelines by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and approved by the Institute’s animal ethical committee.

**Experimental design**

The study was planned to assess the dynamic changes in the serum concentration of UCHL-1, S100β, IL-6, corticosterone, and β-endorphin at 24 and 72 h following mild, moderate, and severe FPI. For this purpose, 11 weeks old C57BL/6J male mice were divided into four groups (n=8 each group), control, mild, moderate and severe. Following the FPI, serum was collected retro-orbital on 24 h and 72 h for the analysis of biomarkers, and brain samples were collected for evaluation of qualitative and quantitative histopathology.

**Induction of traumatic brain injury**

The mice were deeply anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) cocktail at a final volume of 0.1 ml/25 g. A midline scalp incision was made from the eyes to the neck to expose the right parietal bone to provide a clear surgical field for the craniectomy. During the surgical procedure, an artificial tear lubricant eye ointment (Akorn A, Switzerland) was applied over the eyes to avoid any possible damage to the cornea due to dryness. Craniectomy of 2.5 mm diameter was performed manually over the right parietal bone using a trephine as described previously (Aleem et al., 2020). The craniectomy site was over the right parietal bone, halfway between bregma and lambda, and between the sagittal suture and lateral ridge. To stabilize the trephine during the craniectomy, a piece of solid ny-
Serum enzyme-linked immunosorbent assays

The blood samples were collected on 24 h and 72 h following FPI. The samples were collected retro-orbitally, using micro-hematocrit capillary, in a silica-coated microcentrifuge tube. A topical ophthalmic anesthetic, proparacaine (0.5% w/v) was applied prior to the sampling. The serum samples were left over to coagulate and centrifuged at 3000 g for 15 min at 4°C. After centrifugation, serum samples were stored at -80°C until the analysis. UCHL-1, S100β (FineTest, Wuhan, China), corticosterone (Arbor Assays, Michigan, USA), and IL6 (Enzo Life Sciences, Lausen, Switzerland) sandwich ELISAs, and β-endorphin (competitive ELISA) (FineTest, Wuhan, China) were carried out using standard 96-well ELISA microplate according to the manufacturers’ protocols provided. To meet the required amount of serum for the five parameters, the samples of eight mice from each group were pooled in pair (two mice) resulting four sample for each group. Signal intensity, developed with TMB substrate measured at 450 nm using a spectrophotometer with an inbuilt ELISA plate reader (Multiskan GO, Thermo-Fischer, Vantaa, Finland). Target contents of concerned endpoints were determined from standards with a 5-parameter curve (SPL) fitting using SkanIt 5.0 software. Values were expressed either in ng/ml or pg/ml and compared with control as well as among the groups at different time points.

Histology

After harvesting, brain tissue samples were fixed in 10% buffered formalin for 24 h and then cut at a 10 µm thickness with an automated vibratome (Leica VT1200S Vibratome; Nussloch, Germany). Six sections from each mouse’s brain were cut near bregma (~2.12 mm, AP) and interaural (1.68 mm), with inter-distance of 50 µm among the sections. Sections were further processed for hematoxylin and eosin (H&E) staining to detect tissue morphological alterations, and neuronal damages, and death.

Images were photographed at 40X magnification using LMI BM-Prime Microscope (LMI, England). Quantification of dead and degenerating neurons in H&E stained sections was carried out in a 300 × 300 µm² area of cortex below the injury site. ImageJ software (National Institutes of Health, Bethesda, MD) was employed to count the number of dead and degenerating neurons.

Statistical analysis

All the results presented as the mean ± standard error of the mean (SEM). Differences among the groups and the days were evaluated by one-way analysis of variance (ANOVA) and Sidak’s multiple comparisons test post hoc analysis. For this purpose, Graph pad Prism version 8.0 (Graph Pad Software, La Jolla, CA) was used. Concentrations of target proteins/contents were determined from standards with a 5-parameter logistic curve (SPL) fitting. Statistical significance was taken at the 95% confidence intervals (P<0.05).
RESULTS

