Experimental Trial

Intervertebral Foramen Injection of Ozone Relieves Mechanical Allodynia and Enhances Analgesic Effect of Gabapentin in Animal Model of Neuropathic Pain

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Background: In a 5-year follow-up study in a hospital in southern China, it was shown that intervertebral foramen (IVF) injection of ozone at the involved segmental levels could significantly alleviate paroxysmal spontaneous pain and mechanical allodynia in patients with chronic, intractable postherpetic neuralgia (PHN) and improve the quality of life. However, so far no proof-of-concept studies in animals have been available.

Objective: This study was designed to investigate whether IVF ozone has an analgesic effect on animal models of neuropathic and inflammatory pain.

Study Design: Experimental trial in rats.

Setting: Institute for Biomedical Sciences of Pain.

Methods: By IVF injection, a volume of 50 µl containing 30 µg/mL ozone-oxygen mixture or 50 µl air was carried out on male Sprague-Dawley rats of naïve, inflammatory pain states produced by injections of either bee venom or complete Freud's adjuvant, and neuropathic pain state produced by spared nerve injury, respectively. The effects of IVF ozone on pain-related behaviors were evaluated for 2 weeks or one month. Then combined use of gabapentin (100 mg/1 kg body weight) with IVF ozone was evaluated in rats with neuropathic pain by intraperitoneal administration 5 days after the ozone treatment. Finally, the analgesic effects of another 4 drugs, AMD3100 (a CXCR4 antagonist), A-803467 (a selective Nav1.8 blocker), rapamycin (the mTOR inhibitor), and MGCD0103 (a selective histone deacetylase inhibitor) were evaluated for long term through IVF injection, respectively.

Results: (1) IVF injection of ozone at L4-5 was only effective in suppression of mechanical allodynia in rats with neuropathic pain but not with inflammatory pain; (2) the analgesic effects of IVF ozone lasted much longer (> 14 days) than other selective molecular target drugs (< 48 hours) inhibiting or antagonizing at Nav1.8 (A-803467), CXCR4 (AMD3100), mTOR (rapamycin), and histone deacetylase (MGCD0103); (3) combined use of systemic gabapentin and IVF ozone produced a synergistic analgesic effect in rats with neuropathic pain.

Limitations: Evaluation of the possible analgesic effects of the intraplantar injection of ozone was not performed.

Conclusions: In the present study, we provided a line of evidence for the first time that IVF injection of ozone selectively relieved neuropathic pain but not inflammatory pain, and enhanced the analgesic effect of gabapentin.

Key words: Chronic pain, neuropathic pain, inflammatory pain, ozone therapy, interventional therapy, gabapentin, spared nerve injury, bee venom, complete Freud's adjuvant

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Neuropathic pain is newly defined as pain arising as a direct consequence of a lesion or disease affecting the somatosensory system (1). Neuropathic pain presents unique diagnostic and therapeutic challenges, affecting 3.0% to 8.0% of the population (2-5). However, available treatments, either of pharmacological or nonpharmacological (including interventional) therapies provides only insufficient symptomatic relief (6-8). Being recommended as first-line drugs, gabapentinoids, the Federal Drug Administration (FDA)-approved anticonvulsants, have been frequently used to manage neuropathic pain in clinics for many years. However, ineffectiveness and tolerance have also been noted with long-term use of the drugs. Nonetheless, so far, the phenomenon of gabapentinoid insensitivity has been greatly neglected and underestimated. Recently, Moore and colleagues (9) found that about 35% of participants achieved about 50% of pain relief with gabapentin, while over half of those treated with it had no worthwhile pain relief, suggesting a problem of tolerance or insensitivity for gabapentinoid analgesia. In animal studies, although short-term administration did not reveal gabapentinoid insensitivity, long-term use of the drugs definitely produced such a phenomenon (10-12). For example, it has been found that repeated treatment with gabapentin in a rat model of central post-stroke pain for at least 4 weeks resulted in a distinct decrease in analgesic effectiveness (13). The weak and negative effects of gabapentinoids have also been indicated in some peripheral neuropathic pain models (14,15). Because it has been demonstrated that the $\alpha 2\delta$–1 subunit of the voltage-gated calcium channel, the selective binding site of gabapentinoids, was up-regulated in the early stage of central post-stroke pain but followed by dramatic down-regulation, an off-target effect was proposed to be associated with gabapentinoid insensitivity in animals (16-19). To provide neuropathic pain treatment, new therapeutic approaches that can enhance analgesic effectiveness or decrease the daily dose of gabapentinoids are critically needed and require proof-of-concept preclinical studies.

Interventional therapies have long been used for management of chronic pain. However, in a recent review article published by the neuropathic pain special interest group (NeuPSIG) of the International Association for the Study of Pain (IASP), it was stated that none of the existing interventional therapeutic approaches could be strongly recommended for management of chronic neuropathic pain due to the paucity of high-quality clinical trials (6). Nevertheless, in a 5-year follow-up study in a hospital in southern China, intervertebral foramen (IVF) injection of ozone at the involved segmental levels has been shown to significantly alleviate paroxysmal spontaneous pain and mechanical allodynia in patients with chronic intractable postherpetic neuralgia (PHN) and improve the quality of life (20). More interestingly, after IVF ozone, the daily dose of gabapentin or pregabalin could also be reduced to a tolerable level (20). Ozone therapy started in the beginning of the nineteenth century (21-24). Because pure ozone does not exist under natural or artificial conditions, it is usually referred to as oxygen-ozone mixture that can be generated by an ozone generator. Ozone therapy has been most commonly used for the treatment of intervertebral disc herniation (25) and some conditions in the field of stomatology (26). More recently, some physicians also tried to use ozone therapy to treat lower back pain and knee osteoarthritis through intraforaminal or intraarticular injection (27,28).

To get insight into the underlying mechanisms of ozone therapy in the treatment of neuropathic pain, the aims of the current study were (1) establishment of an intervertebral foramen route for delivery of ozone or other drugs in rats; (2) assessment of the analgesic effects of IVF ozone in animals of naïve, neuropathic and/or inflammatory pain conditions; (3) assessment of the analgesic effects of combined use of IVF ozone and intraperitoneal (i.p.) injection of gabapentin in animal model of neuropathic pain.

Methods

Animals

The experiments were performed on male Sprague-Dawley rats weighing 160 – 250 g. The animals were provided by the Laboratory Animal Center of the Fourth Military Medical University (FMMU). The use of animals was reviewed and approved by the Animal Care and Use Committee of the FMMU. All experiments on animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996), and followed the ethical guidelines for pain research in conscious animals of IASP. Rats were housed in a climate-controlled room (22 – 26 °C) under a light/dark cycle of 12 hour/12 hour with access to food and water ad libitum. The light was on at 08:00 and all somatic functional evaluations were carried out between 09:00 and 18:30.

The rats were acclimated to test boxes for at least 30 minutes each day before the first testing. The number of animals used and their suffering was minimized.

Experimental Design and Preparation of Pain Models

Experimental Design

In the present study, 3 models were used: a spared nerve injury (SNI) model was used as a chronic neuropathic pain model (29); while a bee venom (BV) model (30,31) and complete Freund's adjuvant (CFA) model (32) were used as acute and chronic inflammatory pain models, respectively. The rats were randomly divided into 6 groups: (1) rats with SNI not receiving any therapeutic treatment (n = 15), receiving single IVF injection of ozone (n = 15) or air (n = 15); (2) naïve rats receiving a single IVF injection of ozone (n = 8) or air (n = 8); (3) rats with intraplantar (ipl) BV injection not receiving any therapeutic treatment (n = 9), receiving a single IVF injection of ozone (n = 9) or air (n = 9); (4) rats with ipl CFA injection not receiving any therapeutic treatment (n = 9), receiving a single IVF injection of ozone (n = 9) or air (n = 9); (5) rats with SNI receiving vehicle (n = 8) or IVF injection of drugs including AMD3100, a CXCR4 antagonist (n = 8) (33,34); A803467, a selective Nav1.8 blocker (n = 8) (33); rapamycin, the mTOR inhibitor (n = 8) (35); and MGCD0103, a selective class I histone deacetylase inhibitor (n = 8) (36); which have been demonstrated to be effective in suppression of pathological pain, respectively and served as positive controls in the current study; (6) rats with SNI receiving combined use of a single IVF injection of ozone plus i.p. injection of gabapentin (n = 8), with a single IVF injection of ozone plus i.p. injection of saline (n = 8), i.p. saline (n = 8), or i.p. gabapentin (n = 8) being used as controls.

