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Epigallocatechin gallate enhances the motor neuron survival and functional recovery after brachial plexus root avulsion by regulating FIG4

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Abstract

The survival of motor neurons (MNs) is the key to recovery of the motor function after brachial plexus root avulsion (BPRA). (–)-epigallocatechin-3-gallate (EGCG) exerts neuroprotective roles in neurons under different pathological conditions. However, the role of EGCG in regulating motor neurons under BPRA remains to be unclear. In the present study, we investigated the functional role of EGCG both in vitro and in vivo. In an in vitro study, we observed that EGCG obviously increased the cell survival rate of MNs and FIG4 protein levels compared with the vehicle control, with a peak level observed at 50 μM; EGCG can also upregulate FIG4 to reduce the cell death of MNs and increase the neurite outgrowth under oxidative stress; moreover, EGCG can upregulate FIG4 to promote the functional recovery and the survival of MNs in the ventral horn in mice after BPRA. These combined results may lay the foundation for EGCG to be a novel strategy for the treatment of BPRA.

Key words: *motor neurons, brachial plexus root avulsion, (–)-epigallocatechin-3-gallate (EGCG), neurodegenerative diseases, FIG4.*

Introduction

Brachial plexus injury is a very serious peripheral nerve injury that is common in clinical practice. It is considered to be one of the world's clinical medical treatment problems, and its incidence has been increasing [10,13]. Adult brachial plexus injury often occurs in accidental traffic accidents caused by external factors [14], resulting in the loss of the upper limb motor function, and its clinical treatment effect is not optimistic. Not only does it cause great pain to the individual, it brings heavy economic pressure to the family, but also adds a huge burden to the society. Therefore, the effective treatment of brachial plexus avulsion and its mechanism of action are extremely urgent scientific issues.

Brachial plexus root avulsion is a complex type of nerve injury involving the central and peripheral nervous system. It separates the spinal nerve from the spinal cord and causes serious damage to itself and related spinal segments, resulting in the damage to motor neurons and peripheral nerves. Separation of glial cells causes motoneurons in related segments to undergo a series of damages such as morphological

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changes, gene expression disorders, biochemical disorders, and metabolic changes [7], resulting in massive motor neuron death [38]. In the clinical treatment and animal model study of brachial plexus avulsion injury, replantation of avulsion nerve roots improves the survival rate of motor neurons after avulsion to a certain extent, and promotes the re-innervation and recovery of the motor function. However, replantation surgery does not completely restore the motor function of the upper limbs. The main reason is that before the target muscle atrophy of the upper limbs, the spinal motor neurons are unable to re-do the corresponding muscles because the axon growth rate is too slow. A large number of deaths of motor neurons after radiculopathy are considered to be important obstacles to the recovery of the motor function [12]. Therefore, the survival of motor neurons is considered to be an important prerequisite for the recovery of the motor function after brachial plexus injury [24]. In order to maintain the motor function of the upper limbs, after the injury, the motor neurons from the steady state to resist apoptosis and try to regenerate, but due to the adverse factors after the injury, the motor neurons eventually undergo irreversible death [11]. Therefore, further exploration of important signalling molecules and mechanisms to promote the survival and regeneration of motor neurons after injury will be of great significance for the prevention and treatment of brachial plexus avulsion.

(–)-epigallocatechin-3-gallate (EGCG), the predominant active polyphenol isolated from green tea, has been extensively investigated in a great deal of studies and considered to be a promising therapeutic agent for the treatment of diseases associated with chronic inflammation and oxidative damage [1,30,39]. EGCG inhibits the induction of iNOS transcription in lipopolysaccharide (LPS)-activated macrophages [26]. EGCG reduces airway inflammation [15,35,41], free radical generation, and tumor necrosis factor α (TNF-α) release [2] in animal models undergoing allergen-induced allergic diseases. EGCG targeting HO-1 reduces contrast-induced kidney damage through anti-oxidative stress and anti-inflammatory pathways [17]. Previous studies have also shown that Erk1/2 signalling cascades exert a key role in the regulation of gene expression and prevention of apoptosis [29]. It is also reported that activation of the MAPK/Erk can promote the differentiation and survival of neurons [34,42].

