PERSISTENT NEUROINFLAMMATION AND NEURODEGENERATION AFTER BRAIN INJURY

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ABSTRACT

INTRODUCTION: Traumatic brain injury (TBI) is a major global health issue in both civilians and war-affected military personnel and has no proper cure or treatment. Survivors of TBI exhibit long-term cognitive dysfunction and even permanent disabilities. As TBI patients exhibit multiple pathological conditions and the exact mechanism of neuroinflammation and neurodegeneration is still remain elusive. Therefore, we assess the prolonged effects of penetrating brain injury in animal model to further explore the neuroinflammation-associated neuropathological conditions in clinical studies.

OBJECTIVE: To determine the long term neuroinflammatory and neurodegenerative effects after penetrating brain injury in preclinical model.

METHODOLOGY: In the present study, we developed a penetrating brain injury and examined compare the neuroinflammatory effects after seven days and two months following brain injury in animal model. We used confocal laser microscopy to assess the expression of neuroinflammatory mediators in the mice group.

RESULTS: These findings provide evidence that penetrating brain injury have prolonged effects after two months. Using confocal laser microscopy, we found that the expression of GFAP (reactive astrocytes marker) and IL-1β (inflammatory marker) in the mice brain were significantly sustained after two months following brain injury, suggesting the prolonged neuroinflammatory effects after penetrating brain injury. Here, we found fascinating results that severity of neuroinflammation was higher at day 7 as compare to two months in animal brains.

CONCLUSION: These findings provide evidence that penetrating brain injury is critically involved in prolonged neuroinflammation which effect the cognitive ability and neurological impairments in the brain. Furthermore, these findings would be supportive in the preclinical and clinical settings.

KEY WORDS: inflammation, Traumatic brain injury, Astrocytes, interleukin beta.

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INTRODUCTION

Traumatic brain injury (TBI) is a serious global health problem that involves brain damage with excessive reactive oxygen species generation, persistent neuroinflammation, amyloid beta (Aβ) accumulation, increased expression of phosphorylated tau protein, and memory loss, leading to long-term disabilities.[1,2] There is strong evidence from animals and human studies that a single TBI can lead to a long-term neurodegenerative process and distressing the regulation of central body functions.[3,4] Traumatic brain injury elicits abrupt neuroinflammatory responses that may contribute to several brain disturbances with neuropsychiatric and cognitive problems.[5] Studies reported long term, life altering concerns in children after TBI.[6] Furthermore, studies reported astrocytes and microglia activation in a rodent model of brain injury.[7] To date, there is a severe paucity of therapies with several failed clinical trials for TBI.[8] This lack of effective therapies is mainly due to complex pathophysiology of TBI and less knowledge about the exact molecular pathology and the prolong effects after brain injury. Thus, an urgent demand exists to understand the injury mechanisms and to identify multi-targeted therapeutic agents that exert significant neuroprotection against the devastating neurodegenerative conditions of

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TBI. To come up with an effective treatment, there is urgent demand to explore the molecular mechanism and the prolonged effects associated with brain injury.

Neuroinflammation is a critical injury mechanism which is critically engaged to on-going neurodegeneration and neuronal abnormalities associated with brain injury. Neuroinflammation is characterized by astrocytes and microglial activation, leukocyte recruitment, and upregulation of inflammatory mediators.[9]

The aim of the present study was to investigate prolonged effects of brain injury-associated neuroinflammation in animal model. We compare the neuroinflammation associated inflammatory mediators at day 7 and at day 60 in animal brains. We found persistent neuroinflammation in animal brain following penetrating brain injury. It was interesting to find that the severity of neuroinflammation was higher at day 7 as compared to 60 days post brain injury. Further study is needed to explore the long term consequences and pathologies associated with TBI and the other broad range of neuroinflammatory mediators needs to be explored.

METHODOLOGY

Animals and treatment

Male C57BL/6N mice, 8-10 weeks of age with an average body weight of 30 g (Samtako Bio, Korea) were kept in the animal care center of Gyeongsang National University, South Korea. The experiments and animals experimental procedures in the present study were approved (Approval ID: 125) by the animal ethics committee (IACUC) of the Division of Life Science, Department of Biology, Gyeongsang National University, Republic of South Korea. The animals included in this study were carefully handled and acclimatized for two weeks in the university animal house before starting the experimental procedure as according to the animal ethics committee (IACUC) of the Division of Life Science, Department of Biology, Gyeongsang National University, Republic of South Korea.