In rats with SNI, IVF injection of ozone was administered 2 weeks after the surgical preparation when pain hypersensitivity reached its peak and was stable. In this set of experiments, the mechanical pain sensitivity was measured before and after model preparation, followed by 28 days of measurements (post-IVF injection day 1, 3, 5, 7, 10, 14, 21, and 28).

In rats with acute inflammatory pain, IVF injection of ozone was given one day prior to ipl BV treatment. Immediately after BV treatment, persistent spontaneous nociceptive behavior (number of paw flinches) was scored for 60 minutes, followed by measurement of paw withdrawal mechanical threshold (PWMT) and paw withdrawal thermal latency (PWTL) 2 – 4 hours after ipl BV injection.

In rats with chronic inflammatory pain, IVF injection of ozone was given 3 days after ipl CFA treatment when pain hypersensitivity had been well established. The mechanical and thermal pain sensitivity were evaluated before and after CFA treatment followed by 2 weeks of measurements (post-IVF injection day 1, 3, 5, 7, 10, and 14).

In rats with SNI receiving both IVF injection of ozone and i.p. administration of gabapentin, i.p. treatment of gabapentin was administered at post-IVF ozone day 5 when the analgesic effect of ozone had reached its peak and was stable. The synergistic effect of ozone and gabapentin was evaluated for 5 hours (post-i.p. gabapentin hour 0.5, 1, 1.5, 2, 3, and 5).

Pain Models

The BV model is a well-established animal model of acute inflammatory pain (30,31). The model was established by injecting a volume of 50 µl BV solution containing 0.2 mg BV (Sigma) dissolved in 50 µl physiological saline, into the center of the plantar surface of left hind paw of rats with slight restraint (31).

The CFA model is also a well-established animal model of chronic inflammatory pain (37). A volume of 100 µl CFA (Sigma, each mL of solution contains 1 mg of heat-killed and dried Mycobacterium tuberculosis, 0.85 mL paraffin oil, and 0.15 mL of mannide monooleate) was injected into the center of the plantar surface of one hindpaw of rats, with slight restraint (32).

The SNI model of neuropathic pain was prepared as described previously (29). In brief, under 6% chloral hydrate (300 mg/kg, i.p.) anesthesia, the left sciatic nerve and its 3 terminal branches—the sural, common peroneal, and tibial nerves were exposed. The common peroneal and tibial nerves were tight-ligated with 5.0 silk thread followed by removal of 2 – 4 mm of nerve stump, leaving the sural nerve intact. Then, the muscle and skin were closed layer by layer.

Quantitative Score of Pain-related Behaviors in Awake Animals

Persistent Spontaneous Nociceptive Behavior

According to previous works in our laboratory, a 30 × 30 × 30 cm transparent plastic box was placed on a supporting frame of 30 cm high above the experimental table. The rat was placed in the test box for at

least 30 minutes before administration of any chemical agents. After the acclimation period, an ipl injection of BV was made as described above. Quantitative score of persistent spontaneous nociception was obtained by counting the number of paw flinches occurring at each 5-minute interval for one hour following ipl BV injection.

Mechanical Pain Sensitivity

For determination of the threshold of paw withdrawal after a mechanical stimulus, the rats were placed on an elevated wire grid covered with a transparent plastic chamber (20 × 20 × 25 cm). A series of increasing force von Frey monofilaments (0.8, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0, 25.0, 30.0, 45.0, and 60 g) were used to bilaterally stimulate the plantar surface of the rat's hind paws. The PWMT was taken as the lowest forces that induce at least 5 brisk withdrawal responses in 10 repetitive stimuli. The interval between each stimulus is no less than 5 seconds. The interval between stimuli of left and right hind paw is no less than 5 minutes.

Thermal Pain Sensitivity

The thermal pain sensitivity was determined by measuring the withdrawal latency of the hind paws in response to radiant heat. Rats were placed in a plastic chamber on the surface of a 2 mm thick glass plate and the sensitivity to heat stimuli by a TC-1 radiant heat stimulator (new generation of RTY-3 made in Xi'an Bobang Technologies of Chemical Industry Co. Ltd., China, 10 V) was measured. The heat stimuli were applied to both the injection site and the corresponding area of the contralateral paw, and the latency was determined as the duration from the beginning of heat stimuli to the occurrence of a marked withdrawal reflex. Five stimuli were repeated for each site, and the latter 3 values were averaged as mean PWTL. A cut-off of 30 seconds was chosen to prevent tissue damage. The inter-stimulus interval for each heat test was more than 15 minutes at the same region and 10 minutes at the different paws.

Intervertebral Foramen Route of Drug Delivery

Procedures for Intervertebral Foramen Route of Drug Delivery

The design of IVF route of drug delivery was based upon methods and experimental procedures in rats published previously (38,39) and modified as in Fig. 1. In brief, after shaving the fur over the lower back, the animals were anesthetized by 6% chloral hydrate (i.p.) and placed over a small cylinder to elevate the lumbar region. For L5-foramen, the point of skin puncture was defined at a crossing point 'b' of a para-posterior midline of the vertebral column (see a dashed line 1.5 cm laterally to the vertical solid line in Fig. 1A) and a subhorizontal line (see a dashed line 0.5 cm caudal to the solid inter-iliac crest line in Fig. 1A). For L4-foramen, the puncture point was crossing point 'a' about 1 cm rostral to crossing point 'b' (Fig. 1A). To facilitate the penetration of the injecting needle through the skin, an initial puncture with a larger needle (1.2 × 38 mm, 18G) was made. In sequence, the injecting needle (0.3 × 21 mm, 30G) is inserted at an angle of 45° both to the horizontal and sagittal plane to approach the L5-foramen (Fig. 1B) through the punctured skin, towards the intervertebral foramen between the L5 and L6 vertebrae (or inter L4-5 foramen), until the tip touched the lateral region of the vertebrae. To localize the inter L5-6 or inter L4-5 intervertebral foramen, delicate movements of the needle were made until the bone resistance was diminished and a paw flinch reflex was observed. The paw flinch reflex was used as a sign that the needle tip entered the intervertebral foramen.

Drugs and Agents

An accurate concentration of ozone (μg/mL: μg of O_3 per mL of O_2) was obtained from clinical grade oxygen using a medical ozone generator (Medozon Compact, Germany). Because 10 – 30 μg/mL ozone-oxygen has been commonly used as safe and effective therapeutic concentrations for treatment of patients (40), and one report indicated that concentrations over 40 μg/mL would be toxic in vitro (41), a dose of 30 μg/mL ozone-oxygen in 50 μl volume was chosen for IVF injections. To confirm the localization of this delivery route, intraganglionar injection of DiI (2.5%) was administered in 5 rats through IVF route, the presence of DiI was confirmed in each rat by exposure of the lumbar dorsal root ganglia (DRG) 15 minutes after injection. The results showed the existence of DiI-labeling only in L4 and L5 DRGs (Fig. 1C). Gabapentin was a gift from Enhwa Pharmaceutical Group (Xuzhou, Jiangsu Province, P. R. China) and 100 mg per 1 kg body weight was used through the whole experiment. IVF injection of AMD3100, a CXCR4 antagonist (Sigma, 100 μg/10 μl dissolved in 0.9% sterile saline); A803467, a selective Nav1.8 blocker (Abcam, 30 μg/10 μl dissolved in DMSO);

