Gardenoside combined with ozone inhibits the expression of P2X3 and P2X7 purine receptors in rats with sciatic nerve injury

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Received December 22, 2016; Accepted September 1, 2017

DOI: 10.3892/mmr.2018.8803

Abstract. Neuropathic pain is a severe health problem for which there is a lack of effective therapy. Ozone and *Gardenia* fruits have been used separately in pain relief for many years; however, their underlying mechanisms remain unclear. To investigate the pain-relieving effects of combined ozone and *Gardenia*, a chronic constriction sciatic nerve injury (CCI) rat model was constructed and treated with ozone and gardenoside (Ozo&Gar), which is a compound found in *Gardenia* fruits. A total of 70 rats were randomly divided into five groups: Control (Ctrl), Ctrl + Ozo&Gar, Sham, CCI, and CCI + Ozo&Gar. The rats in the Ctrl + Ozo&Gar and CCI + Ozo&Gar groups were administered an intravenous injection of 30 μg/ml ozone and 300 μmol/l gardenoside. The rats in the Ctrl, Sham and CCI groups were administered the same volume of saline. Pain behavior, mechanical hyperalgesia, thermal hyperalgesia, and the protein expression levels of P2X3 and P2X7 purine receptors in L4-L5 dorsal root ganglion (DRG) were determined 15 days post-surgery. The results demonstrated that treatment with a combination of ozone and gardenoside increased mechanical withdrawal threshold and thermal withdrawal latency, thus confirming their pain-relieving effects. In addition, a significant increase in the mRNA and protein expression levels of P2X3 and P2X7 was detected in the DRG of rats in the CCI group compared with in the control groups; however, following treatment with a combination of ozone and gardenoside, the mRNA and protein expression levels of P2X3 and P2X7 receptors were significantly reduced compared with in the CCI group. These results indicated that the mechanism underlying the pain-relieving effects of ozone and gardenoside may be mediated by inhibition of P2X3 and P2X7 purine receptors in the DRG. This finding suggested that ozone and gardenoside may be considered potential drug candidates that target P2X3 and P2X7 purine receptors.

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Key words: sciatic nerve injury, P2X3, P2X7, ozone, gardenoside

Introduction

Neuropathic pain is defined as pain that arises as a direct consequence of a lesion or disease affecting the nervous system, which is caused by an injury to the peripheral or central nervous system (1,2). Characteristic features of neuropathic pain include hyperalgesia, allodynia and spontaneous pain (3). Neuropathic pain may develop in response to sciatic nerve injury. The sciatic nerve is the largest and longest peripheral nerve in the human body, which is formed from the anterior and posterior divisions of the L4, L5, S1 and S2 spinal nerves, and the anterior division of the S3 spinal nerve. In clinical practice, the treatment of neuropathic pain, particularly sciatic nerve pain, remains a prevalent and persistent challenge (2,3); therefore, more efficient novel treatments require further exploration.

Ozone (O_3) has been used in pain treatment for >30 years (4). On the basis of suggestions made by Wolff (5), ozone therapy has been used by practitioners in an empirical fashion. The issues regarding how its toxicity can be controlled and how its therapeutic effects are exerted have been fully clarified (6-8). At present, the clinical use of ozone is very popular and is promising compared with surgical procedures, and it has been used to treat sciatic nerve injury. It has previously been reported that a single subcutaneous injection of ozone in mice with spared nerve injury of the sciatic nerve decreased neuropathic pain-type behavior (9). The mechanism underlying this action remains unclear; however, ozone has been observed to regulate the expression of genes that serve vital roles in the onset and maintenance of allodynia (9). Another substance that is often used in clinical pain treatment is *Gardenia* fruit extracts. *Gardenia* fruits are widely used in Chinese traditional medicine, since they are thought to exert homeostatic, hepatoprotective, analgesic, antiphlogistic, antipyretic and hypolipidemic effects (10). In addition, *Gardenia* is widely used in modern clinical medicine for the treatment of numerous diseases, including acute viral hepatitis, esophagitis, canker sores, coronary heart disease, neurasthenia and insomnia (11). Previous studies have confirmed that iridoid constituents and crocins were the most effective and major chemical components of *Gardenia* fruits; among these, gardenoside has been reported to possess the largest therapeutic effect in sciatic nerve injury (12,13).

