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Post-ischemic treatment of WIB801C, standardized *Cordyceps* extract, reduces cerebral ischemic injury *via* inhibition of inflammatory cell migration

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ABSTRACT

Ethnopharmacological relevance: Anti-inflammatory therapy has been intensively investigated as a potential strategy for treatment of cerebral stroke. However, despite many positive outcomes reported in animal studies, anti-inflammatory treatments have not proven successful in humans as yet. Although immunomodulatory activity and safety of *Cordyceps* species (Chinese caterpillar fungi) have been proven in clinical trials and traditional Asian prescriptions for inflammatory diseases, its anti-ischemic effect remains elusive.

Aim of the study: In the present study, therefore, we investigated the potential therapeutic efficacy of WIB801C, the standardized extract of *Cordyceps militaris,* for treatment of cerebral ischemic stroke.

Materials and methods: The anti-chemotactic activity of WIB801C was assayed in cultured rat microglia/macrophages. Sprague-Dawley rats were subjected to ischemic stroke *via* either transient (1.5-h tMCAO and subsequent 24-h reperfusion) or permanent middle cerebral artery occlusion (pMCAO for 24-h without reperfusion). WIB801C was orally administered twice at 3- and 8-h (50 mg/kg each) after the onset of MCAO. Infarct volume, edema, blood brain barrier and white matter damages, neurological deficits, and long-term survival rates were investigated. The infiltration of inflammatory cells into ischemic lesions was assayed by immunostaining.

Results: WIB801C significantly decreased migration of cultured microglia/macrophages. This anti-chemotactic activity of WIB-801C was not mediated *via* adenosine A3 receptors, although cordycepin, the major ingredient of WIB801C, is known as an adenosine receptor agonist. Post-ischemic treatment with WIB801C significantly reduced the infiltration of ED-1-and MPO-positive inflammatory cells into ischemic lesions in tMCAO rats. WIB801C-treated rats exhibited significantly decreased infarct volume and cerebral edema, less white matter and blood-brain barrier damages, and improved neurological deficits. WIB801C also improved survival rates over 34 days after ischemia onset. A significant reduction in infarct volume and neurobehavioral deficits by WIB801C was also observed in rats subjected to pMCAO.

Conclusions: In summary, post-ischemic treatment of WIB801C reduced infiltration of inflammatory cells into ischemic lesions *via* inhibition of chemotaxis, which confers long-lasting histological and neurological protection in ischemic brain. WIB801C may be a promising anti-ischemic drug candidate with clinically relevant therapeutic time window and safety.

1. Introduction

Among various injury pathways involved in ischemic cascades, post-ischemic inflammation has emerged as one of promising therapeutic targets to prevent the expansion of irreversible brain damage into the penumbra (Iadecola and Anrather, 2011). Inflammation has a significant impact on delayed ischemic damage including reperfusion injury, blood-brain-barrier disruption, and hemorrhagic transformation, which occur several hours to days

Abbreviations: A3AR, A3 adenosine receptor; APP, amyloid precursor protein; BBB, blood-brain barrier; CBF, cerebral blood flow; EB, evans blue; MBP, myelin basic protein; MCAO, middle cerebral artery Occlusion; MCP-1, monocyte chemoattractant protein-1; PFA, paraformaldehyde; TTC, 2, 3, 5-triphenyltetrazolium chloride

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post-ischemia (Khatri et al., 2012). In addition, it is recognized as a key determinant of clinical outcome and long-term prognosis in stroke patients (McColl et al., 2009). Therefore, a neuroprotective therapy targeting post-ischemic inflammation possesses great promise to ensure improved clinical outcome along with prolonged therapeutic time window.

Post-ischemic inflammation develops through not only activation of resident microglia but also infiltration of peripheral inflammatory cells (Iadecola and Anrather, 2011). The peripheral inflammatory cells release cytotoxic mediators (*e.g.*, pro-inflammatory cytokines, free radicals, and proteases), thereby further enhancing inflammation and secondary brain damage. Previously, we and other researchers demonstrated that selective inhibition of infiltration of peripheral inflammatory cells into ischemic lesions reduced ischemic injury in rodent brains (Choi et al., 2011; Jickling et al., 2015; Murikinati et al., 2010; Zhang et al., 2007). Similarly, depletion of peripheral granulocytes or genetic depletion of their adhesion ability reduced infarct volume and improves outcomes including mortality in rodent ischemic models (Kitagawa et al., 1998).

The *Cordyceps* species (Chinese caterpillar fungi), a parasite that forms its scleotium in the insect larvae, has been widely used as an herbal medicine for inflammatory diseases in humans (Das et al., 2010; Yue et al., 2013). A number of clinical trials have demonstrated the pharmacological usefulness and acceptable safety of *Cordyceps* species, especially *Cordyceps militaris* and its substitute, *Cordyceps sinensis*, in chronic bronchitis, influenza A viral infection, or an adjunctive treatment by immunosuppressive therapy in renal transplantation (Gai et al., 2004; Li et al., 2009; Wang et al., 2007). The extract of *Cordyceps* species and its major active constituent, cordycepin (3′-deoxyadenosine) have been documented for anti-inflammatory, anti-oxidant, and anti-thrombotic activities in *in vitro* and *in vivo* studies (Cho et al., 2007; Jeong et al., 2010; Kim et al., 2006; Noh et al., 2009; Won and Park, 2005; Yu et al., 2006; Zhou et al., 2009). Such biological activities may render *Cordyceps* species as a promising therapeutic treatment candidate for stroke patients. Indeed, recent preclinical studies have provided evidence that *Cordyceps* extracts or their components protect the brain against ischemic injury (Cai et al., 2013; Cheng et al., 2011; Hwang et al., 2008; Liu et al., 2010; Wang et al., 2012). However, these studies employed pretreatments or concurrent treatments for a long-time, which are not practical in clinical setting and cannot efficiently alleviate pathology phases targetable at feasible therapeutic time window.