After acclimatization, the animals were randomly divided into two groups (n=12). The treatment groups were as follows:

Saline-treated group

TBI group

Cortical stab wound

Eight- to 10 weeks-old wild-type mice were anesthetized using Rompun and Zoletil via intraperitoneal injection and were carefully placed on the stereotaxic instrument. The skull was exposed by midline skin incision and, using an electric drill the skull was removed very carefully in the right hemisphere, 2mm lateral to the midline and 1.5mm posterior to the bregma in the cortical region of the mouse brain and left the dura undisturbed. The animal with severe bleeding was excluded from the experiment. The animals received 3mm stab wound in the cortical region with sharp scalpel adjusted in the stereotaxic arms. After the injury, the skin was carefully closed using standard silk suture with continuous heating with control heating lamp. The animals of the saline-treated group were subjected to the same procedure with stab wound injury. Following injury, all the animals were visually monitored every 10 mints until there safe recovery.

Tissue collection and sample preparation

For brain section staining, the male mice (n=5) were anesthetized and transcardially perfused with saline to wash away the blood followed by fixation with 4% paraformaldehyde. The brain was carefully dissected and post-fixed with 4% paraformaldehyde for 48 h followed by post-fixation in a 20% sucrose solution for 72 hr. The brains were frozen in OCT compound (A.O USA). The frozen blocks of OCT compound were cut into 14-µm-thick sections using a vibratome (Leica, Germany) and were thaw-mounted on Probe-On Plus-charged slides (Fisher) and stored at −80 °C.

Assessment of brain lesion volume

The slides containing the tissues were kept at room temperature until dry and stained with cresyl violet. Digital camera photographs of the TBI and treated mouse groups were taken and analyzed with ImageJ software. The injured and recovered areas of the brain sections were carefully marked; the lesion volume was calculated by multiplying the sum of the areas in the ipsilateral hemispheres by the distance between the sections.

Immunofluorescence analysis.

The immunohistological analysis was performed as previously described in the reference [10, 11] with modifications. Briefly, the slides of the treated mouse brains were dried overnight and washed (2 time, 8min) in phosphate buffer saline (PBS, 0.01 mM), incubated with proteinase k (5 min) and rinsed with PBS. The slides were blocked in Goat/Rabbit serum (2% in PBS) containing 0.1% Triton X-100 for 60 min. Next, the slides were incubated with primary antibodies (Table 1), overnight at 4 °C followed by incubation with tetramethylerhodamine isothiocyante–fluorescein isothiocyante (FITC)-labeled secondary antibodies at room temperature. The fluorescent images of the sections were captured using confocal laser-scanning microscope (FluoView FV 1000 MPE, Olympus, Japan).

Nissl staining

For histological analysis the brain sections were Nissl stained as previously described with minor modification[12]

Statistical analysis

The histological analysis were performed through Sigma gel software (SPSS Inc.) and ImageJ software respectively. All the data are expressed as mean ± S.E.M and analyzed through one-way ANOVA followed by post-hoc analysis. A P value less than 0.05 was considered statistically significant. The symbols *P < 0.05 and **P < 0.01, **P < 0.001 showing significant differences between control and TBI.

RESULTS

Effects of Traumatic brain injury-induced neuroinflammation

Traumatic brain injury induces chronic neuroinflammation. Here, we examined the prolong effects of penetrating brain injury in animal model. We found the sustained neuroinflammation at day 60 as revealed by the active astrocytes and neuroinflammatory mediator IL-1β in the ipsilateral cortical region of the mice brain. We compared the severity on day 7 (Figure 2) and on day 60 (Figure 3) post brain injury. It was interesting to observe that the severity was more prominent on day 7 as compared to day 60. These results indicated that astrocytes are active at day 60 which continuously contribute to cytokines production in brain following brain injury.

Figure 1. A. Representative sketch of the injury site of the TBI mouse brain. The sketch represent Cortex, Cornu Ammonis 1 (CA1), Cornu Ammonis 3 (CA3) and dentate gyrus (DG) regions. Injury was limited to the cortical region. B. Crossly violet-stained (gray) coronal section from TBI.

