

Contents lists available at [SciVerse ScienceDirect](http://SciVerse.Sciencedirect.com)

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Anti-inflammatory properties of rose oxide

Fabiana Regina Nonato ^a, Danielle Gomes Santana ^b, Flavielle Martins de Melo ^c,
Gisele Graça Leite dos Santos ^a, Danielle Brustolim ^a, Enilton A. Camargo ^b, Damião P. de Sousa ^b,
Milena Botelho Pereira Soares ^{a,d}, Cristiane Flora Villarreal ^{a,c,*}

^a Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão 121, CEP 40296-710 Salvador, Bahia, Brazil

^b Universidade Federal de Sergipe, Departamento de Fisiologia, São Cristóvão, Sergipe, Brazil

^c Faculdade de Farmácia, Universidade Federal da Bahia, Rua Barão de Geremoabo s/n, CEP 40170-290 Salvador, Bahia, Brazil

^d Centro de Biotecnologia e Terapia Celular, Hospital São Rafael, Av. São Rafael 2152, CEP 41253-190 Salvador, Bahia, Brazil

ARTICLE INFO

Article history:

Received 26 June 2012

Received in revised form 28 September 2012

Accepted 18 October 2012

Available online 1 November 2012

Keywords:

Essential oils

Monoterpenes

Anti-inflammatory

Cytokines

ABSTRACT

Rose-oxide is a fragrance found in roses and rose oil. There are no reports about the pharmacological activity of this molecule. The present study was undertaken to evaluate whether rose-oxide (RO) has anti-inflammatory properties and to investigate possible mechanisms involved with its effects. The anti-inflammatory activity of RO was first suggested by the formalin test in mice, an inflammatory pain model, because intraperitoneal (i.p.) administration of RO (50 and 100 mg/kg) inhibited only the late phase of this test. To further investigate the anti-inflammatory properties of RO, the complete Freund's adjuvant (CFA)- and carrageenan-induced paw inflammation models were used. Pre-treatment with RO (50 and 100 mg/kg) significantly reduced paw edema at 4, 6 and 24 h after the CFA injection. In addition, RO (100 mg/kg) reduced the IL-1 β , but not TNF- α , local production induced by CFA. Administration of RO (25–100 mg/kg) decreased the paw edema induced by carrageenan in rats, which was more evident at 3 and 4 h after induction. In addition, neutrophil migration to the hind paw was measured by MPO assay after the carrageenan injection. The MPO activity was significantly inhibited by RO at 25–100 mg/kg, 4 h after stimulus. In another experimental set, administration of RO (25–100 mg/kg) significantly reduced the leukocyte migration in the carrageenan-induced peritonitis model in mice. The results described here are the first report of pharmacological properties of RO and strongly suggest that RO possesses anti-inflammatory activity related to its ability to inhibit the IL-1 β production and the leukocyte migration.

© 2012 Elsevier B.V. Open access under the [Elsevier OA license](http://creativecommons.org/licenses/by/3.0/).

1. Introduction

Inflammation is derived from the Latin word *inflammatio*, which means to set alight. It is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. Thus, inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. On the other hand, the outcome may be deleterious if it leads to chronic inflammation without resolution of the underlying injurious process. The clinical treatment of inflammatory diseases is dependent on non-steroidal or steroidal chemical therapeutics [1]. Nonsteroidal antiinflammatory drugs (NSAID) reduce the pain and inflammation by blocking the metabolism of arachidonic acid by cyclooxygenase enzyme (COX), and thereby the production of prostaglandin [2]. Since prostaglandins are cytoprotective, long term administration of

NSAID may induce gastro-intestinal ulcers, bleeding, and renal disorders due to their non-selective inhibition of both isoforms of the COX enzyme, the constitutive (COX-1) and the inducible (COX-2) isoforms [3]. On the other hand, fully selective and reversible COX-2 inhibitors with reduced gastro-intestinal toxicity have been associated with adverse cardiovascular effects [4]. Furthermore, the use of steroidal drugs as antiinflammatory agents is also becoming highly controversial due to their multiple side effects [5,6].

There is currently strong interest in the development of new anti-inflammatory agents, with potentially less side effects, based on natural products. Rose-oxide is an organic compound of the monoterpene class and a fragrance found in roses and rose oil such as the Bulgarian rose, Geranium, Damask rose, *Laggera* spp., *Eucalyptus citriodora*, and *Dracocephalum heterophyllum* [7–9]. Rose-oxide also contributes to the flavor of some fruits such as lychee and can be industrially produced based on the photooxygenation of citronellol [10]. Due to its commercial application, many studies evaluating the use of rose-oxide in the production of flavorings have been reported. However, as the time of writing, there are currently no reports about its pharmacological activities. The present study was undertaken to evaluate whether rose-oxide (RO) has anti-inflammatory properties

* Corresponding author at: Laboratório de Engenharia Tecidual e Imunofarmacologia, Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Waldemar Falcão 121 Candeal CEP 40296-710 Salvador, BA, Brazil. Tel.: +55 71 31762260; fax: +55 71 31762327.

E-mail address: cfv@ufba.br (C.F. Villarreal).

and to investigate the possible mechanisms involved with its effects. To the best of our knowledge, this is the first report of pharmacological properties of rose-oxide.

