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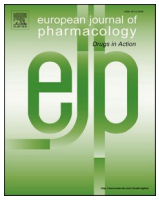

Investigation of the participation of the TRPV1 receptor in the irritant effect of dithranol in mice

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ABSTRACT

Dithranol is one of the most effective topical medications for treating plaque psoriasis. However, its clinical use is limited by irritative adverse reactions to the skin, such as oedema, erythema, and pruritus, caused by poorly understood mechanisms. Because TRPV1 activation mediates skin irritation caused by several drugs, we conducted blind and randomised experiments in male and female C57BL/6 mice to elucidate the role of TRPV1 in dithranol-induced irritation. Dithranol (0.01%–0.5%) or vehicle was applied topically to the right ear of the animals. Oedema, erythema, and pruritus were monitored from 2 h to 6 days after application. Treatment with 0.5% dithranol caused oedema and erythema, but not pruritus, starting at 6 h, reaching its highest point at 1 day, and persisting up to 6 days after treatment, mainly in male mice. The 0.1% dose induced erythema but not oedema. Interestingly, topical application of 1% capsaicin was shown to defunctionalise TRPV1-positive fibres and did not influence early irritation caused by dithranol (2 h–2 days). However, it increased the late phase of irritation (5–6 days). Similarly, salicylate did not reduce the early irritation caused by dithranol but also increased the late phase. Antagonism by SB366791 and 4-tert-butylcyclohexanol did not alter skin irritation. Our results suggest that, contrary to our initial hypothesis, TRPV1 appears to act protectively against skin irritation caused by dithranol, particularly in the late stage.

1. Introduction

Psoriasis is a chronic inflammatory skin disease. The most common form, known as vulgar or plaque psoriasis, is characterised by thick, scaly plaques resulting from abnormal keratinocyte proliferation and immune cell infiltration in the dermis and epidermis (Korman, 2020). Psoriasis is estimated to affect approximately 1%–3% of the population, presenting with both cutaneous and systemic manifestations and significantly impacting patients' quality of life (Greb et al., 2016). Approximately 15% of patients experience a severe form of psoriasis, which can be effectively managed with systemic agents such as anti-IL-17 or anti-IL-23 inhibitors (Hawkes et al., 2018). However, about 85% of patients have a mild to moderate form of the disease, which can be treated with safe and highly effective topical agents (Lebwohl, 2018).

Dithranol is a therapeutic agent considered effective and safe for the topical treatment of psoriasis (Benezeder et al., 2020) and alopecia areata (Barton et al., 2022; Wu et al., 2018). However, early adverse effects can occur, including burning and irritation of the skin in the perilesional region (van de Kerkhof et al., 2006), as well as erythema, oedema, and itching at the application site, with some reactions peaking after treatment (Swinkels et al., 2002). The mechanisms underlying the adverse effects of dithranol are unclear; it is believed that oxidation of the molecule, generating reactive oxygen species (ROS), may cause damage to biological tissues, leading to intense irritation, a burning sensation, and itching (Savian et al., 2015). Thus, receptors sensitive to ROS could be the molecular targets responsible for dithranol's irritative effects, such as the transient receptor potential vanilloid type 1 (TRPV1) ion channel.

TRPV1 is a sensor for irritating substances (including capsaicin, ROS, and certain drugs) that is highly expressed in sensory neurons; its stimulation, similar to that caused by dithranol, results in a burning sensation and skin irritation (Stanford et al., 2019). TRPV1 is predominantly expressed by a subset of peripheral nerve endings in sensory neurons (TRPV1-positive neurons) that detect pain and pruritus and induce neurogenic inflammation (Caterina and Pang, 2016; Cevikbas et al., 2014; Wilzopolski et al., 2021). In addition to sensory neurons, TRPV1 can also be expressed by various skin cells, including

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Abbreviations

ANOVA	Analysis of Variance
B	Baseline
CEUA	Ethics Committee for the Use of Animals
ARRIVE	Animal in Research Reporting in vivo Experiments
DRG	Dorsal Root Ganglion
4-TERT	4-tert-butylcyclohexanol
μg	micrograms
DTN	dithranol
CAP	capsaicin
NCBI	National Center for Biotechnology Information
Actb	actin beta
Hprt	hypoxanthine phosphoribosyltransferase 1
Calca	calcitonin-related polypeptide alpha
S.E.M	Standard Error of the Mean
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid 1
ROS	reactive oxygen species

keratinocytes, mast cells, and dendritic cells (Basu and Srivastava, 2005; Ständer et al., 2004). Highlighting its clinical relevance in inflammatory skin diseases, elevated TRPV1 expression has been observed in conditions such as rosacea, atopic dermatitis, and psoriasis (Caterina and Pang, 2016; Nattkemper et al., 2018). Moreover, several drugs used to treat psoriasis, particularly topical treatments, may exert both therapeutic and adverse effects through direct interaction with sensory neurons (Lee et al., 2020) and with TRPV1, including vitamin A and D analogues, capsaicin, salicylates, and calcineurin inhibitors (Kita et al., 2019; Long et al., 2020; Ohta et al., 2009; Yin et al., 2013). Finally, the topical application of TRPV1 antagonists has been shown to reduce inflammatory and sensory symptoms in skin diseases such as rosacea, perioral dermatitis, and sensitive skin (Schoelermann et al., 2016; Srour et al., 2020; Sulzberger et al., 2016). Thus, the aim of the present study was to investigate the role of the TRPV1 receptor in the irritative effect of dithranol in mice.

2. Materials and methods

2.1. Materials

Capsaicin (N-vanillylnonanamide, a TRPV1 agonist), SB366791 and 4-tert-butylcyclohexanol (TRPV1 antagonists), dithranol (anthralin), and salicylic acid were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The powders were solubilised in propylene glycol, purchased from Vetec Química (Duque de Caxias, RJ, Brazil), following pharmacopoeial methodology (w/v). The vehicle cream (Lanette®) used for preparing topical formulations was purchased from a compounding pharmacy (Bio Extract, Florianópolis, SC, Brazil). Creams containing the active ingredients were prepared following pharmacopoeial methodology based on the calculated percentage concentration (%) (w/w). The inhalational anaesthetic isoflurane was obtained from Cristália Produtos Químicos Farmacêuticos (Itapira, SP, Brazil), while ketamine and xylazine were sourced from Syntecvet (Tamboré, SP, Brazil). Primers were purchased from Síntese Biotecnologia (Belo Horizonte, MG, Brazil) under Integrated DNA Technologies. DNase I was obtained from Invitrogen®, the high-capacity cDNA reverse transcription kit from Applied Biosystems®, and the GoTaq® qPCR Master Mix from Promega®.

2.2. Ethical approval, study design, and experimental animals

All experimental procedures were authorised by the Ethics Committee for the Use of Animals of the Federal University of Santa Catarina (Protocol 6048210720) and conducted in accordance with current regulations of CONCEA, CEUA/UFSC, and the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). Several measures were applied to increase reproducibility and minimise experimental bias, including pre-study calculations of the number of animals per experimental group, blinding, and randomisation. Two experimenters (RGS and MAF), aware of the randomisation, applied the treatments, while one blinded experimenter (AMS), unaware of the randomisation, measured the outcomes. Allocation concealment was performed using prior randomisation; numbers were generated online on the Random.org website, assigning treatment groups in experimental blocks. Experiments were conducted in two or three blocks on different days to enhance reproducibility. All experiments took place between 8:00 and 17:00 h.