The present study aimed to investigate the pain-relieving effects of a combination of gardenoside and ozone, and to elucidate the mechanism underlying their function. Therefore,

a chronic constriction sciatic nerve injury (CCI) rat model was generated, and the effects of gardenoside and ozone were examined. A combination of gardenoside and ozone markedly increased mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL), thus confirming their pain-relieving effects. The mRNA and protein expression levels of P2X3 and P2X7 purine receptors were significantly decreased in the dorsal root ganglion (DRG) following gardenoside and ozone cotreatment, thus suggesting that the mechanism underlying their pain-relieving effects may be mediated by inhibiting P2X3 and P2X7 receptors in the DRG. The present study is the first, to the best of our knowledge, to demonstrate the mechanism underlying the pain-relieving effects of gardenoside and ozone cotreatment, and may provide a novel direction for further studies regarding the treatment of neuropathic pain.

Materials and methods

Animals. A total of 70 healthy male Sprague-Dawley rats (8 weeks old; weight, 200-250 g) were purchased from the Experimental Animal Center of Suzhou Aiermaite Technology Co., Ltd. (Suzhou, China). The rats were housed in a specific pathogen-free animal facility, at 22-24˚C, 50-60% humidity and a 12 h light/dark cycle. The rats had free access to food and water prior to experimentation. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Weifang People's Hospital (Weifang, China).

CCI model construction. A total of 70 male Sprague-Dawley rats were randomly divided into the following five groups (n=14 rats/group): The control group (Ctrl), the ozone and gardenoside treatment control group (Ctrl + Ozo&Gar), the sham surgery group (Sham), the CCI model group (CCI) and the ozone and gardenoside treatment CCI model group (CCI + Ozo&Gar). The rats in each group were treated as follows. After environmental adaptation for ≥5 days, two groups (CCI and CCI + Ozo&Gar groups; total number of rats, 28) were used to generate the CCI model. For 24 h prior to surgery, these rats were provided only water, with no food. The CCI model is well characterized for the study of neuropathic pain (14,15). Briefly, rats were anesthetized with 10% chloral hydrate (350 mg/kg, intraperitoneal), fixed in the supine position and were subjected to an aseptic operation. The right legs were shaved and sterilized with 70% ethanol and betadine antiseptic solution. Following exposure of the thigh muscle, the sciatic nerve was separated and loosely ligated with sterile 4-0 catgut thread at four consecutive sites, with an interval of ~1 mm. The nerve was carefully ligated, so as not to completely block peripheral blood flow in the nerves. In addition, a sham surgery was performed in the Sham group, in which the sciatic nerve was exposed, but not ligated. Finally, the wound was sutured in layers and was cleaned with iodine, followed by an intramuscular injection of penicillin (40,000 U/mouse). Rats were then maintained in the same manner as the rats in the control group. The two control groups (Ctrl and Ctrl + Ozo&Gar) did not undergo surgery.

Drug application. The rats in the Ctrl + Ozo&Gar and CCI + Ozo&Gar groups were administered an intravenous injection of 30 μg/ml ozone (Chengdu Must Biotechnology Co., Ltd., Chengdu, China) and 300 μmol/l gardenoside (Chengdu Must Biotechnology Co., Ltd.). The rats in the Ctrl, Sham and CCI groups were administered the same volume of saline. The drugs were delivered intravenously once daily for 14 days, beginning on day 1 after CCI. Specimens were collected from the L4-L5 DRG on the 15th day for analysis.

Evaluation of pain behavior. The five groups of rats were raised in separate cages after the operation. MWT and TWL were measured prior to the operation (day 0), and 1, 3, 5, 7, 9, 11 and 14 days after the operation. The time of each daily measurement, the room temperature and other conditions were similarly maintained across all measurements.

Detection of mechanical hyperalgesia. BME-403 Von Frey fine thread (Beijing Jinuotai Technology Development Co. Ltd., Beijing, China) was used to measure MWT. The rats were maintained in a transparent plexiglass box (20x30x30 cm); the bottom of the plexiglass box was made of wire netting (1x1 cm). The rats were maintained in the plexiglass box for 15 min prior to the measurement. The bending forces of the wires inserted through the wire netting used were as follows: 0.13, 0.20, 0.33, 0.60, 1.30, 3.60, 5.00, 7.30, 9.90 and 20.1 g, and tolerance of bending forces >20.1 g were recorded as 20.1 g. The interval between two stimulations was 20 sec or until the stimulation-induced reactions, such as licking feet and paw withdrawal. The test was stopped once paw withdrawal was induced. Each test was performed from the smallest to the largest bending force until the frequency of withdrawal was ≥50%. From this, the MWT value was determined. The test was repeated three times and the mean was calculated.

