ORIGINAL ARTICLE

Effects of semi-refined carrageenan (food additive E407a) on cell membranes of leukocytes assessed *in vivo* and *in vitro*

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ABSTRACT

Aim To assess the impact of semi-refined carrageenan (E407a) on hydrophobic regions of phosphololipid bilayer in cell membranes of leukocytes collected from rats orally administered this food additive and white blood cells incubated with E407a.

Methods Fluorescent probes (*ortho*-hydroxy derivatives of 2,5-diaryl-1,3-oxazole) were used to estimate the state of lipid bilayer in leukocytes obtained from rats orally exposed to the food additive E407a and in white blood cells incubated with E407a.

Results No noticeable changes in the physico-chemical properties were observed in the lipid membranes of leukocytes in the region where the probes locate in response to oral intake of semi-refined carrageenan. Incubation of leukocytes with E407a solutions resulted in a decrease in polarity and proton-donor ability of leukocytes in the area of carbonyl groups of phospholipids and in the area of hydrocarbon chains of phospholipids near the polar region of the bilayer.

Conclusion Membrane fluidity abnormalities found in cells exposed to E407a are similar to those observed in patients with IBD suggesting that contribution of carrageenan to IBD development may be partially explained by leukocyte membrane modifications.

Key words: fluorescence, membrane fluidity, phospholipids, polysaccharides, rats

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INTRODUCTION

Both animal and epidemiological studies have provided compelling evidence that dietary habits influence inflammatory bowel disease (IBD) development, including Crohn's disease (CD) and ulcerative colitis (UC), and intensity of intestinal inflammation (1,2). The CD and UC are debilitating chronic inflammatory disorders of the gut clinically characterized by fatigue, diarrhoea, pain in the abdomen, loss of weight, and rectal bleeding (3,4). Inflammation in UC is usually limited to the large bowel and affects mucosal and submucosal layers. CD, in its turn, can affect any region of the gut transmurally (5). Europe and North America have a higher prevalence of IBD compared to other regions of the world. However, the trend towards an increase in the number of CD and UC cases has been observed worldwide. In particular, the average prevalence rate of IBD increased from 79.5/100,000 persons in 1990 to 84.3/100,000 persons in 2017 (6).

Of note, IBD etiopathogenesis is complex. It comprises a combination of genetic predisposition with bacterial and environmental (primarily dietary) factors. In a simplified way, IBD can be considered an alteration of gut homeostasis (7). Recent genome-wide association studies (GWAS) have shed light on genetically determined factors that might increase risks for IBD development. IBD susceptibility single nucleotide polymorphisms (SNPs) have been identified and allowed confirming the involvement of autophagy, IL-17, IL-23, and type 3 innate lymphoid cells in IBD development (8). However, even prior to the implementation of GWAS approach that has revolutionized our understanding of associations between particular loci and diseases, SNPs in the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) gene were associated with susceptibility to IBD (9-11). NOD2 is an intracellular protein receptor activated upon exposure to bacterial components and its downstream effects are activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and release of cytokines (12,13). The NOD2 protein is expressed in monocyte/macrophage lineage cells, Paneth cells and intestinal epithelial cells playing a crucial role in the maintenance of gut homeostasis and intestinal microbiota-host interactions (12,14,15). The recognized impact of NOD2 gene mutations in IBD aetiology supports the view on IBD as a disease developing as a result of impaired intestinal homeostasis. Furthermore, there is accumulating evidence that environmental exposures contribute to IBD pathogenesis via modifying intestinal microbiota (16).

