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Acute Spinal Cord Injury in Rats Induces Autophagy Activation

Sıçanlarda Akut Omurilik Hasarı Otofaji Aktivasyonunu İndükler

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ABSTRACT

AIM: Autophagy is an important process that balances cellular protein synthesis and degradation and is involved in many physiological and pathological conditions. However, the precise role of autophagy has not yet been defined in the model of spinal cord injury (SCI).

MATERIAL and METHODS: Here, we utilized a hemisection model of acute SCI to elucidate the role of autophagy in the pathological processes underlying SCI.

RESULTS: LC3B-II, a well-known marker of autophagy, was immunohistochemically detected 4H after SCI, peaked at 3D, and decreased at 21D. Hematoxylin-eosin (HE) staining confirmed accurate spinal cord hemisection, which was accompanied by both neuronal swelling and shrunken neurons with darkly stained, condensed nuclei. These findings suggest that the process of autophagy is related with pathological changes following SCI.

CONCLUSION: Our results indicate autophagy is involved in the pathological changes after SCI, and potential therapies to promote neuronal regeneration following SCI should target the mechanism of autophagy.

KEYWORDS: Acute spinal cord injury, Autophagy, LC3, Neuronal regeneration, HE staining, Rat

ÖZ

AMAÇ: Otofaji, hücre protein sentezini ve degradasyonunu dengeleyen önemli bir süreçtir ve birçok fizyolojik ve patolojik durumla ilişkilidir. Ancak otofajinin omurilik yaralanması modelinde tam rolü tanımlanmamıştır.

YÖNTEM ve GEREÇLER: Burada otofajinin omurilik yaralanmasının temelindeki patolojik süreçlerdeki rolünü açıklığa kavuşturmak üzere akut omurilik yaralanması için bir hemiseksiyon modeli kullandık.

BULGULAR: Otofajinin iyi bilinen bir işareti olan LC3B-II, omurilik yaralanmasından 4 saat sonra immünohistokimyasal olarak saptandı, 3 günde tepe düzeye çıktı ve 21 günde azaldı. Hematoksilen eozin (HE) boyama hassas omurilik hemiseksiyonunu doğruladı ve beraberinde hem nöronal şişme hem de koyu boyanan, kondanse çekirdekli küçülmüş nöronlar vardı. Bu bulgular otofaji sürecinin omurilik yaralanmasında patolojik değişikliklerle ilişkili olduğunu düşündürmektedir.

SONUÇ: Sonuçlarımız otofajinin omurilik yaralanması sonrasında patolojik değişikliklerle ilgili olduğuna işaret etmektedir ve omurilik yaralanması sonrasında nöron rejenerasyonunu desteklemek amaçlı potansiyel tedaviler otofaji mekanizmasını hedeflemelidir.

ANAHTAR SÖZCÜKLER: Akut omurilik yaralanması, Otofaji, LC3, Nöron rejenerasyonu, HE boyama, Sıçan

INTRODUCTION

Autophagy is a cellular "self-eating" of damaged organelles and long-lived proteins (15,17) and is known to participate in various diseases, such as cancer (17), infection (12), heart disease (1), and vascular disease (9). Autophagy is a static metabolism process during periods of nutrient availability, when it eliminates dysfunctional or damaged organelles and long-lived proteins. During times of nutrient deprivation, autophagy increases and recycles aging proteins back to their amino acid and fatty acid constituents to sustain the cell (25). In addition, autophagy can suppress tumor development, eliminate bacterial infection, and is involved in ischemia-reperfusion injury. Recently, many studies have investigated autophagy activation during neuronal regeneration. For example, autophagy is deregulated with aging, and enhanced autophagy may slow down the symptoms of Alzheimer's disease (7,20,27). Conversely, in a model of Parkinson's disease (4,5,11,30), the expression of autophagy-related proteins was upregulated, suggesting that the process was enhanced.