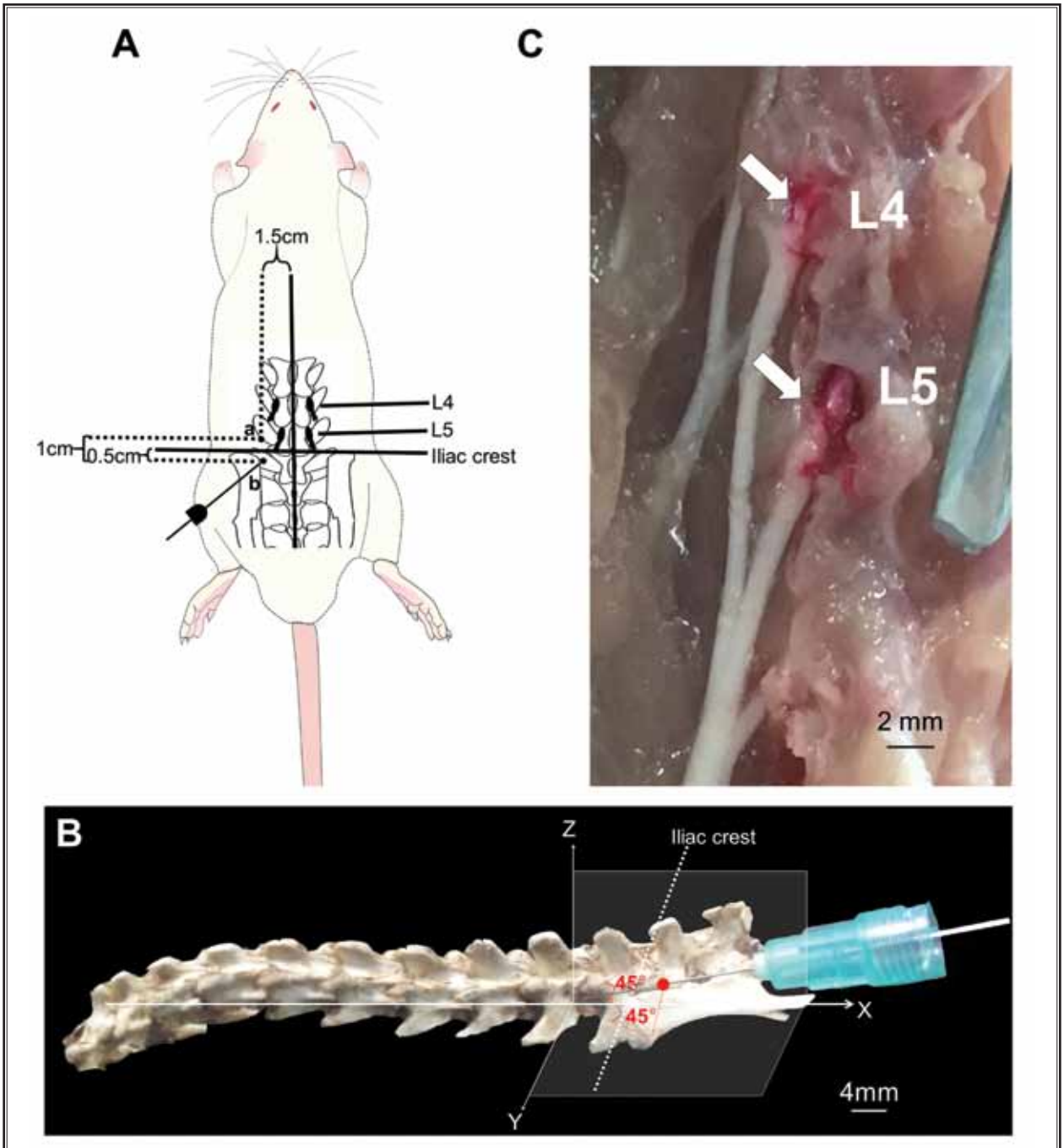

Fig. 1. *IVF injection of ozone. (A), crossing point 'a' and 'b' are the insertion positions of the L4-5 and L5-6 intervertebral foramens, respectively. The L5-6 foramen is accessed by introducing the needle to crossing point 'b' of a para-posterior midline of the vertebral column (dashed line 1.5 cm laterally to the vertical solid line) and a sub-horizontal line (dashed line 0.5 cm caudal to the solid inter-iliac crest line), then toward the space between the lateral processes of the L5 and L6 lumbar vertebrae. For L4-5 foramen, the puncture point was crossing point 'a' which was about 1 cm rostral to crossing point 'b'. (B), the injecting needle is inserted at an angle of 45° both to the horizontal and sagittal plane to approach the L5-6 foramen and L4-5 foramens. Red solid circle, namely, the point 'b' in 1A, is the insertion position of the L5-6 intervertebral foramen. The origin of the axis is the position of L5 dorsal root ganglion (DRG), where the drug is injected. Dashed line, inter-iliac crest line. Scale bar, 4 mm. (C), the sites of IVF injection of ozone at the L4 and L5 DRGs were localized by IVF injection of 2.5% DiI injections. Scale bar, 2 mm.*

rapamycin, the mTOR inhibitor (Chengdu YATU biotechnology CO., 2.5 μg/10 μl dissolved in DMSO); and MGCD0103, a selective histone deacetylase inhibitor (Selleck Chemicals, 50 μg/10 μl dissolved in DMSO), respectively, served as positive control of IVF injection of ozone.

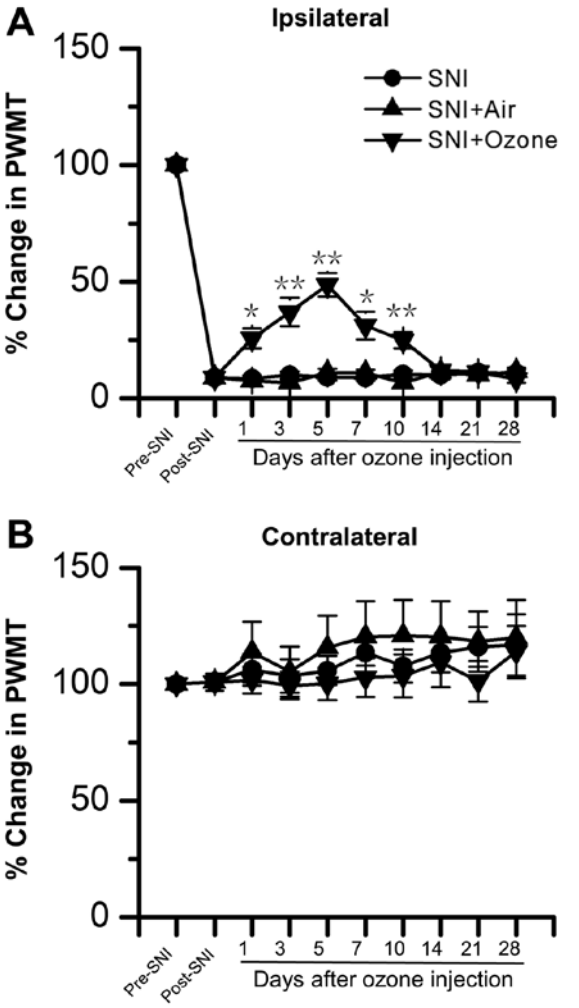

Fig. 2. *Effects of IVF injection of ozone in rats with chronic neuropathic pain. (A), 28 days time course observation for the percent changes in ipsilateral PWMT in rats with SNI, SNI treated with IVF injection of air (SNI+Air), and SNI treated with IVF injection of ozone (SNI+Ozone). (B), 28 days of time course observation for the changes in contralateral PWMT in rats with SNI, SNI+Air, and SNI+Ozone. **P < 0.01, *P < 0.05, Ozone vs. Air. PWMT, paw withdrawal mechanical threshold. n = 15 for each group.*

Statistical Analysis

Statistical analyses were carried out using SPSS v19.0 (SPSS, Chicago, IL, USA). All data were expressed as mean ± SEM. To explore enhanced effect of ozone on the analgesic effect of gabapentin, the percent maximum possible effect (% MPE) in 2 situations (ozone pretreated or not) was calculated: % MPE = ($PWMT_{h\ post\text{-}GBP}$ – $PWMT_{pre\text{-}GBP}$) / ($PWMT_{baseline}$ – $PWMT_{pre\text{-}GBP}$) × 100%. A 2-way analysis of variance for repeated measures was used to evaluate the overall effect of the treatment compared with other groups followed by Dunnett's test or a post hoc Student's t-test when appropriate. Value of $P < 0.05$ was considered significant.