2. Material and methods

2.1. Animals

Experiments were performed on male Swiss Webster mice (22–28 g) obtained from the Animal Facilities of Centro de Pesquisas Gonçalo Moniz or male *Wistar* rats (150–200 g) obtained from the Animal House of the Federal University of Sergipe. Animals were housed in temperature-controlled rooms (22–25 °C), under a 12:12 h light–dark cycle, with access to water and food ad libitum until use. All behavioral tests were performed between 8:00 a.m. and 5:00 p.m., and animals were only used once. Animal care and handling procedures were in accordance with the Institutional Animal Care and Use Committee of the Gonçalo Moniz Research Center (FIOCRUZ L-029/2009). Every effort was made to minimize the number of animals used and any discomfort. Behavioral tests were done without knowing to which experimental group each mouse belonged. Results shown are from two independent experiments performed.

2.2. Formalin test

Mice were placed in an open Plexiglas observation chamber for 30 min to accommodate to their surroundings, and then removed for formalin administration. Mice were gently restrained while the hind paw received an intraplantar administration of 20 μ L of 2.5% formalin (1:100 dilution of stock formalin solution, 37% formaldehyde in 0.9% saline), using a 30 gauge needle. Following injection, mice were returned to the observation chamber for a 30 min observation period. Mice were observed from 0 to 10 min (early phase) and from 10 to 30 min (late phase), and a nociception score was determined for each period by counting the time that the animal spent licking the injected paw during the observation time [11]. Mice were treated with RO (25–100 mg/kg), vehicle (Tween 20, 5% in saline; control group), or indomethacin (10 mg/kg; reference drug), by intraperitoneal (i.p.) route 30 min before formalin.

2.3. Paw inflammation induced by complete Freund's adjuvant

Mice were slightly anesthetized with halothane and received 20 μ L of complete Freund's adjuvant (CFA; 1 mg/mL of heat killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate) in the right hind paw, according to a method previously reported [12]. Mice were injected with RO (25–100 mg/kg) or vehicle (Tween 20, 5% in saline; control group) by the i.p. route 30 min before CFA. Dexamethasone (2 mg/kg), the reference drug, was administered by subcutaneous route (s.c.) 4 h before stimulus. Paw edema and local levels of cytokines were measured by plethysmometer and ELISA, respectively, as described below.

2.4. Paw edema test

The volume of each mouse paw was measured with a plethysmometer (Ugo Basile, Comerio, Italy) before (V_0) the i.p. injection of CFA and 2, 4, 6 and 24 h after the CFA stimulus (VT), as described previously [13]. The amount of paw swelling was determined for each mouse and the difference between VT and V_0 was taken as the edema value (mm^3/paw).

2.5. Cytokine measurements

Skin tissues were removed from the paws 2 h after the i.p. injection of CFA, in mice terminally anesthetized with isoflurane and

posterior cervical dislocation from each experimental group. Tissue proteins were extracted from 100 mg of tissue/mL of PBS to which 0.4 M NaCl, 0.05% Tween 20 and protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A/100 mL) were added. The samples were centrifuged for 10 min at 3000 g and the supernatant was frozen at -70 °C for later quantification [14]. Interleukin 1 β (IL-1 β) and tumor necrosis factor α (TNF- α) levels were estimated using commercially available immunoassay ELISA kits for mice (R&D System, Minneapolis, MN), according to the manufacturer's instructions. The results are expressed as picograms of cytokine per mL of protein solution. As controls, the concentrations of these cytokines were determined in vehicle-treated mice and dexamethasone (positive control).

2.6. Paw inflammation induced by carrageenan

Rats were anesthetized with inhaled isoflurane and received an intraplantar injection of carrageenan 1% or sterile saline in the right paw in a final volume of 100 μ L. RO (25–100 mg/kg) or vehicle (Tween 20, 5% in saline; control group) was administered by the i.p. route 30 min before carrageenan. Dexamethasone (2 mg/kg) was administered by s.c. route 1 h before stimulus. The paw volume was assessed by plethysmometer, as described above. The area under curve (AUC_{0–4 h}) was also calculated using the trapezoidal rule. In another set of experiments, the myeloperoxidase (MPO) activity in rat paw was measured. Skin tissues were removed from the paws 4 h after the injection of carrageenan in rats terminally anesthetized with isoflurane and posterior cervical dislocation. The tissue was immediately collected and placed in a test tube in the presence of 0.5% of hexadecyltrimethylammonium bromide in 50 mmol/L potassium phosphate buffer (pH 6.0). Each tissue sample was homogenized and the homogenate was centrifuged at 12,000 g for 5 min. The supernatants were collected and the MPO assay was performed using a microliter plate scanner, according to Bradley et al. [15] with minor modifications. This consisted of mixing 10 μ L of sample with 200 μ L of *o*-dianisidine solution (0.167 mg/mL of *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide) before reading the plate. The changes in absorbance were measured at 460 nm for 15 s over a period of 5 min. The MPO activity was expressed as MPO units (U MPO) per milligram of tissue. One unit of MPO activity was defined as that degrading 1 μ mol of peroxide per minute at 25 °C [16].

