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Original Article

Catalpol induces oligodendrocyte precursor cell-mediated remyelination in vitro

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Abstract: In demyelinating diseases such as multiple sclerosis, one of the treatment strategies includes remyelination using oligodendrocyte precursor cells (OPC). Catalpol, the extract of radix rehmanniae, is neuroprotective. Using an OPC culture model, we showed that 10 μM catalpol promotes OPC proliferation, cell migration and differentiation into mature oligodendrocytes. The 10 μM catalpol displayed stronger effects on OPCs migration and oligodendrocyte differentiation. These results suggest that catalpol has a potential role in promoting remyelination in demyelinating diseases, and is of therapeutic interest.

Keywords: Oligodendrocytes precursor cell, catalpol, differentiation, proliferation

Introduction

Multiple sclerosis (MS) is an autoimmune and inflammatory demyelinating disease of the central nervous system (CNS). It is the major cause of neurological disabilities in young adults [1, 2]. Its pathological features include lymphocyte infiltration, myelin breakdown, oligodendrocyte apoptosis and axonal injuries [3]. Currently available treatments include nonspecific immunoregulation and immunosuppression such as high-dose hormone therapy, β-interferon (IFN-β) immune therapy and mitoxantrone chemotherapy, with efficacies ranging between 30% and 50% [4]. The major pathological signs of MS include loss of myelin and oligodendrocyte apoptosis. Therefore, strategies to promote remyelination are actively sought. Oligodendrocytes are involved in CNS myelination [5], and oligodendrocyte progenitor cells (OPC) are the precursor cells of oligodendrocytes. One of the strategies involves intrinsic repair mechanisms to promote the survival, proliferation and differentiation of OPC for myelin repair [6].

Rehmannia is a flowering plant belonging to Scrophulariaceae family, which is endemic to China. It contains a wide range of biological and pharmacological activities, including anti-aging, anti-tumor, purgative, sedative and hepatoprotective effects. Catalpol, an iridoid glycoside extracted from the root of *Rehmannia*, has been shown to be neuroprotective in CNS. For example, catalpol protects primary cultured cortical neurons induced by Aβ1-42 via a mitochondrial-dependent caspase pathway [7]. It down-regulates nitric oxide in mesencephalic neurons induced by rotenone via extracellular regulated kinase (ERK) signaling [8] and it increases hippocampal neuroplasticity and up-regulates protein kinase C (PKC) and brain-derived neurotrophic factor (BDNF) in the aged rats [9]. Furthermore, pre-treatment with catalpol decreases the production of nitric oxide and reactive oxygen species in lipopolysaccharide and interferon-gamma treated astrocyte primary culture, suggesting an anti-inflammatory function [10]. Catalpol prevents oligodendrocyte death and myelin breakdown in a rat model of chronic cerebral hypoperfusion [11]. In this

study, we aimed to evaluate the effect of catalpol on OPCs.

Materials and methods

Materials

Catalpol (purity > 98%, analytical grade) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). It was dissolved in 0.01 M phosphate buffered saline (PBS). 3-(4,5-Dimethylt-hiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), poly-D-lysine and triiodothyronine (T3) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium with Ham's nutrient mixtures F12 (DMEM/F12), fetal bovine serum (FBS), basic fibroblast growth factor (bFGF) and platelet derived growth factor-alpha (PDGFα) were purchased from Gibco BRL (Grand Island, NY). Monoclonal antibody against glial fibrillary acidic protein (GFAP), polyclonal antibodies against Olig1, 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) and 5-Bromo-2-Deoxyuridine (BrdU) were acquired from Abcam (Cambridge, UK).

