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Original Article

Protective Role of Autophagy in Dorsal Root Ganglion to the Mechanical Hyperalgesia Induced By Oxaliplatin in Rats

Yan Kong*, Yingqi Weng*, Xinran Hou, and Qulian Guo

ABSTRACT

Background: Oxaliplatin, a platinum-based chemotherapeutic agent, usually causes an acute peripheral neuropathy. Some research revealed autophagy decreases central sensitization, enhances synaptic plasticity, and relieves the mechanical hyperalgesia in some pathological pain models.

Methods: We utilized a rat model of acute oxaliplatin induced neuropathy with a single dose of 6 mg/kg and 12 mg/kg oxaliplatin intraperitoneal injection to determine a proper dose. Rats were randomly divided into 3 groups: O+DMSO group, O+Rap group and sham+DMSO group. The rats in three groups were tested mechanical withdrawal threshold the day before oxaliplatin injection and for consecutive 7 days after injection. After finishing behavioral assessment on the 3rd day after injection, some of the rats were sacrificed and the dorsal root ganglion (L4 and L5) were collected for further experiment and measured by Western-blot and immunofluorescence to detect the expression of Beclin 1, LC3-II, and cleaved caspase 3.

Results: The lower dose showed less severe toxicity but also induced significant mechanical hyperalgesia so as being adopted for the following experiment. The autophagy was activated in rats with oxaliplatin injection, and pre-treatment with rapamycin, a strong inducer of autophagy, enhanced the activity of autophagy and ameliorated mechanical hyperalgesia induced by oxaliplatin. This beneficial effect of rapamycin may be attributed to the suppression of apoptosis.

Conclusion: A single administration of oxaliplatin induced autophagy activation with mitochondrial dysfunction, and enhanced autophagy exerted a protective role on mechanical hyperalgesia induced by oxaliplatin. (Funded by the National Natural Science Foundation of China.)

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Oxaliplatin is one of the most effective drugs successfully employed as first- line treatment in chemotherapy for common cancers. However it often causes severe peripheral neurotoxicity, which makes chemotherapy- induced peripheral neurotoxicity (CIPN) as its common side effect and limits its use (1). Though the mechanisms involved in CIPN have been unclear yet, studies suggested that structural deficits in dorsal root ganglion (DRG) and sensory neurons which lack a blood brain barrier contributed to the symptoms such as sensory loss, paresthesia, dysaesthesia and numbness (2).

Autophagy is a common molecular mechanism for maintaining cellular homeostasis and functioning. Basal autophagy is usually at a low level and activated by much stimuli, such as nutrient starvation, hypoxia, stress, medicine stimulation, and biological agents like rapamycin (3). Autophagy is involved in many physiological and pathological conditions including tumor, inflammation, neuropathy and infection (4, 5). There are studies showing that autophagy plays an important role in neuropathic pain, and increased autophagy activity can attenuate neuropathic pain in the SNL model (6) and diabetic neuropathy (7).

Chemotherapy drugs induce apoptosis in cancer cells (8), and activate autophagy in some conditions (9).The relationship between apoptosis and autophagy is complicated, though generally speaking, autophagy inhibits apoptosis and promotes cell survival (10). In CPIN animal model, the apoptosis of neurons in DRG and spinal cord increased ,which contributed to the induction and maintenance of neuropathic pain that could be relieved by the inhibition of neuron apoptosis (11). A recent study found that melatonin, another widely- used autophagy inducer, could promote neuroprotection in a CIPN model (12), however, the role of autophagy in CIPN is still unclear. To explored this, we utilized a rat model of neuropathic pain induced by a single dose of oxaliplatin (13), and assessed the autophagy level and evaluated the effect of rapamycin on the mechanical hyperalgesia.

METHODS

Animals

Adult male Sprague-Dawley rats were used (Hunan SLAC Laboratory Animal Co., LTD, China, weighting 200-220 g). The animals were housed in a temperature-controlled (23-25 °C) room on a 12- hour light/dark cycle with food and water ad libitum. All procedures were approved by the Institutional Ethics Committee of Central South University, China, and followed the ethics rules of the International Association for the Study of Pain (14).

Assessment of General Toxicity

Body weight was measured daily during 7:00-8: 00 AM the day before and 7 days after modeling. All the rats were examined daily for symptoms, such as appetite and piloerection (13).