Detection of thermal hyperalgesia. Thermal hyperalgesia was measured using an automatic thermalgia stimulator system (BME-410C; Institute of Bioengineering, Chinese Academy of Sciences, Beijing, China) to irradiate the paws. The plexiglass box was placed on a glass plate (3 mm thick), and the rats were maintained in the box for 30 min prior to the experiment. The time it took for the rats to withdraw their paws from the thermal stimulus was considered the TWL. The cut-off time was 30 sec, in order to prevent tissue damage. Each rat was measured three times and the mean value was taken as the threshold value.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To measure the mRNA expression levels of P2X3 and P2X7 receptors, rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (350 mg/kg) and the L4 and L5 DRG was harvested on the 15th day post-surgery, and was used for total RNA isolation using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Subsequently, 1,000 ng total RNA was used as a template for RT using the Applied Biosystems RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer instructions. β-actin was used as the internal reference gene. The primer sequences are listed in Table I. The SYBR-Green master kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) PCR products were amplified using the following cycling

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primers	Primer length (bp)
P2X3 receptor	F: 5'-CAACTTCAGGTTTGCCAAA-3'	519
	R: 5'-TGAACAGTGAGGGCCTAGAT-3'	
P2X7 receptor	F: 5'-GTTTGACATCATCCAGTTGGTTGT-3'	566
	R: 5'-ATCTTACTGAAGAGCTCAGAGGTA-3'	
β-actin	F: 5'-TAAAGACCTCTATGCCAACACAGT-3'	240
	R: 5'-CACGATGGAGGGGCCGGACTCATC-3'	

F, forward; R, reverse.

parameters: 94˚C for 5 min, followed by 35 cycles at 94˚C for 45 sec, 53˚C for 30 sec and 72˚C for 40 sec, and a final step at 72˚C for 5 min. Calculated relative expression of the target gene expression was calculated with the $2^{-\Delta\Delta Cq}$ method (16).

Western blot analysis. For western blot analysis, rats were deeply anesthetized with diethyl ether and were sacrificed by decapitation. The L4 and 5 DRG was rapidly removed and lysed with 20 mM Tris-HCl buffer (pH 8.0), containing 1% NP-40, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% β-mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase and phosphatase inhibitors (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Protein concentration was determined using the bicinchoninic acid protein assay method with bovine serum albumin (Beyotime Institute of Biotechnology, Shanghai, China) as a standard. β-actin served as an internal control. Protein samples (60 μg/lane) from DRG were separated by 8% SDS-PAGE and were then electrotransferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing Tween 20 (50 mM Tris, 150 mM NaCl and 0.1% Tween 20 v/v, pH 7.4) for 2 h at room temperature, and were then incubated at 4˚C overnight with P2X3 (cat no. sc-12215) and P2X7 (cat no. sc-25698) receptor antibodies (1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and β-actin antibody (cat no. sc-130656; 1:1,000; Santa Cruz Biotechnology, Inc.). The membrane was subsequently incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (cat no. sc-2005; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. The signal was detected using the Amersham enhanced chemiluminescence system (Amersham; GE Healthcare, Chicago, IL, USA). The relative expression levels of P2X3 and P2X7 receptors were densitometrically semi-quantified using Image-Pro Plus (Media Cybernetics, Inc., Rockville, MD, USA), and were calculated according to the reference bands of β-actin.

Statistical data analysis. Statistical analysis was performed using SPSS statistical software, version 19.0 (IBM Corp., Armonk, NY, USA). Data are expressed as the mean ± standard deviation. Experiments were repeated three times. Statistical analyses were performed using one-way analysis of variance followed by a least significant difference post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Mechanical hyperalgesia measurement. MWT was determined prior to the operation (day 0), and 1, 3, 5, 7, 9, 11 and 14 days after the operation. On days 0 and 1, there was no significant difference between the rats in all five groups (P>0.05; Table II). However, from day 3, MWT values were significantly lower in the CCI and CCI + Ozo&Gar groups compared with in the control groups (P<0.01). At all time points, there was no significant difference between the Ctrl, Ctrl + Ozo&Gar and Sham groups. MWT remained significantly reduced in the CCI group between days 3 and 14, thus confirming that the CCI model was successfully generated.

