(-)-Epigallocatechin Gallate Attenuates Spinal Motoneuron Death Induced by Brachial Plexus Root Avulsion in Rats

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Research Article

Keywords: root avulsion, brachial plexus, epigallocatechin gallate, c-jun phosphorylation, motoneuron death

Posted Date: April 30th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-442780/v1

Abstract

Background

Recent studies have indicated that epigallocatechin gallate (EGCG) benefits a variety of neurological insults. This study was performed to investigate the neuroprotective effect of EGCG after brachial plexus root avulsion in rats.

Methods One hundred twenty rats were randomized into the following three groups: an EGCG group, an Avulsion group, and a Sham group. EGCG (100 mg/kg, i.p.) or normal saline was administered to rats immediately following the injuries. The treatment was continued from day 1 to day 7, and the animals were sacrificed on days 3, 7, 14 and 28 for Nissl staining, immunohistochemistry and western blot analysis.

Results We determined that EGCG significantly increased the survival ratio of motoneurons and inhibited the cell apoptosis. The level of phospho-c-jun was reduced at 3d and 7d after the injury.

Conclusions Our results indicated that motoneurons were protected by EGCG against the cell death induced by brachial plexus root avulsion,and this effect was correlated with inhibiting c-jun phosphorylation.

Introduction

Brachial plexus root avulsion is a neurological complication of shoulder injury, most frequently occurring in traffic accidents(1).Brachial plexus root avulsion often involves avulsion of multiple nerve roots of the cervical spinal cord, resulting in mass death of motor neurons and permanent paralysis of innerved muscles(2–6). After avulsion of brachial plexus, the pain involved peripheral and central tissues. Thus, it is characterized by a mixed (central and peripheral) neuropathic pain syndrome(7). It places an enormous burden on individuals, families and society. Therefore, the effective treatment of brachial plexus avulsion injury and the exploration of its mechanism are very urgent scientific topics(8).

Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea(9). Several epidemiological studies in animal models have shown that EGCG benefits a variety of disorders that range from cancer to weight loss(10–12). It has been shown that EGCG can pass the blood-brain barrier to reach the brain parenchyma(13–17). Recent research has shown that EGCG has a potential neuroprotective effect in various pathological states in the nervous system(18–24). However, the mechanisms behind these actions have not been fully elucidated.

The survival of spinal motoneurons is the key to the recovery of motor function(25).Given the demonstrated effects of EGCG on neuroprotection, we explored whether EGCG administration had any beneficial effects on the prevention of the degeneration of motoneuron following brachial plexus injury and further investigated its underlying mechanism.

Materials And Methods

Animal Preparation

A total of 120 healthy SD rats were used for this study. These animals were randomly allocated into the following three groups: an EGCG group, an Avulsion (control) group, and Sham group. There were 40 rats in each group. The animals in each group received daily doses of 100 mg/kg EGCG (Hangzhou Gosun Biotechnologies Co., Ltd., China) as described in our previous studies(26) or normal saline i.p. from day 1 to day 7. The animals received treatment immediately following the injuries. Five rats were randomly selected from each group on days 3, 7, 14 and 28 post-surgery for the harvesting of spinal cord samples for Nissl staining, immunohistochemistry and western blot analysis. All the experiments were performed in conformity with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were conducted in accordance with the relevant guidelines and regulations. The experimental studies followed the recommendations in the ARRIVE guideline. All experiments were approved by the Ethics Committee of the Fourth Affiliated Hospital of China Medical University. All of the experimental animals were housed in the same conditions (i.e., a controlled temperature of 22°C on a 12-h light/dark cycle) and food and water were provided to the rats.

Surgical Procedures

The root avulsions of the left brachial plexus were performed according to the procedures described in previous publications(27-31). Briefly, the animals were anesthetized with 350 mg/kg of 10% chloral hydrate via intraperitoneal injections. Following anesthesia, the skin was shaved and cleaned with povidone iodine, and the surgeries were performed under sterile conditions.The left brachial plexus was exposed, and the left cervical C5- T1 nerve roots were isolated under a surgical microscope in the supine position. Extravertebral root avulsion was performed by pulling the spinal nerves out one by one with microhemostatic forceps. The avulsed ventral and dorsal roots, together with the dorsal root ganglia, were cut away from the distal ends of the spinal nerves and confirmed under the microscope. For the sham-operated controls, similar procedures were performed until the left brachial plexus was exposed and identified, but not damage to the nerves was inflicted. The surgical wounds were closed in layers. The animals were allowed to recover, and they were returned to their cages upon awakening.

Perfusion and tissue preparation

At the end of the survival time, the animals were deeply anesthetized with an overdose of 10% chloral hydrate and perfused transcardially with 100 ml of salinefollowed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. After perfusion, the C6- C8 segments were quickly removed and postfixed for 24 hours in the same fixative at 4°C overnight. The C6- C8 segments were defined as the region between the uppermost root of the C6 nerve and the lowermost root of the C8 nerve of the contralateral cord. The fixed segments were dehydrated and embedded in paraffin sections. Serial 10-μm thick sections of the segments were cut transversely. Every fifth section from each animal was used for Nissl staining and immunohistochemisty analysis.