Given protective roles of EGCG in multiple diseased systems, the neuroprotective effects of EGCG on motor neurons (MNs) were never proposed. Moreover, little is known about the underlying mechanism of EGCG on oxidative stress and inflammation in MNs *in vitro*. Therefore, the aim of this study is to investigate the effect of EGCG on brachial plexus root avulsion (BPRA), focusing on its anti-oxidative stress and neuroinflammation properties.

Material and methods

Animals

Four-week-old C57BL/6 mice obtained from the Guangdong Medical Laboratory Animal Centre (PR China) were maintained on a 12 h light/12 h dark cycle, and afforded food and water *ad libitum*. One male and 3 female mice were mated in each cage, and the offspring on P0 were used for primary cultures of MNs. The Laboratory Animal Ethics Committee of the Xiangnan University approved all experimental protocols conducted on animals.

Primary culture of motor neurons and treatments

The primary culture of MNs was performed according to the previous studies with mirror modifications [6,20,23,40]. The spinal cord tissues from 0-day mice pups were dissected on the ice, and then digested with Hank's balanced salt solution (HBSS; 14175079, Thermo Fisher Scientific) containing 0.125% trypsin (25200056, Thermo Fisher Scientific) for 20 min at 37°C, 5% CO_2 atmosphere. After trituration, the purified dissociated cells were resuspended with DMEM/F-12 culture medium (SH30023, Solarbio Biotech Corp, Beijing, China) containing 10% foetal bovine serum (FBS; F8245-100, Hangzhou Sijiqing bioengineering Materials Co., Ltd, Hangzhou, China) and 1% Penicillin-Streptomycin (P/S; P1400, Solarbio Biotech Corp). The well-separated MNs were seeded in poly-D-lysine (PDL; D6790, Solarbio Biotech Corp)-coated 96-well plates at a concentration of 1×10^4 cells per well. After incubation for 4 h to allow the cell to adhere, the culture medium was replaced with Neurobasal-A medium (10888022, Thermo Fisher Scientific) supplemented with 2% B-27 (17504044, Life Technologies) and 1% P/S. The MNs were then incubated at 37°C in 5% CO_2 atmosphere overnight.

To test the effect of EGCG on the MNs, cells were utilized with two different cell models: i) MNs were treated with Neurobasal-A medium containing 0, 0.5, 5, 50 or 500 μM EGCG for 48 h, followed by MTT assay; ii) MNs were exposed to 20 μM H_2O_2 for 2 h and then maintained in Neurobasal-A medium containing 50 μM EGCG with or without the FIG4 antibody for 48 h; iii) cells were treated with 50 μM EGCG with or without the FIG4 antibody for 2 h prior to co-incubation with 1 μg/ml LPS for 24 h. Finally, cell viability and ELISA assays were performed.

Cell viability assay

Cell viability assay was performed according to the previous studies with minor modifications [5,23]. The Cell Counting Kit (CCK-8; HY-K0301, MedChem Express, China) was used to evaluate cell viability. At the indicated time points, 10 μl of CCK-8 solution was added to each well in 96-well culture plate, respectively, following a 2-h incubation at 37°C. And then, absorbance was measured in a multi-well plate reader (Tecan Infinite® M1000 Pro) at 490 nm.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits manufactured by Hanhong Biochemical Company (Sino Biological, China) was used for measuring the FIG4 concentration in the primary cultured MNs. The 450 nm absorbance was measured by an ELISA reader (Tecan Infinite® M1000 Pro).

Brachial plexus root avulsion and re-implantation model and grouping

The surgical procedures were performed according to the previous studies with minor modifications [5,14,37]. Mice were anesthetized by avertin (T48402, Sigma-Aldrich) at a concentration of 13 μl/g and placed prone on a surgical table. After retracting the paravertebral muscles, the C5-7 segments of the spinal cord were easily identified. After right hemi-laminectomies at C4 to C6 and opening the dura, the C5-7 dorsal and ventral roots at the right side were avulsed. To build the re-implantation models, the C6 ventral root was immediately re-implanted to the exact detachment point (on the pial surface) after the avulsion [4]. After muscles and skin were sutured in layers, all animals were recovered from anaesthesia and returned to their cages.