Compared with in the CCI group, MWT was significantly increased in the CCI + Ozo&Gar group between days 5 and 14 (P<0.05; Table II). The MWT in the CCI + Ozo&Gar group was lowest on day 3, and continued to increase on the following days, until it was similar to that in the Ctrl, Ctrl + Ozo&Gar and Sham groups (Table II). These findings indicated that gardenoside and ozone cotreatment was able to increase MWT in rats suffering from neuropathic pain.

Thermal hyperalgesia measurement. TWL was measured on day 0, and on days 1, 3, 5, 7, 9, 11 and 14 after the operation. On days 0 and 1, there was no significant difference between the rats in all five groups (P>0.05; Table III). However, from day 3, TWL values were significantly lower in the CCI and CCI + Ozo&Gar groups compared with in the control groups (P<0.01). At all time points, there was no significant difference between the Ctrl, Ctrl + Ozo&Gar and Sham groups. TWL was significantly lower in the CCI group between days 3 and 14, thus confirming that the CCI model was successfully generated.

Compared with in the CCI group, TWL was significantly increased in the CCI + Ozo&Gar groups between days 5 and 14 (P<0.05; Table III). The TWL in the CCI + Ozo&Gar group was lowest on day 3, after which it continued to increase, until it was similar to that in the Ctrl, Ctrl + Ozo&Gar and Sham groups (Table III). These findings indicated that gardenoside and ozone cotreatment was able to increase TWL in rats suffering from neuropathic pain.

Alterations in the mRNA expression levels of P2X3 receptor in the DRG. RT-qPCR was used to detect the mRNA expression

Table II. MWT measurement of the rats in each group.

	MWT (g)							
	Day							
Group	0	1	3	5	7	9	11	14
Ctrl	20.1	20.1	20.1	20.1	20.1	20.1	20.1	20.1
Ctrl + Ozo&Gar	20.1	20.1	20.1	20.1	20.1	20.1	20.1	20.1
Sham	20.1	20.1	18.0±1.4	18.6±1.4	19.0±1.3	19.1±1.4	19.4±1.7	19.5±1.5
CCI	20.1	18.9±1.3	9.9±1.3[a]	8.9±1.4[a]	7.1±1.5[a]	6.7±1.2[a]	7.5±1.9[a]	8.9±1.2[a]
CCI + Ozo&Gar	20.1	18.8±1.5	10.9±1.7[a]	12.9±1.5[b]	14.2±1.4[c]	15.3±1.5[c]	16.9±1.4[c]	18.5±1.6[c]

Data are presented as the mean ± standard deviation. [a]P<0.01 vs. the Ctrl, Ctrl + Ozo&Gar and Sham groups; [b]P<0.05, [c]P<0.01 vs. the CCI group. CCI, chronic constriction sciatic nerve injury; Ctrl, control; Gar, gardenoside; MWT, mechanical withdrawal threshold; Ozo, ozone.

Table III. TWL measurement of rats in each group.

	TWL (sec)							
	Day							
Group	0	1	3	5	7	9	11	14
Ctrl	23.3±2.3	22.4±2.5	23.7±1.9	24.2±1.8	23.4±2.1	22.5±2.4	21.9±2.5	23.2±2.6
Ctrl + Ozo&Gar	22.7±1.8	21.8±1.7	21.9±2.1	22.7±1.9	22.5±1.8	21.4±1.9	22.4±1.8	22.6±2.1
Sham	22.1±1.8	20.3±2.1	19.3±2.5	20.8±2.9	21.2±2.4	22.6±2.3	23.1±2.1	22.7±1.9
CCI	23.4±2.4	22.4±1.9	14.3±2.3[a]	12.7±2.4[a]	10.2±1.9[a]	11.3±2.4[a]	11.7±2.3[a]	11.1±2.2[a]
CCI + Ozo&Gar	22.7±2.1	21.7±1.9	15.3±2.1[a]	17.2±2.2[b]	18.7±2.0[c]	19.4±2.1[c]	21.2±1.7[c]	21.8±1.8[c]