2.7. Leukocyte migration to peritoneal cavity

Mice were pretreated with RO (25–100 mg/kg) or vehicle (Tween 20, 5% in saline; control group) by the i.p. route 30 min before the i.p. injection of carrageenan (1%; 250 μ L). Four hours after, the mice were euthanized under isoflurane anesthesia and 2 mL of a phosphate-buffered saline (PBS, pH 7.4) containing heparin (5 UI/mL) solution was injected into the peritoneal cavity. The abdomen was carefully massaged and the fluid was withdrawn, placed in polypropylene centrifuge tubes and centrifuged at 1000 g for 10 min. The resulting cell pellet was gently resuspended in 1.0 mL of PBS–heparin solution and the total and differential cell counts were assayed. Total cell counts were done using a Neubauer chamber while differential counts were carried out on a minimum of 200 cells using a cytospin preparation stained with Diff-Quick. The cells were classified as polymorphonuclear (neutrophils and eosinophils) or mononuclear (macrophages, mast cells and lymphocytes) based on normal morphological criteria.

2.8. Exudate macrophages

Peritoneal exudate cells were obtained by washing with cold Hank's balanced salt solution (HBSS; Life Technologies, GIBCO-BRL,

Gaithersburg, MD) the peritoneal cavity of mice 4 days after injection of 3% thioglycollate (Sigma-Aldrich, St. Louis, MO, USA) in saline (3.0 mL per mouse). Mice were treated with RO (50 and 100 mg/kg) or vehicle (Tween 20, 5% in saline; control group) by i.p. route 30 min before peritoneal lavage. Peritoneal exudate cells were plated in 24-well plates at 1×10^6 cells/well, in RPMI medium supplemented with 10% FCS. After 2 h of incubation at 37 °C, nonadherent cells were removed by washing with complete RPMI. Cultures were then stimulated with 500 ng/mL lipopolysaccharide (from *Escherichia coli* serotype 0111:B4, Sigma). Cell-free supernatants were collected at 6 h for determination of IL-1 β concentration by ELISA.

2.9. Drugs

(+)-Rose oxide (RO) was purchased from Fluka (Steinheim, Germany), with 99.0% of purity. Among the known isomers, the isomer (+)-rose oxide is the least studied on biological activities, therefore, this compound was selected for pharmacological evaluation [17,18]. Indomethacin, dexamethasone, carrageenan, complete Freund's adjuvant (CFA), phosphate buffered saline (PBS), Tween 20, phenylmethylsulphonyl fluoride (PMSF), benzamethonium chloride, EDTA, aproptinin A and 3,3',5,5'-tetramethylbenzidine (TMB), were obtained from Sigma Chemical Company (St. Louis, MO, USA). Indomethacin was dissolved in Tris HCl 0.1 M pH 8.0 plus saline. Dexamethasone (1 mg/mL) was dissolved in ethanol (10% in normal saline). Remaining drugs were dissolved directly in saline.

2.10. Data analysis

Data are presented as means \pm SEM of measurements made on 6–9 animals in each group. Comparisons across three or more treatments were made using one-way ANOVA with Tukey's post hoc test or repeated measures two-way ANOVA with Bonferroni's post hoc test, when appropriate. All data were analyzed using the Prism 5.01 computer software (GraphPad, San Diego, USA). Statistical differences were considered to be significant at $p < 0.05$.

3. Results

The anti-inflammatory properties of RO were evaluated initially using the formalin test in mice, an inflammatory pain model. The injection of formalin in control animals induced a biphasic licking response, with the early phase ranging from 0 to 10 min (Fig. 1a) and the late phase from 10 to 30 min (Fig. 1b) after the injection. Treatment with RO (50 and 100 mg/kg, i.p.) or indomethacin (10 mg/kg, i.p.), 30 min before the formalin, inhibited only the late phase of the formalin test ($p < 0.05$). Next, the effects of treatment with RO were assessed in the complete Freund's adjuvant (CFA)-induced paw inflammation

model. Paw edema and local levels of inflammatory cytokines were evaluated throughout the experimental period. The administration of RO (50 and 100 mg/kg, i.p.) 30 min before CFA reduced significantly ($p < 0.01$) the paw edema at 4, 6, and 24 h after the CFA injection (Fig. 2). The results obtained with control groups supported the effects of RO, since the vehicle (Tween 20, 5% in saline) had no activity, and the paw edema was strongly inhibited by dexamethasone (2 mg/kg, s.c.). In addition, the pre-treatment with RO (100 mg/kg) reduced the IL-1 β , but not TNF- α , local production induced by CFA (Fig. 3). Dexamethasone (2 mg/kg, s.c.), the reference drug, reduced both the IL-1 β and the TNF- α concentrations on inflamed paws.

Next, a possible direct action of RO on inflammatory cells was evaluated using exudate macrophages obtained by a peritoneal lavage of RO-treated animals. Upon stimulation with lipopolysaccharide, macrophage cultures from RO (100 mg/kg)-treated mice produced significantly less IL-1 β than macrophages obtained from vehicle-treated animals ($p < 0.05$; Fig. 4).