Here, we determined whether autophagy was activated in a model of acute spinal cord injury (SCI). Light chain 3 (LC3) was used to assess the time course of autophagy following spinal cord hemisection at different time points. Hematoxylin-eosin (HE) staining was performed to investigate the relationship between autophagy and pathological changes. These results are to elaborate the role of autophagy in the process of pathological changes after SCI.

MATERIAL and METHODS

Animals' Preparation and Operation

All procedures were in compliance with the guidelines for animal scientific procedures approved by the host institution's ethical committee. A total number of 48 Sprague Dawley rats weighing 200-250g were randomly divided into two groups: control (laminectomy only) and SCI group, which included five sub-groups, 4H (hour), 3D (day), 7D, 14D and 21D after injury. Before surgery, all animals were housed three or four per cage for 1 week to adapt to the new environment (25°C on a 12-h light/dark cycle).

Mice were anesthetized with 10% pentobarbital sodium (300 mg/kg, intraperitoneal [i.p.]) (28). The skin was sterilized, and an incision was made to expose the dorsal muscles, which were then divided in layers. A laminectomy was performed at the T9-T10 level with the help of a dissecting microscope to expose the spinal cord. A dorsal hemisection (right side) was performed at T9-T10 (16), and residual fibers were removed from the lesion site. After that, the muscles and skin were sutured in layers. During surgery, body temperature was recorded and maintained at 37°C with a heating pad. Following surgery, the bladder was manually expressed three times a day until self-voiding bowel function recovery. The control group was also operated on, but the spinal cord was not hemisected.

Tissue Preparation

After surgery, animals at each time point were transcardially perfused with physiological saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). About 1.5 centimeters of spinal cord around the lesion site was collected and immersed in the same fixative for further sectioning. The spinal cord samples were then postfixed in 30% sucrose in phosphate-buffered saline (PBS) overnight until the tissue sank. Next, the samples were frozen, and serial 20-μm transverse and longitudinal sections were taken around the SCI epicenter and mounted on slides.

Immunohistochemistry

For further immunohistochemical staining, the samples were washed in PBS three times for 5 min each and boiled in 0.1% Trisodium citrate for 15 min for antigen retrieval. Next, the samples were incubated with blocking reagent for 1 h at room temperature and further incubated with anti-LC3 polyclonal rabbit antibody (1:200, Sigma, St. Louis, MO, USA) at 4°C overnight. Next, sections were washed with 0.01% Tween20 in PBS and then immersed with TRITC goat-rabbit IgG secondary antibody for 1 h. Then, the sections were counterstained with DAPI to identify cell nuclei. After the slides were sealed, the sections were imaged with a confocal microscope. LC3-positive cells were counted in 100 sections per animal and then the percentage of LC3-positive cells was averaged in all 8 animals.

HE Staining

The procedures were performed following the manufacturers' guidelines. In brief, the sections were washed with PBS three times for 5 min each, followed by hematoxylin for 5 minutes and eosin for another 5 min at room temperature. After three more 5-min washes in PBS, sections were quickly differentiated in 95% alcohol, made transparent in dimethylbenzene, and sealed with neutral resin. Finally, the percentage of damaged cells were counted in 100 sections per animal and averaged for further analysis in all 8 animals.

Statistical Analysis

All images were analyzed using Image pro plus software. Data were reported as Mean±Standard Deviation (SD). Significant differences among time points were assessed by analysis of variance (ANOVA) with SPSS software17.0, and $p<0.05$ was considered statistically significant (* and ** indicate $p<0.05$ and $p<0.01$, respectively).

RESULTS

LC3 Upregulation After Acute SCI

Over time, the percentage of cells with punctate LC3B-II gradually increased near the wound site (Figure 1A,B). In the control group, LC3B-II remained at basal level; there were only a few cells with punctate LC3B-II immunoreactivity. However, in the acute SCI group, the percentage of LC3B-II positive cells was higher than the control group for all time points. At 4H after injury, the percentage of positive cells was increased; it peaked at 3D, and began to decrease at 7D and 21D after SCI. Collectively, the results indicate that the potential primary and secondary pathological mechanisms of SCI activated the process of autophagy.