Data are presented as the mean ± standard deviation. [a]P<0.01 vs. the Ctrl, Ctrl + Ozo&Gar and Sham groups; [b]P<0.05, [c]P<0.01 vs. the CCI group. CCI, chronic constriction sciatic nerve injury; Ctrl, control; Gar, gardenoside; Ozo, ozone; TWL, thermal withdrawal latency.

level of P2X3 receptor in the DRG on day 15 post-surgery (Fig. 1). Compared with in the Ctrl group, no significant alterations in the mRNA expression levels of P2X3 receptor were detected in the Ctrl + Ozo&Gar and Sham groups (P>0.05). However, the mRNA expression levels of P2X3 receptor were significantly higher in the CCI (P<0.01) and CCI + Ozo&Gar groups (P<0.05) compared with in the other three groups. Compared with in the CCI group, the CCI + Ozo&Gar group exhibited a significant reduction in P2X3 receptor mRNA expression (P<0.05). These results suggested that the mRNA expression levels of P2X3 receptor were increased in the CCI group, but were decreased following gardenoside and ozone cotreatment.

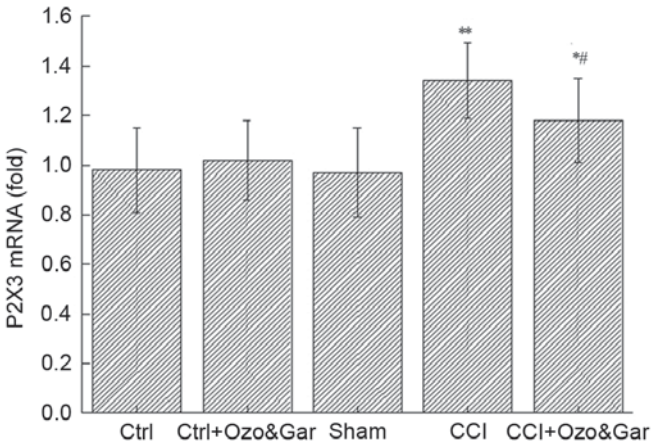

Figure 1. Effects of CCI surgery and/or gardenoside and ozone cotreatment on the mRNA expression levels of P2X3 receptor in the DRG. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. the Ctrl, Ctrl + Ozo&Gar and Sham groups, #P<0.05 vs. the CCI group. CCI, chronic constriction sciatic nerve injury; Ctrl, control; Gar, gardenoside; Ozo, ozone.

Alterations in the mRNA expression levels of P2X7 receptor in the DRG. RT-qPCR was also used to detect the mRNA expression levels of P2X7 receptor in the DRG on day 15 post-surgery (Fig. 2). Compared with in the Ctrl group, no significant alterations in mRNA expression levels of P2X7 receptor were observed in the Ctrl + Ozo&Gar and Sham groups (P>0.05). However, the mRNA expression levels of P2X7 receptor were significantly higher in the CCI (P<0.01) and CCI + Ozo&Gar groups (P<0.05) compared with in the other three groups. Compared with in the CCI group, the CCI + Ozo&Gar group exhibited a significant reduction in P2X7 receptor mRNA

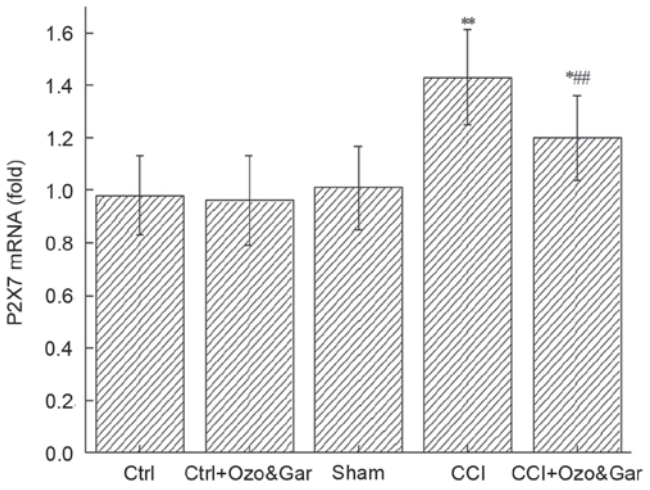

Figure 2. Effects of CCI surgery and/or gardenoside and ozone cotreatment on the mRNA expression levels of P2X7 receptor in the DRG. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. the Ctrl, Ctrl + Ozo&Gar and Sham groups, $^{\#\#}$P<0.01 vs. the CCI group. CCI, chronic constriction sciatic nerve injury; Ctrl, control; Gar, gardenoside; Ozo, ozone.