The established *a priori* exclusion criteria were animals weighing <20 g or showing signs of skin injuries (e.g., scratches, ear peeling). Clinical and behavioural investigation scores were used to assess weight, lethargy, piloerection, tremors, periorbital exudate, respiratory distress, and diarrhoea; these were considered humane outcomes, so removing animals from the experimental blocks was unnecessary.

The sample size (*n*) was calculated from a pilot experiment based on measurements of ear oedema using a digital micrometer as the primary outcome. The calculation was conducted using the mean of the control group to assess a 30% reversal of oedema, yielding an effect size of 1.5, with a significance level of 5%, test power of 95%, and a two-tailed hypothesis test, resulting in a total of 10 animals per group.

The experiments utilised C57BL/6 male and female mice (18–30 g, bred in-house, 8–12 weeks old). Only animals raised and maintained in the vivarium of the UFSC Experimental Pharmacology Laboratory were used. Two to six animals were housed in a micro-isolator rack system in small transparent polypropylene boxes (35 × 20 × 13 cm) with filtered water and *ad libitum* food, under controlled light/dark (12/12 h) and temperature (22 °C ± 2 °C) cycles. PVC tubes (5 cm diameter × 10 cm length) and paper were provided as environmental enrichment.

2.3. Assessment of topical irritation induced by dithranol

Oedema was measured as an increase in ear thickness following the application of chemical irritants (Inoue et al., 1995). To obtain this measurement, ear thickness was recorded before (baseline) and after induction of the inflammatory response using a digital micrometer (Mitutoyo® S-293) in animals anaesthetised with isoflurane. The micrometer was applied near the tip of the ear, distal to the cartilaginous sulcus, and was cleaned between animals. Thickness was recorded in millimeters. To minimise variations, all measurements were performed by a single blinded researcher throughout each experiment.

The level of erythema was assessed using a comparison table of red spots, generating an erythema scale where 0 indicated none; 1, slight; 2, moderate; 3, severe; and 4, very severe, as described by van der Fits et al. (2009).

Von Frey filaments were used to evaluate pruritic behaviour evoked by external touch, as described by Huang et al. (2019). Each mouse was anaesthetised with 2% isoflurane, and the fur behind the ear was carefully shaved. For familiarisation and habituation, the animals were placed on the von Frey platform for 1 h on the day before the start of the test and for 30 min each day during evaluation. A von Frey filament (0.02 g) was applied to the dorsal part of the ear, and the incidence of scratching responses was recorded in 10 trials per animal. The filament was applied with an interval of 1 min between attempts. In the absence of a pruritic response, it was recorded as negative. The movement of withdrawing the filament with the front paw or shaking behaviour immediately upon or following stimulation was not considered a positive response. If 10 positive responses occurred following the first application, the test concluded with a 100% response.

2.4. Determination of the minimum irritating dose of dithranol

To assess the irritative effect of dithranol, the animals were anaesthetised with ketamine (100 mg/kg) and xylazine (10 mg/kg) in 0.9% saline, administered intraperitoneally in the lower left quadrant of the abdomen. Anaesthesia allowed dithranol treatment for 30 min, followed by removal, and prevented the animals from spreading the cream to other body parts. The animals were kept on a heating blanket during the dithranol application to prevent possible anaesthetic-induced hypothermia.

Different concentrations of dithranol cream were used to evaluate the minimal dose causing effective biological activity with minimal irritation: a minimum dose of 0.01%/ear, an intermediate dose of 0.1%/ear, and a maximum dose of 0.5%/ear, based on Lowe and Breeding (1981). The cream was applied evenly across the animal's ear in an amount of approximately 6 μg/cm^2 with the aid of a spatula. After 30 min, the drug was removed using cotton wool, water, and soap suitable for animal use (Ashton et al., 1983). In control animals, only the vehicle cream was applied. The development of oedema, erythema, and pruritic behaviour was monitored from 6 h to 6 days after treatment.

2.5. Determination of TRPV1 and inflammatory mediator mRNA levels

Biological samples from the right ear, right auricular lymph node, and cervical dorsal root ganglion (DRG) were collected 24 h and 7 days after dithranol treatment. DRG RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and the PureLink® kit (Invitrogen). For ear and lymph node samples, we used the ReliaPrep™ RNA Tissue MiniPrep System kit (Promega Corporation, Madison, WI, USA). cDNA was synthesised from 180 ng (DRG) and 1000 ng (ear and lymph node) using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA). Quantification of specific products was performed by qPCR using the Power SYBR™ Green PCR Master Mix (Applied Biosystems). Double-stranded products were amplified with specific primers on the StepOne Plus Real-Time PCR System (Applied Biosystems). Relative amounts of target genes were calculated by normalising to the expression of the hypoxanthine phosphoribosyltransferase (*Hprt*) and β-actin (*Actb*) reference genes. RNA levels were compared using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primer specificity in all samples was confirmed by single peak performances of qPCR products during melting curve analysis. RNA expression levels and specific primer sequences are provided in Table 1.

2.6. Defunctionalisation of TRPV1

To induce defunctionalisation of TRPV1-positive skin fibres, animals were pre-treated topically on the right ear on days 1, 3, and 7 with either vehicle cream (control, 6 μg/cm^2) or cream containing 1% capsaicin (6 μg/cm^2), as described by Inoue et al. (1997) with modifications. Eight days after the start of the applications, animals in both groups received treatment with a cream containing dithranol at a concentration of 0.5%, as previously described. The development of oedema, erythema, and pruritic behaviour was then monitored for 6 days (Silva et al., 2011).

2.7. Effects of the combination of dithranol and salicylic acid

Considering that dithranol is commonly combined with salicylic acid in clinical practice (de Mare et al., 1988) and that salicylates can desensitise TRPV1 receptors (Maurer et al., 2014; Ohta et al., 2009), we decided to test a combination of dithranol and salicylic acid. A group of animals was topically treated as described in Section 2.4 with either vehicle cream or cream containing 0.5% dithranol combined with 2% salicylic acid (Benezeder et al., 2020). This treatment was administered only once. Oedema, erythema, and pruritus were evaluated from 2 h to 6 days after treatment.