Drugs Preparation and Administration

Oxaliplatin (Sanofi- Aventis, Paris, France) was freshly dissolved in 5% glucose solution to the concentration of 2 mg/ml. Rats in sham group, O6 group and O12 group received a single intraperitoneal (i.p.) administration of 5% glucose solution (3 ml/kg), oxaliplatin (6 mg/kg) and oxaliplatin (12 mg/kg) respectively (n=10 per group).

Rapamycin (Medchem, New Jersey, USA) was freshly dissolved in 5% dimethyl sulfoxide (DMSO) to the concentration of 1 μg/μl. The intrathecal administration of rapamycin or DMSO was carried out 0.5-1 h before the i.p. injection with oxaliplatin (6 mg/kg), or glucose solution (3 ml/kg).

For intrathecal injection, rats were anesthetized with isoflurane. Briefly, a small skin incision was made and a 24G needle was inserted between the L4 and L5 vertebrae, and 10 μl of the solution containing 10 μg rapamycin (O + Rap group) or vehicle (O + DMSO group and sham + DMSO group) was injected in 30 s with syringe respectively (n=16 per group).

Mechanical Hyperalgesia

Von Frey filaments (Stoelting, USA) ranging from 1.0g to 26 g was used to measure paw withdrawal mechanical threshold (PWMT). Briefly, rats were placed in a clear transparent polymethyl methacrylate box and allowed to adapt for 30 min before test. Von Frey was used to apply mechanical stimuli to the left hind paw the day before and consecutive 7 days after oxaliplatin injection. 10 rats in the sham + DMSO

group, O + DMSO group and O + Rap group respectively were sacrificed on the 3rd day after PWMT assessment, and the rest of the rats were evaluated PWMT until the 7th day.

Transmission Electron Microscopy (TEM)

Two rats in the sham group, O6 group, and O12 group respectively were anesthetized by 10% chloral hydrate and sacrificed on the 3rd day after injection. L4 DRG was quickly harvested. Briefly, the tissues were fixed in 2.5% glutaraldehyde for 48 h at 4°C, postfixed in 1% (w/v) osmium tetroxide in PBS for 1 h, dehydrated through a graded series of acetone, flat embedded in Epon epoxy resin, and polymerized at 60° C for 24 h. 50 nm ultrathin sections were completed and stained by lead citrate and uranyl acetate, and examined using a FEI Tecnai G2 Spirit transmission electron microscope.

Western blot

Briefly, the proteins from total tissue lysates were separated by standard SDS-PAGE and then transferred to a PVDF membrane (Millipore). The membrane was washed, blocked, and incubated with specific primary antibodies: rabbit anti-Beclin 1(1:1000, CST), rabbit anti-LC3B (1:1000, Abcam), rabbit anti-cleaved caspase 3(1:1000, CST) and rabbit anti-GAPDH (1:2000, Santa-Cruz). The secondary antibody is HRP-conjugated secondary antibodies (1:1000, Millipore). Images were acquired on a luminescence image analyzer using Image Lab software (Bio-Rad, Universal Hood III, USA).

Immunofluorescence

Rats were anesthetized with 10% chloral hydrate, and perfused with ice-cold PBS followed by 4% paraformaldehyde. Briefly, L4 and L5 DRG were dissected out, post fixed for 2 hours and transferred to 30% sucrose in PBS at 4° C overnight, which were stained with anti-LC3B (1:100, Abcam), and donkey antibody conjugated to Alexa Fluor 594 (1:400, Jackson Lab). The images were captured using a Leica DM5000B microscope (Leica, Germany).

Statistics Analysis

Data were expressed as mean ± SEM. Two way repeated measure ANOVA was used for behavioral testing with Bonferroni posttests. Other data were done using one-way ANOVA with Boneffoni's Multiple Comparison Test. $P<0.05$ was considered significantly different.

RESULTS

Assessment of general toxicity

Two rats in the O12 group died on the 4th day and the 5th day respectively after injection. Administration of 6 mg/kg oxaliplatin showed no severe toxicity. Rats in O6 and O12 group developed a significant mechanical hyperalgesia and no significant difference was detected between two groups. Considering the toxicity, the dose of 6 mg/kg was determined to use for the following experiment.

No rats receiving rapamycin or DMSO pretreatment died during the period of the experiment. During the first 24 h after oxaliplatin injection, rats in O + DMSO group and O + Rap group showed a transient anepithymia, but recovered soon. Between groups, no significant difference in body weight was observed, no deterioration in general status was observed, and clinical status remained good.

