A repositioning approach: nitazoxanide inhibits inflammation and nociceptive response in mice models via a reduction of paw oedema, cellular migration and early TNF-α production

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SUMMARY

Introduction: Various studies have evaluated the *in vitro* anti-inflammatory effect of nitazoxanide (NTZ), suggesting new therapeutic functions for this drug. **Aims:** To evaluate the *in vivo* anti-inflammatory and antinociceptive activities of NTZ in acute mice models. **Methods:** Mice models of paw oedema, abdominal writhing, formalin and the rota-rod test were used. **Results:** Oral treatment with NTZ induced inhibition of paw oedema (60.00% and 66.67% at doses of 10 and 30 mg/kg, respectively) in the first hour after inflammatory stimulus, carrageenan (Cg). There was also a significant inhibition of 60.71% and 40.00% at the 30 mg/kg dose after 4h and 6 h, respectively after inflammation. Four hours after inflammation, the histological analysis of the footpad of animals treated with 30 mg/kg of NTZ showed a reduction in the migration of inflammatory cells by 65.77%. It is also important to highlight that there was a significant reduction of tumor necrose factor-alfa (TNF-α) in the initial phase of inflammation, 2 h after administration of the Cg. There was an inhibition in abdominal contortions by 54.14% and 56.21% at 30 and 90 mg/kg doses,

respectively. In the formalin test only the dose of 90 mg/kg showed antinociceptive action (54.85%; first phase and 45.67%; second phase). The results from rota-rod test showed that motor coordination was not affected with NTZ. **Conclusions:** This anti-inflammatory activity of NTZ appears to be a consequence of its ability to reduce the levels of an important mediator of the inflammatory response and pain the TNF-α.

Keywords: Nitazoxanide, repositioning, anti-inflammatory, antinociceptive, paw oedema, tumour necrosis factor-alpha.

RESUMEN

Un enfoque de reposicionamiento: la nitazoxanida inhibe la inflamación y la respuesta nociceptiva en modelos de ratones mediante una reducción del edema de la pata, la migración celular y la producción temprana de TNF-α

Introducción: Diversos estudios han evaluado el efecto antiinflamatorio *in vitro* de la nitazoxanida (NTZ), sugiriendo nuevas funciones terapéuticas para este fármaco. **Objetivos:** Evaluar las actividades antiinflamatorias y antinociceptivas *in vivo* de NTZ en modelos de ratones agudos. **Métodos:** Se utilizaron modelos en ratones de edema de pata, contorsiones abdominales, de formalina y prueba de rota-rod. **Resultados:** El tratamiento oral con NTZ indujo la inhibición del edema de la pata (60,00% y 66,67% a dosis de 10 y 30 mg/kg, respectivamente) en la primera hora después del estímulo inflamatorio con carragenano (Cg). También hubo una inhibición significativa del 60,71% y 40,00% con la dosis de 30 mg/kg después de 4 h y 6 h, respectivamente, después de la inflamación. Cuatro horas después de la inflamación, el análisis histológico de la almohadilla plantar de los animales tratados con 30 mg/kg de NTZ mostró una reducción de la migración de células inflamatorias del 65,77%. También es importante resaltar que hubo una reducción significativa del factor de necrosis tumoral alfa (TNF-α) en la fase inicial de la inflamación, 2 h después de la administración del Cg. Hubo una inhibición en las contorsiones abdominales de 54,14% y 56,21% con dosis de 30 y 90 mg/kg, respectivamente. En la prueba de formalina sólo la dosis de 90 mg/kg mostró acción antinociceptiva (54,85%; primera fase y 45,67%; segunda fase). Los resultados de la prueba rota-rod

mostraron que la coordinación motora no se vio afectada con NTZ. **Conclusiones:** Esta actividad antiinflamatoria de NTZ parece estar relacionada con su capacidad para reducir los niveles de un importante mediador de la respuesta inflamatoria y del dolor el TNF-α.

Palabras clave: Nitazoxanida, reposicionamiento, antiinflamatorio, antinociceptivo, edema de pata, factor de necrosis tumoral alfa.

Resumo

Uma abordagem de reposicionamento: a nitazoxanida inibe a inflamação e a resposta nociceptiva, em modelos com camundongos através da redução do edema da pata, da migração celular e da produção precoce de TNF-α

Introdução: Diversos estudos avaliaram o efeito antiinflamatório *in vitro* da nitazoxanida (NTZ), sugerindo novas funções terapêuticas para esta droga. **Objetivos:** Avaliar as atividades anti-inflamatória e antinociceptiva *in vivo* da NTZ em modelos agudos com camundongos. **Métodos:** Foram utilizados modelos de camundongos com edema de pata, contorções abdominais, teste de formalina e teste rota-rod. **Resultados:** O tratamento oral com NTZ induziu inibição do edema de pata (60,00% e 66,67% nas doses de 10 e 30 mg/kg, respectivamente) na primeira hora após o estímulo inflamatório, carragenina (Cg). Houve também uma inibição significativa de 60,71% e 40,00% na dose de 30 mg/kg após 4h e 6h, respectivamente, após a inflamação. Quatro horas após a inflamação, a análise histológica da pata dos animais tratados com 30 mg/kg de NTZ mostrou redução na migração de células inflamatórias em 65,77%. É importante destacar também que houve redução significativa do fator de necrose tumoral alfa (TNF-α) na fase inicial da inflamação, 2 horas após a administração do Cg. Houve inibição nas contorções abdominais em 54,14% e 56,21% nas doses de 30 e 90 mg/kg, respectivamente. No teste da formalina apenas a dose de 90 mg/kg apresentou ação antinociceptiva (54,85%; primeira fase e 45,67%; segunda fase). Os resultados do teste rota-rod mostraram que a coordenação motora não foi afetada com NTZ. **Conclusões:** Esta atividade anti-inflamatória da NTZ parece ser consequência da sua capacidade de reduzir os níveis de um importante mediador da resposta inflamatória e da dor o TNF-α.