2.8. Antagonism of TRPV1

Selective antagonists for this receptor were used to assess the involvement of the TRPV1 receptor in dithranol-induced irritation. Animals were pre-treated with 4-tert-butylcyclohexanol 0.4% (Kueper et al., 2010), SB366791 0.04% (Andrade et al., 2008), or vehicle cream. After 1 h, a cream containing 0.5% dithranol was applied topically in all groups. The dithranol treatment protocol was performed as described in Section 2.4. Treatment with antagonists or vehicle was reapplied daily at 24-h intervals, and parameters of oedema and erythema were evaluated from 2 h to 6 days after dithranol treatment, with evaluations conducted 1 h after treatment with antagonists.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0® software (La Jolla, CA, USA). The Grubbs test was applied to detect outliers (Outlier Calculator [graphpad.com]). Data are presented as mean ± standard error of the mean (SEM). Oedema, erythema, and scratching data were analysed by two-way analysis of variance (ANOVA) followed by Šídák's post hoc test, or by three-way ANOVA followed by Tukey's post hoc test to compare males and females. Dose-response results were analysed by one-way ANOVA, followed by Dunnett's post hoc test. Gene expression levels were analysed by multiple t-tests, with data presented as fold change and log-transformed before analysis. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Dithranol 0.5% caused both oedema and erythema

Initially, we determined the minimum irritating doses (the lowest dose that caused statistically significant oedema and erythema) for dithranol at the peak of these effects. Representative photographs of skin irritation treated with dithranol 24 h later at different doses are shown in Fig. 1A. The 0.1% dithranol dose was sufficient to induce statistically significant erythema. By contrast, only the 0.5% dose was able to induce oedema (Fig. 1B), while this higher dose also caused more pronounced erythema (Fig. 1C). None of the tested doses caused touch-evoked scratching in the treated ears (Fig. 1D). Because ear oedema was chosen as our primary outcome (being an objective and quantitative measure), we used the 0.5% dose. We assessed the animals for up to 6 days throughout the study.

Table 1
Forward and reverse primers used for RT-qPCR assays and their respective sequences.

Gene	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Accession number
Actb	CATTGCTGACAGGATGCAGAAGG	TGCTGGAAGGTGGACAGTGAGG	NM_007393
Hprt	GCAGACTTTGCTTTCCTTGG	CAACAACAAACTTGTCTGGA	NM_012583
Calca	GGACTTGGAGACAAACCACCA	GAGAGCAACCAGAGAGGAACTACA	NM_007587.2
S100a8	TGTCCTCAGTTTGTGCAGAATATAAAT	TTTATCACCATCGCAAGGAACTC	NM_013650.2
S100a9	GGCAAAGGCTGTGGGAAGT	CCATTGAGTAAGCCATTCCCTTTA	NM_009114.3
Trpv1	CCCGGAAGACAGATAGCCTGA	TTCAATGGCAATGTGTAATGCTG	NM_001001445.2

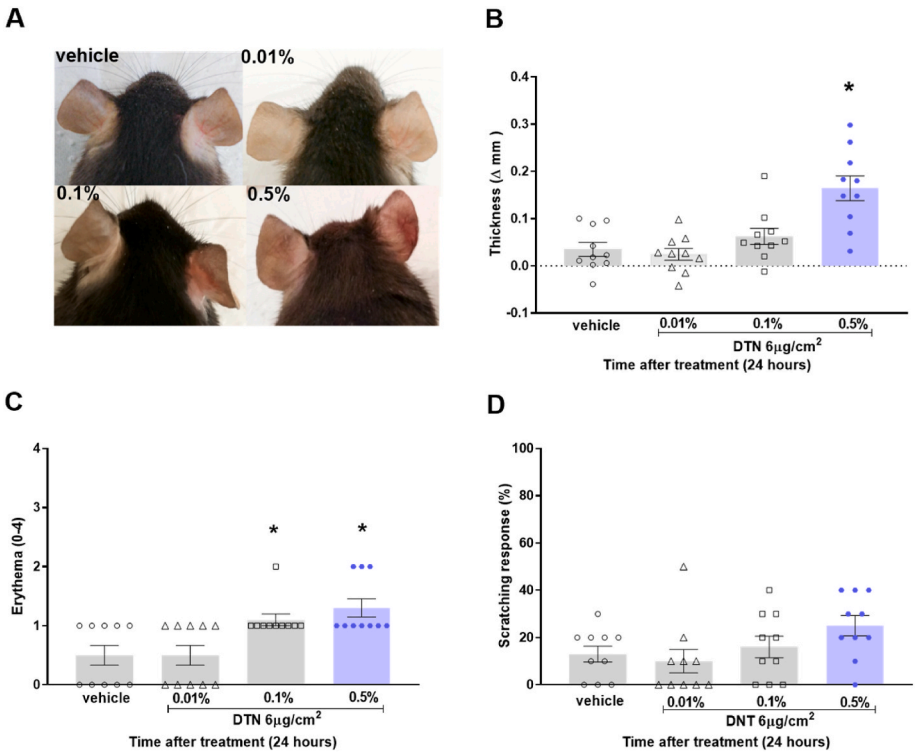

Fig. 1. Determination of minimum irritating dose of dithranol in male mice. Male mice were treated with vehicle cream or cream containing dithranol at concentrations of 0.01%, 0.1%, and 0.5%, applied once for 30 min followed by cleaning ($n = 10$). Photographs representing (A) irritation, (B) oedema, (C) erythema, and (D) pruritus measurements 24 h after treatment are shown. One-way ANOVA followed by Dunnett's post hoc test; $p < 0.05$ compared with the control group. Columns and vertical lines represent the mean ± SEM.

3.2. Dithranol 0.5% caused oedema and erythema in male and female mice

Next, we investigated the temporal course and sexual dimorphism in the irritation caused by a clinically relevant dose of dithranol (0.5%). The experimental design for assessing oedema, erythema, and scratching in male and female mice is shown in Fig. 2A. Representative photographs of skin irritation on the right ear treated with vehicle cream or cream containing 0.5% dithranol at different time points are presented in Fig. 2B. Compared with vehicle cream, topical treatment with 0.5% dithranol caused oedema and erythema (Fig. 2C), but not pruritus (Fig. 2E), beginning at 6 h, peaking at 1 day, and persisting up to 6 days after a single application in male mice (Fig. 2C). In female mice, the 0.5% dithranol cream also induced oedema and erythema, but with a delayed response, becoming significant after 1 day (Fig. 2C–E).

To confirm these findings, we applied a three-way ANOVA with time, treatment, and sex as factors. The interaction of all these factors was significant for oedema (sex × time × treatment, F [7143] = 2.683, $p = 0.01$) but not for erythema (sex × time × treatment, F [7144] = 0.8050, $p = 0.58$). Despite the irritation persisting, we euthanised the animals 6 days after treatment to prevent unnecessary discomfort because the ears showed intense scaling (Fig. 2B). We continued our study by examining skin irritation only in male mice.

3.3. Topical 0.5% dithranol induced an increase in TRPV1 in the lymph node and DRG but not in the skin

We also analysed the expression of mRNA encoding TRPV1 and the antimicrobial peptides S100a8 and S100a9 in the right ear (Fig. 3A and D) and the ipsilateral auricular lymph node (Fig. 3B and E) of animals 24 h and 7 days after treatment with 0.5% dithranol or vehicle. In the DRG, we evaluated the expression of TRPV1 and the neuronal marker *Calca* (Fig. 3C and F). Real-time qPCR analysis revealed that compared with tissues from vehicle-treated animals, dithranol treatment induced an increase in TRPV1 expression in the draining lymph node and DRG at both 24 h and 7 days, without affecting its expression in the skin.

Additionally, there was an increase in mRNA levels of the antimicrobial peptide S100a8 in the ear skin at both 24 h and 7 days post-treatment. The mRNA levels of the neuronal marker *Calca* were detected in the DRG of vehicle-treated animals and remained unchanged following dithranol treatment.

