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# Intrathecally injected tramadol reduces articular incapacitation and edema in a rat model of lipopolysaccharide (LPS)-induced reactive arthritis

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ABSTRACT

*Aims:* Intrathecal injection of morphine presents analgesic and antiedematogenic effects in rats. However, it is unknown whether tramadol, which possess a mixed mechanism of action, can also produce analgesic and antiedematogenic effects similarly.

*Main methods:* Male Wistar rats received carrageenan and LPS in the right knee joint. Tramadol (10 μg) was injected intrathecally 20 min before articular LPS injection. Incapacitation and articular edema were measured 5 h after LPS stimulation. Synovial fluid was collected for leukocyte counting and western blot analysis. Whole joint and lumbar spinal cord were also collected for histology and immunohistochemistry, respectively. Intrathecal pretreatments groups were with the NKCC1 blocker bumetanide, $TRPV_1$ agonist resiniferatoxin, μ-opioid receptor antagonist CTOP and serotonergic neurotoxin 5,7-DHT, all previously to tramadol.

*Key findings:* Tramadol treatment caused the reduction of incapacitation and edema. It also reduced c-Fos protein expression in the spinal cord dorsal horn and slightly reduced TNF-α levels in synovial fluid, but neither reduced cell migration nor tissue damage. Bumetanide and resiniferatoxin prevented the analgesic and antiedematogenic effects of tramadol. CTOP prevented the analgesic and the antiedematogenic effects, but 5,7-DHT prevented only tramadol-induced analgesia.

*Significance:* Spinal NKCC1 cotransporter and peptidergic peripheral afferents seem to be important for the analgesic and antiedematogenic effects of tramadol, as well as μ-opioid receptor. However, the monoamine uptake inhibition effect of tramadol seems to be important only to the analgesic effect.

## 1. Introduction

Tramadol is an opioid agonist often known as a weak opioid, since it has proportionally low affinity for the μ-opioid receptor and pretreatment with naloxone only partially inhibits its analgesic effect, indicating it is not restricted to the opioid system [1–3]. In fact, many studies support a monoamine reuptake inhibitor effect of this drug [4,5]. The affinity of tramadol for noradrenaline transporters (NET) and serotonin transporters (SERT) can enhance monoamine levels in the spinal cord, which also accounts for the analgesic effect. This analgesic profile may explain the reduced overall opioid-related side effects, and its increasing clinical popularity ranging from acute postoperative pain to chronic cancer pain management [6], and osteoarthritis [7,8].

The spinal cord can modulate not only nociceptive transmission from the periphery, but also the neurogenic component of peripheral inflammation, probably by the regulation of the dorsal root reflex (DRR), a phenomenon importantly implicated in pain and inflammation which was revised by Willis [9]. DRR is the result of a complex interaction among the primary afferent nociceptor, spinal cord GABAergic interneurons, descending brain stem projections and dendrites of spinal transmission neurons, all arranged in a synaptic glomeruli, which can modulate the state of sensitization of the central terminal of nociceptors. Primary afferent nociceptor presents an enhanced expression of the $NKCC_1$ cotransporter, which maintains a higher $Cl^-$ concentration inside the cell [10,11]. Normally, afferent action potentials reaching the spinal cord can stimulate the release of GABA in the synaptic glomeruli,

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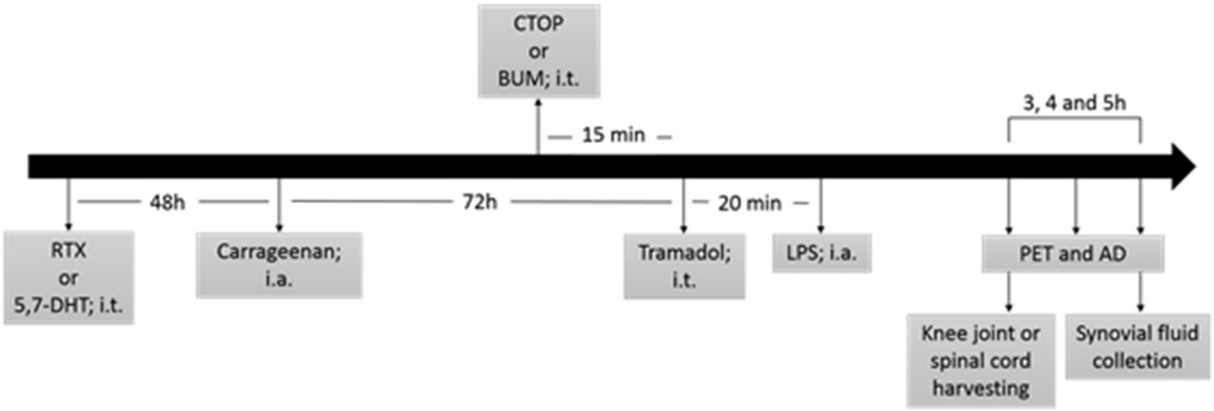


**Fig. 1.** Flowchart of the experimental design. RTX = resiniferatoxin; 5,7-DHT = 5,7-dihydroxytriptamine; CTOP = D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2; BUM = bumetanide; LPS = lipopolysaccharide; PET = paw elevation time measurement; AD = articular diameter measurement.

which leads to $Cl^-$ efflux from nociceptors central terminals via $GABA_A$ receptors, causing a partial depolarization and the consequent reduction in the probability that nociceptive potentials reach second order nociceptive transmission neurons [9,12].

In persistent nociceptive conditions, however, the primary afferent stimuli on GABAergic interneurons is temporally intensified, leading to faster $Cl^-$ outward current. Thus, provoking a depolarization fast enough to elicit action potentials in the primary afferent central terminals, which allows the antidromic firing and the consequent release of pro-inflammatory neuropeptides in the peripheral side [9,13]. Such a model of DRR-dependent neurogenic component of inflammation has allowed the testing of several hypothesis, which focused on intrathecal injection of drugs targeting to different signaling systems to reduce both nociception and inflammation in animal models of arthritis [14–18]. Morphine was seen to potently reduce peripheral inflammatory edema after intrathecal administration [17,18].

