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Oleic acid exhibits an expressive anti-inflammatory effect in croton oil-induced irritant contact dermatitis without the occurrence of toxicological effects in mice

Natháli Schopf Pegoraro [a], Camila Camponogara [a], Letícia Cruz [b], Sara Marchesan Oliveira [a,*]

[a] Graduate Program in Biological Sciences: Biochemistry Toxicology, Center of Natural and Exact Sciences, Federal University of Santa Maria, Santa Maria, RS, Brazil
[b] Graduate Program in Pharmaceutical Sciences, Center of Health Sciences, Federal University of Santa Maria, Santa Maria, RS, Brazil

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ABSTRACT

Ethnopharmacological relevance: Cutaneous inflammatory diseases, such as irritant contact dermatitis, are usually treated with topical corticosteroids, which cause systemic and local adverse effects limiting their use. Thus, the discovery of new therapeutic alternatives able to effectively treat skin inflammatory disorders, without causing adverse effects, is urgently needed.

Aim of the study: To investigate the topical anti-inflammatory effect of oleic acid (OA), a monounsaturated fatty acid, into Pemulen® TR2-based semisolid dosage forms, employing a croton oil-induced irritant contact dermatitis model in mice.

Materials and methods: Male *Swiss* mice were submitted to skin inflammation protocols by acute and repeated applications of croton oil. The anti-inflammatory activity of Pemulen® TR2 hydrogels containing OA was evaluated by assessing oedema, inflammatory cell infiltration, and pro-inflammatory cytokine IL-1β levels. The mechanisms of action of OA were evaluated using cytokine IL-1β application or pretreatment with the glucocorticoid antagonist mifepristone. Possible toxic effects of OA were also assessed.

Results: Pemulen® TR2 3% OA inhibited the acute ear oedema [maximal inhibition (I_{max}) = 76.41 ± 5.69%], similarly to dexamethasone (I_{max} = 84.94 ± 2.16%), and also inhibited ear oedema after repeated croton oil application with I_{max} = 85.75 ± 3.08%, similar to dexamethasone (I_{max} = 81.03 ± 4.66%) on the day 7 of the experiment. Croton oil increased myeloperoxidase activity, which was inhibited by Pemulen® TR2 3% OA (I_{max} = 71.37 ± 10.97%) and by 0.5% dexamethasone (I_{max} = 96.31 ± 3.73%). Pemulen® TR2 3% OA also prevented the increase in pro-inflammatory cytokine IL-1β levels induced by croton oil (I_{max} = 94.18 ± 12.03%), similar to 0.5% dexamethasone (I_{max} = 87.21 ± 10.58%). Besides, both Pemulen® TR2 3% OA and 0.5% dexamethasone inhibited IL-1β-induced ear oedema with an I_{max} of 80.58 ± 2.45% and 77.46 ± 1.92%, respectively. OA and dexamethasone anti-inflammatory effects were prevented by 100% and 91.43 ± 5.43%, respectively, after pretreatment with mifepristone. No adverse effects were related to Pemulen® TR2 3% OA administration.

Conclusions: OA demonstrated anti-inflammatory efficacy similar to dexamethasone, clinically used to treat skin inflammatory conditions, without presenting adverse effects.

Abbreviations: ALT, glutamic pyruvic transaminase; ANOVA, analysis of variance; AST, glutamic oxaloacetic transaminase; CONCEA, Brazilian Council of Animal Experimentation; E_{max}, maximal effect; HTAB, hexadecyltrimethylammonium bromide; ICD, irritant contact dermatitis; I_{max}, maximum inhibition; IL-1β, interleukin 1 beta; MPO, myeloperoxidase; NF-κB, nuclear factor-kappa B; OA, oleic acid; OD, optical density; Pemulen® 0.3% OA, Pemulen® TR2-based semisolid containing 0.3% oleic acid; Pemulen® 1% OA, Pemulen® TR2-based semisolid containing 1% oleic acid; Pemulen® 3% OA, Pemulen® TR2-based semisolid containing 3% oleic acid; s.c., subcutaneous injection; SEM, standard error of the mean; TMB, tetramethylbenzidine; TNF-α, tumour necrosis factor-alpha; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ω-9, omega 9.

* Corresponding author. Department of Biochemistry and Molecular Biology, Federal University of Santa Maria, Av. Roraima 1000, Camobi, Zipcode 97105-900, Santa Maria, RS, Brazil.

E-mail address: saramarchesan@ufsm.br (S.M. Oliveira).

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1. Introduction

The skin tissue consists of the interface between the external and internal environments of the human body. It is composed of a complex structure with multiple cellular and non-cellular components that play the first line of body defence. Due to its role as the organism's interface, the skin is exposed to and affected by different challenges, including chemical, physical, and biological agents that are capable of damaging it. The damage provoked by these agents could evoke inflammatory skin diseases such as irritant contact dermatitis (ICD) (Serhan et al., 2008; Nestle et al., 2009; Eberting et al., 2014; Pasparakis et al., 2014).

ICD is a skin inflammatory response, associated with innate immune system activation in response to contact with an external stimulus that occurs without a prior sensitisation by the external agent (Eberting et al., 2014). This agent can be a caustic or irritating substance or a physical agent whose contact with the skin results in a direct cytotoxic effect, associated with skin barrier disruption, cellular alterations, and the release of pro-inflammatory substances (Eberting et al., 2014).

Nowadays, cutaneous inflammatory disorders as ICD are treated with topical corticosteroids (Frosch and John, 2010; Brasch et al., 2014). However, these drugs can cause severe adverse effects like skin atrophy, rosacea, and purpura development, increased blood glucose levels, and the rebound effect (Coondoo et al., 2014; Barnes et al., 2015), directly compromising their clinical use. These disadvantages reinforce the need for the discovery of new effective therapeutic alternatives to treat these inflammatory skin diseases with less potential to cause adverse effects.

Natural compounds, *e.g.* plant derivatives, have been employed by humans for thousands of years to treat a variety of diseases, including cutaneous disorders. Nowadays, they remain excellent options for medicine development due to the variety of biologically active compounds they contain and the possibility of treatment with fewer adverse effects (Yuan et al., 2016).

Several natural compounds also present effectiveness in treating skin inflammation (Dawid-Pać, 2013;Camponogara et al., 2019a,c). This pharmacological effect is possible due to the ability of these compounds to act through inflammation-associated pathways, reducing the inflammatory process (Calixto et al., 2004; Maione et al., 2015; Arulselvan et al., 2016).

Many essential and widely clinically used drugs were discovered in natural products and based on their popular use, such as morphine, codeine, and acetylsalicylic acid (Calixto, 2019). An example of a medicinal plant is *Cordia verbenacea* A. DC. (Boraginaceae family) (known as 'erva baleeira' or 'maria-milagrosa' or 'whaling herb'), popularly used in Brazil to treat tumours and inflammation. The anti-inflammatory effects of *C. verbenacea* were observed in pre-clinical and clinical studies, attracting the interest of a pharmaceutical company. A semisolid preparation containing *C. verbenacea* essential oil resulted in the development of a topical anti-inflammatory medicine called Acheflan® (Calixto, 2005; Balbani et al., 2009).

A natural compound that attracted our interest was a compound of the omega-9 (ω-9) family. Oleic acid (OA) is a fatty acid naturally found in vegetable oils and foods, such as cod and oilseeds (Roncero et al., 2016; Viola and Viola, 2009). OA also occurs naturally in the human body, as it is part of the cell membrane and participates in hormone synthesis (Tvrzicka et al., 2011).

In Brazil, more specifically, in the rich plant diversity Amazonian region, natives employ several vegetable oils with therapeutic purposes (Burlando and Cornara, 2017). It is important to highlight that literature data report the ethnopharmacological use of vegetable oils containing OA as one of their major components to treat skin diseases as well as for skincare (Burlando and Cornara, 2017). Among these oils, we highlight babassu oil (from *Orbignya phalerata* Mart; Arecaceae family) (Souza et al., 2011), andiroba oil (from *Carapa guianensis* Aubl.; Meliaceae family) (Pesso, 2011), olive oil (from *Olea europaea* L.; Oleaceae family) (Donato-Trancoso et al., 2016), and grape seed oil (from *Vitis vinifera* L.; Vitaceae family) (Shivananda Nayak et al., 2011).