3.4. Topical treatment with capsaicin induced oedema and erythema, but not pruritus

Like dithranol, topical treatment with capsaicin (1%, a TRPV1 receptor agonist) induced oedema and erythema but did not induce pruritus when compared with vehicle-treated animals (Fig. 4B–D). Although capsaicin produced effects similar to dithranol, both oedema and erythema appeared much earlier (within 30 min after application) and were transient, no longer being detectable after 3 h (Fig. 4A–D).

Next, we induced the defunctionalisation of TRPV1-positive fibres by repeated application of capsaicin (Fig. 5A). We found that unlike the first application, both the second and third applications of capsaicin did not induce oedema, erythema, or scratching (Fig. 5B–E).

After pre-treatment with vehicle or capsaicin, the animals were treated with dithranol (Fig. 6). Contrary to our initial hypothesis, animals with TRPV1 fibre defunctionalisation showed no change in the initial phase of skin irritation (between 6 h and 4 days) but exhibited worsened irritation in the late phase (between 5 and 6 days) induced by dithranol.

3.5. The combination of dithranol and salicylic acid increased the intensity of irritation in the late phase

Given that salicylates are commonly combined with dithranol in

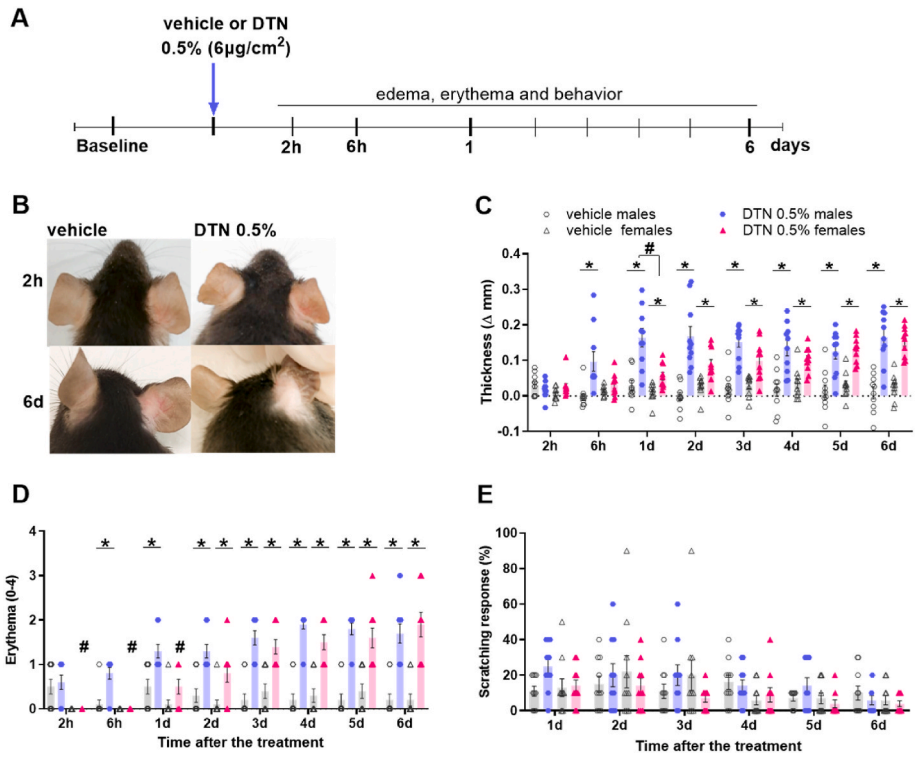

Fig. 2. Time course of skin irritation following 0.5% dithranol application in male and female mice. Male and female mice were treated with either vehicle cream or cream containing 0.5% dithranol ($n = 10$). (A) Animals received a single topical application of vehicle cream or cream containing 0.5% dithranol (6 µg/cm^2) for 30 min, followed by cleaning. Oedema and erythema were evaluated from 2 h to 6 days after treatment. Pruritus was assessed 24 h after treatment. Photographs represent (B) irritation, (C) oedema, and (D) erythema measurements taken 2 h to 6 days after treatment. (E) The incidence of pruritus induced by mechanical stimulation on the treated skin was evaluated from 24 h to 6 days after treatment. Three-way ANOVA (time, treatment, and sex as factors) with repeated measures (time factor), followed by Tukey's post hoc test; $p < 0.05$ compared with the control group and $\#p < 0.05$ when comparing the female 0.5% dithranol group with the male 0.5% dithranol group. Columns and vertical lines represent the mean ± SEM.

clinical practice and can desensitise TRPV1 receptors, we examined the effect of adding salicylate on the irritant potential of our topical formulation (Fig. 7). Unlike dithranol and capsaicin, the application of a cream containing salicylic acid alone (2%) did not induce oedema, erythema, or pruritus at any of the time points analysed. Although the addition of salicylate to dithranol cream did not affect the initial phase (from 6 h to 4 days), the combination of the two agents increased the intensity of irritation in the late phase (5–6 days), an effect similar to that observed following defunctionalisation of TRPV1-positive fibres.

3.6. Treatment with TRPV1 antagonists did not reduce skin irritation caused by dithranol

At last, we evaluated whether treatment with TRPV1 antagonists—SB366791 0.04% and 4-tert-butylcyclohexanol 0.4%—could prevent skin irritation caused by dithranol. No significant differences in oedema were observed in either treatment group (Fig. 8B). Two-way ANOVA indicated that the interaction between time and treatment factors was not significant (F [7126] = 1.186; $p = 0.32$). Similarly, no significant differences in skin erythema were detected in the groups treated with antagonists (Fig. 8C) (F [14,189] = 0.6713; $p > 0.05$).

4. Discussion

Dithranol is recognised as one of the most effective topical treatments for plaque psoriasis and is also relevant in treating alopecia areata (Ngwanya et al., 2017). However, its application is limited by skin irritation, characterised by oedema, erythema, and touch-evoked scratching. The mechanisms that trigger these reactions are not yet fully understood. In our study, we observed that topical application of dithranol to the ears of mice induced acute inflammatory responses, with the development of oedema (minimum dose of 0.5%) and erythema (minimum dose of 0.1%), which worsened in the late phase, 5–6 days after treatment. However, pruritus was not detected.

Our results align with a clinical trial involving 55 patients who used dithranol cream (0.25%–2%) or calcitriol (3 µg/g) to treat plaque psoriasis. This study showed that the most common adverse effect of dithranol was skin irritation, reported by 39 patients (72%) and characterised by erythema, oedema, and pruritus (Hutchinson et al., 2000). Previous studies also corroborate our findings, indicating that dose is a critical factor for both the duration and intensity of the irritation caused by dithranol (Kemény et al., 2002).

