Anti-inflammatory effect of a sulphated polysaccharide fraction extracted from the red algae Hypnea musciformis via the suppression of neutrophil migration by the nitric oxide signalling pathway

Tarcisio Vieira de Britoa, Rafael da Silva Prudêncioa, Adriano Bezerra Salesa, Francisco das Chagas Vieira Júniora, Starley Jone Nogueira Candeiraa, Álvaro Xavier Francob, Karoline Sabóia Aragãob, Ronaldo de Albuquerque Ribeirób, Marcellus Henrique Loiola Ponte de Souzab, Luciano de Sousa Chaves, Ana Lúcia Ponte Freitasc, Jand-Venes Rolim Medeirosa and André Luiz dos Reis Barbosaa

Keywords
inflammatory process; nitric oxide; polysaccharide

Correspondence
André Luiz dos Reis Barbosa,
BIOTEC/LAFFEX/UFPI, Av. São Sebastião, n°2819, CEP 64202-020, Parnaíba, PI, Brazil.
E-mail: andreluiz@ufpi.edu.br

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Abstract

Objectives The aim of this study was to evaluate the anti-inflammatory effect of a sulphated polysaccharide fraction (PLS) extracted from the alga Hypnea musciformis and investigate the possible involvement of the nitric oxide (NO) pathway in this effect.

Methods The anti-inflammatory activity of PLS was evaluated using inflammatory agents (carrageenan and dextran) to induce paw oedema and peritonitis in Swiss mice. Samples of paw tissue and peritoneal fluid were removed to determine myeloperoxidase (MPO) activity, NO3/NO2 levels, and interleukin-1β (IL-1β) level. The involvement of NO in the modulation of neutrophil migration in carrageenan-induced paw oedema or peritonitis was also investigated.

Key findings Compared with vehicle-treated mice, mice pretreated with PLS (10 mg/kg) inhibited carrageenan-induced and dextran-induced oedema; it also inhibited total and differential peritoneal leucocyte counts in a model of peritonitis. These PLS effects were reversed by l-arginine treatment and recovered with the administration of a NO synthase blocker (aminoguanidine). Furthermore, PLS reduced the MPO activity, decreased IL-1β levels, and increased NO3/NO2 levels in the peritoneal cavity.

Conclusions PLS reduced the inflammatory response by modulating neutrophil migration, which appeared to be dependent on the NO pathway.

Introduction

The search for natural products with pharmacological properties has significantly contributed to the discovery of substances with important applications.[1,2] Thus, marine algae are valuable sources of diverse structurally bioactive compounds such as carotenoids, pigments, polyphenols, enzymes, and diverse functional polysaccharides.[3,4]

Many species of seaweed (marine macroalgae) are used as food and they have also found use in traditional medicine because of their perceived health benefits. Seaweeds are rich sources of sulphated polysaccharides, including some that have become valuable additives in the food industry because of their rheological properties as gelling and thickening agents (e.g. carrageenan).[5]

The red seaweed Hypnea musciformis synthesizes sulphated polysaccharide fraction (PLS), which is composed of a backbone structure of alternating β-D-galactosyl and α-D-galactosyl residues. The carrageenan from H. musciformis has been reported as being a κ-carrageenan, a family of
carrageenans which has a sulphate group at the carbon 4 of the β-D-galactopyranosyl residue, and a 3,6-anhydro bridge on α-D-galactose.\(^\text{[6,7]}\)

Many types of sulphated polysaccharides are recognized as having a number of biological activities, including anticoagulant, gastroprotective, antinociceptive, and anti-inflammatory, which might give them relevance in pharmaceutical applications.\(^\text{[8–10]}\) However, despite the wealth of marine flora, the anti-inflammatory activity of sulphated polysaccharides from marine organisms have not been well explored.

