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Research report

Icariin enhances remyelination process after acute demyelination induced by cuprizone exposure

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ABSTRACT

Pathology are still progressive and cumulative in the remission course of relapsing-remitting MS (RRMS), thus drug treatment during the remission period may play a great role for the regeneration of the myelin sheath. C57BL/6 mice were fed with cuprizone (CPZ, 0.2% w/w) for 5 weeks to induce acute demyelination and oligodendrocytes degeneration, after which CPZ was withdrawn to allow recovery. Icariin (ICA, 6.25, 12.5 and 25 mg/kg/day), vehicle (0.5% sodium carboxymethyl cellulose solution) or water was administrated orally to mice for 1 week after CPZ withdrawal. Luxol-fast blue (LFB), immunohistochemical or immunofluorescence staining was used to detect morphological and biological changes in the brains. CPZ administration for 5 weeks resulted in completely demyelination and remyelination occurred when CPZ was withdrawn. ICA treatment during the recovery period for 1 week significantly improved myelin restoration, enhanced NF200-positive axons repair, increased the number of $APC^+/Olig2^+$ mature oligodendrocytes and prevented neuron-derived neurotrophic factor such as nerve growth factor (NGF) loss. Our results demonstrated that ICA treatment during the recovery period promotes remyelination and axon rewrapped, at least, in part, by promoting oligodendrogenesis and neurotrophic factor production.

1. Introduction

Multiple sclerosis (MS), a classical demyelination disease that affects the central nervous system (CNS), is characterized by pathological changes, including inflammation, demyelination, and axon injury (Kipp et al., 2009; Scolding and Franklin, 1998). It is the most common neurological disorder in young adults in the Western hemisphere, being more common in females. The remission period of classical relapsing-remitting MS (RRMS) has been characterized as a critical time for the repair of damaged myelin sheaths and axons. Demyelinated axons would be rewrapped by the regenerated myelin sheath, thus ameliorating axonal dysfunction. The remission period is also considered the period of remyelination (Fox et al., 2015; Kister et al., 2013). During the remission course of RRMS, pathology are still progressive and cumulative, thus the remission period is a key time point for the regeneration of the myelin sheath. Treated RRMS patients with drugs during the remission period may improve adequate levels of remyelination and further benefit MS patients.

The mechanism of RRMS is complicated and largely unknown. There is no drug currently used to treat patients ultimately cure the disease, just controlling the relapse times. Investigation of traditional Chinese medicine may lead to the discovery and development of novel drugs with potentially less adverse side effects than current treatments. Epimedium, as one of the members of the traditional Chinese herbs, its use dates back to more than 2000 years ago when it was documented in the *Shen Nong's Materia Medica*. Bushen Gusui Tablet, in which epimedium accounted for the pivotal component, have the certain effect on both MS patients and experimental allergic encephalomyelitis animals (Liu and Sun, 2001). Epimedium flavonoids (EF), the main component extracted from *Epimedium sagittatum*, which including 56.7% icariin (ICA) have therapeutic benefit for the treatment of MS (Liang et al., 2015; Yin et al., 2012). It has been reported that ICA has extensive pharmacological effects (Chen et al., 2005; Li et al., 2010). However, it is unknown the effect of purify compound ICA on de- or remyelination process in diseases of the CNS such as MS.

The cuprizone (CPZ) model offers the opportunity to study MS-related lesions. CPZ (0.2%, w/w) mixed in basic chow repre-

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sents a highly reproducible de- or remyelination of distinct brain regions, including both the cerebral cortex and corpus callosum (CC) (Kipp et al., 2009; Stidworthy et al., 2003; Torkildsen et al., 2008). After 5–6 weeks of cuprizone treatment, the CC is almost completely demyelinated, a process called "acute demyelination". Acute demyelination is followed by spontaneous remyelination during subsequent weeks when mice are fed normal chow. The remyelination animal model could mimic the remission process in RRMS patients. The significant demyelination can be evaluated by Luxol-fast blue (LFB) staining or immunohistochemistry against the myelin components myelin basic protein (MBP). In the CNS, the myelin sheath is formed by cholesterol-rich specialized membranes of oligodendrocytes, compactly enwrapped around axons. The toxic copper chelator CPZ could impair the mature oligodendrocytes due to its high metabolic demands (Buschmann et al., 2012; Kipp et al., 2009; Praet et al., 2014) and neurofilament heavy chain (NF200)-positive axons were declined in demyelinated CPZ-fed mice (Sun et al., 2016). Olig2-positive cells are frequently found within the CC and the adjacent neocortex (Zhang et al., 2012). The myelin status was also assessed by the number of anti-adenomatous polyposis coli (APC)-positive mature oligodendrocytes. Neurotrophins such as nerve growth factor (NGF) have a dramatic effect on neuron and oligodendrocytes survival, stimulating axonal regeneration and remyelination.

Therefore, in the present study, we intend to identify whether ICA can promote remyelination in the remission period or not. We also investigate the effect of ICA on NF200-positive axons, mature oligodendrocytes and NGF expressions. The aim is to provide a new insight in the role of ICA during remyelination and suggest a promising treatment for RRMS during remission period.