Given the mixed pharmacology of tramadol, the present study was designed to evaluate whether this drug also produces an anti-edematogenic effect, in parallel to its analgesic effect, when administered by intrathecal route in a model of reactive arthritis.

## 2. Methods

### 2.1. Animals

The experiments were performed on 152 male Wistar rats (*Rattus norvegicus*), aged 90 to 110 days old, weighting 300–370 g, bred in the university's animal facility. They were housed in a temperature-controlled room (20 ± 1 °C) under a 12 h light/dark cycle with free access to water and food. Animals came from the central vivarium of the institution (UFSC) housed in groups of five animals per home cage lined with sawdust. This study followed the ethical guidelines of the International Association for the Study of Pain (IASP), was previously approved by the local ethics committee for animal use (CEUA-UFSC: PP3007200716) and complies with the ARRIVE guidelines.

### 2.2. LPS-induced monoarthritis

The lipopolysaccharide (LPS)-induced monoarthritis model was described in detail previously [19]. First, the right knee joint was primed with an intra-articular (i.a.) injection of a boiled carrageenan solution (BDH Chemicals Ltd., UK), suspended in 0.9% sterile saline (300 mg/20 μL/knee). A second challenge was made 72 h after priming with *Escherichia coli* lipopolysaccharide (LPS) serotype 055:B5 (DIFCO, USA) diluted in physiological saline (30 ng/50 μL/knee; i.a). LPS induces an intense inflammatory reaction in the knee-joints primed with carrageenan. Incapacitation and articular diameter measurements were made at 3 h from the LPS injection, and hourly after.

### 2.3. Intrathecal injections and treatments

Intrathecal drug injections were performed at the lumbar level of the spinal cord according to the method previously described [20]. The animals were anesthetized with isoflurane (2% in oxygen) and a 29-gauge needle was carefully inserted between the L5–L6 vertebrae space until a flick of the rat's tail was observed, indicating the needle was in the spinal channel. Individual injections did not exceed 20 μL.

Neurotoxins 5,7-dihydroxytryptamine (5,7-DHT; 20 μg/20 μL; ascorbic acid 2% in PBS) and resiniferatoxin (RTX; 3.8 μg/kg/10 μL; Tween 20 7% in PBS), or their vehicles, were injected 48 h before the first intra-articular challenge with carrageenan. After 72 h, tramadol (10 μg/20 μL) or saline were injected 20 min prior to the LPS stimuli. This dose of tramadol was seen to produce maximal analgesic effect after spinal injection in a previous study [21]. Treatments with D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP;15 μg/10 μL; saline) or bumetanide (BUM; 60 μg/10 μL; 1.29% sodium bicarbonate in PBS, pH 7.4, kept slightly warm to avoid precipitation) were made on the day of the experiment, 35 min prior to the LPS stimuli, i.e. 15 min before tramadol or saline i.t. injections (Fig. 1).

### 2.4. Articular incapacitation measurement

Articular nociception was evaluated by the rat knee-joint incapacitation test [22]. The rats were placed on a revolving cylinder (30 cm diameter; 3 rpm) for 1 min periods, while a computer-assisted device measured the total time during which the right hind paw was not in contact with the cylinder surface, representing the paw elevation time (PET, s). Before the carrageenan/LPS stimulus, PET was 10s on average. Carrageenan/LPS increases PET only in the affected limb [19]. In the present study, baseline PET was assessed just before LPS stimulation, and incapacitation was registered at 3, 4 and 5 h after. PET values are represented as the mean ± SEM of these three measurements.

### 2.5. Articular diameter increase

Inflammatory edema induced by LPS was quantified by taking the articular diameter (AD) using a micrometer. The animals were restrained in the supine position and the micrometer was placed at three consecutive points along the proximodistal direction. The highest value was chosen. AD was assessed on the day of the experiment immediately before LPS injection (baseline) and after each PET measurement. The differences between the baseline AD and the subsequent measurements ($\Delta AD = AD_{baseline} - AD_{hour\ 3,\ 4\ or\ 5}$) were calculated and the data are

expressed as the mean ± SEM of those three differences.

### 2.6. Synovial leukocyte counts

As reported in detail elsewhere [19], 5 h after LPS injection, just after the last PET and AD measurements, knee-joint synovial fluid (5 μL) was collected for smear slide preparation and stained with May-Grünwald and Giemsa. Mononuclear and polymorphonuclear leukocytes were counted under 100× optical magnification. Synovial lavage was obtained with 100 μL of 4% EDTA in physiological saline +1% protease inhibitor. A 20 μL aliquot of this lavage was diluted in Turk's solution (1:20) for 5 min for total leukocyte count in a Neubauer chamber (TL; cells/$mm^3$). The remaining synovial lavage was used for western blotting analysis. Naïve samples were collected from the contralateral knee joints.

### 2.7. Western blot

The electrophoresed gel was blotted onto a nitrocellulose membrane (pore size, 0.1 mm) in blotting buffer (3.03 g Tris base, 14.4 g glycine and 200 mL methanol per liter) at 100 V for 1 h, at 4 °C. The membrane was then rinsed in PBS (pH 7.4), soaked in 5% skimmed milk in PBS for 1 h and rinsed in PBS for 5 min. The membrane was incubated with anti-TNF primary antibody (1:1000; SC-52746, mouse IgG, Santa Cruz) for 1 h, rinsed three times in PBS for 5 min, and then treated with the secondary antibody (1:5000; anti-mouse IgG-horseradish peroxidase conjugate, Sigma-Aldrich) for 1 h. The membrane was rinsed three times in PBS for 5 min and the bands were then visualized by chemiluminescence with the help of a photo-documenter.