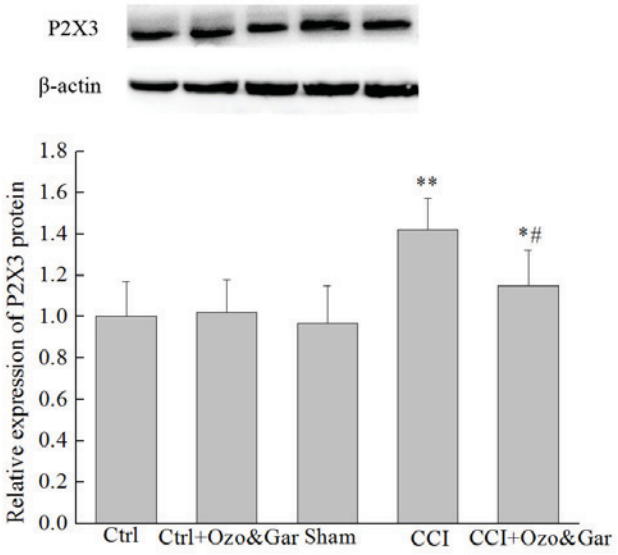

Figure 3. Protein expression levels of P2X3 receptor in the rats of the five groups with or without CCI and/or gardenoside and ozone cotreatment, as determined by western blotting. β-actin was used as a control. P2X3 receptor expression was significantly higher in the CCI group compared with in the Ctrl, Ctrl + Ozo&Gar and Sham groups. However, in the CCI + Ozo&Gar group, P2X3 receptor expression was significantly lower than in the CCI group. *P<0.05, **P<0.01 vs. Ctrl, Ctrl + Ozo&Gar and Sham groups; $^{\#\#}$P<0.01 vs. the CCI group. CCI, chronic constriction sciatic nerve injury; Ctrl, control; Gar, gardenoside; Ozo, ozone.

expression (P<0.01). These results suggested that the mRNA expression levels of P2X7 receptor were increased in the CCI group, but were decreased following gardenoside and ozone cotreatment.

Alterations in the protein expression levels of P2X3 receptor in the DRG. Western blotting was used to detect the protein expression levels of P2X3 receptor on day 15 post-surgery. As presented in Fig. 3, there was no significant difference in the protein expression levels of P2X3 receptor between the Ctrl, Ctrl + Ozo&Gar and Sham groups (P>0.05). However, the expression levels of P2X3 receptor in the CCI group were significantly higher compared with in the three control groups. Conversely, the protein expression levels of P2X3 receptor were significantly reduced in the CCI + Ozo&Gar group compared with in the CCI group. These results indicated that P2X3 receptor protein expression was increased in the CCI group, but was decreased following cotreatment with gardenoside and ozone.

Alterations in the protein expression levels of P2X7 receptor in the DRG. Western blotting was used to detect the protein expression levels of P2X7 receptor on day 15 post-surgery. As shown in Fig. 4, there was no significant difference in the protein expression levels of P2X7 receptor between the Ctrl, Ctrl + Ozo&Gar and Sham groups (P>0.05). However, the expression levels of P2X7 receptor in the CCI group were significantly higher compared with in the three control groups. Conversely, the protein expression levels of P2X7 receptor were significantly lower in the CCI + Ozo&Gar group compared with in the CCI group. These results indicated that P2X7 receptor protein expression was increased in the CCI group, but was decreased following cotreatment with gardenoside and ozone.