Nissl staining

After deparaffinization and rehydration, the sections were stained with warmed 0.5% cresyl violet solution (10 minutes) as described(32). Next, the sections were dehydrated in increasing concentrations of ethanol and cleared via immersion in xylene for 2 min before cover slipping with Permount. Both sides of the motor neurons located in the anterior horn that had maximum diametersof no less than 20 μm and contained a well-defined nucleolus and a soma rich in Nissl bodies, were counted by two investigators who were blinded to the grouping(33). The numbers of surviving motoneurons on the injured side were described quantitatively as the percentage of the surviving motoneurons on the contralateral side in the same section(28,29,33).

IHC staining

An avidin-biotin kit was used for the immunohistochemical staining. Briefly, the sections were deparaffinized and treated with 3% H_2O_2 for 15 min to block the endogenous peroxidase. The sections were exposed to normal bovine serum for 30 min and then incubated with the primary antibodies overnight at 4°C. These antibodies included rabbit monoclonal anti-p-JNK antibody (diluted 1:50, Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal anti-p-c-Jun(Ser73) antibody (diluted 1:50, Cell Signaling Technology, Danvers, MA, USA) and rabbit monoclonal anti-caspase-3 antibody (diluted 1:200, Cell Signaling Technology, Danvers, MA, USA). After washing 3 times with 0.01M PBS (PH 7.4), the sections were incubated with the appropriate secondary antibody (Boster, China) for 15 min at 37°C. The immunolabeling was visualized as brown using diaminobenzidine, and counterstaining was performed with hematoxylin. Sections stained without primary or second antibodies served as negative controls. The motoneurons with yellowish-brown nuclei in the p-JNK or p-c-Jun(ser73) immunohistochemical sections were counted as a positive cells, and motoneuron with yellowish-brown cytoplasm in the caspase-3 immunohistochemical sections were counted as positive cells. The results were counted by two observers (who were blind to the groups) and averaged to obtain the final countfor each section. The numbers of IHC-positive motoneurons in each rat are expressed as the total numbers in 10 serial IHC-stained sections(27,34).

Western blot

For the western blot assays, the spinal cord (C6-C8) was quickly removed and preserved in liquid nitrogen for further analysis. After homogenization in RIPA buffer, the samples were centrifuged at 12,000 g for 30 min at 4°C. The protein concentrations of soluble materials were determined by the Coomassie G250 binding method. The protein samples were separated on 10% polyacrylamide gels containing 0.1% SDS, followed by transfer to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skimmed milk for 2 h and then incubated with primary anti-JNK antibody (dilution 1:000,Santa Cruz Biotechnology, Burlingame, CA, USA), anti-p-JNK antibody (dilution 1:000, Cell Signaling Technology, Danvers, MA, USA), anti-c-Jun antibody (dilution 1:000, Cell Signaling Technology, Danvers, MA, USA), anti-p-c-Jun(Ser73) antibody (dilution 1:000, Cell Signaling Technology, Danvers, MA, USA) or β-actin

(dilution 1:2000,Santa Cruz Biotechnology, Burlingame, CA, USA) at 4°C overnight. The appropriate secondary antibodies (goat anti-rabbit or -mouse IgG conjugated with horseradish peroxidase) were subsequently used at room temperature for 1 h. Finally, the EC3 Imaging System (UVP Inc., Upland, CA, USA) was used to quantify the p-JNK, JNK, p-c-Jun, c-Jun and β-actin protein bands. Quantification was performed using an ImageJ software (NIH, Bethesda, MD, USA) on a computer.

Statistical Analyses

The data are presented as the mean ± SD and were analyzed using the SPSS 20.0 software. The statistical analyses were performed with one-way analyses of variance (ANOVA) followed by Student-Newman-Keuls tests. P values less than 0.05 were considered significant. The data quantification and analysis were performed by two independent persons who were blinded to the injury.

Results

Survival of the Injured Motoneurons

The effects of EGCG on motoneuron survival were investigated after the avulsion of the brachial plexus roots. Significant decreases of motoneurons in the spinal cords were observed from days 14 to 28 after the injury. EGCG treatment caused significant increases in the percentage of surviving motoneurons at days 14 (control: 65.68±4.05%, EGCG-treated: 77.98±2.35%, n=5, $P<0.05$) and 28 (control: 43.47±3.13%, EGCG-treated: 60.39±4.02%, n=5, $P<0.05$) compared to the control animals (Fig. 1). No significant differences between the control and EGCG-treated rats were present at days 3 or 7 based on the Nissl staining results.

Apoptosis in Motoneurons

As shown in Fig. 2, the number of caspase-3-positive motoneurons was significantly increased in the Avulsion group compared to the Sham group at 3d, 7d, 14d and 28d after the injury. The numbers of caspase-3-positive motoneurons increased from day 3 (control: 105.6±3.6, EGCG-treated: 84.4±3.3) , with peak at day 7 (control: 134.6±4.7, EGCG-treated: 95.0±3.4), and then descended sharply to day 28 (control: 61.8±2.2, EGCG-treated: 59.2±2.2). Fewer caspase-3 positive motoneurons were observed in the EGCG-treated animals than in the control animals. At days 3 and 7 after avulsion, the numbers of caspase-3-positive motoneurons in the EGCG-treated animals were significantly fewer than in the control animals (n=5, all $P<0.05$), whereas at days 14 and 28 after avulsion, no significant difference was observed between the two groups.

Effects of EGCG on phospho-JNK expression

The numbers of p-JNK-positive motoneurons were significantly increased in the Avulsion group compared to the Sham group at 3d and 7d after injury (n=5, all $P<0.05$). Subsequently, gradual decreases in the p-JNK positive motoneurons were observed from days 3 to 28. The numbers of p-JNK-positive motoneurons were lower in the EGCG-treated rats than in the control rats at each time point as shown in

Fig 3 (B-C). Statistical analyses revealed that there were no significant differences between the Avulsion group and the EGCG-treated group after injury at any time point.

