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Relationship between circulating IGF-1 levels and traumatic brain injury-induced hippocampal damage and cognitive dysfunction in immature rats

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ABSTRACT

It is well known that traumatic brain injury (TBI) induces the cognitive dysfunction resulting from hippocampal damage. In the present study, we aimed to assess whether the circulating IGF-I levels are associated with cognition and hippocampal damage in 7-day-old rat pups subjected to contusion injury. Hippocampal damage was examined by cresyl violet staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Spatial memory performance was assessed in the Morris water maze. Serum IGF-1 levels decreased in both early and late period of TBI. Decreased levels of serum IGF-1 were correlated with hippocampal neuron loss and spatial memory deficits. Circulating IGF-1 levels may be predictive of cognitive dysfunction resulted from hippocampal damage following traumatic injury in developing brain. Therapy strategies that increase circulating IGF-1 may be highly promising for preventing the unfavorable outcomes of traumatic damage in young children.

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Traumatic brain injury (TBI) is a leading cause of death and disability in childhood [1]. Its long-term squeal consequences are often more devastating in children due to their age and developmental potential. The cognitive dysfunctions are usually the most disabling squeal following traumatic injury [7,16].

TBI can be classified as primary, which occurs immediately after trauma, and secondary, which may appear several hours or even days later [6]. Two pathogenic mechanisms have been described for secondary brain injury: excitotoxicity and apoptosis. Apoptosis is a form of cell death that serves to eliminate dying cells in proliferating or differentiating cell populations [19]. TBI has been demonstrated to overactivate physiological apoptotic mechanisms in the developing brains. The immature brain is exceedingly vulnerable to apoptotic neurodegeneration triggered by head trauma [8,9]. This developmental vulnerability period coincides with the brain growth spurt period, which occurs in the first two postnatal weeks in the rats and in the first 2 years old in humans [12].

Two brain structures prominently affected in TBI are the cortex and the hippocampus [34]. Hippocampal neurons normally play a crucial role in the processing of spatial memory and learning [22]. Memory function, in particular, is at risk following head trauma due to the selective vulnerability of the hippocampus [23,31].

IGF-1 plays a major role in brain development and survival [2]. IGF-1 has been implicated as a potential neuroprotective agent in brain injuries [4,18,21,27]. The expression of hippocampal IGF-1 was demonstrated to be increased in a developmental rodent model of TBI [30]. Recently, activation of the IGF-1R antiapoptotic pathway by mild traumatic brain injury has been suggested to be part of a neuroprotective process aimed at maintaining normal brain function [27].

In addition to brain, all organs produce IGF-1, with the liver contributing around 70% of the total circulating IGF-1 [35]. IGF-1 is one of the growth factors found to enter the brain from the circulation [25], while the relationship between the circulating and brain IGF-1 remains unclear until now; a recent study has shown that circulating IGF-1 regulates hippocampal IGF-1 levels and brain gene expression in adolescent rats [37]. Reduced circulating IGF-1 level has been suggested to be an important factor in the development of cognitive dysfunction in adults [2]. Many clinical and experimental studies have suggested that circulating IGF-1 levels are related with cognitive deficits in the aging and amnesic models. Recently, meta-analysis showed an overall relationship between low levels of circulating IGF-1 and cognitive functioning in healthy elderly [5]. In a large cohort of children, it was demonstrated that there was a

