Iranian Journal of Basic Medical Sciences

ijbms.mums.ac.ir

IJBMS

Neuroprotective effects of gallic acid in a rat model of traumatic brain injury: behavioral, electrophysiological, and molecular studies

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Feb 6, 2018
Accepted: May 7, 2018

Keywords:
Brain inflammation
Gallic acid
Long-term potentiation
Memory
Rat
Traumatic brain injury

ABSTRACT

Objective(s): Traumatic brain injury (TBI) is one of the main causes of intellectual and cognitive disabilities. Clinically, it is essential to limit the development of cognitive impairment after TBI. In the present study, the neuroprotective effects of gallic acid (GA) on neurological score, memory, long-term potentiation (LTP) from hippocampal dentate gyrus (hDG), brain lipid peroxidation and cytokines after TBI were evaluated.

Materials and Methods: Seventy-two adult male Wistar rats divided randomly into three groups with 24 in each: Veh + Sham, Veh + TBI and GA + TBI (GA; 100 mg/kg, PO for 7 days before TBI induction). Brain injury was made by Marmarou's method. Briefly, a 200 g weight was fallen down from a 2 m height through a free-falling tube onto the head of anesthetized animal.

Results: Veterinary coma scores (VCS), memory and recorded hDG -LTP significantly reduced in Veh + TBI group at 1 and 24 hr after TBI when compared to Veh + Sham (P<0.001), respectively, while brain tissue content of interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and malondialdehyde (MDA) were increased significantly (P<0.001). Pretreatment of TBI rats with GA improved clinical signs, memory and hDG-LTP significantly (P<0.001) compared to Veh + TBI group, while brain tissue content of IL-1β, IL-6, TNF-α and MDA were decreased significantly (P<0.001).

Conclusion: Our results propose that GA has neuroprotective effect on memory and LTP impairment due to TBI through decrement of brain lipid peroxidation and cerebral pro-inflammatory cytokines.

► *Please cite this article as:*
Mirshekar MA, Sarkaki AR, Farbood Y, Gharib Naseri MK, Badavi M, Mansouri MT, Haghparast A. Neuroprotective effects of gallic acid in a rat model of traumatic brain injury: behavioral, electrophysiological, and molecular studies. Iran J Basic Med Sci 2018; 21:1056-1063. doi: 10.22038/IJBMS.2018.29639.7165

Introduction

Traumatic brain injury (TBI) is a main health problem in world affecting millions of people. Great number of deaths and cases of permanent disability are caused by TBI every year (1, 2). It was suggested that TBI as significant health trouble, leads to a potentially catastrophic weakening medical emergency with poor prediction and long-term disability (3).

Brain injury due to TBI going on at the moment of impact, which has not appear therapeutic feedback and physiological and pathological reactions activates subsequently, these situations provide a chance for clinical remediation.

These phenomena include release of excitatory neurotransmitters, production of free radicals and activation of inflammatory cytokines that contribute to delayed neuronal cell death, which last for days or months (4).

It was well established that many disabilities such as locomotor activity, intellectual and cognition are manifested in both short and long-term regardless of the severity of the TBI (5-8).

Alterations in synaptic role within a neural network and subsequent adjustment of cell action have been theorized as a neuronal substrate for cognition. Electrical discharge designs of a single neuron or a group of neurons encode sensory or suitable signals related to learning and memory (9). The most famous cellular mechanism underlying memory is long term potentiation (LTP), which correspond to activity based on synaptic efficacy (10).

There is a suggestion that pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) engage in TBI conditions and cerebral ischemic. These cytokines are important mediators of infection-related disorders (11). Initiation of inflammatory pathway delayed responses to TBI. Inflammatory mediators such as transforming growth factor- β (TGF-β) and IL-1β are attenuated by anti-inflammatory factors (12).