Oxaliplatin induced mechanical hyperalgesia which could be ameliorated by rapamycin pretreatment

There were no significant differences in the baseline of PWMT between all groups. Compared with sham group, PWMT was significantly decreased ($P<0.05$) after oxaliplatin injection during day 2 to 6 in O6 group and day 2 to 7 in the O12 group (Figure 1A).

Compared with the sham + DMSO group, PWMT in O + DMSO and O + Rap group was significantly decreased ($P<0.05$) from the 1st day to the day 7th after oxaliplatin injection. Compared with the O + DMSO group, only on the 4th day after injection PWMT in O + Rap group was significantly different ($P<0.05$). Compared with sham + DMSO group, on the 3rd day after injection PWMT in the O + Rap group was significantly different ($P<0.01$, Figure 1B).

Results of Transmission Electron Microscopy (TEM)

Compared with that of the sham group, in the

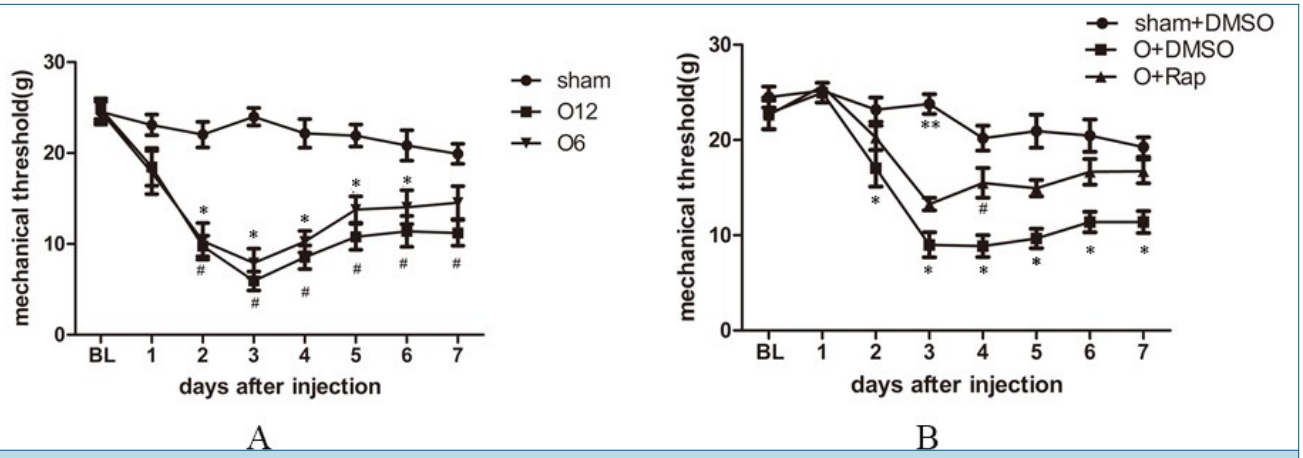

Figure 1. Paw Withdrawal Mechanical Threshold (PWMT) in All Groups.
(A) PWMT in sham group, O6 group and O12 group (n=10 from BL to the 3rd day per group; n=8 from the 4th day to the 7th day in sham group and O6 group; n=6 from the 4th day to the 7th day in O12 group). Values are mean ± SEM. *P, # P<0.05, O6 group or O12 group vs. sham group. (B) PWMT in sham+DMSO group, O+DMSO group and O+Rap group (n=16 from BL to day 3 per group, n=6 from day 4 to day 7 per group). Values are mean ± SEM. *P<0.05, sham+DMSO vs. O+DMSO, # P<0.05,O+DMSO vs. O+Rap; **P<0.01, sham+DMSO vs. O+Rap.