To investigate the neuroprotective role of EGCG after BPRA, 10 mice per group with re-implantation were randomly divided to 3 groups: (A) BPRA, (B) BPRA + EGCG and (C) BPRA + EGCG + FIG4 antibody. The treatment groups were intraperitoneally injected with 100 μl of phosphate buffer saline (PBS) or EGCG (the final concentration diluted in blood is 50 μM) with or without the FIG4 antibody once daily for 7 days after the surgery. The mice without BPRA or EGCG treatment were used as Sham control (n = 10/group). No animals died during the 12-week survival time, after which the animals were sacrificed.

Behaviour tests

The grooming test, grasping test and cylinder test were performed according to the previous studies with minor modifications [5,9,16,37,43].

The TGT was performed at 6 weeks and 12 weeks after the surgery to evaluate the recovery of the motor function of the injured forelimb. 1-3 ml of purified water was sprayed on mice snout, and the movements of the forepaw to clear the water were videotaped for more than 5 min each time. The test scores were graded based on the position of the right forepaw [3]: 0 – no movement; 1 – elbow flexion but cannot touch the snout; 2 – elbow flexion and can touch the snout; 3 – elbow flexion and can reach below the eye; 4 – elbow flexion and can reach the eye; and 5 – elbow flexion and can reach the ear or back of the ear.

Generally, the grasping test was performed using the grip strength meter (GSM Grip Strength Meter 47200, Italy). The tail of the mice was gently lifted until only the tested paw grasped a grid connected to an ordinary electronic balance. And then, the mice were lifted further by the tail with the paw firmly grasping the grid. At the moment the paw lost its grip, the value shown by the electric balance was recorded. 5 measurements per forepaw were performed and recorded. The time interval between each measurement was 5 min. The highest value in grams (g) was selected for the grasping strength for each mouse.

Briefly, the mice were placed in the CylinderScan (CSI, Gene&I, Beijing, China), the times for the left and right paws to touch the wall of the CylinderScan were recorded, respectively. And the total times for the left and right paws were 20.

Tissue preparation and immunofluorescence staining

Tissue preparation and immunofluorescence staining were performed according to the previous studies with minor modifications [5]. Mice (n = 5/group) were anesthetized deeply with isoflurane at 12 weeks post-surgery and transcardially perfused through the left ventricle with PBS followed by 4% paraformaldehyde (PFA) in PBS. The C5-7 segments of spinal cords were dissected and harvested. After being post-fixed in 4% PFA for 24 h at 4°C, spinal cord tissues were transferred to PBS containing 15%, 30% sucrose at 4°C, respectively, and each for 24 h. The sinking tissues were cut into sections at the thickness of 15 µm on a sliding microtome (CM 1950, Leica, Germany). The C5-7 segments of each mouse were allocated to 4 alternating series of 10 sections each. The slides were blocked with 10% normal donkey serum (NDS) in PBS at RT for 1 h, following the incubation overnight at 4°C with the following primary antibody: goat anti-choline acetyltransferase (ChAT, 1 : 500, AB144p, Millipore). After rinsing with PBS, immunoreactivity was visualized by incubation with Alexa Fluor 546 fluorescent secondary antibody (1 : 1000, Invitrogen). The sections mounted using ProLong® Gold Anti-fade reagent with 4',6-diamidino-2-phenylindole (P36935, Gibco; Thermo Fisher Scientific) were imaged under an AxioObserver A1 microscope (Carl Zeiss, Oberkochen, Germany) using AxioVision 4.6 software (Carl Zeiss).

Statistics

All statistical analyses were performed by using GraphPad Prism 6 software. Data were reported as mean ± standard deviation (SD) and analysed using ANOVA followed by the post-hoc Bonferroni test. $p < 0.05$ was considered statistical significance.