Primary cultures of OPCs

All animal care and surgical procedures were in strict accordance with the governmental policies and regulations set by the Animal Care Committee of Capital Medical University of Beijing, China. Briefly, two day-old Sprague-Dawley (SD) neonatal rats were euthanized with an overdosed injection of sodium pentobarbital (50 mg/kg, intraperitoneally). The animals were then sprayed with 70% ethanol and decapitated on ice block. The following procedures were performed in sterile environment. Under dissecting microscope (Leica Microsystems AG, Heerbrugg, Switzerland), brains of neonatal rats were dissected, meninges were completely removed and cortices were isolated. The cerebral cortices were washed in cold Hank's balanced salt solution (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), cut into small pieces and triturated into cell suspension. The cells were collected through a 70-mm strainer (170 μm/pore) and centrifuged (130 × g, 10 minutes at 4°C). The pellet was resuspended in basic culture medium consisting of DMEM supplemented with 10% FBS, 0.6% glucose, 4 mmol/L L-glutamine, 5 mmol/L sodium pyruvate, 50 U/ml penicillin, and 5 μg/ml streptomycin. The cells were plated onto coated 25-cm^2 flasks at 1-2 × 10^6 cells/flask and cultured in humidified incubator (37°C, 5% CO_2). The cultures were left untouched for the first 3 days, and replenished with basic culture medium every other day for 9 days. The flasks were vortexed to remove microglia. After 24 hr of incubation with fresh medium, the flasks were shaken at 180 revolutions/min and 37°C for 1-2 hr. The flasks were washed with 0.01 M PBS and supplied with fresh medium. Two hours later, the flasks were subjected to shaking overnight (200 revolutions/minute, 37°C, 18-20 hr). On the next day, the supernatant containing OPCs was collected and centrifuged. The pellet was resuspended and cultured in OPC culture medium consisting of DMEM/F12 supplemented with 0.5% FBS, 50 μg/ml transferrin, 5 μg/ml insulin, 30 nmol/L sodium selenite, 10 nmol/L bFGF, 10 nmol/L PDGF-α, 4 mmol/L L-glutamine, 5 mmol/l sodium pyruvate, 50 U/ml penicillinum, and 50 mg/ml streptomycin. OPC culture medium was used for subsequent experiments unless otherwise specified.

Immunofluorescence and morphology

To confirm the identity of OPC, cells were seeded into 24-well plates and cultured for 72 hr. The cells were then fixed for 15 min with 4% paraformaldehyde (PFA) at room temperature. After washes with 0.01 M PBS, cells were immunostained with oligodendrocyte markers Olig1 (ab104581, Abcam) and CNPase (MAB326, Millipore) and astrocyte marker GFAP (G3893, Sigma). Briefly, cells were blocked with 10% normal serum with 0.2% Triton-X 100 and incubated with primary antibodies (Olig1, 1:200; CNPase, 1:200 and GFAP, 1:1000) overnight at 4°C. After washing with PBS, the cells were incubated with corresponding fluorescent secondary antibodies for 1 h at room temperature. After extensive washing, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) to label nuclei for 3 min. The wells were mounted with anti-fade mounting medium and pictures were taken with an inverted microscope connected to a CCD camera.

Proliferation of OPCs

OPC proliferation was evaluated by BrdU incorporation. Briefly, cells were seeded into 24-well plates and cultured for 72 hr, followed by addi-