The Western blot (WB) analyses produced the representative gels for JNK, p-JNK and β-actin that are shown in Fig 4 (A). Semiquantitative changes in the level of p-JNK normalized to JNK expression after root-avulsion were determined by OD measurements. As shown, the ratio of p-JNK/JNK was lower in the Sham group following injury than in the Avulsion group at 3d and 7d after injury (n=5, all $P<0.05$). However, there were no significant differences between the Avulsion group and the EGCG group at any time point ($p>0.05$).

Effect of EGCG on phospho-c-jun expression

The IHC result for phospho-c-jun are shown in Fig.5. At 3d, 7d and 14d after injury, the numbers of p-c-jun-positive motoneurons were significantly increased in the Avulsion group compared with the Sham group. The numbers were significantly lower in EGCG-treated group compared with the Avulsion group at 3d and 7d after injury ($p<0.05$), whereas at days 14 and 28 after avulsion, no significant differences were observed between the two groups.

Representative WB gels for p-c-jun, c-jun and β-actin are shown in Fig. 6. Semiquantitative changes in the level of phospho-c-jun normalized to c-jun expression after root-avulsion were determined by OD measurements. As shown, the ratio of p-c-jun/c-jun in the EGCG group exhibited drops compared to the Avulsion group at 3d and 7d after injury ($p<0.05$, Fig. 3 A,C). However, at 14d and 28d after injury, there were no significant differences between the two groups.

Discussion

Approximately 70% of severe brachial plexus injuries in humans involve avulsion of one or more of the roots(35). Brachial plexus avulsion leads to serious conditions. It is known that numerous morphological, physiological and biosynthetic changes occur in the damaged neurons following peripheral nerve injury(36–40). Following avulsion injuries, massive motoneuron death occurs(27, 34).

Presently, strategies for the treatment of brachial plexus avulsion include nerve grafting, nerve transfer, nerve suture (neurorrhaphy), neurolysis and late peripheral nerve reconstruction(41–46). However, the overall outcomes of these surgical strategies regarding upper limb and hand function remains poor. One possible reason is the death of a major neuronal pool(47, 48). There are not sufficient regenerating motoneurons that send axons to innervate the peripheral targets to achieve significant functional recovery. Therefore, the enhancement of the survival of damaged neurons after injury is a determinant of surgical effectiveness(3, 48).

EGCG is the main catechin polyphenol of tea and has been found to easily pass the BBB and penetrate into the CNS(13–17). It has also been reported that EGCG exhibits neuroprotective actions against a variety of injuries(49–55). In our previous studies of EGCG treatments of central nervous system injuries,

100 mg/kg doses of EGCG were used and exhibited good efficacy(48). Therefore, we chose a 100 mg/kg does of EGCG for the treatment of brachial plexus avulsion in the current study. In the present study, we found that the motoneurons were protected by EGCG against the death induced by brachial plexus root avulsionas evidenced by our present data.

The data from the present study revealed significant increases in phospho-c-jun in the spinal cords following brachial plexus root avulsion. Moreover, the expression of p-JNK temporarily increased at 3d and 7d, which corresponded to the changes of c-jun phosphorylation. In contrast, there were significant changes in the levels of phospho-jun but not phospho-JNK between the Avulsion and EGCG groups.

Previous studies revealed that there are three different sets of c-jun signaling effects: the classical JNK to c-jun signaling, the non-c-jun-mediated effects of JNK and the JNK-independent effects of c-jun(56). Moreover, there are three JNK (JNK1, JNK2, and JNK3) genes, and each specific isoform can play differential roles in neuronal function(57–59). In the present study, the phosphorylation of c-jun might have been activated by specific JNKs following brachial plexus root avulsion. However, our primary antibody for JNK could not detect changes in specific JNK isoform levels in the IHC and Western blot studies, because it cannot differentiate the three JNK isoforms. In the present study, it was difficult to determine which isoform of JNK was responsible for the changes in c-jun, and this issue requires further research.

C-jun is a functional component of the AP-1 complex, and the response of this gene to axonal insult is early and consistent. It has been suggested that the c-jun gene in the mammalian nervous system exerts dual functional regulation in both neuronal death and survival[24]. A series of studies has suggested the contributions of c-jun to neuroprotection and nerve regeneration(33, 37, 60–64). These studies support the hypothesis that the up-regulation of c-jun is helpful for improving the viability of motoneurons following axonal injury.

Although the molecular mechanisms behind these actions have not been fully elucidated, other studies have shown that the c-jun acts as a “killer protein” in several neuronal injury models and in developing neurons and is involved in the processes of programed cell death(60, 65–67). C-jun has been shown to be associated with neuroprotection and nerve regeneration,and the down-regulation of c-jun exerts positive effects in the protection of spinal motoneurons after injury(68–70). Previous studies have also indicated that the phosphorylation of c-jun is associated with neuronal death following axonal injury and that the inhibition of the phosphorylation of c-jun reduces motoneuron death. Phosphorylated c-jun triggers apoptosis in motoneurons, and the prevention of the phosphorylation of c-jun in the early phase of root avulsion can prevent the death of motoneurons(37, 67, 71, 72).

In the current study, both IHC and Western blot methods showed that the level of phosphorylated c-jun was significantly increased from 3d to 14d after the injury, while the treatment of EGCG could obviously decrease its level. The results indicated that neuroprotective effect of EGCG after brachial plexus root avulsion might though decrease phosphospho-c-jun level.