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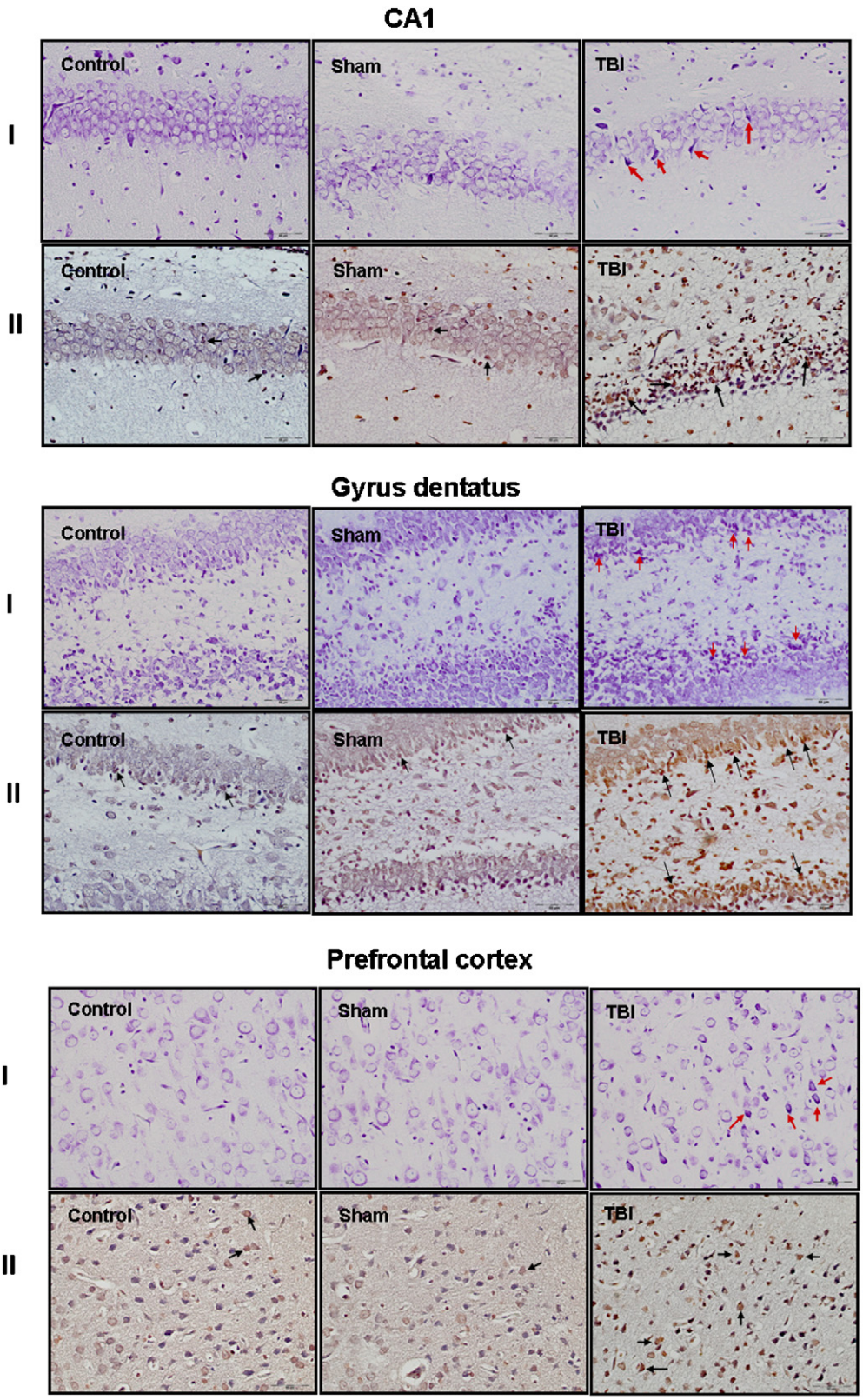


Fig. 1. Representative photomicrographs of terminal transferase dUTP nick end-labeling (TUNEL)-stained sections. *Upper*: Cresyl violet staining. The morphology of pyramidal neurons was normal in control and sham groups. In TBI group; among the normal pyramidal neurons are interspersed condensed dark neurons (red arrows). *Lower*: Representative photomicrographs of TUNEL-positive cells (black arrows). (A) TUNEL(+) cells in CA1, (B) TUNEL(+) cells in gyrus dentatus, (C) TUNEL(+) cells in prefrontal cortex, (D) the percentage of TUNEL + neurons. $^{*}p < 0.001$ compared to other groups. GD: gyrus dentatus; PREF: prefrontal cortex; PC: parietal cortex; TBI: traumatic brain injury; line I: cresyl violet staining of brain regions; Line II: TUNEL(+) cells. *Scale bar* 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

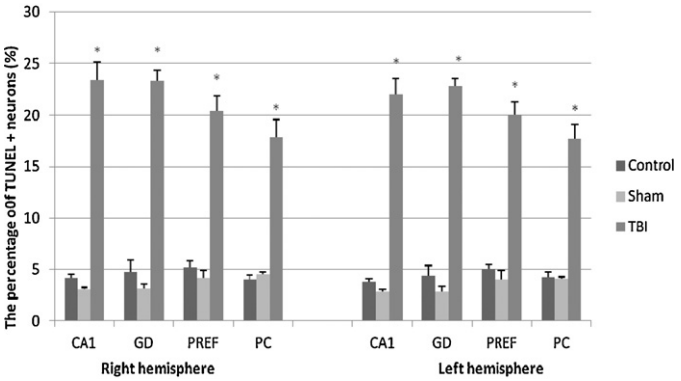


Fig. 1. (*continued*)

significant positive association between serum IGF-1 levels and IQ, supporting a role of IGF-1 in brain development [15].