Recently, our research group showed the effective anti-inflammatory action of OA to treat UVB radiation-induced sunburn (Pegoraro et al., 2019). However, neither study demonstrated its effectiveness as a topical anti-inflammatory agent against ICD. In skin inflammatory disorders, such as ICD, oedema is considered the first sign and a classical marker of skin inflammation, resulting in increased vascular permeability and the proliferation of epidermal keratinocytes (Medzhitov, 2008; Xu et al., 2016). Another event that occurs during skin inflammation and that is also a result of increased vascular permeability is leukocyte infiltration to damaged sites (Stanley et al., 1991; Ortega-Gómez et al., 2013; Xu et al., 2016). These processes are the result of the release of inflammatory mediators at the inflamed site after contact with a harmful stimulus, including proinflammatory cytokines [like interleukin 1 beta (IL-1β) and tumour necrosis factor-alpha (TNF-α)], and chemokines (Turner et al., 2014). These mediators are responsible for attracting inflammatory cells to inflamed sites, amplifying and sustaining inflammation (Kolaczkowska and Kubes, 2013).

In this sense, we aimed now to evaluate the anti-inflammatory efficacy of OA by employing an ICD model induced by croton oil. Croton oil, by its main constituent 12-*O*-tetradecanoylphorbol-13-acetate (TPA), has been recognised as a compound able to experimentally induce ICD in rodents, promoting erythema, oedema, and polymorphonuclear leukocyte infiltration (Stanley et al., 1991; Bald et al., 2016; Piana et al., 2016; Camponogara et al., 2019a).

We employed this irritant agent to induce an inflammatory process in the mouse ear, and we evaluated inflammatory markers to assess the efficacy of the natural compound OA in treating cutaneous inflammatory disorders, such as ICD. Moreover, we investigated possible mechanisms of action and the adverse effects of the OA-containing semisolid dosage form.

2. Material and methods

2.1. Materials

Pemulen® TR2 was donated by Noveon (Cleveland, USA). Oleic acid (OA) (about 78% purity) was obtained from LabSynth (Diadema, Brazil). Croton oil, hexadecyltrimethylammonium bromide (HTAB), tetramethylbenzidine (TMB), and mifepristone were purchased from Sigma Aldrich (São Paulo, Brazil). Dexamethasone acetate was purchased from Nova Derme (Santa Maria, Brazil). Ketamine (Dopalen®) and xylazine (Anasedan®) were purchased from Ceva (Paulínia, Brazil). Formaldehyde, ethanol, sodium citrate, acetone, and acetic acid were purchased from Vetec (Rio de Janeiro, Brazil). Hematoxylin-eosin and paraffin were obtained from Merck (Darmstadt, Germany). Enzyme-linked immunoassay for IL-1β measurement was purchased from Peprotech (São Paulo, Brazil). Laboratory kits for biochemical tests were obtained from Labtest Diagnóstica (Lagoa Santa, Brazil). All other reagents and solvents were of analytical grade and used as received.

3. Methods

3.1. Preparation of semisolid formulations

Semisolid dosage forms containing OA or dexamethasone acetate, as well as the vehicle, were prepared as previously described by Pegoraro et al. (2019).

3.2. Animals

Male *Swiss* mice (25–30 g; 4–5 weeks of age) were produced and provided by the Federal University of Santa Maria and used in all experiments. Animals were kept in suitable cages, under controlled temperature (22 ± 2 °C) and a 12 h light-dark cycle, and fed with standard laboratory food and water *ad libitum*. The animals were acclimatised to the experimental room for at least 1 h before performing the

experiments. All experiments were carried out between 8:00 a.m. and 5:00 p.m., and they were performed following national legislation (Guidelines of Brazilian Council of Animal Experimentation – CONCEA) and followed the Animal Research: Reporting *In Vivo* Experiments ARRIVE guidelines (McGrath and Lilley, 2015) and the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals – PHS Policy). All conducted procedures were approved by the Institutional Committee for Animal Care and Use of the Federal University of Santa Maria (protocol numbers 7412190319/2019 and 5582261018/2018). The number of animals and the intensity of stimuli were the minimum necessary to demonstrate the consistent effects of treatments.

3.2.1. Acute croton oil application-induced irritant contact dermatitis

Acute ear oedema was induced by a croton oil single topical application (1 mg/ear dissolved in acetone; 20 μL/ear) given in the right mouse ear. After croton oil application, mice's ears were topically treated with the semisolid developed formulations or dexamethasone (0.5%; employed as positive control). Ear thickness was measured before and 6 h after the croton oil or croton oil plus treatment application. Next, mice were euthanised to collect ear biopsies for further analysis (Brum et al., 2016; Piana et al., 2016; Rigon et al., 2019).

3.2.2. Repeated croton oil application-induced irritant contact dermatitis

Skin inflammation by croton oil multiple topical applications (0.4 mg/ear) in the mouse right ear, on days 1, 3, and 5 of the experimental protocol was also induced. Topical treatments with semisolid dosage forms were applied to the same ear twice a day from the day 5 until the day 9 of the experimental protocol. The ear thickness was measured once a day, during the experimental period. On the last day of the experiment (day 9), the animals were euthanised, and ear biopsies were collected for further analysis (Horinouchi et al., 2013; Camponogara et al., 2019a,b).

3.2.3. Formulation administration and experimental design

Mice were divided in eight groups containing seven animals each and classified as it follows: naïve; croton oil (1 mg/ear); croton oil + Pemulen® TR2 vehicle; croton oil + Pemulen® TR2 0.3% OA; croton oil + Pemulen® TR2 1% OA; croton oil + Pemulen® TR2 3% OA; croton oil + 0.5% dexamethasone acetate (positive control). Topical treatments (15 mg/ear) were applied in the mouse ear after croton oil application, according to the experimental groups described above.

3.2.4. Ear oedema measurement

Mouse ear oedema was evaluated through the measurement of the ear thickness, before (basal measure) and after the croton oil application. An increase in ear thickness after croton oil application when compared to the basal value was considered as indicative of ear oedema. The ear thickness was evaluated using a digital micrometer (Digimess, São Paulo, Brazil) in animals previously anaesthetised, as described previously (Silva et al., 2011; Pegoraro et al., 2017, 2019). Ear thickness was expressed in μm, as the difference between basal thickness and ear thickness at every time point. A single investigator performed all the measurements to minimize the variation.

3.2.5. Assessment of inflammatory cells infiltration

3.2.5.1. Myeloperoxidase (MPO) activity measurement. The inflammatory cells infiltration to the inflamed tissue was evaluated from MPO activity determination since its measure is directly related to the number of neutrophils in the tissue. Six hours after the irritant agent and treatment application, ear samples were collected, homogenised in acetate buffer (80 mM, pH 5.4) containing 0.5% HTAB and centrifuged at 16.000×*g* at 4 °C for 30 min, as previously described (Oliveira et al., 2014). Supernatants were incubated with acetate buffer and TMB solution (18.4 nM) at 37 °C for 10 min. Samples were spectrophotometrically analysed at 630 nm and the results were expressed as optical density (OD)/mL of the sample.

3.2.5.2. Histopathological analyses. Complementary to the MPO activity measurement, the leukocytes infiltration to the inflamed tissue was assessed by histopathological analyses. Separate groups of mice were used to evaluate histopathological changes into ear tissue at 6 h after receiving the croton oil or croton oil plus topical treatment application. Six hours after ear oedema assessment, mice were euthanised, and the right ear was collected and fixed in Alfac solution (16:2:1 mixture of ethanol 80%, formaldehyde 40%, and acetic acid). The samples were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin. A representative area was selected and the quantitative analysis of the number of leukocytes was performed using 20x objectives (Piana et al., 2016) and qualitative histopathological changes (hydropic swelling, exocytosis, spongiosis, and spongiotic vesicles) were assessed from 40x objectives (Willis, 2006; Park et al., 2011). These analyses were performed blindly to minimize the source of bias. The leukocyte quantification was performed by counting the cells per field using the Image J software, and 3 fields from 6 distinct histological slides of each group were analysed (Camponogara et al., 2019a,b).