It is worth noting that IBD prevails in urban Westernized societies where the Western-style diet characterized by high intake of pro-inflammatory processed foods is common. This type of diet is associated with consumption of food rich in saturated fats, carbohydrates with high glycaemic index, and xenobiotics such as food additives (17). One of such food additives that have drawn a lot of attention recently is carrageenan, which is a linear polymer of carbohydrate nature isolated from marine red algae, primarily *Kappaphycus alvarezii* (18). Structurally, carrageenans are composed of D-galactose and 3,6-anhydrogalactose monosaccharide units linked with α1,3- and β1,4-glycosidic bonds. There are three major types of carrageenans (kappa, lambda and iota). They differ in the number of sulfate groups and sulfate ion sites (19). Carrageenans in food industry are available as E407 (refined food-grade carrageenan) and E407a (semi-refined carrageenan). Both food additives are used primarily to improve the texture of processed foods, as well as thickeners and emulsifiers (18,19). According to WHO and FDA, the consumption of carrageenans should be limited to 75 mg/kg of weight daily, i.e. 5.25 g per an average 70-kg adult (20,21). This temporary limitation has been recently introduced due to a number of papers reporting on intestinal and extraintestinal inflammatory effects of orally consumed E407 and E407a (19,22,23). There is accumulating evidence that TLR4 and Bcl10 pathways are involved in carrageenan-induced inflammation (24,25). The possible role of carrageenans in IBD is confirmed by the similarity of intestinal morphological alterations caused by the intake of carrageenan and IBD (17,26). In addition, a recently conducted randomized trial has demonstrated that carrageenan-free diet maintains remission in IBD (27).

Another factor that may affect the course of IBD is impaired enterocyte transcytosis that may promote the transfer of luminal antigenic molecules into the sterile lamina propria contributing to antigenic overload. Cell membrane abnormalities have been reported to alter transcytosis (28). Surprisingly, data on cell membrane alterations

in IBD is extremely scarce. It has been shown that lipid composition of cell membranes in red blood cells (RBCs) and colonic mucosa even in uninflamed tissue in IBD is affected (29,30). Furthermore, cell membrane fluidity in erythrocytes has been reported to be reduced in CD (30). Carrageenan has been also demonstrated to affect cell membrane lipid order in RBCs and enterocytes upon oral exposure by experimental animals (31,32). However, there are no reports concerning the effects of carrageenan intake on phospholipid bilayer of leukocytes.

The aim of this study was to investigate the effects of carrageenan oral intake on cell membranes of leukocytes using fluorescent probes.

MATERIALS AND METHODS

Study design

The study was performed at Kharkiv National Medical University and V.N. Karazin Kharkiv National University (Kharkiv, Ukraine) in December 2019.

A total of 24 female WAG rats weighing 160-190 g. were used, 16 of which were randomly divided in into two groups: experimental (n=8) and control (n=8). The animals from the experimental group ingested a solution of semi-refined carrageenan at a final concentration of dry components of 140 mg/kg of weight. The control animals received no semi-refined carrageenan. The access to food and drinking water was *ad libitum*. The rodents were housed in standard conditions. Acclimatization period lasted for 2 weeks.

When animals were anesthetized and sacrificed, their blood was collected into sterile dipotassium ethylenediaminetetraacetate (EDTA)-containing vacutainer tubes; 100 mL blood from each animal of experimental and control groups was lysed and washed in accordance with a protocol (described below) to obtain leukocyte suspensions. In addition, blood was incubated with semi-refined carrageenan. Briefly, 2 mL blood was collected into the K2 EDTA vacutainer tubes to prevent clotting. This volume obtained from each rat was equally distributed between four capped polystyrene test tubes, i.e. 500 μL blood per sample; 100 μL of phosphate buffered saline (PBS, pH 7.4) (Becton Dickinson, USA), 1%, 2% and 5% solutions of semi-refined carrageenan were added to the corresponding test tubes for a 4h incubation.

All experimental procedures, accommodation and care of animals were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (EST 123), Directive 2010/63/EU for the Protection of Animals Used for Scientific Purposes, and Recommendation 2007/526/EC regarding guidelines for the accommodation and care of animals used for experimental and other scientific purposes. The study design was approved by the Bioethics Committee of the Kharkiv National Medical University (Kharkiv, Ukraine).

