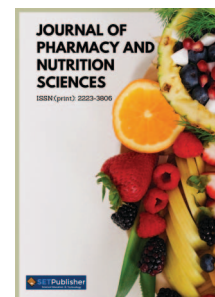




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Semi-Refined Carrageenan Attenuates Lipopolysaccharide-Induced Reactive Oxygen Species Production in Peripheral Blood Mononuclear Cells and Cell Membrane Alterations in Leukocytes

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Abstract:

Aim: To assess the effects of semi-refined carrageenan (E407a) on lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) generation in peripheral blood mononuclear cells (PBMCs) and LPS-mediated cell membrane alterations in leukocytes.

Methods: Blood samples collected from 8 intact rats were incubated with E407a (10 mg/ml), E407a (50 mg/ml), LPS (1 µg/ml), E407a (10 mg/ml) + LPS (1 µg/ml), E407a (50 mg/ml) + LPS (1 µg/ml) and without those compounds (controls) for 2 h in RPMI 1640 medium enriched with 5% fetal bovine serum. ROS generation in PBMCs obtained from the incubated samples was estimated by flow cytometry using 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) staining. The impact of E407a, LPS and their mixture on leukocyte cell membranes was evaluated spectrofluorimetrically using the fluorescent probe 2-(2'-hydroxy-phenyl)-5-phenyl-1,3-oxazole.

Results: Expectedly, incubation with LPS induced ROS generation in PBMCs and decreased the lipid order of cell membranes in leukocytes. E407a alone was found to alter neither ROS production in PBMCs, nor membrane lipid order in leukocytes. Semi-refined carrageenan partially reduced LPS-mediated ROS overproduction in PBMCs and cell membrane alterations in leukocytes.

Conclusion: E407a attenuates LPS-induced alterations of redox homeostasis in rat PBMCs and LPS-mediated modifications of cell membrane lipid order in leukocytes.

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INTRODUCTION

Natural additives have been gaining popularity for decades due to the awareness about their benefits both among industrial companies and consumers [1]. However, even legally sanctioned food additives originating from naturally occurring sources don't meet high safety standards. Based on these premises, the major European regulatory body the European Food Safety Authority (EFSA) has been carrying out the re-evaluation of currently recognized as safe food additives since 2012. Among multiple food additives whose safety for consumers is controversial, carrageenans (registered as E407 and E407a) are of great concern [2]. Carrageenans, which are also referred to as Irish moss, are highly sulfated polyanionic marine polysaccharides with no nutritional value. These polymers are made up of alternating 3-O-substituted β -D-galactopyranosyl rings and 4-O-substituted α -D-galactopyranosyl monomers [3]. It is important to note that only high-molecular-weight carrageenans (200-800 kDa) are officially permitted to be used in the food industry as thickeners, gelling agents, emulsifiers and texture improvers (E407 – food-grade carrageenan or E407a – semi-refined carrageenan). At the same time, there is compelling evidence that their low-molecular-weight counterparts called degraded carrageenans and poligeenans are toxic and, thus, their use in foodstuffs is officially prohibited [4].

Experimental data indicate that carrageenans induce intestinal inflammation despite their poor or no absorption in the gut [5, 6]. It should be mentioned that several mechanisms have been suggested to be implicated in the carrageenan-induced intestinal inflammation. In particular, carrageenans have been shown to upregulate interleukin-8 in colonic epithelial cells through the pro-inflammatory bcl10 (B-cell lymphoma/leukemia 10) / NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway [7-10]. In addition to the NF- κ B-mediated synthesis of pro-inflammatory cytokines, it has been proven that carrageenans can promote upregulation of interleukin-1 β via the NLRP3 (NLR family pyrin domain containing 3) inflammasome pathway in macrophages [11]. Furthermore, several studies have suggested the involvement of toll-like receptors (TLRs) in the carrageenan-triggered inflammation [12, 13]. TLRs are crucial for the innate immune response providing the recognition of pathogen-associated molecular patterns (PAMPs). This TLR+PAMP receptor-ligand binding triggers intracellular signal transduction culminating in

the upregulation of pro-inflammatory factors [14]. According to data available, dietary carrageenans can alter the cellular redox homeostasis both upon direct exposure and after their ingestion by experimental animals [15, 16].