The aim of the present study was to assess whether the circulating IGF-1 levels are associated with cognition and hippocampal damage induced by traumatic insult in infant rats.

All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylul University, School of Medicine. Wistar Albino rats with dated pregnancies were maintained at the same center and housed in individual cages with free access to water and laboratory chow. Forty-two litters delivered spontaneously were reared with their dams until the time of experimentation at 7 days of postnatal age. All rats were maintained on a constant 12-h-light/dark cycle at constant room temperature (21 °C), and humidity (60%). Rats were divided into three groups: (1) control ($n = 14$), (2) sham ($n = 14$) and (3) TBI ($n = 14$).

We used a modification of a well-described percussion trauma model in immature rats, in an attempt to model infant and early childhood head trauma [8]. The contusing device consisted of a hollow tube 40 cm long, perforated at 1-cm intervals to prevent air compression. The device was kept vertical to the surface of the skull and guided a falling weight onto a circular footplate resting upon the surface of the parietal bone. The center of the footplate was stereotaxically positioned 3 mm anterior and 2 mm lateral to the lambda and was fixed in place under ether anesthesia. A force of 160 g cm produced by a 10-g weight was selected to produce brain contusion. Experiments were carried out between 9.00 and 11.00 am in a sound-attenuated and air-regulated experimental room. All the pups were kept on a heating pad until returned to their mothers at 4 h after the trauma or sham surgery. In order to compare acute and long-term alterations, half of the animals in each group ($n = 7$/group) were sacrificed 7 days and the remaining animals ($n = 7$/group) 3 weeks after trauma or sham surgery.

After a light ether anesthesia, the blood of the subjects was drawn with cardiac puncture for serum IGF-1 measurement in both early (7 days after) and late period of TBI (3 weeks after). IGF-1 protein levels in the serum were measured by commercially available ELISA kits specific for rat IGF-1 (Catalog Number EK0377, Boster Immunoleader, Wuhan China, with assay sensitivity <5 pg/ml and range 62.5–4000 pg/ml) according to the manufacturer's instructions.

The brains were sectioned coronally into sequential 6 μm slices using a rat brain slicer. Each sample was subjected to the estimation of neuron number by taking three coronal sections through the hippocampus that corresponded approximately to Plates 21, 23, 25 in the rat atlas of Paxinos and Watson [24]. All sections were stained by cresyl violet. The images were analyzed by using a computer assisted image analyzer system consisting of a microscope (Olympus BH-2 Tokyo, Japan) equipped with a high-resolution video camera (JVC TK-890E, Japan). The numbers of CA1, CA2, CA3, gyrus dentatus (GD), prefrontal cortex (PFC) and parietal cortex neurons were counted by help of a 6000 μm^2 counting frame viewed through a 20× Nikon lens at the monitor. The counting frame was placed randomly five times on the image analyzer system monitor and the numbers of neurons were counted (UTHSCA Image Tool for windows version 3.0 software) and the average was taken. Neuron densities were calculated.

To detect DNA fragmentation in cell nuclei, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction was applied to the paraffin sections. DeadEnd Colorimetric TUNEL system kit (Roche, Germany) was used for apoptotic cell detection. Serial 5 μm thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol, and pretreated in proteinase K for 15 min. After washing in phosphate-buffered saline (PBS), the specimens were incubated with fluorescein-labeled deoxy-UTP and TdT at 37 °C for 60 min. Then, the converter POD solution was applied to the slides. Sections were stained with DAB and counter-stained with mayer hematoxylin and analyzed using a light microscope.

For quantitative measurement of the number of cells that underwent apoptosis, 1000 cells were randomly counted in these different areas and the percentages of the apoptotic cells were calculated.

The spatial memory performance was evaluated 3 weeks after trauma or sham surgery using a Morris water maze [22]. Water maze was 140 cm in diameter and 75 cm in height. The water level in the tank was 75 cm, which was 1.0 cm above the height of the escape platform. The pool was filled with opaque water to prevent visibility of the platform in the pool. The animals were tested for 5 days in a water maze task. On each day, rats were placed in the water (22 ± 1 °C) and allowed to swim (maximum swim time 60 s) until they located a hidden, but fixed escape platform, using extra maze cues. The escape platform was placed in the middle of one of the random quadrants of the pool. If the animal failed to locate the platform in 60 s, the experimenter placed the rat on the platform and left it there for 20 s. Each rat was tested for five consecutive trials per day, with an inter-trial interval of 60 s. Each rat was exposed to the task for four consecutive days (a total of 20 trials), and on day 5 a probe trial was run in which the platform was removed from the pool, and rats were placed into the pool and swam for 60 s. The data were analyzed for latency to find the platform and time spent in the correct quadrant. Morris water maze training was recorded using HVS image tracking system.