Methods

Lyse/wash protocol. To analyse the state of leukocyte membranes by fluorescent probes, leukocyte suspensions were prepared from freshly collected blood samples in accordance with the lyse/wash protocol provided by Becton Dickinson (2002). Briefly, 2 mL 1x FACSLyse solution (Becton Dickinson, USA) was added to 100 μL blood placed in capped test tubes. To provide lysis of erythrocytes, incubation for 15 minutes at 24 °C was performed. After centrifugation at 500g for 5 minutes, the supernatant was discarded. Then solutions were washed twice with 2 mL PBS. Cell pellets were resuspended in PBS to form suspensions to which fluorescent probes O6O and PH7 were added.

Fluorescent probes O6O and PH7. The cells were fluorescently labelled by the same procedure: an aliquot of the probe stock solution in acetonitrile was added to the white blood cell (WBC) suspension to achieve a final probe concentration of ~5×10^{-6} mol/L. Lipid-to-probe molar ratio was ~200:1. Before fluorescence measurements, the cell suspensions were incubated with the probe at room temperature for 1 hour.

The fluorescence spectra for leukocyte suspensions obtained from blood extracted from animals treated and untreated with E407a were recorded on a Thermo Scientific Lumina fluorescence spectrometer (Thermo Fisher Scientific, Waltham, USA) in the range of 350-630 nm, with an increment of 0.1 nm. Data were collected with 0.02 s interval. The slits on the excitation and emission monochromators were 5 and 10 nm, respectively. The excitation wavelength was 330 nm.

For WBC suspensions incubated with semi-refined carrageenan, the fluorescence spectra were measured on a fluorometer (Hitachi F850, Tokyo, Japan) in the range of 350-630 nm, with an increment of 2 nm. The excitation wavelength was 330 nm. The excitation and emission slits were 5 nm.

Fluorescent probes O6O (2-(2'-hydroxy-phenyl)-5-(4'-biphenyl)-1,3-oxazole) and PH7 (2-(2'-hydroxy-phenyl)-phenanthro[9,10-d]-1,3-oxazole) were used, since its fluorescence parameters depend upon the polarity and proton-donor ability of the microenvironment (33-36).

Probe O6O is located (Figure 1) in the area of carbonyl groups of phospholipids and in the area of hydrocarbon chains of phospholipids near the polar region of the bilayer, while probe PH7 is localized (Figure 1) in the area of hydrocarbon chains of phospholipids closer to the centre of the lipid bilayer (36).

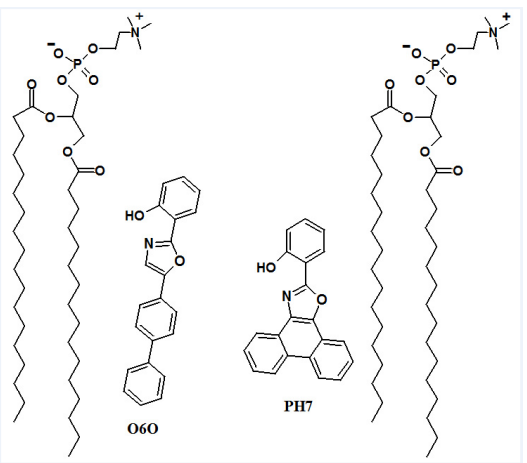

Figure 1. Localization and orientation of fluorescent probes O6O and PH7 in phospholipid membranes. Two molecules of phosphatidylcholine from the outer leaflet are shown to denote the localization of the probe (Adapted from Posokhov and Kyrychenko) (36)

When the probes O6O and PH7 are in the excited state, the excited state proton transfer reaction occurs (33-36). As a result of this reaction, photo tautomer form (T*) is formed. The photoproduct is fluorescent in significantly longer wavelengths in comparison with the initial (or so-called "normal") form (N*) (33-36).

The presence of two-band fluorescence enables us to conduct ratiometric measurement, i.e. to use the ratio of the photo tautomer form and the initial form fluorescence intensities (I_{T*}/I_{N*}) as a parameter for estimation of the changes in physical and chemical properties of the microenvironment: e.g. with growth of polarity and/or proton-donor ability of the media, the ratio I_{T*}/I_{N*} decreases (34-36).