### 2.8. Histology

In a different experimental group, the ipsi and contralateral knee joints were collected 3 h after LPS injection in order to evaluate the histological features. They were fixed in 10% formalin for 24 h, followed by dehydration in a graded series of alcohol, diaphanization in xylol and embedding in histologic paraffin. An Olympus Cut 4055® microtome was used to make 7 μm-thick sagittal plane slices, which were stained with hematoxylin and eosin (HE). The slides were photographed in an Olympus DP71® microscope using 400× and 1000× magnification. For histomorphological analysis, the articular cartilage and synovial membrane were observed to analyze structure and cellular organization.

### 2.9. Immunohistochemistry

In another experimental group, neuronal activation in the dorsal horn was assessed by immunohistochemistry of the c-Fos protein expression using the free-floating protocol as described in detail previously [23]. Three hours after LPS injection, rats were anesthetized with xylazine:ketamine (1:1; 5 mg/kg: 90 mg/kg; 1.2 mL) and perfused with 9.25% sucrose and 4% paraformaldehyde (PFA) to collect the lumbar region of the spinal cord, which were cut into 50 μm-thick coronal slices. Free-floating slices were rinsed three times with PBS + 0.3% Triton-X (PBST) for 5 min and then bathed in a methanol + $H_2O_2$ solution for 30 min. Unless indicated otherwise, slices were rinsed three times with PBST for 5 min between all incubations. After blockade of endogenous peroxidase, slices were incubated with PBST + 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) for 60 min and immediately followed by overnight incubation with the anti-Fos primary antibody (SC-52, rabbit IgG, Santa Cruz, 1:2000) at 4 °C. After washing, these slices were then incubated for 90 min with a biotinylated secondary antibody (anti-rabbit, 1:1000) and then incubated for 2 h with biotinylated avidin enzyme complex (Vectastain Elite ABC Kit VectorLabs, in PBST, 1:500). Final staining was obtained by bathing the slices in a 0.05% 3,3′-diaminobenzidine-solution (DAB, Sigma-Aldrich, St. Louis, MO), in 0.03% $H_2O_2$-PBST) for 10 min. Stained slices were mounted in gelatin-coated slides, air-dried, dehydrated in a graded series of alcohols and xylenes and finally cover slipped with DPX mounting medium (Sigma-Aldrich, St. Louis, MO). Immunoreactive c-Fos cells were counted in two random slices within laminae I and II of the dorsal horn. Using the ImageJ software, the photomicrographs were converted into binary form to exclude subthreshold nuclei, and a square area was selected also using ImageJ. c-Fos positive nuclei were then manually counted within this area, with the total cell x area was calculated with the aid of ImageJ.

### 2.10. Statistical analysis

Statistical analyses were carried out using one-way ANOVA, two-way ANOVA, or non-parametric analysis of variance Kruskal-Wallis, accordingly, followed by the Newman-Keuls or Dunn post hoc test when a P level of $< 0.05$ was detected. Data are expressed as the mean ± SEM as shown in the figures. The behavior experimenter was blinded to all treatments, that were all randomized by two other experimenters during the intrathecal injections. The animals were treated in a sequence which allowed that in each cage would have at least one representant of each treatment group.

## 3. Results

### 3.1. Incapacitation and articular diameter

Fig. 2 shows the effect of intrathecally injected tramadol (10 μg/20 μL) on the articular incapacitation and diameter parameters. Intrathecal injection of tramadol was able to reduce both PET and AD when compared with saline-treated animals injected by the same route. However, when the same dosage of tramadol was injected by the subcutaneous route, it did not reduce either of the two parameters (Fig. 2A and B), suggesting the effects observed after intrathecal injection were specific to the injection site and cannot be attributed to the diffusion of tramadol out the spinal channel.

### 3.2. Synovial leukocytes, TNF-α measurement and histological features

The polymorphonuclear and mononuclear leukocyte content in the synovial fluid collected 5 h after LPS injection were not significantly different between the saline- and tramadol-treated groups (Fig. 2C and D). The TNF-α immunocontent assayed at this time-point was higher in the saline-treated group than in naive group. Tramadol-treated animals showed a tendency to lower levels of the cytokine than saline group (Fig. 2E and F).

Fig. 3 shows the morphological aspects of knee-joints. In naive animals, thickness either of the articular cartilage or synovial membrane was normal. In the saline-treated group, there was a loss of cellular organization, flocculation, discontinuity of the tidemark and decreased chondrocyte counts in the articular cartilage, with altered morphology and intense inflammatory cells infiltration in the synovial membrane, which was predominant in the sub-intima. Presence of fibrous tissue, decreased number and size of adipocytes, and a large number of inflammatory cells in the articular cavity were also observed in the saline group. Treatment with intrathecal tramadol was not able to modify the histological features of the synovial membrane nor the cartilage of the inflamed joint, presenting a degradation of the joint similar to the saline group (Fig. 3A–G).

### 3.3. c-Fos immunoreactivity

The number of immunoreactive c-Fos cells in the laminae I and II at the lumbar region of the dorsal horn of the spinal cord (Fig. 4C–E) was higher (Fig. 4B), when compared to naive animals that did not receive an i.a. injection of carrageenan/LPS nor any pharmacological

