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Anticoagulant activity, paw edema and pleurisy induced carrageenan: Action of major types of commercial carrageenans

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ABSTRACT

Considering the use of commercials carrageenans as a model for inflammation, the aim this work is a comparative study these compounds in the pro-inflammatory action by ear edema and pleurisy and to analyze their anticoagulant activity. Paw edema was induced by injecting kappa, iota and lambda carrageenans in saline solution into the hind paw of male Wistar rats ($p < 0.05$). The three types of carrageenans showed different volume in pleural exudates, characterized by fluid accumulation, a large number of neutrophils and raised NO production ($p < 0.001$). The activated partial thromboplastin time (aPTT) for kappa and iota carrageenans (100 μg) was 240 s and 132 s, respectively. Lambda carrageenan was the most potent anticoagulant at 240 s (20 μg). These carragenans demonstrated no anticoagulant action using the PT test in vitro. Histological analysis in paw edema demonstrated that iota and lambda carrageenans showed major cellular infiltration in relation to kappa. Thus, quantitative evaluation of inflammation demonstrated that iota and lambda carrageenans have higher inflammatory potential than do kappa carrageenans.

1. Introduction

Carrageenan is a generic name for a family of linear, sulfated galactans, obtained from certain species of marine red algae. They are widely used as texturizing, viscosity-building and gel-forming ingredients in the food and pharmaceutical industry (Navarro & Stortz, 2005). They represent a multi-million-dollar market that is growing steadily (Velde, de Knutsen, Usov, Rollema, & Cerezo, 2002). The backbone of the polysaccharide is composed of D-galactose units (G) linked alternately to α-(1 → 3) and β-(1 → 4) linkages. The β-linked residue always belongs to the D-series, the α-linked residues may be either D- or L-galactose units, partially occurring as 3,6-anhydrogalactopyranosyl moieties. Carrageenans are classified according to the presence of 3,6-anhydro-D-galactose (AG) in the 1,4-linked residue and the position and number of sulfate groups (de Ruiter & Rudolph, 1997; Falshaw et al., 1996). Since natural carrageenan is a mixture of non-homologous polysaccharides, the term disaccharide-repeating unit refers to the idealized structure (Velde et al., 2002). The most common types of carrageenans are traditionally identified by a Greek letter. The three main industrial types are kappa, iota and lambda carrageenans. The kappa (κ) and iota (ι) forms are gelling polymers, whereas lambda (λ) is a non-gelling, thickening agent (Usov, 1998). Several studies have shown that iota carrageenans, with additional sulfate groups in the anhydrous galactose residues, display greater hydrophilic properties than those of kappa carrageenans (Stanley, 1990).

In recent years, the medical potential of seaweed polysaccharides has increasingly attracted the attention of researchers. Antitumor, antivirus, anti-hyperlipidemia and anticoagulant biological activities have been found in seaweed polysaccharides (Carlucci et al., 1997; Girond, Crance, Van Cuicky, Renaudet, & Delaince, 1991; Zhou, Sheng, Yao, & Wang, 2006). Studies have demonstrated that the sulfate concentration and molecular weight of seaweed polysaccharides influence their biological activities (Franz & Alban 1995).

The inflammatory process is characterized by pain, redness of the skin, edema and heat. The production of protaglandins, leukotrienes, histamine, bradykinin, nitric oxide, PAF and by the release of cells from other chemical compounds. Paw edema induced carrageenans represent a classical inflammation model that has been extensively used in nonsteroidal anti-inflammatory drug development. The inflammatory response is usually quantified by an increase in paw size (edema after carrageenan injection) and is modulated by inhibitors of specific molecules within the inflammatory cascade. The level of pro-inflammatory response of these three polysaccharides can be determined by simultaneous quantitative temporal measurement of the number of inflammatory cells

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mobilized by fluid exudation, leading to a local swelling formation consisting of leukocytes (Cuzzocrea et al., 1998).

Thus, the aim of this comparative study was to analyze the pro-inflammatory action of the main types of commercial carrageenans by ear edema and pleurisy and to measure their anticoagulant activities.

2. Experimental

2.1. Animals

The male Wistar rats ($n = 6$, 6–8 weeks old, 140–180 g body weight) used in this study were housed in a temperature controlled (24 ± 2 °C) and light controlled room (12 h light/dark cycle), with free access to water and food. The number of animals were $n = 6$ by groups in studies of carrageenan induced pleurisy and paw edema. All procedures were in accordance with NIH Animal Care Guidelines.