Conclusions

In our investigation, we observed that the motoneurons that were injured due to brachial plexus root avulsion were protected against death by EGCG, as evidenced by the main results. Moreover, the EGCG treatments significantly decreased the level of phosphorylated c-jun following brachial plexus root avulsion. The present results imply that a neuroprotective effect was exerted by EGCG and that this effect was possibly mediate by inhibiting phosphorylation of c-jun. Future studies should employ JNK isoformspecific knockout rats to confirm which JNK isoforms are mediated by EGCG.

Declarations

Acknowledgments

This study was supported by the General program of China Postdoctoral Science Foundation(2019M66166) and Liaoning Provincial Doctoral Fund (20180540065) for the writing and publication of the manuscript.

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Figures

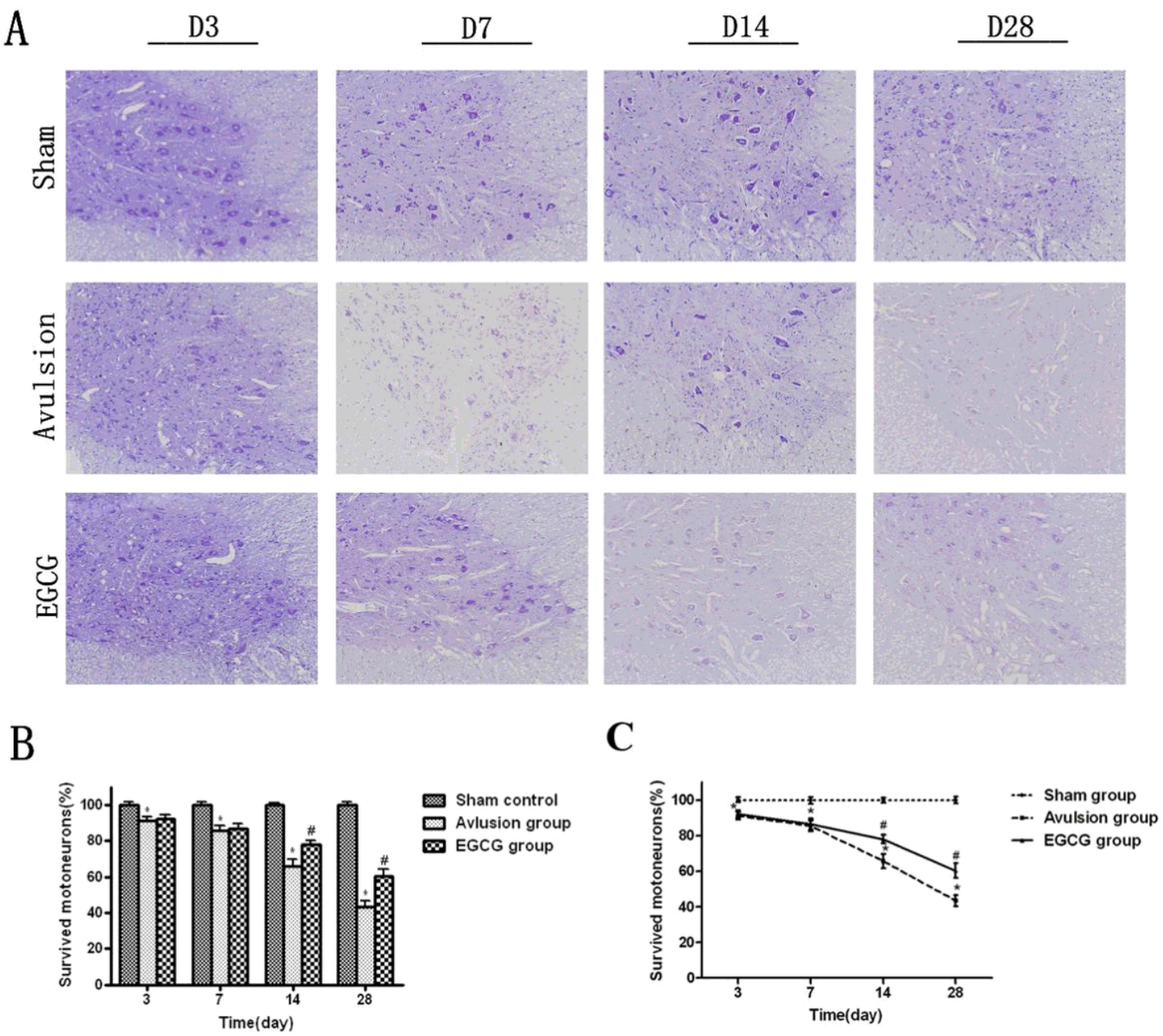

Figure 1

(A) Nissl staining of transverse sections of the spinal cord. (B,C) Percentages of surviving motoneurons in the spinal cord sections of the rats.The bars represent the means±the SDs (n=5 every group, *P<0.05 between the Sham and Avulsion groups, #P<0.05 between the Avulsion and EGCG groups; D14, 14 days after injury; D28, 28 days after injury; Scale bar = 100μm).