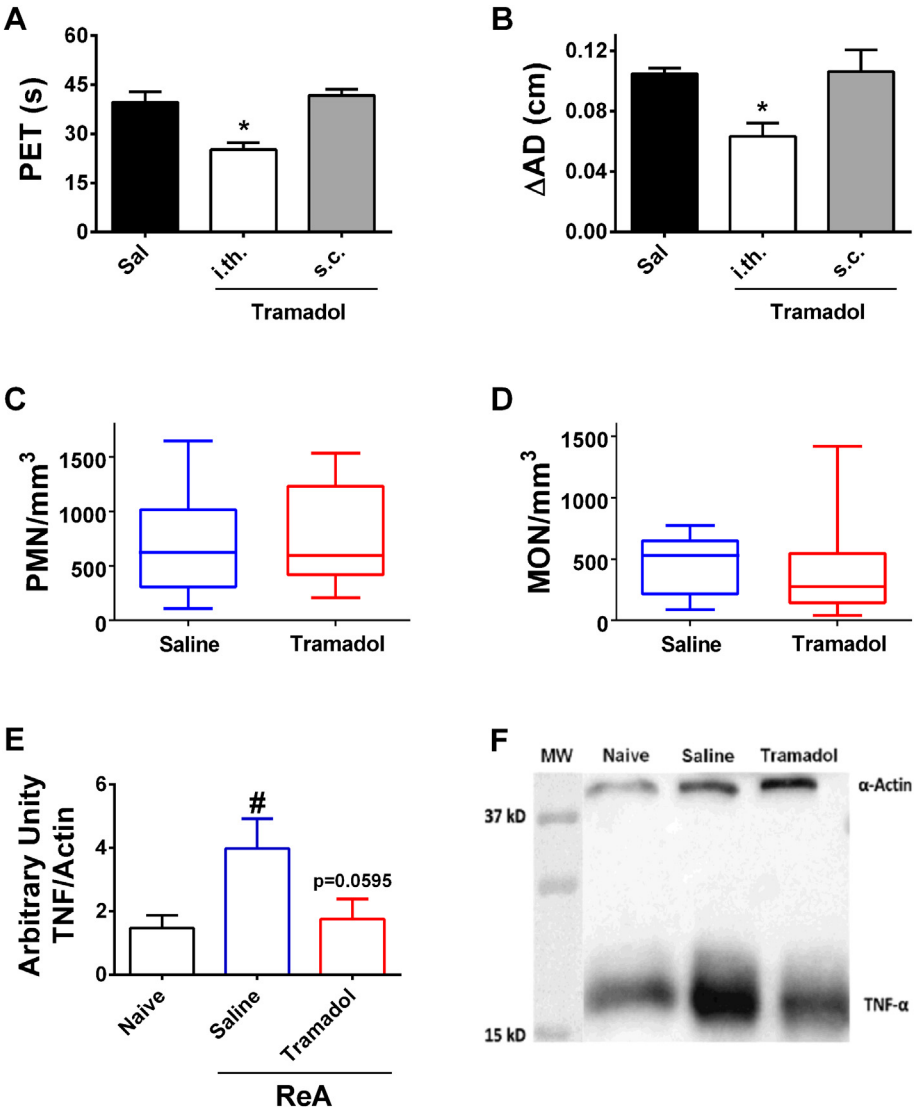


**Fig. 2.** Effect of tramadol (10 μg/20 μL) administered intrathecally (i.th.) or subcutaneously (s.c.) 20 min before intra-articular LPS (30 ng/50 μL). Control group (Sal) received physiological saline (20 μL) intrathecally. Naïve group did not receive carrageenan/LPS intra-articular injection or any other treatment. Paw elevation time (A) and articular diameter (B) were measured hourly after LPS injection, from hour 3 to 5. Synovial fluid was collected from i.th. and Sal groups after the last PET and AD measurement for polymorphonuclear (PMN) (C) and mononuclear (MON) (D) leukocyte counts, as well as TNF (E–F) quantification. MW = molecular weight. PET (n = 8), AD (n = 8) and TNF (n = 4) data are represented as mean ± SEM, PMN and MON (n = 8) data are represented as median & Interquartile range (IQR) *P < 0.05 compared with control group, #P < 0.05 compared with naïve group (one-way analysis of variance followed by Newman-Keuls post hoc test).

treatment. When treated with tramadol this number was reduced to almost the same content as naive animals, i.e. the reduction of c-Fos positive cells indicates that intrathecal injection of tramadol is able to reduce neuronal activity in the observed region. Furthermore, motor neurons in lamina IX at the ventral horn of the spinal cord of all three experimental groups presented similar results (Fig. 4F–H), indicating that neuronal inhibition was restricted to the area of injection, which was the area with the highest density of primary afferents and projection neurons synapses.

### 3.4. Bumetanide effects

The $NKCC_1$ selective blocker bumetanide (60 μg/10 μL) was injected by the intrathecal route 15 min prior to tramadol, i.e. 35 min before LPS challenge. Bumetanide itself has analgesic and antiedematogenic activities, reducing both incapacitation and articular diameter, as shown by the first blue bar in Fig. 5A and B. The treatment with tramadol inhibited incapacitation and articular diameter increase in the vehicle, but not in bumetanide pretreated groups (Fig. 5A and B). Bumetanide treatment also tended to reduce the mononuclear infiltrate to the joint when compared to the vehicle + saline group, and the treatment with tramadol did not potentiate this effect (data not shown).

### 3.5. Resiniferatoxin effects

Intrathecal injection of low doses of the TRPV1 agonist RTX are known to desensitize the central terminal of TRPV1-expressing fibers without impairing their peripheral activity. In animals pretreated with RTX (3.8 μg/kg/10 μL) the levels of PET achieved during the arthritic process was lower than in animals receiving vehicle intrathecally. The subsequent treatment with tramadol did not produce a further antinociceptive effect (Fig. 5C). The augmented articular diameter induced by arthritis, however, was not different between RTX- and vehicle-pretreated groups, yet the subsequent intrathecal tramadol administration was not able to reduce this parameter (Fig. 5D). Even with a low dose, some RTX-pretreated animals had severe hypothermia after injection of the toxin and did not survive, resulting in less animals per group after this treatment. Total and differential leukocyte counts did not change in any of the groups (data not shown).