Aiming to further evaluate the anti-inflammatory properties of RO the carrageenan-induced paw inflammation model was also used. The intraplantar injection of carrageenan increased the rat paw volume with maximal effect 3 h after stimuli. Pretreatment of rats with RO (25–100 mg/kg, i.p.) decreased the paw edema induced by carrageenan (Fig. 5a), which was more evident 3 and 4 h after induction. Rats pretreated with dexamethasone (2 mg/kg) also had a significant reduction of edema. The $AUC_{0-4\text{ h}}$ analysis of the relationship between paw volume (mm^3/paw) \times time (h) clearly showed the anti-edematogenic action of RO. Pretreatment with RO at 25, 50 or 100 mg/kg significantly reduced the $AUC_{0-4\text{ h}}$ (2.6 ± 0.3 , $p < 0.05$; 2.4 ± 0.4 , $p < 0.01$ or 2.2 ± 0.2 , $p < 0.01$, respectively) when compared with the vehicle-treated group (3.8 ± 0.2), an effect also observed when dexamethasone was given as pre-treatment (1.3 ± 0.1 , $p < 0.001$). In addition to the paw edema, neutrophil migration to the hind paw was measured by MPO assay after the carrageenan injection. A high activity of MPO in the hind paw plantar tissues was detected after the carrageenan injection (Fig. 5b). The carrageenan-induced MPO activity was significantly inhibited by RO at 25 ($p < 0.05$), 50 ($p < 0.001$) or 100 mg/kg ($p < 0.01$). The MPO activity was also decreased by dexamethasone treatment ($p < 0.01$).

In another set of experiments, the effects of the treatment with RO were investigated in a carrageenan-induced peritonitis model in mice (Table 1). The intraperitoneal injection of carrageenan induced a marked leukocyte migration, characterized mainly by neutrophils, in the peritoneal cavity of mice. Eosinophils were virtually absent in all groups analyzed. The previous administration of RO significantly reduced the total leukocyte number at 25 ($p < 0.05$), 50 ($p < 0.01$) or 100 mg/kg ($p < 0.001$). This was also observed for polymorphonuclear (neutrophil) cell counts at all doses used. Mice treated with dexamethasone (2 mg/kg) also showed a significant decrease in the total

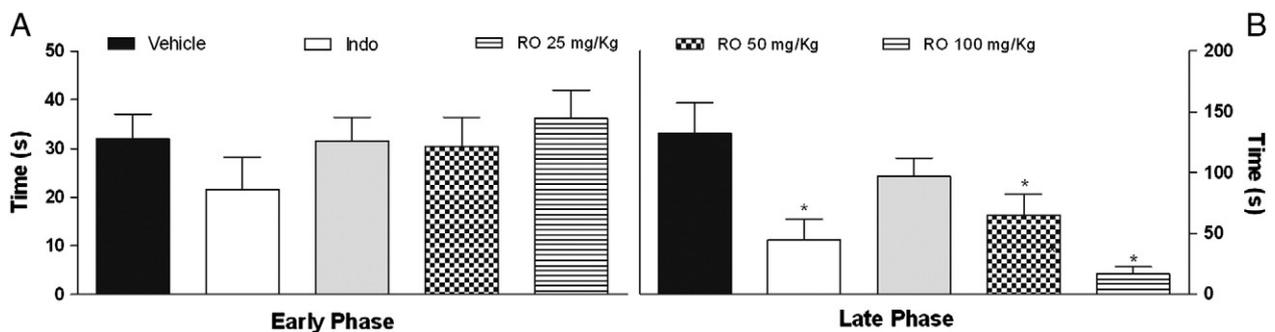


Fig. 1. Effects of the rose-oxide (RO) treatment on inflammatory pain induced by formalin. Panels (A) and (B) represent effects of RO on the early and late phases of formalin-induced nociception in mice, respectively. Mice were treated with RO (25, 50 and 100 mg/kg) or vehicle (Tween 20, 5% in saline; control group) by the i.p. route 30 min before formalin (injected at time zero). Indomethacin (In; 10 mg/kg, i.p.) was the positive control. Data are expressed as means \pm SEM of 6 mice per group. *Significantly different from the control group ($p < 0.05$) as determined by ANOVA followed by Tukey's test.

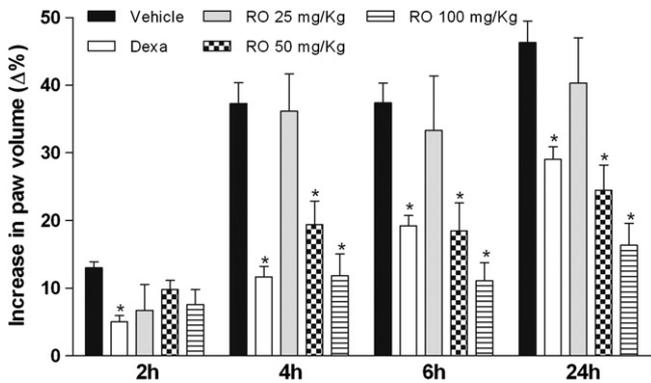


Fig. 2. Effects of the rose-oxide (RO) treatment on complete Freund's adjuvant (CFA)-induced paw inflammation measured at 2, 4, 6 and 24 h after the CFA stimulus. Mice were injected with RO (25, 50 and 100 mg/kg) and vehicle (Tween 20, 5% in saline; control group) by the i.p. route 30 min before CFA and dexamethasone (2 mg/kg; positive control) by the subcutaneous route 4 h before CFA (injected at time zero). Data are expressed as means \pm SEM of 6 mice per group. *Significantly different from the control group ($p < 0.01$). Two-way ANOVA followed by the Bonferroni's test.

and polymorphonuclear cell counts. Mononuclear cell counts were not affected by pretreatment with RO at 25 or 50 mg/kg or dexamethasone, but were significantly reduced by RO at 100 mg/kg ($p < 0.05$).