Results

EGCG increases the survival rate and the FIG4 expression in motor neurons in a dose-dependent manner

To investigate the effect of EGCG on the primary cultured MNs, the cell viability assay and ELISA assay were performed after the MNs were treated with EGCG at various concentrations (0, 0.5, 5, 50, 500 µM) for 48 h.

We observed that the survival rate was increased in MNs after the treatment of EGCG in a dose-dependent manner, with a maximum effect observed at a concentration of 50 µM (Fig. 1A). A similar pattern for the FIG4 levels in MNs was also observed (Fig. 1B).

EGCG increases the survival rate of motor neurons under H_2O_2-induced oxidative stress and neuroinflammation via FIG4

To investigate the effect of EGCG on the survival of MNs under oxidative stress and neuroinflammation, the cell viability assay was performed after the MNs were treated with 20 µM H_2O_2 for 2 h and

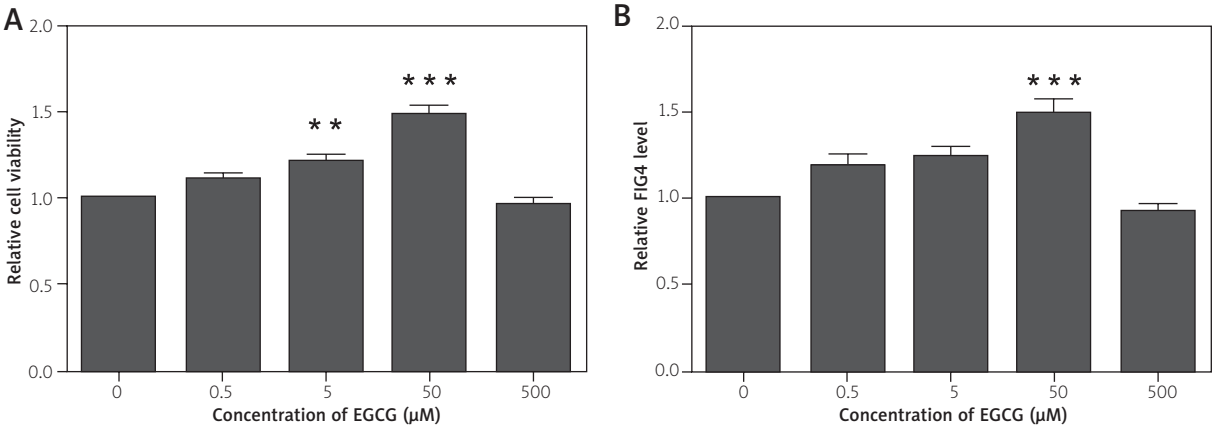

Fig. 1. Effect of EGCG on the cell viability and FIG4 expression in MNs in a dose-dependent manner *in vitro*. EGCG increased the peak levels of MNs survival and FIG4 expression at a concentration of 50 µM (**A**, **B**) (**$p < 0.01$, ***$p < 0.0001$, $n = 6$).