The inflammatory process consists of diverse physiological and pathological activities. A key characteristic of the inflammatory reaction is the migration of leucocytes from the blood into the tissues, which occurs in a sequence of steps. Locally produced inflammatory mediators, including tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), activate vascular endothelial cells and upregulate key adhesion molecules that mediate tethering, rolling, cell adhesion, and extravasation of leucocytes. The leucocytes then migrate towards the site of tissue inflammation.\(^\text{[11,12]}\)

However, the nitric oxide (NO) released by either constitutive NO synthase (cNOS) or inducible NO synthase (iNOS) during the inflammatory process down-modulates the migration of neutrophils to inflammatory sites as a result of decreased rolling and adhesion of the neutrophils on the endothelium. In addition, the released NO plays an important major role in the apoptosis of migrated neutrophils.\(^\text{[13,14]}\)

Given that marine sulphated polysaccharides are important sources of new chemical substances that may have anti-inflammatory activity and therapeutic effects, this study aimed to evaluate the anti-inflammatory effect of a sulphated polysaccharide fraction (PLS) extracted from the red algae \textit{H. musciformis} and to investigate the possible involvement of the NO signalling pathway in this effect.

**Materials and Methods**

**Extraction of the polysaccharide fraction of \textit{H. musciformis}**

The marine red algae \textit{H. musciformis} was collected at Flechearias Beach, Trairí, Ceará, Brazil. The samples were cleaned of epiphytes, washed with distilled water and stored at −20°C. The extraction procedure of polysaccharides was performed according to Farias \textit{et al.}\(^\text{[15]}\). The dried tissue (5 g) was milled and suspended in 250 ml 0.1 M sodium acetate buffer (pH 6.0) containing 510 mg papain (E. Merck, St Louis, MO, USA), 5 mM ethylene diamine tetra acetic acid (EDTA), 5 mM cysteine and incubated at 60°C for 12 h. The residue was removed by filtration and centrifugation (2700g for 25 min at 4°C) and the sulphated polysaccharides were precipitated by addition of 48 ml 10% cetylpyridinium chloride (CPC, Sigma Chemical, St Louis, MO, USA). The mixture was centrifuged (2700g for 25 min at 4°C) and the polysaccharides (κ-carrageenan) in the pellet were washed with 200 ml 0.05% cetylpyridinium chloride solution, dissolved in 174 ml 2 M NaCl/ethanol (100 : 15, v/v) solution, and precipitated with 200 ml 70% ethanol (v/v) for 12 h at 4°C. After further centrifugation (2700g, 4°C; 25 min) the precipitate was washed twice with 200 ml absolute ethanol and dried with acetone under hot air flow (60°C).

**Chemical characterization of the polysaccharide fraction extracted from \textit{H. musciformis}**

The chemical characterization had been determined previously.\(^\text{[6,7]}\) Total sugar content of each fraction was determined according to the method of Dubois \textit{et al.}\(^\text{[16]}\). Protein content was measured by the Bradford method.\(^\text{[17]}\) Sulphate content in polysaccharides was determined by the barium chloride-gelatin method and the monosaccharide composition of red seaweed galactans was obtained by reductive hydrolysis.\(^\text{[18,19]}\)

**Animals**

Male Swiss mice (25–35 g) were from the Central Animal Facility of the Federal University of Piauí. Experiments were approved under N\textit{°}23111.011979/11-80 in 2011 by the Ethics Committee of the Federal University of Piauí, Brazil.

**Drugs and reagents**

The following drugs and reagents were used: carrageenan (Sigma Aldrich, St Louis, MO, USA), dextran sulphate (Sigma Aldrich), indometacin, aminoguanidine and L-arginine (Sigma Aldrich). These drugs were dissolved in sterile saline (0.9% NaCl).

**Carrageenan-induced or dextran-induced paw oedema**

Doses of PLS (2.5, 5 or 10 mg/kg, i.p.) were administered to the mice. One hour later, carrageenan (500 μg per paw; 50 μl) or dextran (500 μg per paw; 50 μl) were administered by subplantar injection into the right paw.

Paw volume was measured with a plethysmometer (Ugo-Basile 7140) immediately before injections (basal volume) and then 1, 2, 3, and 4 h later for carrageenan or 30 min, 1, 2, 3, and 4 h later for dextran injections. Results are expressed as the change in paw volume (ml), calculated by subtracting the basal volume from the volume measured at the indicated times. The percentage of inhibition in increase of paw volume for each group was calculated by the following formula:\(^\text{[20]}\)
% inhibition of oedema
\[
\frac{(V_t - V_o)_{\text{Control}} - (V_t - V_o)_{\text{Treated}}}{(V_t - V_o)_{\text{Control}}} \times 100
\]

where V_o is the basal volume and V_t is the final volume measured at the indicated times.