Gallic acid (GA), as a phenolic compound, is a natural product that is used in chemical industries such as dye making and tanning (13). Also, it has a broad range of biological properties such as antioxidant and anti-

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Figure 1. Plan of experimental program and intervals for estimation of various parameters

inflammatory activities (14). GA, as form of gallate, is generally utilized as antioxidant by food supplements and pharmaceutical companies (15). Anti-tyrosine action of GA has been reported (16), so it has a protective effect on brain by increasing antioxidant enzymes and reducing inflammation in cerebral hypo-perfusion (17, 18).

Herein, this study designed to investigate the effects of GA on memory, LTP, malondialdehyde (MDA) and pro-inflammatory brain cytokines (IL-1β, IL-6 and TNF-α) in response to administration of GA seven days before TBI in male rats.

Materials and Methods

Agents

GA (purity≥98%), Evans blue, Triton-X100 and protease inhibitor cocktail obtained from Sigma-Aldrich Co (St Louis, MO, USA). Tris base, sodium phosphate, sodium chloride, potassium phosphate, potassium chloride and all other chemicals prepared from Merck Company (Darmstadt, Germany).

Animals

Seventy-two adult male Wistar rats (280-320 g) were purchased from animal care and breeding center, Ahvaz Jundishapur University of Medical Sciences (AJUMS), Ahvaz, Iran. They were kept under standard condition, temperature-controlled room (20±2 ˚C), a 12 hr light/dark cycles and with food and water access *ad libitum*. All experimental procedures were confirmed by AJUMS Ethical Committee (REC. 1392. 363), in agreement with the internationally accepted ethics codes for the care and use of laboratory animals.

Experimental plan

Animals were randomly allocated into three main groups (n=24) as follows: 1) Veh+Sham; sham operated received normal saline (10 ml/kg once daily, gavage) for seven consecutive days before TBI induction. 2) Veh+TBI; TBI group received normal saline (10 ml/kg once daily, gavage) for 7 successive days before TBI induction. GA+TBI; TBI group received GA (100 mg/kg/10 ml once daily, gavage) (19) for same duration before TBI induction.

GA was dissolved in normal saline; freshly prepared daily (20). Each main group divided into 3 subgroups (n=8) for behavioral, electrophysiological and molecular assessment. Experimental schedule and the intervals for estimation of various parameters are shown in Figure 1. All procedures were completed during light phase between 8:00–12:00 a.m.

TBI induction

With the aim of making experimental model of TBI, rats were intubated tracheally under anesthesia. TBI model was made by using a domestic instrument equipped in Ahvaz Physiology Research Center (Ahvaz-Iran) according to Marmarou's method with some modification (21). Briefly, rats were anesthetized with ketamine/xylazine (90/10 mg/kg, IP), and a 200 g weight was fallen down from a 2 m height through a vertical tube onto the head of animal while a stainless steel disc (r=5 mm & h=3 mm) was attached to its skull. After then, the animal was instantly connected to the small animal ventilator (UGO Basile, Italy) and at the moment of starting the spontaneous breathing, it was disconnected from ventilator and returned to the cage to be cared.

Assessment of neurological outcome

Neurological scores were estimated in awakened TBI animals according to veterinary coma scale (VCS) (22). Scoring range was between 3 -15 as follows: the sum of motor response (1-8), visual response (1-4) and respiratory response (1-3). An elevated score was assigned as a better neurological outcome, while lower score indicates injury severity. VCS was assessed at one hour before, and 1, 24 and 48 hr after TBI induction. Based on the VCS score, the severity of head injury can be categorized into mild (13–15), moderate (9 –12), and severe (8 or less).