Results

Effects of IVF Injection of Ozone in Rats with Chronic Neuropathic Pain

As shown in Fig. 2A, single IVF injection of ozone-oxygen on the side of the nerve injury 14 days after establishment of the SNI model resulted in long-term relief of mechanical allodynia relative to IVF injection of air or non-treatment control. Time course of 28 days of observations showed that the significant therapeutic analgesic effect occurred on the post-IVF ozone day one, followed by gradual increase in effectiveness on the following day 3 and reached peak on day 5 (Fig. 2A). The analgesic effect of IVF ozone began to decline on day 7 and disappeared on day 14 (Fig. 2A). Unilateral IVF injection of ozone between L4-5 and L5-6 levels didn't affect PWMT on the contralateral side of the hindpaw (Fig. 2B).

To further confirm whether IVF injection of ozone would change the basic pain sensitivity, the same dose of ozone was adminsitered in naïve rats. Time couse of 14 days of observations showed no change in either PWMT or PWTL on either side of rat's hindpaws following unilateral IVF injection of ozone or air (Fig. 3).

Effects of IVF Injection of Ozone in Rats with Acute and Chronic Inflammatory Pain

Following ipsilateral IVF injection of ozone, no analgesic effects were found in either the acute inflammatory pain model induced by ipl BV (Fig. 4A-D) or chronic inflammatory pain model induced by ipl CFA (Fig. 4E-F). Moreover, ipsilateral IVF injection of ozone did not relieve either spontaneous pain behaviors (Fig. 4A-B) or mechanical and thermal pain hypersensitivity in either of the 2 inflammatory pain models (Fig. 4C-F).

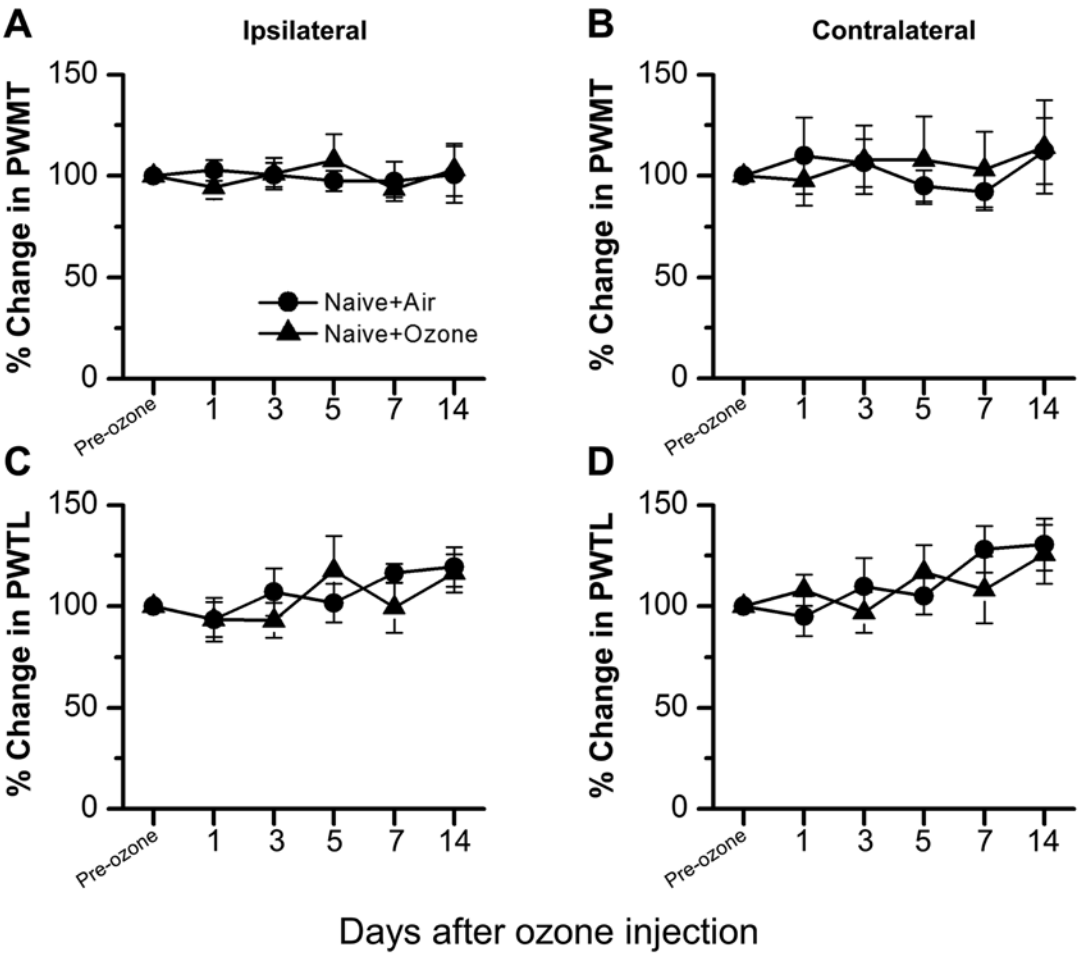

Fig. 3. *Effects of IVF injection of ozone on the basic pain sensitivity. (A)-(B), 14 days time course observation for the percent changes in ipsilateral (A) and contralateral (B) PWMT in naïve rats. (C)-(D), 14 days time course observation for the percent changes in ipsilateral (C) and contralateral (D) PWTL in naïve rats. PWMT, paw withdrawal mechanical threshold; PWTL, paw withdrawal thermal latency. n = 8 for each group.*

Analgesic Effects of IVF Delivery of Some Molecular Target Drugs in Rats with Neuropathic Pain

IVF deliveries of 4 drugs antagonizing at SDF1-CXCR4 signaling with AMD3100 (n = 8), blocking Nav1.8 subunit of voltage-gated sodium channels with A803467 (n = 8), inhibiting mTOR with rapamycin (n = 8), and inhibiting class I histone deacetylase with MGCD0103 (n = 8), respectively, were shown to produce significant analgesic effects in rats with SNI (Fig. 5). However, compared to the longer (14 days) effect of IVF injection of ozone (Fig. 2A), the analgesic effects of the above 4 drugs were much more short-lasting in duration (less than 48 hours utmost) (Fig. 5). AMD3100 had the longest analgesic effect among the 4 drugs which began to produce analgesia at 2 hours, peaked at 6 hours, and vanished within 48 hours after IVF administration. A803467 was the one with the shortest analgesic effect among the 4 drugs which began to produce analgesia at 2 hours, peaked at 4 hours, and vanished within 8 hours after IVF administration. Rapamycin also produced short-lasting analgesia for less than 8 hours after IVF administration, displaying a peak at 4 hours and vanished within 8 hours. MGCD0103 had a long delay in effectiveness which began to produce analgesia at about 8 hours, peaked at 24 hours, and vanished at about 96 hours after IVF administration.

