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Research report

Cordycepin attenuates traumatic brain injury-induced impairments of blood-brain barrier integrity in rats

Jing Yuan[a], Aihua Wang[a], Yan He[a], Zhihua Si[a], Shan Xu[a], Shanchao Zhang[a], Kun Wang[b,*], Dawei Wang[c,*], Yiming Liu[d,*]

[a] Department of Neurology, Qianfoshan Hospital Affiliated to Shandong University, 16766 Jingshi Road, Jinan 250014, PR China
[b] Department of General Surgery, Qianfoshan Hospital Affiliated to Shandong University, 16766 Jingshi Road, Jinan 250014, PR China
[c] Department of Orthopedics, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jingwu Road, Jinan 250021, PR China
[d] Department of Neurology, Qilu Hospital of Shandong University, 107 West Wenhua Road, Jinan 250012, PR China

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ABSTRACT

Loss of blood-brain barrier (BBB) integrity is a downstream event caused by traumatic brain injury (TBI). BBB integrity is affected by certain physiological conditions, including inflammation and oxidative stress. Cordycepin is a susbtance with anti-inflammatory and anti-oxidative effects. Therefore, it is necessary to investigate whether cordycepin affects TBI-induced impairments of BBB integrity. Using TBI rats as the *in vivo* model and applying multiple techniques, including stroke severity evaluation, Evans blue assessment, quantitative real-time PCR, Western blotting and ELISA, we investigated the dose-dependent protective effects of cordycepin on the TBI-induced impairments of BBB integrity. Cordycepin treatment attenuated the TBI-induced impairments in a dose-dependent manner, and played a role in protecting BBB integrity. Cordycepin was able to alleviate TBI-induced loss of tight junction proteins zonula occludens protein-1 (ZO-1) and occludin, which are important for BBB integrity. Moreover, cordycepin suppressed pro-inflammatory factors, including IL-1β, iNOS, MPO and MMP-9, and promoted anti-inflammation-associated factors arginase 1 and IL-10. Furthermore, cordycepin inhibited NADPH oxidase (NOX) expression and activity following TBI, probably through NOX1, but not NOX2 and NOX4. Cordycepin has protective effects against brain damages induced by TBI. The protection of cordycepin on BBB integrity was probably achieved through recovery of tight junction proteins, inhibition of local inflammation, and prevention of NOX activity.

1. Introduction

Traumatic brain injury (TBI) is a type of severe brain injuries, which results from direct blows to the head during relative head-brain movements. Sometimes brain damage-induced TBI can result in long-term cognitive and motor deficits even in young people. Researchers have extensively studied TBI-caused molecular and anatomical changes, however the consequences of these changes to normal neuronal functions in the cortex were seldom investigated (Carron et al., 2016). Loss of blood-brain barrier (BBB) integrity is a serious event caused by TBI, after which the BBB can no longer be a flawless protective barrier between the intravascular compartments and the brain, and its dysfunctions lead to leakage of fluid, proteins and immune cells (Alluri et al., 2015). Tight junctions have a major role in protecting the BBB integrity by holding together brain endothelial cells. Tight junctions are composed of transmembrane protein complexes and cytoplasmic accessory proteins, including zonula occludens protein-1 (ZO-1) and occludin. ZO-1 and occludin are known to be critical for BBB integrity (Liu et al., 2014). In addition, BBB integrity is also affected by certain physiological conditions, including inflammation and oxidative stress (Shetty et al., 2014). Local inflammatory responses can result in BBB disruption (Huang et al., 2013). During the process, pro-inflammatory factors, including IL-1β, iNOS (Wang et al., 2014), MPO (Chen et al., 2008) and MMP-9 (Shao et al., 2014), are induced by brain injury. Meanwhile, anti-inflammation-associated factors arginase 1 (Hu et al., 2012) and IL-10 (Fouda et al., 2013) are also stimulated for protection purpose. It has also been shown that excessive reactive oxygen species (ROS) after stroke contributes to

Abbreviations: BBB, loss of blood-brain barrier; TBI, traumatic brain injury; ZO-1, zonula occludens protein-1; NOX, NADPH oxidase.

* Corresponding authors.
E-mail addresses: qywangkun1981@sohu.com (K. Wang), wangdwsd@126.com (D. Wang), yimingliusdql@yeah.net (Y. Liu).

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the disruption of BBB integrity, and NADPH oxidases (NOXs) are the main sources of ROS (Carone et al., 2015; Wang et al., 2014).

Cordycepin is extracted from a species of fungus named *Cordyceps militaris*, and has been recognized as a susbtance with anti-inflammatory and anti-oxidative properties (Park et al., 2014). For instance, cordycepin has been reported to significantly attenuate the levels of proinflammatory and inflammatory factors, including iNOS, PGE2, TNF-α and IL-1β, and increase the expression of anti-inflammatory factors, such as interleukin-10 protein (Tuli et al., 2013). Moreover, cordycepin reduces age-related oxidative stress and improves antioxidant capacity in rats (Ramesh et al., 2012). In addition, it has also been shown that cordycepin has neuroprotective effects. Cordycepin protects hippocampal cells against neurotoxicity through its anti-oxidant properties, and inhibits apoptosis of the cells (Jin et al., 2014). Therefore, it is necessary and informative to investigate whether cordycepin affects TBI-induced impairments of BBB integrity.

In our study, we utilized TBI rats as the *in vivo* models to investigate neuroprotective effects of cordycepin treatment on the TBI-induced impairments of BBB integrity. We hereby present results demonstrating a role of cordycepin treatment in protecting BBB integrity, which supports its future clinical application in post-TBI recovery.