Palavras-chave: Nitazoxanida, reposicionamento, antiinflamatório, antinociceptivo, edema de pata, fator de necrose tumoral alfa.

Introduction

Drug repositioning refers to the identification of new therapeutic indications for drugs already used in clinical practice and their application in the treatment of diseases that were not initially indicated. The advantages of this practice are evident: the drug has already been tested in all preclinical and clinical phases of drug development, has been used by humans and has well-established toxicology, pharmacology and safety data. The targeting of approved drugs is believed to be one of the most efficient and economical strategies for new drug development [1-3].

Nitazoxanide (NTZ) and tizoxanide (TIZ) are synthetic thiazolide derivatives discovered by Jean Francois Rossingnol [4]. NTZ is an anti-protozoal medicine clinically approved in the USA since 2002 and in Brazil since 2004 for treating various parasitic diseases in adults and children [5-10], being well tolerated, even at high doses [11, 12], with mild adverse reactions [5, 12, 13].

In addition to having a broad antiparasitic activity, NTZ and its circulating active metabolite, TIZ, have also been shown to have antimicrobial activity against various viruses [7-9, 14-20] and various aerobic and anaerobic bacteria [16, 21, 22].

Due to the broad spectrum of pathogens targeted by NTZ and TIZ, thiazolides might possess other pharmacological properties in addition to antimicrobial activity [23-26]. Although several *in vitro* studies have already demonstrated the anti-inflammatory effect of NTZ and its main active metabolite TIZ, the potential effect of these compounds on inflammation response, mainly *in vivo* inflammatory mouse models, has not been widely investigated [23-26]. There is only one *in vivo* study evaluating specifically the anti-inflammatory effect of NTZ in mice models of inflammation. In this study the NTZ reduced the plasma interleukin (IL)-6 levels in a mice model of thioglycollate-induced inflammation, but other inflammatory parameters were not evaluated [26].

The inflammatory response is a fundamental biological process that not only protects the host against infections by pathogens, but also promotes repair and healing after an injury [27, 28]. Failing to properly regulate inflammation results in a multitude of complications that are extremely harmful to the pulmonary, cerebrovascular and cardiovascular systems [27, 29-31]. The inflammatory process is part of the pathophysiology of several clinically important chronic diseases, such as asthma, obesity, cancer, cardiovascular diseases and diabetes [27, 29, 30, 32].

Non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are the most commonly used drugs for the treatment of various types of inflammation and pain.

However, the chronic use of NSAIDs and glucocorticoids commonly induces gastro-intestinal, cardiovascular, renal, hepatic, cerebral and pulmonary problems [33, 34]. In turn, analgesic opioids can cause constipation, nausea, vomiting, sedation, respiratory depression and dependence [35].

It is necessary to search for safer alternative therapies, since the inflammatory process is part of the pathophysiology of several important diseases [29, 30, 36]. Thus, the present study aimed to investigate the potential anti-inflammatory and antinociceptive activities of NTZ using *in vivo* mice models.

Materials and methods

Animals

Experiments were conducted using adult male Swiss (28–30 g) mice. Animals were housed in temperature-controlled rooms (22–25 °C), under a 12:12 h light-dark cycle, with free access to food and water, maintained for at least 7 days for acclimatization before the experiments. The feed was suspended 2 h before each experiment, leaving free access to water. The animals were randomly allocated to groups of six and seven animals each, for anti-inflammatory and antinociceptive tests, respectively. Each mouse was used only once during the study. All experimental protocols were by the guidelines adopted by the International Association for the Study of Pain and the Brazilian National Council for the Control of Animal Experimentation. The experiments were approved by the Ethics Committee in Animal Experimentation of the Federal University of São João del-Rei, Brazil (CEUA/UFSJ, protocol 016/2020 on 10/07/2020 and protocol 1699010321, on 04/01/2021). All efforts were made to minimize the number of animals and their suffering.

Anti-inflammatory activity

Carrageenan-induced mouse paw oedema

The antioedematogenic effect of NTZ was evaluated using Carrageenan (Cg)-induced mouse paw oedema. The Swiss mice were pre-treated orally with the vehicle (10 mL/kg, control group), NTZ (3, 10 and 30 mg/kg) or indomethacin (Ind; 10 mg/kg), 1 h before the intraplantar injection of Cg (400 µg/paw, 30 µL) into the left hind paw. The paw volume was measured by a blind evaluator using a plethysmometer (Insight®, Brazil) before treatment (basal) and 1, 2, 3, 4 and 6 h after injection of the Cg. The difference between paw volume before and after inflammatory stimulus injection was taken as the volume of oedema (µL). The percentage of oedema inhibition in treated mice was calculated in comparison with the respective the control group [37]. Carrageenan

λ type IV (22049) and indomethacin (I7378) were purchased from Sigma-Aldrich, Inc. (MO, USA) and NTZ (DCB 06413) was supplied by Eurofarma (SP, Brazil). Ind and Cg was dissolved in phosphate buffer saline (PBS) and Tween 20 solution and NTZ was diluted in PBS. The naive and control groups were treated with PBS.