Accumulating evidence on food-grade carrageenan toxicity [5, 6, 10, 17-21] has established a basis for setting up a programme by the EFSA for the risk assessment of carrageenan consumption. In 2018, the EFSA released a call for technical and toxicological data on carrageenan (E 407) for uses in foods for all population groups including infants below 16 weeks of age (EFSA-Q-number: EFSA-Q-2018-00771).

In contrast to the studies outlined above, some researchers have claimed that carrageenans exert no cytotoxic effects towards different types of cells [22-25]. In particular, a study performed on human intestinal and hepatic cell lines revealed that food-grade carrageenans neither upregulated pro-inflammatory cytokines via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway, nor affected the intracellular redox homeostasis [24]. Semi-refined carrageenan has been found to be incapable of triggering apoptosis in leukocytes, but slightly upregulated anti-apoptotic bcl-2 protein in lymphocytes [26]. Moreover, it has been experimentally shown that carrageenans don't stimulate TLR4 signaling [27].

Such inconsistency in experimental data may be explained by either confusion in carrageenan types used in the studies [23], since this group of marine carbohydrates is extremely diverse and heterogeneous, or the fact that cell culture studies exclude the complex cell-cell interactions in the body and the impact of gut microbiota, which can modify the ingested carrageenans. Several studies have suggested that there are links between carrageenan consumption and bacterial intestinal inflammation [28, 29]. However, the mechanisms by which carrageenans may aggravate bacteria-mediated gut inflammation are still poorly understood and required to be scrutinized.

The study was intended for assessing the ability of E407a to aggravate LPS-induced alteration of redox homeostasis and cell membrane structural features in leukocytes.

MATERIALS AND METHODS

1. Blood Collection and Preparation of Samples

A total of 8 adult 5-month-old female WAG rats were selected in the study. Sterile vacutainer test tubes

(IMPROVACUTER Evacuated EDTA K₂ Spray Dried PET Tubes, Guangzhou, China) containing dipotassium ethylenediaminetetraacetate (K₂EDTA) were used to collect samples. Aliquots of blood (100 µl) obtained from each animal were incubated with 10 ml RPMI-1640 medium with stable glutamine (product code: L0498-100 Biowest, France) containing 5% fetal bovine serum (FBS, BioWhittaker® reagents, Lonza, Belgium) for 2 h. The following sample groups were allocated: 1) control samples; 2) E407a at a concentration of 10 mg/ml; 3) E407a at a concentration of 50 mg/ml; 4) LPS (L2630-10MG, lipopolysaccharides from *Escherichia coli* O111:B4 purified by phenol extraction, Sigma Aldrich, Israel) whose final concentration was 1 µg/ml; 5) both E407a (10 mg/ml) and LPS (1 µg/ml); 6) E407a (50 mg/ml) and LPS (1 µg/ml). The study design is available in Figure 1. LPS was added 30 minutes before the end of incubation.

Incubation was followed by the preparation of leukocyte suspensions for further evaluation of the redox state of PBMCs and features of leukocyte cell membranes. Erythrocytes were lysed using BD Pharm Lyse™ Lysing Buffer (Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA) in accordance with the standard protocol with a double wash procedure using phosphate-buffered saline (PBS, pH 7.4, BD™ CellWash, Becton, Dickinson and Company, BD Biosciences, Poland).

The research was carried out in accordance with the EU Directive 2010/63/EU on the protection of animals

used for scientific purposes and the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123). The Committee of Bioethics (Kharkiv National Medical University, Kharkiv, Ukraine) approved the study design.

