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ORIGINAL ARTICLE

Antinociceptive effects of cefadroxil and ceftriaxone in experimental animal models of pain.

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Abstract

BACKGROUND: As an "off-target" effect, cephalosporins can enhance glutamate transporter-1 expression in astrocytes to recycle glutamate from synaptic cleft, and exhibited analgesic properties in animals and humans with chronic pain.
METHODS: In the present study, we focused on making a side-by-side comparison of the analgesic potentials of cefadroxil and ceftriaxone, using rodent models of peripheral neuropathic pain, inflammatory pain and incisional pain. Microdialysis technique was adopted to validate the *in vivo* glutamate regulatory properties of these two drugs in central nervous system.
RESULTS: We have shown that cefadroxil and ceftriaxone are beneficial in a variety of pain scenarios, without inducing observable side effects. The two cephalosporins worked better on neuropathic pain, rather than inflammatory pain or incisional pain, suggesting nociceptive system was differentially affected. Further, microdialysis has confirmed that cephalosporins can effectively reverse the elevated levels of glutamate in brain of animals with neuropathic pain.
CONCLUSIONS: The outcome of this study may guide us to identify a molecular skeleton derived from cefadroxil, based on which we could possibly develop new non-antibiotic analgesic compounds with glutamate recycling properties.

BACKGROUND

Management of chronic pain is one of the most pressive clinical challenges (Treede *et al.* 2019). Current medication of chronic pain is primarily based on anticonvulsants, tricyclic antidepressants, and opioidergic drugs. However, their therapeutic efficacy is either unsatisfactory (Gordh *et al.* 2008; Moore *et al.* 2015; Rice & Maton 2001; Serpell 2002), or hindered with severe side effects, such as tolerance, dependence, and addiction (Birnbaum *et al.* 2011; Mercadante *et al.* 2019; Till *et al.* 2019; Vowles *et al.* 2015).

Excitotoxicity is the key element for establishment and maintenance of pain (Gegelashvili & Bjerrum 2019). The release of glutamate in pain relaying neuronal axis is increased after peripheral nerve injury or inflammation, to generate rapid excitatory postsynaptic potentials (Inquimbert *et al.* 2018; Latremoliere & Woolf 2009; Yam *et al.* 2018). Excessive glutamate subsequently activates NF-κB in microglias and astrocytes in the dorsal horn of spinal cord (Nicholson *et al.* 2014), to further intensify glutamate release and sensitize the NMDA and AMPA receptors (also modulating their trafficking to the membrane) in a manner of an ever-exacerbating spiral (Inquimbert *et al.* 2018; Latremoliere & Woolf 2009).

Accordingly, effective removal of the excessive glutamate from synaptic cleft, by activating its recycling system (to promote the reuptake of glutamate by its transporters) may reverse the glutamate-induced central excitation and disinhibition, therefore is a new therapeutic approach for pain (Grace *et al.* 2014). For such purpose, we turned our interest to glutamate transporter-1 (GLT-1).

GLT-1 is predominately expressed in astrocytes (Lee *et al.* 2008), and together with glutamate and aspartate transporter (GLAST), responsible for almost 95% of the extracellular glutamate reuptake in central nervous system (CNS) (Gegelashvili & Bjerrum 2019). Therefore, GLT-1 plays an important role in maintaining extracellular glutamate homeostasis.

GLT-1 expression can be modulated by chemicals. As an "off-target" effect, beta-lactam antibiotics has been reported to induce GLT-1 expression in astrocytes (Lee *et al.* 2008; Rothstein *et al.* 2005). Such effect is thought to be dependent on the transcriptional activation of GLT-1 through an interaction of NF-κB subunits with specific sites in the GLT-1 promoter (Ghosh *et al.* 2011).

In animal studies, ceftriaxone has been reported to have an anti-nociceptive role in models of chronic neuropathic pain (Hu *et al.* 2010), streptozocin-induced neuropathic pain (Gunduz *et al.* 2011), and nerve root compression induced radicular pain, by reducing spinal astrocyte activation and neuronal hyperexcitability (Bajrektarevic & Nistri 2017). Furthermore, ceftriaxone also exerted therapeutic effects on a wide range of experimental neuronal disorders by spinal upregulation of GLT-1 (Ramos *et al.* 2010).

In human, single dose of the ceftriaxone caused analgesia in patients with post-operative pain, whereas cefazolin was inactive, correlating with animal studies using inflammatory or postsurgical pain models (Macaluso *et al.* 2013). In addition, there is a case report (McGill University Hospital, Canada) indicated a female patient who had treatment-refractory complex regional pain syndrome for 6 years took cefadroxil for a minor infection. While cefadroxil reduced the patient's pain and motor dysfunction within days; the pain and motor disorder returned when cefadroxil was discontinued; and both again abated when cefadroxil was re-instituted (Ware & Bennett 2014).

Although evidences supported that cephalosporins may produce analgesic effects. Still there are several reasons hampered its further application as a painkiller. Firstly, knowledge about the antinociceptive spectrum of cephalosporins are inadequate. Secondly, taking beta-lactam antibiotics in a long run can cause nausea, vomiting, gastrointestinal side effects, and even contribute to the development of antibiotic-resistant bacteria (Bush & Bradford 2016; Lagacé-Wiens & Rubinstein 2012). Thirdly, there is no *in vivo* data could confirm the glutamate modulatory properties of cephalosporins, and demonstrate that they can reverse the imbalanced excitatory and inhibitory neurotransmission in CNS (Das *et al.* 2015; Jiang *et al.* 2022).

Therefore, in the present study, we focused to make a side-by-side comparison of the analgesic potential of cefadroxil and ceftriaxone, using different pain animal models. Specifically, the spared nerve injury (SNI) model was used for evaluating the effects of cephalosporins on neuropathic pain (Guida *et al.* 2020). Analgesic effects on inflammatory pain were evaluated using the carrageenan induced inflammation model (Malik *et al.* 2021; McCarson & Fehrenbacher 2021). For testing the analgesic effects on acute pain occurs following surgery, we adopted the incisional pain model (Pogatzki-Zahn *et al.* 2021). In addition, we used microdialysis technique to validate the in vivo glutamate regulatory properties of these two cephalosporins in CNS.

METHODS

Animals

Rats and mice were used in animal experiments. Sprague-Dawley rats, male, weighing 250-350 g (housed 4 per cage), and C57BL/6 mice, male, weighing 20–30 g (housed 5 per cage), were obtained from Beijing Vital River Laboratory Animal Technology, China. All animals were kept at a constant room temperature of 22 °C in a 12:12 h light-dark cycle with *ad libitum* access to food and water.