Histopathological analysis

The animals were treated orally as follows: naive (PBS), control (PBS), NTZ (30 mg/kg) or Ind (10 mg/kg). One hour after oral treatment, Cg (400 µg/paw, 30 µL) was administered to the left hind paw of the animals, except for the naive group, which received an intraplantar injection of PBS. Samples of the footpad tissue were collected 4 h after the inflammatory stimulus and fixed in 4% Paraformaldehyde solution for 24 h. Paraffin blocks were then prepared, cut into 4 µm thick tissue sections and subsequently stained with hematoxylin-eosin (H&E) [38]. Images of the infiltration of inflammatory cells were obtained using a conventional optical microscope (Motic®) coupled to a capture system (Moticam 3000®), amplified 40x and 400x, and evaluated by two investigators using ImageJ® software, version 1.44 (Research Services Branch, U.S. National Institute of Health, Bethesda, MD, USA). The photomicrographs were used for the quantitative analysis of cell infiltration. The inflammatory cells were identified based on their characteristic morphology, in six different regions randomly selected for each animal, at 400x magnification. Subsequently, the total number of leukocytes was expressed as the number of cells/area analysed in μm^2.

Tumour necrosis factor alpha (TNF-α) levels in the tissue

The animals were treated orally as follows: naive (PBS), control (PBS), NTZ (30 mg/kg), Ind (10 mg/kg) or Dexamethasone (Dex; 1.5 mg/kg). One hour after treatment, Cg (400 µg/paw, 30 µL) was administered to the left hind paw of the animals, except for the naive group, which received an intraplantar injection of PBS. Samples of the plantar pads were collected 2 h after the inflammatory stimulus. The tissues from the pads were homogenised in 1 mL of cytokine extraction solution containing protease inhibitors (1 mL of solution for 50 mg of tissue) and 0.05% Tween 20. The samples were immediately ground and centrifuged at 3000 rpm for 8 minutes. The supernatant was then collected and stored in a -80 °C freezer until required. The TNF-α levels were determined using an enzyme-linked immunosorbent assay (ELISA), according to the kit manufacturer's instructions (eBioscience - 88-7324-88, San Diego, CA, USA) [39]. Dexamethasone (DCB02817) was purchased from Aché (SP, Brazil) and used as a commercial suspension.

Antinociceptive activity

Acetic acid-induced abdominal writhing test

The animals were treated orally as follows: control (PBS), NTZ (10, 30 and 90 mg/kg) or Ind (10 mg/kg). One hour after treatment, 1% acetic acid (10 mL/kg) was administered intraperitoneally (*ip*). The amount of abdominal writhing (abdominal contraction and/or simultaneous contraction of the abdomen with stretching of the hind limbs) was recorded 10 minutes after administration of the nociceptive stimulus, over 20 minutes, by a blind evaluator [40]. Acetic acid 96% (695092) was supplied by Merck KGaA (Darmstadt, Germany) and was diluted in PBS.

Formalin-induced nociception test

The animals were treated orally as follows: control (PBS), NTZ (10, 30 and 90 mg/kg) or Ind (10 mg/kg). Morphine (Mor; 7.5 mg/kg) was administered via *ip* injection. One hour after the oral treatments and 30 minutes after the *ip* treatment, 20 μL of 2.50% formaldehyde (formalin) was injected into the subplantar region of the left hind paw. The animals were then placed individually in an acrylic box and observed by a blind evaluator in two stages: 0-5 minutes (the first, neurogenic phase) and 15-30 minutes (the second, inflammatory phase). The time that the animal spent licking, biting or shaking their paw was timed and considered to be indicative of nociception [41]. Morphine (DCB 06090) sulphate was obtained from Cristália (SP, Brazil) and was used as a commercial injectable solution. Formaldehyde 37% (252549) were supplied by Merck KGaA (Darmstadt, Germany) and was diluted in PBS.

Rota-rod test

The day before the experiment, each animal was placed on a rotating bar at a speed of 24 rpm/min, using a cut-off time of 120 seconds (s). The time that each animal remained on the bar was recorded (the basal time). The following day, the animals were treated orally as follows: control group (PBS), NTZ (10, 30 and 90 mg/kg) or alprazolam (2 mg/kg). One hour after oral administration, the animals were placed on the rotating bar at the same speed, and the time the animals remained on the device was recorded at 30, 60, 120 and 150 minutes after treatments. Time until the first fall was recorded in seconds with a cut-off time of 120 s [42]. Alprazolam (DCB 00597) was supplied by Eurofarma (SP, Brazil) and was diluted in PBS.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Statistical significance between groups was determined by one-way analysis of variance (ANOVA)

followed by Bonferroni's test. The level of significance (p<0.05, 0.01 or 0.001) was employed for each experimental model. The analyses were carried out using GraphPad Prism™ software, version 5.01 (GraphPad® Software Inc., San Diego, CA).