In male mice, skin irritation began after 6 h, peaked at 1 day, and remained severe for up to 6 days after treatment. In females, oedema and erythema appeared later, beginning after 1 day. Dithranol is known to be one of the few compounds capable of causing delayed skin irritation in humans and rodents, with a maximal inflammatory response occurring between 24 and 48 h and lasting up to 1 week following a single dose (Juhlin, 1981; Sohl et al., 2022). The mechanism underlying this delayed inflammation is not fully understood. Initially, it was thought to be related to the formation of ROS during dithranol autoxidation (Yamamoto and Nishioka, 2003). Later observations revealed that late-stage necrosis and apoptosis of keratinocytes, melanocytes, and Langerhans cells occur 24 h after dithranol application in healthy human skin (Kanerva, 1990). Correspondingly, *in vitro* studies demonstrated that dithranol induces apoptosis through cytochrome *c* release, caspase activation, and DNA fragmentation, confirming mitochondrially regulated apoptosis in keratinocytes caused by dithranol (George et al., 2013; McGill et al., 2005).

The prolonged irritant effect of topical dithranol has also been

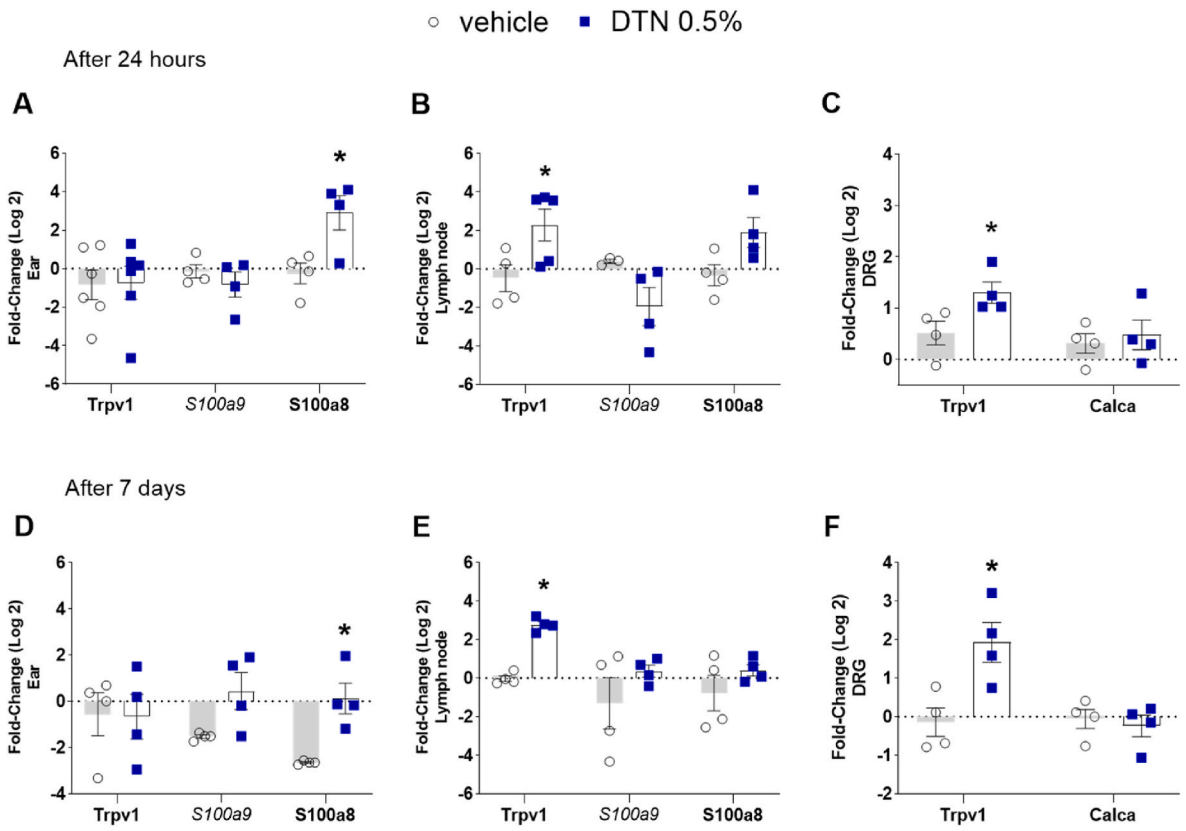

Fig. 3. Evaluation of TRPV1 mRNA levels and inflammatory mediators in mice 24 h and 7 days after treatment with dithranol. The expression of mRNA encoding TRPV1 (*Trpv1*) and antimicrobial peptides (*S100a8* and *S100a9*) was determined in the (A, D) right ear and (B, E) ipsilateral auricular lymph node. (C, F) Additionally, the expression of the neuronal marker (*Calca*) was evaluated in the ipsilateral DRG of animals treated with vehicle cream or cream containing 0.5% dithranol (6 μg/cm^2) 24 h or 7 days after treatment (n = 4–6 per group). Relative expression values ($2^{-\Delta\Delta CT}$) were normalised to the fold change, with the control value set to 1. Holm-Šídák multiple comparison tests; $p < 0.05$ compared with the control group.

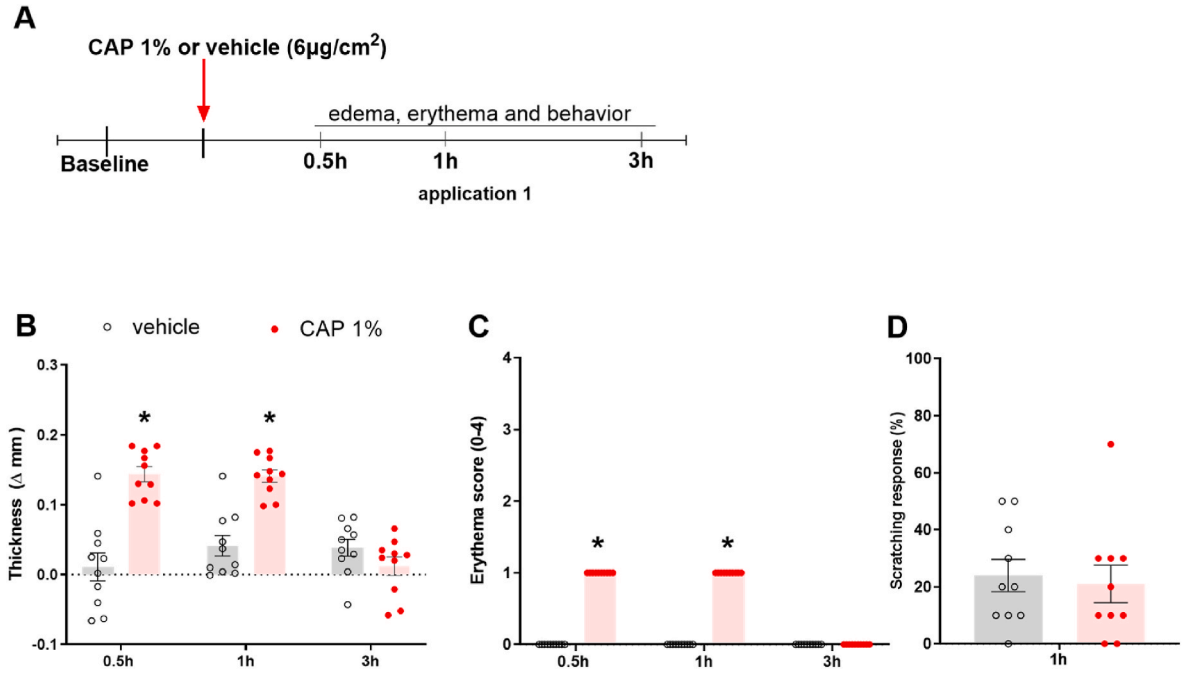

Fig. 4. Ear oedema and erythema following treatment with 1% capsaicin (CAP). (A) Male mice were treated with either vehicle cream or cream containing 1% capsaicin ($n = 10$). (B) Oedema and (C) erythema were measured at 30 min, 1 h, and 3 h after treatment. The incidence of itching induced by mechanical stimulation on the treated skin was evaluated 1 h after treatment (D). Two-way ANOVA with repeated measures, followed by Šídák's post hoc test; $p < 0.05$ compared with the control group. Each column represents the mean ± SEM.