2. Evaluation of Redox Homeostasis Alterations

To detect ROS production in living leukocytes, the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) was employed. This ROS-sensitive dye is cleaved by intracellular esterases to form 2',7'-dichlorodihydrofluorescein, which is converted into highly fluorescent 2',7'-dichlorofluorescein (DCF) whose fluorescence degree reflects the intracellular ROS levels. According to the staining protocol used, leukocytes resuspended in 100 µl PBS were stained with 10 µl APC-Cy™ 7 mouse anti-rat CD45 (BD Pharmingen, USA) for 15 minutes for discrimination of leukocytes, 5 µl 7-aminoactinomycin D (7-AAD, BD Pharmingen, USA) for 15 minutes to detect living cells and freshly prepared 5 µM H₂DCFDA (Invitrogen™, USA) working solution in PBS. The fluorescence data were acquired by FACS Canto II flow cytometer (BD Biosciences, USA). DCF was excited by the 488 nm laser and its fluorescence was detected at 535 nm (FL1). 7-AAD was excited using the same laser, while emission was measured at 647 nm (FL3). Apy-Cy7 was excited by the 633 nm laser and the emitted fluorescence was detected at 760 nm.

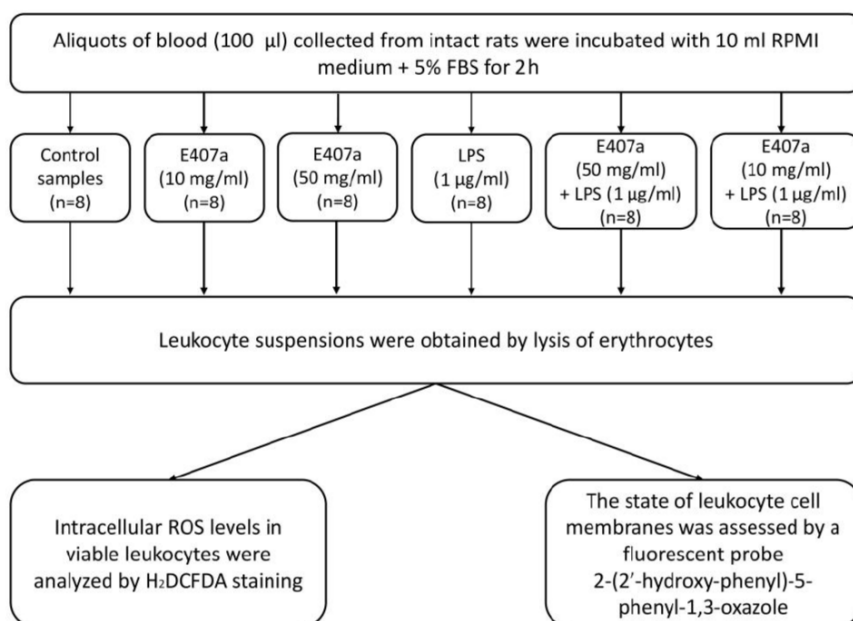


Figure 1: Schematic diagram of experimental design.