3.2.6. Interleukin (IL)-1β pro-inflammatory cytokine levels

Six hours after the croton oil application, animals were euthanised, and the right ear was collected and prepared according to Walker et al. (2013). After centrifugation, the supernatant obtained was used to determine the IL-1β levels using an enzyme-linked immunoassay kit (Peprotech®, Brazil). The results were expressed as ng cytokine/mL of sample.

3.2.7. Skin inflammation model induced by IL-1β application

IL-1β (10 ng/ear; 20 μL acetone) was topically applied in the mice's right ear to cause acute ear oedema. The topical treatments were administrated immediately after the IL-1β application. The ear oedema was measured as described above, before and at 1 h after ear oedema induction and topical treatment application (Camponogara et al., 2019a, b).

3.2.8. Oleic acid anti-inflammatory activity via glucocorticoid receptors

We also verified if the OA anti-inflammatory activity is dependent on the glucocorticoid receptors. Animals were pre-treated with a glucocorticoid receptor antagonist, mifepristone (50 mg/kg; s. c.; dissolved in saline containing 10% ethanol) 15 min before the croton oil administration and topical treatments. Ear thickness was measured before and at 6 h after receiving croton oil. The ear oedema was expressed in μm, as described above (Mendes et al., 2016; Camponogara et al., 2019b; Pegoraro et al., 2019).

3.2.9. Biochemical markers of toxicity

Alanine transaminase (ALT) and aspartate transaminase (AST) activities, glucose, urea, and creatinine levels were employed as indicators of hepatic, pancreatic, and renal alterations, respectively (Badalov et al., 2007; Ozer et al., 2008; Van Meer et al., 2014; McGill, 2016; Chu et al., 2016; Sharma et al., 2020). On the day 9 after the repeated application of croton oil or croton oil plus topical treatments, the animals were euthanised, and blood samples were collected by the cardiac punch. Blood samples were centrifuged at 3.000 rpm for 10 min to obtain the serum. AST and ALT activities and the glucose, urea, and creatinine serum levels were assessed by spectrophotometry using Labtest® kits according to the manufacturer's specifications (Labtest Diagnostica, Brazil) (Camponogara et al., 2019a).

3.3. Statistical analysis

The results are presented as the mean + standard error of the mean (SEM) and are reported as geometric means plus its respective 95% confidence limits. The maximum inhibitory effect (I_{max}) was calculated based on the response of the control groups, considered as 100% of the effect. Statistical significance between groups was assessed by one-way or two-way (repeated measures) analysis of variance (ANOVA) followed by Tukey's and Dunnett's post hoc tests. P-values less than 0.05 ($p < 0.05$) were considered as indicative of significance. All statistical tests were carried out using GraphPad Prism 6.00 Software (San Diego, USA).

4. Results

4.1. Oleic acid reduces the croton oil-induced acute ear oedema and inflammatory cells infiltration

Croton oil increased the mice's ear thickness with a maximum effect (E_{max}) of 87 ± 6 μm when compared to the naïve group. Pemulen® TR2-based semisolids containing OA at 0.3% and 1% reduced acute ear oedema with an I_{max} of 36.59 ± 5.81% and 50.64 ± 5.83%, respectively. Dexamethasone acetate (0.5%), inhibited the acute ear oedema with an I_{max} of 84.94 ± 2.16%. The inhibitory effect showed by Pemulen® TR2 3% OA (I_{max} = 76.41 ± 5.69%) was similar to that presented by the positive control (Fig. 1).

Additionally, we verified if the topical treatments can reduce the inflammatory cells infiltration to the injured tissue through the MPO enzyme activity measurement. The croton oil increased the neutrophils infiltration to the ear tissue evaluated at 6 h after its administration when compared to the naïve group. Topical treatments with Pemulen® TR2-based semisolids containing OA at 0.3%, 1%, and 3% reduced the MPO activity with an I_{max} of 42.89 ± 12.33%, 78.57 ± 4.50%, and 71.37 ± 10.97%, respectively, while 0.5% dexamethasone acetate reduced this parameter by 96.30 ± 3.73% (Fig. 2). No statistical difference was verified when comparing topical treatment with semisolids containing the three oleic acid concentrations with dexamethasone acetate.

We also demonstrated the reduction of leukocytes infiltration into the damaged tissue employing histological counting evaluated at 6 h after the croton oil administration (Fig. 3). We confirmed that the topical treatments with oleic acid-containing semisolid dosage forms reduced the leukocytes infiltration to the inflammatory site. Croton oil increased the number of leukocytes (70 ± 6 cells per field) when compared to the naïve group (34 ± 6 cells per field). Pemulen® TR2 3% OA inhibited this inflammatory signal with higher I_{max} (100%) than 0.5% dexamethasone acetate (I_{max} = 95.82 ± 9.37%); however, there was no statistical significance between both experimental groups. Also, no statistical difference was observed when comparing the lower oleic acid concentrations (Pemulen® TR2 0.3% OA and Pemulen® TR2 1% OA) with the dexamethasone acetate group.

The histopathological features were also assessed by histological analysis in mouse ear at 6 h after croton oil application or croton oil plus treatments. Qualitative tissue changes croton oil-induced are presented in Fig. 4. Hydropic swelling, exocytosis, spongiosis, and spongiotic vesicles were observed in mouse ear tissue after croton oil application.

4.2. Oleic acid reduces ear oedema induced by repeated application of croton oil

Besides the acute ear oedema model induced by croton oil, we evaluated the anti-oedematogenic effect of OA in reducing the oedema induced by multiple croton oil applications. Multiple topical