2.2. Materials

Carrageenans: Iota (Type V, C3799), Kappa (Type III, C1263), Lambda (Type VI, C3889), Zymosan, KBr, sodium diclofenac and D_2O_2 were obtained from Sigma Chemical Co. (St. Louis, MO, EUA). aPTT and PT kits were obtained from Labtest, São Paulo, SP/Brazil.

2.3. Chemical analysis

Total hexose was measured by Dubois, Gilles, Hamilton, Rebers, and Smith (1956 method), total sulfate was estimated by the $BaCl_2$ gelatin method (Dodgson & Price, 1962) and protein content was measured by Spector's, technique (1978). Standard curves for hexose and sulfate were constructed from galactose and Na_2SO_4, respectively.

2.4. FIT-IR spectroscopy

A FT-IR ABB Bomem MB 104 spectrometer was used; 32 scans per spectrum; nominal resolution: 4 cm^{-1}; scan speed: 20 scans per minute. All the spectra were recorded in the 4000–400 cm^{-1} range and KBr pellets sample were prepared.

2.5. ^{13}C NMR Spectroscopy

^{13}C NMR spectra were obtained at 500 MHz using a Bruker AMX500 spectrometer. Chemical shifts were given in values relative to DSS at 0 ppm. The signals in the ^{13}C NMR spectrum were assigned based on literature data for correlated compounds.

2.6. Anticoagulant effect "in vitro"

The plasma was obtained by centrifugation (1000 g) of a mixture of human blood and citrate (9:1). Part of this plasma (90 μl) was then homogenized with 10 μl of carrageenan solution at different concentrations and incubated for 3 min (37 °C) with 0.1 ml liquid cephalin. The activated partial thromboplastin time (aPTT) was determined using normal citrated human plasma according to the manufacturer's specifications (Labtest, São Paulo, SP, Brazil). For the prothrombin time (PT) assay, 90 μl of normal citrated human plasma was mixed with 10 μl of carrageenan solution at different concentrations and incubated for 1 min at 37 °C. The PT assay reagent (200 μl), preincubated for 3 min at 37 °C, was then added and the clotting time recorded with a Quick Times coagulometer (Drake Ltd., São Paulo, SP, Brazil).

2.7. Carrageenan-induced paw edema

The animals were lightly anaesthetized with ethyl ether and injected intradermally with 100 μl of carrageenan in the right hind paw. The left paw received 100 μl of saline and was used as control. The carrageenans were dissolved in saline solution. Each type of carrageen was applied in different concentrations (0.1%, 0.2%, 0.5% and 1%). Zymosan (1 mg) was diluted in 100 μl of 0.9% saline. The paw edema and the temperature were measured immediately after carrageenan injection and at 1, 2, 3, 4, 8, 12 and 24 h. The difference in volume between the right and left hind paws was considered paw edema and was measured with a pachymeter. Six rats per group were used in the experiments.

2.8. Carrageenan-induced pleurisy

Carrageenan-induced pleurisy was measured as previously described (Cuzzocrea et al., 1998). The rats ($n = 6$) were lightly anaesthetized under ether and submitted to a skin incision at the level of the sixth left intercostal space. The underlying muscles were dissected and 0.2 ml saline, alone or containing 1% carrageenans (iota, kappa and lambda), was injected into the pleural cavity. The skin incision was closed with a suture and the animals were allowed to recover. The animals were killed 4 h after carrageenan injection. The chest was carefully opened and the pleural cavity washed with 2 ml of saline solution with heparin (5 U/ml) and indomethacin 10 mg/ml). The exudates and washing solution were removed by aspiration and the total volume measured. Blood-contaminated exudates were discarded. The results were calculated by subtracting the volume injected (2 ml) from the total volume recovered. The number of leukocytes in the exudates was suspended in phosphate buffer saline and counted with an optical microscope in a Neubauer chamber after staining with Türck solution.

2.9. Measurement of nitrate/nitrite

Nitrite and nitrate production, an indicator of NO synthesis, was measured in the supernatant samples, as previously described (Cuzzocrea et al., 1998). First, nitrate in the supernatant was reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 mM) at room temperature for 3 h. After this time, nitrite concentration in the samples was measured using the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulfanilamide in 5% concentrated H_3PO_4; vol. 1:1) to 100 μl samples. The optical density at 540 nm was measured using ELISA microplate reader. Nitrate concentrations were calculated by comparison with OD 540 nm of standard solutions of sodium nitrite.

2.10. Histological examination

For histological examination, paw biopsies were taken 4 h after carrageenan-induced edema. Tissue slices were fixed in 10% neutral-buffered formaldehyde, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin.

2.11. Statistical analysis

Values are presented as mean ± SEM. Analysis of variance (ANOVA, Bartlett's method and Tukey–Kramer test) were used for data evaluation and $p < 0.05$ was accepted as statistically significant.