The data from water maze testing was analyzed by using GLM repeated measure and ANOVA post hoc Bonferroni. Differences in the histopathological parameter (cell number) between groups were tested by ANOVA post hoc Bonferroni. Correlations

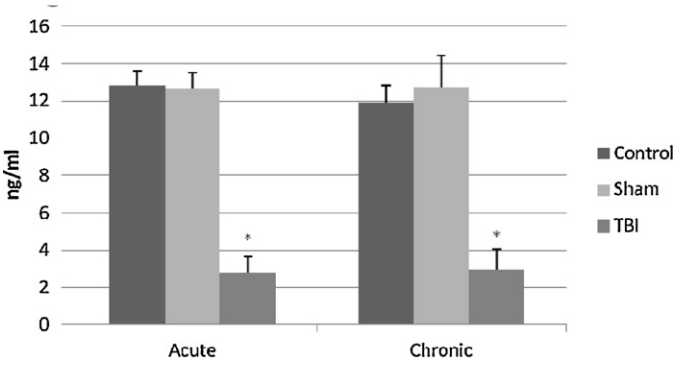


Fig. 2. The blood IGF1 levels of groups in early (7 days after) and late period of TBI (3 weeks after). TBI-rats had a lower serum IGF1 levels than other-animals in both 7 days and 3 weeks after ($p < 0.001$). TBI: traumatic brain injury.

among groups were calculated using Spearman correlation analysis. Results are presented as mean ± S.E.M. We declared significant level at $p < 0.05$.

At 7-day after trauma or sham surgery, the density of hippocampal neurons was significantly less in the ipsi and contra-lateral PFC of TBI-rats in comparison with other groups (both, $p < 0.05$) (Table 1). TUNEL-positive neurons were detected in both ipsi and contra-lateral parietal-PFC and hippocampal regions of TBI-rats (all regions, $p < 0.001$) (Fig. 1). TBI-rats had a lower serum IGF-1 levels than other-animals in 7 days ($p < 0.001$) (Fig. 2). There were strong negative correlations between serum IGF-1 levels and the number of apoptotic cells (PFC, $r = -0.924$, $p = 0.000$; parietal cortex, $r = -0.969$, $p = 0.000$; CA1, $r = -0.921$, $p = 0.000$; GD, $r = -0.944$, $p = 0.000$).

Three weeks after trauma, the mean latency to find the platform declined progressively in water maze performance of all animals. However, TBI-rats had longer escape latencies in second, third and fourth training days than other groups ($p < 0.05$) (Fig. 3A). TBI-rats spend significantly less time in the target quadrant ($p < 0.05$), more time in the opposite quadrant than other groups ($p < 0.001$) (Fig. 3B). There were no any differences about swimming speeds between groups in training trials and probe trial ($p > 0.05$). The density of neurons was significantly less in the contra- and ipsi-lateral hippocampal CA1, dentate gyrus, PFC and parietal cortex of TBI-rats ($p < 0.05$) (Table 1). TBI-rats had a lower serum IGF1 levels than other-animals in third-week ($p < 0.001$) (Fig. 2). There was a strong positive correlation between the time spent on target quadrant and serum IGF1 levels ($r = 0.728$, $p = 0.005$). There was also a very strong negative correlation between the time spent on opposite quadrant and serum IGF-1 levels ($r = -0.828$, $p = 0.000$).

The present study demonstrated that decreased levels of circulating IGF-1 were related with hippocampal neuron loss and cognitive dysfunction caused by traumatic injury in the immature rats. Neuronal apoptosis plays a crucial role in the pathogenesis of traumatic damage in developing brains, which is involved in the delayed and progressive degenerative response to trauma [6]. The severity of the trauma-induced apoptotic neurodegeneration in the brains of 3–30-day-old rats had been demonstrated to be age dependent and highest in 7-day-old animals. Thus, apoptotic neurodegeneration has been suggested to contribute in an age-dependent fashion to neuropathological outcome of head trauma. The possible explanations have been postulated for the developmental vulnerability to apoptotic neurodegeneration: (1) the neurons and glia may be most vulnerable to apoptosis, (2) the differences in the degree of myelinization and brain water content will allow traumatic forces to transmit easier to deeper brain structures in the immature brain [9,13]. An important consideration of hippocampal damage following traumatic injury is the unique

Table 1
The number of neurons of hippocampal regions, prefrontal and parietal cortex in acute and late period of traumatic brain injury. GD: gyrus dentatus; PREF: prefrontal cortex; PC: parietal cortex; TBI: traumatic brain injury.