Rodent model of spared nerve injury

Method for producing spared nerve ischemic injury (SNI) in rodents has been described previously

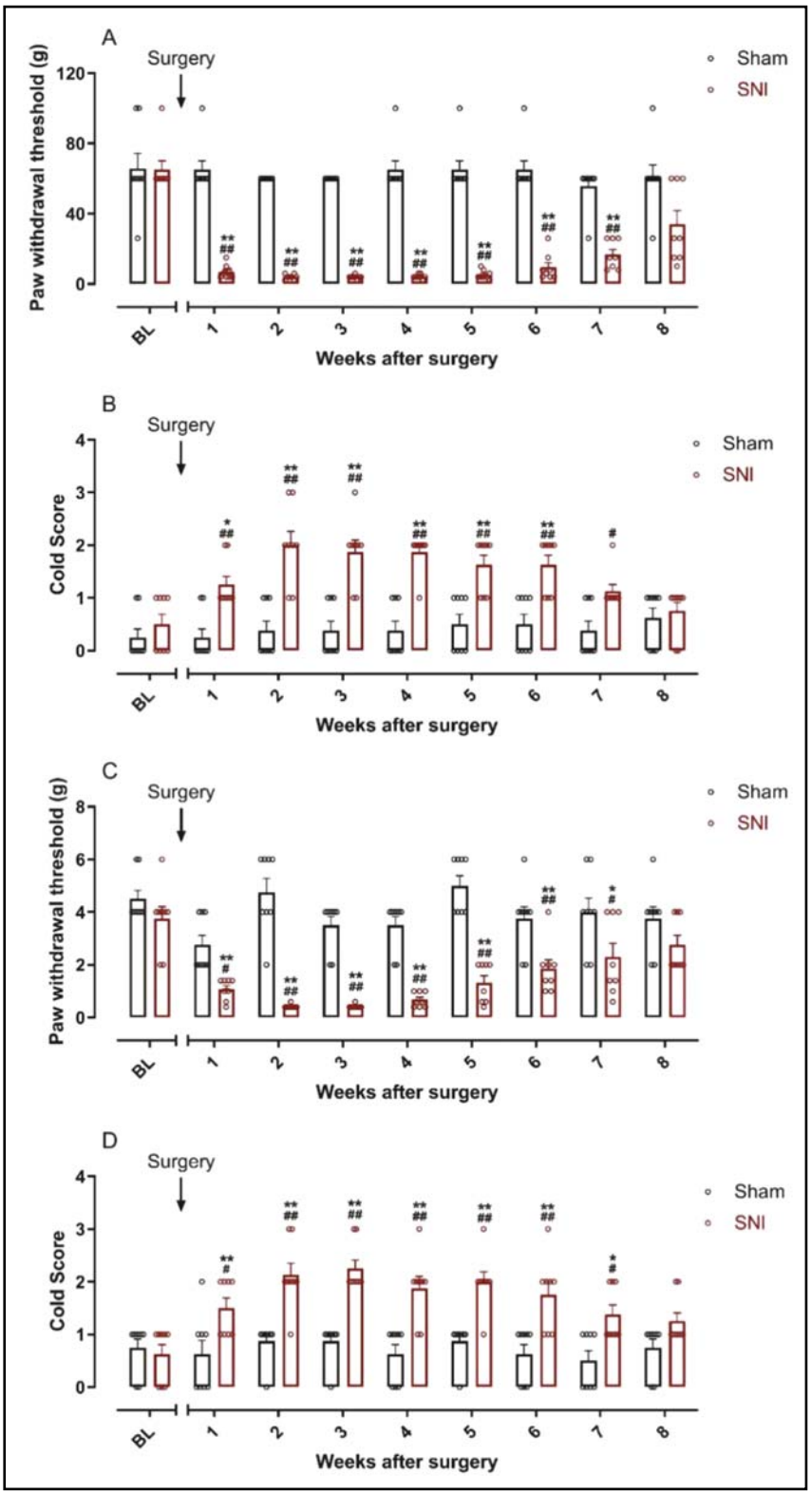

Fig. 1. The development of mechanical and cold hypersensitivities in rats (A, B) or mice (C, D) after spared nerve injury (SNI). N=8 animals for each group. Kolmogorov-Smirnov test revealed SNI and Sham groups are normally distributed both in (B, C, D) but not in (A). *P<0.05, **P<0.01, mechanical (A, C) or cold (B, D) thresholds from Week 1 to Week 8 post surgery were compared with baseline values at Week 0 (BL), using Wilcoxon signed rank test (A) or Dunnett's multiple comparisons test following ANOVA (B, C, D); #P<0.05, ##P<0.01, values of animals with SNI were compared with values of animals receiving sham operations at each time point, using Mann-Whitney U test (A) or Bonferroni's multiple comparisons test following ANOVA (B,C,D).

(Decosterd & Woolf 2000). The SNI procedure comprised an axotomy and ligation of the tibial and common peroneal nerves, while leaving the sural nerve intact. The common peroneal and the tibial nerves were tight-ligated and sectioned distal to the ligation, removing 4 mm (in rats) or 2 mm (in mice) of the distal nerve stump. Caution was taken not to stretch or contact the intact sural nerve.

Rodent model of carrageenan induced inflammation

As reported in our earlier studies (Gao *et al.* 2013), a volume of 60 µl or 20 µl λ-carrageenan (2%, Sigma-Aldrich, Germany) was subcutaneously (s.c.) injected into the plantar surface of one hind paw of rats or mice respectively. Cardinal signs of inflammation—edema, hyperalgesia, and erythema were developed within short period (2 h) following subcutaneous injection.

Rodent model of incisional pain

We adopted a model of incisional pain that has been reported previously in rodents (Brennan *et al.* 1996; Cowie & Stucky 2019) In brief, a 1 cm or a 5 mm longitudinal incision was made through the skin, fascia and muscle from the plantar aspect of the hind paw in rats or mice respectively. The surgery wound was covered with an antibiotic ointment (Bacitracin Zinc Ointment, Actavis Pharma, Inc., USA), to prevent infections.

Behavioral tests in rats and mice

For measurement of mechanical hypersensitivity, rats or mice were placed in plastic cages with a metal mesh floor. After habituation for 1 h, the plantar surface of the hind paw was stimulated with a set of calibrated von Fray hairs (Stoelting, USA) with increasing forces. Each filament was applied 5 times and the response threshold was determined when the animal withdrew the paw at least 3 times. The cut-off value was 60 g for rats and 6 g for mice. The stimuli were applied at the frequency of 1/s.

Heat hyperalgesia was measured by adopting a radiant heat source

(Ugo Basile, Italy) that applying heat stimulation on the plantar surface of hind paw. The hind paw withdrawal latency was automatically recorded. The intensity of the stimulation was adjusted so that the baseline latency for normal animals were between 2 to 6 s and the cut-off value was set at 20s for rats and at 15 s for mice.

The response to cold was tested by applying a drop of acetone to the plantar surface of the hind paw. The immediate response after acetone application was observed and scored for both mice and rats as follows: 0=no response, 1=startle response without evident paw withdrawal, 2=withdrawal of the stimulated hind paw, 3=sustained withdrawal of the simulated hind paw with flinching or licking.