Neuronal death and degeneration

Quantitative evaluation of dead and degenerating neurons following FPI was carried out in H&E stained sections in the ipsilateral cortex corresponding to the injury site. The count of dead and degenerating neurons was in proportion to the injury severity at both time points (Figs 2, 3). At 24 h following FPI, the severe FPI group (152.3±4.05) showed a significantly higher (P<0.0001) number of dead neurons in comparison to the control (15.67±1.2), mild (52±2.08), and moderate (88.3±4.17) FPI (Fig. 4A). The count of dead neurons in the moderate FPI group was significantly (P<0.0001; P<0.001) high in comparison to the control and mild FPI respectively (Fig. 4A). A similar pattern was observed at 72 h following FPI, where dead neurons were increased in the proportion of injury severities. The severe FPI group (154.7±5.36) showed a significantly higher number of dead neurons in comparison to control (22±1.53; P<0.0001), mild (99±3.05; P<0.001), and moderate (121.7±2.91; P<0.001) FPI (Fig. 4B). Similarly, the count of dead neurons was significantly higher in the moderate FPI group than in the control and mild FPI. The mild FPI group showed a significantly higher count of dead neurons in comparison to the control group. The qualitative morphometric analysis also showed profound perineuronal vacuolation in injury groups at both time points. The extent of vacuolation was in proportion to the injury severity (Figs 2, 3).

Serum UCHL-1

Fluid percussion-induced neurotrauma significantly (P<0.0001) augmented the serum UCHL-1 at 24 h following the mild (6010±157.6), moderate (6745±560.7), and severe (5611±498.7) TBI (Fig. 1A). Though UCHL-1 showed a multifold increase at all injury levels, the severity-dependent difference at 24 h was not significant. At 72 h, serum UCHL-1 level showed an increasing
trend with the magnitude of TBI severity, and a statistically significant (P<0.05) difference was observed between mild (4687±144.5) and severe TBI (6218±125.9) (Fig. 1A). However, no significant difference in UCHL-1 level was observed between mild and moderate TBI at 72 h (Fig. 1A).

### Serum S100β

Serum S100β level significantly (P<0.05) increased following 24 h FPI in mild (12.87±0.32) and moderate (14.38±1.02) injury groups as compared to control (10.56±0.41) (Fig. 1B). Similarly, at 72 h a significant increase (P<0.05) in serum S100β level was reported in mild (13.60±0.53) and moderate (13.08±0.53) injury group as compared to control (10.56±0.41) (Fig. 1B). Interestingly, the serum S100β level of the severe injury group was significantly not different from the control at both the 24 h and 72 h following FPI. The severity-dependent changes in the S100β level were statistically not significant except that the severe group (10.9±0.82) showed significantly (P<0.05) less value of S100β in comparison to moderate (14.38±1.02) at 24 h (Fig. 1B).

### Serum IL-6

Serum IL-6 level in the mild injury group (88.51±18.13) showed a significant increase (P<0.05) at 24 h following FPI as compared to the control (33.64±1.791) (Fig. 1C). However, no significant changes in serum IL-6 were reported in the moderate (52.87±6.09) and severe injury (40.37±2.192) groups as compared to the control. The value of serum IL-6 level in the mild injury (88.51±18.13) group was significantly higher than the moderate (P<0.05)

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**Fig. 2.** Representative photomicrographs of H&E stained brain sections depicting neuronal damage following 24 h of FPI. A1-A3: Control; B1-B3: Mild injury; C1-C3: Moderate injury D1-D3: Severe injury. The black arrow indicates dead neuron and red arrow indicate vacuolation. The black dotted square box represents the quantitation area of the cerebral cortex ipsilateral to FPI.
and severe group (P<0.01) (Fig. 1C). At 72 h following FPI, a reversal trend was observed where moderate (63.45±7.36; P<0.05) and severe (69.42±10.53) injury groups showed significant (P<0.05) increase as compared to the mild injury (36.44±1.27) group (Fig. 1C). At 72 h, the serum IL‑6 level in the moderate (63.45±7.36; P<0.05) and severe (69.42±10.53; P<0.01) injury group was also significantly high from the control (33.64±1.791).