3. Results and discussion

3.1. Chemical analysis

Chemical tests were performed to characterize the three carrageenans. Quantitative determination showed that iota carrageenan has lower sulfate content (27.20 ± 2.01%) than that of lambda carrageenan (33.38 ± 0.87%), while kappa carrageenan (17.89 ± 1.81%) has the lowest of the three. Chemical analysis revealed kappa, iota and lambda levels of 60.26 ± 0.11%, 64.50 ± 0.23% and 61.20 ± 0.81% for total sugars. All the carrageenans showed low protein contamination (Table 1 all).

3.2. FT-IR spectroscopy

The Fourier transformed infrared (FT-IR) of the commercial carrageenans showed a typical absorption band around 1250 cm^{-1}, corresponding to the ester sulfate groups (S=O), whose intensity decreases from the lambda carrageenan (high values) to the iota and kappa carrageenan (lower bands) (Fig. 1A–C). The IR spectra of kappa and iota carrageenan display one band at 845 cm^{-1} arising from the galactose-4-sulfate (Fig. 1A and B). In addition, they have strong, broad absorption bands, characteristic of all polysaccharides, in the 1000–1100 cm^{-1} region (Turquois, Acquistapace, Vera, & Welti, 1996). The iota carrageenan had an additional characteristic band around 805 cm^{-1}, associated to the sulfate group, given the 3,6-anhydrogalactose-2-sulfate structure (Fig. 1B). Band absorbance provides information on the presence in polymers of 3,6 anhydrogalactose at 930 cm^{-1},galactose-4-sulfate at 845 cm^{-1}, galactose-4-sulfate at 830 cm^{-1}, galactose-6-sulfate at 820 cm^{-1} and 3,6-anhydrogalactose-2-sulfate at 805 cm^{-1} (Volery, Besson, & Schaffer-Lequert, 2004; Černá et al., 2003). As expected, the absorbance of this band is much higher for lambda (Fig. 1C) than for kappa and iota carrageenan. All these spectra display an absorbance band at 2920 cm^{-1} due to C—H content and at 1250 cm^{-1} due to total sulfate.

3.3. ^{13}C NMR spectroscopy

NMR spectroscopy (both ^{1}H and ^{13}C NMR) is one of the standard tools used for determining the chemical structure of carrageenan samples. NMR experiments were carried out at an elevated temperature to reduce the viscosity of the lambda carrageenan solution. In this paper the ^{13}C NMR spectroscopy of galactans from red algae was highly regular. The chemical shifts of kappa, iota and lambda carrageenans are given in Table 2. It should be noted that these assignments are closely related to the literature values. (Bhattacharjee, Yaphe, & Hamer, 1978, Usov et al., 2004; Usov, 1984). However, a few signals attributed to lambda carrageenan differ from those observed in this paper (Falshaw & Furneaux, 1994; Navarro & Stortz, 2005). According to Noseda & Cerezo, 1993, such divergences are common and are a result of the various temperatures used in the NMR and the MW of the polymers. The anomeric carbon signals of 104.7 and 96.4 ppm are characteristic of kappa carrageenan and attributed to C-1 of β-galactose and