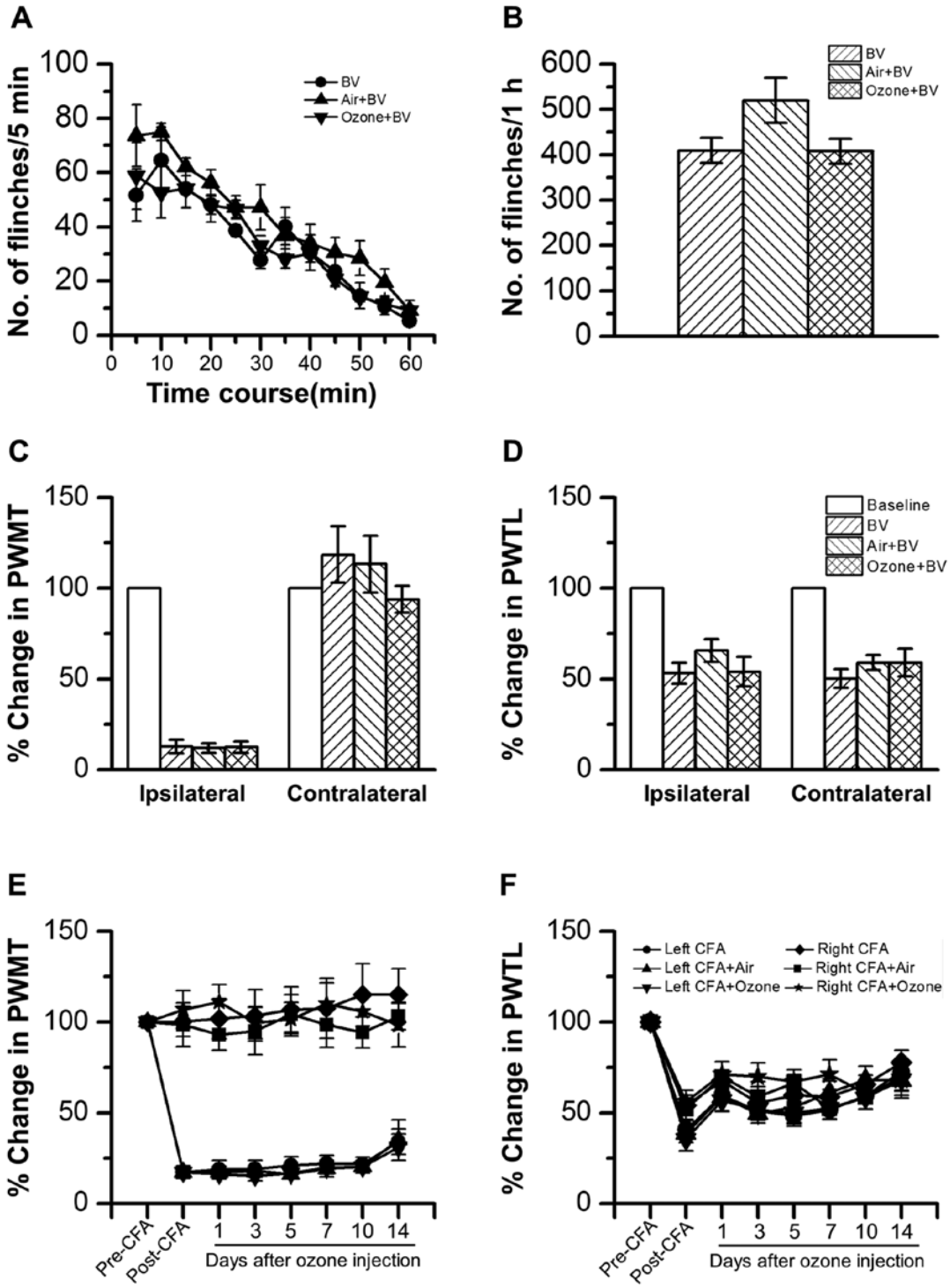

Fig. 4. *Effects of IVF injection of ozone in rats with acute and chronic inflammatory pain. (A)-(B), showing one hour time course observation of the effect of IVF ozone on persistent spontaneous nociception (A) and the mean total numbers of paw flinches averaged from one hour period following ipl bee venom (BV) injection (B). (C)-(D), exhibiting the effects of IVF ozone on the BV-induced mechanical and thermal hypersensitivity. (E)-(F), exhibiting the effects of IVF ozone on the complete Freund's adjuvant (CFA)-induced mechanical and thermal hypersensitivity. PWMT, paw withdrawal mechanical threshold; PWTL, paw withdrawal thermal latency. n = 9 for each group.*

Synergistic Effect of IVF Ozone with Systemic Gabapentin in Rats with Neuropathic Pain

Similar to most of previous reports, i.p. administration of gabapentin alone (SNI+GBP, n = 8) only produced mild to moderate (20% to 40%) relief of mechanical pain hypersensitivity in rats with SNI relative to saline control (n = 8) (Fig. 6A-B). The analgesic effect of gabapentin alone could be detected on both sides of the tested hindpaws, which began to appear at about 0.5 to 1 hour and reached peak at 2 hours and vanished within 5 hours after i.p. administration (Fig. 6A-B). However, i.p. administration of gabapentin 5 days after IVF delivery of ozone (peak time for analgesia) produced a synergistic analgesic effect in rats with SNI, showing more than 80% relief of mechanical pain hypersensitivity (Fig. 6A). In contrast, i.p. saline 5 days after IVF delivery of ozone only produced at most 48% of the relief of mechanical pain hypersensitivity in rats with SNI, although the level of analgesia remained unchanged during the whole time course of observation (Fig. 6A). Moreover, analysis of the results from SNI rats receiving gabapentin alone and gabapentin plus ozone showed that there was no statistically significant difference in analgesic effects on the contralateral side, suggesting that synergistic analgesic effect of gabapentin and ozone could only occur at the side of IVF delivery of the gas (Fig. 6B). Areas under percent maximum possible effect (% MPE) curves showed that combined use of both IVF ozone and systemic gabapentin resulted in at least 2- to 4-fold of the improvement produced by i.p. gabapentin alone (Fig. 6C). The relative value of areas under curves was 231.20 ± 65.35 vs. 57.48 ± 15.30.

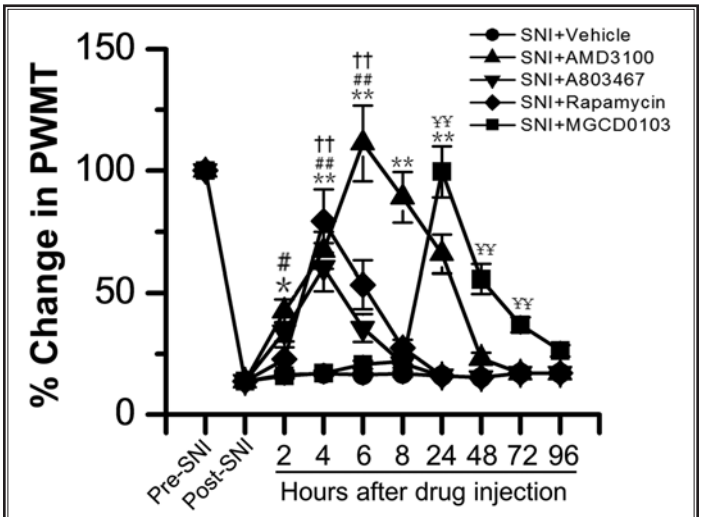

Fig. 5. *Effects of IVF delivery of 4 molecular target drugs in rats with neuropathic pain. Ninety-six hour time course observation of the effects of AMD3100, a selective CXCR4 antagonist; A803467, a selective Nav1.8 blocker; rapamycin, a selective mTOR inhibitor; and MGCD0103, a selective class I histone deacetylase.* $^{**}P < 0.01$, $^{*}P < 0.05$, *AMD3100 vs. vehicle;* $^{\#\#}P < 0.01$, $^{\#}P < 0.05$, *A803467 vs. vehicle;* $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$, *rapamycin vs. vehicle;* $^{\text{¥¥}}P < 0.05$, *MGCD0103 vs. vehicle. PWMT, paw withdrawal mechanical threshold; SNI, spared nerve injury. n = 8 for each group.*

Discussion

IVF Injection of Ozone Relieves Mechanical Allodynia and Enhances Analgesic Effect of Gabapentin in Animal Model of Neuropathic Pain

In the current study, we provided a new line of experimental evidence showing that IVF delivery of ozone can selectively relieve neuropathic pain but not acute or chronic inflammatory pain in rats. Compared to other drugs targeting a single molecular site, the analgesic effect of IVF injection of ozone was long-lasting and sustained for at least 14 days after a single treatment. The analgesia produced by IVF ozone was thought to be a local effect rather than systemic effect because no analgesia was found on the contralateral side. No analgesic effects were found in naïve rats either. Furthermore, the stable presence of IVF ozone-produced analgesia could be achieved 2 weeks after well establishment of neuropathic pain condition. It is therefore suggested that IVF ozone treatment may have a strong therapeutic effect in relieving neuropathic pain as seen in a 5-year follow-up study of IVF administration of ozone for the treatment of elderly patients with intractable chronic PHN (20).