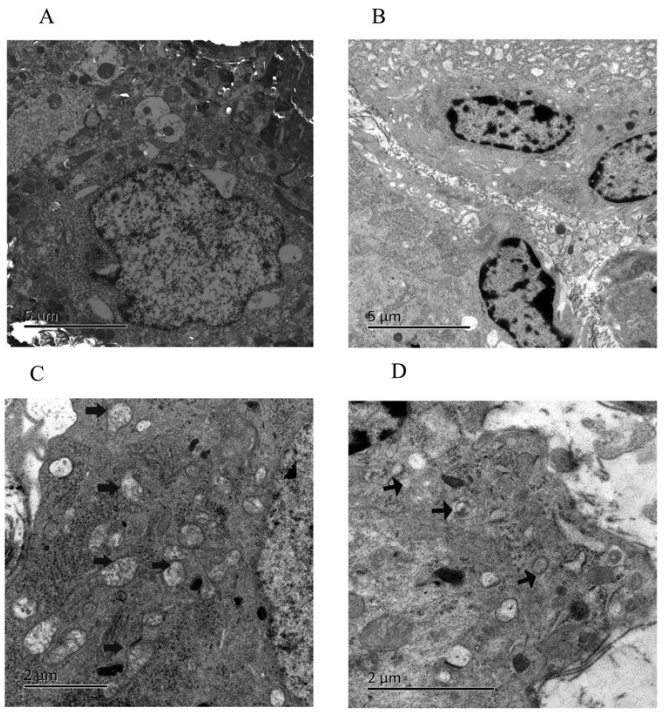

Figure 2. TEM Analysis on DRG Neurons (n = 2).
(A) The neuron in sham group had a good shaped nucleus; (B) The shrunken nucleus in neurons was observed in O6 group; (C) Damaged mitochondria with edema and vacuolation was observed in O6 group (arrow indicated damaged mitochondria); (D) Autophagosomes in DRG was observed in O6 group (arrow indicated autophagosome).

DRG neurons from rats in O6 group the quantity of mitochondria increased, and a large number of damaged mitochondria existed with severe edema and vacuolation; the quantity of autophagosomes significantly increased (Figure 2). In the sham group, the image of TEM showed the DRG neurons was normal.

Expression of L3- II and Beclin 1 in DRG by Western Blot

Compared with the sham + DMSO group, L3-II and Beclin 1 in O + DMSO and O + Rap group were significantly increased ($P<0.05$), and the level of L3-II and Beclin 1 in O+Rap group was significantly higher than that in O + DMSO group ($P<0.05$)(Figure 3).

Expression of LC3 in DRG by Immunofluorescence

In the sham+DMSO group, LC3 was sparse and dispersive distributed in DRG. In O + DMSO group, LC3 was increased compared with sham + DMSO group and clustered distributed. In O + Rap group, the red immunofluorescent signal of LC3 was mostly strong, with clustered or circular distribution. In three group, LC3 positive cells were morphologically observed and mainly in neurons (Figure 4).

Expression of Cleaved Caspase 3 in DRG by Western Blot

Compared with the sham + DMSO group, cleaved caspase 3 in O + DMSO and O + Rap group was significantly increased ($P<0.05$), but rapamycin pre- treatment significantly attenuated the augment of cleaved caspase3 when compared with that in O + DMSO group ($P<0.05$, Figure 5).

DISCUSSION

A single injection of 6mg/kg oxaliplatin in rats caused no other deterioration in general health condition except transient anepithymia and all rats survived during the experiment. In the O12 group, 2 rats died during the course of the experiment, and the cause of the death is considered to be the toxicity of oxaliplatin, which was somehow more severe than what was reported in some other studies, the reasons might be the different sources of oxaliplatin or the vendor difference in rats. Both doses of oxaliplatin induced severe mechanical hyperalgesia, and the PWMT was lower in rats receiving higher dose of oxaliplatin, but with no significance. Thus we adopted 6 mg/kg as the dose for the following experiment. After a single i.p. injection of 6 mg/kg oxaliplatin in rats, PWMT of rats was decreased since the first 24h after injection, and sustained at least 7 days with a slight but insignificant recovery, which was consistent with the model of acute oxaliplatin induced neuropathy described by Ling (13). As the PWMT touched the bottom at the 3rd day after oxaliplatin injection, we harvested DRG on the day after behavior tests.

Oxaliplatin exerts toxic changes on the nuclei of DRG sensory neurons and alters energy mechanisms in some intracellular organelles such as mitochondria. Some studies showed oxaliplatin could induce oxidative stress in mitochondria which caused the accumulation of reactive oxygen species (ROS) and mitochondria dysfunction (15). Functional deficits in peripheral nerve mitochondria in rats with oxaliplatin evoked painful peripheral neuropathy (16). Mitochondria dysfunction deteriorates energy supply which induces the compensatory increase of damaged mitochondria and exacerbates the survival environment of neurons (17). Consistent with those theories, the analysis of TEM showed that mitochondria in DRG neurons increased, and obvious edema and vacuolation of mitochondria was detected. Besides that, the increase of autophagosomes in DRG neurons was observed, indicating an enhanced level of autophagy. Autophagy is a mechanism for maintaining cellular homeostasis and functioning via the clearance of damaged organelle and other cellular structure, but its role in oxaliplatin induced neuropathy has been rarely investigated. The fact that the mechanical hyperalgesia induced by oxaliplatin was attenuated by pre-treat-