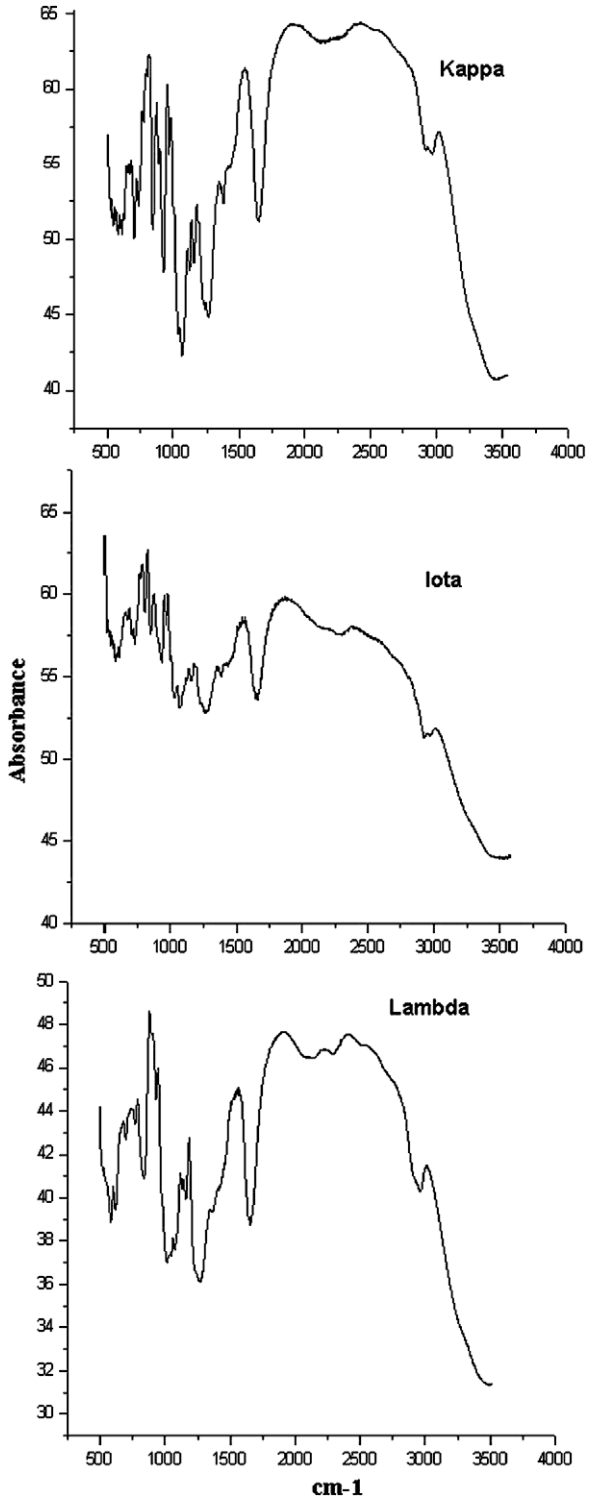

Fig. 1. Infra red spectra (400–4000 cm^{-1}) of polysaccharide, kappa, iota and lambda carrageenans.

Table 1
Chemical composition of kappa, iota and lambda commercial carrageenans.

Carrageenans	Protein (%)	Total sugars (%)	Sulfate (%)*
Kappa	0.2 ± 0.09	60.26 ± 0.11	17.89 ± 1.81
Iota	0.1 ± 0.23	64.50 ± 0.23	27.20 ± 2.01
Lambda	0.1 ± 0.15	61.20 ± 0.81	33.38 ± 0.87

The value are mean ± (SD).
* Expressed as SO_3Na.

C-1 of α-galactose linked ß-galactose 2-sulfate, values that are in agreement with the data reported by Usov, Velde, Knustsen, Rollemah, & Cerezo, 2002 (Fig. 2A). The signal at 93.8 ppm is typical of iota carrageenan linked to anhydrogalactose. The anomeric signals at 105.61 and 93.8 ppm are characteristic of lambda carrageenan (Fig. 2B). The spectra of lambda carrageenan showed anomeric signals at 103.9 attributed to C-1 of β-galactose 2-sulfate linked to reductor α-anhydrogalactose (Fig. 2C). The signal at 101.9 ppm is

Table 2
Anticoagulant activity of commercials carrageenans by aPTT assay (A) and PT assay (B).

	Concentration (μg)							
	0	5	10	15	20	40	60	100
Polysaccharides								
A								
Heparin	34.3	240	240	240	240	240	240	240
Carragenan kappa	34.3	35.1	50.2	51.8	55.7	88.3	108.7	132.3
Carragenan iota	34.3	34.6	40.1	40.2	36.1	54.1	87.9	240
Carragenan lambda	34.3	47.87	70.7	116.8	240	240	240	240
B								
Heparin	0	120	120	120	120	120	120	120
Carragenan κ	0	15.2	n.d. [a]	15.9	16.93	23	25.33	31.07
Carragenan ι	0	18.1	18.9	21.3	25.4	25.53	27.87	30.5
Carragenan λ	0	9.53	14.13	83.57	120	120	120	120

[a] Not determinated.

indicative of C-1 of ß-galactose 2-sulfate linked to α-3,6 anhydrogalactose (Noseda and Cerezo,1993, van de Velde, Pereira, & Rollema, 2004).