Since the increase in the hydration of the lipid bilayer leads to an increase in proton-donor ability and polarity of the membrane medium (37,38), the probes can be used to detect the changes in the hydration of lipid membranes (33). Taking into account that the changes in membrane hydration in their turn are linked with the changes of the membrane lipid order (39-41), the probes can indicate the latter.

Statistical analysis

An assessment of the normality of data was performed by Shapiro-Wilk test. Based on its results, non-parametric tests were used to compare independent groups of variables. To analyse the results of an *in vivo* study, the Mann-Whitney U test was used. To evaluate differences between four independent groups in an *in vitro* study, the Kruskal–Wallis one-way analysis of variance was selected. It was followed by the Dunn's multiple comparisons test. $p<0.05$ was statistically significant.

RESULTS

The spectra of fluorescent probe PH7 in leukocyte cell membranes differed negligibly between leukocytes treated and untreated with semi-refined carrageenan (Figure 2). Comparison of numerical values of I_{T*}/I_{N*} ratios demonstrated no statistically significant difference ($p>0.05$) (Table 1) indicating that incubation of blood with E407a did not

Table 1. The ratio of the fluorescence intensities of the photo tautomer and normal forms (I_{T*}/I_{N*}) of probes O6O and PH7 in cell membranes of intact leukocytes incubated with different concentrations of E407a

Animal group	Probe 060		Probe PH7	
	I_T*/I_N* ratio (median; (interquartile range)	p*	**I_T*/I_N* ratio** (median; (interquartile range)	p*
Intact leukocytes untreated with semi-refined carrageenan (n=8) (control)	3.9 (3.6-4.2)		3.4 (3.0-3.8)	
Intact leukocytes treated with 1% E407a solution (n=8)	5.5 (5.1-5.7)	p<0.0001	3.6 (3.0-3.9)	p>0.05
Intact leukocytes treated with 2% E407a solution (n=8)	4.8 (4.7-5.0)	p<0.05	3.5 (3.30-3.7)	p>0.05
Intact leukocytes treated with 5% E407a solution (n=8)	5.8 (5.4-6.1)	p<0.0001	3.7 (3.1-3.9)	p>0.05

*p as comparing to control group;

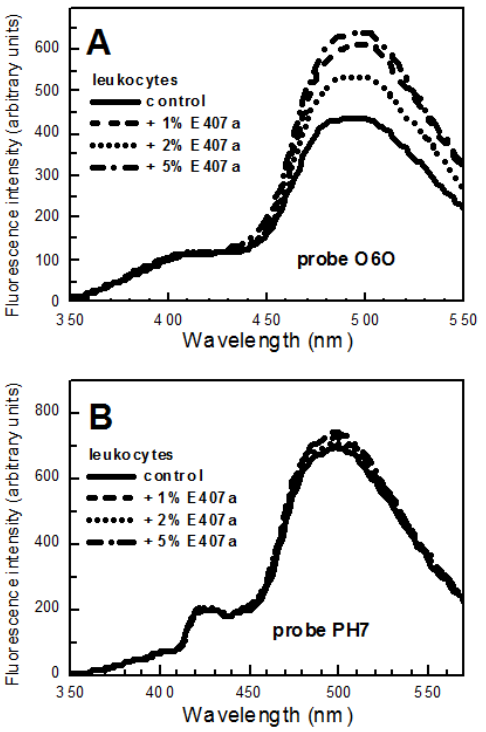

Figure 2. Representative fluorescence spectra of A) probes O6O and B) PH7 in leukocyte suspensions for the control group of rats (solid line), white blood cells exposed to the 1% solution of E407a (dashed line), leukocytes treated with the 2% E407a solution and white blood cells incubated with the 5% E407a solution (dash-dot line)