### 3.6. CTOP effects

Pretreatment with the selective antagonist of μ-opioid receptors CTOP (15 μg/10 μL) slightly diminished the PET response elicited by the arthritic stimulus, although it was not statistically significant. The subsequent treatment with tramadol did not produce antinociception in the group pretreated with CTOP, as observed in the vehicle-treated

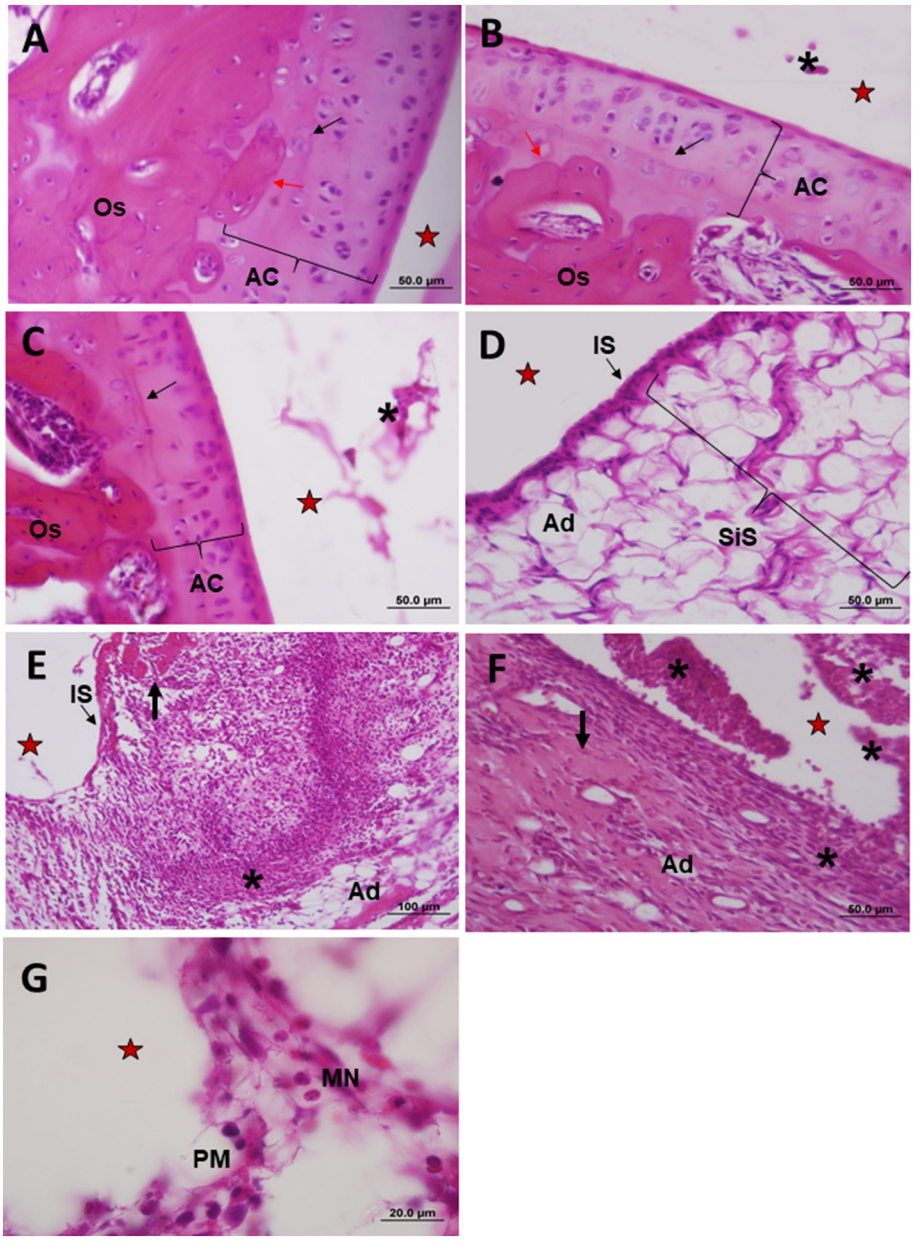


**Fig. 3.** Effect of tramadol (10 µg/20 µL) administered intrathecally 20 min before intra-articular LPS (30 ng/50 µL) on articular morphology using hematoxylin and eosin staining of sagittal sections. Joints were collected after the last PET and AD measurement. (A–C) Photomicrographs of the femoral articular cartilage. In (A) Naive group: articular cartilage (AC); subchondral bone (SB); articular cavity (red star); tidemark (arrow); invagination of the subchondral bone (red arrow). In (B) Saline group and in (C) Tramadol group: inflammatory infiltrate in the articular cavity (asterisk). (D–G) Photomicrographs of the synovial membrane of the femoral-tibial joint. In (D) Naive group: synovial membrane with normal morphological aspect, with the surface of the synovial intima (SI); synovial subintima (SSi); adipocytes (Ad); articular cavity (red star). In (E) Saline group: synovial membrane with altered morphology, with inflammatory cells in the intima and subintima and presence of fibrous tissue (black arrow) and large amounts of inflammatory cells in the articular cavity (asterisk). In (F and G) Tramadol group: with inflammatory characteristics similar to (E), and in (G) details of the inflammatory infiltrate in the joint cavity of the Tramadol group: mononuclear cells (MON); polymorphonuclear (PMN) cells (n = 4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

group (Fig. 6A). Similarly, the results from the articular diameter shows that the antagonism of µ-opioid receptors with CTOP completely blocked the antiedematogenic effect of intrathecally injected tramadol (Fig. 6B). Total and differential leukocyte counts did not change in any of the groups (data not shown).

### 3.7. 5,7-DHT effects

Pretreatment with the neurotoxin 5,7-DHT (20 µg/20 µL) caused a slight reduction in PET when compared to the vehicle + saline group, although it was not statistically significant. The subsequent treatment with tramadol did not produce antinociception in the group pretreated with 5,7-DHT, as observed in the vehicle-treated group (Fig. 6C). Nonetheless, intrathecal treatment with tramadol still produced an antiedematogenic response, even in the group previously treated with the neurotoxin (Fig. 6D). Total and differential leukocyte counts did not change in any of the groups (data not shown).

## 4. Discussion

The main findings of the present study were that a low intrathecal dose of tramadol reduced the incapacitation and articular diameter increase induced by carrageenan/LPS, supporting an analgesic and antiedematogenic effects, respectively. Either the analgesic or the antiedematogenic effects of tramadol were suggested to be mediated by µ-opioid receptor, while serotonin seemed to be involved only in the analgesic one. Furthermore, both effects seem to depend on a fully functional peptidergic afferent fibers and NKCC1 cotransporter. Intrathecal tramadol also caused a reduction of c-Fos, within laminae I and II of the spinal cord dorsal horn, and TNF-α immunoreaction in the synovial fluid.