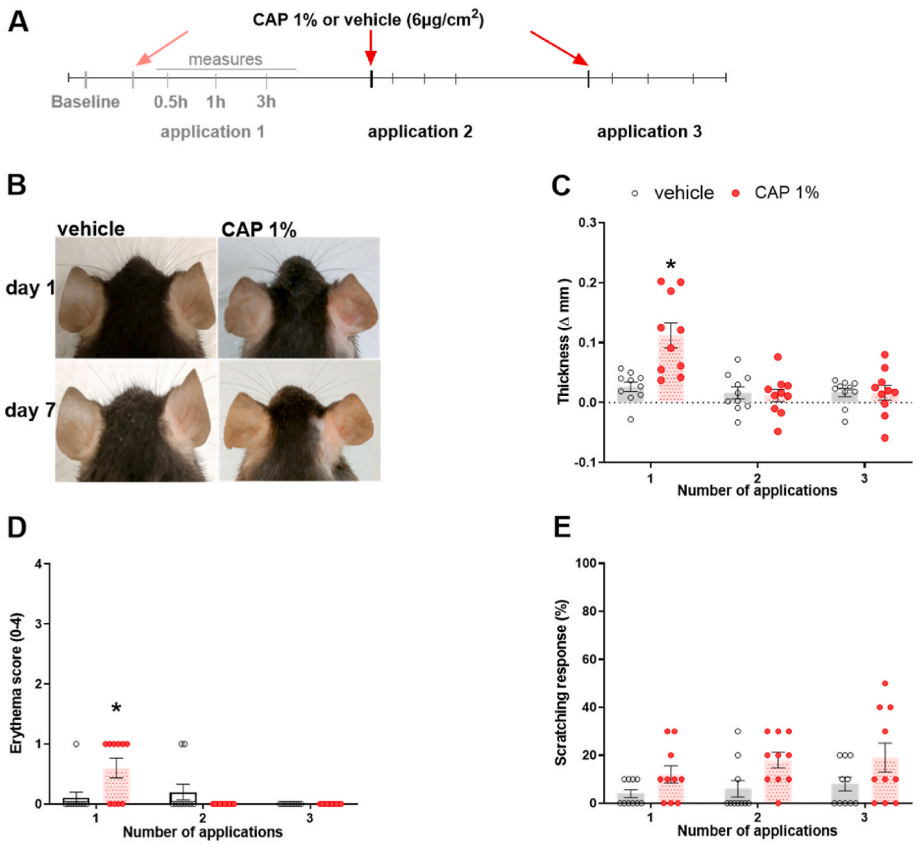

Fig. 5. Effect of repeated topical applications of 1% capsaicin (CAP). (A) To induce defunctionalisation of TRPV1-positive skin fibres, male mice were treated topically on the right ear with vehicle cream or 1% capsaicin three times every other day ($n = 10$). Photographs representing (B) irritation, (C) oedema, (D) erythema, and (E) pruritus measurements were taken 1 h after each treatment. Two-way ANOVA with repeated measures, followed by Šídák's post hoc test; $p < 0.05$ compared with the control group. Columns and vertical lines represent the mean ± SEM.

observed on healthy human skin, lasting up to 8 days and associated with epidermal proliferation, increased T lymphocytes and macrophages, and reduced Langerhans cells (Swinkels et al., 2002). Even after 4–5 days, the increased thickness of the ear may indicate both oedema and hyperplasia (Viluksela, 1991), which aligns with the desquamation observed after 5 days of application.

We tested dithranol in both male and female mice to obtain more reliable results and to validate treatments across sexes (Springate et al., 2017). Regarding sexual dimorphism, a clinical study found no differences between men and women in the intensity of oedema induced 48 h after topical application of dithranol (Lawrence et al., 1986). To the best of our knowledge, no studies have investigated sex as a factor for dithranol-induced irritation in mice. However, several studies have used females to characterise the model (Viluksela and Kosma, 1991).

Next, we investigated the involvement of TRPV1 in the skin irritation produced by dithranol because TRPV1 is clinically relevant to skin inflammatory diseases. In fact, TRPV1 expression is upregulated, and TRPV1 antagonism has been shown to be beneficial in patients with skin inflammatory diseases, including psoriasis, rosacea, and dermatitis (Nattkemper et al., 2018; Schoelermann et al., 2016; Srour et al., 2020). On the other hand, stimulation of TRPV1 by drugs used to treat skin inflammatory diseases, such as retinoids, causes skin irritation as an adverse effect (Yin et al., 2013). Therefore, TRPV1 antagonism could also be clinically beneficial for reducing the adverse effects of some topical anti-inflammatory drugs, such as dithranol.

To investigate the factors mediating dithranol-induced skin inflammation, the expression of mRNAs, including TRPV1, was analysed by real-time qPCR. This revealed that dithranol treatment induced an increase in TRPV1 expression in lymph nodes and the DRG, without changes in the ear skin. TRPV1 mRNA is highly expressed in the cell bodies of DRG sensory neurons, and its expression is increased by peripheral inflammation (Malin et al., 2006; Xu et al., 2009). In this context, increased expression of TRPV1 in the DRG appears to be relevant to neurogenic skin inflammation (Xu et al., 2009) and may amplify skin irritation produced by dithranol. In addition to sensory neurons, TRPV1 can also be expressed (at both the mRNA and protein levels) by various skin cells, especially keratinocytes (Ständer et al., 2004). However, we did not detect changes in TRPV1 mRNA in the skin 24 h or 7 days after dithranol application. Interestingly, we observed an increase in TRPV1 mRNA in the draining lymph node of ears treated with dithranol. A similar increase was reported in the lymph nodes of mice repeatedly treated with the irritating agent formaldehyde (Saito et al., 2011). The sources of this TRPV1 mRNA increase could include dendritic cells, which are known to express this receptor. TRPV1 stimulation is relevant to dendritic cell activation and migration to draining lymph nodes (Basu and Srivastava, 2005). Notably, dithranol application on human skin activates dendritic cells, which are thought to be involved in the late phase of skin irritation (Swinkels et al., 2002). Thus, TRPV1 expression in the draining lymph node could be related to the long-lasting irritation produced by a single application of dithranol in the mouse ear. Moreover, because TRPV1 mRNA is usually efficiently translated into protein in mice (Megat et al., 2019), our findings could be further validated at the protein and functional levels to confirm our hypotheses.