In the present study, we also provided another line of experimental evidence showing that synergistic analgesic effect could be obtained in neuropathic painful rats by combined use of IVF ozone and i.p. gabapentin. Moreover, combined use of IVF ozone and i.p. gabapentin can achieve at least 2- to 3-fold improvement over a single treatment of the 2 therapies. The synergistic analgesic effect produced by the combined use of IVF ozone and i.p. gabapentin observed in rats was also true for PHN patients who received only one or two treatments with IVF ozone followed by daily use of oral gabapentin (20). The

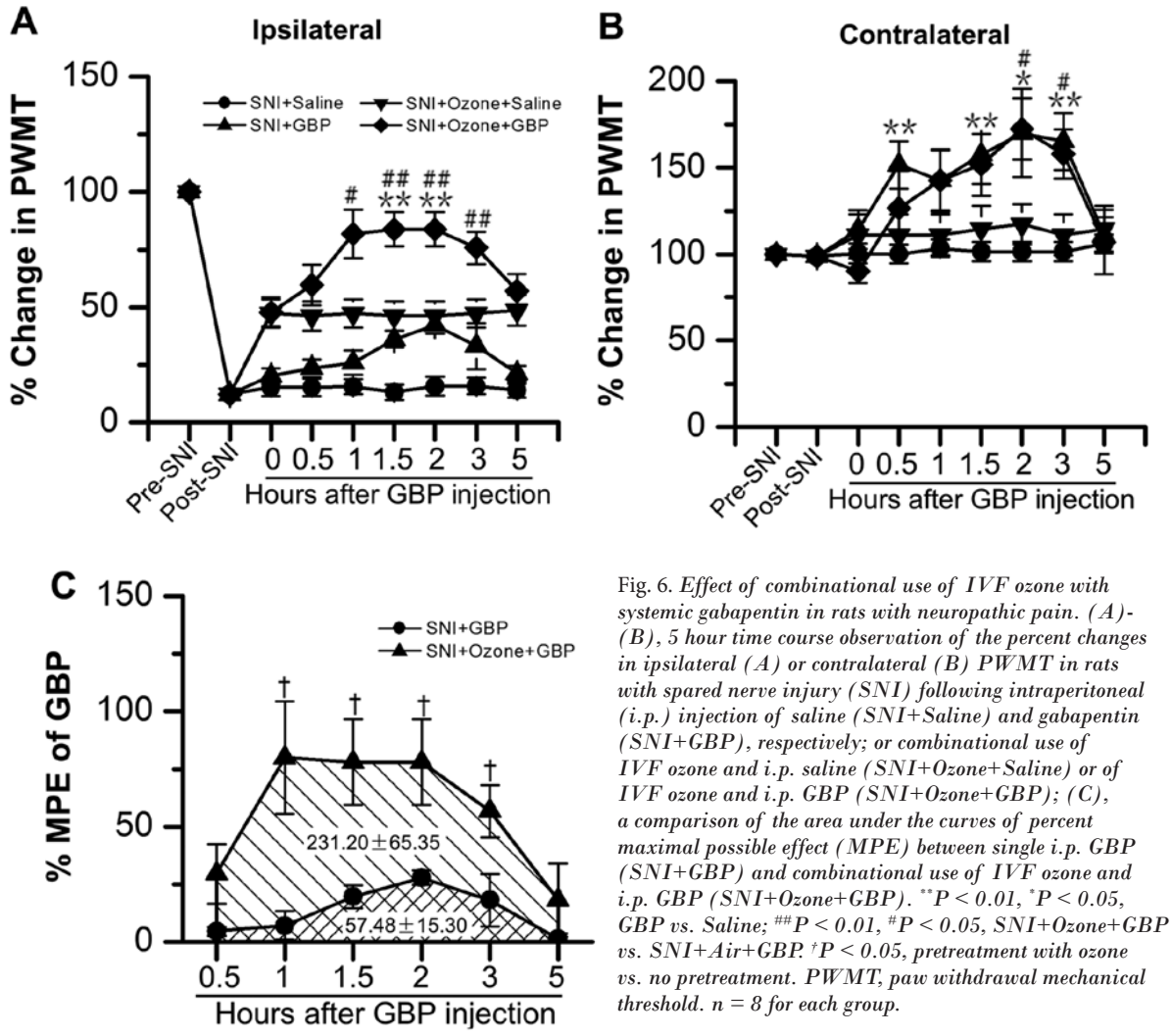

Fig. 6. *Effect of combinational use of IVF ozone with systemic gabapentin in rats with neuropathic pain. (A)-(B), 5 hour time course observation of the percent changes in ipsilateral (A) or contralateral (B) PWMT in rats with spared nerve injury (SNI) following intraperitoneal (i.p.) injection of saline (SNI+Saline) and gabapentin (SNI+GBP), respectively; or combinational use of IVF ozone and i.p. saline (SNI+Ozone+Saline) or of IVF ozone and i.p. GBP (SNI+Ozone+GBP); (C), a comparison of the area under the curves of percent maximal possible effect (MPE) between single i.p. GBP (SNI+GBP) and combinational use of IVF ozone and i.p. GBP (SNI+Ozone+GBP). $^{**}P < 0.01$, $^{*}P < 0.05$, GBP vs. Saline; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$, SNI+Ozone+GBP vs. SNI+Air+GBP. $^{\dagger}P < 0.05$, pretreatment with ozone vs. no pretreatment. PWMT, paw withdrawal mechanical threshold. n = 8 for each group.*

total amount of daily dose of gabapentin (or pregabalin) could be decreased to 600 – 900 mg from 1200 mg – 1800 mg (20). Collectively, this proof-of-concept study highly supports the clinical use of IVF ozone in the management of neuropathic pain in general and PHN in particular, and calls for a multiple center assessment of the therapeutic effect of the approach.

IVF Route of Ozone Delivery Is Appropriate for Treatment of Neuropathic Pain but Not Inflammatory Pain Conditions

In the current study, it is interesting to find that IVF ozone-induced analgesia was likely to be present only under neuropathic pain conditions caused by SNI but not inflammatory pain conditions caused by ipl injection of BV and CFA. So far, the underlying mechanisms of IVF ozone-induced analgesia under neuropathic pain conditions remain unclear and are worthy of being further studied in both basic and clinical settings.

However, in some clinical studies, ozone therapy has been demonstrated to be effective in the treatment of some inflammatory pain conditions, such as knee osteoarthritis, low back pain, and diabetic foot (24,28,42-46). One animal study also suggests a possible analgesic effect on inflammatory pain in a model of capsaicin-induced edema (47). The discrepancy in results between these previous clinical and basic investigations and our current study is probably due to different routes for the delivery of ozone. In the previous studies, ozone was mostly injected into the sites where inflammatory

responses were occurring, while in our current study ozone was injected through an IVF route from which the loci of inflammatory responses caused by subcutaneous injection of BV or CFA was far away. Thus, the results from the present study only demonstrate that the IVF route for ozone delivery is likely to be selectively effective in treatment of neuropathic pain but not inflammatory pain. This result also implicates that local administration of ozone at the site of tissue injury and inflammatory responses might be effective in the treatment of inflammatory pain which requires further testing in different animal models.

Synergistic Analgesic Effect of Combined Use of IVF Ozone and Systemic Gabapentin for Treatment of Neuropathic Pain

It is more intriguing to find that combined use of IVF ozone and systemic gabapentin can produce a synergistic analgesic effect in rats with neuropathic pain. The similar synergistic analgesic effect has also been noted in a clinical setting for treatment of PHN by IVF ozone and oral gabapentin or pregabalin (20). In a 5-year follow-up study, the total amount of daily use of gabapentin was reduced from 1200 – 1800 mg to 600 – 900 mg after administration of one or two doses of CT-guided IVF ozone (unpublished data) (20). So far, the finding of synergistic analgesia caused by a combined use of IVF ozone and systemic gabapentin for the treatment of neuropathic pain is novel and there is no study available to explain such a therapeutic effect.