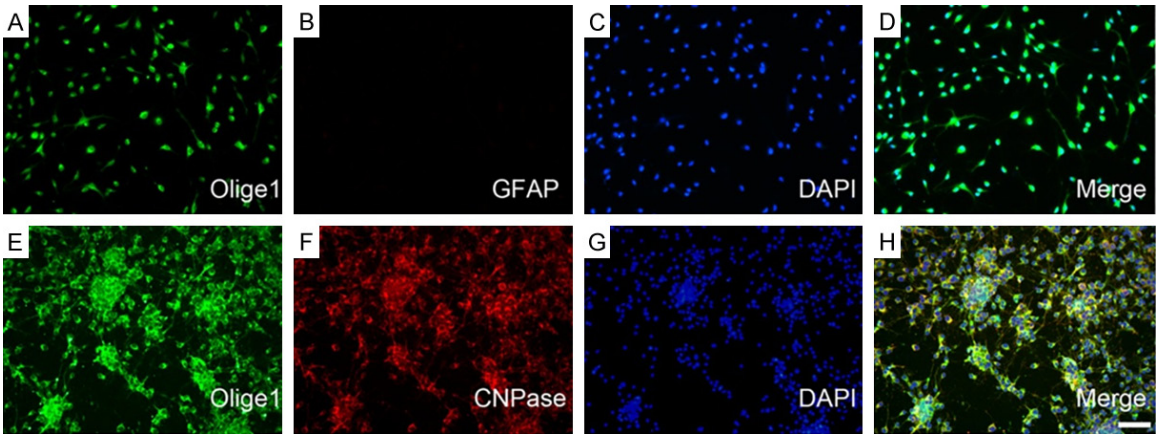

Figure 1. Photomicrographs showing the morphologies of OPCs cultured for 3 days. Cells were circular or ovoid with 2-3 neurites, and often in the proliferating stages of cell division. CNPase and Olig1 double immunofluorescence-labeled cells. In (A and B), Green Olig1 is positive, and red GFAP is negative. In (C), Blue DAPI staining shows the nucleus. In (E and F), Red CNPase and green Olig1 were positive. In (G), Blue DAPI staining shows the nucleus. GFAP and Olig1 double immunofluorescence-labeled cells in (D and H). These results indicate that the cells were OPCs. (Scale bars: 50 μm).

tion of 10 μM BrdU into each well and cultured for an additional 16 hr. At the end of the survival period, cells were fixed with 4% PFA for 15 min at room temperature. After washing with PBS, the cells were treated with 1 N hydrochloric acid (HCl) at 37°C for 20 min and treated with boric buffer (pH 8.5) for 30 min to neutralize the acid. After additional washes with PBS, the cells were processed immunohistochemically as described above. Briefly, after blocking with normal goat serum, cells were incubated with anti-BrdU primary antibody (1:200, 11170376001, Roche) overnight at 4°C. The cells were then rinsed twice with PBS and subsequently incubated with fluorescent conjugated secondary antibody at 37°C for 1 hr. Finally, the sections were incubated with DAPI for 3 min after washing twice with PBS. The images were taken under 20 × objective on an inverted microscope connected to a CCD camera. Using Image J, the number of DAPI-labeled nuclei and BrdU immunopositive cells were counted in three independent wells, five fields per well.

Migration of OPCs

Around 30-40 OPC spheres from culture flasks were seeded into each well of 24-well plates pre-coated with 0.01% poly-D-lysine. After culturing with Catalpol or vehicle for 3 days, OPCs were found migrating out of the spheres. The spheres were randomly selected under light microscope and photographed using a CCD camera. The distance between the cell migration front and the center and the radius of the sphere were measured using Image J. The ratio of migration front distance to the sphere radius was compared between control and treatment groups.

Differentiation of OPCs

To examine OPC differentiation, cells were seeded into 24-well plates. After culturing in differential medium consisting of DMEM/F12 supplemented with 0.5% FBS, 30 nmol/L triiodothyronine (T3), 30 nmol/L sodium selenite, 50 μg/ml transferrin, 5 μg/ml insulin, 5 mmol/l sodium pyruvate, 50 U/ml penicillin, 50 mg/ml streptomycin and Catalpol for 9 days, cells were fixed with 4% PFA at room temperature for 15 min. After washing with PBS, cells were incubated with primary antibodies (Olig1, 1:200; GFAP, 1:1000) overnight at 4°C. After extensive washing with PBS, cells were incubated in corresponding fluorescent conjugated secondary antibodies (1:500, A11029; A11036, Invitrogen) at room temperature for 1 hr. DAPI was added to label nuclei after washes and mounted with anti-fade mounting medium. Pictures were taken under 20 × objective on an inverted microscope connected to a CCD camera, covering five fields per well and at least 3 wells per treatment group. Number of GFAP positive astrocytes and Olig1-positive oligodendrocytes were counted in Image J.