Passive avoidance memory evaluation

In the first subgroup, the passive avoidance task (PAT) was accomplished using shuttle box 48 hr after TBI. The apparatus comprised two illuminate and dark chambers and a slipping door (Borj Sanaat Co, Tehran-Iran). In order to adapt the instrument, rat were located in the light compartment while the sliding door was slipped up and allowed to explore into all parts of device for 5 min. After 10 min, the rat was re-located in the light compartment again facing away from the closed sliding door and the door was opened 10 sec later. Delay of rat entrance into the dark part was recorded as initial latency (IL). As soon as the animals entered the dark compartment, the door was closed and an unescapable electrical foot-shock (50 Hz, 1.2 mA for 3 sec) was delivered through the grid floor with a stimulator. The retention test was performed 24 hr later again using the same paradigm without the foot-shock. Step-through latency (STL) as memory trial was recorded. Cut – off time to avoid into dark section entry was 300 sec (23).

Electrophysiological study

In the 2nd subgroup, two days after TBI induction, heads of anesthetized rats were fixed again in a stereotaxic device with the purpose of electrodes implantation and field excitatory post synaptic potential (fEPSP) recording. The bipolar microelectrodes (metal wire, tungsten wire, 50 µm in diameter, tip separation 1 mm, and stainless steel, 100 µm in diameter, tip separation 500 µm (CFW, USA)) were placed in the granular cells of DG (AP=-3.8 mm from bregma; ML=-2.3 mm; DV=-3.5 mm from dura) and in the perforant pathway (PP) (AP: -7.5 from bregma, ML:-4, DV:-3.9 mm from the dura), respectively (24, 25). The electrodes were pulled down very slowly (0.1 mm/30 sec) from dura to the PP in order to minimize trauma to brain tissue. Establishment of electrodes at the correct

position was determined by fEPSP recording (26).

LTP induction and recording

Single monopolar pulses (length 50 µs) were passed through stimulating microelectrode at 30 sec intervals. Different intensities were used to record fEPSP with 40% of its maximum amplitude by input/output (I/O) curve. The signal was amplified (×1000), filtered (0.1 Hz-3 KHz), digitized at 2 KHz and stored on a PC. LTP was induced 24 hr later in rats with weak anesthesia following high-frequency stimulation (HFS) including 6 trains of 6 pulses (50 µs) at 400 Hz, 100 ms between each train by repetitive 6 times at a 20 sec interval (e-Probe 4Ch. 12R. Partoye Danesh Co. Iran). LTP was recorded 0.25, 0.5, 1, 3 and 24 hr later. Amplitude (Amp), area under curve (AUC) of population spikes (PSs) and fEPSP slope were measured. The recorded PS was analyzed as percentage increase of baseline fEPSP (23).

Biochemical assay

Two days after TBI induction, rats in the 3rd subgroup were anesthetized irreversibly with an overdose of sodium thiopental (80 mg/ kg, IP) and perfused intracardially with normal saline (pH: 7.4) for 1 min to eliminate the intracerebrovascular blood (27, 28). The brains were taken out immediately and stored in -80 ˚C. The whole brain tissues were homogenized in a tissue protein extraction reagent (0.5% Triton X-100, 150 mM/l NaCl, 50 mM/l Tris) and a protease inhibitor cocktail (500 mg tissue per 1 ml of the reagent). Samples were shaken for 90 min, centrifuged (4˚C and 4000 rpm, 15 min) and homogenate supernatant was collected.

Brain tissue cytokines assessment

ELISA kit for IL-1β, IL-6 and TNF-α was obtained from eBioscience (San Diego, USA) and assay was performed according to the manufacturer's recommended procedures. Concentration of cytokines was measured as a picogram of antigen per milligram of brain tissue (29).

Measurement of brain MDA content

MDA measurement was performed according to Rao *et al*. 1989 method by using thiobarbituric acid (TBA). Light absorbance of samples was read at 534 nm wave-length by spectrophotometer (Biowave II, UK), and MDA concentration was reported as nM/mg protein (30).

Statistics

Data presented as mean±SEM and their normalcy was checked with Kolmogorov–Smirnov test. VCS and LTP measures at different times were analyzed by repeated measure (RM)-ANOVA, while the STL data in passive avoidance test and biochemical assays were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $P<0.05$ was assigned as significant difference.