Drugs

Ceftriaxone injectable solution is prepared by dissolving ceftriaxone (sodium salt, MedChemExpress LLC, China) in saline to a concentration of 50 mg/ml, with the aid of ultrasonic water bath. Vehicle for ceftriaxone treatment is saline solution. Cefadroxil oral solution is prepared by mixing cefadroxil (MedChemExpress LLC, China) with H_2O with the aid of ultrasonic water bath. Vehicle for cefadroxil treatment is H_2O. Based on our earlier lab experience and previous studies (Gunduz *et al.* 2011; Ochoa-Aguilar *et al.* 2018), the dose of cefadroxil is 150 mg/kg daily, and administered orally (p.o.); the dose of ceftriaxone is 200 mg/kg daily, and administered intraperitoneally (i.p.).

Pharmacological study design

In this study we used both rats and mice for the model development and for the pharmacological experiment. The main reason for this is trying to exclude the species related potential variances in the model induced hypersensitivities and drug responses.

After SNI, hypersensitivity to mechanical or cold stimuli, will be developed. Here, as a part of SNI model validation, we performed behavioral tests to monitor the development of mechanical allodynia and cold hypersensitivity in rats and mice (N=8 animals) for 8 consecutive weeks following nerve injury. Since we have clearly demonstrated that mechanical and cold hypersensitivities would be developed in the ipsilateral paw of rats and mice, within 10 days post SNI surgery, and could last to about 7 weeks (when mechanical or cold threshold of SNI animals returned to normal after week 8, we stopped testing). Pharmacological study was performed between 2–4 weeks after SNI surgery.

This study investigated whether repeated dosing (1/day, administered immediately after surgery and continued for 8 days, from day 0–7) of cefadroxil or ceftriaxone, could reverse the mechanical allodynia and cold hyperalgesia induced by SNI. Animals that successfully developed pain behaviors were randomized to different groups. N=5 animals for vehicle treatment groups and N=8 animals for drug treatment groups. Behavioral tests were performed for 11 consecutive days. Baselines were registered at day 0. The antinociceptive effects of each drug were tested 2 hours post drug administration from day 1 to 7. After cessation of drug application, the prolonged potencies of repeated dosages were measured from day 8 to day 10.

Plantar injection of 2% λ-carrageenan was used to create a rat / mouse model of inflammatory pain. As indicated in pilot studies, the animals may have the greatest degree of inflammatory pain in the injection area of foot, 24 hours after injection, with the animal's mechanical threshold (measured with the Von Frey Filament), and the threshold to thermal stimulation (measured with the Hargrave radiant device) significantly reduced. As a part of model validation, we performed behavioral tests to monitor the development of mechanical allodynia and heat hypersensitivity in rats and mice (N=8 animals) for 7 consecutive days following carrageenan injection. This study also investigated whether single dose of cefadroxil or ceftriaxone (administered 24 h after carrageenan injection), could reverse the mechanical allodynia and heat hyperalgesia induced by carrageenan. Animals that successfully developed pain behaviors were randomized to different groups. N=5 animals for vehicle treatment groups and N=8 animals for drug treatment groups. Behavioral tests will be performed for 240 minutes (post drug administration) to evaluate the antinociceptive effects of each drug.

Postoperative pain was induced by an incision (epidermal and dermis) on the plantar area of the anesthetized rat/mice. After 24 hours, mechanical allodynia and cold hyperalgesia are normally developed around the wound. As a part of model validation, we performed behavioral tests to monitor the development of mechanical allodynia and heat hypersensitivity in rats and mice (N=8 animals) for 7 consecutive days following incision. This study also investigated whether repeated dosing (administered immediately after surgery, once a day, continuously for 4 days, from day 0–3) of cefadroxil (150 mg/kg daily, p.o.) or ceftriaxone (200 mg/kg daily, i.p.), could reverse the mechanical allodynia and cold hyperalgesia induced by incision. Animals that successfully developed pain behaviors were randomized to different groups. N=5 animals for vehicle treatment groups and N=8 animals for drug treatment groups. Behavioral tests were performed for 8 consecutive days. Baselines were registered at day 0. The antinociceptive effects of each drug are tested 2 h post drug administration from day 1 to 3. After cessation of drug application, the prolonged potencies of repeated dosages are measured from day 4 to day 7.

For all pharmacological studies, we used N=5 animals for vehicle treatment groups and N=8 animals for drug treatment groups. This is mainly based on the bigger variation in the treated groups than the control groups.