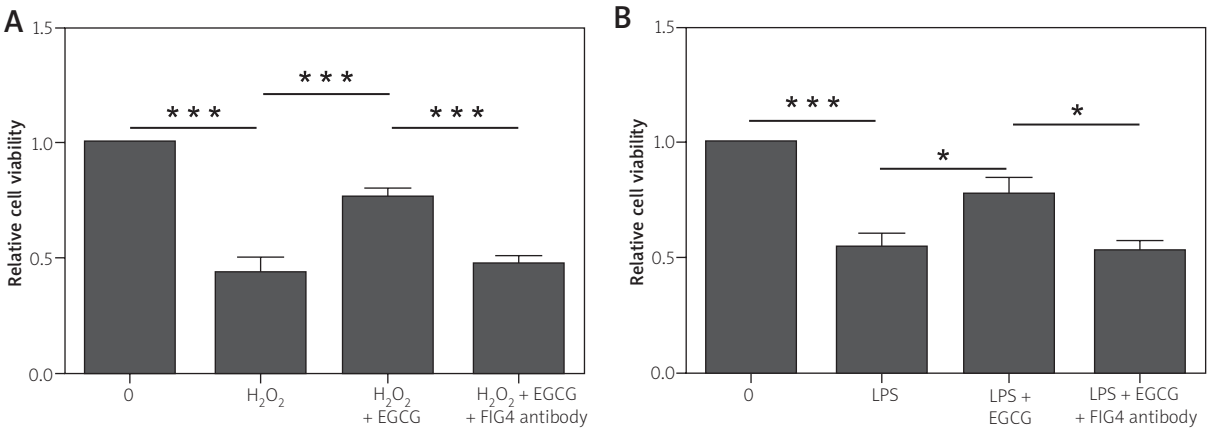

Fig. 2. Effect of EGCG on MNs survival under oxidative stress and neuroinflammation *in vitro*. **A**) EGCG protected against the cell death of MNs induced by H_2O_2. **B**) EGCG protected against the cell death of MNs induced by LPS (*$p < 0.05$, ***$p < 0.0001$, $n = 6$).

following the treatment of 50 μM EGCG for 48 h or pretreated with 50 μM EGCG for 2 h and then co-cultured with 2 μg/ml LPS for 24 h.

We found that treatment of H_2O_2 and LPS decreased the cell survival rates of MNs, whereas EGCG increased the cell survival of MNs induced by H_2O_2 and LPS; moreover, after treating the cells with the FIG4 antibody, EGCG cannot increase the cell survival of MNs induced by H_2O_2 and LPS (Fig. 2A, B).