**Myeloperoxidase activity**

The extent of neutrophil accumulation in the mouse paw was measured by myeloperoxidase (MPO) activity evaluation. Briefly, 50–100 mg hind paw tissue was homogenized in 1 ml potassium buffer with 0.5% hexadecyltrimethylammonium bromide for each 50 mg tissue. The homogenate was centrifuged at 40 000 g for 7 min at 4°C. MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm using o-dianisidine dihydrochloride and 1% hydrogen peroxide. The results were reported as the MPO units/mg tissue. A unit of MPO (UMPO) activity was defined as that converting 1 μmol hydrogen peroxide to water in 1 min at 22°C.

**Measurement of cytokine IL-1β**

Samples of peritoneal fluid was collected and the level of IL-1β was evaluated using sandwich enzyme-linked immunoabsorbent assay (ELISA). ELISA kits for IL-1β were from the National Institute for Biological Standards and Control (Potters Bar, UK).

These ELISA methods consistently detected a level of IL-1β over 4000 pg/ml and did not cross-react with other cytokines. The results were expressed as picograms (pg/ml) of each cytokine per peritoneal cavity washed.

**Peritonitis model in mice**

Mice were injected intraperitoneally with 250 μl sterile saline or indomethacin 10 mg/kg or PLS 10 mg/kg. One hour later, the animals were injected intraperitoneally with 250 μl carrageenan (500 μg per cavity) into the peritoneal cavity. Mice were killed by decapitation under anaesthesia 4 h later and the peritoneal cavity was washed with 1.5 ml heparinized phosphate buffered saline (PBS) to harvest peritoneal cells. Total cell counts were performed in a Neubauer chamber, and differential cell counts (100 cells total) were carried out on cyt centrifuge slides stained with haematoxylin and eosin. The results were presented as the number of neutrophils per ml of peritoneal exudate.

**Actions of aminoguanidine and L-arginine on the inhibitory effect of PLS on peritonitis induced by carrageenan**

Mice were then co-treated subcutaneously with L-arginine (500 mg/kg; 250 μl) plus aminoguanidine (50 μg/kg; 250 μl) or aminoguanidine (50 mg/kg; 250 μl) only, and 30 min later carrageenan (500 μg per cavity) was injected intraperitoneally and the neutrophil migration was determined. The determination of neutrophil migration into the peritoneal cavity was done as described previously in the peritonitis model in mice.

**Actions of aminoguanidine and L-arginine on the inhibitory effect of PLS on paw oedema induced by carrageenan**

Mice were injected orally with 500 μl sterile saline or PLS 10 mg/kg. Animals were then co-treated subcutaneously with L-arginine (500 mg/kg; 250 μl) plus aminoguanidine (50 mg/kg; 250 μl) or treated subcutaneously with aminoguanidine (50 mg/kg; 250 μl) only, and 30 min later carrageenan (500 μg per paw) was injected into the right plantar surface and the paw oedema was determined. The measurement of volume of the legs was done as described in carrageenan-induced or dextran-induced paw oedema.

**Action of PLS on peritoneal fluid levels of nitric oxide (NO₃/NO₂) in peritonitis model**

The animals received injections of 250 μl sterile saline or PLS 10 mg/kg into the peritoneal cavity. One hour later, the animals were injected intraperitoneally with 250 μl carrageenan (500 μg per cavity) into the same cavity. Four hours later mice were killed by decapitation under anaesthesia and the peritoneal cavity was washed with 1.5 ml PBS to harvest samples of peritoneal fluid. Peritoneal fluid of animals was incubated in a microplate with nitrate reductase (0.016 U per well) for 12 h to convert NO₃ to NO₂. Nitric oxide production was determined by measuring nitrite concentrations in an ELISA plate reader at 540 nm using the Griess method.[21] Results were expressed as micromoles of nitrite using the internal standard curve.

**Statistical analysis**

Results are expressed as mean ± SEM from at least five animals per group. Statistical analysis was performed using analysis of variance followed by Bonferroni post hoc test, when appropriate. Statistical significance was set at P < 0.05.