Results

Anti-inflammatory activity

Antioedematogenic effect of NTZ

The NTZ showed antioedematogenic activity at a dose of 10 mg/kg from the first hour (60.00%, p<0.001) to the fourth hour (21.43%, p<0.05) compared to the control group. At the 30 mg/kg dose, the inhibition of oedema occurred from the first hour (66.67%, p<0.001) and remained until the sixth hour (40.00%, p<0.001). Ind (10 mg/kg) inhibited oedema by 66.67% in the first hour (p<0.001), which was similar to the effect produced by NTZ at a dose of 10 mg/kg (Fig. 1a). In Fig.1b it is possible to confirm the antioedematogenic effect of NTZ compared to the control group. A greater thickening of the dermis and epidermis is evident in the control group in relation to the NTZ, Ind and naive groups. It is important to highlight that the antioedematogenic effect of NTZ was more noticeable compared to Ind.

*Effect of NTZ on inflammatory infiltrate*

In Fig. 2a, only discrete leukocytes are present in the naive group, probably indicating defence cells residing in the plantar tissue. On the other hand, the footpads of the control group show a significant infiltration of polymorphonuclear leukocytes, probably neutrophils, as they are the first cells to migrate in acute inflammatory processes. NTZ (30 mg/kg) and Ind (10 mg/kg) reduced the accumulation of leukocytes in the footpad. In Fig. 2b, it is important to note that, in comparison to the control group, NTZ reduced leukocyte migration (65.77%, p<0.001) in a similar manner to Ind (64.44%, p<0.001).