2. Methods & materials

2.1. TBI Sprague-Dawley rats with cordycepin treatment

Sprague-Dawley rats were purchased from SLACs (Shanghai, China). Our animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Qianfoshan Hospital Affiliated to Shandong University. TBI rats were established *via* controlled cortical impact (CCI) injury, according to the previous description (Wang et al., 2015). Rats from sham group underwent identical surgical procedures, but without a CCI. TBI rats were treated with either saline (vehicle control group) or cordycepin (1, 5, 10 and 20 mg/kg; Sigma, St. Louis, MO) through intravenous injection 30 min after surgery as a single dose.

2.2. Stroke severity evaluation

Stroke severity evaluation was performed 24 h post-injury. Neurological severity scores used a 10-point scale for 10 functional tasks, 0 point indicates normal function, while 10 points suggest the most severe neurological dysfunctions (Chen et al., 1996). For brain infarct volume measurements, brains were cut into 2-mm-thick slices, which were subsequently analyzed (Wang et al., 2014). Brain water contents were calculated based on Hatashita's wet-dry method (Hatashita et al., 1988). In brief, brain water content (%) = (wet weight − dry weight) × 100/wet weight (Wang et al., 2015).

2.3. Evans blue assessment

BBB disruption was evaluated by Evans blue leakage following surgical procedures (Wang et al., 2014). Briefly, Evans blue (EB, 4 mL/kg, Sigma, St. Louis, MO, USA) was adminstrated with the tail vein injection 24 h post-injury. After 2 h, brain tissue samples were collected and photographed. Tissues were then homogenized and centrifuged to extract supernatants, which were measured at 620 nm to detect the absorbance.

2.4. Quantitative real-time PCR

Twenty-four hours after injury, tissues were homogenized in lysis buffer for RNA isolation. Qiagen kits (Qiagen, Valencia, CA, USA) were used to isolate RNA (RNeasy mini kit), synthesis of cDNA (QuantiTect reverse transcription kit RNA) and the subsequent qPCR (SYBR Green Master Mix). The following primer sequences are utilized: ZO-1 (Forward primers 5′-CGG TCC TCT GAG CCT GTA AG-3′; Reverse primers 5′-GGA TCT ACA TGC GAC GAC AA-3′); occludin (Forward primers 5′-GCT CAG GGA ATA TCC ACC TAT CA-3′; Reverse primers 5′-CAC AAA GTT TTA ACT TCC CAG ACG-3′); IL-1β (Forward primers 5′-CAC CTC TCA AGC AGA GCA CAG-3′; Reverse primers 5′-GGG TTC CAT GGT GAA GTC AAC-3′); iNOS (Forward primers 5′-CCA CAA TAG TAC AAT ACT ACT TGG-3′; Reverse primers 5′-ACG AGG TGT TCA GCG TGC TCC ACG-3′); MMP-9 (Forward primers 5′-TCG AAG GCG ACC TCA AGT G-3′; Reverse primers 5′-TTC GGT GTA GCT TTG GAT CCA-3′); arginase 1 (Forward primers 5′-AAG AAA AGG CCG ATT CAC CT-3′; Reverse primers 5′-CAC CTC CTC TGC TGT CTT CC-3′); GAPDH (Forward primers 5′-ATC ACC ATC TTC CAG GAG CG-3′; Reverse primers 5′-TTC TGA GTG GCA GTG AGG GC-3′).

2.5. Western blotting

Tissues were collected and western blotting method was performed as previously described (Yu et al., 2015) 24 h post-injury. In brief, lysis buffer (10 mM HEPES, pH 7.4, 2 mM EGTA, 0.5% NP-40, protease inhibitors) was used to extract total proteins from tissues. Samples (20 μg proteins) were separated by SDS-PAGE, then blotted onto nitrocellulose membranes. Membranes were incubated with specific primary antibodies against ZO-1 (#ab59720, Abcam, Cambridge, MA, USA), occludin (#ab31721, Abcam) and β-actin (#ab8226, Abcam), respectively, The application of corresponding HRP-conjugated secondary antibodies (KPL, USA) was followed to visualize immunoreactive bands using chemiluminescence (Sigma). Image J was applied to quantify the intensities of protein bands.

2.6. ELISA

Total proteins were extracted (see western blotting method) 24 h post-injury. 20 μg total proteins were applied for IL-10 detection according to the manufacturer's instruction (Wuhan Boster Biological Technology LT, Wuhan, China). Final results were reported as pg/mg.

2.7. Evaluation of the activities of MPO and NOX

The MPO activity was measured as previously described (Pazar et al., 2016) in the tissues 24 h post-injury. In brief, 0.3 mL homogenized brain tissues were mixed with 2.3 mL reaction mixture, which included 50 mM PB, *o*-dianisidine and 20 mM H_2O_2. Enzyme activity was reflected by absorbance at 460 nm. The NOX activity was detected as previously reported (Liu et al., 2008). In brief, 20 μL supernatant of homogenized and centrifuged brain samples was added into a 96-well luminescence plate, then mixed with 80 μL phosphate-buffered saline and 6.25 μL l M lucigenin. NADPH was added to start the reaction, and photoemission was determined by the absorbance at 340 nm, which is monitored every 30 s for 5 min.

2.8. Statistics

All data were statistically analyzed by GraphPad Prism software (GraphPad Software, USA), using one-way ANOVA analysis followed by a Tukey's post hoc test. P-values less than 0.05 should be considered as statistical significant. At least three independent repeats were performed for each experiment.