**Results**

**Effect of PLS on carrageenan-induced paw oedema in mice**

Table 1 shows that the administration of carrageenan into the plantar surface (500 μg per paw; into the plantar...
surface) induced severe paw oedema within 1 h of injection and was maintained until 4 h after injection. Indomethacin (10 mg/kg) administration significantly decreased paw oedema throughout the experimental period (*P < 0.05), with maximal inhibition of 95.74%. Similarly, PLS (5 and 10 mg/kg, i.p.) induced long-lasting inhibition of paw oedema at all intervals. At 3 h, compared with the carrageenan group, the animals pretreated with 5 and 10 mg/kg PLS showed 74.46% and 93.62% reduction in oedema, respectively. Table 1 shows that PLS prevented carrageenan-induced paw oedema (500 μg per paw/50 μl) in a dose-dependent manner, with maximal inhibitory effect exerted at a dose of 10 mg/kg (3 h: 0.006 ± 0.006 ml; 4 h: 0.012 ± 0.005 ml). Therefore, this dose was selected for studying the possible mechanisms of action involved in PLS-mediated decrease in inflammatory response.

**Effect of sulphated polysaccharide on paw oedema induced by dextran**

Figure 1 shows that injection of dextran (0.0960 ± 0.010 ml) resulted in an increase in oedema over time, peaking approximately 30 min after injection. By contrast, saline injected in the paw did not induce paw oedema. Pretreatment of the animals with PLS (10 mg/kg) 30 min before dextran injection effectively inhibited oedema (inhibition at 30 min: 97.91%; Figure 1).

**Effect of sulphated polysaccharide fraction on carrageenan-induced myeloperoxidase activity in paw tissue**

Figure 2 shows that PLS (10 mg/kg) inhibited neutrophil infiltration, which was evident from the MPO activity measured in the mouse paws. The carrageenan subplantar determined MPO activity in the concentration of 37.39 ± 8.23 UMPO/mg plantar tissue, while the group treated with PLS presented an activity of 4.73 ± 0.68 UMPO/mg plantar tissue, which was equivalent to a reduction of 87.34%.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Paw oedema (ml) 1 h</th>
<th>Paw oedema (ml) 2 h</th>
<th>Paw oedema (ml) 3 h</th>
<th>Paw oedema (ml) 4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (carrageenan)</td>
<td>5</td>
<td>0.086 ± 0.004</td>
<td>0.088 ± 0.008</td>
<td>0.094 ± 0.002</td>
<td>0.104 ± 0.004</td>
</tr>
<tr>
<td>Saline</td>
<td>2.5</td>
<td>0.008 ± 0.003*</td>
<td>0.012 ± 0.005*</td>
<td>0.008 ± 0.003*</td>
<td>0.004 ± 0.002*</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>0.022 ± 0.005* (74.42%)</td>
<td>0.004 ± 0.040* (95.44%)</td>
<td>0.004 ± 0.022* (95.74%)</td>
<td>0.016 ± 0.005* (84.61%)</td>
</tr>
<tr>
<td>PLS</td>
<td>2.5</td>
<td>0.036 ± 0.010* (58.14%)</td>
<td>0.066 ± 0.019 (25.00%)</td>
<td>0.060 ± 0.011* (36.17%)</td>
<td>0.080 ± 0.018 (23.076%)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.036 ± 0.008* (58.13%)</td>
<td>0.018 ± 0.008* (79.54%)</td>
<td>0.024 ± 0.009* (74.46%)</td>
<td>0.018 ± 0.009* (82.69%)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.030 ± 0.008* (65.12%)</td>
<td>0.008 ± 0.003* (90.91%)</td>
<td>0.006 ± 0.006* (93.62%)</td>
<td>0.012 ± 0.005* (88.46%)</td>
</tr>
</tbody>
</table>

PLS, sulphated polysaccharide fraction. Values of paw oedema are expressed as mean ± SEM (n = 5). The % inhibition of paw oedema is indicated in parentheses. *P < 0.05 compared with control (one-way analysis of variance followed by the Bonferroni post hoc test).