An antiedematogenic effect of tramadol was only reported in two other studies, one using intra-articular injections [24] directly at the injured site and the other was a systemic treatment by intraperitoneal administration [25], raising the question of how intrathecal injection of such a small dose of tramadol, similar to the one used in this work, was able to reduce both nociception and edema.

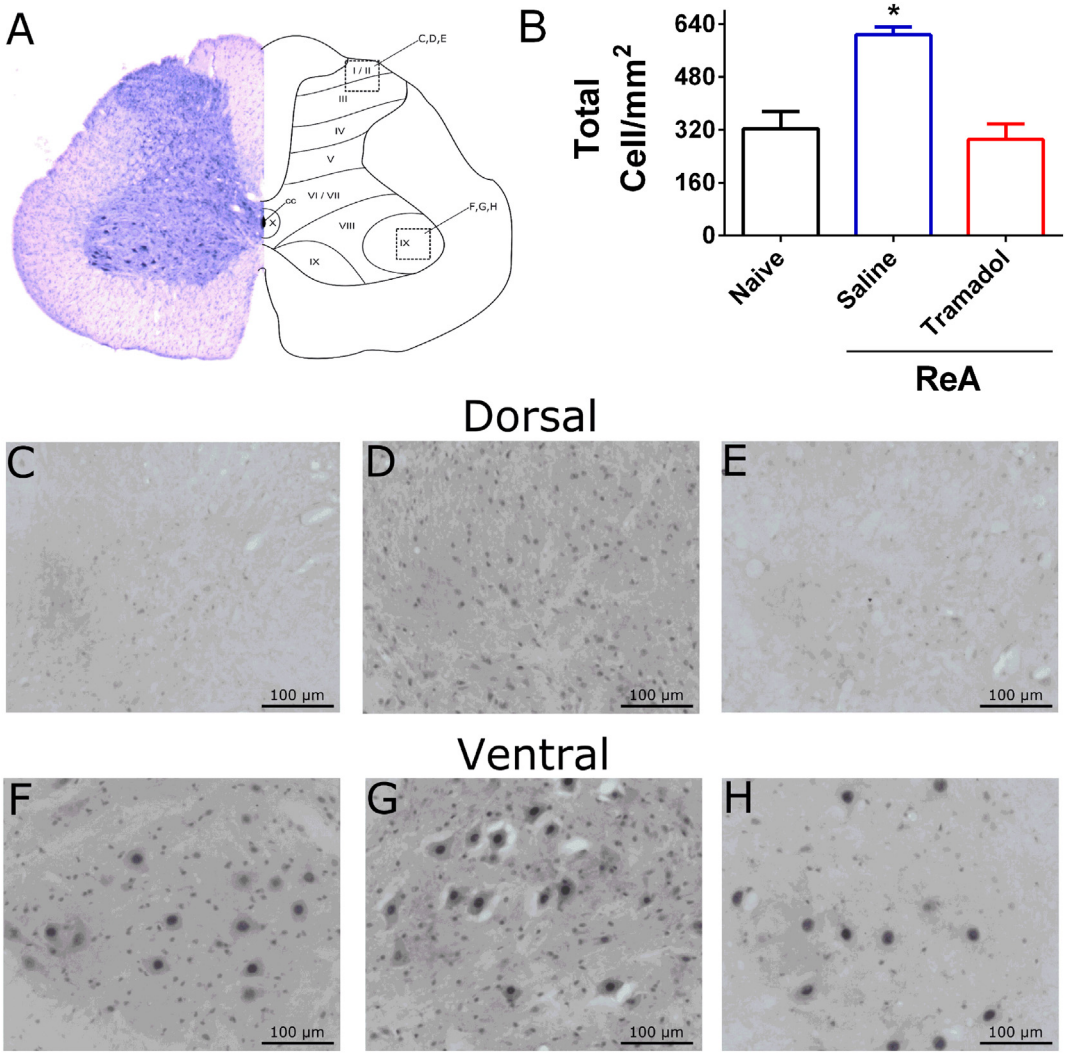


**Fig. 4.** Effect of tramadol (10 µg/20 µL) administered intrathecally 20 min before intra-articular LPS (30 ng/50 µL) on c-Fos protein staining in spinal cord dorsal horn (A). Spinal cord tissue was collected 3 h after LPS injection. (B) is the graphic representation of the cell counting, showing a reduction of c-Fos positive cells after tramadol treatment in arthritis-induced (ReA) animals. Naive group did not receive carrageenan/LPS intra-articular injection or any other treatment. (C–E) Photomicrographs of dorsal horn laminae I and II of naive (C), saline (D) and tramadol (E) groups. (F–H) Photomicrographs of ventral horn of naive (F), saline (G) and tramadol (H) groups. Data are represented as mean ± SEM (n = 4) with *P < 0.05 compared with naive group (one-way analysis of variance followed by Newman-Keuls post hoc test).

The available data shows that intrathecal injection of tramadol reduces neuronal activity in the spinal cord, which was not reverted by naloxone injected either systemically or intrathecally [26–28]. Also, intraperitoneal injection of tramadol was able to reduce the excitatory currents and enhance inhibitory currents in the rat dorsal horn laminae I and II [29], implying that even when systemically injected the tramadol-induced effect seems to be due to spinal cord neuronal activity modulation. Staining for c-Fos in laminae I and II, as well as glial activation and IL-1β levels were reduced after tramadol intrathecal injection in neuropathic rats [30,31]. The same reduction in c-Fos was observed in the present work, which was limited to laminae I and II, indicating that this treatment reduces the neuronal activity only at a nociceptive transmission area, contrasting with previous electrophysiological studies with higher doses of tramadol [26–28].