Effect of NTZ on TNF-α levels in the tissue

The level of TNF-α significantly increased in the footpads of the control group animals following Cg-induced inflammation. Treatment with NTZ (30 mg/kg) promoted a local decrease of TNF-α in the initial phase of the inflammatory response, 2 h after Cg, reducing the TNF-α level by 63.96% (p<0.001) (Fig. 3).
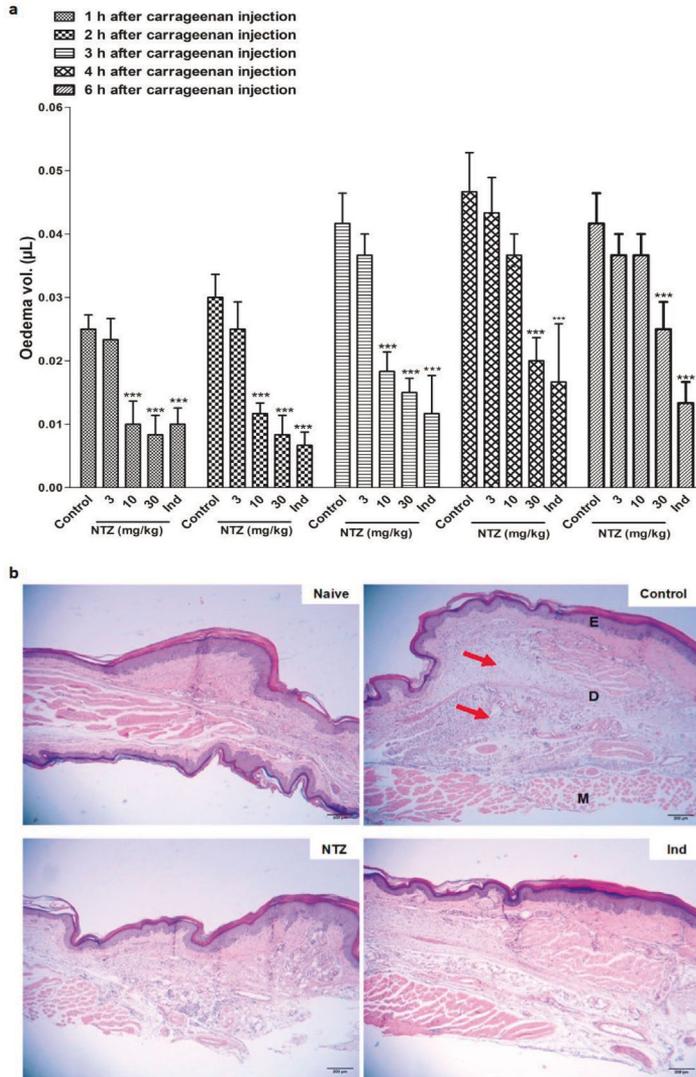
Fig. 1. a) Effect of NTZ (3, 10 and 30 mg/kg) and Ind (10 mg/kg) on Cg-induced paw oedema. NTZ and Ind were administered orally and Cg was administered via intraplantar. Each value represented as mean ± SEM (n = 6 per group). *p<0.05, and ***p<0.001 versus the respective control group (one-way ANOVA followed by Bonferoni's test). b) Effect of oral treatment with NTZ and Ind on paw oedema and leukocyte migration 4 hours after Cg stimulus. Representative histologic images from different experimental groups: Naive; Control; NTZ (30 mg/kg) and Ind (10 mg/kg). The arrows indicate the areas of oedema in the dermis and epidermis leading to increased thickness of the footpad. E: epidermis, D: dermis, M: muscle. Staining: haematoxylin-eosin (H&E). Magnification: 40x. Bar: 200 µm.
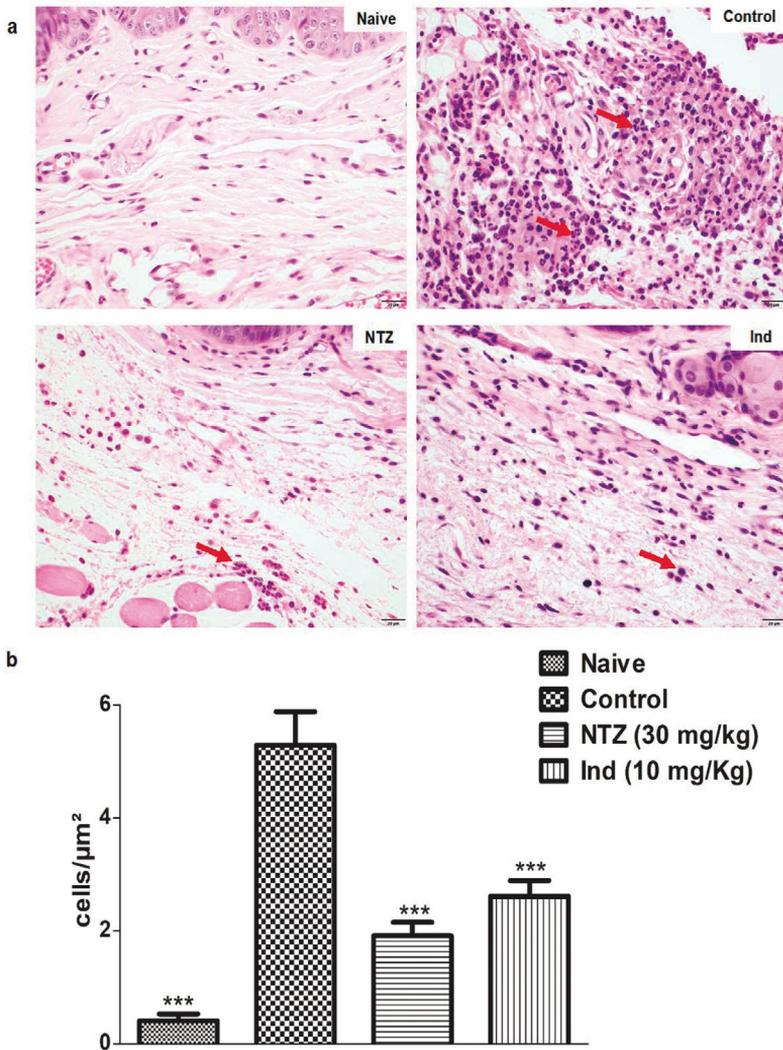
Fig. 2. a) Histopathological evaluation of mouse footpad tissue sections 4 h after the Cg intraplantar injection, demonstrating the effect of NTZ (30 mg/kg) and Ind (10 mg/kg), administered orally, on cellular migration. The arrows indicate leukocytes. Colour: haematoxylin-eosin (H&E). Magnification: 400x. Bar: 20 μm. b) Quantitative analysis of footpad sections collected 4 h post Cg injection, demonstrating the effect of NTZ (30 mg/kg) and Ind (10 mg/kg) on cellular migration. Each value represented as mean ± SEM (n = 6 per group). ***p<0.001 versus the control group (one-way ANOVA followed by Bonferroni's test).
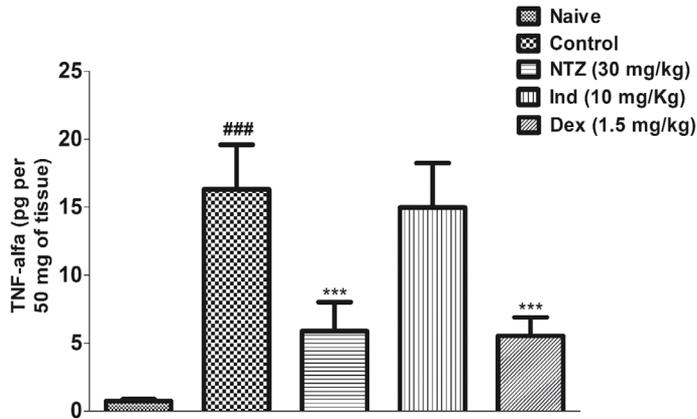
Fig. 3. Effect of NTZ (30 mg/kg), Ind (10 mg/kg) and Dex (1.5 mg/kg), administered orally, on TNF-α levels in footpads, 2 h following Cg-induced inflammation. Each value represented as mean ± SEM (n = 6 per group) ###p<0.001 control versus naive group; ***p<0.001 NTZ versus the control group (one-way ANOVA followed by Bonferroni's test).

Antinociceptive activity

*Effect of NTZ on abdominal contortions*

As the lowest dose of NTZ (3 mg/Kg) showed no activity on Cg-induced paw edema, doses of 10, 30 and 90 mg/Kg were used to assess antinociceptive activity. The administration of NTZ at doses of 30 and 90 mg/kg induced a statistically significant reduction in the number of abdominal contortions induced by acetic acid, with reductions of 54.14% (p<0.001), and 56.21% (p<0.001), respectively, compared to the control group. Meanwhile, Ind (10 mg/kg) caused a decrease of 73.20% (p<0.001) compared to the control group (Fig. 4).