3.4. Anticoagulant effect "in vitro"

There is ample evidence that inflammation and coagulation are intricately related processes that may considerably affect each other. This interaction occurs in platelet activation, fibrin formation, and resolution as well as in the physiological anticoagulant pathways (Levi, van der Poll, & Büller, 2004). Based on this fact, we determined the anticoagulant activity of these sulfated polysaccharides. The anticoagulant activity (aPTT and PT) of kappa and iota carrageenans was assessed using human plasma from healthy donors. The mechanism of the anticoagulant activity of carrageenans can be shown via thrombin inhibition. The anticoagulant properties of the three carrageenans were compared with unfractionted heparin. The results obtained in an aPTT assay showed that carrageenans had very low anticoagulant activity when compared to heparin from bovine lung (Table 2A). Kappa and iota carrageenans exhibited anticoagulant activity of 132.2 and 240 s at a concentration of 100 μg. Although lambda carrageenans displayed elevated anticoagulant activity (240 s at 20 μg), this value is much lower than that of heparin (250 s at 2.5 μg). The compounds may act only on the intrinsic pathways of the blood coagulation system and not on the extrinsic pathways (Table 2B). According to Boisson-Vidal et al., 2000, polysaccharides in which the native sulfated pattern was intact were more potent than polymers of equivalent molecular weight and more potent than the overall degree of sulfation in which this pattern had been disrupted by partial desulfation. The main basis of the anticoagulant activity of carrageenans appears to be an anti-thrombic property (Shanmugam & Mody, 2000). We observed that carrageenans extracted from the red algae *Hypnea musciformis* act on inductions of heparan sulfate with antithrombotic properties (results not shown). However, algal heparinoids are natural marine products and as such do not pose a risk of contamination by prions and viruses (Leite et al., 1998). The anticoagulant activity mechanism of carrageenans can be shown via thrombin inhibition, given that compounds have negative charges.

3.5. Carrageenan-induced paw edema

Carrageenan-induced inflammation in the rat paw represents a classical model of edema formation and hyperalgesia that has been extensively used in the development of nonsteroidal anti-inflammatory drugs. The inflammatory response is usually quantified by an increase in paw size (edema post carrageenan injection) and is modulated by inhibitors of specific molecules within the inflammatory cascade. In this research, paw swelling was induced with different carrageenans. The kappa carrageenan showed high edema in the first 5 h and a prolonged effect after 2–8 h when compared to other carragenans at 1% (Fig. 3A). Lambda carrageenans, although difficult to dissolve in water, demonstrated less action than that of kappa on paw edema at 1% (Fig. 3A and C). The kappa, iota and lambda carrageenans at 1% increased paw edema by 3.7, 4.0 and 4.2 mm (see Fig. 3A, B and C) with ($p < 0.0001$) when compared to the control group. When compared to zymosan, a polysaccharides with pro-inflammatory properties, paw edema increased 10% in relation to the carrageenans analyzed in this study. (Fig. 4A). The anti-inflammatory sodium dicofenac decreased the inflammatory effect in 40% (Fig. 4B).

3.6. Carrageenan-induced pleurisy

The injection of several carrageenans into the pleural cavity of rats elicited an acute inflammatory response, characterized by the accumulation of fluid (Table 4) containing large amounts of polymorphonuclear cells (PMNs). A high concentration of fluid was observed in the lambda carrageenan in relation to the kappa and iota carrageenans. The degree of exudation and PMN (Fig. 5) was very significant for kappa with 38×10^6 ($p < 0.010$), iota $52 \times 10^6 \pm 1.70$ ($p < 0.001$) and lambda $47 \times 10^6 \pm 0.47$ ($p < 0.001$) when compared to saline ($20 \times 10^6 \pm 0.41$).

3.7. Measurement of nitrate/nitrite

NO levels were significantly increased ($P < 0.001$) in the exudates of carrageenan-treated rats (Fig. 6). NO is a readily diffusible and highly reactive radical that can attack a wide range of molecules. NO appears to modulate edema formation not only by increasing local blood flow but also by stimulating prostaglandin formation at the inflammation site (Usov et al., 2002, Salvatore, Zingarelli, Gilad, Salzman, & Szabó, 1997). Nitrite/nitrate production, an indicator of NO synthesis inflammatory modulator, was measured in pleural exudate. The nitrite/nitrate level for these polysaccharides was 21.04 ± 0.72, 33.17 ± 0.89 and 63.48 ± 2.58 nmoles for kappa, iota and lambda carrageenans, respectively. The NO counts were high for iota and lambda carrageenan in relation to the groups of animals that received saline. However, the nitrite/nitrate level of lambda in relation to kappa increase in 67%.

