Catechin Attenuates Traumatic Brain Injury-induced Impairments of Blood-brain Barrier Damage and Improve Longer-term Neurological Outcomes in Rats

Zhixian Jiang, Jinning Zhang*, Yonghui Cai, Jiaxin Huang, Lingtong You

Quanzhou 1st Hospital Affiliated to Fujian Medical University, Chendong Area, 10th floor district N13, Inpatient Department, Quanzhou 362000, Fujian, China

*Corresponding author: Jinning Zhang, Quanzhou 1st Hospital Affiliated to Fujian Medical University, Chendong Area, 10th floor district N13, Inpatient Department, Quanzhou 362000, Fujian, China

E-mail: zjn00131@sina.com

Running title: Neuroprotective effects of catechin after TBI

Keywords: Traumatic brain injury; catechin; blood-brain barrier; inflammation; neuroprotection

Total number of words: 2909

Total number of references: 23
New findings

What is the central question of this study?

The present study therefore investigated the potential neuroprotective effects of catechin after TBI and explored the underlying mechanisms.

What is the main finding and its importance?

Catechin treatment possessed neuroprotective effects in a rat model of TBI, and these effects may be through intervention in the self-perpetuating process of BBB disruption and excessive inflammation reaction.

ABSTRACT

Traumatic brain injury (TBI), resulting from external force on the head usually lead to long-term deficits in motor and cognitive functions. Catechin has shown neuroprotective effect in neurodegenerative diseases and ischemia models. The present study therefore investigated the potential neuroprotective effects of catechin after TBI and explored the underlying mechanisms. Male rats were subjected to controlled cortical impact injury and then treated with catechin. Brain damages, motor and cognitive functions, blood-brain barrier (BBB) integrity and neuro-inflammation were examined. Catechin treatment ameliorated brain damage and motor and cognitive deficits after TBI. Catechin was shown to protect BBB
integrity, alleviate TBI-induced loss of junction proteins, occludin and zonula occludens protein-1 (ZO-1) and suppress local inflammatory reactions. Catechin treatment possessed neuroprotective effects in a rat model of TBI, and these effects may be through intervention in the self-perpetuating process of BBB disruption and excessive inflammation reaction.

**INTRODUCTION**

Traumatic brain injury (TBI) is a type of brain injury which results from external force on the head, usually caused by traffic accidents, falls, et al. In both human and animal models, TBI was reported to result in motor deficit and cognition deficits such as disturbed spatial orientation and memory, which could become obvious as early as 48 hours after TBI and last for as long as 2 weeks, or even persist for lifetime (Alves, 2014). Blood-brain barrier (BBB) dysfunction is a significant event caused by TBI, with disrupted tight junctions and increased paracellular permeability (Neuwelt et al., 2008). The main components for the BBB are brain capillary endothelial cells, sealed by tight junction networks, and TBI was reported to damage the tight junction networks by a number of literatures (Alluri et al., 2015; Corrigan et al., 2016). As a result, blood-born substances well as ionic imbalance occur in the central nervous system (CNS) and activate astrocytes and microglia (Thal & Neuhaus, 2014).

Activation of these cells results in inflammatory activation and triggers the release of several pro-inflammatory cytokines, which could facilitate influx of even more extracerebral inflammatory cells (Cederberg & Siesjö, 2009). Excessive inflammation could further
compromise BBB integrity (Alves, 2014). These self-perpetuating processes contribute to the development of secondary brain damage that could result in coma or death. Thus targets for BBB stabilization might be considered as a potential target for therapeutic intervention after TBI.

Catechin is a flavonoid which is especially concentrated in green tea. Previous studies reported that catechin displayed neuroprotective properties in neurodegenerative diseases such as Alzheimer and Parkinson diseases, via its transitional metal (iron and copper) chelating property and antioxidant activity (Mandel et al., 2005; Mandel et al., 2008). Meanwhile, catechin has also been revealed to display anti-inflammatory property (Hirao et al., 2010). These properties suggested that catechin might also play a neuroprotective role in brain injuries. It was reported that pretreatment of catechin hydrate displayed neuroprotective effects through NF-kB mediated downregulation of inflammatory response in a rat model of middle cerebral artery occlusion (Ashafaq et al., 2012). To our knowledge, the potential neuroprotective effect of catechin in TBI and the underlying mechanisms remains unclear.