**Effect of sulphated polysaccharide fraction on carrageenan-induced cytokine production in peritonitis**

The intraperitoneal administration of carrageenan was found to induce a marked increase in IL-1β concentrations in the peritoneal exudate (Figure 3). The level of IL-1β in the peritoneal cavity of control animals (saline group) was 713 ± 35.3 pg/ml and increased to 1230.0 ± 76.94 pg/ml after carrageenan injection. Compared with the carrageenan group, the animals pretreated with PLS (10 mg/kg, i.p.) showed significantly decreased IL-1β peritoneal concentration (621.1 ± 9.2 pg/ml).
Anti-inflammatory effect of sulphated polysaccharide fraction on carrageenan-induced peritonitis in mice

As shown in Figure 4a, compared with the carrageenan group, the PLS-administered group showed significantly reduced peritoneal leucocyte count ($7970.0 \pm 1078.0$ vs $550.0 \times 10^3 \pm 97.47 \times 10^3$ cells/ml). Furthermore, the same

Figure 2 Effect of sulphated polysaccharide fraction on carrageenan-induced myeloperoxidase activity in paw tissue. Saline (s.c.) or carrageenan (500 μg per paw) was injected into the plantar surface of mice. One hour before this injection, animals had been treated with indometacin (IND; 10 mg/kg, i.p.) or sulphated polysaccharide fraction (PLS; 10 mg/kg, i.p.). Myeloperoxidase (MPO) activity was detected in the paw tissue after 4 h. The results are expressed as the mean ± SEM MPO units (UMPO/mg of tissue). *P < 0.05 compared with carrageenan group; #P < 0.05 compared with saline plus carrageenan group with saline group. Statistical analysis was performed using analysis of variance followed by Bonferroni post hoc test.

Figure 3 Effect of sulphated polysaccharide fraction on carrageenan-induced cytokine production in peritonitis. The level of interleukin (IL)-1β in the peritoneal cavity was measured 4 h after carrageenan injection. Mice were orally administered sulphated polysaccharide fraction (PLS; 10 mg/kg) or indometacin (IND; 10 mg/kg), followed by injection of 250 μl carrageenan (500 μg per cavity, i.p.) after 1 h. Each point represents the mean ± SEM values obtained from five animals. *P < 0.05 compared with carrageenan group; #P < 0.05 compared with saline group. Statistical analysis was carried out using one-way analysis of variance followed by Bonferroni post hoc test.

Figure 4 Anti-inflammatory effect of sulphated polysaccharide fraction on carrageenan-induced peritonitis in mice. (a) Total count of leucocytes. (b) Count of neutrophils per cavity. Mice were administered 250 μl saline (i.p.), indometacin (IND; 10 mg/kg, p.o.), or sulphated polysaccharide fraction (PLS; 10 mg/kg, p.o.), followed by injection of 500 μg carrageenan diluted in 250 μl saline solution (i.p.) after 1 h. Mice were killed 4 h later, and the peritoneal cavity was washed with 1.5 ml heparinised phosphate-buffered saline (PBS) to harvest the peritoneal cells. The white bars represent the peritoneal neutrophils in the saline-injected mice (control group). The values are represented as mean ± SEM. *P < 0.05 compared with carrageenan group; #P < 0.05 compared with saline group. Statistical analysis was performed by analysis of variance followed by Bonferroni post hoc test.
dose of PLS significantly reduced neutrophil migration into the peritoneal cavity (208.6 × 10^3 ± 46.84 × 10^3 cells/ml; Figure 4b) compared with that in the carrageenan group (4679.0 × 10^3 ± 317.3 × 10^3 cells/ml). This result was consistent with the fact that neutrophils are the most abundant cells in primary inflammatory exudates.

**Effect of aminoguanidine and L-arginine on the inhibitory effect of sulphated polysaccharide fraction on neutrophil migration**

Figure 5a shows that aminoguanidine when co-administered with PLS plus carrageenan increased the leucocyte count (4800 × 10^3 ± 738.7 × 10^3 cells/ml) relative to that in the PLS plus carrageenan group (1325.0 ± 78.26 × 10^3 cells/ml). L-Arginine when co-administered with PLS and aminoguanidine decreased the leucocyte count (425.0 × 10^3 ± 156.1 × 10^3 cells/ml) relative to that in the aminoguanidine plus PLS group (4800 × 738.7 × 10^3 cells/ml). The same dose of aminoguanidine also significantly increased neutrophil migration into the peritoneal cavity (Figure 5b; 4788.0 × 737 ± 10^3 cells/ml) relative to that in the PLS plus carrageenan group (1290.0 ± 85.73 × 10^3 cells/ml). When co-administered with PLS and aminoguanidine, L-arginine resulted in a decrease in the neutrophil count (424.0 × 154.1 × 10^3 cells/ml) relative to that in the aminoguanidine plus PLS group (4788.0 × 737 ± 10^3 cells/ml).