The SNI model represents an animal model of persistent peripheral neuropathic pain (14,29). Its most notable feature is long-term (at least 9 weeks) mechanical pain hypersensitivity which can mimic the clinical symptom of mechanical allodynia (29). It has been shown that both peripheral (DRG) and spinal levels can be changed by this type of peripheral nerve injury that involves changes in both expression and transcriptional levels of a variety of molecular signaling pathways including cytokines, chemokines, lipid mediators, NaV1.8, voltage-gated Ca^{2+} channel subunit $\alpha2\delta$-1, and opioid receptors, etc. (13,16,19,48,49). Among these molecular targets, $\alpha2\delta$-1 has been demonstrated to be the binding sites of gabapentin (16-18). It has been demonstrated that up-regulated expression of spinal $\alpha2\delta$-1 at the central terminals of DRG nociceptor cells can be caused by neuropathic pain conditions (16,17,19). It has been thus proposed that the binding of gabapentin with $\alpha2\delta$-1 would block the trafficking of more $\alpha2\delta$-1 subunits from cytosol to presynaptic membrane, leading to inhibition of neurotransmitter release (16,17,19). How ozone synergizes the therapeutic effect of gabapentin is not known; the possible effects of ozone on the function and structure of $\alpha2\delta$-1 at both transcriptional and translational levels should be carefully examined in near future.

What Are the Possible Underlying Mechanisms of IVF Ozone-induced Analgesia under Neuropathic Pain Conditions?

It has been traditionally proposed that ozone therapy has anti-inflammatory action through reactivation of the innate antioxidant system, resisting against the oxidative stress (45). The biological effects of ozone are believed to be indirectly elicited by reactive oxygen species (ROS) and lipid oxidative products (LOPs) that are produced immediately after ozone is dissolved in biological water (physiological saline, plasma, lymph, urine, interstitial fluid) (40). ROS (H_2O_2 and hydroxyl radicals, etc.) and LOPs have been shown to induce activation of nuclear transcriptional factors, such as nuclear factor-erythroid 2-related factor 2 (Nrf2) (45) to alleviate pain (50,51). However, due to the paucity of experimental studies (52), the biological and pharmacological effects of ozone on the nervous system are not known. Pharmacologically, for example, whether ozone has direct or indirect actions on transmembrane proteins (receptors and ion channels), intracellular cascades, and nuclear transcriptions is yet unclear. Biologically, it is also interesting to examine whether ozone has repairing and restoring functions in the nervous system. Getting insight into the biological and pharmacological actions of ozone in the nervous system would shed new light on the therapy of neuropathic pain.

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References

1. Haanpaa M, Attal N, Backonja M, Baron R, Bennett M, Bouhassira D, Cruccu G, Hansson P, Haythornthwaite JA, Iannetti GD, Jensen TS, Kauppila T, Nurmikko TJ, Rice AS, Rowbotham M, Serra J, Sommer C, Smith BH, Treede RD. NeuPSIG guidelines on neuropathic pain assessment. *Pain* 2011; 152:14-27.
2. Bouhassira D, Lanteri-Minet M, Attal N, Laurent B, Touboul C. Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain* 2008; 136:380-387.
3. Gustorff B, Dorner T, Likar R, Grisold W, Lawrence K, Schwarz F, Rieder A. Prevalence of self-reported neuropathic pain and impact on quality of life: A prospective representative survey. *Acta Anaesthesiol Scand* 2008; 52:132-136.
4. Torrance N, Smith BH, Bennett MI, Lee AJ. The epidemiology of chronic pain of predominantly neuropathic origin. Results from a general population survey. *J Pain* 2006; 7:281-289.
5. van Hecke O, Austin SK, Khan RA, Smith BH, Torrance N. Neuropathic pain in the general population: A systematic review of epidemiological studies. *Pain* 2014; 155:654-662.
6. Dworkin RH, O'Connor AB, Kent J, Mackey SC, Raja SN, Stacey BR, Levy RM, Backonja M, Baron R, Harke H, Loeser JD, Treede RD, Turk DC, Wells CD. Interventional management of neuropathic pain: NeuPSIG recommendations. *Pain* 2013; 154:2249-2261.
7. Finnerup NB, Sindrup SH, Jensen TS. The evidence for pharmacological treatment of neuropathic pain. *Pain* 2010; 150:573-581.
8. Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, Gilron I, Haanpaa M, Hansson P, Jensen TS, Kamerman PR, Lund K, Moore A, Raja SN, Rice AS, Rowbotham M, Sena E, Siddall P, Smith BH, Wallace M. Pharmacotherapy for neuropathic pain in adults: A systematic review and meta-analysis. *Lancet Neurol* 2015; 14:162-173.
9. Moore RA, Wiffen PJ, Derry S, Toelle T, Rice AS. Gabapentin for chronic neuropathic pain and fibromyalgia in adults. *Cochrane Database Syst Rev* 2014; 4:CD007938.
10. Camara CC, Araujo CV, de Sousa KK, Brito GA, Vale ML, Raposo RS, Mendonca FE, Mietto BS, Martinez AM, Oria RB. Gabapentin attenuates neuropathic pain and improves nerve myelination after chronic sciatic constriction in rats. *Neurosci Lett* 2015; 607:52-58.
11. Hooker BA, Tobon G, Baker SJ, Zhu C, Hesterman J, Schmidt K, Rajagovindan R, Chandran P, Joshi SK, Bannon AW, Hoppin J, Beaver J, Fox GB, Day M, Upadhyay J. Gabapentin-induced pharmacodynamic effects in the spinal nerve ligation model of neuropathic pain. *Eur J Pain* 2014; 18:223-237.
12. Lin HC, Huang YH, Chao TH, Lin WY, Sun WZ, Yen CT. Gabapentin reverses central hypersensitivity and suppresses medial prefrontal cortical glucose metabolism in rats with neuropathic pain. *Mol Pain* 2014; 10:63.
13. Yang F, Fu H, Lu YF, Wang XL, Yang Y, Yang F, Yu YQ, Sun W, Wang JS, Costigan M, Chen J. Post-stroke pain hypersensitivity induced by experimental thalamic hemorrhage in rats is region-specific and demonstrates limited efficacy of gabapentin. *Neurosci Bull* 2014; 30:887-902.
14. Decosterd I, Allchorne A, Woolf CJ. Differential analgesic sensitivity of two distinct neuropathic pain models. *Anesth Analg* 2004; 99:457-463.
15. Kim JS, Bashford G, Murphy TK, Martin A, Dror V, Cheung R. Safety and efficacy of pregabalin in patients with central post-stroke pain. *Pain* 2011; 152:1018-1023.
16. Bauer CS, Nieto-Rostro M, Rahman W, Tran-Van-Minh A, Ferron L, Douglas L, Kadurin I, Sri Ranjan Y, Fernandez-Alacid L, Millar NS, Dickenson AH, Lujan R, Dolphin AC. The increased trafficking of the calcium channel subunit alpha2delta-1 to presynaptic terminals in neuropathic pain is inhibited by the alpha2delta ligand pregabalin. *J Neurosci* 2009; 29:4076-4088.
17. Bauer CS, Rahman W, Tran-van-Minh A, Lujan R, Dickenson AH, Dolphin AC. The anti-allodynic alpha2delta ligand pregabalin inhibits the trafficking of the calcium channel alpha2delta-1 subunit to presynaptic terminals in vivo. *Biochem Soc Trans* 2010; 38:525-528.
18. Li KW, Yu YP, Zhou C, Kim DS, Lin B, Sharp K, Steward O, Luo ZD. Calcium channel $\alpha 2\delta 1$ proteins mediate trigeminal neuropathic pain states associated with aberrant excitatory synaptogenesis. *J Biol Chem* 2014; 289:7025-7037.
19. Yang Y, Yang F, Yang F, Li CL, Wang Y, Li Z, Lu YF, Yu YQ, Fu H, He T, Sun W, Wang RR, Chen J. Gabapentinoid insensitivity after repeated administration is associated with down-regulation of the alpha(2)delta-1 subunit in rats with central post-stroke pain hypersensitivity. *Neurosci Bull* 2016; 32:41-50.
20. Wang JS, Wei X, Bao JJ, Du WQ, Tang DC, Wu QY, Zhang XF, Huang HX. A combined ozone interventional therapy for intractable post herpetic neuralgia – a five-year follow up study. *Chinese Journa1 of Pain Medicine* 2016; 22:34-40.
21. Bocci V. Autohaemotherapy after treatment of blood with ozone. A reappraisal. *J Int Med Res* 1994; 22:131-144.
22. Coppola L, Giunta R, Verrazzo G, Luongo C, Sammartino A, Vicario C, Giugliano D. Influence of ozone on haemoglobin oxygen affinity in type-2 diabetic patients with peripheral vascular disease: In vitro studies. *Diabete Metab* 1995; 21:252-255.
23. Magalhaes FN, Dotta L, Sasse A, Teixera MJ, Fonoff ET. Ozone therapy as a treatment for low back pain secondary to herniated disc: A systematic review and meta-analysis of randomized controlled trials. *Pain Physician* 2012; 15:E115-E129.
24. Wainstein J, Feldbrin Z, Boaz M, Harman-Boehm I. Efficacy of ozone-oxygen therapy for the treatment of diabetic foot ulcers. *Diabetes Technol Ther* 2011; 13:1255-1260.
25. Andreula CF, Simonetti L, De Santis F, Agati R, Ricci R, Leonardi M. Minimally invasive oxygen-ozone therapy for lumbar disk herniation. *AJNR Am J Neuroradiol* 2003; 24:996-1000.
26. Stubinger S, Sader R, Filippi A. The use of ozone in dentistry and maxillofacial surgery: A review. *Quintessence Int* 2006; 37:353-359.
27. Bonetti M, Fontana A, Cotticelli B, Volta GD, Guindani M, Leonardi M. Intraforaminal O(2)-O(3) versus periradicular steroidal infiltrations in lower back pain: randomized controlled study. *AJNR Am J Neuroradiol* 2005; 26:996-1000.
28. Hashemi M, Jalili P, Mennati S, Koosha A, Rohanifar R, Madadi F, Razavi SS, Taheri F. The effects of prolotherapy with hypertonic dextrose versus prolozone (intraarticular ozone) in patients with knee osteoarthritis. *Anesth Pain Med* 2015; 5:e27585.
29. Decosterd I, Woolf CJ. Spared nerve injury: An animal model of persistent peripheral neuropathic pain. *Pain* 2000; 87:149-158.
30. Chen J, Guan SM, Sun W, Fu H. Melittin, the major pain-producing substance of bee venom. *Neurosci Bull* 2016; 26:3428-3436.