In addition to examining the putative role of NO, pleural inflammation was elicited in rats by the intrapleural injection of carrageenan (1%). A pleural exudate and cellular influx developed, peaked at 24 h and was generally resolved by 72 h. The pleural inflammation provoked by these polysaccharides quickly

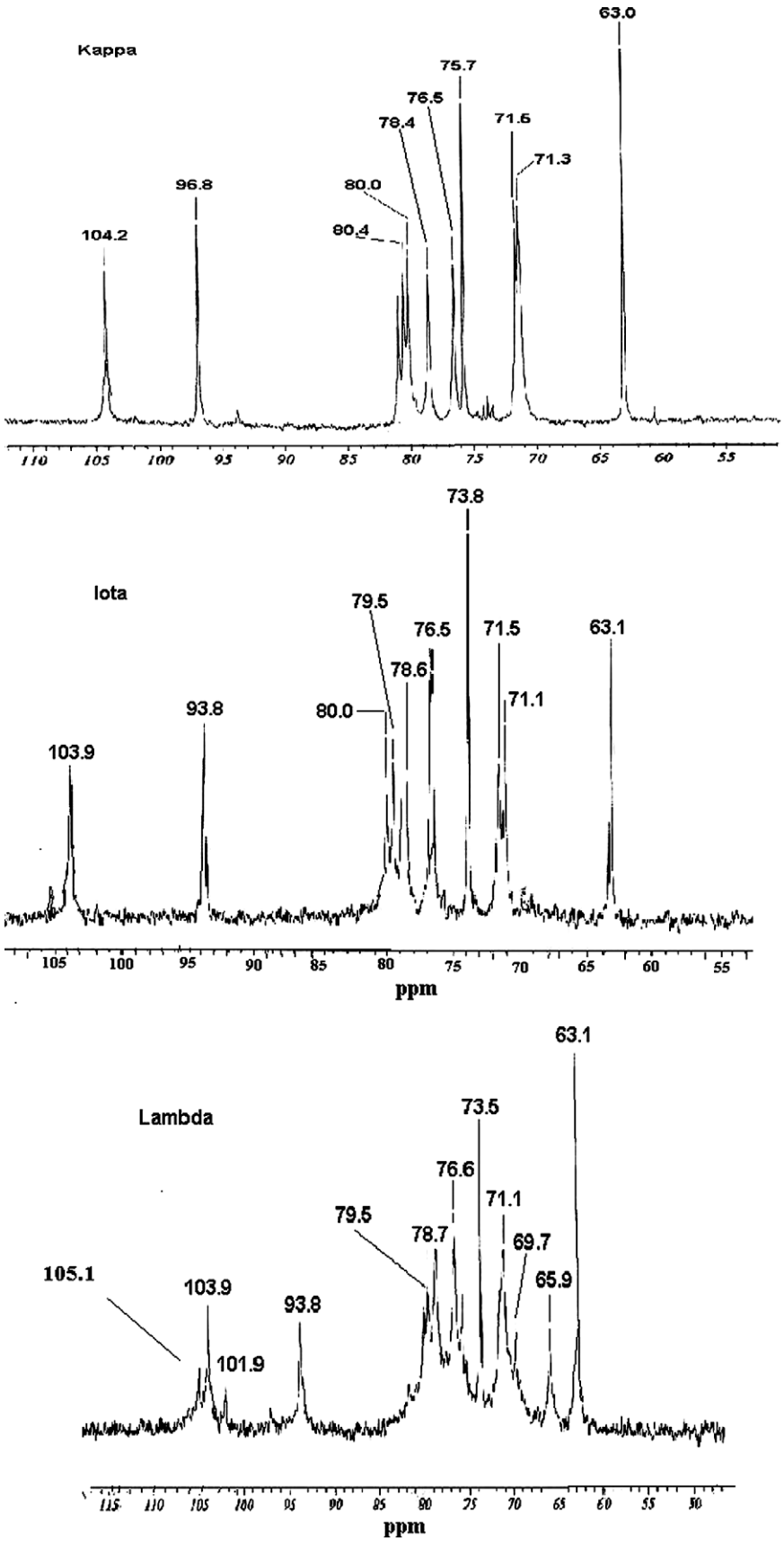

Fig. 2. ^{13}C NMR spectra (500 MHz) of polysaccharide, kappa, iota and lambda carrageenans. The samples were deuterium-exchanged several times by freeze-drying from $^{2}H_2O$ and then examined in solution (50 mg/ml) in 99.98% $^{2}H_2O$. Chemical shifts are given in p.p.m., using DMSO (^{13}C) as references.