Results

Neurological score

Clinical scores of tested groups are presented in Figure 2. VCS scores revealed a significant reduction in both Veh+TBI and GA+TBI groups at 1 hr after TBI when compared to Veh+Sham ($P<0.001$). Scores in GA+TBI group were significantly greater than Veh+TBI at 4, 24 and 48 hr after TBI ($P<0.001$).

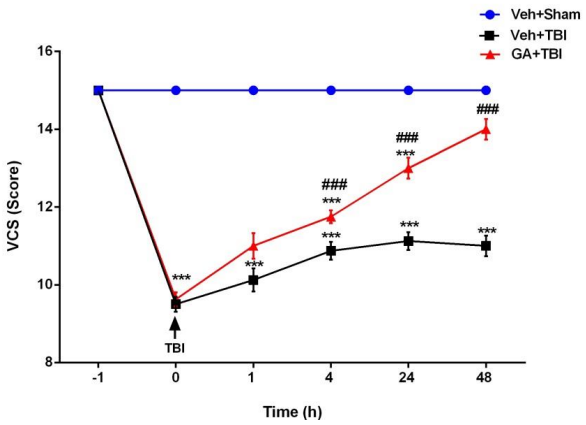

Figure 2. Administration of gallic acid (GA) for 7 days before traumatic brain injury (TBI) on neurological scores after induction of TBI in male rats. Data presented as mean±SEM, n=72. (*** Significant difference between GA+TBI and vehicle (Veh)+Sham groups ($P<0.001$). ### Significant difference of GA+TBI with Veh+TBI group ($P<0.001$). RM-ANOVA followed by Tukey's *post hoc* test)

Passive avoidance memory

The STL in Veh+Sham, Veh+TBI and GA+TBI groups are shown in Figure 3. There were no significant alterations in all groups during IL. STL was significantly decreased ($P<0.001$) during memory test at 24 hr after shock delivery to foot paw in Veh+TBI group (20.75±6.71 sec) compared to Veh + Sham (50.62±4.17 sec). STL was significantly improved ($P<0.001$) in GA+TBI group (44.37±8.16 s) in comparison with Veh+TBI.

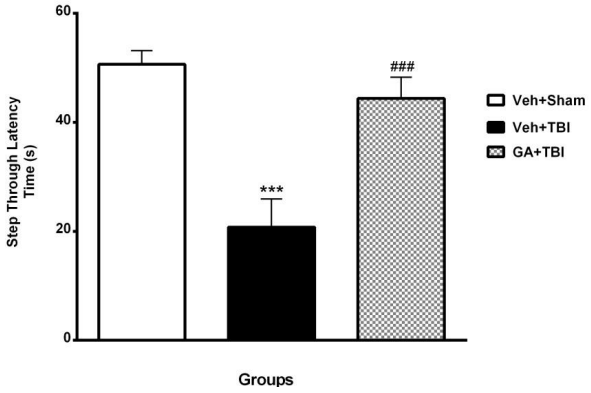

Figure 3. Mean±SEM. of step-through latency (STL) in vehicle (Veh)+Sham, Veh+traumatic brain injury (TBI) and gallic acid (GA)+TBI groups during passive avoidance test (***$P<0.001$ vs Veh+ham and ### $P<0.001$ vs TBI group, n=8 in each group, one way ANOVA followed by Tukey's *post hoc* test)

Improvement of electrophysiological indexes by GA

PS amplitude

As shown in Figure 4A, the PS Amp (mv) reduced significantly ($P<0.001$) in Veh+TBI during all recording times post HFS compared to Veh+Sham group, while it was improved significantly ($P<0.001$) at the same times in GA+TBI group when compared to Veh+TBI.

fEPSP slope

As shown in Figure 4B, the slope of fEPSP (v/s) in Veh+TBI group was significantly less ($P<0.001$) than Veh+Sham group during all recording times, while it