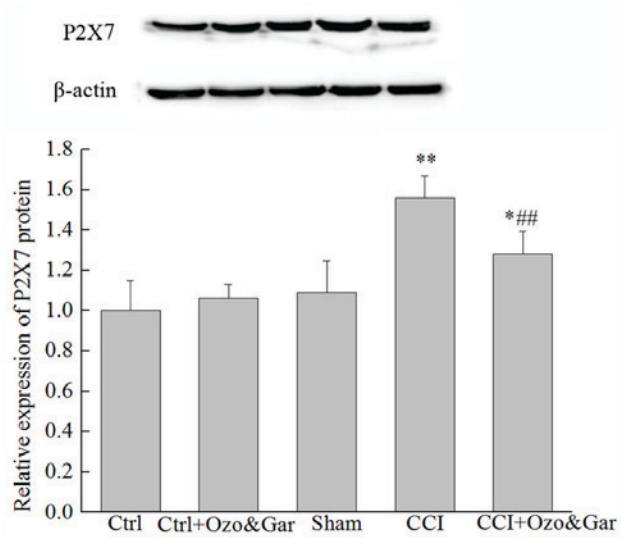

Figure 4. Protein expression levels of P2X7 receptor in the rats of the five groups with or without CCI and/or gardenoside and ozone cotreatment, as determined by western blotting. β-actin was used as a control. P2X7 receptor expression was significantly higher in the CCI group compared with in the Ctrl, Ctrl + Ozo&Gar and Sham groups. However, in the CCI + Ozo&Gar group, P2X7 receptor expression was significantly lower than in the CCI group. *P<0.05, **P<0.01 vs. Ctrl, Ctrl + Ozo&Gar and Sham groups; $^{\#\#}$P<0.01 vs. CCI group. CCI, chronic constriction sciatic nerve injury; Ctrl, control; Gar, gardenoside; Ozo, ozone.

Discussion

The present study used gardenoside combined with ozone to treat rats following the generation of a CCI model. The treatment proved to be effective with regards to pain relief, and decreased the mRNA and protein expression levels of P2X3 and P2X7 receptors in the DRG. The DRG is an anatomically discrete structure that forms part of the peripheral nervous

system, and is located laterally to the spine. The DRG is recognized as one of the organs that may be damaged in response to peripheral sensory neuropathic pain (17). The results of the present study indicated that the mechanism underlying the pain-relieving effects of gardenoside and ozone cotreatment may be associated with the inhibition of the mRNA and protein expression levels of P2X3 and P2X7 receptors in the DRG. The present study is the first, to the best of our knowledge, to experimentally demonstrate the mechanism underlying the effects of gardenoside and ozone on neuropathic pain.

P2X receptors are a family of cation-permeable ligand-gated ion channels that open in response to the binding of extracellular adenosine 5'-triphosphate, which results in the generation and transmission of pain and inflammatory nociceptive signals (18,19). It has been suggested that P2X3 homotrimers are responsible for acute pain, whereas P2X2/3 heterotrimers mediate chronic pain (20); therefore, P2X receptors have long been considered potential therapeutic targets for the treatment of inflammatory pain (21). P2X7 receptor is the most intensively investigated, and numerous pharmaceutical companies have synthesized small molecules that potently and selectively block its expression (21). In addition, there has also been significant progress in the specific targeting of the P2X3 homotrimeric and P2X2/3 heterotrimeric receptors (22). The findings of the present study suggested that novel chemicals, including gardenoside and ozone, may potentially target P2X3 and P2X7 receptors.

As an exploratory experiment, the present study may be considered successful. However, there are numerous questions that must be addressed and require follow-up studies. For example, it remains unclear whether gardenoside was oxidized into geniposide during treatment. Gardenoside and geniposide exist in *Gardenia* fruits (23,24); geniposide also exhibits a wide range of pharmacological activities, including hepatoprotective (25), hypoglycemic (26), insulin resistance-alleviating (27), antiproliferative (28), antioxidant (29), and antioxidant and neuroprotective effects (30). It is also used as a cross-linker to generate polymeric material in biomedical applications (31). In addition, it remains unclear as to whether the pain-relieving effects were mainly induced by ozone or gardenoside, or whether both were equally important. It also remains unclear as to whether ozone or gardenoside inhibited the mRNA and protein expression levels of P2X3 and P2X7 receptors in the DRG. Therefore, more experiments are required to address these questions.

Taken together, the present study confirmed the pain-relieving effects of gardenoside and ozone cotreatment, and also revealed a possible mechanism underlying this effect, which may be mediated by the inhibition of P2X3 and P2X7 receptors in the DRG. Therefore, gardenoside and ozone may be considered novel drug candidates that target P2X3 and P2X7 receptors.

In conclusion, the present study demonstrated that gardenoside combined with ozone was able to increase the MWT and TWL of rats that suffered from neuropathic pain, thus suggesting that this treatment could alleviate chronic neuropathic pain. The effects of gardenoside and ozone may be mediated by the inhibition of P2X3 and P2X7 receptor expression in the DRG.

Competing interests

The authors declare that they have no competing interests.

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