Statistical analysis

All the experiments were performed in triplicate and data are shown as mean ± S.D. based on

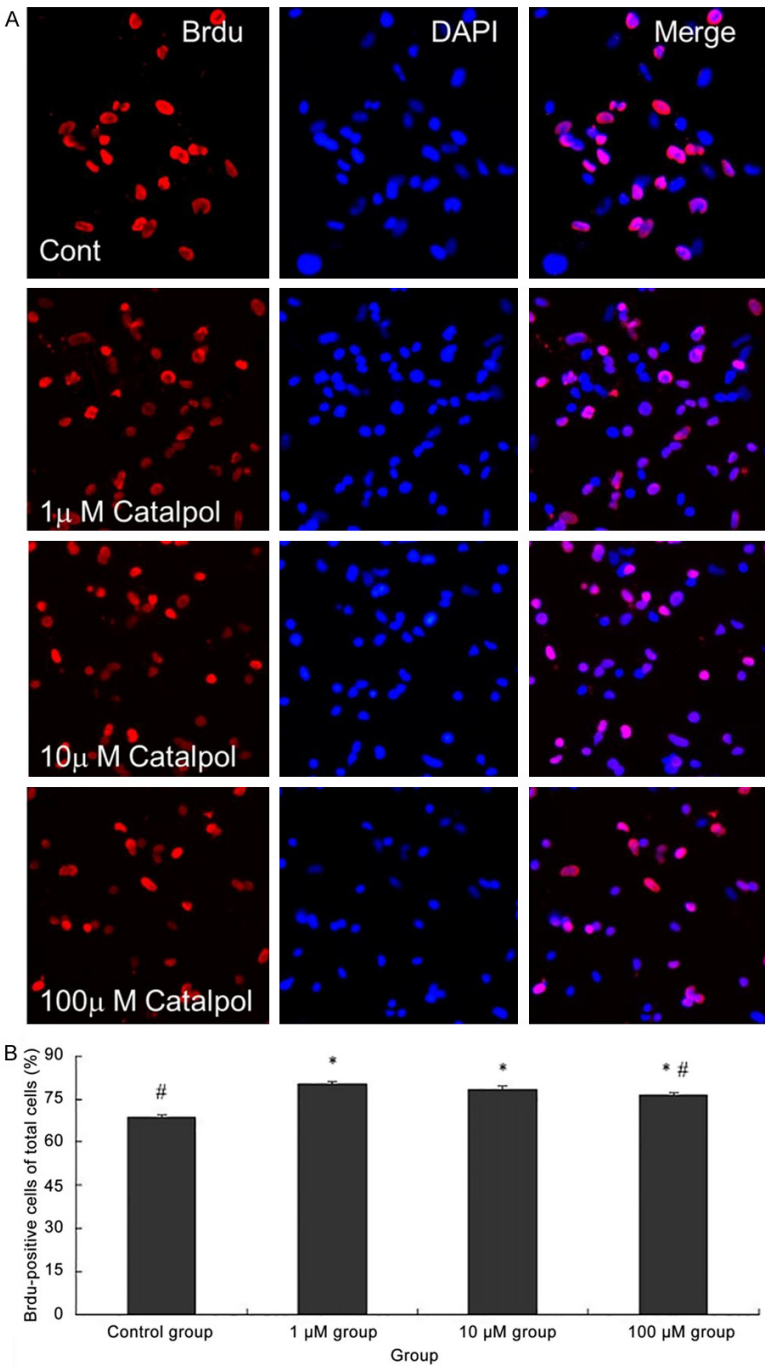

Figure 2. A: Effect of Catalpol on OPCs proliferation. BrdU-labeled cells. Nucleus was labeled in blue, BrdU-positive cells were labeled red. Cells with overlapping blue and red colors represent OPCs in the state of proliferation. B: Catalpol at all concentrations increased OPCs proliferation in vitro. Number of BrdU-positive cells was quantified using the Image J as described in Methods. *Indicates difference with control where $P \leq 0.05$, #indicates difference with 1 μM where $P \leq 0.05$, using Least-Significant Difference (LSD) and Dunnett-t test after one-way ANOVA analysis. (Scale bars: 50 μm).

three separate experiments. Statistical analysis was performed according to Least-Significant Difference (LSD) and Dunnett-t test after one-way ANOVA analysis. A *P* value < 0.05 was considered as significant.