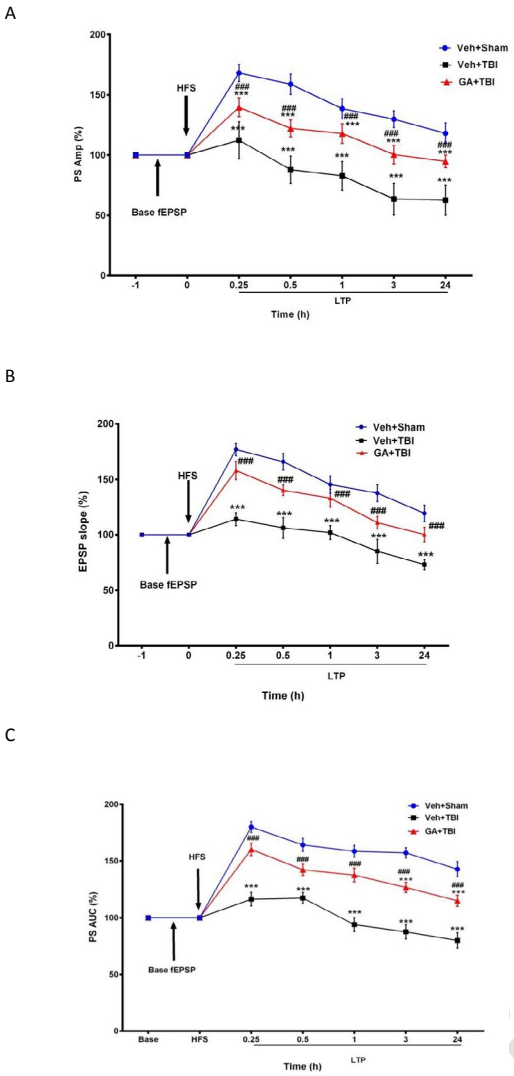

Figure 4. Mean ± SEM of percentages of amplitude (A), area under curve (B) of population spikes (PSs) and slope of fEPSP (C) in different groups during basal fEPSP and long-term potentiation (LTP) recorded from hippocampal DG at 0.25, 0.5, 1, 3, and 24 hr after high frequency stimulation (HFS) to brain PP (Repeated measures two- way ANOVA, followed by HSD *post hoc* test, n=8, ** P<0.01 and *** P<0.001, vehicle (Veh)+traumatic brain injury (TBI) and gallic acid (GA)+TBI vs. Veh+Sham, ### P<0.001, other groups vs Veh+TBI). Veh + Sham: sham operated received normal saline, Veh+TBI. TBI rats received normal saline, GA+TBI. TBI rats pretreated with 100 mg/kg GA. Base excitatory post synaptic potential (EPSP). EPSP recorded one hour before HFS, LTP 0.25-24. LTP recorded during different times after HFS.was increased significantly (P<0.001) at similar times in GA+TBI group when compared to Veh+TBI

PS AUC

Figure 4C shows no significant decay in AUC (v,s) of population spike in Veh+Sham group, while it decreased significantly in Veh+TBI group during all recording times (P<0.001). Pre-TBI treatment with GA caused significant AUC augmentation (P<0.001) during all recording times.

Decrement of brain IL- 1β content by GA

The effect of pre-TBI administration of GA for 7 days on IL-1β of brain tissue at 48 hr after TBI is shown in Figure 5. IL-1β content in Veh+TBI animals (185.37± 7.51 pg/ml) was significantly greater than Veh + Sham group (120.46±5.50 pg/ml) (P<0.001). Pretreatment with GA caused a significant decrease (P<0.01) of IL-1β content (149.37±6.54 pg/ml) compared to Veh+TBI group.