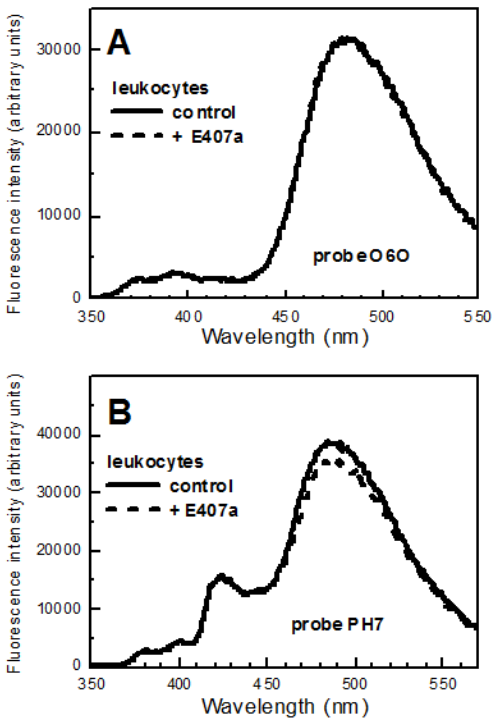

Figure 3. Representative fluorescence spectra of probes O6O (panel A) and PH7 (panel B) in leukocyte suspensions: A) control group of rats (solid line), B) animals orally exposed to E407a during two weeks (dashed line). For better comparison, the spectra were normalized to the fluorescence intensity of the normal form

affect the polarity and the proton-donor ability in rat WBC cell membranes in the membrane area, where probe PH7 locates, i.e. in the hydrophobic region closer to the centre of the lipid bilayer.

In the case of probe O6O, statistically significant changes in the spectra of the probe were detected (Figure 2). The I_{T*}/I_{N*} ratios of probe O6O for WBC suspensions treated with E407a solutions were statistically significantly higher compared with the control samples (Table 1). Any concentration of semi-refined carrageenan used for incubation affected the fluorescence of probe O6O. Thus, our *in vitro* experiment demonstrated that E407a influenced the state of phospholipid bilayer in the regions, where probe O6O locates: i.e. in the area of carbonyl groups of phospholipids and in the area of hydrocarbon chains of phospholipids near the polar region of the bilayer.

The spectra of fluorescent probe O6O bound to the WBCs extracted from the rats treated with semi-refined carrageenan during two weeks coincided with the corresponding spectrum for the control group of animals (Figure 3). Hence, the difference between the ratios of the fluorescence intensities of the photo tautomer and normal forms (I_{T*}/I_{N*}) for probe O6O was found to be statistically insignificant (Table 2). This suggests that oral intake of E407a promoted no changes in the polarity and the proton-donor ability in the lipid membranes of WBCs in the regions, where probe O6O locates.

Table 2. The ratio of the fluorescence intensities of the phototautomer and normal forms (I_{T*}/I_{N*}) of probes O6O and PH7 in cell membranes of leukocytes obtained from rats orally administered semi-refined carrageenan

Animal group	Probe 060		Probe PH7	
	I_T*/I_N* (median; (interquartile range)	p*	I_T*/I_N* (median; (interquartile range)	p*
Control group (n=8)	10.1 (9.4-10.5)		2.5 (2.3-2.6)	
Rats orally exposed to E407a during 2 weeks (n=8)	9.9 (8.9-11.2)	p>0.05	2.3 (2.2-2.5)	p>0.05

*p as comparing to control group;

The same trend was observed for probe PH7. The difference between the spectra of the probe bound to the WBCs obtained from the rats orally

administered E407a during a fortnight was found to be negligible from the corresponding spectra of the probe embedded in the cell membranes of leukocytes from the control group (Figure 3). In the case of probe PH7, the ratios of the fluorescence intensities of the photo tautomer and normal forms (I_{T*}/I_{N*}) did not differ significantly ($p>0.05$) from the corresponding ratios calculated for the control group (Table 2). Thus, in comparison with the control group, no significant changes in the polarity and the proton-donor ability were observed in the experimental group in the membrane area, where probe PH7 locates.