In the present study, therefore, we investigated the anti-ischemic effect of post-ischemic treatment of *Cordyceps militaris* extracts. *C. militaris* extracts possess cordycepin as a bioactive component. Cordycepin has been reported as an A3 adenosine receptor (A3AR) agonist, which was proven to have neuroprotective effect *via* modulation of peripheral inflammatory cells (Chen et al., 2006; Choi et al., 2011; Von Lubitz et al., 1994). In the present study, we used a preparation of cordycepin-enriched *C. militaris* extract, WIB801C, which is standardized based on the level of cordycepin (8.2%, w/w). The yield of cordycepin in WIB801C was significantly increased through defined mycelia fermentation process when compared with that in whole fruiting body mycelia of *C. militaris* (0.16%, w/w; Lee et al., 2014). We investigated whether WIB801C inhibits the recruitment of microglia/macrophages into ischemic lesions, an underlying mechanism of A3AR agonists for anti-ischemic effects. Additionally, the pharmacological efficacy of WIB801C was thoroughly evaluated according to the Stroke Therapy Academic Industry Roundtable (STAIR) criteria for successful translation to future human clinical trials (Fisher, 2011; Fisher et al., 2009).

2. Materials and methods

2.1. Preparation of standardized Cordyceps militaris extract, WIB-801C

Lyophilized powders of the standardized *C. militaris* (Cavicipitaceae) extract, WIB801C, was kindly provided from Whanin Pharmaceutical Co., Ltd. (Seoul, Korea) using the fungus strain *C. militaris* , from the Dongchong Xiacao collection of Whanin Pharm Co., Ltd. (Seoul, Korea) as previously described (Lee et al., 2014, 2015). In brief, the fermented culture media cultivated with the mycelia of *C. militaris* -hypha were concentrated at 60 °C with a rotary vacuum evaporator (Eyela N3000, Rikakikai Co., Ltd, Tokyo, Japan), extracted with n-butanol twice and filtered. The extracts were then evaporated at 40 °C and lyophilized to yield WIB801C. The amounts of adenine, adenosine, and a major component, cordycepin were analyzed by high performance liquid chromatography (Alliance HPLC system Co., Ltd., MA) using YMC hydrosphere C18 column (4.6 mm × 250 mm analytical, 5 μm; at 25 °C; Waters Chromatography Division, Milford, MA) on Waters instrument equipped with Waters CapLC 2695 and 2998 photodiode assay detector (Waters Chromatography Division). The sample extracts and standards were prepared in 50% methanol and filtered through 0.2 μm membrane filter. The mobile phase was composed of 15% methanol and KH_2PO_4 (0.01 M). The HPLC analyses were performed at a flow rate of 1.0 ml/min and recorded at 254 nm. The content of cordycepin and adenine in WIB801C used in the present study was standardized to 82.0 ± 1.4 mg/g-WIB801C and 16.2 ± 0.3 mg/g-WIB801C, respectively (Lee et al., 2014). Cordycepin and adenine were purchased from Sigma (St. Louis, MO).

2.2. Primary microglial cultures

Pure microglial cells were prepared from primary mixed glial cell culture. Cerebral cortices from neonatal Sprague-Dawley rats 1–2 days old) were triturated to single cells and plated into poly-l-lysine (1 μg/ml; Sigma-Aldrich)-coated 175 cm^2 T-flasks and maintained in modified Eagle's medium (MEM) containing 10% fetal bovine serum. At 7 or 8 days after plating, microglia were detached from the flasks by mild shaking (37 °C, 30 min at 200 rpm) and plated onto the experimental plates or used for chemotaxis assay.

2.3. Chemotaxis assay

For quantitative analysis, monocyte chemoattractant protein-1 (MCP-1) was placed at the bottom chamber of a chemotaxis chamber (Neuroprobe, Cabin John, MD) and microglia (1×10^4 cells/well in serum-free MEM) in the upper chamber were allowed to migrate to the bottom part for 2-h, through membrane pore (8 mm^2 filter area; Neuroprobe, Cabin John, MD). Migrated cells on the bottom side filter were nuclei-stained with Harris hematoxylin (Sigma-Aldrich) and then counted. In a subset of experiments to determine the involvement of adenosine receptors, cells were pretreated with either an adenosine A3 receptor antagonist, MRS1523 (1 μM) or an adenosine A2A receptor antagonist, SCH58261 (100 nM) for 20 min and allowed to migrate for 2-h in the absence or presence of WIB801C (either 100 or 300 μg/ml). An A3 receptor agonist, LJ-529 (1 μM) was used as a positive control (Choi et al., 2011). At doses used in this study, antagonists alone did not show any effect on migration.

2.4. Animals

Male Sprague-Dawley rats weighing between 260 and 300 g were purchased from Charles River Laboratories (Seoul, Korea) and