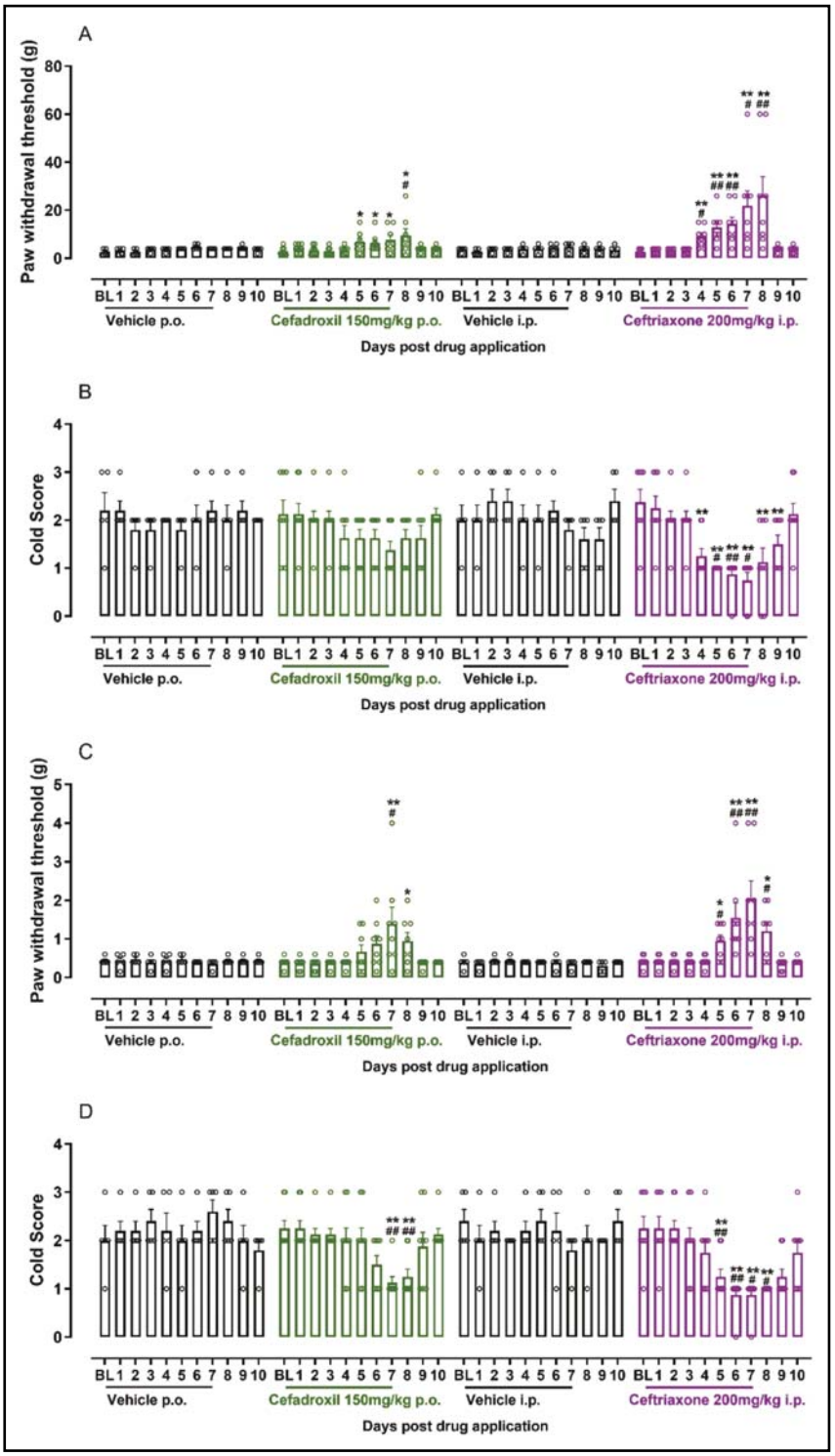

Fig. 2. The effects of cefadroxil and ceftriaxone on mechanical and cold hypersensitivities in rats (A, B) or mice (C, D) with spared nerve injury (SNI) induced peripheral neuropathic pain. N=5 animals for vehicle treatment groups and N=8 animals for drug treatment groups. Drug / Vehicle were repeatedly administered (1/day) for 7 days. Kolmogorov-Smirnov test revealed that groups are not normally distributed in (A, C) but are normally distributed in (B, D). **P*<0.05, ***P*<0.01, mechanical (A) or cold (B) thresholds from Day 1 to Day 10 post drug / vehicle administration were compared with baseline values at Day 0 (BL), using Wilcoxon signed rank test (A, C) or Dunnett's multiple comparisons test following ANOVA (B, D); #*P*<0.05, ##*P*<0.01, values of SNI animals receiving cefadroxil (150mg/kg, p.o.) or ceftriaxone (200 mg/kg, i.p.) treatments were compared with values of SNI animals receiving vehicle treatments at each time point, using Mann-Whitney U test (A, C) or Bonferroni's multiple comparisons test following ANOVA (B, D).

Brain microdialysis surgery and microdialysate sampling

Methods for brain microdialysis surgery and microdialysate sampling has been reported previously (Gao *et al.* 2020). Rats were kept in free-moving condition for 1 h prior to the initiation of sample collection for reaching equilibrium. Animals received corresponding drugs followed by a 5 h dialysate sampling. The dialysates were collected at 30 min intervals using a CMA470 micro-fraction collector (CMA Microdialysis, Sweden). The levels of glutamate in the rat brain dialysate were detected at 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min post drug administration.

Determination of glutamate levels by HPLC-Fluorescence Analysis

The glutamate quantitative analysis was carried out using a high-performance liquid chromatography (HPLC) system including Aglient1200 (Aglient, USA) with G1321A fluorescence detectors (Aglient, USA) were applied. Chromatographic separation was performed on a column (Eclipse AAA, 4.6 mm × 150 mm, 5 μm; Aglient, USA). Data acquisitions and analysis are performed using ChemStation B.04.01 software (Aglient, USA).

Statistics

All the experiments were blindly performed. Data were presented as mean ± standard error of mean (SEM). Normality of the data was assessed by Kolmogorov-Smirnov test. Dunnett's multiple comparisons test following two-way ANOVA with repeated measures / Wilcoxon signed rank test was used for comparing values at different time points with baseline values, Bonferroni's multiple comparisons test following two-way ANOVA with repeated measures / Mann-Whitney U test was used to compare effect of model / drug group versus sham / vehicle group respectively at each time points. Extracellular glutamate levels represented by area under curve were analyzed using one-way ANOVA, followed by Tukey's multiple

comparisons test to examine the significance between different groups.

RESULTS

Development of mechanical and cold hypersensitivities in rats and mice with SNI

Both rats and mice exhibited significant pain behaviors following surgery. After SNI, rats developed both mechanical and cold hypersensitivities from week 1 (compared with sham control rats), which lasted for at least 7 weeks for mechanical allodynia (Fig.1A), and 7 weeks for cold allodynia (Fig.1B). Similarly, after SNI, mice also developed both mechanical and cold hypersensitivities from week 1, and lasted for 7 weeks (Fig.1C). The pain behaviors of SNI rats and mice showed a gradual trend of recovering after 5 weeks, such result is correlated with our previous experience (Gao *et al.* 2013; Gao *et al.* 2019).

The antinociceptive effects of cefadroxil and ceftriaxone on SNI induced peripheral neuropathic pain

In SNI rats, repeated dosages of cefadroxil and ceftriaxone generated antinociceptive effects against SNI induced mechanical allodynia (Fig.2A). Such effects were not immediate, but emerged after 4 or 5 days of continuous drug application, and lasted longer the cessation of drug treatment (from day 4 or 5 to day 8). However, continuous drug administrations of cefadroxil and ceftriaxone produced divergent effects against hypersensitivity to cold stimulation (Fig.2B). In which, repeated ceftriaxone produced significant analgesic effect against cold pain from day 4–9 (even lasted 2 days longer the cessation of drug treatment), while repeated cefadroxil treatment were not effective.

Similar as in SNI rats, cefadroxil and ceftriaxone also produced antinociceptive effects against mechanical allodynia in SNI mice (Fig.2C). Such effects were also emerged after continuous drug applications (7 days required for cefadroxil and 5 days required for ceftriaxone), and lasted longer the cessation of drug treatment (for 1 day). Intriguingly, differed from SNI rats, cefadroxil exhibited analgesic effect against cold pain, with duration coincided with analgesia against mechanical allodynia (from day 7–8, Fig 2D). Resembling the effect in SNI rats, repeated drug administration of ceftriaxone also produced antinociceptive effect against hypersensitivity to cold stimulation, which even lasted 1 day longer the cessation of drug treatment (from day 5–8).

Development of mechanical and cold hypersensitivities in rats and mice after intra-plantar carrageenan injection

To our surprise, in rats after λ-carrageenan injection, no significant difference was detected in mechanical (Table 1) or heat (Table 2) thresholds between carrageenan injected rats and rats receiving sham treatments.