Results

Identification of OPCs

Primary cultured OPCs maintained their identity in multiple passages. They showed extended 2 to 3 neurites when adhering to the bottom of the culture flask. The identity of OPCs was confirmed by immunostaining the cells with OPC-specific antibodies, i.e., CNPase and Olig1. Results showed that the cells were CNPase and Olig1-positive (**Figure 1A**, **1E**, **1F**) but GFAP negative (**Figure 1B**), suggesting that they were OPCs.

Catalpol increases OPCs proliferation

To examine whether catalpol increased proliferation of OPCs, we added various doses of catalpol and vehicle into OPCs. After 3 days in culture, BrdU was added for 16 hr to allow cells to incorporate BrdU into their DNA during mitosis. BrdU-positive cells indicate OPCs in a state of proliferation (**Figure 2A**). Results showed that different levels of catalpol increased the proliferation of OPCs (**Figure 2B**). The percentage of BrdU-positive cells was 80.37 ± 0.73%, 78.47 ± 0.93% and 76.26 ± 0.79% at a concentration of catalpol of 1, 10 and 100 μM respectively, which were significantly higher than control (68.67 ± 0.85%, $P < 0.05$). At a catalpol concentration of 1 μM, BrdU-positive cells significantly increased ($P < 0.05$) relative to that of control and 100 μM.

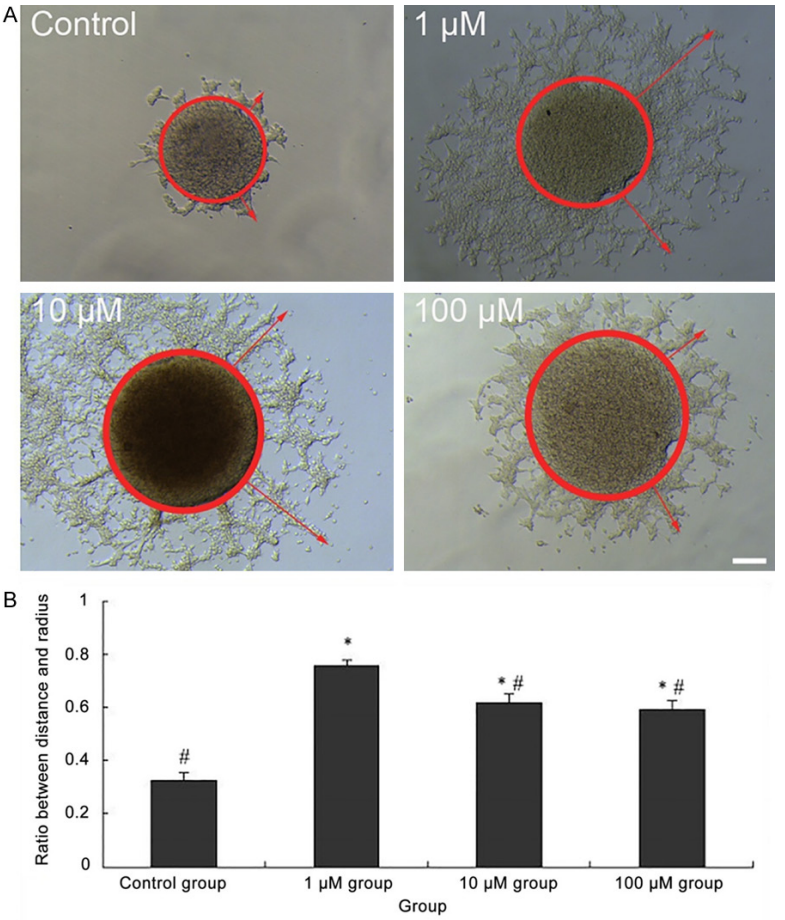

Figure 3. A: Effect of Catalpol on OPCs migration. Spheres were seeded in 24-well plates. OPCs migrated out of the spheres. Images taken at 4 days are presented. Cells grew from each of the spheres, clearly showing a radial migration as highlighted by red arrows. B: Catalpol at all concentrations promoted outward migration of cells as indicated by the longer migration front. Extent of cell migration was quantified using the Image J. The ratio increased significantly after 4 days of Catalpol treatment. *indicates significant difference from control where P < 0.05 using Least-Significant Difference (LSD) and Dunnett-t test after one-way ANOVA analysis. (Scale bars: 200 μm).