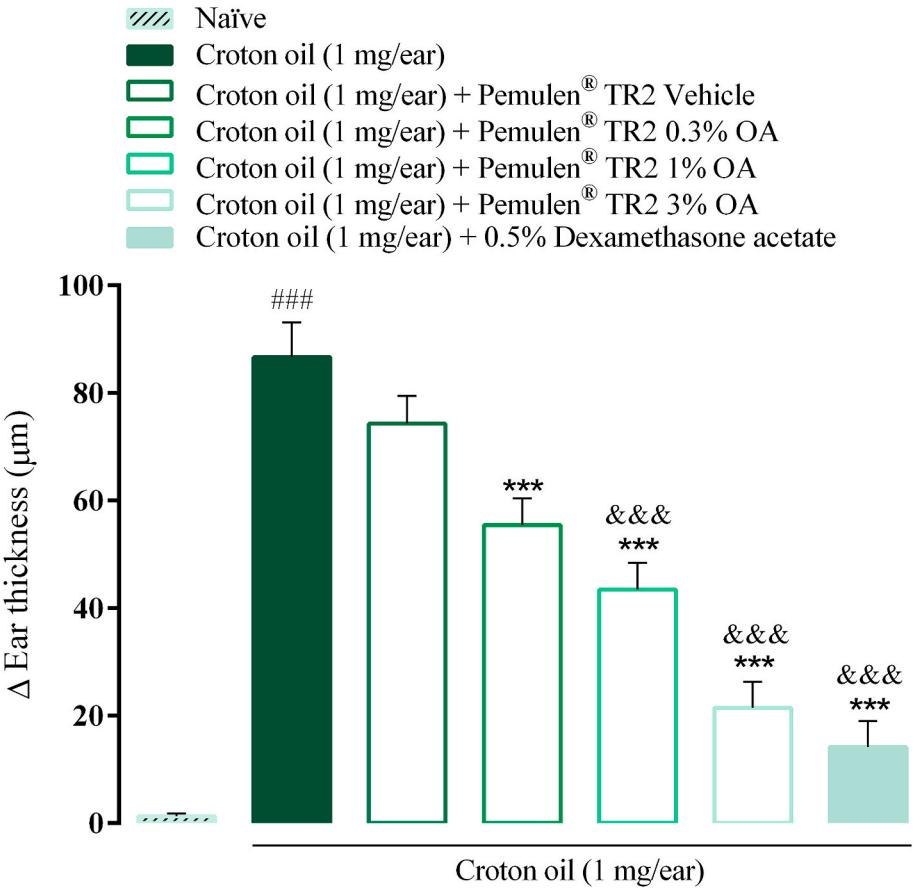

Fig. 1. Anti-oedematogenic effect of semisolid dosage forms containing oleic acid (0.3–3%) and dexamethasone acetate (0.5%) topically applied (15 mg/ear) on the acute croton oil application-induced irritant contact dermatitis in mice. All formulations were topically applied (15 mg/ear) immediately after mouse received croton oil. Ear thickness was measured at 6 h after croton oil or croton oil plus topical treatment application. Each bar represents the mean + SEM ($n = 7$); $^{###}p < 0.001$ shows a significant difference when compared to the naïve group; $^{***}p < 0.001$ shows a significant difference when compared to the croton oil group; $^{\&\&\&}p < 0.001$ shows a significant difference when compared to the Pemulen® TR2 vehicle group. One-way ANOVA followed by post hoc Tukey's test.

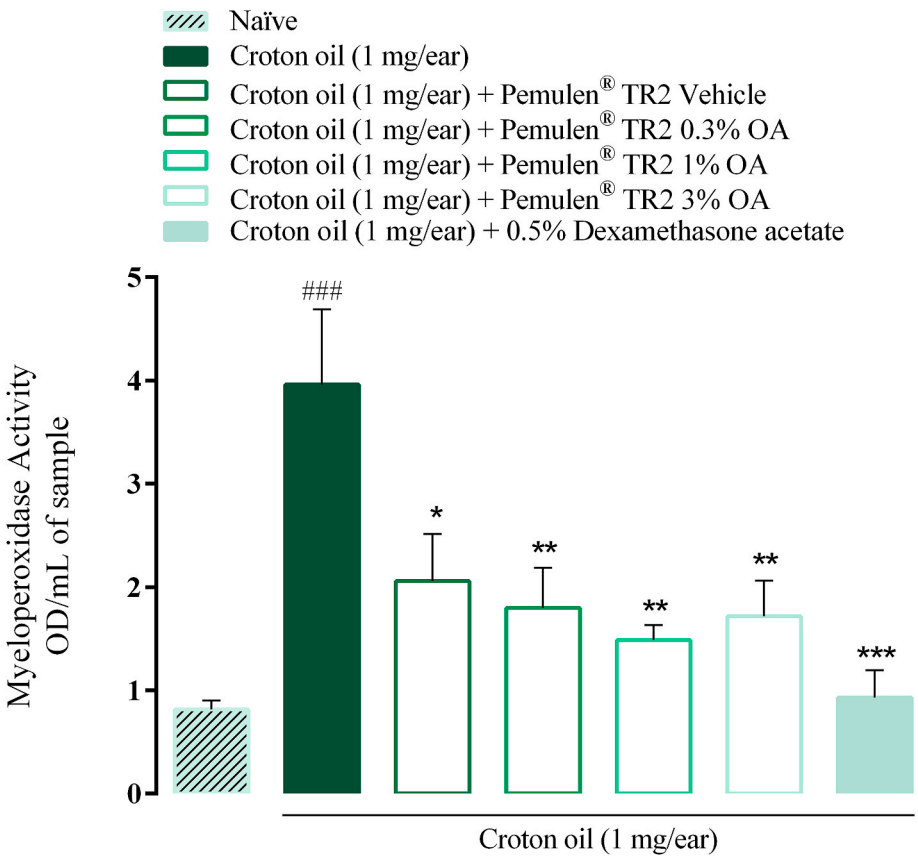

Fig. 2. Effect of semisolid dosage forms containing oleic acid (0.3–3%) and dexamethasone acetate (0.5%) topically applied (15 mg/ear) on the MPO enzyme activity after acute croton oil application-induced irritant contact dermatitis in mice. MPO activity was measured at 6 h after croton oil and the croton oil plus treatment application. Each bar represents the mean + SEM ($n = 7$). $^{###}p < 0.001$ indicates a significant difference when compared to the naïve group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ denotes significant difference when compared with the croton oil group. One-way ANOVA followed by post hoc Tukey's test.

applications of croton oil increased the ear thickness with E_{max} of 227 ± 24 μm on the day 6 of the experiment when compared to the naïve group. Pemulen® TR2 3% OA inhibited the croton oil-induced ear oedema from the day 6 until day 9 (last day) of the experiment, with an I_{max} of 85.75 ± 3.08% on the day 7. Similarly, 0.5% dexamethasone acetate inhibited the ear oedema from the day 6 up to the day 9 of the experiment with an I_{max} of 81.03 ± 4.66% on the day 7 (Fig. 5).

4.3. Oleic acid effectively reduces the croton oil-induced increase on IL-1β cytokine levels and the ear oedema IL-1β-induced

The croton oil (1 mg/ear) increased the cytokine IL-1β levels in mouse inflamed ear when compared to the naïve group. Effectively, Pemulen® TR2 3% OA reduced the IL-1β levels with an I_{max} of 94.18 ± 12.03%, which was similar to that showed by 0.5% dexamethasone acetate (I_{max} = 87.21 ± 10.58%) (Fig. 6).

Since IL-1β tissue levels was reduced by OA topical treatment, this inflammatory cytokine was employed to induce an acute inflammatory process in mouse skin. Topical IL-1β application promoted a skin inflammation evidenced by local oedema. IL-1β increased mice's ear thickness with an E_{max} of 73 ± 4 μm at 1 h after its application. Pemulen® TR2 3% OA reduced the IL-1β-induced ear oedema with an I_{max} of 80.58 ± 2.45%, which was similar to that presented by the positive control 0.5% dexamethasone acetate (I_{max} = 77.46 ± 1.92%) (Fig. 7).

4.4. Oleic acid anti-inflammatory effect is glucocorticoid receptor-dependent

Croton oil caused ear oedema, which did not alter by the pretreatment with the glucocorticoid antagonist mifepristone. Pemulen® TR2 3% OA and 0.5% dexamethasone acetate reduced the croton oil-induced ear oedema with an I_{max} of 84.72 ± 4.93% and 83.51 ± 5.69%, respectively. Mifepristone prevented both Pemulen® TR2 3% OA and 0.5% dexamethasone anti-oedematogenic effect by 100% and 91.43 ± 5.43%, respectively (Fig. 8).

4.5. Oleic acid into semisolid dosage forms does not cause adverse effects in vivo

We also investigated if OA leads to the occurrence of some adverse effects after nine days of its repeated application. Initially, we observed that none of the treatments caused mice body weight loss (data not shown) neither behavioural alterations as immobility nor in the locomotion pattern.

Neither Pemulen® TR2 3% OA nor 0.5% dexamethasone altered AST and ALT enzymes activities and urea and creatinine levels, used as toxicity indicators. On the other hand, Pemulen® TR2 vehicle and 0.5% dexamethasone acetate increased the blood glucose levels when compared to the naïve group. Importantly, increased blood glucose levels were not observed in animals topically treated with Pemulen® TR2 3% AO (Table 1).

5. Discussion

The human skin provides defence against the environment due to its anatomical location at the interface between the human body and the outside environment (Pasparakis et al., 2014). The human skin has a well-developed immune system that acts with coordinated mechanisms to respond to harmful stimuli and restore skin homeostasis after an injury (Nestle et al., 2009; Kabashima et al., 2019).