4. Discussion

The present study has demonstrated, for the first time, the anti-inflammatory properties of rose-oxide. The systemic administration of rose-oxide inhibited key events related to inflammation, namely edema, local increase of IL-1 β level, and leukocyte migration, producing consistent anti-inflammatory effects in different models of inflammation in mice and rats.

Because pain is one of the classic signs of the inflammatory process, the properties of RO were evaluated initially using the formalin test in mice, a screening tool for the assessment of new antinociceptive and anti-inflammatory substances [11]. This model has two distinct phases that may indicate different types of pain: the early phase (named nociceptive), which results essentially from the direct stimulation of nociceptors, and the late phase (named inflammatory pain), caused by local inflammation with a release of inflammatory mediators [19]. Treatment with RO caused antinociceptive effects only in the late phase of the formalin test, a profile similar to that of nonsteroidal anti-inflammatory drugs, which seem to suppress only the second phase of formalin test, and is in contrast with the profile of central analgesics, which seem to be antinociceptive for both phases [19,20]. This was a first indication of an anti-inflammatory action by RO.

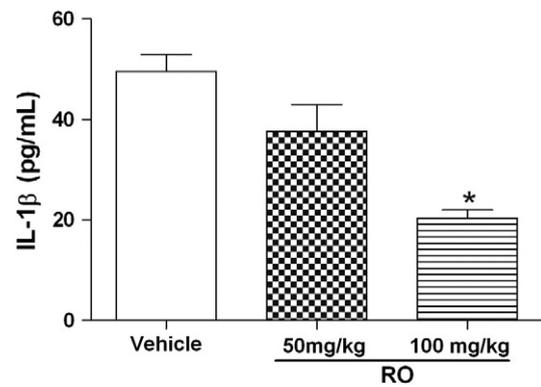


Fig. 4. Reduction of interleukin-1 β (IL-1 β) production by macrophages from rose-oxide treated mice. Mice were treated with rose-oxide (RO; 50 and 100 mg/kg) or vehicle (Tween 20, 5% in saline; control group) by the i.p. route 30 min before peritoneal lavage. Peritoneal exudate macrophages from treated mice were stimulated *in vitro* with lipopolysaccharide. Cell-free supernatants were collected after 6 h and tested for production of IL-1 β by ELISA. Data are expressed as means \pm SEM of 6 experiments. *Significantly different from the control group ($p < 0.001$) as determined by ANOVA followed by Tukey's test.

To confirm this hypothesis, the effects of RO treatment were assessed in the complete Freund's adjuvant (CFA)-induced paw inflammation model. It is now well recognized that the inflammation caused by intraplantar injection of CFA involves the release of multiple inflammatory mediators, such as cytokines and prostanoids, which increase blood supply and accumulation of leukocytes, resulting in local swelling and edema [21,22]. In the present study, the administration of RO resulted in a marked inhibition of two important events, the edema and increase in tissue IL-1 β levels, which are related to the inflammatory response induced by CFA. Nowadays, it is well accepted that cytokines constitute a link between cellular injuries or immunological recognition and the local or systemic signs of inflammation, e.g. cell migration, edema, fever, and hyperalgesia [23–25]. Different cell types, including macrophages, monocytes, and glial cells produce IL-1 β which, in turn, induce the production of other inflammatory mediators involved with cellular recruitment, such as IL-6, IL-8, adhesion molecules, chemokines, nitric oxide synthase, and COX-2, fever, acute phase protein release, and increase of vascular permeability [23,26–29]. Considering the important role of IL-1 β during the inflammatory response, we suggest that the anti-inflammatory effects of RO may be related to its ability to inhibit the release of IL-1 β . In addition, in the present study, a single administration of RO produced an anti-edematogenic effect that lasted 24 h after its administration. This long-lasting anti-inflammatory effect may reflect an interference with the *de novo* synthesis of inflammatory proteins, reinforcing the idea that the