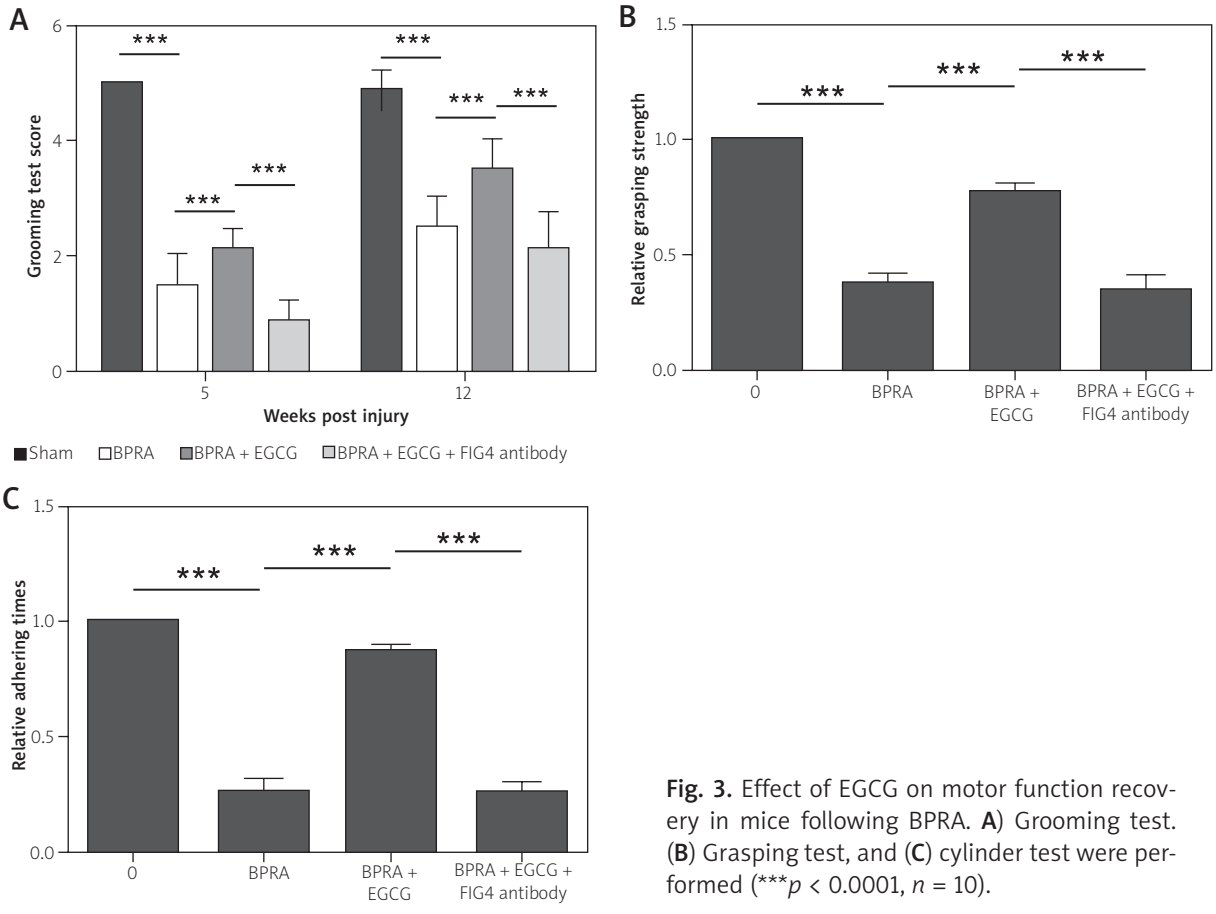

Fig. 3. Effect of EGCG on motor function recovery in mice following BPRA. **A**) Grooming test. (**B**) Grasping test, and (**C**) cylinder test were performed (***$p < 0.0001$, $n = 10$).

EGCG upregulates the FIG4 expression to promote the function recovery in mice following BPRA

To investigate the effect of EGCG on the recovery of the motor function in mice after BPRA, the grooming test, grasping test and cylinder test were performed after the injury at 6 weeks or 12 weeks.

We found that the grooming test score was decreased in response to BPRA, whereas EGCG increased the grooming test score, moreover, after the treatment of the FIG4 antibody, EGCG cannot increase the grooming test score (Fig. 3A). The similar patterns for the grasping test and cylinder test in mice following BPRA were also observed (Fig. 3B,C).

EGCG upregulates the FIG4 expression to increase the survival of motor neurons in the ventral horn in mice following BPRA

To investigate the effect of EGCG on the survival of MNs in mice after BPRA, immunofluorescence staining was performed after injury at 12 weeks.

We found that the cell survival rates of MNs were decreased in response to BPRA, whereas EGCG increased the cell survival of MNs, moreover, after treating the cells with the FIG4 antibody, EGCG cannot increase the cell survival of MNs.

Discussion

In the present study, we observed that EGCG increases the survival rate of MNs after BPRA. Moreover, it also modulates FIG expression to be against the oxidative stress and neuroinflammation. This may suggest that EGCG may modulate FIG4 to promote the motor function recovery in mice after BPRA.

Several experimental studies have shown that EGCG can provide neuroprotection against brain, spinal cord injury and sciatic nerve injury [28,36]. These benefits are mainly due to free radical scavenging or the antioxidant, anti-inflammatory and anti-apoptotic properties of EGCG [32,33]. EGCG was verified to modulate the cell cycle and cell signalling [25] and be against liver injury via its anti-inflammatory and antioxidant effects [27]. In the present study, we indicate