Skin exposure to irritant agents leads to an inflammatory state, which is a body protective reaction aiming to eliminate the inciting stimulus, resulting in tissue repair/healing (Fullerton and Gilroy, 2016). This inflammatory state is characterised by the development of inflammatory signs like erythema, oedema, heat, and pain; another sign that can occur is the loss of function of the affected tissue/limb

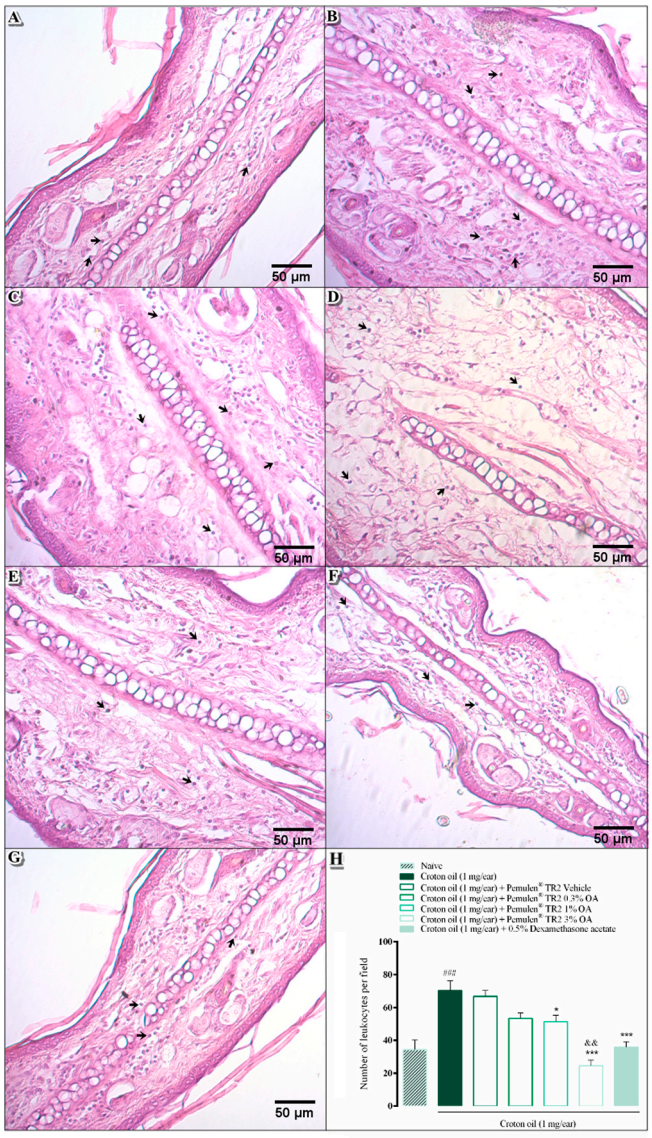

Fig. 3. Effect of the semisolid dosage forms containing oleic acid (0.3–3%) and dexamethasone acetate (0.5%) (15 mg/ear) on acute croton oil application-induced irritant contact dermatitis in mice. Histological changes (A-H; hematoxylin-eosin 10x objectives) of the ear tissue of mice at 6 h after croton oil or croton oil plus treatments. A: naïve; B: croton oil (1 mg/ear) (no treatment); C: croton oil (1 mg/ear) + Pemulen® TR2 vehicle; D: croton oil (1 mg/ear) + Pemulen® TR2 0.3% OA; E: croton oil (1 mg/ear) + Pemulen® TR2 1% OA; F: croton oil (1 mg/ear) + Pemulen® TR2 3% OA; G: croton oil (1 mg/ear) + 0.5% Dexamethasone acetate; H: number of leukocytes per field. The arrows indicate the presence of leukocytes in the ear tissue. Scale bar of 50 μm. Each bar represents the mean + SEM ($n = 7$). $^{###}p < 0.001$ shows a significant difference when compared to the naïve group; $^{*}p < 0.05$ and $^{***}p < 0.001$ show a significant difference when compared to the croton oil group; $^{\&\&}p < 0.01$ indicates significant difference when compared to the Pemulen® TR2 1% OA group. One-way ANOVA followed by post hoc Tukey's test.

(Fullerton and Gilroy, 2016). These inflammatory signals result in the release of several soluble mediators by skin resident cells, *e.g.* cytokines, chemokines, vasoactive amines, and complement system proteins, among others. These mediators evoke alterations to the local vasculature and lead to an increase in blood flow, fluid leakage, and circulating cell infiltration into adjacent tissue, amplifying the inflammatory process (Fullerton and Gilroy, 2016; Schwager and Detmar, 2019).

The therapies available to treat skin inflammatory disorders such as ICD are limited and promote several local and systemic adverse effects, compromising their clinical use. One example is the topical glucocorticoid dexamethasone, an effective drug to treat skin inflammation whose therapeutic use is associated with increased blood pressure and glucose levels, delayed wound healing, decreased bone density, and water retention, among others (Poetker and Reh, 2010). In this sense, the search for effective therapeutic alternatives with fewer adverse effects to treat skin inflammation is of fundamental relevance (Camponogara et al., 2019a,c; Pegoraro et al., 2019).

Our research group recently developed Pemulen® TR2-based hydrogels containing oleic acid with efficacy in treating sunburn-induced skin inflammation (Pegoraro et al., 2019). Here, we reinforced this anti-inflammatory effect of oleic acid employing a croton oil-induced ICD model. Croton oil is an irritant agent that has been employed to experimentally induce skin inflammation in rodents. This biological effect is due to its main constituent, TPA, which causes erythema, oedema, and inflammatory cell infiltration, characterising an inflammatory process (Stanley et al., 1991; Bald et al., 2016; Piana et al., 2016).

Oleic acid presented topical anti-inflammatory activity via glucocorticoid receptors, similar to dexamethasone acetate, on croton oil-induced ICD without increasing blood glucose levels, an important adverse effect related to glucocorticoids like dexamethasone (Badalov et al., 2007; Sharma et al., 2020). This discrepancy between OA and dexamethasone is very important especially in the treatment of inflammatory processes in diabetic patients who present increased glucose levels (Tamez-Pérez et al., 2015). Oleic acid could be employed not only to treat sunburn-induced inflammation but also for the treatment of ICD.

Regarding the toxicity markers, both urea and creatinine are renal toxicity markers (Van Meer et al., 2014). Creatinine is an endogenous cation formed in the liver and muscle by a multistep process and eliminated via the kidney by combining glomerular filtration and active transport; it is the most widely used marker to assess renal injury (Van Meer et al., 2014). Elevated serum creatinine levels are associated with renal injury (Chu et al., 2016). However, there is no evidence in the literature that reduced creatinine levels indicate any toxicity parameter. We verified that the creatinine levels in Pemulen® TR2 3% OA-treated animal group was significantly lower than in the naïve group ($p < 0.05$). Our results are in accordance with that obtained in previous studies by Camponogara and co-workers (2019a,b) that also obtained lower creatinine levels in the vehicle group than in the naïve group.

AST and ALT enzymes are liver toxicity markers, and elevated serum AST and ALT levels are associated with liver injury (Ozer et al., 2008; McGill, 2016). Here, we demonstrated that AST and ALT levels did not differ between the naïve and Pemulen® TR2 3% OA groups. Like creatinine levels, lower values of these biomarkers are not considered indicatives of toxicity. Our results are similar to those found by Camponogara and co-workers (2019), which was observed lower ALT serum activity in dexamethasone-treated topically animals (29 ± 9 U/L) than in naïve animals (36 ± 3 U/L). Considering the above, the topical treatment employing the semisolid dosage forms containing OA can be considered safe according to these preliminary toxicological tests. No indication of hepatic or renal toxicity was observed after topical treatment with OA at the employed doses, enabling its repeated use in the clinic.