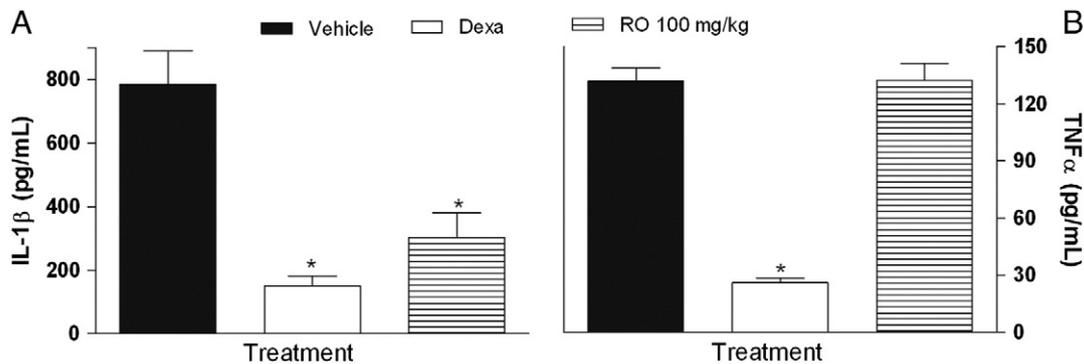


Fig. 3. Effects of the rose-oxide (RO) on inflammatory cytokines on complete Freund's adjuvant (CFA)-induced paw inflammation. Cytokine levels were measured 3 h after the stimulus. (A) Paw interleukin 1 β (IL-1 β) levels. (B) Paw tumor necrosis factor α (TNF- α) levels. Mice were injected with RO at 100 mg/kg and vehicle (Tween 20, 5% in saline; control group) by the i.p. route 30 min before CFA and dexamethasone (2 mg/kg; positive control) by the subcutaneous route 4 h before CFA (injected at time zero). Data are expressed as means \pm SEM of 6 mice per group. *Significantly different from the control group ($p < 0.05$) as determined by ANOVA followed by Tukey's test.

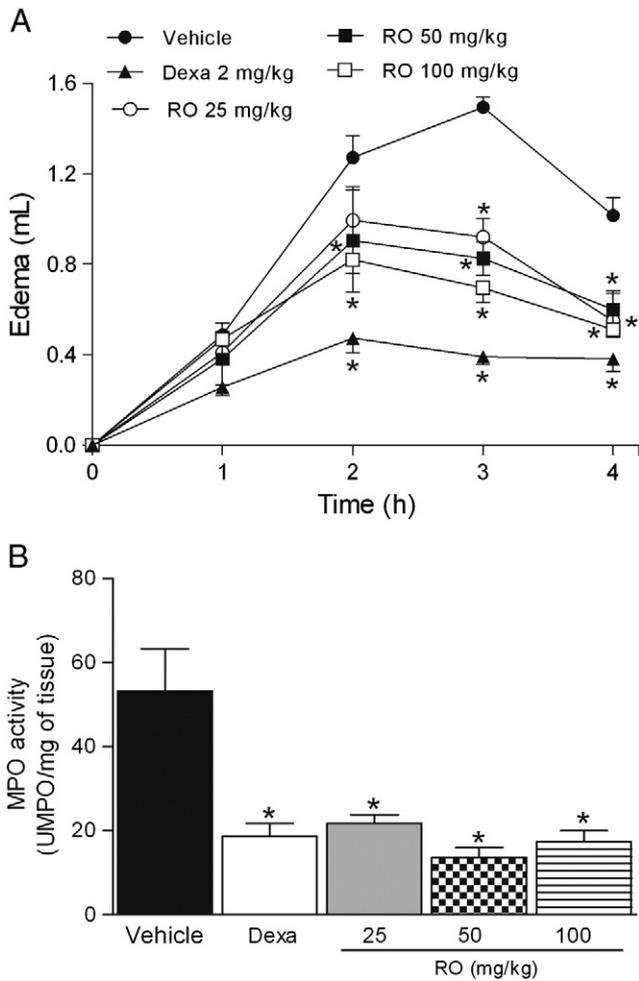


Fig. 5. Rose oxide (RO) reduced carrageenan (CAR)-induced rat paw edema and neutrophil infiltration. Panel (a) shows the time course of rat paw edema in animals treated with vehicle, RO (25–100 mg/kg) or dexamethasone (Dexa, 2 mg/kg) and injected with carrageenan (CAR 1%) after 40 min. Panel (b) shows the myeloperoxidase (MPO) activity determined in the rat paws after the 4th hour. Data are expressed as means \pm SEM of 6 mice per group. *Significantly different from the carrageenan + vehicle group ($p < 0.05$) as determined by ANOVA followed by the Tukey's test.

anti-inflammatory effect of RO may be related to the modulation of cytokine production. The inhibitory effect of RO on IL-1 β production was next confirmed in vitro, in macrophage cultures. Peritoneal exudate macrophages obtained from mice previously treated with RO produced significantly less IL-1 β when stimulated with lipopolysaccharide than macrophages from vehicle-treated animals. This ability of RO to suppress the stimulated release of IL-1 β indicates a direct action of RO on inflammatory macrophages.

Table 1

Total and differential leukocyte counts in the peritoneal cavity of mice pretreated with vehicle, rose oxide (RO) or dexamethasone in the peritonitis model induced by carrageenan.

Group	Cells $\times 10^6$ /peritoneal cavity		
	Total leukocyte	Mononuclear	Polymorphonuclear
Vehicle	15.4 \pm 0.7	4.1 \pm 0.4	11.2 \pm 0.6
RO (25 mg/kg)	10.6 \pm 0.9 ^a	3.6 \pm 0.3	7.0 \pm 0.7 ^b
RO (50 mg/kg)	9.4 \pm 1.3 ^b	3.7 \pm 0.4	5.7 \pm 1.0 ^c
RO (100 mg/kg)	5.4 \pm 0.8 ^c	2.0 \pm 0.2 ^a	3.4 \pm 0.6 ^c
Dexamethasone (2 mg/kg)	6.1 \pm 1.5 ^c	3.0 \pm 0.8	3.1 \pm 0.8 ^c

Results are presented as mean \pm S.E.M. of cells $\times 10^6$ /peritoneal cavity for $n = 5$ mice. ^a $p < 0.05$, ^b $p < 0.01$ and ^c $p < 0.001$ compared to carrageenan + vehicle.