An incubation of WBCs with E407a induced changes in cell membranes in the regions where probe O6O is located without affecting those areas where probe PH7 locates, while oral consumption of this food additive produced no effect on the corresponding regions of leukocyte lipid bilayer.

DISCUSSION

Cell membranes are complex lipid-protein structures acting as semi-permeable barriers. However, in addition to the barrier function, they perform a crucial role in cells mediating interaction and communication with the extracellular environment. Membrane fluidity whose maintenance is of paramount importance for such interactions and, thus, modulation of cellular functions is determined by a number of factors, including fatty acid composition of phospholipids in bilayer and the percentage of cholesterol (42). It is important to note that over 30% of all cellular proteins are membrane-embedded. Thus, changes in membrane fluidity alter lateral mobility of these proteins, protein-protein interactions and protein diffusivity, which affects signalling from membrane-embedded receptors and, hence, response of cells to environmental challenges (42,43). Experimental evidence also supports the role of membrane fluidity in leukocytes. In particular, cell membranes are involved in extravasation of neutrophils, including interactions with vascular endothelium, rolling, adhesion, and diapedesis (44). In addition, there is evidence that changes in membrane lipid order (membrane fluidity) reduce leukocyte motility and, thus, chemotaxis (45).

Thus, our findings suggest a higher dehydration of leukocyte membranes of rats in the area of carbonyl groups of phospholipids and in the area of hydrocarbon chains of phospholipids near the polar region of the bilayer. The dehydration, in its turn, indicates an increase in the lipid order (i.e. a decrease in fluidity) of WBC membranes exposed to high doses of E407a. We believe that the increased lipid order of membranes in WBCs treated with high concentrations of E407a might be attributed to direct electrostatic interactions of negatively charged carrageenan molecules, which are characterized by the high level of sulfate ion with outer leaflets of cell membranes. Furthermore, our observation of membrane dehydration and, thus, the increase in the membrane lipid order, may develop in response to lipid peroxidation (46,47), since polyunsaturated fatty acids (PUFAs) found in cell membranes are prone to free radical oxidation caused by reactive oxygen species (ROS) whose overgeneration by neutrophils under the influence of carrageenan has been reported (48).

Our experimental results in an *in vivo* study indicate that no changes in the membrane hydration were detected in the hydrophobic area adjacent to the polar region and the hydrophobic region closer to the centre of the lipid bilayer of the cell membranes of leukocytes in the rats orally exposed to the common food additive E407a during two weeks. The discussed lack of the changes in the membrane hydration points to the absence of changes in the lipid order of the hydrophobic region of the leukocyte membrane.

However, it is worth mentioning that the absence of structural changes in rather hydrophobic regions of leukocyte lipid bilayer in rats orally exposed to the common food additive E407a during two weeks does not exclude the impact of oral carrageenan consumption on leukocyte membranes. Earlier we demonstrated that oral administration of E407a affected less hydrophobic regions of leukocyte cell membranes (Tkachenko AS, personal communication 2020): the increase in the polarity and the proton-donor ability of the microenvironment of fluorescent probe O1O (2-(2'-hydroxy-phenyl)-5-phenyl-1,3-oxazole) pointed to an increase in hydration in the area of glycerol backbones of phospholipids and thus, suggested the decrease of the membrane lipid order.

In conclusion, E407a has impact on leukocyte membrane both *in vivo* and *in vitro*. It should be noted that in the case of direct exposure to

carrageenan more hydrophobic regions are affected compared with the effects of orally consumed carrageenan. Cell membrane fluidity abnormalities found in this study are similar to those observed in patients with IBD suggesting that the contribution of carrageenan to the IBD development may be explained by leukocyte membrane modifications. However, further experimental and clinical studies are required to elucidate the role of carrageenan in IBD etiopathogenesis.

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TRANSPARENCY DECLARATION

Conflicts of interest**:** None to declare

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