Tab. 1. Development of mechanical hypersensitivity in rats after carrageenan injection (Unit: g; Data presented as Mean±SD)

Group	N	BL	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Carrageenan Injection	8	55.75±12.021	38.875±22.869	45.875±19.788	50.125±18.52	50.125±18.52	45.875±19.788	50.125±18.52	51.5±15.739
Sham	8	55.75±12.021	60.75±19.826	51.5±15.739	59.375±22.747	56.5±23.342	55.75±12.021	51.5±15.739	60.75±19.826

Tab. 2. Development of heat hypersensitivity in rats after carrageenan injection (Unit: s; Data presented as Mean±SD)

Group	N	BL	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Carrageenan Injection	8	8.663±1.813	7.413±2.101	7.963±1.937	7.913±1.705	7.838±1.514	7.813±1.495	7.625±1.589	8.225±1.757
Sham	8	8.463±2.211	8.613±1.711	8.6±1.716	8.6±1.579	8.4±1.488	8.588±1.076	8.038±1.514	7.788±1.844

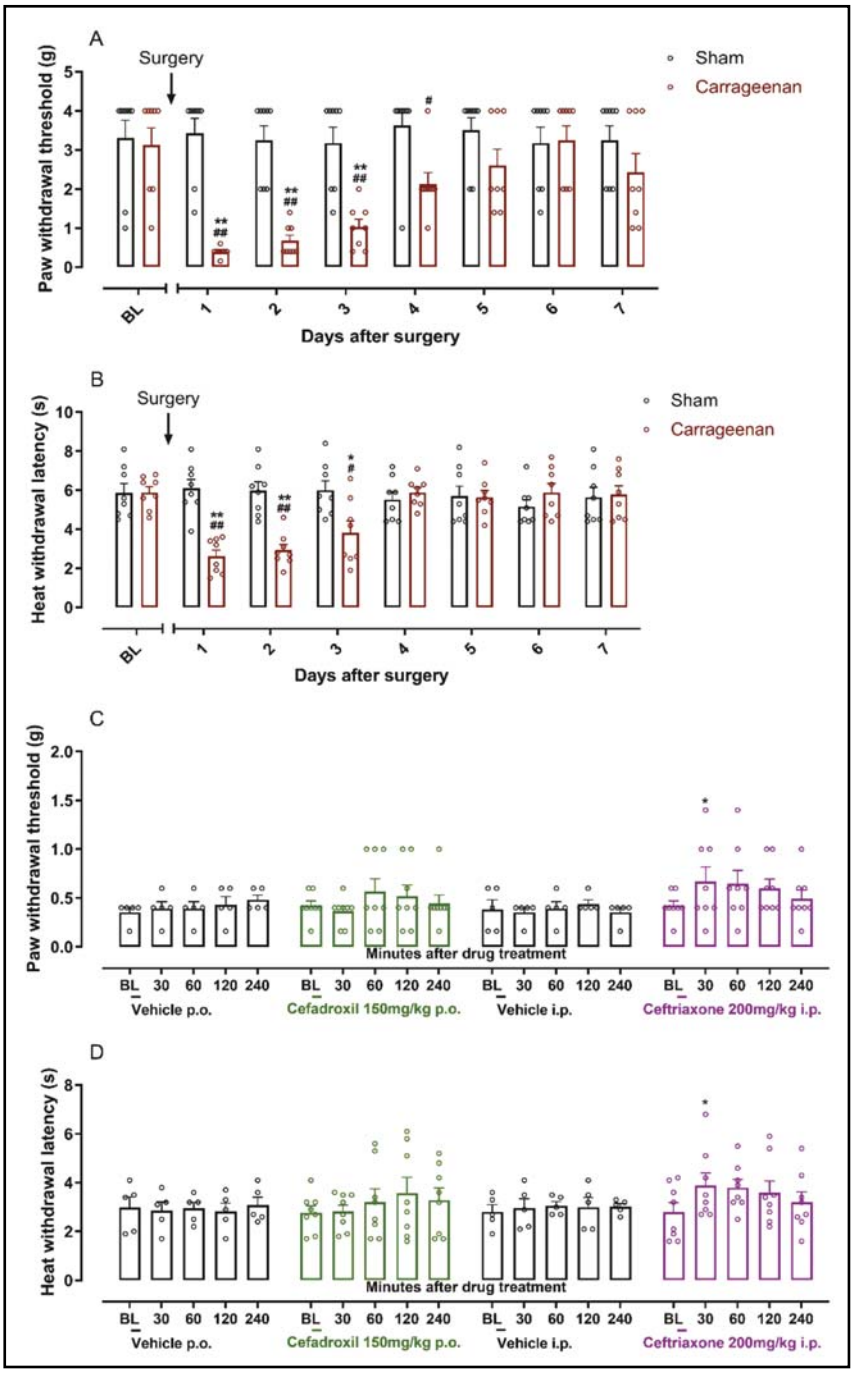

Fig. 3. The development of mechanical (A) and heat (B) hypersensitivities in mice after intraplantar Carrageenan injection. N=8 mice; The effects of cefadroxil and ceftriaxone on mechanical (C) and heat (D) hypersensitivities in mice with carrageenan induced inflammatory pain. N=5 mice for vehicle treatment groups and N=8 mice for drug treatment groups. Kolmogorov-Smirnov test revealed that groups are normally distributed in (A, C, D) but are not normally distributed in (B). #P<0.05, ##P<0.01, values of mice with carrageenan injection were compared with values of mice with sham operations at each time point, using Bonferroni's multiple comparisons test following ANOVA (A) or Mann-Whitney U test (B). There was no significant difference detected in mechanical (C) or heat (D) thresholds between carrageenan injected mice receiving cefadroxil (150mg/kg, p.o.) or ceftriaxone (200 mg/kg, i.p.) treatments and mice with carrageenan injection receiving vehicle treatments, at any time point, using Bonferroni's multiple comparisons test following ANOVA.

However, after intra-plantar injection of carrageenan, mice developed both mechanical and heat hypersensitivities from day 1 (compared with sham control mice), which lasted for 4 or 3 days for mechanical or heat allodynia respectively (Fig.3A and B), indicating a successful establishment of inflammatory pain in mice but not rats.

By so far, we have not found in any literature that could explain why rats injected with carrageenan did not manifest mechanical / heat hyperalgesia. But we think 3 parameters might be relevant to this, which are genomic background of the animals, the method of carrageenan operation, and the time point of behavioral testing.

Although the carrageenan induced inflammatory pain model has been previously shown in the literature to present a different time point of peak hyperalgesia (usually, 3–4 hours after plantar injection is the peak time point for hyperalgesia), in our study, animals showed peak hyperalgesia around 24 hours post carrageenan injection (and following inflammatory cascades). This is also the case in our previous study (Gao *et al.* 2020). We think differences in animal's genomic background, environmental factors such as housing condition, and the method of carrageenan operation / injection could be partially accounted for such variance.

Therefore, the analgesic effects of cefadroxil and ceftriaxone against inflammatory pain were determined solely using the mouse model of carrageenan induced inflammation, 24 hours post carrageenan injection.

The antinociceptive effects of cefadroxil and ceftriaxone on carrageenan induced inflammatory pain

In mice with carrageenan induced inflammatory pain, at 30 min (post drug application) time point, single dosage of ceftriaxone generated antinociceptive effects against mechanical (Fig.3C) and heat (Fig.3D) allodynia. While single dose of cefadroxil produced no therapeutic effect on mechanical or heat pain.