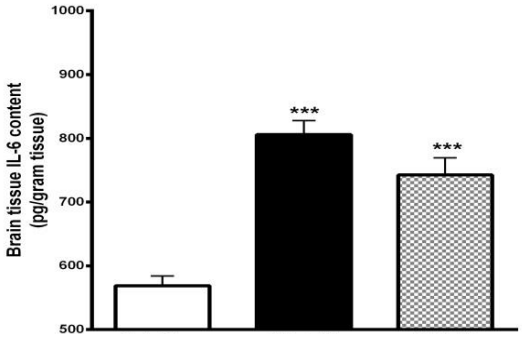

Figure 5. Effect of gallic acid (GA) administration for 7 days before traumatic brain injury (TBI) on brain interleukin-1β (IL-1β) content (pg/ml) in male rats (n= 8 in each group). *P<0.05, ***P<0.001, for vehicle (Veh)+TBI and GA+TBI vs. Veh + Sham, ## P<0.01, for GA + TBI vs. Veh+TBI. One way ANOVA followed by Tukey's *post hoc* test

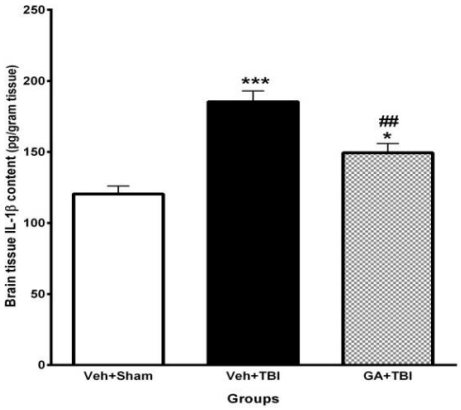

Figure 6. Effect of gallic acid (GA) administration for 7 days before traumatic brain injury (TBI) on brain interleukin-6 (IL-6) content (pg/ml) in male rats (n= 8 in each group). **P<0.01, ***P<0.001, for vehicle (Veh)+TBI and GA+TBI vs. Veh + Sham, # P<0.05, for GA+TBI vs. Veh+TBI. One way ANOVA followed by Tukey's *post hoc* test

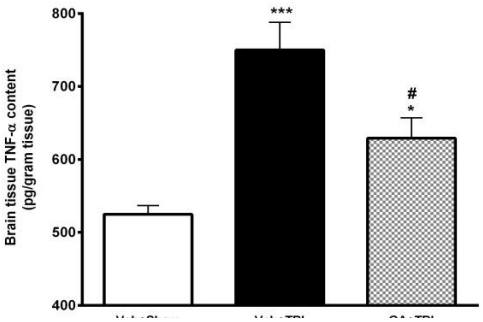

Figure 7. Effect of gallic acid (GA) administration for 7 days before traumatic brain injury (TBI) on brain levels of tumor necrosis factor-α (TNF-α) (pg/ml) in male rats (n= 8 in each group). ***P<0.001, for vehicle (Veh)+TBI vs. Veh + Sham, ## P<0.01, for GA+TBI vs. Veh+TBI. One way ANOVA followed by Tukey's *post hoc* test

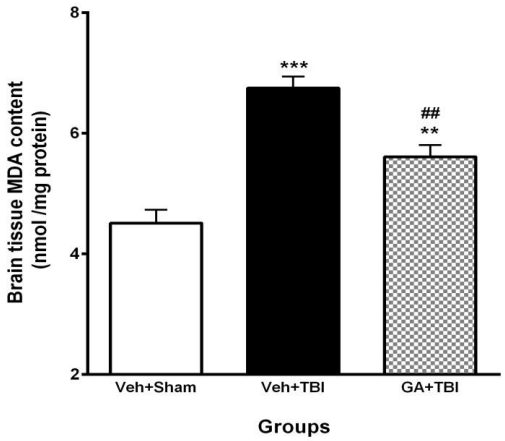

Figure 8. Effect of gallic acid (GA) administration for 7 days before traumatic brain injury (TBI) on brain malondialdehyde (MDA) content (nM/mg protein) in male rats (n=8 in each group). ***P*<0.01, ****P*< 0.001, for vehicle (Veh)+TBI vs. Veh+Sham, ## *P*<0.01, for GA+TBI vs. Veh + TBI. One way ANOVA followed by Tukey's *post hoc* test