S100 proteins are upregulated in response to injury and play a role in modulating and binding innate and adaptive immune responses (Kennedy-Crispin et al., 2012). We analysed *S100a8* and *S100a9* expression as a positive control to evaluate local tissue damage, as they are recognised as early indicators of cellular stress and tissue damage, making them suitable candidates for the early and sensitive detection of

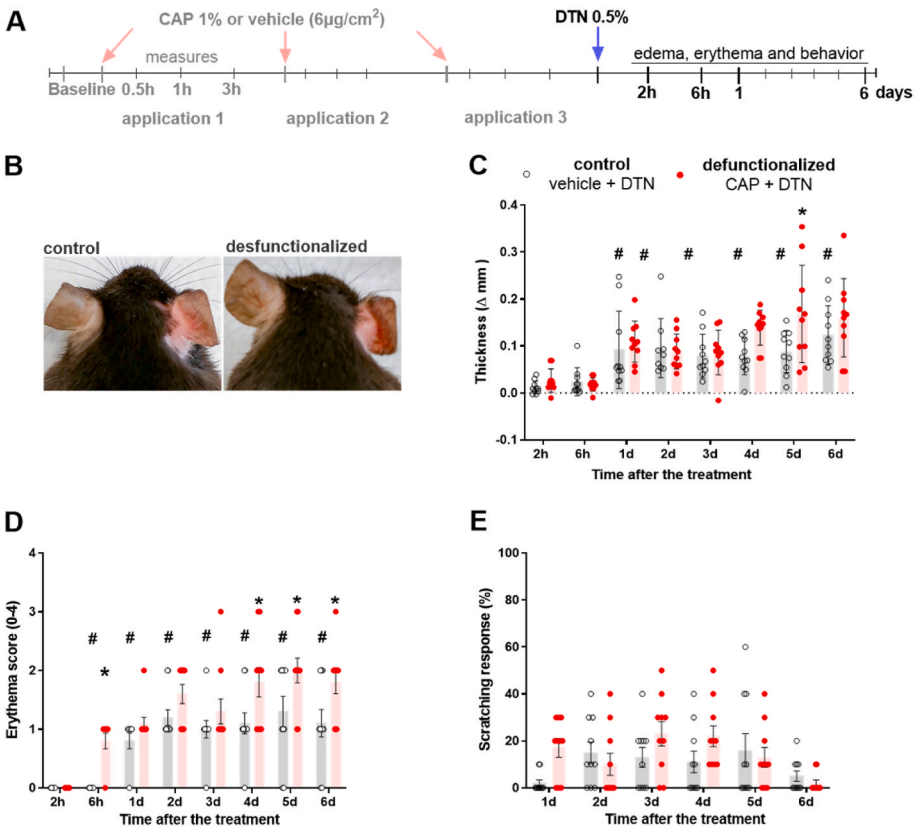

Fig. 6. Treatment with 0.5% dithranol after defunctionalisation of TRPV1+ fibres. (A) All animals were treated with cream containing 0.5% dithranol (6 µg/cm^2) following pre-treatment with either vehicle cream or 1% capsaicin (CAP) ($n = 10$). (B) Representative photos of irritation 6 days after treatment. (C) Oedema and (D) erythema measurements were taken from 2 h to 6 days after treatment. (E) The incidence of pruritus induced by mechanical stimulation on the treated skin was evaluated from 24 h to 6 days after treatment. Two-way ANOVA with repeated measures, followed by Dunnett's post hoc test (vs. 2 h) or Šídák's post hoc test; $p <$ 0.05 compared with the control (vehicle + DTN) group and $\#p < 0.05$ compared with 2 h after treatment. Columns and vertical lines represent the mean ± SEM.

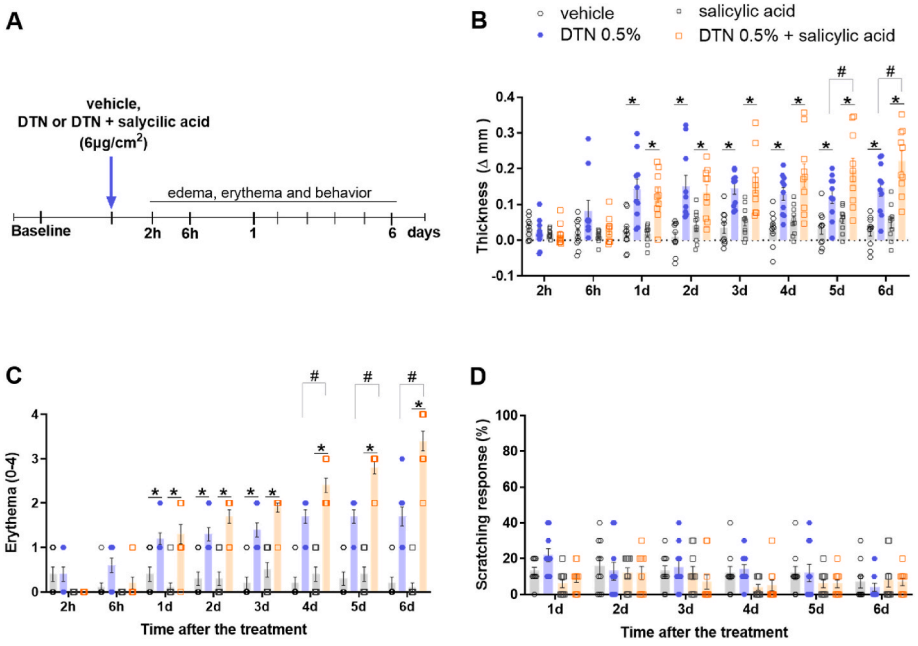

Fig. 7. Treatment with 0.5% dithranol combined with 2% salicylic acid. (A) Male mice were treated with vehicle cream, cream containing 2% salicylic acid, cream containing 2% salicylic acid + 0.5% dithranol, or 0.5% dithranol alone (n = 10). (B) Oedema and (C) erythema measurements were taken from 2 h to 6 days after treatment. (D) The incidence of pruritus induced by mechanical stimulation on the treated skin was evaluated from 24 h to 6 days after treatment. Two-way ANOVA with repeated measures, followed by Tukey's post hoc test; $p < 0.05$ compared with the control group and $\#p < 0.05$ when comparing dithranol vs. salicylic acid + dithranol. Columns and vertical lines represent the mean ± SEM.