Effect of NTZ on formalin-induced nociception

The Fig. 5 shows that only the group treated with the highest dose of NTZ (90 mg/kg) was able to significantly reduce the reaction time of the animals, by 54.85% (p<0.01) and 45.67% (p<0.05) in the first and second phases of the test, respectively, compared to the control group. As expected, Ind (10 mg/kg), being a NSAID, showed a reduction in reaction time only in the inflammatory phase, by 59.48% (p<0.05), while Mor (7.5 mg/Kg), being an opioid, was able to act in the first and second phases of the formalin test (p<0.001).
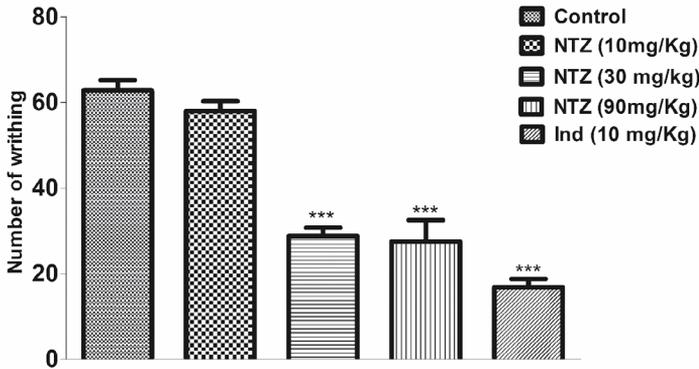
Fig. 4. Effect of NTZ (10, 30 and 90 mg/kg) and Ind (10 mg/kg), administered orally, on acetic acid-induced abdominal writhing. Each value represented as mean ± SEM (n = 7 per group). ***p<0.001 versus the control group (one-way ANOVA followed by Bonferroni's test).
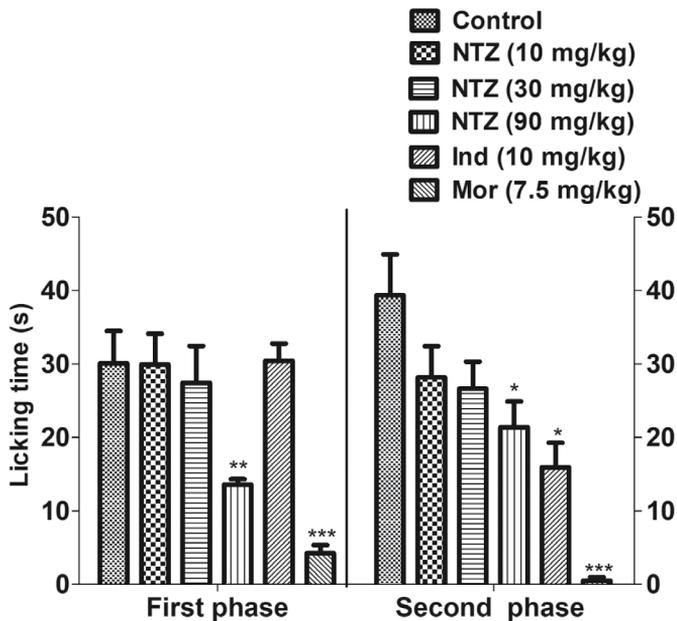
Fig. 5. Effect of NTZ (10, 30 and 90 mg/kg), Ind (10 mg/kg), administered orally, and Mor (7.5 mg/kg), via intraperitoneal, on the animals' reaction time in the formalin test. Each value represented as mean ± SEM (n = 7 per group). *p<0.05, **p<0.01 and ***p<0.001 versus the respective control group (one-way ANOVA followed by Bonferroni's test).

Effect of NTZ on motor coordination and balance

The results showed no statistical difference between the control groups and all tested doses of NTZ (10, 30 and 90 mg/kg) at any of the analysed times, after oral treatments (30, 60, 120 and 150 minutes). In contrast, animals treated with alprazolam remained on the bar for a significantly shorter period in seconds (s) (p<0.001) compared to the control group, as shown in Table 1.

Table 1. Effect of NTZ on motor coordination

Treatment	Time after treatment (min)			
	30	60	120	150
	Time on rod (s)	Time on rod (s)	Time on rod (s)	Time on rod (s)
Control	120±0.00	120±0.00	120±0.00	120±0.00
NTZ (10 mg/kg)	120±0.00	120±0.00	120±0.00	120±0.00
NTZ (30 mg/kg)	120±0.00	120±0.00	120±0.00	120±0.00
NTZ (90 mg/kg)	120±0.00	120±0.00	120±0.00	113.714±6.286
Alprazolam (2 mg/kg)	3.000±0.707*	14.250±3.425*	26.000±10.384*	28.250±16.332*

Each value represented as mean ± SEM (n = 7 per group). *p<0.001 versus the control group (one-way ANOVA followed by Bonferroni's test).

Discussion

The present study demonstrates that NTZ has an anti-inflammatory and antinociceptive activity in these *in vivo* models of inflammation and pain. NTZ was able to inhibit oedema and reduce leukocyte migration and TNF-α levels in tissue inflamed with Cg. NTZ also reduced the number of abdominal contortions induced by acetic acid and reduced the reaction time in the formalin-injected paw model, without altering motor function or balance.

Several previous works have demonstrated the ability of NTZ and TIZ to modulate the inflammatory response due to their anti-inflammatory activity. However, most of these studies evaluated the anti-inflammatory or modulatory effect of NTZ or TIZ in cell cultures [5, 14, 23-26, 43, 44], contributing to the decision to evaluate the possible anti-inflammatory and antinociceptive effect of NTZ using *in vivo* mice models.