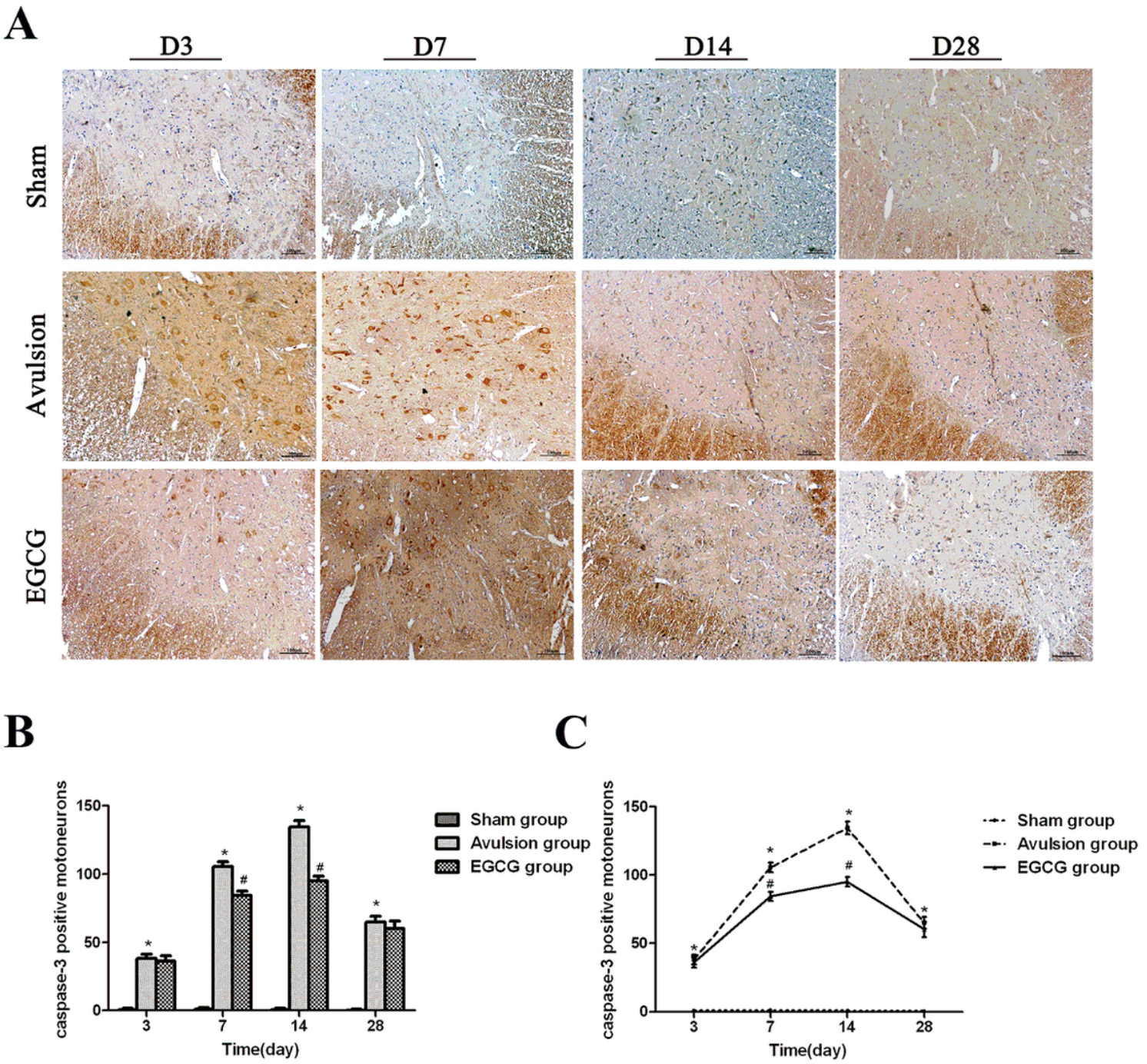

Figure 2

(A) Immunohistochemical images of caspase-3-positive motoneurons. (B,C) Percentages of caspase-3-positive motoneurons in the spinal cord sections of the rats at the indicated times.The bars represent the means±the SDs (n=5 every group, *P<0.05 between the Sham and Avulsion groups, #P<0.05 between the Avulsion and EGCG groups; D3, 3 day after injury; D7, 7 days after injury; D14, 14 days after injury; D28, 28 days after injury; Scale bar = 100μm).