In the central nervous system, the $NKCC_1$ cotransporter is mainly expressed in nociceptive afferent fibers such as C-fibers and dorsal root ganglia (DRG) [32,33], being pivotal to the generation of dorsal root reflexes (DRR) and the consequent neurogenic inflammation [9]. In the present work, inhibition of this cotransporter with bumetanide reduced both incapacitation and edema, suggesting both depends on the ongoing DRR activity in the central terminal of the primary afferent in the present model. Bumetanide pretreatment also prevented the antinociceptive and antiedemadogenic effects of tramadol, which is consistent to suppose the inhibitory effects due to the spinal injection of tramadol were due to the inhibition of DRR generation.

Intrathecal injection of low doses of resiniferatoxin causes desensitization of the central terminal of a population of nociceptive primary afferent fibers-expressing $TRPV_1$, which are thought to be important for DRR transmission and the neurogenic component of inflammation [9], although maintaining their cell bodies and peripheral branch intact [34]. The results obtained here indicate that the incapacitation and edema need a functional population of peptidergic primary afferents to be elicited in this model, which is also consistent that both depends on DRR generation. Again, tramadol was ineffective after resiniferatoxin, which is consistent with an inhibitory action on DRR.

Tramadol injection not only reduced articular diameter, but also reduced TNF-α content in the synovial fluid; however, without changing the leukocyte content. There is only one other work reporting TNF-α reduction after chronic orally-given tramadol in humans [35]. Intraperitoneal injection of tramadol also did not change leukocyte counts

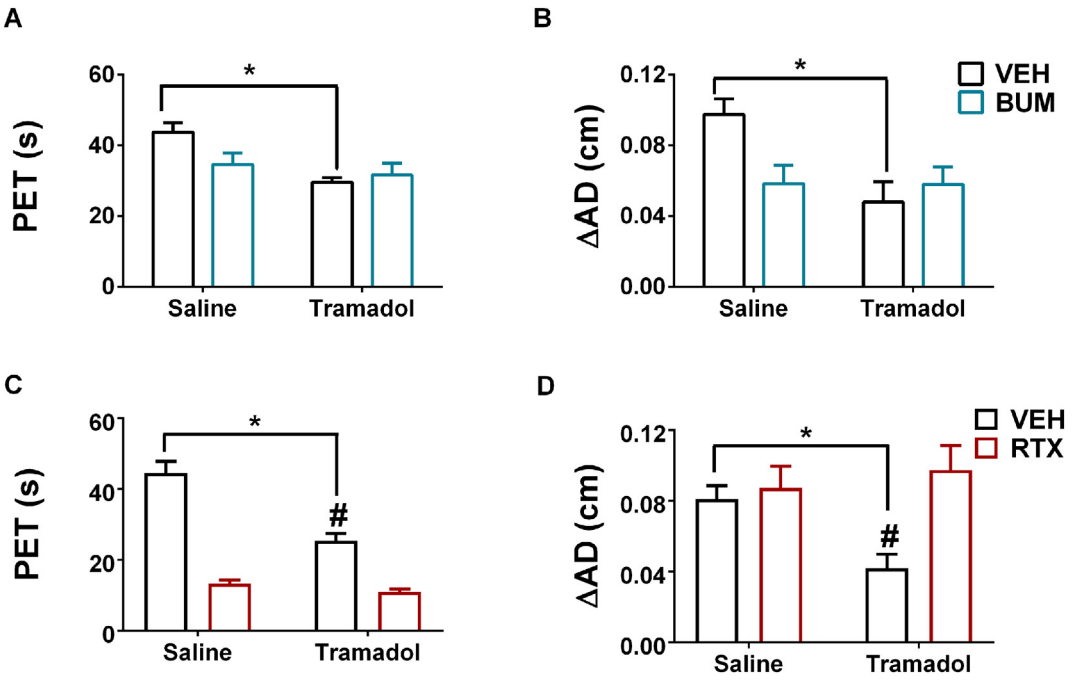


**Fig. 5.** Effect of intrathecal bumetanide (BUM; A–B) and resiniferatoxin (RTX; C–D) on the activity of tramadol (10 µg/20 µL) administered intrathecally 20 min before intra-articular LPS (30 ng/50 µL). BUM was injected 15 min before tramadol (35 min before LPS) and RTX was injected 5 days before tramadol together with their vehicles: sodium bicarbonate-containing PBS solution at 1.29% w/v and PBS + 7% Tween 20, respectively. Saline group received physiological saline (20 µL) intrathecally. Paw elevation time (A and C) and articular diameter (B and D) were measured hourly after LPS injection, from hour 3 to 5. Data are represented as mean ± SEM. $NKCC_1$ experiments used n = 8, RTX experiments used n = 7 for Veh group and n = 5 for RTX group. *P < 0.05 compared with Veh + Saline group, $^{\#}$P < 0.05 compared with RTX + Tramadol group (two-way analysis of variance followed by Newman-Keuls post hoc test). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