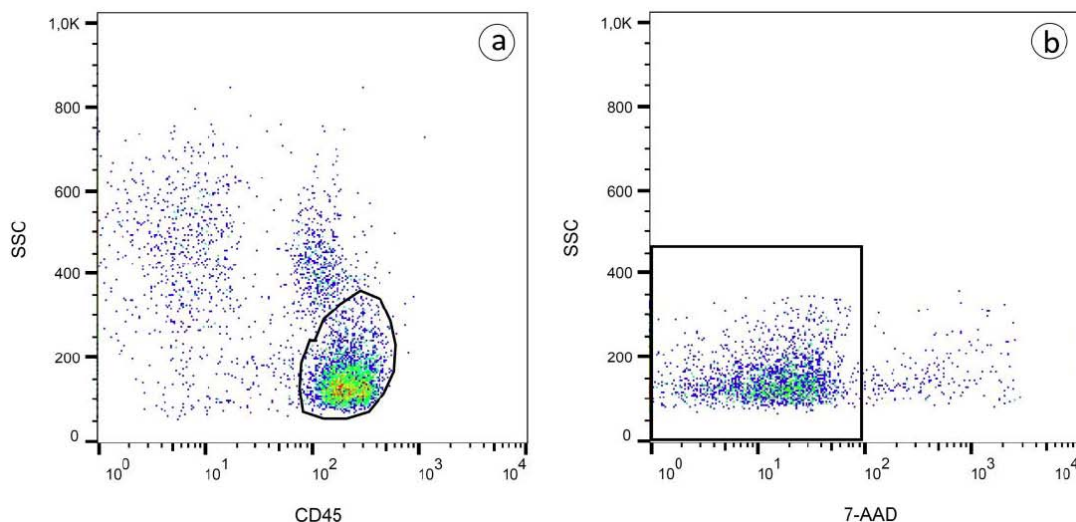


Figure 2: Gating strategy. Firstly, the population of CD45⁺ cells was identified in a FL6 vs. side scatter (SSC) plot (panel a). Secondly, viable CD45⁺ cells were selected in a FL3 vs. SSC plot based on 7-aminoactinomycin D (7-AAD) staining (panel b).

Debris was excluded and populations of firstly PBMCs and then viable PBMCs were isolated using the gating strategy available in Figure 2.

3. Assessment of the State of Cell Membranes in Leukocyte by a Fluorescent Probe

Cell fluorescent labelling was performed in the following way. To prepare a working solution of probe 2-(2'-hydroxy-phenyl)-5-phenyl-1,3-oxazole, its stock solution in acetonitrile was used. The chemical synthesis of this probe was described earlier [30]. The final probe concentration in leukocyte suspensions was approximately $5 \cdot 10^{-6}$ mol/L. This corresponded to the lipid/probe molar ratio of approximately 200:1. Before proceeding to the measurements, the leukocyte suspensions were incubated with the probes at 24 °C for 60 min. The fluorescence data were acquired using a fluorescence spectrometer "PerkinElmer FL8500" in the range of 340-550 nm, with an increment of 0.1 nm. The emission scan speed was 240 nm/min. The excitation wavelength was 330 nm. The excitation and emission slits were 5 nm.

Fluorescent probe O1O 2-(2'-hydroxy-phenyl)-5-phenyl-1,3-oxazole was used in our research due to the fact that the fluorescence indices of this probe are dependent on the polarity and proton-donor ability of its microenvironment [31-34].

The localization of 2-(2'-hydroxy-phenyl)-5-phenyl-1,3-oxazole is available in Figure 3. It is located in the region of glycerol heads of phospholipids (closer to the center of the lipid bilayer), in the area of carbonyl groups and hydrocarbon chains of phospholipid

molecules near the region of the carbonyl groups of phospholipids [34].

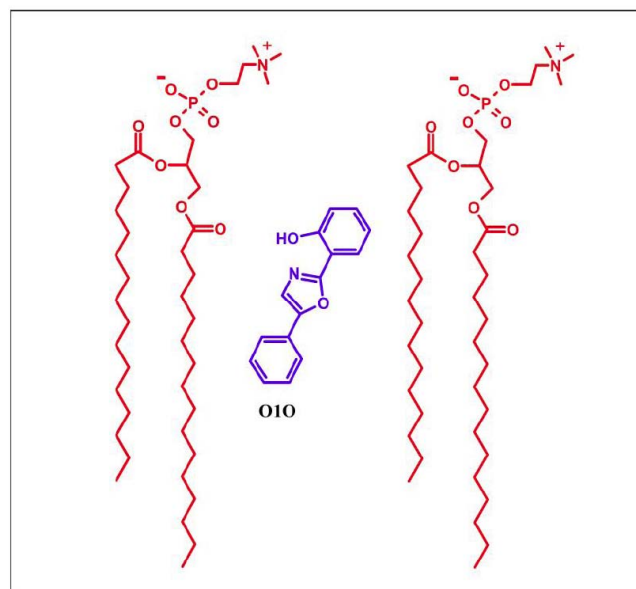


Figure 3: Image that demonstrates the site of fluorescent probe O1O location in cell membranes. Two molecules of 1,2-dipalmitoyl-phosphatidylcholine located in the outer leaflet of phospholipid bilayer are visualized to show the localization (Adapted from [34]).