In the present study, we first investigated neuroprotective effects of catechin treatment on the TBI-induced brain damage and deficit in long-term motor and cognitive behaviors. We further examined two potential mechanisms, preservation of BBB integrity and inflammation inhibition, which might underlie the neuroprotective effect of catechin on TBI.
MATERIALS AND METHODS

Ethical Approval

All animal handling procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of Quanzhou 1st Hospital Affiliated to Fujian Medical University (approval number: #2016-JC-202). All efforts were made to minimize the number of the animals used and their suffering. At the end of experimental period, the rats were anesthetized with ketamine (80 mg/kg) and xylazine (12 mg/kg) by intraperitoneal injection, then sacrificed by exsanguinations.

Animal Model of Traumatic Brain Injury

Adult male Sprague-Dawley (SD) rats weighing 220 ± 20 g were purchased from Shanghai SLAC Laboratory Animal Co. Ltd., China. Animals were housed in a colony room under controlled temperature (23°C ± 2°C) and 12 h light/dark schedule (light on at 7:00 a.m.) with free access to food and water. To avoid the interference of gender on the observed results, only male rats were employed.

TBI was induced via unilateral controlled cortical impact injury (CCI), according to previous description (Shi et al., 2015). In brief, animals were anesthetized with 4% isoflurane and maintained with 2% isoflurane during surgery in an oxygen-air mixture using a gas anesthesia mask in a stereotaxic frame. CCI was produced using a pneumatic controlled...
cortical impact device (TBI 0310; Precision Systems and Instrumentation, Fairfax Station, VA) with a 5-mm diameter flat-tipped impactor. The depth of the impact was 3.2 mm with dwell time 500 ms and velocity 4.0 m/second. Animals were maintained under 37°C during the experiment. Rats from sham group underwent the same surgical procedures without a CCI. Catechin treated TBI rats were given catechin dissolved in 0.5% dimethyl sulfoxide (DMSO) at a dose of 1, 5, 10, 20 or 30 mg/kg bw once per day via gavage. Vehicle control group received 0.5% DMSO at a dose of 1 mg/kg bw once per day via gavage. Of note, DMSO at this dose had no observed toxicity in our pilot study.

**Determination of Brain Infarct Volume and Brain Edema**

Animals were sacrificed 24 h after surgery. Determination of brain infarct volume and brain edema was carried out following previous study (Wang *et al.*, 2014). For brain infarct volume assessment, brains were cut into 2-mm thick slices, stained with 1% triphenyl tetrazolium chloride (TTC, Sigma, USA) and further analyzed. For brain edema assessment, brains were removed and weighed, and then dried at 88°C for 48 h and reweighed. Brain water content (%)= (wet weight – dry weight) × 100 / wet weight.

**Behavioral Test**

The foot-fault test was carried out to measure the deficits in motor function due to brain injury, referring previous study (Shi *et al.*, 2015). Animals were tested using a horizontal...
ladder elevated 30 cm above the ground. The rungs were regularly spaced at 2 cm intervals.

Animals were trained for 3 consecutive days before surgery and the score on the last day was used as baseline. The test was further carried out at 3rd, 5th, 7th, 14th, 21st and 28th after surgery. Animals were tested for 3 trials per day and scores were determined as the average of 3 trials. The types of foot placement on the rungs were rated using a 7-category scale (Metz & Whishaw, 2002).