**Effect of aminoguanidine and L-arginine on the inhibitory effect of sulphated polysaccharide fraction on carrageenan-induced paw oedema**

As shown in Figure 6, when co-administered with PLS, aminoguanidine increased the volume of paw oedema (first hour: 0.0575 ± 0.0014 ml; second hour: 0.0500 ± 0.009 ml; third hour: 0.0460 ± 0.006 ml) relative to the volumes in the PLS plus carrageenan group (first hour: 0.002 ± 0.001 ml; second hour: 0.000 ± 0.000 ml; third hour: 0.000 ± 0.000 ml). L-Arginine when co-administered with PLS and aminoguanidine decreased the volume of paw oedema (first hour: 0.005 ± 0.004 ml; second hour: 0.004 ± 0.002 ml; third hour: 0.0025 ± 0.002 ml) relative to the volumes in the aminoguanidine plus PLS group.

**Effect of sulphated polysaccharide fraction on peritoneal fluid levels of nitric oxide (NO3/NO2) in a peritonitis model**

As shown in Figure 7, the PLS plus carrageenan group showed an increased level of NO3/NO2 in peritoneal fluid (0.1022 ± 0.006 μM) relative to that in the carrageenan group (0.756 ± 0.003 μM) or the saline group (0.0778 ± 0.005 μM).

**Discussion**

Natural products of algae origin are used in folk medicine all over the world and exhibit a wide range of pharmaco-
logical activities. Over the years, natural products have contributed enormously to the development of important therapeutic drugs used currently in modern medicine.\(^\text{[22,23]}\)

Polysaccharides extracted from algae can play a relevant role in biomedical and pharmaceutical applications, particularly in the field of drug delivery. In this study, we sought to investigate the possible anti-inflammatory effect of PLS extracted from the marine red algae *H. musciformis* by using pharmacological tools and molecular procedures.

Our results clearly demonstrated that PLS has an anti-inflammatory effect in mice models of inflammation (paw oedema, peritonitis, MPO, cytokine levels). The administration of PLS (5 and 10 mg/kg, i.p.) induced long-lasting inhibition of paw oedema at all intervals. PLS prevented carrageenan-induced and dextran-induced paw oedema. Carrageenan-induced paw oedema involves several chemical mediators, including histamine, serotonin, bradykinin, and prostaglandins.\(^\text{[24,25]}\) In this model, the oedema is believed to be biphasic, with the first phase being mediated by the release of histamine and serotonin, followed by the subsequent release of bradykinin, NO, and prostaglandins. The late oedema phase is dependent on cytokine production by resident cells and neutrophil infiltration.\(^\text{[26–28]}\) In contrast, dextran-induced paw oedema is mediated by increased vascular permeability induced by mast cell degranulation of histamine and serotonin.\(^\text{[29]}\) The oedematous fluid induced by dextran injection contains little protein and few neutrophils.\(^\text{[30]}\) Therefore, we can infer that the anti-oedematogenic action of PLS might be because of the differential inhibition of the mediators involved in inflammatory events and modulation of neutrophil infiltration into the inflamed plantar tissue.

The paw inflammatory response induced by carrageenan was accompanied by an intense neutrophil infiltration.\(^\text{[31,32]}\) MPO activity is commonly considered an indicator of neutrophil infiltration. Our results demonstrated that PLS (10 mg/kg) inhibited neutrophil infiltration, which was evident from the reduced MPO activity measured in the mouse paws. These results suggested that the anti-oedematogenic effect of PLS was related to inflammatory events involving neutrophil migration.

The late oedema phase induced by carrageenan is known to be dependent on cytokine production by resident cells and neutrophils.\(^\text{[26–28]}\) Our results demonstrated that PLS decreased the level of IL-1\(\beta\) in the peritoneal fluid.