Our study showed that OA inhibited ear oedema, the first inflammatory sign, after acute and repeated applications of croton oil. These results ensure that this proposed treatment could be employed to treat short and long-term inflammatory processes. Our results are in agreement with literature data, which demonstrate that the anti-oedematogenic effect of vegetable oils may be associated with OA. Lescano et al. (2015) showed that the anti-inflammatory effect of bocaiuva oil [extracted from *Acrocomia aculeata* (Jacq.) Lodd. Ex Mart.; Arecaceae family; known as 'coco-de-espinho'], employing a model of carrageenan-induced paw oedema in *Wistar* rats, may be related to OA, the main constituent of this oil. Further, açaí oil (from *Euterpe oleracea* Mart.; Arecaceae family) presents anti-inflammatory activity in croton

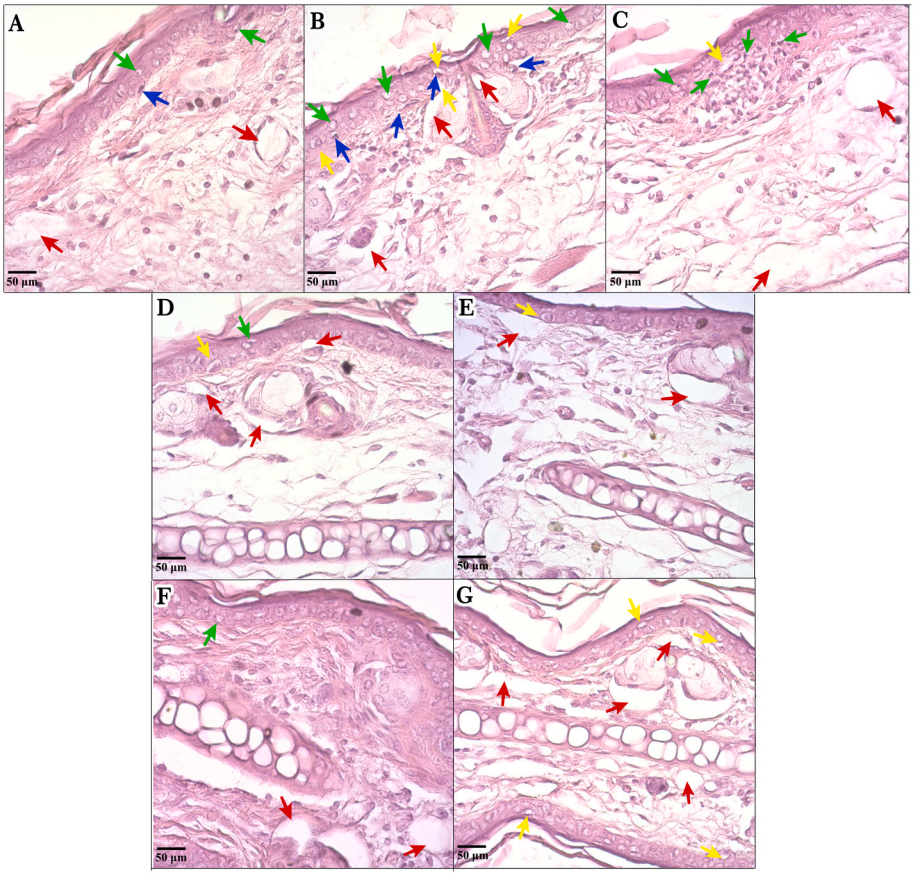

Fig. 4. Qualitative histopathological analyses (A-G; hematoxylin-eosin; 40x objectives) into mice's ears tissue observed in the acute croton oil application-induced irritant contact dermatitis model. Arrows indicate the histopathological changes as follow: green arrows – hydropic swelling (epidermis); blue arrows – exocytosis (epidermis and dermal-epidermal junction); yellow arrows – spongiosis (epidermis); red arrows – spongiotic vesicles (dermis). A: naïve; B: croton oil (1 mg/ear) (no treatment); C: croton oil (1 mg/ear) + Pemulen® TR2 vehicle; D: croton oil (1 mg/ear) + Pemulen® TR2 0.3% OA; E: croton oil (1 mg/ear) + Pemulen® TR2 1% OA; F: croton oil (1 mg/ear) + Pemulen® TR2 3% OA; G: croton oil (1 mg/ear) + 0.5% Dexamethasone acetate. Scale bar, 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

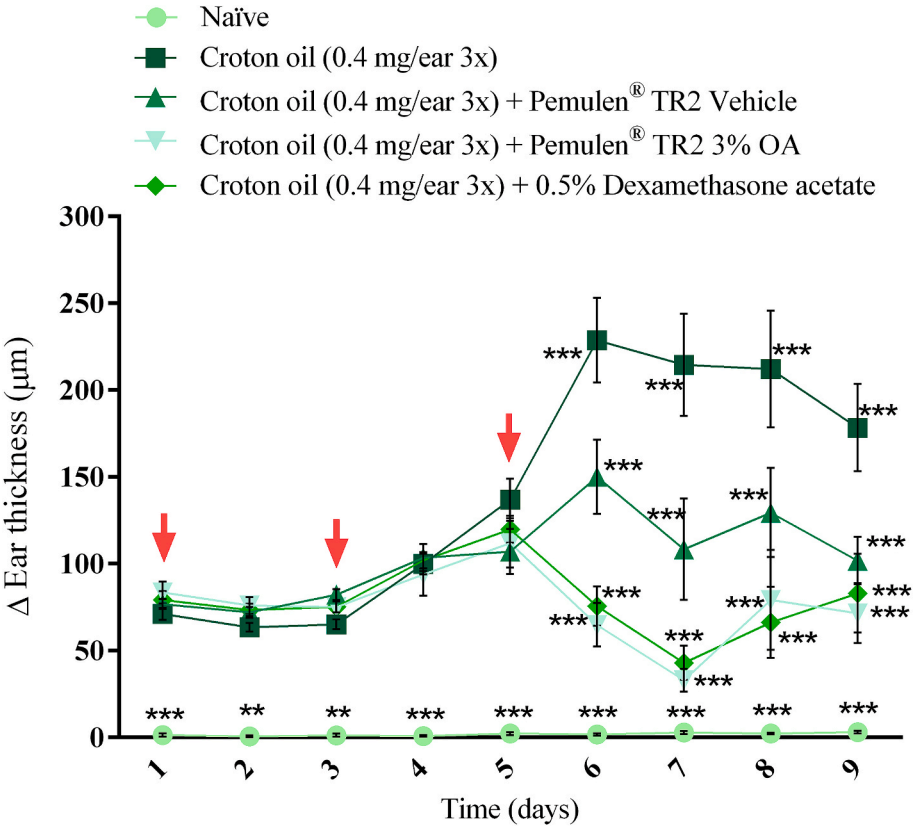

Fig. 5. Anti-oedematogenic effect of semisolid dosage forms containing oleic acid (3%) and dexamethasone acetate (0.5%) topically applied (15 mg/ear) on the ear oedema induced by croton oil repeated application-induced irritant contact dermatitis in mice. Ear oedema was measured once a day for 9 days. The red arrows indicate the days when animals received croton oil administration (0.4 mg/ear). Treatments were topically applied twice a day, starting on day 5 of the experiment. Each line represents the mean + SEM for 7 animals. ***$p < 0.001$ indicates a significant difference when compared with the croton oil group. Two-way (repeated measures) ANOVA followed Dunnett's post hoc test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