Morris water maze was carried out to evaluate spatial learning and memory ability at day 21 – 25 after CCI, referring to a previous study (Shi et al., 2015). The water maze consisted of a black circular tank (180 cm in diameter, 60 cm in height) with an escape platform (15 cm in diameter) submerged 2 cm below the water surface. Each animal was placed in the water at different starting positions for 4 trials per day. Animals were allowed 60 s to locate the hidden platform. If the animal failed to find the platform within 60 s, it was gently guided to the platform and allowed to stay on it for 30 s. A video-tracking system (ANY-maze, Stoelting, USA) recorded the performance of these animals. The latency to platform was recorded for each trial and average of 4 trials per day was used for analysis. A single probe test was carried out 24 h after the last trial. The platform was removed from the tank and each animal was allowed to swim for 60 s in the water. Time spent in the target quadrant was recorded for analysis.
Evans Blue Assessment

Blood-brain barrier disruption was determined by measuring Evans blue (EB) leakage, as described previously (Wang et al., 2014). 2% EB (4 ml/kg, Sigma, St. Louis, MO, USA) was injected via the tail vein 24 h after CCI. 2 h later, brains were removed and photographed. Brain tissues were homogenized with 50% trichloracetic acid and centrifuged. Supernatants were collected and measured at 620 nm to detect the absorbance.

Quantitative RT-PCR Analysis

24 h after CCI, the ipsilateral cortex tissues were harvested, homogenized, and total RNA was extracted with Trizol RNA extraction reagent (Life Technologies, Pleasanton, CA, USA). cDNA was synthesized using reverse transcriptase (Promega, USA). q-PCR was performed using SYBR Premix Ex Taq (Takara Bio Inc, Japan) in an ABI 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Referring previous studies (Shi et al., 2015; Yuan et al., 2016), primers for each gene were as following: ZO-1 (5’-CGG TCC TCT GAG CCT GTA AG-3’; 5’-GGA TCT ACA TGC GAC GAC AA-3’); occludin (5’-GCT CAG GGA ATA TCC ACC TAT CA-3’; 5’-CAC AAA GTT TTA ACT TCC CAG ACG-3’); IL-1β (5’-CAC CTC TCA AGC AGA GCA CAG-3’; 5’-GGG TTC CAT GGT GAA GTC AAC-3’); iNOS (5’-CCACAA TAG TAC AAT ACT ACT TGG-3’; 5’-ACG
AGGTGT TCA GCG TGC TCC ACG-3’; IL-6 (5’-CCAAAGTTCTGACTTGT TTG-3’, 
5’-GTTGTCTTCACAAACTCC-3’; arginase 1 (5’-AAG AAA AGGCCG ATT CAC 
CT-3’; 5’-CAC CTC TGC TGT CTTCC-3’); GAPDH (5’-ATC ACC ATC TTC CAG 
GAGCG-3’; 5’-TTC TGA GTG GCA GTG AGG GC-3’). The relative amount of target gene 
was calculated using the 2-\(\Delta\Delta^{CT}\) method.

**Western-blotting**

24 h after CCI, the ipsilateral cortex tissues were collected. Total proteins were extracted 
using lysis buffer (10 mM HEPES, pH7.4, 2 mM EGTA, 0.5% NP-40, protease inhibitors). 
Protein sample extract (20 µg) was subjected to SDS-PAGE and then transferred to a PVDF 
membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim 
milk for 1 h and then incubated with primary antibodies against ZO-1 (Abcam, Cambridge, 
MA, USA), occludin (Abcam) and β-actin (Abcam), respectively, overnight at 4°C. The 
membranes were washed with TBST, incubated in appropriate horseradish peroxidase (HRP) 
conjugated secondary antibodies (KPL, USA) for 1 h at room temperature, and then 
developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, WI, USA). The 
bands on the film were scanned and analyzed using Image J software.
**Data analysis**

All the values are expressed as mean ± standard deviation (S.D.). To determine the difference among groups, data were analyzed by one-way ANOVA followed by a Tukey’s post hoc test. Significance level was set at p<0.05 for all these analyses.

**RESULTS**

**Catechin treatment dose-dependently attenuates TBI-induced impairments**

As shown in Fig. 1, our results found that compared with sham-operated animals, TBI animals had more brain water content (Fig. 1a) and larger brain infarct volumes (Fig. 1b). Catechin treated TBI animals showed less brain water content and smaller brain infarct volumes, compared with vehicle treated animals, indicating that catechin treatment attenuated TBI-induced impairments. Moreover, catechin treatment acted in a dose-dependent manner.