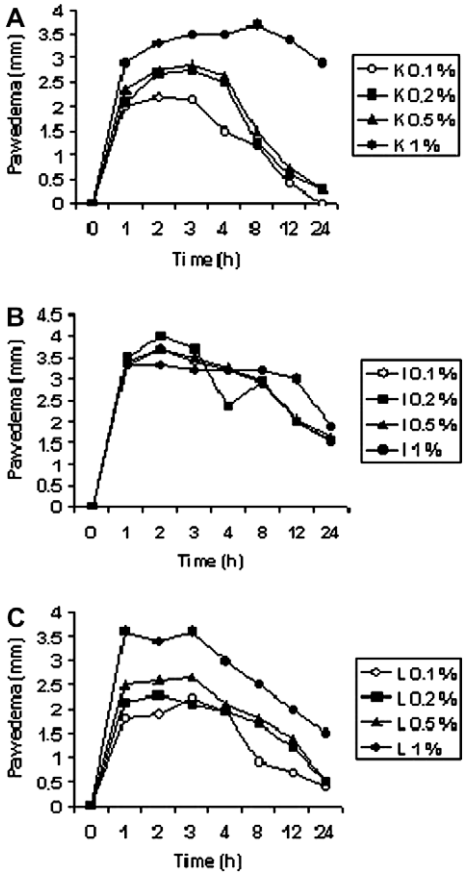

Fig. 3. Paw edema (mm) induced with concentrations (0, 1-1%) of (A) kappa, (B) iota and (C) lambda carrageenans.

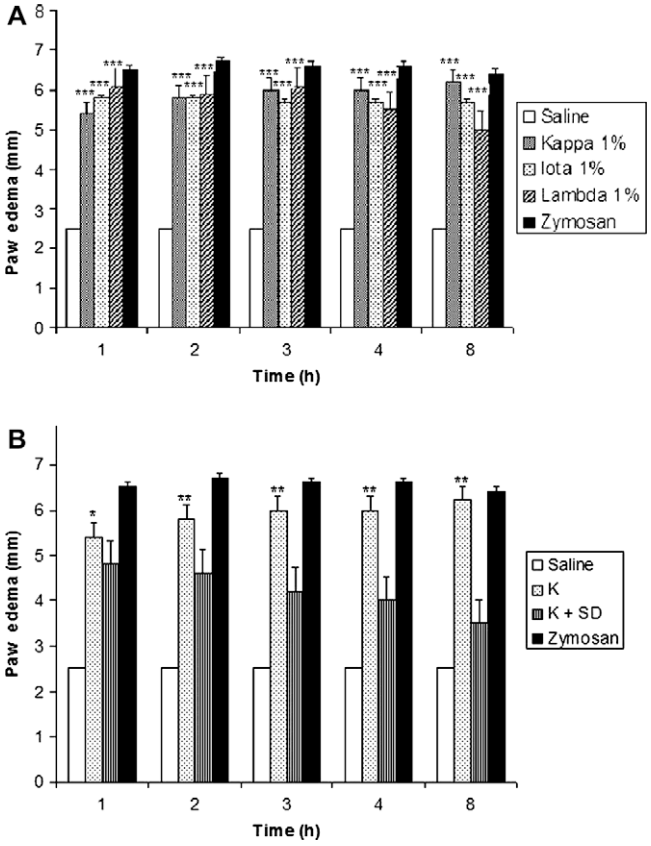

Fig. 4. (A) Paw edema (mm) induced with carrageenans (kappa, iota and lambda). Control (saline solution), zymosan (positive control). A value of $p < 0.001$(***) was considered statistically significant. (B) Effect of kappa carrageenan (1%), zymosan (1 mg/100 μl) and sodium diclofenac (5 mg kg^{-1}) on paw edema in Wistar rats. The differences between treatment and control were tested using by ANOVA. A value of $p < 0.01$(**) was considered statistically significant.

mobilized target systems, affecting the lymphonodes and increasing the number of leukocytes. The cellular influx was primarily composed of polymorphonuclear cells for the first 3–5 h, followed by macrophages in the subsequent 24 h. The highest volume of pleural exudates was observed in the group that was administered lambda carrageenans (1.83 ± 0.13 ml) in relation to the kappa (1.26 ± 0.01 ml) and iota (1.40 ± 0.03 ml) groups. These results are in accordance with those of the literature (Cuzzocrea et al., 1997).

3.8. Histological examination

Histological examination of paw edema sections revealed a significant increase in infiltration damage by PMNs, a common occurrence with inflammation (Fig. 7). Thus, paw edema sections taken from saline-treated animals, and histological examination of the sections of rats treated with carrageenans showed edema, tissue injury and a high level of PMNs. Furthermore, injection of three carrageenans into the paw of rats ($n = 6$) elicited an acute inflammatory response characterized by an accumulation of fluid (edema) that contained large amounts of PMNs. This effect was high for iota and lamda carrageenans in relation to kappa carrageenans. The iota and lambda polysaccharides are more pronounced than the kappa carrageenans.