Given are the confocal laser microscopy images of GFAP (Green) and IL-1β (Red) in the ipsilateral cortical region of the brain injured mouse brain (n=5). The images were quantified with ImageJ software. (Scale bar: 50). The data are the mean ± S.E.M. One-way ANOVA. P< 0.05 was considered statically significant. ***P < 0.001, control versus TBI,

Given are the confocal laser microscopy images of GFAP (Green) and IL-1β (Red) in the ipsilateral cortical region of the brain injured mouse brain (n=5). The images were quantified with ImageJ software. (Scale bar: 50). The data are the mean ± S.E.M. One-way ANOVA. P< 0.05 was considered statically significant. **P < 0.01, control versus TBI.

Traumatic brain injury exhibites sustained neurodegenration

In an examination of our morphological data, we observed a significant increase in the lesion volume in the TBI mouse cortical region at day 7 (Fgure 1 B). Further, we examined the neurodegenrative conditions in mice brain at day 7 and at day 60 using nissil staing. Our results clearly indicated that penetrating brain injury contributes to sever neurodegneraive conditions in the mice brain (Figure 4). It might be due to the sustained neuroinflammation in mice brain following penetrating brain injury. However, like immunoreactivity of the GFAP and IL-1β, the intensity of the neurodegenration was higher at day 7 as compare to day 60. This clearly indicated that with the reversal of neuroinflammation the reduction in neurodegenration was significantly observed.

The Nissl-stained (for the identification of extent of neuronal damage) coronal section represents the cellular density of the TBI. Histological examination revealed a greater cellular density in control animal. However, decreased cellular density was observed at day 7 and day 60 (n=5). The images were quantified with ImageJ software. (Scale bar: 50). The data are the mean ± S.E.M. One-way

FIGURE 1: ????????

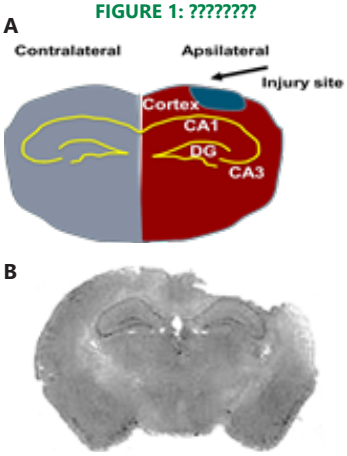


FIGURE 2: Active astrocytes (GFAP) and interleukine-1 beta on day 7 post brain-injury

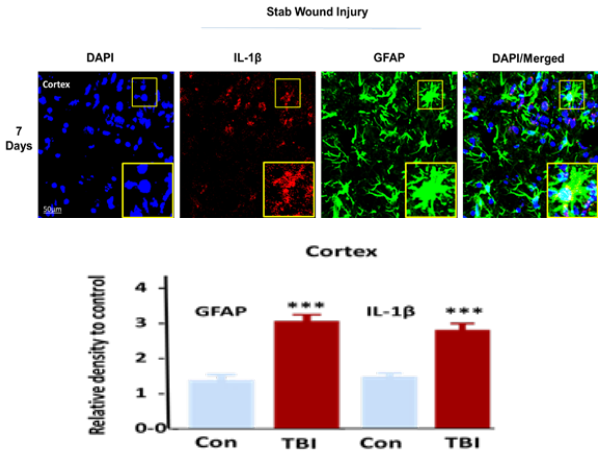


FIGURE 3: Active astrocytes (GFAP) and interleukine-1 beta on day 60 post brain-injury

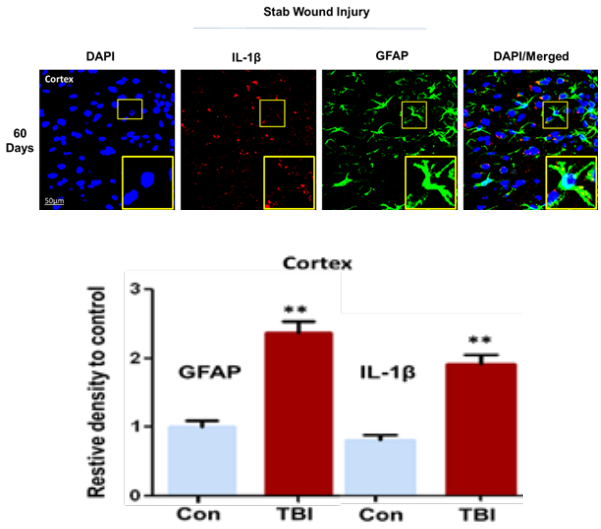
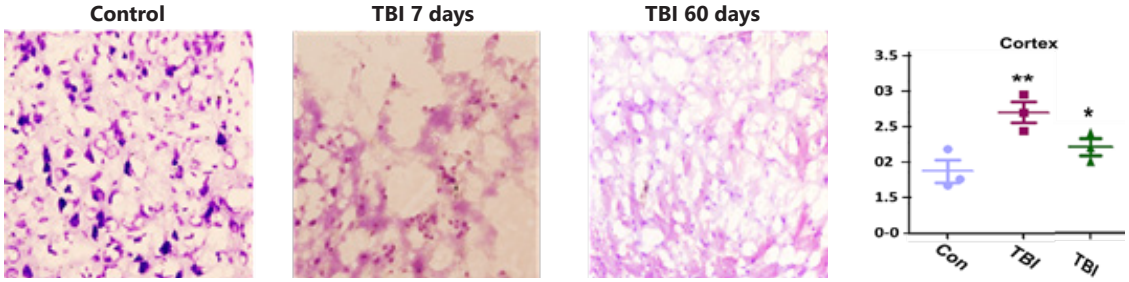