Confirming the anti-inflammatory activity of RO, the edema induced by carrageenan in rats was inhibited to a significant extent by this compound. This result suggests that RO affect the vascular permeability, leading to decreased leakage in rat paws. The intraplantar injection of carrageenan in rodents induces edema associated with the production of inflammatory mediators and leukocyte migration [30–34]. Thus, the potential usefulness of RO was tested as a pharmacological tool to inhibit neutrophil migration induced by inflammation. MPO activity is widely used in experimental studies as a marker of the neutrophil content in various tissues, such as skin [35], paw [16], and pancreas [36], among others. We found that the administration of RO decreased the MPO activity, indicating that RO influences the paw neutrophil migration induced by carrageenan. This inhibitory effect of RO on leukocyte migration was further investigated in the peritonitis model induced by carrageenan in mice, which is well described as a flogistic agent that increases the total leukocyte counts in peritoneal cavity, mainly due to a neutrophil influx [37,38]. In agreement with our previous results, we observed a marked reduction of carrageenan-induced neutrophil recruitment to the peritoneal cavity in mice pretreated with RO. This result may also be associated with the ability of RO to reduce the IL-1 β levels, since it is known that IL-1 β is a potent inducer of leukocyte infiltration into the tissues during the acute phase of an inflammatory reaction [39–41]. A recent report has suggested a role of emigrating neutrophils in the production and/or release of PGE₂ during inflammation [42]. Considering that this prostanoid may be crucial for edema formation [43], the reduction of neutrophil infiltration may also contribute to the anti-edematogenic effect of RO.

The results presented herein strongly suggest that rose-oxide possesses anti-inflammatory activity related to its ability to inhibit the IL-1 β production and the leukocyte migration. However, the precise mechanisms through which RO exerts its action are currently under investigation. These results highlight the need for further investigations of the possible use of RO as a prototype compound for the development of new drugs to treat inflammatory conditions.

References

- Rainsford KD. Anti-inflammatory drugs in the 21st century. *Subcell Biochem* 2007;42:3–27.
- Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 1971;231:232–5.
- Tapiero H, Ba GN, Couvreur P, Tew KD. Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. *Biomed Pharmacother* 2002;56: 215–22.
- Dogné JM, Supuran CT, Pratico D. Adverse cardiovascular effects of the coxibs. *J Med Chem* 2005;48:2251–7.
- Schäcke H, Döcke WD, Asadullah K. Mechanisms involved in the side-effects of glucocorticoids. *Pharmacol Ther* 2002;96:23–43.
- Reinke P, Bevilacqua M, Tryon V, Cheronis J, Volk HD. Immune monitoring of glucocorticoid therapy. *Ernst Schering Res Found Workshop* 2002;40:25–37.
- Babu KGD, Singh B, Joshi VP, Singh V. Essential oil composition of Damask rose (*Rosa damascena* Mill.) distilled under different pressures and temperatures. *Flav Fragr J* 2002;17:136–40.
- Kuiate JR, Bessièrè JM, Zollo PHA. Composition of the essential oils from three *Laggera* spp. from Cameroon. *Flav Fragr J* 2002;17:105–8.
- Taneja SC, Sethi VK, Andotra SS, Koul S, Qazi GN. Rose oxides: a facile chemo and chemo-enzymatic approach. *Synth Commun* 2005;35:2297–303.
- Alsters PL, Jary W, Nardello-Rataj VR, Aubry JM. “Dark” singlet oxygenation of β -citronellol: a key step in the manufacture of rose oxide. *Org Process Res Dev* 2010;14:259–62.
- Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* 1977;4:161–74.
- Kassuya CA, Silvestre AA, Rehder VL, Calixto JB. Anti-allodynic and anti-oedematogenic properties of the extract and lignans from *Phyllanthus amarus* in models of persistent inflammatory and neuropathic pain. *Eur J Pharmacol* 2003;478:145–53.
- Winter CA, Risley EA, Nuss GW. Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. *Proc Soc Exp Biol Med* 1962;111:544–7.
- Santiago HC, Braga Pires MF, Souza DG, Roffè E, Côrtes DF, Tafuri WL, et al. Platelet activating factor receptor-deficient mice present delayed interferon-gamma upregulation and high susceptibility to *Leishmania amazonensis* infection. *Microbes Infect* 2006;8:2569–77.