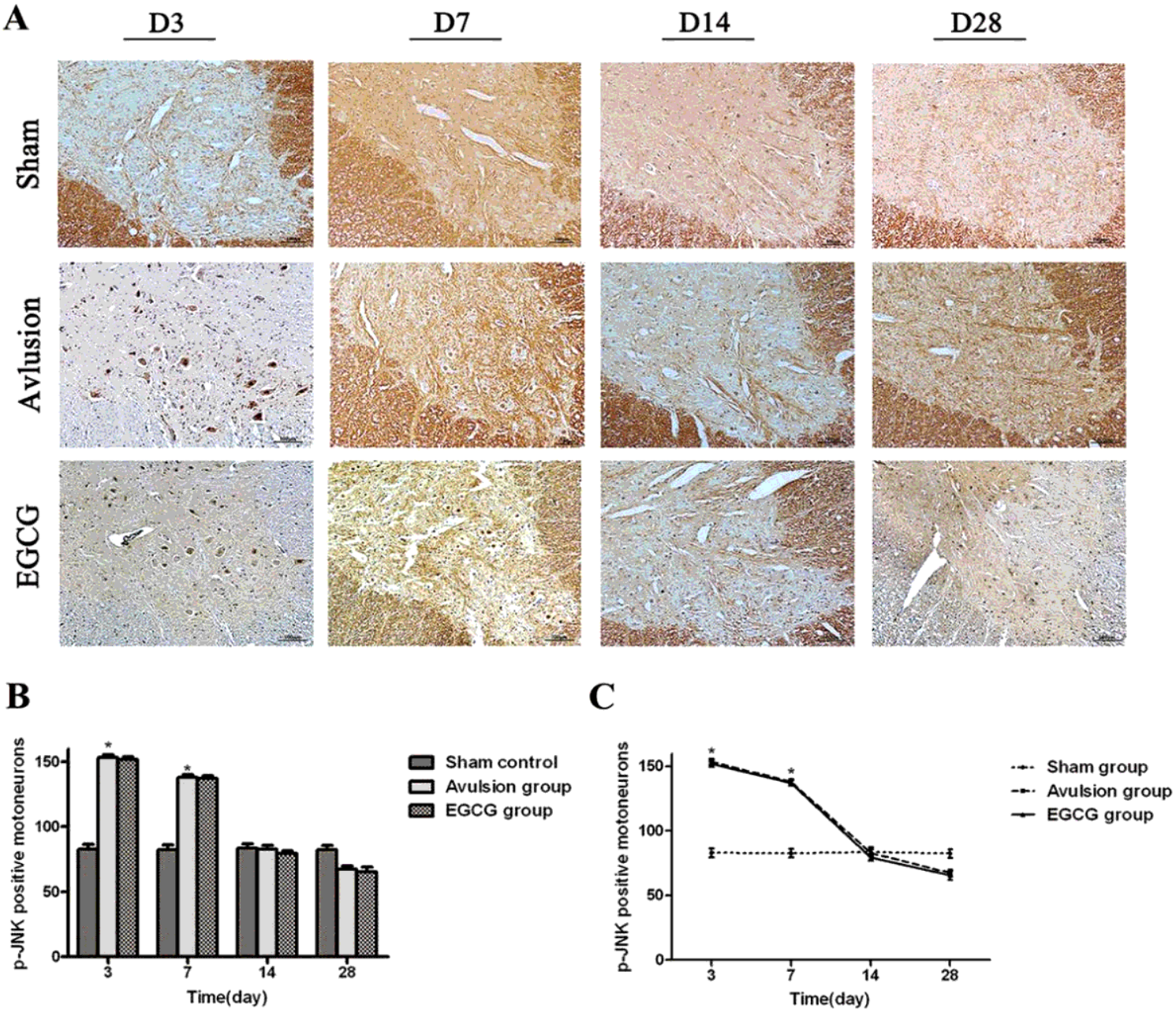

Figure 3

(A) Immunohistochemical images of phospho-JNK-positive motoneurons. (B,C) Percentages of phospho-JNK-positive motoneurons in the spinal cord sections of the rats at the indicated times.The bars represent the means±the SDs (n=5 every group, *P<0.05 between the Sham and Avulsion groups; D3, 3 day after injury; D7, 7 days after injury; D14, 14 days after injury; D28, 28 days after injury; Scale bar = 100μm).