2. Materials and methods

2.1. Materials

Icariin, with a molecular weight of 676.66 ($C_{33}H_{40}O_{15}$), was purchased from Shanxi Baoji Chenguang Biotechnology Co. Ltd (CAS489-32-7) and dissolved immediately in 0.5% carboxymethylcellulose before experiments and administered intragastrically. The purity of icariin is over 99% as determined by a high-performance liquid chromatography assay.

2.2. Animals and cuprizone treatment

Female C57BL/6 mice aged six-week old (weighing between 12–17 g) were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China), and housed in groups of 5 per cage under standard laboratory conditions with a 12/12 h light-dark cycle, constant room temperature of 24 ± 1 °C, and 50 ± 5% humidity. All mice were weighed and examined weekly. All procedures in this protocol were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of Xuan Wu Hospital of Capital Medical University.

After acclimation for one week, the mice from CPZ group were fed with rodent chows containing 0.2% CPZ (bis(cyclohexanone) oxaldihydrazone) (w/w), Sigma-Aldrich, St. Louis, MO, USA) for 5 weeks to induce acute demyelination, meanwhile, the control group were fed normal chows without CPZ mixture. During remyelination, mice from control group still received normal rodent-chow, however, CPZ treatment was stopped, returned to normal chows and allowed to recover for 1 week with water, vehicle or ICA (6.25, 12.5 or 25 mg/kg/day). The volume for the gastrointestinal treatment was 0.1 mL/10 g. While control mice were treated with vehicle (water or 0.5% sodium carboxymethyl cellulose solution). Mixed chows were produced by Experimental Animal Center of Military Medical Science Academy (Beijing, China).

2.3. Tissue preparation

To harvest tissue for histological analysis, animals were anesthetized with chloral hydrate (400 mg/kg) and perfused intracardially with normal saline followed by 4% paraformaldehyde (PFA) in phosphate buffer (3.12 g/L of NaH_2PO_4, 28.64 g/L of $Na_2HPO_4 \cdot 12H_2O$, 30 g/L sucrose, pH-7.4). The brains were post-fixated in 4% PFA in phosphate buffer saline (PBS) at 4 °C overnight, followed by cryoprotection in 30% sucrose in PBS for 3 days. In order to evaluate demyelination or specific protein expression, serial coronal sections in frozen (20 and 40 μm) were taken between levels 1 and 0 mm bregma for LFB and between levels −1.5 and −2.5 mm bregma for immunohistochemical staining. Correspondingly, sections (20 μm-thick) between levels 1 and 0 mm bregma were taken for immunofluorescence.

2.4. Histological staining

For LFB staining, sections were immersed in 95% ethanol for 5 min at room temperature (RT) and were immersed overnight in LFB solution. Next morning, sections were then immersed in 95% ethanol for 3 min to remove excess stain following by being rinsed in deionized water. Afterward, sections were immersed in hematoxylin solution and were then re-rinsed in both 70% hydrochloric acid ethanol and deionized water. Finally, sections were dehydrated and coverslipped.

For immunohistochemistry, sections were pretreated with 3% hydrogen peroxide in 0.01 M PBS for 30 min at RT for quenching endogenous peroxidase activity, then blocked with 10% goat serum in PBST (0.3% Triton X-100 in PBS) for 2 h at 37 °C. Subsequently, the sections were incubated with primary antibody to MBP (1:200, Millipore) in the blocking solution at 4 °C overnight. After rinsing in PBS for three times then incubated with the appropriate biotinylated secondary antibody (1:100, ZSGB-BIO) for 1 h at 37 °C, followed by rinsing in PBS and streptavidin-labeled third antibody (1:100, ZSGB-BIO) for 1 h at 37 °C. A final incubation in diaminobenzidine was performed for visualization. All sections were washed in PBS, mounted on amino propyltriethoxy silane-coated slides, dried, dehydrated with a graded series of ethanol washes, cleared in xylene, and coverslipped.

For immunofluorescence, sections were blocked with 10% donkey serum in PBS for 2 h and then incubated with primary antibody to NF200 (1:200, Sigma), adenomatous polyposis coli protein (APC, 1:200, Millipore), OLIG2 (1:500, Millipore), NG2 (1:200, Millipore), nerve growth factor (NGF, 1:100, Santa Cruz) and NeuN (1:100, Abcam). Appropriate secondary antibodies labeled with Alexa Fluor 488 or Alexa Fluor 594 were prepared in PBS for 2 h at RT.

2.5. Image analysis

Three-four sections from each mouse (n = 3–4) were selected and digitized by a video camera mounted on an Olympus microscope (Olympus, Richmond Hill, ON, Canada). Pictures were further processed using Image-Pro Plus software (version 5.0) and Photoshop software (Adobe Systems). Results were presented by mean value of integrated optical density (IOD) or immunopositive cell counts in a total area of 512 μm × 386 μm field.

2.6. Statistical analysis

All quantitative data were analyzed by independent-sample *t* test and one-way analysis of variance (ANOVA) followed by