Development of mechanical and cold hypersensitivities in rats and mice after incision

Both rats and mice exhibited significant pain behaviors following surgery. After incision, rats developed both mechanical and cold hypersensitivities from day 1 (compared with sham control rats), which lasted for at least 6 days for mechanical allodynia (Fig.4A), and 2 days for cold allodynia (Fig.4B). Similarly, after incision, mice also developed both mechanical and cold hypersensitivities from day 1. In which, mechanical allodynia appeared at day 1 and 2 (Fig.4C), while cold allodynia emerged at day 1 and 2 (Fig4D). These results demonstrated that the development of incisional pain is differed between rats and mice, and varied in pain modalities.

The antinociceptive effects of cefadroxil and ceftriaxone on incisional pain

In rats or mice with incisional pain, repeated dosages of cefadroxil and ceftriaxone (Fig.5) generated only trivial (for cefadroxil on cold pain, Fig.5B) or even no antinociceptive effects against incision induced mechanical or cold allodynia, indicating beta-lactam antibiotics is not favorable for treating postoperative pain under current paradigm, irrespective of their advantage in preventing postoperative wound infection.

Modulatory effects of cefadroxil and ceftriaxone on extracellular glutamate levels in CNS of rats with SNI induced peripheral neuropathic pain

Compared with vehicle control animals, cefadroxil treated rats showed reduced glutamate levels at 30, 90, 120, and 300 min time points (Fig.6A), while ceftriaxone treated rats exhibited reduced glutamate levels at 30, 60, 90, 120, 150, 210, and 300 min time points. When analyzed using Area Under Curve (AUC, from 0–300 min and normalized with vehicle control group), it is apparent that both cefadroxil and ceftriaxone treated group has significant lower overall glutamate

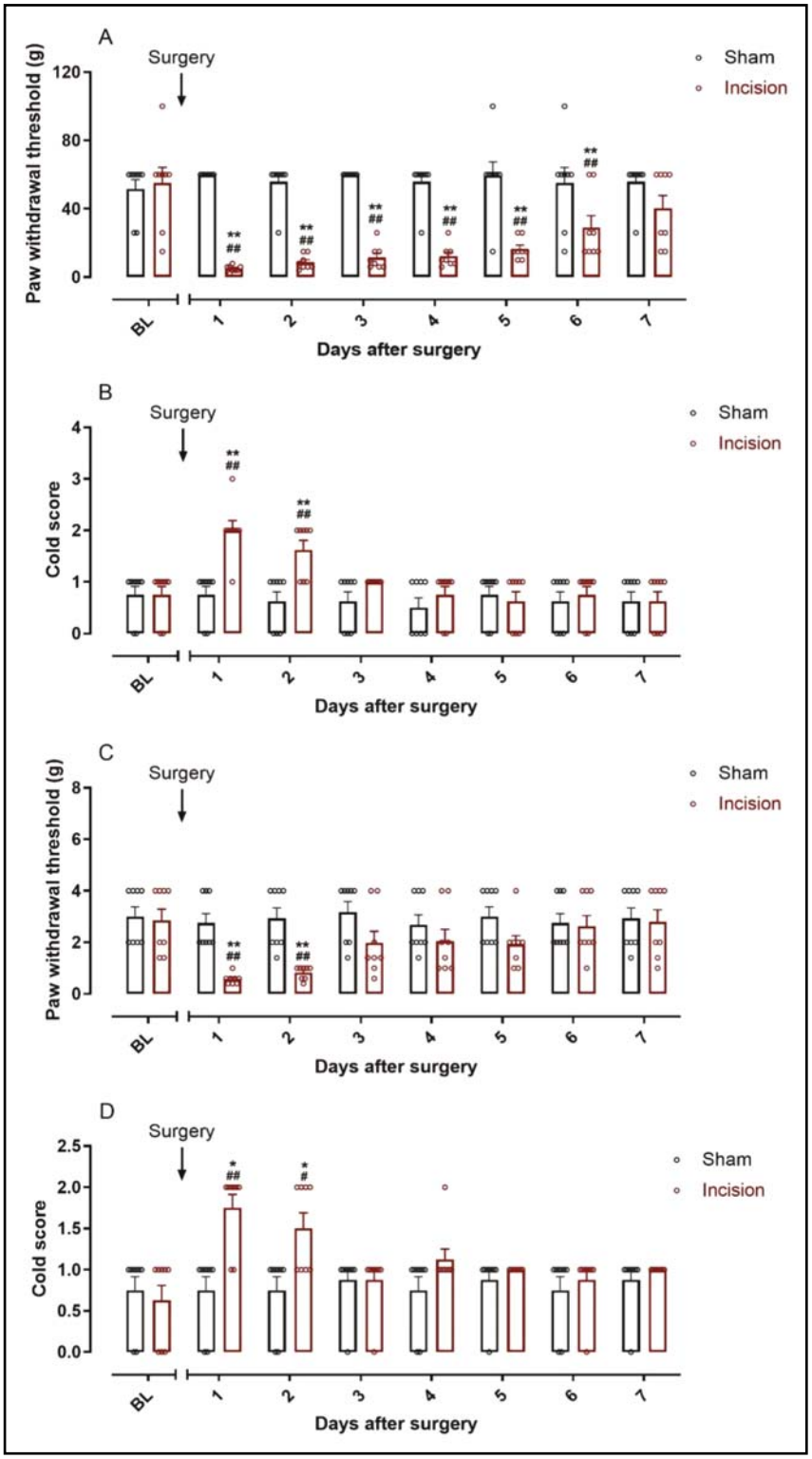

Fig. 4. The development of mechanical and cold hypersensitivities in rats (A, B) or mice (C, D) after incision. N=8 animals for each group. Kolmogorov-Smirnov test revealed groups are normally distributed in (A, C) but are not normally distributed in (B, D). **P*<0.05, ***P*<0.01, mechanical (A, C) or cold (B, D) thresholds from Week 1 to Week 8 post incision were compared with baseline values at Week 0 (BL), using Dunnett's multiple comparisons test following ANOVA (A, C) or Wilcoxon signed rank test (B, D); #*P*<0.05, ##*P*<0.01, values of animals with incision were compared with values of animals receiving sham operations at each time point, using Bonferroni's multiple comparisons test following ANOVA (A, C) or Mann-Whitney U test (B, D).