FIGURE 4: Effects of TBI on neurodegeneration



ANOVA. P< 0.05 was considered statically significant. **P < 0.01, control versus TBI.

DISCUSSION

Neuroinflammation are the critical hallmarks in the neurodegenerative process,[13] including TBI.[14] However, the mechanism neuroinflammation associated with injured brain remains largely elusive. There are several mechanism which contributes in neuroinflammation in brain. Mitogen-activated protein kinase (MAPK) cascades are critically involved in various neurodegenerative diseases which elicit glial cell infiltration of the brain.[15] The importance of the blood brain barrier (BBB) in the brain is widely accepted, and the tight junction proteins are crucial for brain homeostasis. TBI involves severe damage to the BBB integrity with abnormal transcellular transport including cytokine infiltration.[16,17] Our experimental results showed that TBI induces an enlarged contusion volume, which might be associated with elevated levels of neuroinflammation and activation of microglia and astrocytes. Our data clearly indicated that the severity of neuroinflammation was critically sustained for two months following penetrating brain injury as revealed by active astrocyte in the ipsilateral cortical region of the TBI mouse brains. Previous studies also demonstrated that TBI invokes a complex cascade of inflammatory mediators primarily through gliosis in the brain.[18] To examine the effects of TBI-associated neuroinflammation, we examined the expression levels of inflammatory mediators such as IL-1β. Our confocal microscopy analysis revealed increased immunoreactivity of IL-1β in the ipsilateral cortical region of the mice brain following brain injury. The higher expression of the IL-1β was seen at day 7 followed by day 60. Previous literature demonstrated that neuroinflammation after the initial impact may continue up to 17 years post-TBI.[19] Neuroinflammation enhance neurodegeneration via endogenous repair mechanisms and acts through immune cells, chemokines, cytokines, microglia and other inflammatory molecules.[20] After the activation of the inflammatory response in the brain, neutrophils, monocytes, lymphocytes inter the BBB and release pro-inflammatory cytokines, prostaglandins, and other inflammation mediators which further recruit microglia and immune cells by aggregating the expression of chemokines.[21,22] From these observation, we suggested that gradual reduction in the neuroinflammation might be due the regenerative process of the brain, as revealed by previous literature.[23] The aforementioned processes further proliferate progressive neurodegeneration.

It is reported that TBI involves extensive neuronal damage with the severe synaptic loss, resulting in cognitive dysfunction.[24] Therefore, we assessed the neurodegenerative conditions in the mice brain at day 7 and at day 60. We found interesting results that the intensity of the neurodegeneration was higher at day 7 as compared to day 60. Previous preclinical studies indicate that progressive neuroinflammation is critically involved in neurodegeneration in the brain following brain injury.[25] All these observation clearly demonstrated that TBI-evoked neuroinflammation persist for long time in the brain which causes sever neurological abnormalities such as neuronal apoptosis and memory impairments.

CONCLUSION

These data, for the first time, provide evidence that the TBI-evoked neuroinflammation persist for long time which further deteriorates neuronal function in the animal brain. This data further provide evidence that neuroinflammation associated with penetrating brain injury may be a central element of the injury which might be the focus of researchers when developing new treatment strategies.

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NOTES ON CONTRIBUTORS

SUR performed most of the experiments, wrote and finalized the main manuscript. TA interpreted the data. DAK performed statistical analysis. SAH and UF analyzed data and arranged figures.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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