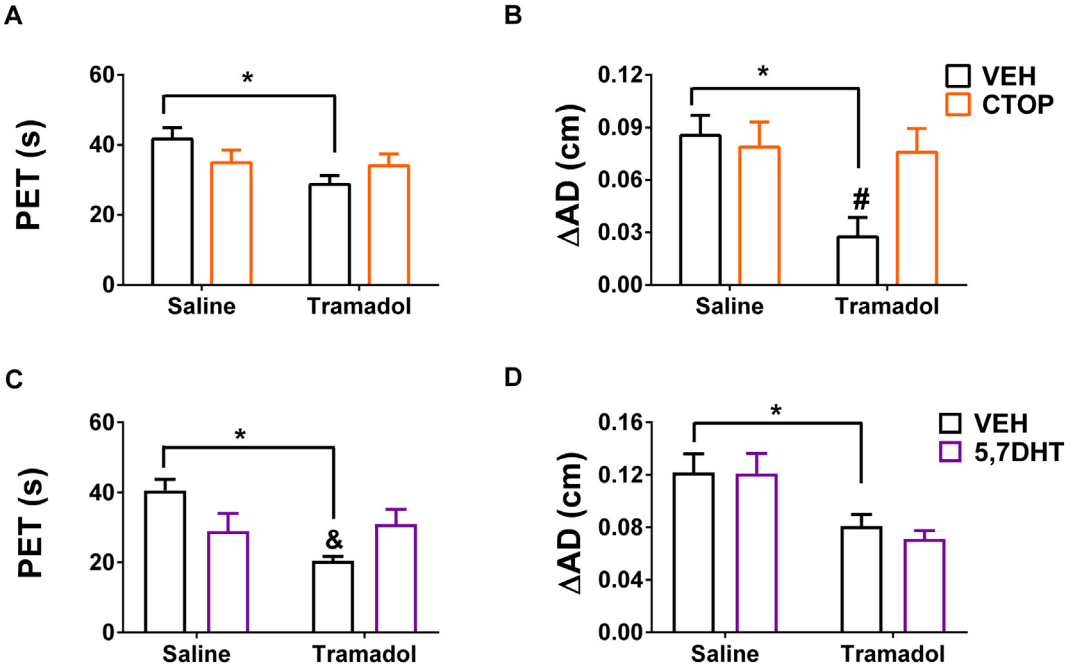


**Fig. 6.** Effect of intrathecal CTOP (A–B) and 5,7-dihydroxytriptamine (5,7-DHT; C–D) on the activity of tramadol (10 µg/20 µL) administered intrathecally 20 min before intra-articular LPS (30 ng/50 µL). CTOP was injected 15 min before tramadol (35 min before LPS) and 5,7-DHT was injected 5 days before tramadol together with their vehicles: saline and ascorbic acid-containing PBS solution at 2% w/v, respectively. Saline group received physiological saline (20 µL) intrathecally. Paw elevation time (A and C) and articular diameter (B and D) were measured hourly after LPS injection, from hour 3 to 5. Data are represented as mean ± SEM (n = 7 for 5,7-DHT groups and 8 for CTOP groups). *P < 0.05 compared with Veh + Saline group, $^{\#}$P < 0.05 compared with CTOP + Tramadol group, &P < 0.05 compared with 5,7-DHT + Tramadol group (two-way analysis of variance followed by Newman-Keuls post hoc test).

nor macrophage chemotaxis, but reduced $PGE_2$ levels in the paw exudate in a model of carrageenan induced inflammation, and enhanced IL-2 levels and natural-killer activity after subcutaneous injection in an equivalent model [36]. Thus, the inhibitory effect on TNF-α content observed here, is consistent with previously reported data by others, and may be related to an effect on the activity of the leukocytes rather than their migration.

In addition, in the present study, tramadol was not expected to be acting directly on leukocytes, since it was administered intrathecally. In this condition, we may suggest that a reduction of the neurogenic component of inflammation, due to the inhibition of DRR activity, could be reducing the secretory activity of pro-inflammatory mediators of leucocytes. In fact, it is known that neuropeptides released from primary afferent terminals can exert important stimulatory effects on leukocytes [37].

The μ-opioid receptor antagonist CTOP was used to discriminate the opioidergic component of the effects produced by tramadol, which seemed to be involved either in the analgesic or in the anti-edematogenic effects. There is substantial data showing morphine reducing paw and articular edema and plasma extravasation when injected systemically or by intrathecal injection, suggesting that it interferes with DRR deflagration [16,17]. However, these effects are not seen when morphine or other μ-opioid agonists are injected at the site of inflammation [38–40], indicating that peripheral μ-opioid receptors are not involved in those effects.

Intrathecal injection of CTOP prevented the antinociceptive effect of tramadol. This result, in fact, is somewhat surprising. The analgesic effect of tramadol is thought to be only partially due to μ-opioid receptor activation [4]. Indeed, an important part of its analgesic effect is reputed to be by inhibition of monoamine transporters [1,5]. Pretreatment with 5,7-DHT also totally reversed the analgesic effect, which is not completely consistent with several reported data. In fact, it has been described that inhibition of serotonergic receptors reduces, but not abolish, tramadol's analgesic effect [1,2,4]. Also, depletion of serotonergic fibers with 5,7-DHT injection in the neonate period reduced tramadol's analgesic efficacy in a variety of nociceptive tests in adult rats [41]. Probably, the present model might not be the best one to discriminate the partial contribution of spinal opioid and serotonergic signaling in the total analgesic effect of tramadol.

However, this model was very discriminative regarding the anti-edematogenic effect induced by this analgesic. It is known that serotonin can modulate the excitability of primary afferent fibers and dorsal horn neurons, influencing DRR [9]. Intrathecally injected serotonin can modify dorsal horn activity, nociceptive responses and also peripheral edema, in a fashion dependent on the receptor activated [14,42,43]. Thus, a balance between the inhibitory and excitatory actions of serotonin receptors is important to the final effect. The 5,7-DHT injection did not prevent the antiedematogenic effect of tramadol, suggesting its pro-serotonergic mechanism does not participate in this effect as in the analgesic one.

Therefore, it is conceivable to suppose that the analgesic activity of tramadol was likely due to a combined action of μ-opioid receptors activation and monoamine reuptake inhibition, while the anti-edematogenic effect seems to be mediated by μ-opioid receptors only.

Considering the relative safety of intrathecal tramadol in humans [44,45], low doses of this drug may be useful in chronic arthritic patients, who present poor outcomes with conventional treatment, or even for post-surgical patients aiming to reduce both pain and swelling. Notwithstanding, the anti-inflammatory action of tramadol herein was inferred only from the articular diameter and TNF-α reduction, so further analysis of other cytokines and inflammatory parameter would be necessary to strengthen these conclusions. In addition, tramadol was injected only once, because the model itself has a timeframe limitation, thus the use of other models that allow a chronic treatment should be considered.

In conclusion, this study showed that a low dose of tramadol injected by intrathecal route is able to reduce not only the incapacitation induced by inflammatory joint pain, but also some inflammatory parameters. Furthermore, the present results indicate that this effect in the periphery may be dependent on the inhibition of the primary afferent terminal activity at the spinal cord. Therefore, treatments targeting the inhibition of the dorsal root reflex deflagration are potential tools for the control of arthritic inflammation.

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## Declaration of competing interest

The authors declare that there are no conflicts of interest.

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