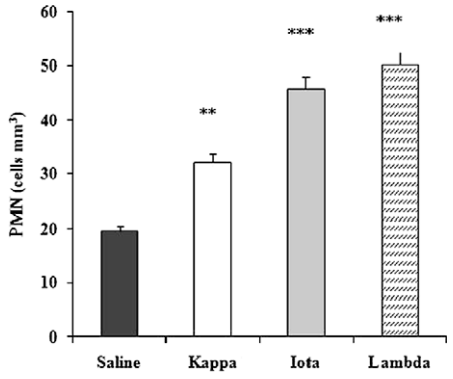

Fig. 5. Polymorphonuclear leukocytes (PMNs × 10^3) after 3 h carrageenan-induced pleurisy. **$p < 0.01$ vs. control ***$p < 0.001$ vs. control.

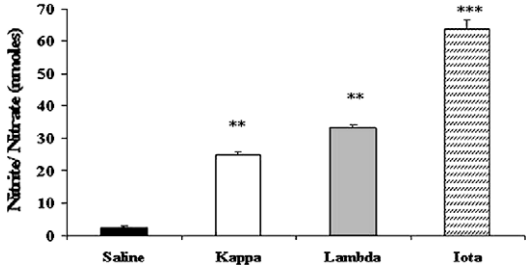

Fig. 6. Effect of kappa, iota and lambda carrageenans on the nitric oxide level.

4. Conclusion

Three primary forms of commercial carrageenans (kappa, iota and lambda) were characterized based on chemical, FIT-IR and NMR spectroscopies. In the present study, our aim was to compare

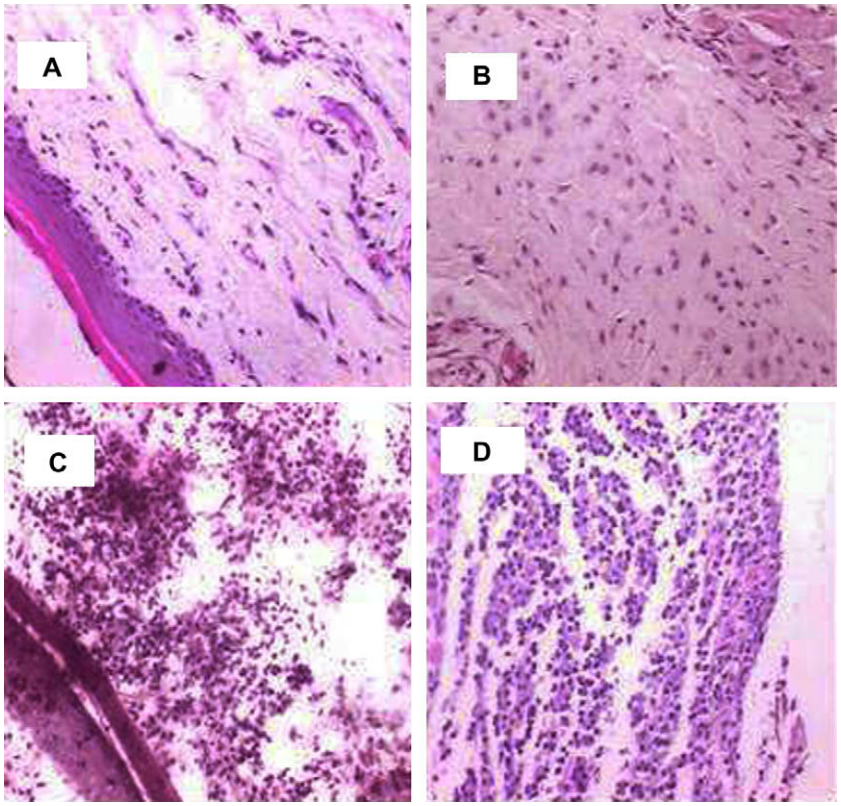

Fig. 7. Histological examination of paw sections after 3 h at 1% carrageenan injection. (A) Control (saline solution), (B) kappa carrageenan, (C) iota carrageenan, (D) lambda carrageenan.

the effect of three carrageenans on the anticoagulant and inflammatory activity of two inflammation models in Wistar rats. Paw edema and pleurisy inflammation were measured in relation to carrageenan concentration. In the inflammation of the pleural model we demonstrated that iota carrageen induced greater inflammation, evidenced by cellular infiltration, and high NO production. Since several sulfated polysaccharides are endowed with anticoagulant properties, the antithrombin activity of these compounds was evaluated concurrently with inflammatory activity. The results lead us to conclude that the high anticoagulant activity (aPTT) of lambda carrageenans (high sulfate) and the low activity of kappa carrageenans (low sulfate) is likely owing to the sulfate content in the carrageenans. Assessment of three carragenans in rats as pro-inflammatory is an important parameter in the evaluation of the effectiveness of anti-inflammatory drugs.

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