- [15] Bradley PP, Priebe DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 1982;78:206-9.
- [16] De Sousa DP, Camargo EA, Oliveira FS, Almeida RN. Anti-inflammatory activity of hydroxydihydrocarvone. *Z Naturforsch C* 2010;65c:543-50.
- [17] Gravel D, Bordeleau J. Photochemical rearrangement of 2-phenylthio-1,3-cyclohexanedioles to deoxysugars. Application to a stereospecific synthesis of (+)-cis-rose oxide. *Tetrahedron Lett* 1998;39:8035-8.
- [18] Sell Charles. Chemistry of essential oils 134p. In: Husnu Can Baser K, Buchbauer Gerhard, editors. *Handbook of essential oils: science, technology, and applications*. CRC Press; 2009.
- [19] Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* 1987;30:103-14.
- [20] Malmberg AB, Yaksh TL. Antinociceptive actions of spinal anti-inflammatory agents on the formalin test in the rat. *J Pharmacol Exp Ther* 1992;263:136-46.
- [21] Woolf CJ, Allchorne A, Safieh-Garabedian B, Poole S. Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. *Br J Pharmacol* 1997;121:417-24.
- [22] Fehrenbacher JC, Vasko MR, Duarte DB. Models of inflammation: carrageenan- or complete Freund's Adjuvant (CFA)-induced edema and hypersensitivity in the rat. *Curr Protoc Pharmacol Mar* 2012 [Chapter 5:Unit5.4].
- [23] Ferreira SH, Lorenzetti BB, Bristow AF, Poole S. Interleukin-1 beta as a potent hyperalgesic agent antagonized by a tripeptide analogue. *Nature* 1988;334:698-700.
- [24] Faccioli LH, Souza GE, Cunha FQ, Poole S, Ferreira SH. Recombinant interleukin-1 and tumor necrosis factor induce neutrophil migration "in vivo" by indirect mechanisms. *Agents Actions* 1990;30:344-9.
- [25] Dinarello CA. Proinflammatory cytokines. *Chest* 2000;118:503-8.
- [26] Dinarello CA. Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. *Int Rev Immunol* 1998;16:457-99.
- [27] Dinarello CA. The biology of interleukin 1 and comparison to tumor necrosis factor. *Immunol Lett* 1987;16:227-31.
- [28] Dejana E, Breviario F, Erroi A, Bussolino F, Mussoni L, Gramse M, et al. Modulation of endothelial cell functions by different molecular species of interleukin 1. *Blood* 1987;69:695-9.
- [29] Cunha FQ, Ferreira SH. Peripheral hyperalgesic cytokines. *Adv Exp Med Biol* 2003;521:22-39.
- [30] Di Rosa M, Giroud JP, Willoughby DA. Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. *J Pathol* 1971;104:15-29.
- [31] Vinegar R, Truax JF, Selph JL, Johnston PR, Venable AL, McKenzie KK. Pathway to carrageenan-induced inflammation in the hind limb of the rat. *Fed Proc* 1987;46:118-26.
- [32] Henriques MGMO, Silva PMR, Martins MA, Flores CA, Cunha FQ, Assreuy-Filho J, et al. Mouse paw oedema. A new model for inflammation? *Braz J Med Biol Res* 1987;20:243-9.
- [33] Posadas I, Bucci M, Roviezzo F, Rossi A, Parente L, Sautebin L, et al. Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. *Br J Pharmacol* 2004;142:331-8.
- [34] Rocha AC, Fernandes ES, Quintão NL, Campos MM, Calixto JB. Relevance of tumour necrosis factor-alpha for the inflammatory and nociceptive responses evoked by carrageenan in the mouse paw. *Br J Pharmacol* 2006;148:688-95.
- [35] Yshii LM, Souza GH, Camargo EA, Eberlin MN, Ribela MT, Muscará MN, et al. Characterization of the mechanisms underlying the inflammatory response to *Polistes lanio lanio* (paper wasp) venom in mouse dorsal skin. *Toxicol* 2009;53:42-52.
- [36] Camargo EA, Zanoni CI, Toyama MH, Muscará MN, Docherty RJ, Costa SKP. Abdominal hyperalgesia in secretory phospholipase A2-induced rat pancreatitis: distinct roles of NK1 receptors. *Eur J Pain* 2011;15:900-6.
- [37] Salvemini D, Wang ZQ, Wyatt PS, Bourdon DM, Marino MH, Manning PT, et al. Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br J Pharmacol* 1996;118:829-38.
- [38] Santos CA, Passos AMPR, Andrade FC, Camargo EA, Estevam CS, Santos MRV, et al. Antinociceptive and anti-inflammatory effects of *Caesalpinia pyramidalis* Tul. (Fabaceae) in rodents. *Braz J Pharmacogn* 2011;21:1077-83.
- [39] Cybulsky MI, Colditz IG, Movat HZ. The role of interleukin-1 in neutrophil leukocyte emigration induced by endotoxin. *Am J Pathol* 1986;124:367.
- [40] Granstein RD, Margolis R, Mizel SB, Sauder DN. In vivo inflammatory activity of epidermal cell-derived thymocyte activating factor and recombinant interleukin 1 in the mouse. *J Clin Invest* 1986;77:1020-7.
- [41] Wankowicz Z, Megyeri P, Issekutz A. Synergy between tumour necrosis factor alpha and interleukin-1 in the induction of polymorphonuclear leukocyte migration during inflammation. *J Leukoc Biol* 1988;43:349-56.
- [42] Cunha TM, Verri Jr WA, Schivo IR, Napimoga MH, Parada CA, Poole S, et al. Crucial role of neutrophils in the development of mechanical inflammatory hypernociception. *J Leukoc Biol* 2008;83:824-32.
- [43] Moncada S, Ferreira SH, Vane JR. Prostaglandins, aspirin-like drugs and the oedema of inflammation. *Nature* 1973;246:217-9.