Catalpol promotes migration of OPCs

To evaluate whether catalpol promoted OPC migration in vitro, we seeded the OPC spheres on the culture dish. Catalpol at concentrations of 1 μM, 10 μM and 100 μM or vehicle was added to the culture. After 3 days in culture, the radius of the sphere and the radius of the migrating front were measured (**Figure 3A**). The results are presented as a ratio of radius of the migrating front to the radius of the sphere. Results showed that catalpol significantly enhanced OPC migration out of the sphere at all concentrations. When catalpol concentration was 1 μM, the ratio was 0.89 ± 0.04, which was higher than the value of control group ($P < 0.05$). The ratios of catalpol concentration in 10 μM and 100 μM were 0.94 ± 0.06 and 0.38 ± 0.05, respectively, which was also higher than the control ($P < 0.05$) (**Figure 3B**).

Catalpol promotes differentiation of OPCs into oligodendrocytes

OPCs differentiate into either type II astrocytes or oligodendrocytes as highlighted by white arrowheads. OPCs were cultured continuously in the differentiation medium for 9 days. Cell body was enlarged and neurites were increased. OPCs were treated with 1, 10 and 100 μM catalpol, respectively for 9 days. The cells were labeled by GFAP and Olig1 double immunofluorescence showed that the proportion of Olig1-positive cells was higher with the increase of catalpol concentration. Therefore, catalpol promoted OPCs differentiation into oligodendrocytes in vitro (**Figure 4A**). catalpol concentration in 100 μM promoted OPCs differentiation to oligodendrocytes, and the ratio was 82.7 ± 2.26%, which was significantly lower than in 10 μM, 1 μM and control groups ($P < 0.05$) (**Figure 4B**).

Discussion

Catalpol is an iridoid glycoside extracted from the root of *Rehmannia*, with neuroprotective effect in central nervous system. Catalpol downregulated nitric oxide in mesencephalic neurons induced by rotenone via ERK signaling pathways [8]. Catalpol protected oligodendrocytes against cell death and prevented myelin breakdown in a rat model of chronic cerebral hypoperfusion [11]. In this study, we focused on OPCs, a cell type which differentiates into astrocytes and oligodendrocytes. Oligoden-