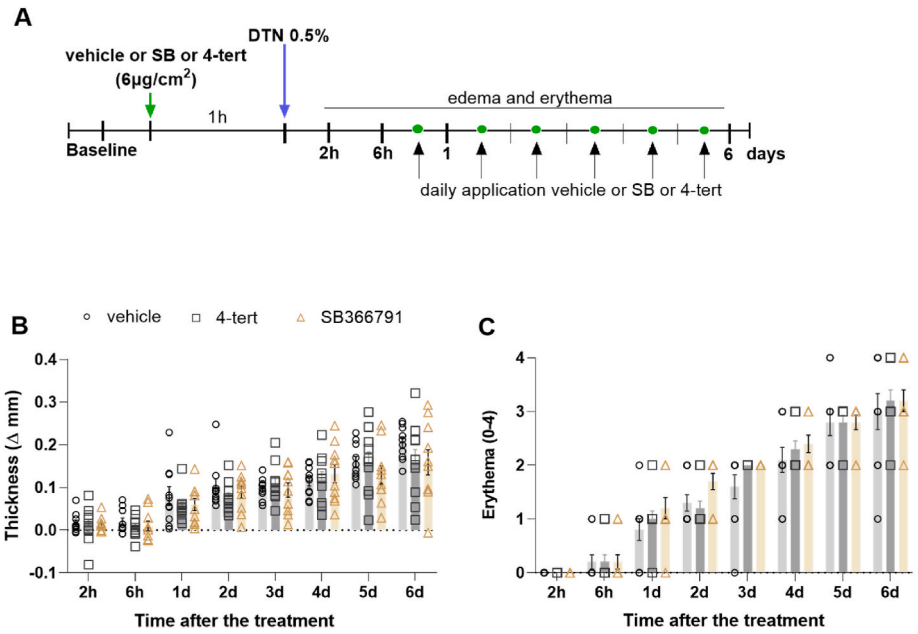

Fig. 8. Treatment with 0.5% dithranol combined with a TRPV1 antagonist. (A) Male mice were pre-treated with vehicle cream or cream containing either 0.04% TRPV1 antagonist SB366791 or 0.4% 4-tert-butylcyclohexanol (6 µg/cm^2). After 1 h, vehicle or 0.5% dithranol was applied topically ($n = 10$). SB366791 or 4-tert-butylcyclohexanol was reapplied daily for 6 days, always 1 h before oedema and erythema measurements. (B) Oedema and (C) erythema measurements were taken from 2 h to 6 days after treatment with dithranol or vehicle. Two-way ANOVA with repeated measures, followed by Šídák's post hoc test. No statistical differences were observed between the groups. Columns and vertical lines represent the mean ± SEM.

developing inflammation (Vogl et al., 2014). Our results show an increased expression of *S100a8* in the skin 24 h and 7 days after dithranol treatment. Similarly, the increase in *S100a8* mRNA in skin treated with dithranol has also been previously observed in human and murine skin (Benezeder et al., 2021).

After the first topical application of a cream containing 1% capsaicin, transient erythema and oedema were observed at 30 min and 1 h but not at 3 h, compared with the vehicle, without inducing pruritus. Our results align with a study by Silva et al. (2011), who observed ear oedema in mice treated with capsaicin diluted in acetone. These findings also support clinical evidence indicating that topical application of capsaicin in healthy subjects results in pain (but not scratching) and erythema, in addition to increasing blood flow at the application site (Mohammadian et al., 1998; Nielsen et al., 2013). Long-term safety evaluations with a patch containing 8% capsaicin in patients with peripheral neuropathic pain also demonstrated that the main adverse effects after treatment were erythema, pain, and swelling at the application site (Simpson et al., 2010).

Repeated application of capsaicin to the skin and other tissues resulted in a gradual insensitivity to capsaicin and other irritants, lasting for days. Subsequent studies in humans revealed that topical applications of capsaicin led to the loss of sensitivity to other chemical stimuli, such as histamine, bradykinin, and menthol (Cliff and Green, 1996; Lo Vecchio et al., 2018; Wallengren and Håkanson, 1992). High doses of topical capsaicin (≥1%) caused a long-lasting loss of sensation that was not readily reversible and could persist for months. This long-term defunctionalisation likely occurs due to the structural ablation of the nerve terminals that express the TRPV1 receptor (TRPV1-positive) (Arora et al., 2021). In our study, as well as in others in the literature (Inoue et al., 1997; Silva et al., 2011), 1% capsaicin did not induce oedema after the second and third applications, indicating the defunctionalisation of TRPV1-positive fibres in the treated skin.

Recently, it was recognised that TRPV1-positive fibres detect noxious stimuli and play a key role in modulating cutaneous immunity through the release of neuropeptides (Baral et al., 2019). In the skin, this subset of neurons can be activated directly by products derived from pathogens (Cohen et al., 2019; Pinho-Ribeiro and Chiu, 2019). Moreover, they play a role in skin inflammatory responses. Our results showed that the defunctionalisation of TRPV1-positive fibres worsened the irritation caused by dithranol, especially in the late phase, suggesting that these fibres may influence skin protection against some irritating substances. Previous studies have already demonstrated a protective role of TRPV1-positive fibres in models of psoriasiform dermatitis (Kemény et al., 2018) and oxazolone-induced allergic contact dermatitis in mouse ears (Bánvölgyi et al., 2005). Furthermore, these fibres exerted a protective role in ear oedema induced by squaric acid dibutyl ester by modulating dermal macrophage function (Feng et al., 2017). However, another study demonstrated that TRPV1 knockout animals had significantly decreased clinical scores in imiquimod-induced psoriasiform dermatitis (Zhou et al., 2018), suggesting that the role of TRPV1 may vary depending on the study model.

Next, we investigated the effect of salicylate on the skin irritation caused by dithranol. We incorporated salicylate into the topical formulation, as it is known in the literature that salicylates can desensitise TRPV1 receptors (Maurer et al., 2014; Ohta et al., 2009). In addition to the deactivation of TRPV1-positive fibres, the incorporation of salicylate (2%) increased the irritation caused by dithranol in the late phase. These results are consistent with clinical studies that demonstrated that adding salicylates does not affect the efficacy of dithranol but increases irritation (de Mare et al., 1988; Prins et al., 1998). Some findings suggest that TRPV1 has an anti-inflammatory effect in contact dermatitis models, with increased TNFα levels in TRPV1 knockout mice (Bánvölgyi et al., 2005). Moreover, TRPV1 exhibits anti-inflammatory properties by inhibiting differentiation, maturation, phagocytosis, and the production of pro-inflammatory cytokines by dendritic cells (Tóth et al., 2009).

Our work also evaluated whether pre-treatment with TRPV1 receptor antagonists, 4-tert-butylcyclohexanol and SB366791, could reduce skin irritation caused by dithranol. We did not observe a reduction in irritation at any of the evaluation times. Clinical studies have found that the topical use of 4-tert-butylcyclohexanol at the same dose used in our study (0.4%) reduced skin irritation induced by capsaicin and some cosmetics (Srour et al., 2020; Sulzberger et al., 2016). However, our results align with a high-throughput screening study that indicated

dithranol was unable to activate TRPV1 expressed in transfected cells *in vitro*, as reported by the National Center for Biotechnology Information (NCBI, 2024). Therefore, contrary to our initial hypothesis, dithranol does not appear to cause skin irritation through the activation of TRPV1.

5. Conclusion

Our results indicate that TRPV1 does not appear to contribute to the initial phase of dithranol-induced irritation and, contrary to our initial hypothesis, may play a protective role against irritation in the late phase. Furthermore, the precise mechanism by which dithranol irritates healthy skin remains unknown, hindering efforts to mitigate this adverse effect.

CRediT authorship contribution statement

Ana Merian da Silva: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Marcella de Amorim Ferreira:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Roberta Giusti Schran:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Debora Denardin Lückemeyer:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Arthur Silveira Prudente:** Writing – review & editing, Writing – original draft, Methodology. **Juliano Ferreira:** Project administration.

Ethics approval

All experimental procedures were authorized by the Ethics Committee for the Use of Animals of the Federal University of Santa Catarina (Protocol 6048210720).

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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Further reading

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