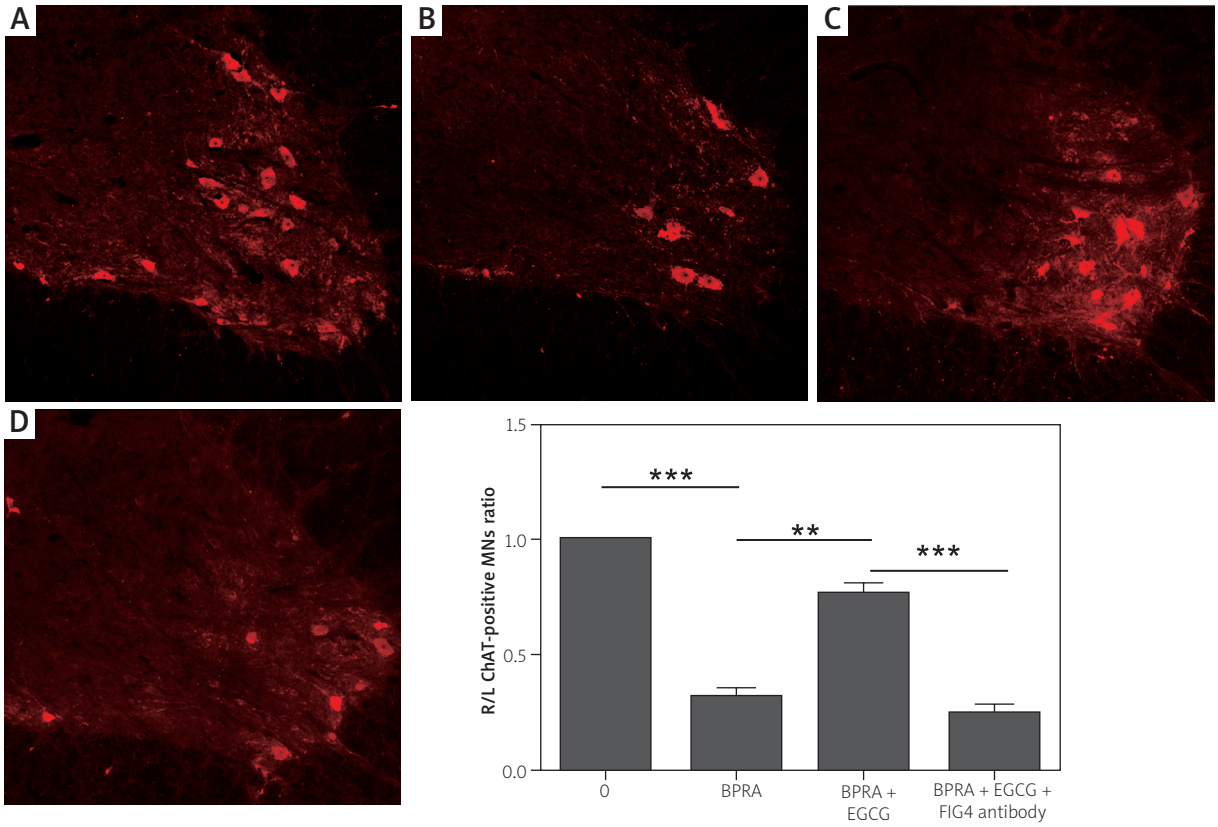

Fig. 4. Effect of EGCG on MN survival in the ventral horn in mice following BPRA. **A-D**) The number of MNs were decreased in response to EGCG treatment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$, $n = 5$).

that EGCG can promote the cell survival of MNs under oxidative stress and neuroinflammation.

FIG4, encoded by the *FIG4* gene located on chromosome 6q21, is a phosphatase interacting with PIKfyve in mammalian cells or phosphatidylinositol-5-phosphate (PI5P) kinase Fab1 in yeast [18]. A protein complex formed by Fig4/PIKfyve and the kinase activators vac7 and 14 in yeast or arPIKfyve in mammalian cells regulates the yield of phophatidylinositol-3,5-diphosphate (PI3,5P2). The loss of function of Fig4 may reduce the levels of PI3,5P2 [21,22]. In the nervous system Fig4/PI3,5P2 signalling seems to be crucial for cell survival. Loss of FIG4 function in patients following Charcot-Marie-Tooth type 4J (CMT4J) may reduce approximately 71% of PI3,5P2 levels and be associated with severe neuronal degeneration [8,31,44]. In addition, vac14 (or arPIKfyve)-knockout mice show an approximately 50% decrease in the PI3,5P2 level and profound neuronal degeneration [19,45].

Taken together, treatment with EGCG partially reverses the damage to MNs after BPRA via regulating FIG4. This may be helpful for the treatment of the neuronal injury, especially for the disorders related to MN survival.

Although the results look promising, our study still had some limitations: in this paper, we mainly concentrated on the survival of MNs according to the previous study [16], further studies are no doubt needed to be performed to evaluate the pathological changes of musculocutaneous nerve and biceps brachii muscles in response to the treatment of EGCG.

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Disclosure

The authors report no conflict of interest.

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