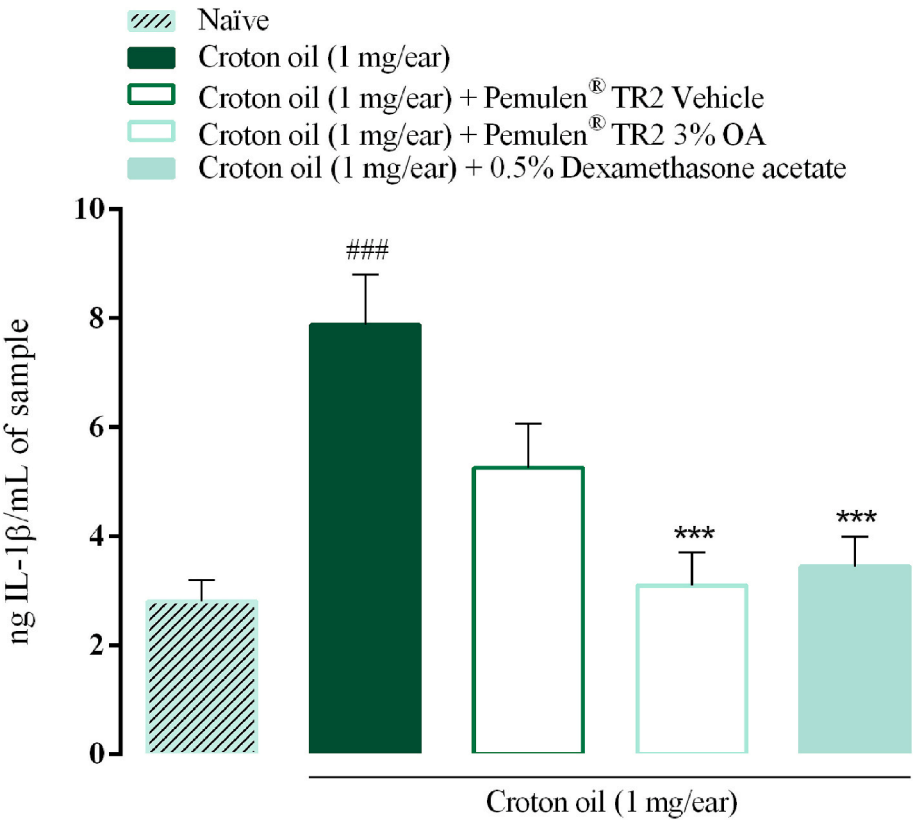

Fig. 6. Effect of the semisolid dosage forms containing oleic acid (3%) and dexamethasone acetate (0.5%) topically applied (15 mg/ear) on the levels of the proinflammatory cytokine IL-1β in the croton oil application-induced irritant contact dermatitis in mice. Each value represents the mean + SEM ($n = 7$). $^{###}p < 0.001$ shows a significant difference when compared to the naïve group; $^{***}p < 0.001$ shows a significant difference when compared to the croton oil group. One-way ANOVA followed by post hoc Tukey's test.

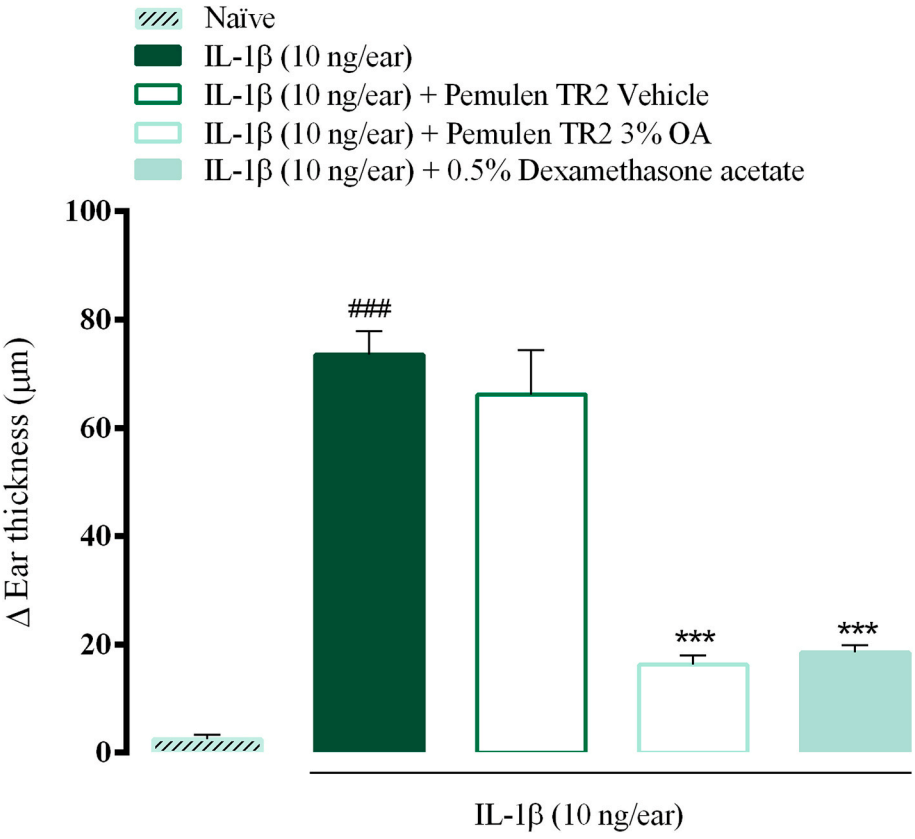

Fig. 7. Anti-oedematogenic effect of the semisolid dosage forms containing oleic acid (3%) and dexamethasone acetate (0.5%) topically applied (15 mg/ear) on the acute ear oedema induced by the IL-1β proinflammatory cytokine. All formulations (15 mg/ear) were topically applied immediately after mice received IL-1β. Ear thickness was measured at 1 h after IL-1β or IL-1β plus treatment application. Each bar represents the mean + SEM ($n = 7$); $^{###}p < 0.001$ shows significant difference when compared to the naïve group; $^{***}p < 0.001$ shows significant difference when compared to the IL-1β group. One-way ANOVA followed by post hoc Tukey's test.

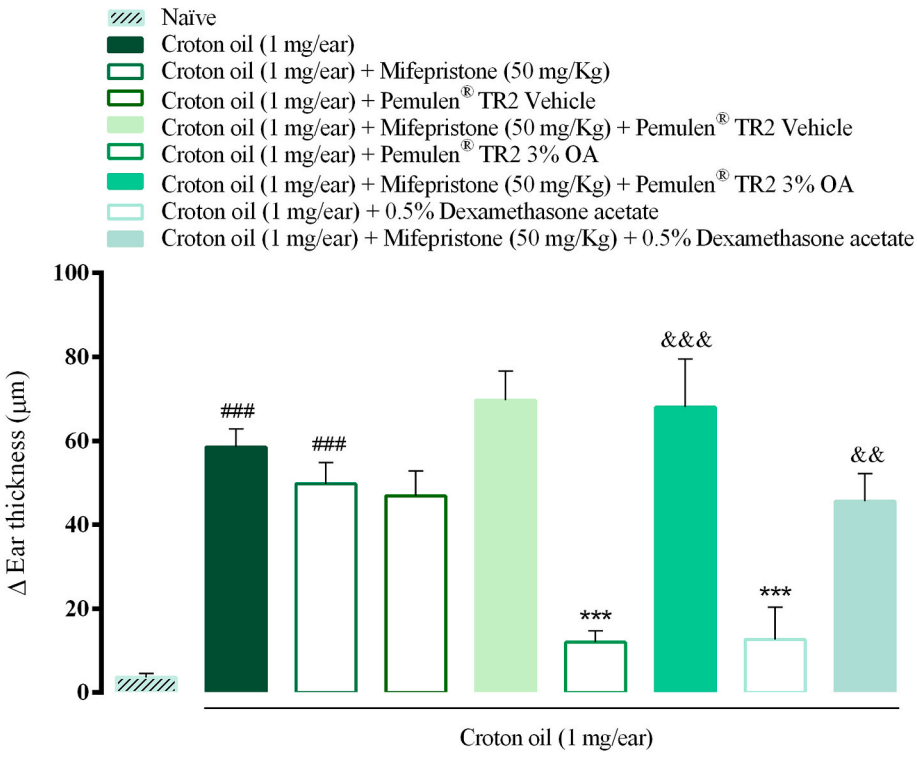

Fig. 8. Reversal of the anti-oedematogenic effect of oleic acid and dexamethasone by the glucocorticoid antagonist mifepristone. Mifepristone (50 mg/kg, s.c.) was administered 15 min before the treatments. Treatments occurred immediately after the topical administration of croton oil. Each bar represents the mean + SEM ($n = 7$); $^{###}p < 0.001$ shows a significant difference when compared to the naïve group; $^{***}p < 0.001$ shows a significant difference when compared to the croton oil group; $^{\&\&}p < 0.01$ and $^{\&\&\&}p < 0.001$ indicate significant difference when compared to the related group that not received the mifepristone. One-way ANOVA followed by post hoc Tukey's test.

Table 1
Effects of oleic acid and dexamethasone-containing semisolid dosage forms on toxicity biochemical parameters.