When the probe O1O gets excited, the excited state proton transfer reaction takes place [30-33]. This reaction implies the formation of phototautomer form (T*), which is fluorescent in significantly longer wavelengths compared with the initial (or so-called "normal") form (N*) [31-34].

The presence of two-band fluorescence provides the opportunity to carry out the ratiometric measurement,

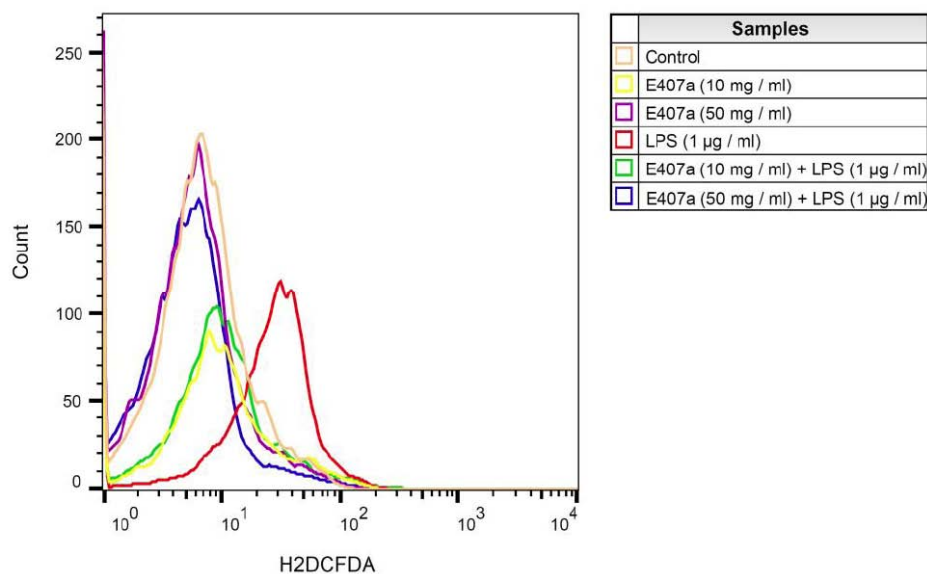


Figure 4: Representative SSC/FL1 histograms that reflect dichlorofluorescein (DCF) fluorescence in peripheral blood mononuclear cells (PBMCs) exposed to semi-refined carrageenan (E407a), lipopolysaccharide (LPS) and their combinations for 2 h.

i.e. to determine the phototautomer form fluorescence intensity-to-the initial form fluorescence intensity ratio (I_T/I_N^*). This index can be used to quantitatively characterize the alterations of physical and chemical properties of the probe microenvironment. For example, the I_T/I_N^* ratio gets reduced in response to the growth of polarity and/or proton-donor ability [31-34].

Given that higher hydration of the phospholipid bilayer promotes an increase in the proton-donor ability and polarity [35, 36], 2-(2'-hydroxy-phenyl)-5-phenyl-1,3-oxazole indicates the modifications of the hydration of lipid membranes [31]. Since the changes in membrane hydration suggest the changes in the membrane lipid order [37-39], the probe can be used to detect them.

4. Statistical Analysis

To test the distribution normality, the Shapiro-Wilk criterion was used. Kruskal-Wallis test followed by *post hoc* Dunn's test was chosen to compare means of six groups of variables, which were not normally distributed. Data were reported as the median and interquartile range. A value of P below 0.05 was statistically significant. To perform statistical analysis, *GraphPad Prism 5.0* software (USA) was used.

RESULTS

Parameters associated with the redox status of viable PBMCs exposed to E407a, LPS and their combinations are compared in Figures 3 and 4.