		Right hemisphere						Left hemisphere					
		CA1	CA2	CA3	GD	PREF	PC	CA1	CA2	CA3	GD	PREF	PC
Control	Acute	42.0 ± 1.3	36.1 ± 1.9	26.0 ± 4.4	90.0 ± 13.7	23.7 ± 1.2	26.8 ± 1.0	42.7 ± 1.2	34.1 ± 2.1	27.1 ± 2.7	90.9 ± 10.6	25.0 ± 1.2	29.3 ± 2.8
	Chronic	31.4 ± 1.3	17.9 ± 0.6	15.1 ± 0.3	62.5 ± 5.6	11.9 ± 0.7	9.4 ± 0.4	33.1 ± 1.8	19.3 ± 1.8	16.8 ± 1.4	68.3 ± 5.8	11.3 ± 0.3	10.7 ± 1.3
Sham	Acute	51.7 ± 7.8	38.8 ± 2.8	32.1 ± 2.4	103.2 ± 11.3	19.3 ± 3.0	31.2 ± 1.6	53.1 ± 8.5	42.1 ± 2.6	33.8 ± 2.4	106.1 ± 14.0	22.0 ± 0.6	36.0 ± 3.5
	Chronic	28.6 ± 1.4	16.4 ± 1.5	12.9 ± 1.1	55.9 ± 3.5	10.4 ± 0.8	7.2 ± 0.8	24.7 ± 1.4	18.8 ± 2.5	13.5 ± 0.9	61.6 ± 2.5	8.7 ± 1.2	9.8 ± 1.2
TBI	Acute	38.8 ± 2.4	36.6 ± 5.7	26.1 ± 2.2	88.3 ± 7.9	13.0 ± 2.1*	25.1 ± 2.3	38.7 ± 1.4	36.6 ± 3.6	25.6 ± 1.3	89.3 ± 7.3	14.5 ± 2.7*	26.8 ± 1.9
	Chronic	18.2 ± 0.8*	17.0 ± 0.8	10.7 ± 1.5	46.2 ± 2.2*	7.4 ± 0.7*	8.9 ± 0.9	20.1 ± 1.7*	19.6 ± 1.6	12.9 ± 1.5	49.1 ± 3.5*	9.7 ± 0.8	10.0 ± 1.5

* $p < 0.05$ compared to other groups.

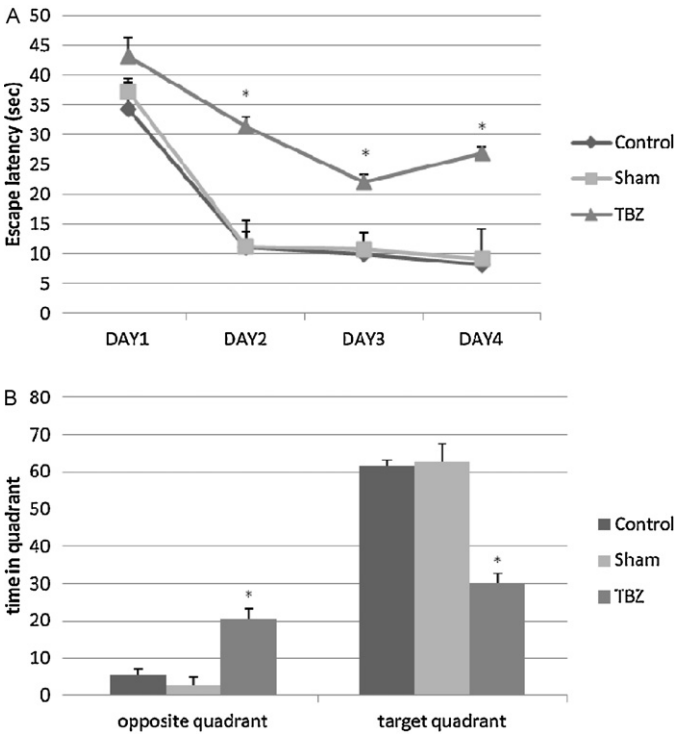


Fig. 3. The Morris Water Maze performance of the groups. TBI: traumatic brain injury. (A) The daily change in escape latency, $^{*}p < 0.05$ compared to other groups. (B) Time spent in target and opposite quadrant in probe trial, $^{*}p < 0.05$ compared to other groups.

pattern of neuron death [20]. In the present study also, the number of apoptotic cells in the PFC, parietal cortex and hippocampal regions were significantly increased in 7 days after traumatic insult, indicating neuronal apoptosis induced by head trauma in the immature rat brains.