Decrement of brain IL- 6 content by GA

The effect of GA administration for 7 days before TBI on the brain content of IL-6 at 48 hr after TBI was shown in Figure 6. IL-6 content in Veh+TBI animals (805.88±21.85 pg/ml) was significantly higher than Veh+Sham group (568.87±15.22 pg/ml) (*P<0.001*). Pretreatment with GA caused a significant decrease (*P<0.01*) of IL-6 content (717.5±30.22 pg/ml) compared to Veh+TBI group.

Decrement of brain TNF-α content by GA

The effect of GA administration for 7 days before TBI on the brain content of TNF-α at 48 hr after TBI was shown in Figure 7. TNF-α content in Veh+TBI animals (687.46±29.34 pg/ml) was significantly higher than Veh+Sham group (524.92±11.82 pg/ml) (*P*<0.001). Pretreatment with GA caused a significant decrease (*P*<0.01) of TNF-α content (591.66±8.73 pg/ml) compared to Veh+TBI group.

Decrement of brain MDA content by GA

Pretreatment effect of GA on the brain content of MDA at 48 hr after TBI was shown in Figure 8. MDA content in Veh+TBI animals (6.75±0.19) was significantly higher than Veh + Sham group (4.51±0.22) (*P*<0.001). Administration of GA for 7 days before TBI caused a significant decrement (*P*<0.01) of MDA content (5.61±0.19) compared to Veh+TBI group.

Discussion

In the present study, cognitive impairment was achieved by TBI, which was shown by decreasing STL in passive avoidance test at 48 hr after TBI. This result was in agreement with the previous investigation (31, 32). It has been recognized that pretreatment with GA (100 mg/kg, PO) seven days pre-TBI could improve memory, evidenced by longer STL at 48 hr after TBI. Furthermore, this effect was associated with enhancement of neurological scores.

The severity of neuroanatomical, neurochemical, and neurological outcomes is related to strictness of the insult measured in depth/velocity for impact models determines. Experimental brain trauma results in both sensorimotor and cognitive behavioral discrepancies (33-37). Our findings are consistent with other investigators results.

In passive avoidance test, the rats in the first trial obtain information to enter into dark chamber. These results in painful experience of electric shock, and the cognitive ability of the animals were reflected by avoidance to entry (a judgment based on successful retention and recall of the acquired information). When injured animals (such as Veh+TBI group in our experiment) were checked in retention trial, there was no increase in STL, which reflects cognitive impairment.

Cortical impact injury is renowned for its ability to model for impaired learning and memory. TBI results in hippocampal degeneration, with grade of atrophy related to severity of brain damage (31). Hippocampus, as an important area of the brain involved in cognition, is very sensitive to injury (38). Hippocampus is characterized by a low capillary mass compared to its other subdivisions (39). Hippocampal pyramidal neurons are more vulnerable and die due to TBI. So, its damage will be observed a few days after injury in rat. Accordingly, hippocampal synaptic transmission and plasticity could be deteriorated and disrupted by TBI (40, 41). Pretreatment of TBI rats with GA causes latency enhancement in a retention trial of avoidance memory; these findings have been observed by other investigators that used different doses of GA in experimental Parkinson's disease induced by 6-hydroxydopamine (19).

Represented data indicate that GA pretreatment increased amplitude, AUC of LTP and slope of fEPSP recorded from DG following HFS. Furthermore, TBI removed PS record from DG area, which is consistent with some earlier findings (42-44).