Expectedly, exposure of cells to LPS increased more than 2.1-fold intracellular ROS levels compared with control untreated samples. The samples incubated with semi-refined carrageenan for 2 h were found to be unaffected in terms of ROS production, evidenced by the absence of statistically significant changes in MFI values of DCF in PBMCs (Figure 5). The effects were not observed even when a higher concentration of the food additive was used. Moreover, the treatment of samples with the mixture of E407a and LPS resulted in no changes in MFI values of DCF compared with the controls. However, a higher concentration of E407a (50 mg/ml) surprisingly reduced the LPS-induced ROS generation suggesting that E407a can partially reduce LPS-induced ROS overproduction (Figure 5).

Outcomes of fluorescence measurements of the spectra of fluorescent probe O1O embedded in cell membranes of leukocytes incubated with E407a, LPS or their mixtures can be found in Figures 6 and 7.

A considerable statistically valid approximately 30% decrease in the fluorescence intensity ratio I_T/I_N^* (I_{475}/I_{370}) was observed in leukocyte suspensions incubated with LPS (Figures 6 and 7) in comparison with the control samples. Such increase in the I_T/I_N^* ratio is indicative of both the increase in polarity and the proton-donor ability of the environment of probe O1O in the leukocyte membranes, suggesting an increase in membrane hydration in the area of the probe location. The increased hydration, in its turn, indicates the decrease in membrane lipid order (Figure 7).

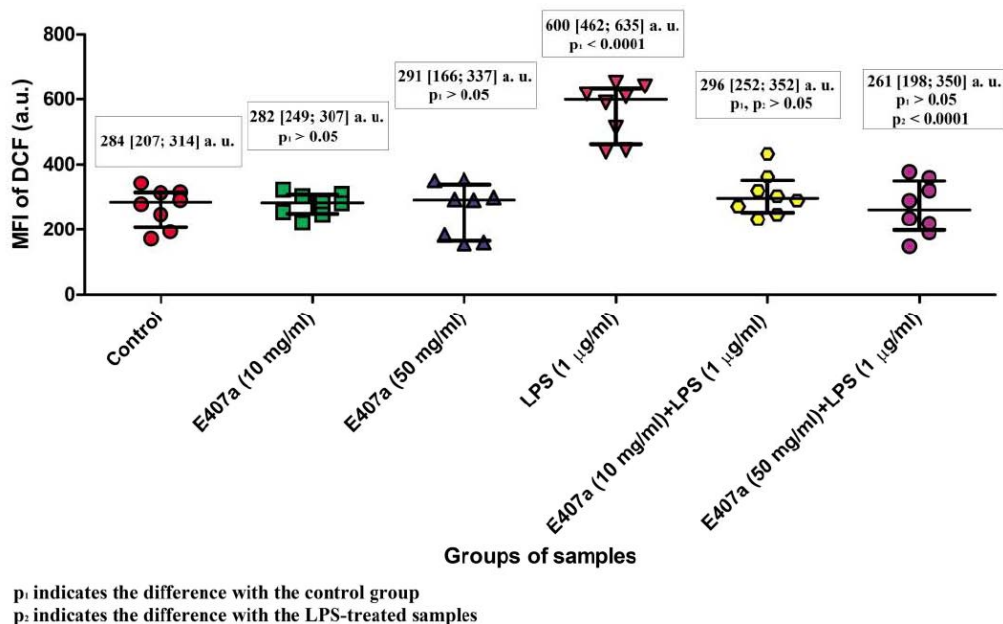


Figure 5: Comparison of mean fluorescence intensities (MFI) of dichlorofluorescein (DCF) in peripheral blood mononuclear cells (PBMCs) exposed to semi-refined carrageenan (E407a), lipopolysaccharide (LPS) and their combinations for 2 h.