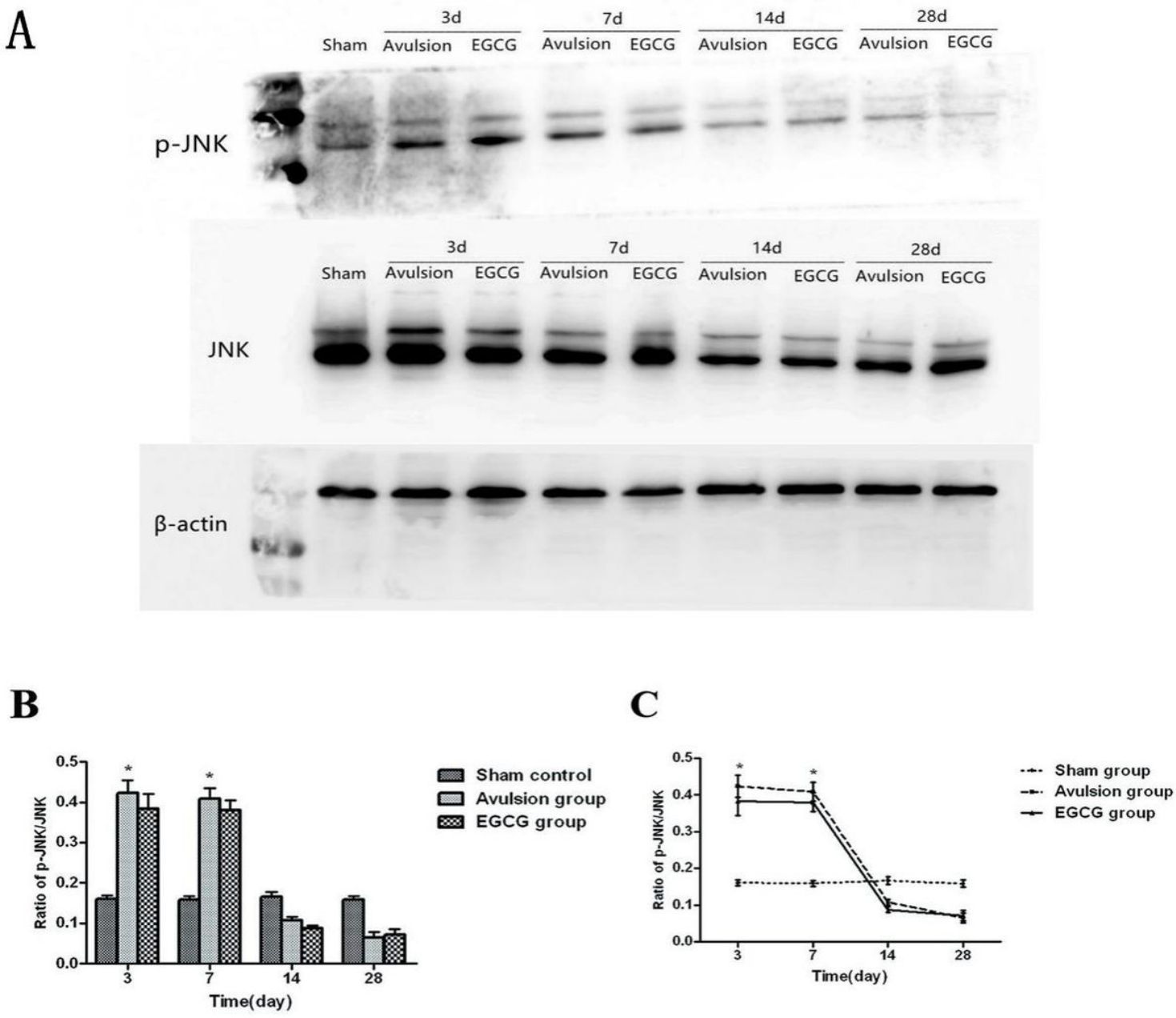

Figure 4

Western blot analyses of the JNK and phospho-JNK levels in the ipsilateral spinal segments following root avulsion of the left brachial plexuses of adult rats. (A) The samples were obtained from rats that were subjected to sham operations or root-avulsions at 3d, 7d, 14 d and 28d post-injury. The optical density(OD) of each protein was measured from the Western blot. (B,C) Semiquantitative changes in the level of phospho-JNK normalized to JNK expression after root-avulsion were determined by OD measurements. The data are presented as the means±the SDs (n=5 every group, *P<0.05 between the Sham and Avulsion groups; D3, 3 day after injury; D7, 7 days after injury; D14, 14 days after injury; D28, 28 days after injury).