To evaluate the anti-inflammatory activity, the Cg-induced paw oedema model was initially used, which is a classic, simple and effective experimental model to study inflammation and inflammatory pain. This model consists of two phases and is associated with the release of several inflammatory mediators. The first, (or initial) phase

mainly involves the release of serotonin, histamine, bradykinin and cyclooxygenase (COX) products. The second (or late) phase is related to leukocyte infiltration, further prostaglandin release, and the production of oxygen-derived free radicals, nitric oxide (NO) and cytokines [38, 45, 46]. Our results showed that NTZ significantly inhibited the formation of paw oedema, indicating that this compound has a potential anti-inflammatory effect since oedema is one of the main signs of inflammation.

To confirm the anti-inflammatory effect of NTZ, cell migration was also evaluated to verify whether the antioedematogenic activity is also associated with a decrease in leukocyte migration. The histological results indicated that Cg-induced inflammation is associated with intense oedema and migration of the infiltrate of inflammatory polymorphonuclear cells, probably neutrophils. This is in line with our previous studies, which also demonstrated an increase in cell migration to the Cg-inflamed site [47, 48]. The NTZ treatment significantly diminished oedema and the cell tissue migration, which is another important parameter of inflammation.

During the acute inflammatory response, leukocytes are important for tissue repair and are an essential part of the immune response. However, during diapedesis, these cells can attack the vessel wall and cause the extravasation of interstitial fluid, inducing the formation of oedema, and their support favours the permanence of cytokines at the site. Therefore, large amounts of leukocytes can have deleterious effects on tissue. Thus, drugs that decrease the passage of blood cells to extravascular tissue may be useful in therapy [28, 49]. Neutrophil migration is considered to be one of the central reactions of inflammation because they are innate effector cells and can respond quickly to danger signals. Neutrophils stimulate the release of several inflammatory mediators, such as NO, attracting more leukocytes to the site of injury, and initiating and maintaining the inflammatory process, thus being an important component of the immune response. Neutrophils can also release TNF-α and other cytokines that can also, indirectly, stimulate the arrival of more neutrophils [50]. The effect of NTZ suggests an association between the reduction of oedema and consequently in tissue cell migration, with a modulation in the production of cytokines.

One *in vivo* study evaluated the potential anti-inflammatory effect of NTZ in a mice model of thioglycollate-induced inflammation; the only anti-inflammatory parameter evaluated was the production of IL-6, which was also shown to be reduced [26].

Cytokines play an important role in the inflammatory response and some, such as TNF-α, deserve to be highlighted. TNF-α creates positive feedback to increase the production of NO, which acts as a vasodilator and induces the activation of adhesion molecules, favouring the continuation and amplification of oedema, cell tissue migra-

tion and inflammation. TNF-α also exerts several other biological effects that influence the development and maintenance of chronic inflammatory conditions, such as activating an important cell signalling pathway, the nuclear factor-kappa B (NF-κB) pathway [51-56]. Previous studies conducted by our group have already demonstrated the important participation of TNF-α in the inflammatory responses induced in this *in vivo* model [47, 48, 57, 58].

The NTZ was also able to reduce tissue TNF-α levels in the early stage of the inflammatory response. This decrease in TNF-α, 2 hours after the Cg inflammatory stimulus, seems to be important for the reduction in inflammatory cell migration observed later, 4 hours after the inflammatory stimulus.

This result is in agreement with several other studies that have also demonstrated the ability of NTZ and/or its active metabolite TIZ to inhibit TNF-α levels and other important inflammatory mediators. One of these studies showed that NTZ inhibited the production of pro-inflammatory cytokines such as TNF-α, IL-5, IL-6 and IL-8 in peripheral blood mononuclear cells [14]. Blum *et al.* also demonstrated a reduction of inflammatory markers such as TNF-α, IL-6 and IL-8 in a small pilot trial in patients with moderate COVID-19 treated with NTZ [20]. In another study on the LPS-induced inflammatory response in cells of microglia, TIZ treatment also decreased the release of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, and chemokines, such as CCL-2 and CCL-3, as well as the extrinsic expression of inducible nitric oxide synthase (iNOS) and COX-2 expression [59, 60]. Fan *et al.* also demonstrated that NTZ reduced the levels of TNF-α, IL-1β and iNOS in the LPS-induced inflammatory response in cells of microglia [61]. Meanwhile, Hong *et al.* demonstrated that NTZ decreased IL-6 production in LPS-stimulated RAW 264.7 cells and peritoneal macrophages, and also showed that NTZ was able to suppress LPS-induced IL-6 messenger ribonucleic acid (mRNA) expression [26]. Salazar *et al.* found that NTZ was able to reduce the concentration of pro-inflammatory cytokines such as IL-2, IL-6 and IL-1β in cultures of human peripheral blood mononuclear cells and decrease the monocyte/macrophage M1/M2 ratio [62]. In addition, Tantawy *et al.* demonstrated that NTZ induces a decrease of IL-6 via the inhibition of JAK 2/STAT3, an important signal transduction pathway in the inflammatory response [63]. Thus, these studies suggest that NTZ has potential immunomodulatory and anti-inflammatory effects by inhibiting cell proliferation and reducing the levels of pro-inflammatory cytokines.