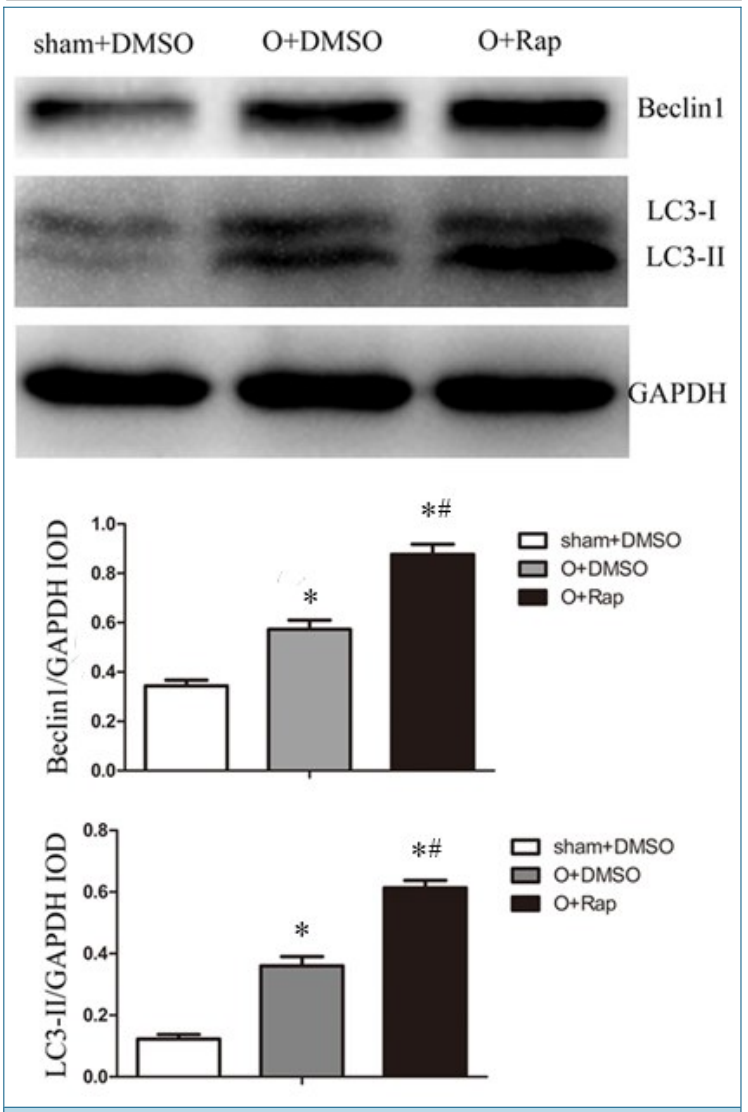

Figure 3. Expression of LC3- II and Beclin 1 in DRG Measured by Western Blot (n = 5).
Values are mean ± SEM. # P<0.05, O+DMSO vs. O+Rap; *P<0.05, vs. sham+DMSO.