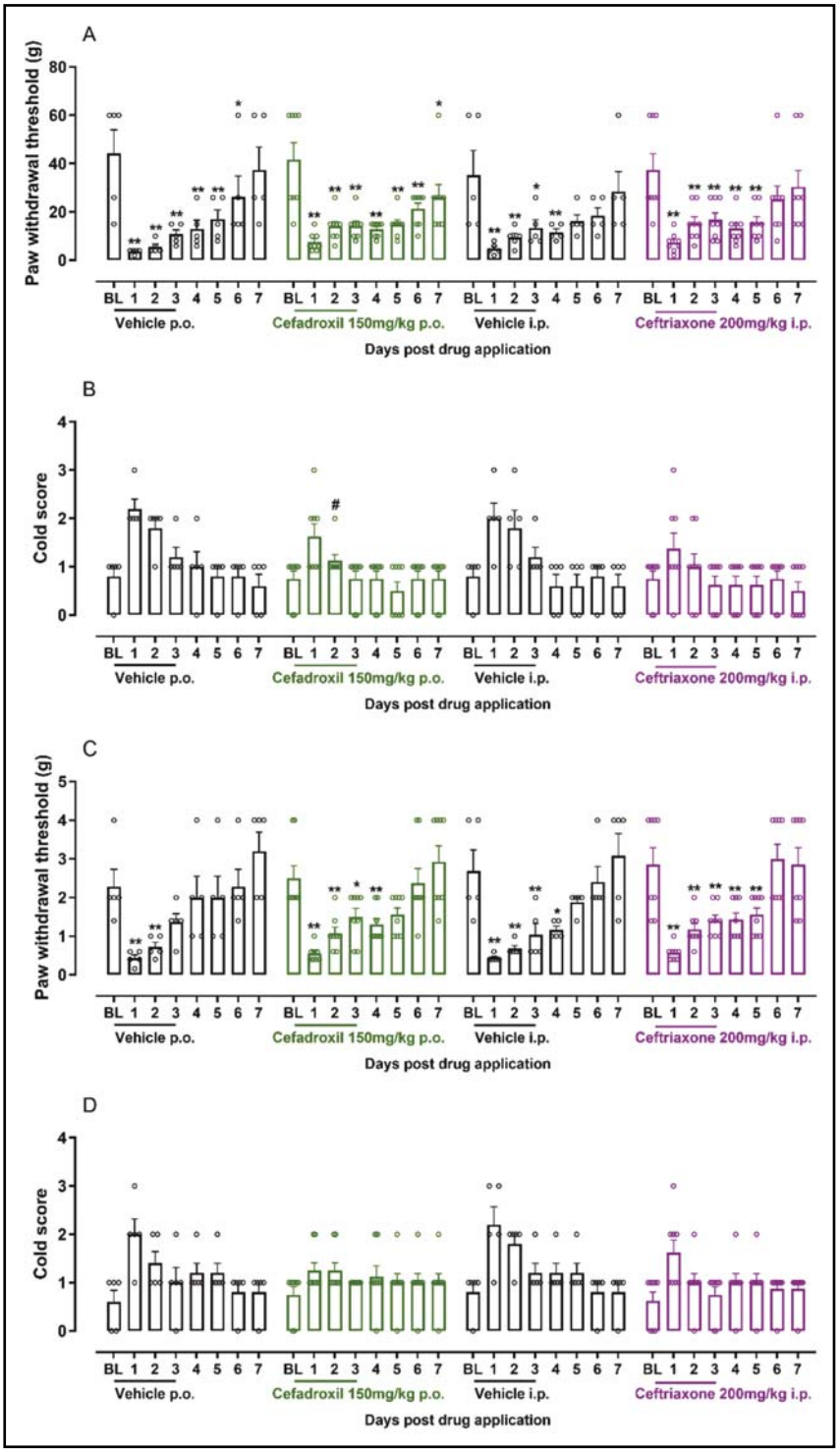

Fig. 5. The effects of cefadroxil and ceftriaxone on mechanical and cold hypersensitivities in rats (A, B) or mice (C, D) with incision induced post-operative pain. N=5 animals for vehicle treatment groups and N=8 animals for drug treatment groups. Drug / Vehicle were repeatedly administered (1/day) for 3 days. Kolmogorov-Smirnov test revealed groups are normally distributed in (A, C) but are not normally distributed in (B, D). **P*<0.05, ***P*<0.01, mechanical thresholds (A) from Day 1 to Day 7 post drug / vehicle administration were compared with baseline values at Day 0 (BL), using Dunnett's multiple comparisons test following ANOVA (A, C). There was no significant difference detected between cold thresholds at different time points and baseline values in B, D, using Wilcoxon signed rank test. #*P*<0.05, values of animals with incision receiving cefadroxil (150 mg/kg, p.o.) or ceftriaxone (200 mg/kg, i.p.) treatments were compared with values of animals with incision receiving vehicle treatments at each time point, using Mann-Whitney U test (B).

levels than the vehicle treated group in CNS (Fig.6B), however no significant difference was detected between cefadroxil and ceftriaxone treated groups. This result confirmed that cephalosporins could reduce the extracellular glutamate levels in CNS of animals with neuropathic pain.

DISCUSSION

In the present study, we have shown that the two cephalosporins worked better on neuropathic pain, rather than on inflammatory pain. Neuropathic pain has an intrinsic "central component", manifested by increased spontaneous neuronal activation, and enlarged peripheral receptive field of dorsal horn neurons and associated pain regions in the CNS (Meacham *et al.* 2017). While, acute inflammatory pain or incisional pain is either orchestrated by a wide range of local inflammatory mediators or induced by surgical damage to peripheral tissues (Brennan *et al.* 2005; Linley *et al.* 2010), that eventually sensitizing peripheral nociceptors (generating peripheral sensitization), however lacking substantial involvement of the "central component". Taking consideration of the fact that cephalosporin reduces astrocyte activation and neuronal excitotoxicity in the central nervous system (Bajrektarevic & Nistri 2017), it is reasonable to postulate that this centrally acting pattern drives a shift in cephalosporin's analgesic spectrum towards a better remedy for the symptom of neuropathic pain, rather than that of inflammatory or incisional pain.

Such differentiation in analgesic effects of cephalosporins between pain types, is similar to naturally derived drugs, which are mainly alkaloids with mild but enduring antinociceptive effects and less adverse reactions (Jiang *et al.* 2022). In addition, the effect of cephalosporins could be accumulated over repeated dosages, which is also similar to naturally derived analgesics (Gao *et al.* 2013; Gao *et al.* 2014). It is possible that, resembling as these alkaloids, combining cephalosporins with