The inhibition of cytokines and their signalling pathways may indicate an effective therapy against inflammatory diseases [64, 65]. Shou *et al.* demonstrated that treatment with TIZ, the active metabolite of NTZ, reduced the inflammatory response in macrophage cell lines by suppressing the NF-κB, mitogen-activated protein kinase

(MAPK) [25] and PI3K/Akt/mTOR [23] signalling pathways. Fan *et al*. also demonstrated that NTZ inhibits NF-κB and PI3K/Akt/mTOR pathway signalling *in vitro* [61]. These signalling pathways are of particular importance in immune and inflammatory responses. Numerous stimuli are responsible for activating these pathways, such as protein kinase C, viruses, oxidants and pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6. Among the genes regulated by these signalling pathways are those involved in the production of pro-inflammatory cytokines, such as TNF-α, chemokines and adhesion molecules. Activation of these pathways therefore leads to a coordinated increase in the expression of numerous genes, whose products are related to the modulation of inflammatory and immune responses [66-70].

In this sense, through modulation of the signal transduction pathways of the inflammatory response, NTZ can reduce the tissue production of numerous inflammatory mediators, such as TNF-α.

The inhibition of inflammatory cytokines by signalling pathway suppression has been identified as a key step in the treatment of inflammation and therefore serves as an important target for drug development [23, 25, 67]. Thus, a good strategy in fighting inflammation is to search for new drugs with a multifactorial mode of anti-inflammatory action [23, 25, 71], and NTZ exhibits polypharmacological actions [11, 16, 72].

As NTZ has been shown to have an anti-inflammatory effect, and pain is a cardinal sign of inflammation, there is also a need to investigate the antinociceptive activity of NTZ, since these properties are shared by several NSAIDs. In our study, the investigation of antinociceptive activity started with a test of the abdominal writhing induced by acetic acid. This is a nonspecific test, in which even weak analgesics can present antinociceptive effects. In addition, with this test, it is not possible to determine the specific pharmacological pathways involved in the effect [73, 74]. The acid induces an increase in the release of inflammatory mediators, such as TNF-α and prostaglandins, which sensitise nociceptive fibers and lead to nociception [51, 73, 75, 76].

Our results demonstrated that NTZ showed significant antinociceptive activity in this model at doses of 30 and 90 mg/kg. This effect may be related to the inhibition of the inflammatory mediators. Thus, at least in part, the antinociceptive effect of NTZ may be a consequence of the suppression of TNF-α. Blum *et al*. evaluated the safety and efficacy of NTZ in patients with moderate COVID-19 and observed that NTZ statistically reduced the levels of TNF-α and IL-8 [20], which cytokines are known to be important for nociception. TNF-α promotes nociception in mice by two independent correlated pathways: (1) stimulation of interleukin (IL-1) release that leads to prostanoid production; and (2) keratinocyte-derived chemokines (KC/CXCL1/IL-8) that induce

both prostanoids and sympathetic amine release [77]. TNF-α can also induce nociception through direct action on the nociceptors, via tumour necrosis factor receptor 1 (TNFR1) activation [78].

The next step was to perform the formalin test, which is a more refined test capable of evaluating the central and peripheral activities of NTZ and confirming whether the antinociception was related to an anti-inflammatory effect. The formalin test is divided into two phases of nociceptive stimulation, the first being neurogenic (central) and the second being the inflammatory (peripheral) phase. The primary or neurogenic phase reflects centrally mediated pain with the release of substance P, being related to the direct stimulation of primary sensory neurons. The secondary or inflammatory phase is associated with peripheral sensitization, when pro-inflammatory cytokines are released, including TNF-α, histamine, serotonin and prostaglandins [79, 80]. Only at the highest dose (90 mg/kg) did NTZ significantly suppress the response in the neurogenic and inflammatory phases, leading to the hypothesis that NTZ has weak central and peripheral antinociceptive activity. Ai *et al.* demonstrated that the intraperitoneal administration of NTZ significantly reversed mechanical hyperalgesia in a model of neuropathic pain in rats [81]. The anti-inflammatory effect of NTZ, previously observed in the present study, corroborates the inhibitory effect of NTZ on the second phase of formalin-induced nociception.

Once the effect of NTZ had been shown in both phases of the formalin test, it was interesting to assess whether this inhibition in response could be due to the muscle relaxant action of NTZ, via its action on the central nervous system. The rota-rod test is widely used to assess central nervous system depression, possible muscle relaxation, and loss of balance [82]. The results of the rota-rod test suggest that NTZ was not able to change the motor capacity or balance of the animals, indicating that the minor antinociceptive effect presented by NTZ in the first phase of formalin test does not interfere with these characteristics.

Conclusion

The present study demonstrated that NTZ exhibits a significant anti-inflammatory activity in *in vivo* acute models of inflammation and pain. This effect appears to be a consequence of its ability to reduce the levels of an important polymorphonuclear leucocyte-derived inflammatory and pain mediator, TNF-α, reducing the oedema and leucocyte migration.

Acknowledgments

Compliance with ethical standards

Ethical approval: The experiments were approved by the ehics committee in animal experimentation of the Federal University of São João del-Rei, Brazil (CEUA/UFSJ, protocol 016/2020 on 10/07/2020 and protocol 1699010321, on 04/01/2021).

Author contributions: All authors contributed to the development, analysis, writing and review of this article.

Conflict of interest

The authors declare that they have no conflict of interest.

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How to cite this article