31. Chen J, Lariviere WR. The nociceptive and anti-nociceptive effects of bee venom injection and therapy: A double-edged sword. *Prog Neurobiol* 2010; 92:151-183.
32. Yu YQ, Zhao F, Guan SM, Chen J. Antisense-mediated knockdown of Na(V)1.8, but not Na(V)1.9, generates inhibitory effects on complete Freund's adjuvant-induced inflammatory pain in rat. *PLoS One* 2011; 6:e19865.
33. Yang F, Sun W, Yang Y, Wang Y, Li CL, Fu H, Wang XL, Yang F, He T, Chen J. SDF1-CXCR4 signaling contributes to persistent pain and hypersensitivity via regulating excitability of primary nociceptive neurons: Involvement of ERK-dependent Nav1.8 up-regulation. *J Neuroinflammation* 2015; 12:219.
34. Yang F, Sun W, Luo WJ, Yang Y, Yang F, Wang XL, Chen J. SDF1-CXCR4 signaling contributes to the transition from acute to chronic pain state. *Mol Neurobiol* 2016; [Epub ahead of print].
35. Lyu D, Yu W, Tang N, Wang R, Zhao Z, Xie F, He Y, Du H, Chen J. The mTOR signaling pathway regulates pain-related synaptic plasticity in rat entorhinal-hippocampal pathways. *Mol Pain* 2013; 9:64.
36. Yang F, Yang Y, Wang Y, Yang F, Li C L, Wang X L, Li Z, Chen J. Selective class I histone deacetylase inhibitors suppress persistent spontaneous nociception and thermal hypersensitivity in a rat model of bee venom-induced inflammatory pain. *Sheng Li Xue Bao* 2015; 67:447-454.
37. Larson AA, Brown DR, El-Atrash S, Walser MM. Pain threshold changes in adjuvant-induced inflammation: A possible model of chronic pain in the mouse. *Pharmacol Biochem Behav* 1986; 24:49-53.
38. Ferrari LF, Cunha FQ, Parada CA, Ferreira SH. A novel technique to perform direct intraganglionar injections in rats. *J Neurosci Methods* 2007; 159:236-243.
39. Sun W, Yang F, Wang Y, Fu H, Yang Y, Li CL, Wang XL, Lin Q, Chen J. Contribution of large-sized primary sensory neuronal sensitization to mechanical allodynia by upregulation of hyperpolarization-activated cyclic nucleotide gated channels via cyclooxygenase 1 cascade. *Neuropharmacology* 2016; [Epub ahead of print].
40. Bocci V, Borrelli E, Zanardi I, Travagli V. The usefulness of ozone treatment in spinal pain. *Drug Des Devel Ther* 2015; 9:2677-2685.
41. Zhou NB, Fu ZJ, Sun T. Effects of different concentrations of oxygen-ozone on rats' astrocytes in vitro. *Neurosci Lett* 2008; 441:178-182.
42. Martinez-Sanchez G, Al-Dalain SM, Menendez S, Re L, Giuliani A, Candelario-Jalil E, Alvarez H, Fernandez-Montequin JI, Leon OS. Therapeutic efficacy of ozone in patients with diabetic foot. *Eur J Pharmacol* 2005; 523:151-161.
43. Re L, Mawsouf MN, Menendez S, Leon OS, Sanchez GM, Hernandez F. Ozone therapy: Clinical and basic evidence of its therapeutic potential. *Arch Med Res* 2008; 39:17-26.
44. Re L, Sanchez GM, Mawsouf N. Clinical evidence of ozone interaction with pain mediators. *Saudi Med J* 2010; 31:1363-1367.
45. Sagai M, Bocci V. Mechanisms of action involved in ozone therapy: Is healing induced via a mild oxidative stress? *Med Gas Res* 2011; 1:29.
46. Verrazzo G, Coppola L, Luongo C, Sammartino A, Giunta R, Grassia A, Ragone R, Tirelli A. Hyperbaric oxygen, oxygen-ozone therapy, and rheologic parameters of blood in patients with peripheral occlusive arterial disease. *Undersea Hyperb Med* 1995; 22:17-22.
47. Ceccherelli F, Gagliardi G, Faggian L, Loprete F, Giron G. Analgesic effect of subcutaneous administration of oxygen-ozone. A blind study in the rat on the modulation of the capsaicin-induced edema. *Acupunct Electrother Res* 1998; 23:171-184.
48. Ellis A, Bennett DL. Neuroinflammation and the generation of neuropathic pain. *Br J Anaesth* 2013; 111:26-37.
49. Lai J, Hunter JC, Porreca F. The role of voltage-gated sodium channels in neuropathic pain. *Curr Opin Neurobiol* 2003; 13:291-297.
50. Fattori V, Pinho-Ribeiro FA, Borghi SM, Alves-Filho JC, Cunha TM, Cunha FQ, Casagrande R, Verri WJ. Curcumin inhibits superoxide anion-induced pain-like behavior and leukocyte recruitment by increasing Nrf2 expression and reducing NF-kappaB activation. *Inflamm Res* 2015; 64:993-1003.
51. Jang M, Lee MJ, Choi JH, Kim EJ, Nah SY, Kim HJ, Lee S, Lee SW, Kim YO, Cho IH. Ginsenoside Rb1 attenuates acute inflammatory nociception by inhibition of neuronal ERK phosphorylation by regulation of the Nrf2 and NF-kappaB pathways. *J Pain* 2016; 17:282-297.
52. Bocci VA. Scientific and medical aspects of ozone therapy. State of the art. *Arch Med Res* 2006; 37:425-435.