In the subsequent experiment, we applied catechin at 20 mg/kg dosage due to its best efficacy of protection.

**Catechin treatment improves long-term neurological outcomes after TBI**

The motor function of rats was assessed using the foot-fault test. Rats subjected to CCI showed significantly reduced left forelimb and hindlimb performance compared with sham-operated animals at 3-28 day after CCI (Fig. 2a, b). Catechin treatment significantly
improved the performance of these limbs at all these time points. These results indicated that catechin treatment could improve motor functions after TBI.

In acquisition process of Morris water maze, as shown in Fig. 2c, the vehicle treated TBI rats showed significantly increased latency to find the hidden platform, compared with sham-operated animals. Catechin treatment significantly reduced latency to platform on last 2 days of acquisition, compared with vehicle treatment. In the probe test, as shown in Fig. 2d, the vehicle treated TBI rats spent significantly less time in the target quadrant than sham-operated animals. Catechin treated rats spent significantly more time in the target quadrant compared with vehicle treated TBI rats. These results indicated that catechin treatment could improve long-term spatial learning and memory following TBI.

**Catechin treatment preserves BBB integrity following TBI**

As shown in Fig. 3a, EB leakage, an indicator of BBB damage, was prominent in ipsilateral brain tissues at 24 h after CCI. Quantification of EB intensities showed that EB content in the ipsilateral brain tissues was significantly higher in vehicle treated TBI rats than that in sham-operated animals. EB content in the ipsilateral brain tissues of catechin treatment TBI rats was significantly lower compared with that in vehicle treated TBI rats (Fig. 3c). There was no difference in EB content in the contralateral brain tissues among all these groups (Fig. 3b). These data indicated that catechin treatment ameliorates BBB damage after TBI.
Catechin treatment alleviates TBI-induced loss of tight junction proteins in the ipsilateral cortex

Relative mRNA expression and protein levels of ZO-1 and occluding in ipsilateral cortex tissues were examined. As shown in Fig. 4, the mRNA expression and protein levels of ZO-1 and occludin were significantly lower in TBI rats (TBI + vehicle and TBI + catechin) compared with that in sham-operated animals (sham and sham + catechin). The mRNA expression and protein levels of ZO-1 and occludin were significantly higher in catechin treated TBI rats compared with that in vehicle treated TBI rats (Fig. 4a, b and c). These data indicated that catechin treatment could alleviate TBI-induced loss of tight junction proteins.

Catechin treatment suppresses local inflammatory in the ipsilateral brains following TBI

To investigate the effect of catechin treatment on cerebral inflammation after TBI, we measured the mRNA expression of a panel of pro-inflammatory factors (IL-1β, iNOS and IL-6) and an anti-inflammation-associated factor (arginase 1). As shown in Fig. 5, the mRNA levels of IL-1β, iNOS, IL-6 and arginase 1 were increased in TBI rats. Compared with vehicle treated TBI rats, catechin treatment significantly reduced the expression of pro-inflammatory factors (IL-1β, iNOS and IL-6) while increased the expression of anti-inflammation-associated factor (arginase 1).
DISCUSSION

Catechin, with its anti-inflammatory and radical-scavenging properties, has been widely studied as a neuroprotective substance. In the present study, we demonstrated that post-injury catechin treatment dose-dependently reduced TBI-induced brain damage, resulting in less brain water content and smaller brain infarct volumes. Catechin was shown to have best protective efficiency at a dose of 20 mg/kg body weight, and thus we used this dosage in the following studies. In support of this, this dosage has also been shown to play protective effect in a brain injury model of middle cerebral artery occlusion (MCAO) and some other disease models (Ashafaq et al., 2012; Uzun & Kalender, 2013).