Histological Changes in SCI

HE staining was used to investigate histological changes after acute SCI. Neurons of gray matter in the control group appeared normal, with intact, round, full nuclei and clear nucleoli. However, SCI induced histological changes near the injury site, including neuronal swelling and shrunken neurons with darkly stained, condensed nuclei. Meanwhile the tissues seemed disorderly and irregularly arranged (Figure 2A,B). In white matter, the glia cells were also damaged after SCI. At 4H after injury, there was evidence of slight neuronal and glia cells losses in the injury site. HE staining showed massive astrocytes infiltration. After 3D, there were significant losses and damage of neurons and glia cells, which were replaced by numerous macrophages. In addition, after 7D and 21 D, with partial blood flow recovery, neurons and glia cells had gradual recovery.

DISCUSSION

Autophagy is characterized by the processes of initiation, elongation, closure (double membranes), maturation (fusion of autophagosome and lysosome), and degradation. Among them, double membrane formation is essential for autophagy activation. The aggregation of Atg8 protein, also known as

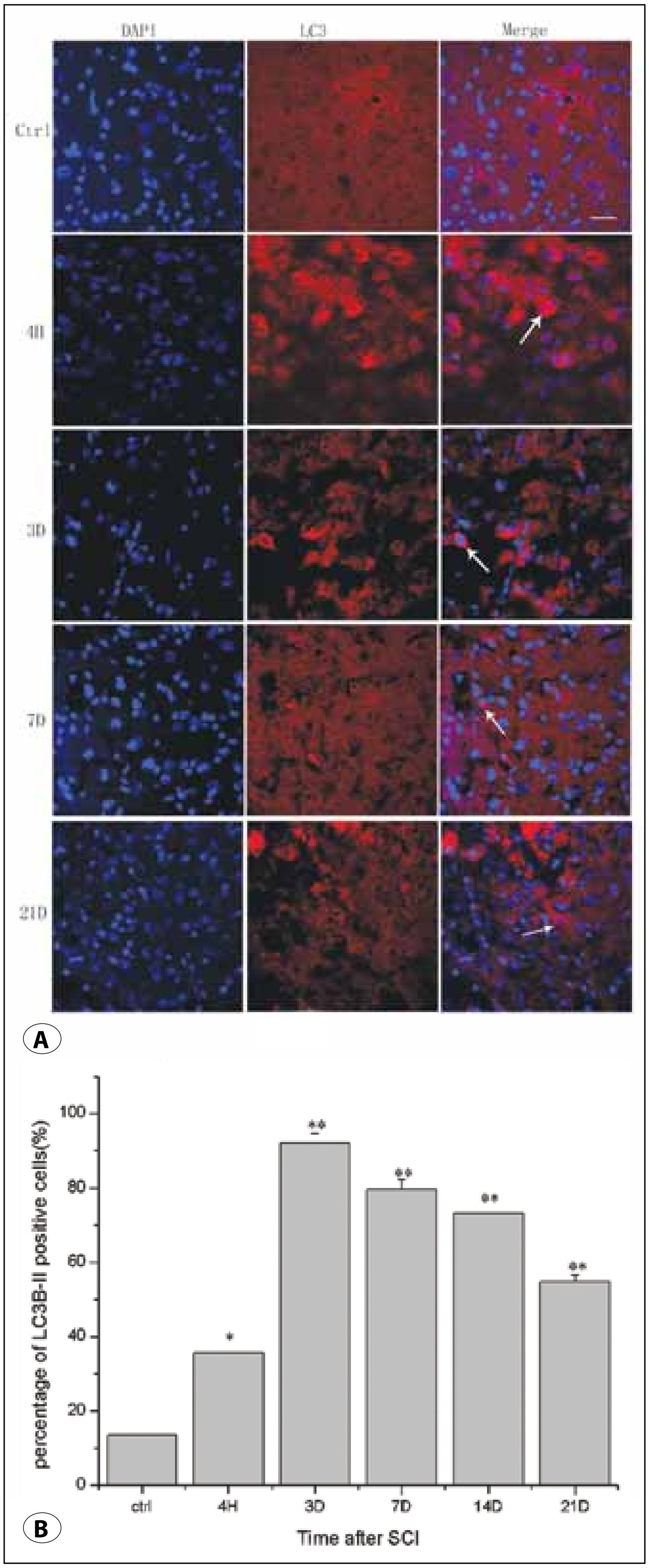

Figure 1: LC3B-II expression near the injury site at each time point. **A)** Cells with punctate LC3B-II expression in the control and SCI groups at 4H, 3D, 7D and 21D after injury (scale bar=20um, arrows show the positive cells). **B)** Quantitative analysis of percentage of LC3B-II positive cells. Values represent the Means±SD. $^{*}p<0.01$,$^{**}p<0.05$.