Serum β‑endorphin

FPI lead to a significant (P<0.05) increase in serum β‑endorphin at 24-h following mild injury (2249±344.9) as compared to the control (1721±192.5) (Fig. 1D). Contrary to the mild injury group, no significant changes were reported in the moderated (1543±91.06) and severe (1301±164) groups as compared to the control. The value of serum β‑endorphin level in the mild injury group was significantly higher than the moderate (P<0.05) and severe group (P<0.01) (Fig. 1D). No significant changes in serum β‑endorphin level were reported at 72 h following the FPI at any level of injury (Fig. 1D).

Serum corticosterone

Serum level of stress hormone corticosterone increased significantly (P<0.05) in mild (168.7±67.58) and severe (196.4±86.22) FPI mice as compared with control mice (15.96±3.44) at 24 h (Fig. 1E). However, no significant difference was observed between control and moderate (122.8±22.27) FPI mice. At 72 h, serum corticosterone level showed a decreasing ten-
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Sensitivity with time in mild (168.7±67.58 to 91.25±30.77), moderate (122.8±22.27 to 28.09±7.17), and severe FPI mice (196.4±86.22 to 72.09±13.61) but changes were statistically not significant in comparison to control (Fig. 1E).

DISCUSSION

Sensitive circulating biomarkers play a significant role in the diagnostic decision-making for the management of TBI in an austere medical setup. Serum-based biomarkers such as UCHL-1, S100β, GFAP, and NSE, have been studied in several clinical studies to categorize the extent of injury (Rothoerl et al., 2000; Woertgen et al., 2002; Svetlov et al., 2010; Liu et al., 2010). Unfortunately, there is no consistency in the results possibly due to greatly varying and unknown factors like the degree of impact, time after injury, age, gender, and concomitant factors like disease, drug, and alcohol (Liu et al., 2010; Goodman et al., 2013; Kawata et al., 2016; Boutté et al., 2016; Ved and Zaben, 2018; Morris et al., 2019). Conversely, animal models-based studies on TBI biomarkers using precise discrete grades of traumatic brain injury are scant. In the present study, while using an FPI model; a diverse panel of serum biomarkers was measured in mild, moderate, and severely injured mice. Since the precision in experimental TBI severity grade is a prerequisite for the evaluation of predictive biomarkers, the current study verified the extent of neuronal damage by qualitative and quantitative histology of the brain. We reported a discrete range of dead and degenerative neurons along with perineuronal vacuolation corresponding to the mild, moderate, and severe FPI.

Our results showed a multifold increase in the serum UCHL-1 level in all grades of injury at 24 h post-FPI. However, UCHL-1 was unable to distinguish the extent of the injury. At 72 h, there was a significant difference in the UCHL-1 level between mild and severe injury group but not with moderate. Earlier clinical and preclinical studies also showed a significant increase in the UCHL-1 level following 4 to 48 h of TBI (Liu et al., 2010; Papa et al., 2016; 2019). However, such results were inconsistent due to varying sam-

Fig. 4. Quantitation of dead and degenerating neurons within the cerebral cortex ipsilateral to FPI at different severity grades. (A) Group comparison of dead and degenerating neurons at 24 h post-FPI. For group comparison at 24 h indicate ****P<0.001, *****P<0.0001 wrt control; ###P<0.001, ####P<0.0001 wrt mild-24h; ####P<0.0001 wrt mod-24 h. (B) Group comparison of dead and degenerating neurons at 72 h post-FPI. For group comparison at 72 h indicate ****P<0.0001 wrt control; **P<0.01, ****P<0.0001 wrt mild-72 h; #ΦΦΦP<0.001 wrt mod-72 h. wrt (with respect to).
plling times after injury. Moreover, UCHL-1 was unable to distinguish moderate to severe injury (Mondello et al., 2016). UCHL-1 is readily detectable in the serum within 2 h after trauma and declines rapidly over 48 h (Liu et al., 2010). Therefore, early detection of UCHL-1 may be useful as an adjunct biomarker for severity grading along with other parameters.