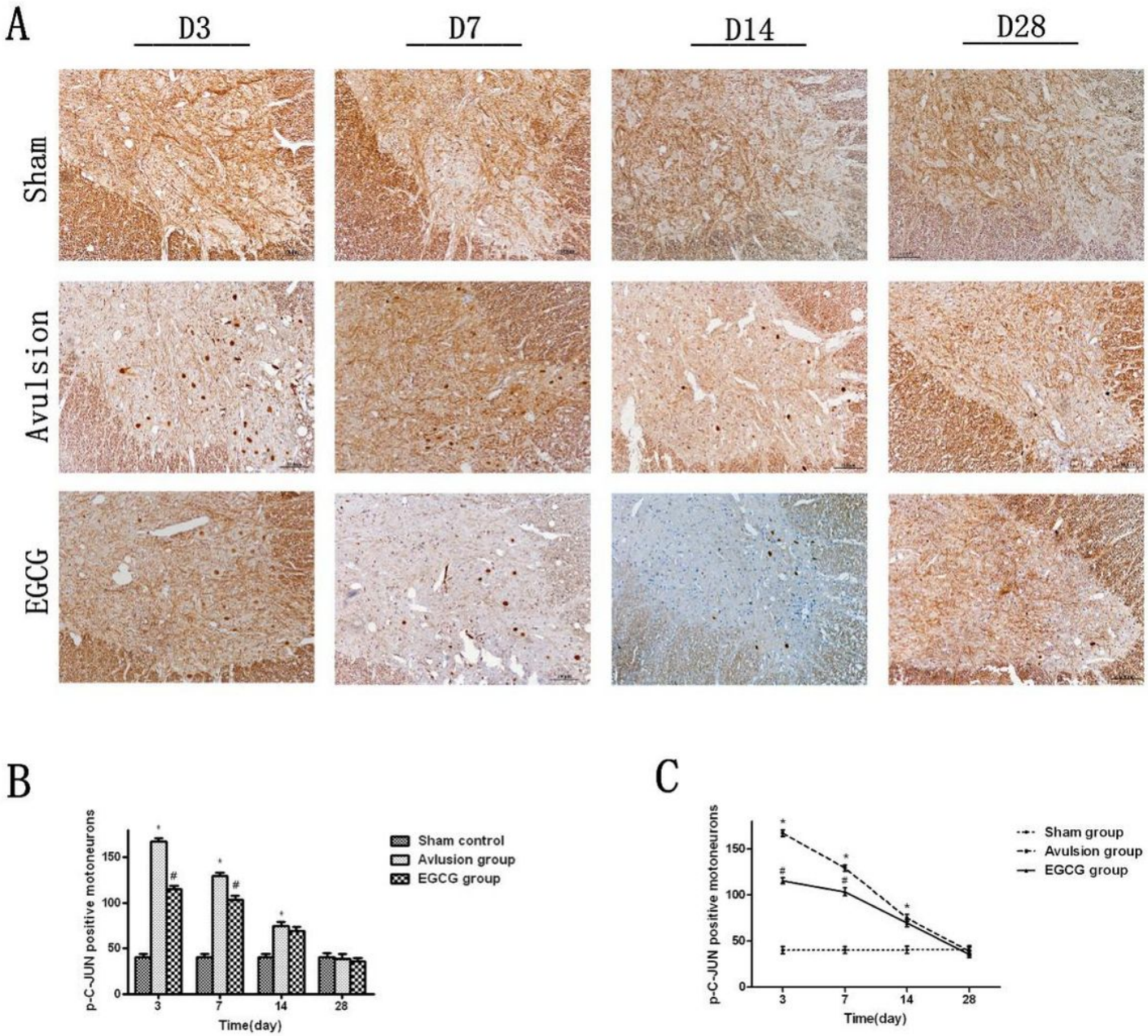

Figure 5

(A) Immunohistochemical images of phospho-c-jun-positive motoneurons. (B,C) Percentages of phospho-c-jun-positive motoneurons in the spinal cord sections of the rats at the indicated times.The bars represent the means±the SDs (n=5 every group, *P<0.05 between the Sham and Avulsion groups; D3, 3 day after injury; D7, 7 days after injury; D14, 14 days after injury; D28, 28 days after injury; Scale bar = 100μm).

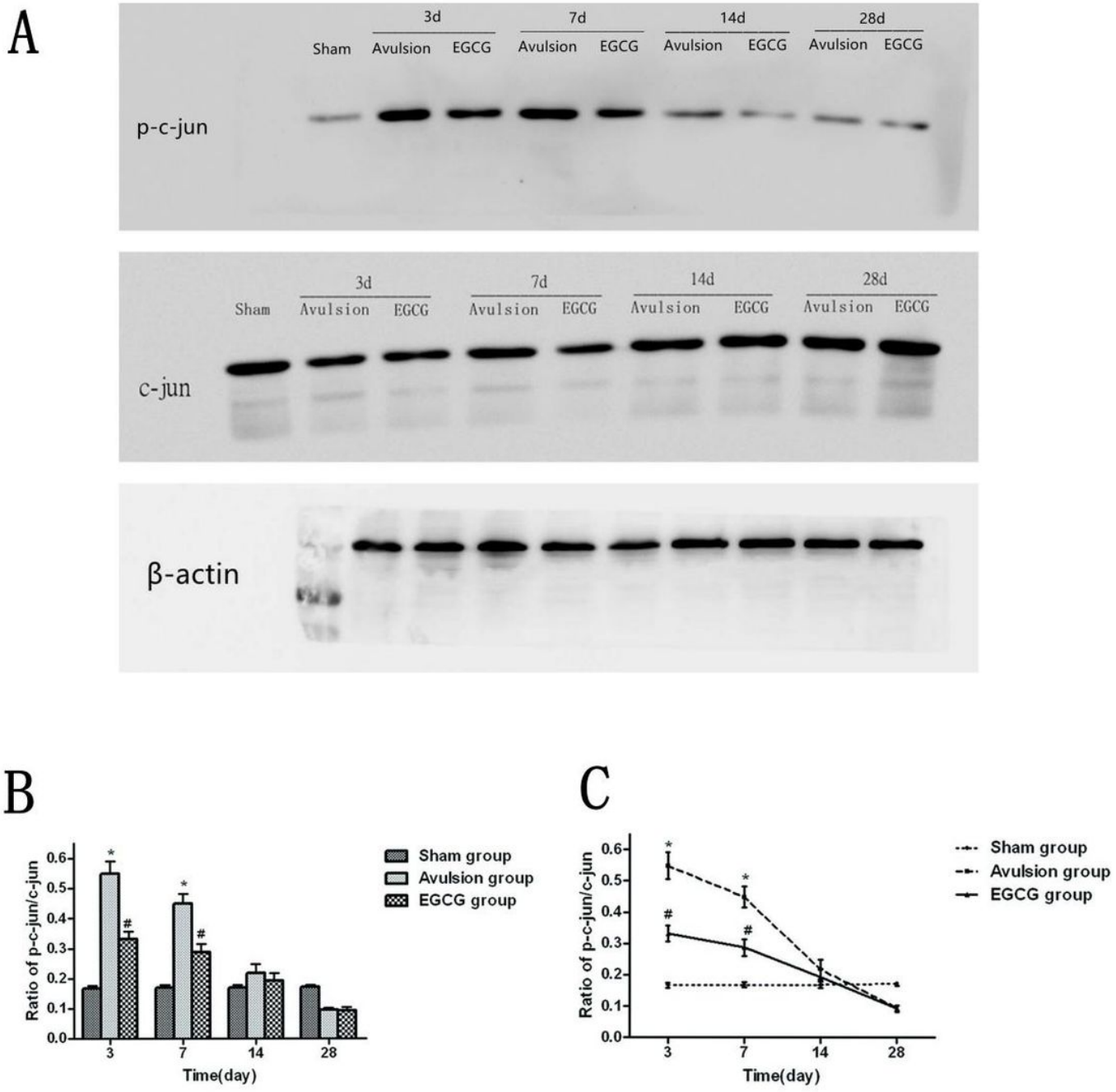

Figure 6

Western blot analyses of the c-jun and phospho-c-jun levels in the ipsilateral spinal segments following root avulsion of the left brachial plexuses of adult rats. (A) The samples were obtained from rats that were subjected to sham operations or root-avulsions at 3d, 7d, 14 d and 28d post-injury. The OD of each protein was measured from the Western blot. (B,C) Semiquantitative changes in the level of phospho-c-jun normalized to c-jun expression after root-avulsion were determined by OD measurements. The data are presented as the means±the SDs (n=5 every group, *P<0.05 between the Sham and Avulsion groups,

#P<0.05 between the Avulsion and EGCG groups; D3, 3 day after injury; D7, 7 days after injury; D14, 14 days after injury; D28, 28 days after injury)