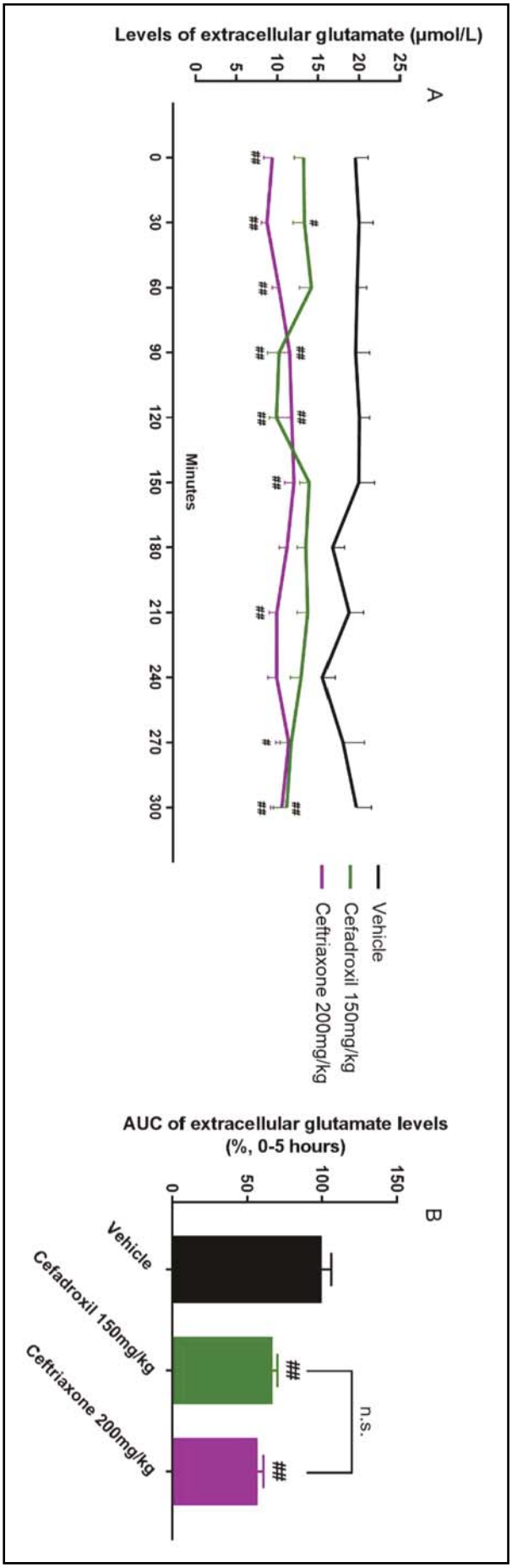

Fig. 6. Levels of extracellular glutamate in brain of rats with peripheral neuropathic pain after repeated treatment of cefadroxil (150 mg/kg/day, p.o.) or ceftriaxone (200 mg/kg/day, i.p.) for 8 days (A), and Area Under Curve (AUC) normalized with vehicle group for the same results (B). N=8 animals for each group. Microdialysis was performed 2 h after final drug / vehicle application at Day 8. Kolmogorov-Smirnov test revealed groups are normally distributed both in (A) and in (B). #P<0.05, ##P<0.01, values of spared nerve injury (SNI) rats receiving cefadroxil (150 mg/kg, p.o.) or ceftriaxone (200 mg/kg, i.p.) treatments were compared with SNI rats receiving vehicle treatments at each time point of microdialysis (A) or as AUC (B), using Bonferroni's multiple comparisons test following two-way ANOVA (A), or Tukey's multiple comparisons test following one-way ANOVA.

clinically available analgesics may generate synergy (Gao *et al.* 2019; Ochoa-Aguilar *et al.* 2018), especially for patients after surgery or with acute local inflammation (considering antibiotic treatment is crucial to prevent / control bacterial infections) (Wu *et al.* 2020).

Interestingly, in rats with SNI induced neuropathic pain, we found repeated dosing of cefadroxil significantly reduced mechanical but not cold hypersensitivity. However, this dissociation was abolished in ceftriaxone treatment groups, indicating the difference between the two cephalosporins in regulating the modality specific pain-relaying neuronal networks. Such dissociations between pain modalities could be also found under physiological modulation of receptors associated with pain transmission (Kayser *et al.* 2007).

Cefadroxil is an orally administered α-amino-containing beta-lactam antibiotic with a broad spectrum of antibacterial activity. It is and has good water solubility and a modest degree of lipid solubility (Huh *et al.* 2013). While ceftriaxone, as an injected third-generation cephalosporin antibiotic, has antimicrobial activity against both gram-positive and gram-negative organisms.

In this study, we have demonstrated for the first time that both cefadroxil and ceftriaxone have *in vivo* glutamate regulatory properties in CNS, presumably based on their ability to enhance GLT-1 expression in astrocytes (Bush & Bradford 2016; Das *et al.* 2015). The probe of microdialysis is inserted to the striatum mainly because we want to confirm if cephalosporin can reduce the overactive CNS glutamate neurotransmission in general. The second reason is technically we need additional effort to be able to correctly place the probe into the region of PAG (which is more relevant to the descending pain pathway). However, in future, it is definitely interesting and meaningful to perform a microdialysis study on the PAG to collect the opioid peptides in the dialysate and provide a better understanding of the effects of cefadroxil and ceftriaxone on the main sites of pain modulation in the CNS.

Cephalosporins are predominately transported to the targeted tissue by H^+/ peptide transporter PEPT2 (Huh *et al.* 2013), which is expressed in astrocytes and neurons in the CNS. Both cefadroxil and ceftriaxone are substrates of PEPT2 (Daniel & Kottra 2004). Further, it is revealed that the affinity between cefadroxil and PEPT2 is much higher than ceftriaxone and PEPT2 (Luckner & Brandsch 2005). This may explain why in our study, orally administered cefadroxil (150 mg/kg)

could reduce the behavioral hypersensitivities and CNS glutamate levels to a similar extent as i.p. injected ceftriaxone (200 mg/kg).

Indeed, long-term use of cephalosporins could raise concerns such as antibiotics may generate resistance and alteration of the intestinal microbiota. Therefore, we have continued our approach to structurally modify cephalosporin based on the tripeptide scheme. Based on the fact that both cefadroxil and ceftriaxone have similar antinociceptive potencies against neuropathic pain. We propose to prioritize structure modification of cefadroxil's molecular skeleton in future study, to generate drug candidates that do not possess antibiotic effects but remaining its analgesic effects.

CONCLUSION

To conclude, we have demonstrated that under repeated administration, both cefadroxil and ceftriaxone can exert analgesic effects especially against neuropathic pain conditions, without generating observable side effects. In addition, using microdialysis, for the first time, we have confirmed that cephalosporins can effectively reverse the elevated levels of glutamate in CNS. Considering cefadroxil has similar potency as ceftriaxone but is orally applicable and more effectively transported by PEPT2, we propose to structurally modify its molecular skeleton, to generate potential novel analgesics with glutamate recycling ability, while abolishing its antibiotic property.

DECLARATIONS

Ethics approval

All animal studies were conducted following the National Guidelines for Housing and Care of Laboratory Animals and performed in accordance with protocol approved by the the Research Ethics Committee at Chinese Academy of Medical Sciences, Beijing, China.

Consent for publication

Not applicable.

Availability of data and materials

The data of this study are available from the corresponding author on reasonable request.

Competing interests

Author Jiang W. are employed by the company Zhejiang Zhenyuan Pharmaceutical Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All authors declare that there is no competing interest.

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Authors' contributions

Chao-Jie Han, Zhen Shen, and Mingze Tang are co-first authors and contributed equally to this work. Tianle Gao directed this research. All authors were involved in establishing the research protocol. All authors were involved in preparing the manuscript. All authors approved the final version of the manuscript.

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