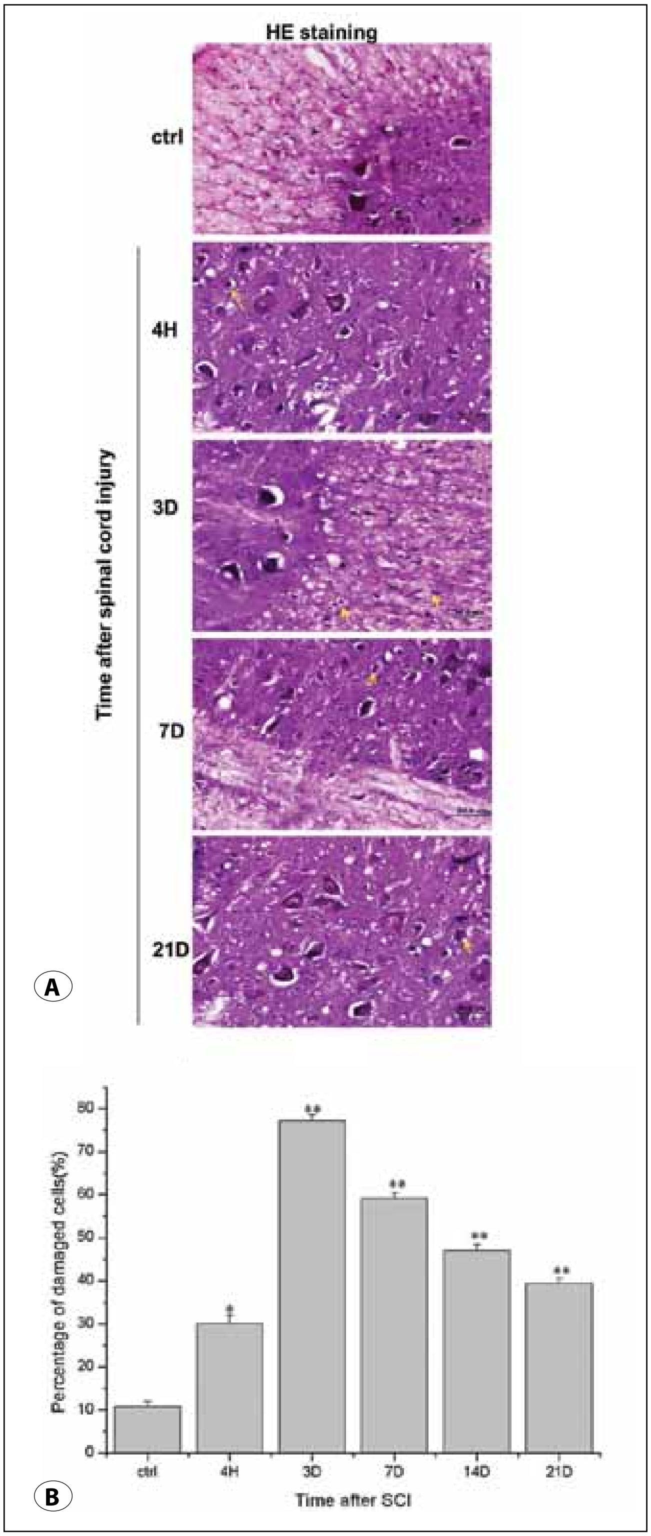

Figure 2: Histopathological changes in the control and SCI groups at 4H, 3D, 7D, and 21D after injury. **A)** At 4H and 3D after surgery, the neurons and glia cells gradually disappeared. At 7D and 21D as blood flow recovered, the neurons and glia cells appeared more normal (scale bar=50um, arrows show the damaged cells). **B)** Quantitative analysis of percentage of damaged cell. $^{*}p<0.01$,$^{**}p<0.05$.

microtubule-associated protein 1 light chain 3 (LC3), is the hallmark of autophagosome formation. LC3 is initially synthesized in an unprocessed form, proLC3, which is converted into a proteolytically processed form lacking C terminus amino acids, LC3-I, and is finally modified into the phosphatidylethanolamine (PE)-conjugated form, LC3-II (LC3-I, unlipidated; LC3-II, lipidated) (18). Previous studies assessed LC3 upregulation to study autophagy induction in various disease models (2,10).

We employed an acute model of SCI to determine whether autophagy was induced. In our model system, LC3B-II expression gradually increased with time. Autophagy induction occurred 4H after injury, peaked at 3D, and declined at 7D and 21D after injury. At the same time, HE staining revealed that the process of autophagy was correlated with the pathological changes. All of these results demonstrate that autophagy activation played an important role in the pathological changes after SCI and may also be involved in the process of neuronal regeneration.

Autophagy has become an important research topic because of the discovery of those components involved in recycling cellular damaged organelles and long-lived proteins (24). This process has an important role in many pathological conditions. In tumor development, autophagy has two different roles; it can promote tumor cells survival and facilitate tumor suppression (13,26,29). In Alzheimer's disease, the expression of proteins involved in