In the present study, we showed that catechin treatment attenuated TBI-induced motor and cognitive deficit, evaluated up to 28th day. In accordance with our findings, previous studies revealed improving effect of catechin on motor and cognitive behaviors in animal models of brain injury and neurodegenerative diseases. Ashafaq et al. found that catechin pretreatment improved muscular coordination skill at 22 h after stroke (Ashafaq et al., 2012). Long-term administration of green tea catechin was shown to prevent spatial learning and memory impairment in senescence-accelerated mouse prone-8 (SAMP8) mice and in an animal model of Alzheimer’s disease, rats infused with Aβ₁-₄₀ (Haque et al., 2008; Li et al., 2009). These effects might be through downregulation of inflammatory response,
upregulating synaptic plasticity-related proteins in the hippocampus, increasing anti-oxidative defenses, et al.

We further investigated potential mechanisms underlying the neuroprotective properties of catechin in TBI. Our result revealed that catechin treatment could attenuate BBB breakdown, which plays a key role in the pathogenesis of TBI (Thal & Neuhaus, 2014). The main components for the BBB are brain capillary endothelial cells, sealed by tight junction networks. Occludin is one of the most important transmembrane proteins contributing to tight junction formation. Zonula occludens protein-1 (ZO-1) binds to occludin and the actin cytoskeleton, serving as a bridge between transmembrane proteins and skeleton proteins (Liu et al., 2012). These proteins are shown to be critical for BBB integrity. It was reported that cerebral ischemia induced disruption of the BBB, along with decreased level of occludin and ZO-1 (Kago et al., 2006). Our results demonstrated that catechin treatment could attenuate TBI-induced BBB leakage, along with an upregulation of occludin and ZO-1 in the ipsilateral cortex. These data indicated that neuroprotective properties of catechin in TBI might be through protecting tight junction and BBB integrity.

Another potential mechanism underlying the neuroprotective properties of catechin in TBI was probably through suppressing inflammatory responses in the brain. The involvement of inflammation in the pathogenesis of TBI has been extensively documented in experimental animal models and clinical observations (Cederberg & Siesjö, 2009). Inflammation was
shown to play a dual role after TBI. Studies on the role of cytokines after TBI have yield conflicting results about whether their action contributes to repair mechanisms or exacerbates the pathophysiology of brain injury (Cederberg & Siesjö, 2009). Appropriate amount of inflammatory cytokines would play a beneficial role in promoting neuronal differentiation and survival, inducing neurotrophic factors, et al. while excessive inflammatory cytokines were associated with secondary brain injury. This process is complex and it was suggested that dosage and timing controlled administration of anti-inflammatory agents might be a therapeutic intervention after TBI (Morganti-Kossmann et al., 2002). It was reported that suppression of pro-inflammatory cytokine by an anti-inflammatory agent decreased brain damage and improved cognitive ability in a mice model of closed head injury (Lloyd et al., 2008). Catechin was shown to display anti-inflammatory properties in previous studies both in vivo and in vitro (Hirao et al., 2010; Abd El-Aziz et al., 2012). In a rat model of MACO, administration of catechin hydrate resulted in alleviated neurological deficit, smaller infarct sizes, along with attenuated brain injury induced inflammatory response (Ashafaq et al., 2012). Our results found that catechin treatment could attenuate local inflammatory response in the ipsilateral brains following TBI. These data indicated that neuroprotective effects of catechin in TBI might also through its anti-inflammatory properties.

In addition, BBB dysfunction is involved in the cascade of excessive inflammatory response, which results in secondary brain damage after TBI. The very early inflammatory
activation after tissue injury is triggered by producing of inflammatory cytokines such as IL-1β, IL-6, et al. by microglia and astrocytes when these cell sense perturbation of tissue homeostasis (Hopkins & Rothwell, 1995; Brown & Neher, 2010; Alves, 2014). These inflammatory molecules might promote BBB disruption, as demonstrated in a previous study showing administration of IL-1β resulted in BBB breakdown and loss of the tight junction proteins, occluding and ZO-1 (Bolton et al., 1998). BBB breakdown can trigger leukocyte recruitment and migration of inflammatory cells leading to neuro-inflammation. Meanwhile, BBB breakdown results in influx of blood-born substances into the tissue and activate more astrocytes and microglia (Thal & Neuhaus, 2014). Together these formed a self-perpetuating process, contributing to the development of secondary brain damage. Our results demonstrated that catechin treatment could prevent TBI-induced BBB breakdown and reduce inflammatory response. Thus these effects might serve as a therapeutic intervention in the self-perpetuating process, resulting in neuroprotective outcome after TBI.