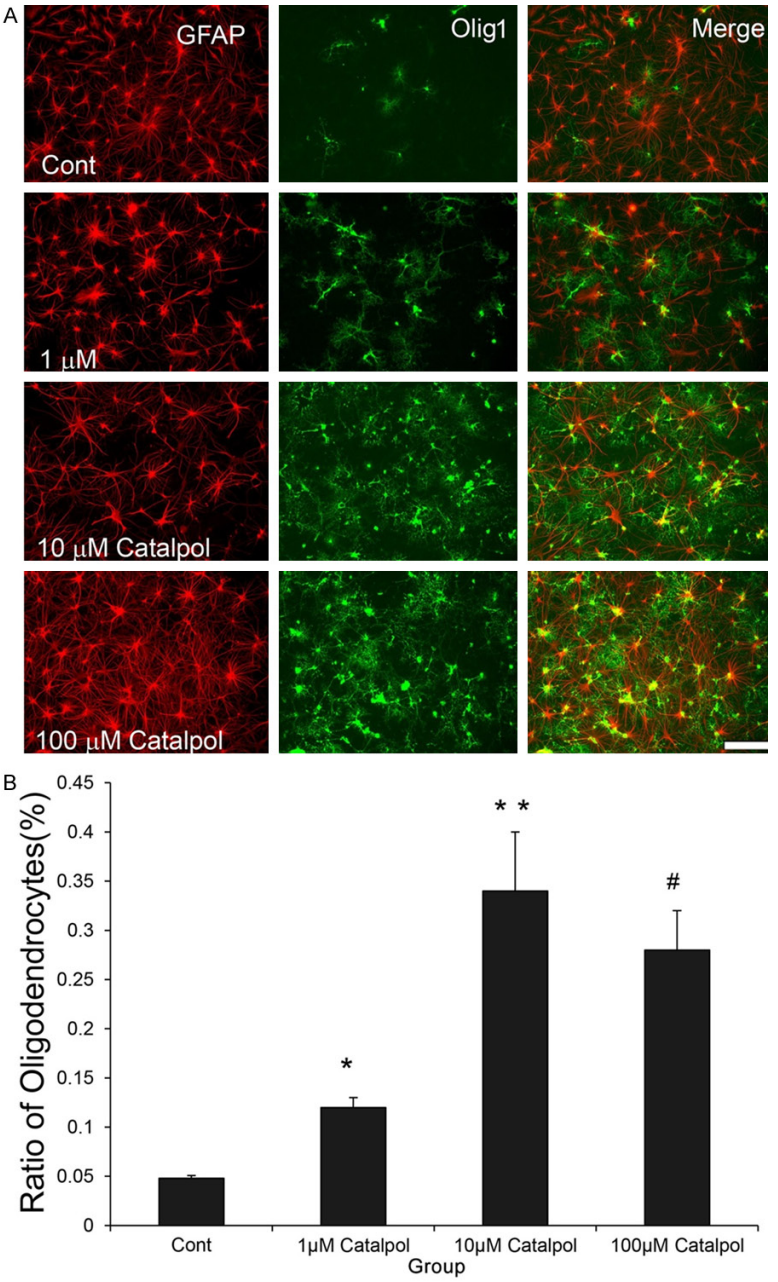

Figure 4. A: Effect of Catalpol on cell differentiation in cultured mouse OPCs. GFAP and Olig1 identified astrocytes and oligodendrocytes, respectively. GFAP positive cells were labeled red and Olig1-positive cells green. Cells showing overlap of red and green colors, indicate the total number of cells. B: Number of Olig1-positive cells was quantified using the Image J. Compared with control group, all concentrations of Catalpol promoted oligodendrocyte differentiation from OPCs. The percentage of oligodendrocyte was the highest in 10 μM-Catalpol treated group. *,**,#Indicates significant difference from control, and $P \leq 0.05$, using Least-Significant Difference (LSD) and Dunnett-t test after one-way ANOVA analysis. (Scale bars: 50 μm).

drocytes play an important role in remyelination of neurons.

The remyelination process is divided into three steps: OPC activation, proliferation and differentiation [12, 13]. Each individual step is closely regulated by extrinsic and intrinsic factors [14, 15]. Upon demyelination, OPCs become active mitotically and induce the expression of oligodendrogenic genes [16-18]. The proliferative stimulus is probably modulated through astrocytes and microglia, which are activated to release mitogens affecting OPCs [19-21]. After successful proliferation, OPCs interact with demyelinated axons, differentiate and express myelin proteins and eventually ensheath the axons [22]. Remyelination is spontaneous but the overall efficiency of remyelination is low. Aging is associated with a lower expression of remyelination-associated growth factors, which resulted in less efficient OPCs recruitment and differentiation [23]. Therefore, promoting either of the three processes in OPCs remyelination is an important therapeutic strategy.

In our study using primary cultured OPCs, we showed that catalpol promoted proliferation of OPCs as evidenced by the increase of BrdU labeling. An increase in the number of OPCs increased the likelihood of differentiation into oligodendrocytes. We also found that the presence of catalpol in the culture medium stimulated OPCs migration, suggesting that catalpol might be a chemotactic agent for OPCs. The newborn oligodendrocytes migrate into the demyelinating sites and complete the remyelination process. Our study also demon-

strated that catalpol promoted the differentiation of OPCs into oligodendrocytes, which increased the number of oligodendrocytes in the demyelination sites, contributing to recovery in demyelinating diseases.

Catalpol is a small molecule, which can be administered orally. We demonstrated here that catalpol enhanced proliferation and migration of OPCs in vitro. Animal models may be used to verify the therapeutic efficacy of the pharmacologically active compound *in vivo*.

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Disclosure of conflict of interest

None.

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