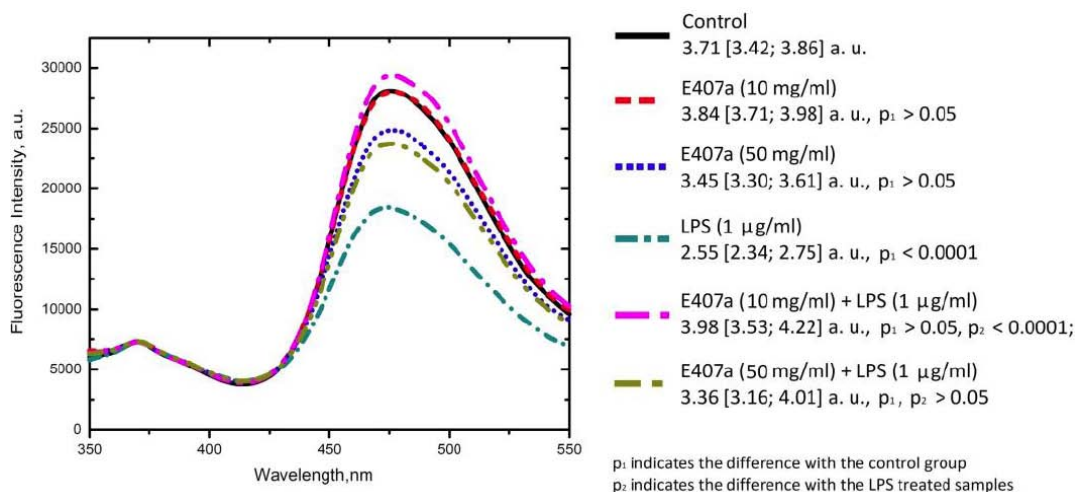


Figure 6: Representative fluorescence spectra of probe O1O in leukocyte suspensions obtained from blood: (a) control samples (black solid line), (b) exposed to 10 mg/ml E407a (red short dash line), (c) exposed to 50 mg/ml E407a (blue short dot line), (d) treated with 1 µg/ml lipopolysaccharide (LPS) (dark cyan short dash dot line), (e) exposed to the mixture of 10 mg/ml E407a and 1 µg/ml LPS (magenta dash dot line), (f) incubated with the mixture of 50 mg/ml E407a and 1 µg/ml LPS (dark yellow dash dot line). To facilitate the comparison, the spectra were normalized to the fluorescence intensity of the normal form. The averaged values of I_{T*}/I_{N*} (I₄₇₅/I₃₇₀) ratio of probe O1O are presented for each group of rats.

On the other hand, in comparison with the corresponding spectra for the control samples, no statistically significant changes in the ratio of the fluorescence intensities of the phototautomer and normal forms (I_{T*}/I_{N*}, i.e. I₄₇₅/I₃₇₀) of probe O1O were detected for the lipid membranes of leukocytes which are exposed to E407a in both concentrations incubated for 2 h (Figures 6, 7). It is important to emphasize that in comparison with the control group, no significant changes in the polarity and the proton-donor ability in

the membrane area, where probe O1O locates, were observed for the suspension exposed to the mixtures of E407a (50 mg per ml) and LPS, evidenced by no statistically significant changes in the ratio of the fluorescence intensities of the probe phototautomer and normal forms (Figure 6). However, the incubation with 10 mg/ml E407a and LPS resulted in a statistically significant increase (p < 0.0001) in the I_{T*}/I_{N*} ratio compared with the samples treated exclusively with LPS.