Experimental groups	ALT (U/L)	AST (U/L)	Urea (mg/mL)	Creatinine (mg/dL)	Glucose (mg/dL)
Naïve	36.88 ± 3.35	22.86 ± 7.16	15.72 ± 4.26	1.56 ± 0.44	72.67 ± 2.80
Croton oil	26.94 ± 2.24	16.59 ± 3.45	14.01 ± 2.22	0.59 ± 0.10	76.97 ± 2.88
Pemulen® TR2 Vehicle	62.76 ± 12.25	13.27 ± 2.08	18.63 ± 0.32	0.86 ± 0.14	103.71 ± 9.94**
Pemulen® TR2 3% AO	28.72 ± 5.67	14.77 ± 1.14	17.00 ± 0.90	0.37 ± 0.13$^{\#}$	81.75 ± 4.89$^{\&\&\&}$
0.5% Dexamethasone acetate	22.01 ± 11.67	13.98 ± 0.90	13.83 ± 2.36	0.53 ± 0.18	138.40 ± 6.09***

Each value represents the mean ± SEM (n = 7). **p < 0.01 and ***p < 0.001 denote significant difference when compared with the naïve group; $^{\&\&\&}$p < 0.001 indicates significant difference when compared to the 0.5% dexamethasone acetate group; $^{\#}$p < 0.05 indicates significant difference when compared to the naïve group. One-way ANOVA followed by post hoc Tukey's test.

oil-induced irritant dermatitis, which may be attributed to the presence of OA, the major constituent in this vegetable oil (Favacho et al., 2011). Oliveira et al. (2010) and Saraiva et al. (2008, 2009) showed the anti-inflammatory effect of pequi oil (extracted from *Caryocar coriaceum* Wittm.; Caryocaraceae family) in several inflammation models, including those induced by croton oil, and attributed this activity to unsaturated fatty acids present in the oil, among them OA.

Besides reducing inflammation-associated oedema, effective treatment to restrain inflammatory cell infiltration to injured tissue is needed since excessive infiltration and activation may lead to an increase in mediator release, contributing to cell chemotaxis and allowing the establishment of chronic inflammation (Nestle et al., 2009; Kolaczkowska and Kubes, 2013). The new therapy to treat skin inflammatory diseases proposed in this study, OA, proved to be effective in also reducing this parameter. Oleic acid reduced myeloperoxidase activity, an indirect measure of neutrophil leukocytes influx (Winterbourn et al., 2000; Kato, 2016), which is the first cellular subtype to infiltrate into damaged sites (Mortaz et al., 2018), confirming the topical anti-inflammatory activity of OA. In addition to that reported by Lescano et al. (2015), our results are also in accordance with those described by Cardoso et al. (2011) who demonstrated reduced inflammatory infiltration in the skin wounds of mice treated with OA. A reduction in the number of leukocytes infiltrating the injured tissue was also confirmed by histology.

Increased cell infiltration occurred after croton oil application. Topical treatment with Pemulen® TR2 OA and dexamethasone reduced this inflammatory parameter, demonstrated by both histological analysis and myeloperoxidase activity. The histological analysis did not enable an exact differentiation between leukocyte types in the tissue. However, since myeloperoxidase activity is an indirect marker of neutrophil infiltration (Winterbourn et al., 2000; Van der Veen et al., 2009; Huang et al., 2016), we showed that most infiltrated cells in the analysed tissue were neutrophils.

Pro-inflammatory cytokines are also important mediators in skin inflammatory processes. They are released by cells activated after inflammatory stimuli and contribute to skin resident cell activation and to the recruitment of leukocytes and other cell types to the skin, besides acting as signalling molecules to mediate and regulate immune and inflammatory processes by altering the expression of inflammation-related genes (Jensen, 2010; Turner et al., 2014; Bou-Dargham et al., 2016). Activated skin resident and recruited cells sustain the inflammatory environment by releasing cytokines (Sokol and Luster, 2015). Important cytokines involved in skin inflammation are members of the IL-1 family, like IL-1β (Jensen, 2011; Fields et al., 2019).

Besides the functions IL-1 family members in pro-inflammatory processes, these mediators also have effects on cell proliferation, differentiation, and apoptosis (Garlanda et al., 2013; Turner et al., 2014). It is already known that IL-1 family members contribute to adaptive immunity by inducing T lymphocyte differentiation into Th17 cells (Louten et al., 2009; Sandquist and Kolls, 2018). Moreover, IL-17 and other Th17

cell-derived cytokines induce granulopoiesis and consequent neutrophil proliferation and accumulation (Cua and Tato, 2010).

Since the cytokine IL-1β is crucial to the establishment of inflammation, a therapeutic alternative capable of reducing its tissue levels is of high relevance. We demonstrated that topically applied croton oil induced an increase in the IL-1β levels in mouse ear tissue. Besides reducing the IL-1β levels in the damaged ear, OA inhibited the ear oedema induced by the topical application of IL-1β, corroborating the effects on ear oedema and inflammatory cell infiltration.

We can hypothesise that the irritant agent croton oil leads to keratinocyte activation, stimulating them to the release cytokines, among then IL-1β, supporting the increased levels observed in mouse ear tissue. Additionally, it could also be hypothesised that this IL-1β induction by croton oil led to increased vascular permeability and consequent leukocyte infiltration into the injured tissue, as observed by histological analysis and myeloperoxidase activity. Damaged tissue inflammatory cells can also be involved in sustaining the number of leukocytes accumulated in the tissue, since skin infiltrating cells also release cytokines, attracting more cells (Oliveira et al., 2016). Furthermore, we speculated that IL-1-induced Th17 responses could contribute to the increased number of inflammatory cells in the damaged tissue by inducing neutrophil proliferation.

It is also known that the dysregulation of pro-inflammatory cytokine signalling leads to aberrant immunity and the development of several diseases (Dinarello, 2011; Hahn et al., 2017). For this reason, skin disease treatments are directed to the discovery of an effective therapy capable of restraining these effects by inhibiting key cytokines (Schlapbach and Navarini, 2016). Cytokine release and binding to their receptors triggers the activation of pro-inflammatory transcription pathways, which can control inflammation-related gene expression (Kataoka, 2009). Thus, inhibiting pro-inflammatory transcription pathway activation is also an important mechanism to suppress inflammation (Rodrigues et al., 2012). Interestingly, the topical therapy Pemulen® TR2 3% OA proposed by us effectively inhibited the increase in IL-1β, indicating that this anti-inflammatory effect could be interesting in clinical practice.

Glucocorticoids can play their biological effects by acting in two different ways at the cellular level: by genomic or non-genomic pathways. In the genomic pathway, the activation of the glucocorticoid receptor results in the transcription of genes with anti-inflammatory functions; the activation of this receptor also negatively regulates the expression of pro-inflammatory players by the transrepression mechanism. The non-genomic pathway involves the modulation of cell activation and responsiveness (Uva et al., 2012). Since our compound was found to activate glucocorticoid receptors, we believe that these mechanisms could play a role in its anti-inflammatory activity.

OA presented anti-inflammatory potential to treat skin inflammatory disorders such as ICD without causing adverse effects, supporting its clinical use. However, further studies evaluating the expression of chemokine and nuclear factor-kappa B (NF-κB) transcription pathway activation could be of great relevance to better understand the mechanisms involved in the anti-inflammatory action of OA.

6. Conclusion

Oleic acid presents *in vivo* anti-inflammatory efficacy in skin inflammation models induced by croton oil in mice, which seems to be glucocorticoid receptor-dependent. OA demonstrated anti-inflammatory efficacy similar to dexamethasone acetate, a clinical medicine widely used to treat skin inflammatory conditions, without causing adverse effects in the preliminary tests evaluated. Therefore, OA represents an attractive therapeutic alternative to treat cutaneous inflammatory disorders such as ICD.

Author contributions

Participated in research design: N.S.P., S.M.O.
Conducted experiments: N.S.P, C.C.
Performed data analysis: N.S.P., S.M.O.
Wrote or contributed to the writing of the manuscript: N.S.P, C.C., L. C., S.M.O.
All the authors reviewed the manuscript.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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