Pivotal role of calcium ion in signaling in all tissues has been detected and it is vital for normal synaptic transmission in CNS. Complicated processes regulate calcium release, and intracellular systems are concerned for responding to a calcium stimulus. Both brain injury and synaptic plasticity are mediated by calcium signaling processes. N-methyl, D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors of glutamate activation lead to calcium signaling and homeostasis (45). Calcium homeostasis obliterated by TBI and lengthened rise of calcium amount lead to some processes like extreme glutamate release and activation of NMDA receptors, creation of free radicals, outward flow of potassium ions, cellular swelling and activation of cytokines such as TNF-α and IL-1β (46).

Tate and Bigler suggested that mentioned disrupted mechanisms achieved by TBI, probably resulted from increasing severe inflammatory responses as well as oxidative stress in rat brains (31). Based on our results, TBI amplified pro-inflammatory cytokines, which confirmed by previous investigations. Moreover, some other investigations showed that release of pro-inflammatory cytokines provoke delayed response to TBI (47, 48). It seems that promoting the inflammation might be mediated by augmentation of brain cytokines.

In current study, we showed that brain content of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α increased after TBI. Increment of TNF-α level following TBI has been reported to be an important reason for brain parenchyma response to the injury (49).

In addition, many published data confirmed that these cytokines are increased during inflammation (50-52). Our results have shown that GA pretreatment decreased brain IL-1β, 48 hr after the TBI. Several lines of studies have reported that changes in the contents of cytokines occur in different models of injury such as frontal brain ischemia (29), injuries induced by fluid percussion (53), spinal cord injury (54) and experimental TBIs (55). Brain cytokines contents change 30 minutes following TBI and last for 24 hr (12). Current data showed that pretreatment with GA reduced IL-6 and TNF-α in brain 48 hr after TBI. Elevation of IL-6 due to TBI induces neuronal death and blood–brain barrier disruption accompanied by severe clinical disorders (51).

The beneficial therapeutic action of phenolic compounds is related to their anti-inflammatory, antioxidant capacity and free radical scavenging activity (56). GA, as a polyhydroxyphenolic compound, is one of the major bioactive compounds isolated and purified from number of plants (57-59). Various pharmacological activities of GA such as anticancer (60) and antioxidant function (60) have been reported. This compound has also been described as an excellent free radical scavenger (61). TBI might induce a significant up-regulation of TNF-α, IL-6 and NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) in animal cell types and is involved in cellular responses to stimuli such as stress, cytokines and free radicals in the rat tissues. NF-kB binding activity was significantly increased from 3 hr to 7 days post-injury, with the maximum at 72 hr. Moreover, TNF-α and IL-6 were significantly increased after TBI and remained elevated on Day 7 post-injury. In addition, there was a positive connection between the expression of NF-kB and the pro-inflammatory cytokines TNF-α and IL-6 (62). In an additional study, it has been established that GA can inhibit TNF-α induced inflammatory role of NF-kB (63). According to previous researches and on base of our results, we hypothesized that pretreatment with GA recovers cerebral inflammation in rats because of its anti-inflammatory and antioxidative actions.

Conclusion

In summary, our results propose that release of cytokines such as IL-1β, IL-6 and TNF-α and also MDA as lipid peroxidation index in brain tissue following TBI cause cognitive impairment and LTP insufficiencies. We found that administration of GA before moderate TBI induction reversed the contents of IL-1β, IL-6, and TNF-α and brain tissue MDA, thereby improves memory and LTP indexes. Further studies are necessary to clarify more neuroprotective mechanisms of GA.

Acknowlegment

This article was extracted from Mohammad Ali Mirshekar's PhD thesis. This project was supported financially by Ahvaz Jundishapur University of Medical Sciences, Physiology Research Center (grant no. APRC-146). We all thanks to Dr. Shahram Mashhadizadeh, Dr. Soltani and Miss Naderi from Physiology Research Center, Kerman University of Medical Sciences for technical assistance and we are also grateful to Dr Ghorbangol Ashabi for editing the manuscript.

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