Our study reported a multifold increase in the values of IL-6 at 24 h in the mild injury group but not in the moderate and severe. Therefore, a significant increase in both the UCHL-1 and IL-6 injury is indicative of mild injury and helps in distinguishing it from moderate and severe. At 72 h, there was a significant difference in the IL-6 level between mild and severe injury group but not with moderate. Surprisingly, IL-6 level at 72 h was just a reversal to the 24 h as the severe injury group showed significantly higher value as compared to the mild. Though there are no sufficient reports on the TBI severity dependent changes in IL-6, an earlier study in the rat model suggested that the serum IL-6 level reached a peak within 90 min post injury and remained higher till 6 h after injury. However, IL-6 level following 24 h was significantly elevated only in the higher injury group as reported in the previous study (Yang et al., 2013). IL-6 plays regulatory effects upon the inflammatory response (Raiovich et al., 1999). Besides being implicated in the inflammatory cascade, IL-6 affords significant neurotrophic effects on neurons (Kushima et al., 1992). IL-6 is produced by both the glia and neuronal cell population (März et al., 1998; Lee et al., 2002). Serum level of IL-6 has been reported to increase between 3 and 8 h in rats following the neurotrauma (Taupin et al., 1993). TBI-induced augmentation of IL-6 was also reported in human CSF, but to a lesser extent in the serum (Kossmann et al., 1995). Therefore, it is clear that serum IL-6 represents a complex pattern following TBI that it cannot be a determinative biomarker for severity grading of neurotrauma but it may supplement with the other biomarkers like UCHL-1.

Similar to the IL-6 level, there was a significant increase in the β-endorphin level at 24 h following the TBI in the mild injury group but not in moderate and severe TBI mice. Taking together, IL-6 and β-endorphin may help in differentiating mild injury to the moderated and severe injury group that otherwise not possible by UCHL-1 alone. Endogenous opioids including β-endorphin have been suggested to be implicated in neurotrauma as secondary pathophysiological factors (Faden, 1984a;b). Brain area-specific perturbation of β-endorphin has been reported in earlier findings but no reports are available for the serum level of β-endorphin in TBI subjects (McIntosh et al., 1986).

TBI-induced rapid acceleration and deceleration of the brain inside the skull results in transient and chronic endocrine dysfunction due to substantial assault on the hypothalamus-pituitary-adrenal (HPA) axis (McAllister, 1992; Masel and DeWitt, 2010). We have reported an elevated level of corticosterone in the mild and severe injury group following 24 h of FPI. Earlier studies using different pre-clinical models also suggested the TBI-induced augmentation in the serum level of corticosterone (McNamara et al., 2010; Kammaksh et al., 2011; Kwon et al., 2011). Since corticosterone level affected only at the early stage in mild and severe TBI group, such a unique trend can be supplemental to the other biomarkers for the severity grading.

Additionally, S100β an astroglial 11 kDa calcium-binding protein is among the most investigated TBI biomarker. In the current study, mild and moderate injury mice showed a significant increase in the S100β level at both the 24 h and 72 h but not in the severe group. Earlier clinical and preclinical studies also reported the TBI-induced augmentation of S100β from 12 h to 48 h following injury (Rothoerl et al., 2000; Blyth et al., 2011; Liu et al., 2015). However, no reports are available to document the role of circulatory S-100β in differentiating the neurotrauma severity. Nevertheless, S100β is a sensitive marker for the prediction of neuropathological aberration revealed by computer tomography and also to assess the post-concussive syndrome in mild neurotrauma (Barbosa et al., 2012; Metting et al., 2012; Johnson et al., 2013; Hemphill et al., 2015; Mckee and Daneshvar, 2015; Kaur and Sharma, 2018).

The present study suggests that UCHL-1 is highly sensitive to neurotrauma at an early stage as it shows a multifold increase following 24 and 72 h of FPI. However, UCHL-1 alone is not sufficient to predict the extent of severity as there was no significant difference among the mild, moderate, and severe TBI groups. Consequently, based on the unique dynamics of each biomarker, a predictive severity scale can be proposed. While using the first letter of biomarkers that significantly augmented at a particular severity extent can be depicted as severity code (Table 1). In the present study, UCIBC, US, and UC represent the mild, moderate and severe at 24 h following TBI. Similarly, at 72 h, US, USI, and UI represent mild, moderate, and severe. Although such a predictive model can be employed as a guiding principle for the diagnosis of clinical subjects, certain more studies are warranted to find out the dynamic changes in biomarkers at other time points following TBI. Moreover, the severity scale needs to be compared using more models of neurotrauma and even polytrauma.
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