IL-1\(\beta\) is a potent pro-inflammatory cytokine that has multiple effects, including activation of inflammatory cells, induction of several inflammatory proteins, cytotoxicity,
oedema formation, and neutrophil migration.\[33\] On these
effects, we could infer that the anti-inflammatory effect of
PLS might occur through the inhibition of cytokines
involved in carrageenan-induced peritonitis.

In the neutrophil migration, our results demonstrated
that PLS significantly reduced peritoneal leucocyte and neu-
trophil counts. Carrageenan induces neutrophil migration
into the mouse peritoneal cavity through an indirect
mechanism that involves the activation of macrophages and
the release of cytokines into the peritoneal cavity.\[34\] In this
study, we have shown for the first time that PLS administra-
tion prevented the carrageenan-induced increase in perito-
neal levels of IL-1β. These results suggested that the anti-
inflammatory effect of PLS may occur through the
inhibition of cytokines involved in carrageenan-induced
peritonitis.

During the inflammatory process, concomitant with the
release of neutrophil chemotactic factors such as cytokines
and chemokines, NO is produced by either cNOS or iNOS
and results in the down-modulation of neutrophil recruit-
ment to the inflammatory site.\[35,36\] Based on this informa-
tion we verified the influence of L-arginine/NOS pathway in
the neutrophil migration-induced by carrageenan. Our
results demonstrated that aminoguanidine when co-
administered with PLS increased the leucocyte and neut-
trophil counts, and L-arginine when co-administered with
PLS and aminoguanidine decreased leucocyte and neu-
trophil counts.

The effects of the NOS inhibitors on neutrophil migra-
tion appear to be a consequence of the inhibition of the
L-arginine/NOS pathway because the enhancement of neu-
trophil migration was reversed by co-treatment with
L-arginine, the NOS substrate. The fact that the inhibition
of NO production enhances neutrophil migration suggests
that during the inflammatory process, simultaneous with
the release of neutrophil chemotactic factors, NO is pro-
duced and that it down-modulates the recruitment of neu-
trophils to the inflammatory site.\[37,38\] On the basis of these
results, we can infer that the L-arginine/NOS pathway par-
ticipated in the anti-inflammatory effect of PLS by modu-
lating the migration of neutrophils into the inflammatory
site.

To strengthen our hypothesis, we performed the
carrageenan-induced paw oedema experiment with
L-arginine/NOS pathway modulation. Our results clearly
demonstrated that aminoguanidine when co-administered
with PLS increased the volume of paw oedema and
L-arginine when co-administered with PLS and aminogua-
nidine decreased the carrageenan-induced paw oedema.

The late oedema phase induced by carrageenan is known
to be dependent on cytokine production by resident cells,
neutrophil infiltration, the release of neutrophil-derived
mediators, as well as the production of neutrophil-derived
free radicals, such as hydrogen peroxide, superoxide, and
hydroxyl radicals, and the production of malondialdehyde
is due to the attack of plasma membranes by free
radicals.\[26–28,39,40\] Our results suggested that the administra-
tion of PLS may lead to increased production of endog-
ogenous NO, which results in a reduced inflammatory
response by decreasing the migration of neutrophils to the
focus of the inflammatory site.

In the evaluation of peritoneal fluid levels of NO (NO3/
NO2) in a mouse peritonitis model, our results demon-
strated that PLS increased serum level of NO3/NO2 in
peritoneal fluid. The measurement of NO3/NO2 levels is a
method that is used to quantify endogenous NO produc-
tion.\[41\] The results obtained from this study suggested that
during the inflammatory process, NO released by either
cNOS or iNOS, during the PLS administration, down-
modulated the migration of neutrophils to inflammatory
sites.

**Conclusions**

PLS effectively reduced carrageenan-induced and dextran-
induced paw oedema in mice, as well as carrageenan-
induced peritonitis. Moreover, PLS modulated the
production and release of pro-inflammatory cytokines and
carried an increase in the levels of NO. This anti-
inflammatory effect of PLS appeared to be mediated by the
activation of the L-arginine/NOS pathway. These observa-
tions raise the possibility that the administration of polysac-
charides may represent new strategies for the treatment of
inflammatory disease.

**Declarations**

**Conflict of interest**

The Author(s) declare(s) that they have no conflicts of
interest to disclose.

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