the autophagic pathway is decreased, and there is evidence that over-activation of autophagy may slow down the symptoms of memory loss and behavioral dysfunction (23,27). In other models of neuronal injury, autophagy was induced and participated in the processes of neuronal regeneration and behavioral recovery (25). In neonatal hypoxia-ischemia-induced brain injury, enhanced beclin1 expression and switching the mechanisms of cell death from apoptosis to necrosis may explain why autophagic processes facilitate neuronal recovery.

In other nervous system injuries (14,19), autophagy was highly activated in both neurons and astrocytes. In a model of closed head injury, Beclin1 was upregulated at cortical injury sites (6). Upregulation of Beclin1 and LC3 in a model of focal cerebral ischemia may represent enhanced autophagy either as a mechanism to discard injured cells or to reduce neuronal damage (22). In traumatic brain injury, elevation of Beclin1 suggests that autophagic pathways are involved in the processes of neuronal loss and regeneration (8).

In neonatal hypoxia-ischemia induced brain injury, the inhibitor of autophagy 3-MA has been used to investigate the role of autophagy in brain injury (3). The results indicate that in the early stage of brain injury, autophagy over-activation may have a potentially protective role. Conversely, in a model of neonatal cerebral ischemia, inhibition of autophagy had a neuroprotective effect (21). Autophagy has been shown to play different roles depending on the models employed and experiment timing (24). In future studies, we hope to elucidate the role of autophagy in acute SCI, specifically its effect on neuronal recovery.

In conclusion, autophagy activation was observed over time in our model of acute spinal cord injury. Cells with punctate LC3B-II appeared at 4H, peaked at 3D, and decreased at 7D and 21D after injury.

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