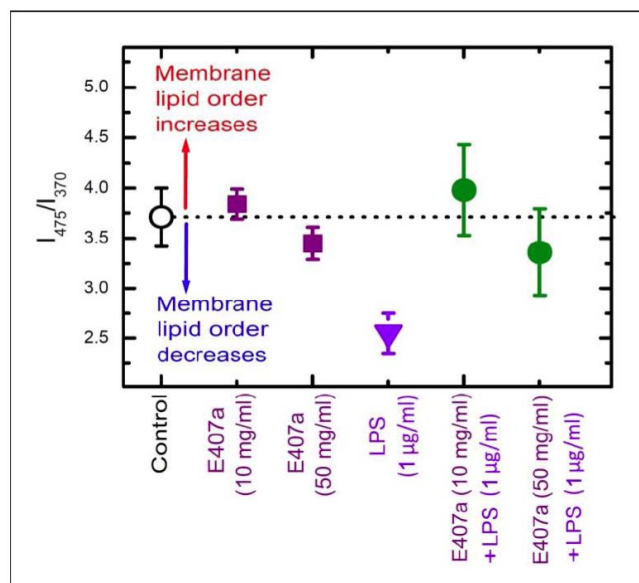


Figure 7: The median fluorescence intensity ratios I_{475}/I_{370} of probe O1O in leukocytes of rats exposed to: (a) E407a at the concentrations of 10 mg/ml and 50 mg/ml (purple squares), (b) LPS (violet triangle), (c) mixtures of E407a (10 mg/ml and 50 mg/ml, respectively) and LPS (filled olive circles), (d) control group of animals (open black circle) are shown to visualize the effects of substances used in this study on the cell membrane lipid order.

DISCUSSION

Bacterial LPS is a well-described component of the cell wall in Gram-negative bacteria, which acts as a PAMP and ligand for receptors of the innate immune system, especially TLR4 [40, 41]. Downstream effects of TLR4-LPS complex formation in immune cells include the activation of NADPH oxidase with the corresponding overproduction of ROS and overexpression of NF- κ B-dependent pro-inflammatory cytokines [42, 43]. It is important to note that LPS is capable of inducing the inflammatory ROS-mediated response in PBMCs [43].

Unsurprisingly, in this study, direct incubation of blood with LPS was found to promote ROS generation in PBMCs and diminish the fluidity of cell membranes in leukocytes, supported by the observed changes in the fluorescence values of fluorescent probe, since the fluidity of phospholipid bilayer decreases in response to free radical-induced peroxidation of polyunsaturated fatty acids, which are essential components of cell membranes responsible for the maintenance of bilayer fluidity [44, 45]. Thus, LPS-mediated changes in the fluidity of cell membranes develop at least partially due to ROS-triggered lipid peroxidation, which causes the reduction of the relative amount of PUFAs in phospholipids. This reduces the lipid order in cell membranes making them more rigid.

In this study, semi-refined carrageenan did not induce ROS generation in leukocytes after incubation for 2 h, even at high concentrations. These data corroborate our earlier findings on the inability of E407a to alter redox homeostasis of rat leukocytes after a short-term incubation [16], but, on the other hand, this contradicts other studies that support the presence of direct pro-oxidant effects of carrageenans on immune cells *in vitro* [15]. However, experimental *in vivo* data provide evidence that carrageenans are known to induce oxidative stress in leukocytes, activate nitric oxide synthesis, cytokine production in macrophages and promote apoptosis of circulating leukocytes [16, 46-49].

Surprisingly, in this study, semi-refined carrageenan was found to partially inhibit LPS-induced ROS generation and LPS-mediated changes in phospholipid bilayers of cell membranes *in vitro*. This is inconsistent with reports stating that carrageenans stimulate LPS-induced generation of TNF- α in leukocytes without affecting the expression of this cytokine alone in the absence of LPS [50]. However, *in vivo* studies demonstrate that carrageenan stimulates macrophages even in a more pronounced way compared with LPS [49]. Moreover, experiments on murine models show that kappa-carrageenan magnifies LPS-induced inflammation via the bcl-10-NF- κ B pathway [29]. These inconsistencies between our findings and literature data may be explained by the direct interactions of LPS and carrageenans, since carrageenan has been shown to directly alter the morphology and, thus, functional activity of LPS suggesting, on the contrary, the protective and inhibitory role of carrageenans in LPS-induced inflammation [51].

Thus, our results don't exclude the synergistic pro-inflammatory effects of carrageenans and LPS *in vivo*. Therefore, future researches should aim at closing the gap in elucidating the crosstalks between the ingested carrageenan, gut microbiome and host immune system. This is especially important for individuals with compromised intestinal barrier integrity and already existing intestinal inflammation.

CONCLUSIONS

Semi-refined carrageenan stimulated neither ROS overproduction in PBMCs, nor changes in the physico-chemical properties of cell membranes in leukocytes directly exposed to this food additive for 2 h. On the contrary, the food additive reduces the LPS-mediated pro-inflammatory effects in leukocytes as a whole and PBMCs in particular.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

FUNDING

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