CONCLUSION

In conclusion, the present study demonstrated that catechin treatment possessed neuroprotective effects in a rat model of TBI, attenuating TBI-induced brain damage and motor and cognitive deficits. Treatment of catechin prevented tight junction and BBB integrity, attenuated post-injury inflammatory responses. These data suggested that
neuroprotective property of catechin in TBI may be through intervention in the self-perpetuating process of BBB disruption and excessive inflammation reaction.

Author Contributions

Zhixian Jiang, Yonghui Cai, Jiaxin Huang, Lingtong You conducted the experiments and analyzed the data. Zhixian Jiang and Jinning Zhang wrote the paper. Jinning Zhang conceived this paper.

Disclosure of potential conflicts of interest

The authors declare that they have no conflict of interest.

Funding

None.

Acknowledgments

None.
REFERENCES


This article is protected by copyright. All rights reserved.


**Figure Legends**

**Fig 1.** Catechin treatment (1, 5, 10, 20, 30 mg/kg, i.v gavage) attenuates TBI dose-dependently 24 h post-injury: the brain water content (a) and brain infarct volume (b).

Data are presented as mean ± SD. Experiments were repeated in four times. *p<0.05, **p<0.01 and ***p<0.001 compared to sham control. #p<0.05 and ##p<0.01 compared to vehicle control.
Fig 2. Catechin treatment improves long-term neurological outcomes after TBI. In the foot-fault tests, catechin treatment (20 mg/kg) significantly improved the scores of left forelimb (a) and hindlimb (b) foot-fault tests, compared to vehicle-treated TBI rats up to three weeks post-injury. “Pre” represents pre-injury levels. In the Morris water maze tests, catechin treatment (20 mg/kg) significantly shortened the escape latency at Days 22-24 post-injury (c) and improved spatial memory at Day 25 post-injury (d), compared to vehicle-treated TBI rats. Data are presented as mean ± SE. n = 10 per group and experiments were repeated in triplicate. *p<0.05, **p<0.01 compared to sham control. #p<0.05 and ##p<0.01 compared to vehicle control.
Fig 3. Catechin treatment (20 mg/kg) defends blood-brain barrier (BBB) integrity following TBI. (a) Evans blue leakage into the ipsilateral brains could be clearly seen in the experimental animals’ wet brain 24 hours post-injury. (b) The contents of Evans blue leakage into the contralateral brains in sham, TBI + veh and TBI + catechin separate groups. (c) The contents of Evans blue leakage into the ipsilateral brains in sham, TBI + veh and TBI + catechin separate groups. Data are presented as mean ± SD. n = 10 per group and experiments were repeated in triplicate. ***p<0.001 compared to sham control. ##p<0.01 compared to vehicle control.
Fig 4. Catechin treatment (20 mg/kg) alleviates TBI-induced loss of junction proteins in the ipsilateral cortex of experimental animals. (a) Relative mRNA expressions of ZO-1 and occludin were analyzed by RT-PCR 24 hours post-injury. GAPDH was used as internal control. (b) Protein expressions of ZO-1 and occludin were analyzed by western blot 24 hours post-injury. β-actin was used as a loading control. (c) Relative expression of ZO-1 and occludin from (c). Data are presented as mean ± SD. Experiments were repeated in triplicate. *p<0.05, **p<0.01 compared to sham control. #p<0.05 and ##p<0.01.
Fig 5. Catechin treatment (20 mg/kg) suppress local inflammatory in the ipsilateral brains following TBI. mRNA expressions of pro-inflammatory genes, including IL-1β, iNOS, IL-6 and anti-inflammatory-associated gene arginase 1 were quantified by RT-PCR. GAPDH was used as internal control. Data are presented as mean ± SD. Experiments were repeated in triplicate. *p<0.05, **p<0.01 and ***p<0.001 compared to sham control. ##p<0.01.