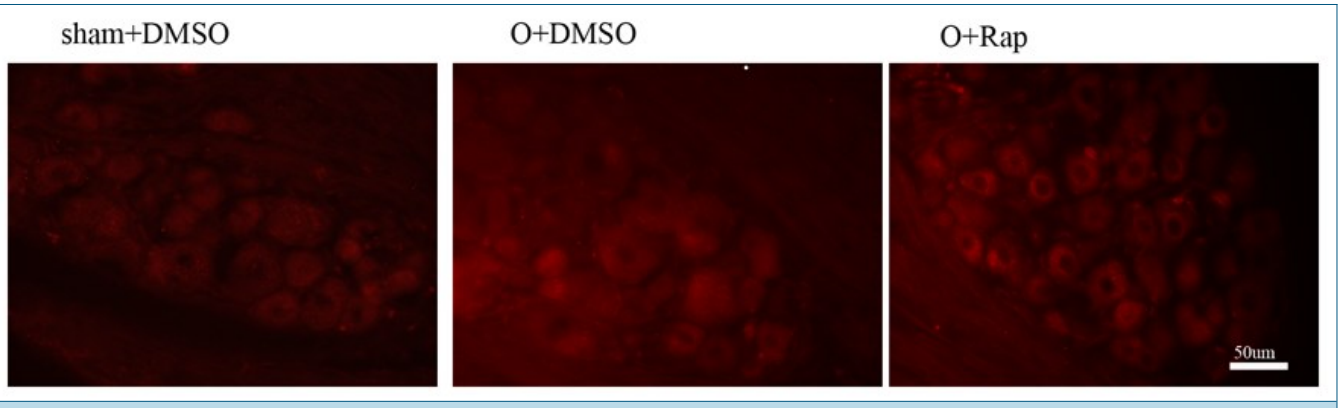

Figure 4. Expression of LC3 in DRG by Immunofluorescence (n = 5).
Red immunofluoresence indicated LC3 (LC3-I and LC3-II). Scal bar = 50 μm.

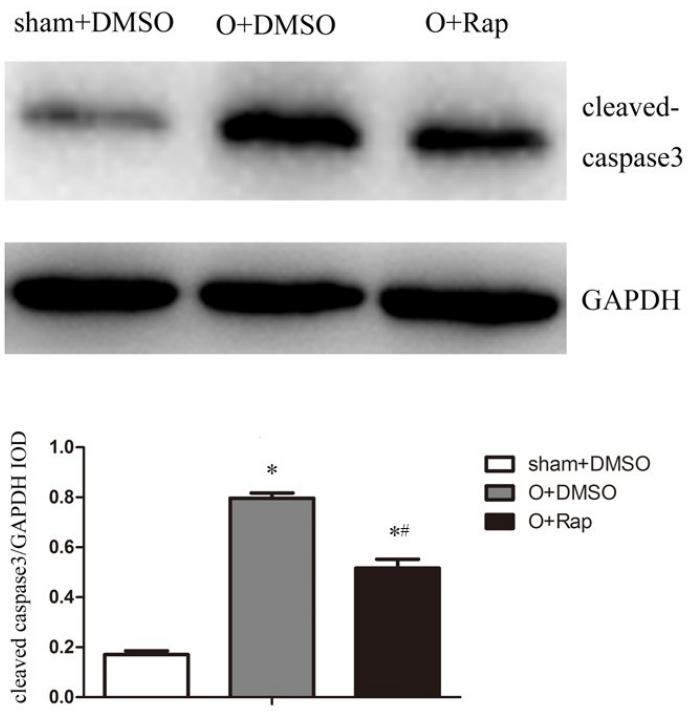

Figure 5. Expression of Cleaved Caspase 3 in DRG by Western Blot (n = 5).
Values are mean ± SEM. # P<0.05, O+DMSO vs. O+Rap; *P<0.05, vs. sham+DMSO.

ment of rapamycin, a strong promotor of autophagy, suggested a beneficial role of autophagy in this type of neuropathy. The significant differences of PWMT between the group of O+rap and O+DMSO were only detected at day 4 after injections, this might because that we administrated rapamycin for only once.

To confirm the autophagy activity in each group, on the 3rd day after injection, the level of LC3-II and Beclin1, the widely used markers for autophagy (18, 19), in DRG were measured by Western Blot. And the distribution pattern of LC3 was observed under immunofluorescence staining. Both these analyses proved that the autophagy was enhanced by oxaliplatin, and rapamycin promoted the augmentation even greater. Under most circumstances, autophagy is activated to maintain cellular homeostasis, prevent apoptosis and promote cell survival (20, 21). It has been reported before that apoptosis in DRG neuron contributed to oxaplatin induced mechanical hyperalgesia (22). It is possible that the protective role of autophagy in oxaliplatin induced neuropathy could be attributed to the prevention of neuron apoptosis. As what was reported by some other studies in acute or chronic oxaliplatin induced neuropathy (23), the expression of cleaved caspase 3 was increased in groups receiving oxaliplatin, indicating a caspase mediated- apoptosis was induced (24). In the rats receiving rapamycin pre-treatment, the increase of cleaved caspase 3 was attenuated, suggesting an alleviation of apoptosis.

In conclusion, our study demonstrates that activation of autophagy in DRG played a beneficial role in oxaliplatin induced neuropathy, and this may be related to the prevention of apopto-

sis. Though some studies reported the enhancement of autophagy improved the anti-cancer effect of oxaliplatin (25), most research showed autophagy contributed to the antitumor drug resistance and promoted the survival rate of tumor cells, therefore, the organ-specific activation of autophagy could be a promising way for the treatment of CIPN.

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The authors declare no other conflicts of interest for this work.

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