IGF-1 had been demonstrated to be neuroprotective against traumatic injury. In adult rats, IGF-1 has been shown to improve neurobehavioral dysfunction after experimental head trauma [18]. Moreover, IGF-1 crosses the blood–brain-barrier and therefore the brain is also influenced by the concentration of circulating IGF-1 [11]. However, both cerebrospinal fluid and serum levels of IGF-1 were demonstrated to be decreased in adult patients with severe head injury [29]. In the present study, we also found that TBI in infant rats caused a significant decrease in circulating IGF-1 levels at both acute and late phase.As mentioned above, developing brain is very susceptible to apoptotic neurodegeneration triggered by traumatic insult. In the present study, we found that there were strong negative correlations between serum IGF-1 levels and the number of apoptotic cells in hippocampal areas at acute phase of traumatic injury. This result can be associated with those of previous studies. Anti-apoptotic effects of IGF-1 have been well documented in *in vitro* systems [28,33].

It is well known that TBI induces the cognitive dysfunction resulted from hippocampal damage [7,16]. We have previously shown that TBI led to cognitive dysfunction and neuron death in hippocampus of immature rats [23]. Several previous studies have shown that traumatic insult causes neuronal injury in the hippocampus [10,34]. The contra-lateral hippocampal regions of injured animals also showed significant neuronal loss, which is consistent with the previous studies [23,34].

Hippocampal neurons normally play a crucial role in the processing of spatial learning and remembering. During the process of spatial learning and remembering, the hippocampal areas function together with entorhinal cortex, PFC, parietal cortex, anterior cingulate cortex, striatum and amygdala [14]. The DG is characterized by the convergence of sensory inputs to create a metric spatial representation and hence is important for spatial pattern separation. The CA3 sub-region is critical for the integration of spatial information. During the learning process, the CA3 region neurons of the hippocampus first process the information and then integrate with the information that comes from the PFC [17]. A healthy GD, CA3 and PFC are prerequisites to find the correct path and the target quadrant during the probe trial [22]. In this study, we found a very strong positive correlation between the time spent on the target quadrant and the number of GD neuron. Also, there is a strong positive correlation between serum IGF-1 levels and the time spent on target quadrant; between serum IGF1 levels and GD neuron numbers.

Hippocampal GD and CA3 region is connected to CA1 region through Schaffer Collaterals. CA1 outputs reach subiculum, entorhinal and PFC [3]. CA1 takes most of the information from CA3, and a smaller portion from the entorhinal cortex [3,26]. CA1 region works as an error detector: it looks for discordance among the information it receives from cortex, CA3 and entorhinal cortex and corrects the stored information [26]. In this study, compared to control and sham rats, TBI-rats spent more time in opposite quadrant in probe trial, meaning they made more mistakes. Furthermore, TBI-rats had also lower number of neurons in ipsi- and contra-lateral CA1. Also we found there is a very strong negative correlation between the time spent in the opposite quadrant and serum IGF-1.

Here, an important question is whether circulating IGF-1 levels are predictive of cognitive dysfunction resulting from hippocampal damage following traumatic injury in developing brain. In animal models, it was shown that decreased serum IGF-1 levels resulted in cognitive deficits [36], and IGF-1 deficiency led to impaired learning and memory in adulthood [32]. In agreement with various experimental studies, we also found that low-serum IGF-I levels were related with cognitive dysfunction following traumatic injury.

In conclusion, our results suggest that circulating IGF-1 levels may have predictive value with regard to cognitive dysfunction resulted from hippocampal damage following traumatic injury in developing brain. Further studies need to be carried out on human subjects or experimental models in order to evaluate the time-course or damage-dependent IGF-1 levels in TBI. Therapy strategies that increase circulating IGF-1 may be highly promising for preventing the unfavorable outcomes of traumatic damage in young children.

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