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Reactive oxygen species (ROS) generation by lymphocytes in rats treated with a common food additive E407a

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ABSTRACT

This research deals with the evaluation of reactive oxygen species (ROS) generation in lymphocytes of rats orally exposed to a thickener and emulsifier E407a (semi-refined carrageenan). There is some evidence that E407a may be toxic and can induce intestinal inflammation. ROS generation in lymphocytes was assessed by flow cytometry using a dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) in 8 rats treated with 140 mg of semi-refined carrageenan per kg of weight daily during two weeks and 8 control animals. A higher number of ROS^{high} viable lymphocytes were found in the group of animals exposed to E407a compared with controls. In addition, mean fluorescence intensity (MFI) in ROS^{high} lymphocytes was elevated, which is indicative of the intensification of ROS production by lymphocytes in response to E407a oral consumption.

Keywords: carrageenan, food additive, reactive oxygen species, lymphocytes, inflammation

Е407А ТАҒАМДЫҚ ҚОСПАСЫН ПАЙДАЛАНУ КЕЗІНДЕ ЕГЕУҚҰЙРЫҚ ЛИМФОЦИТТЕРІНІҢ АКТИВТІ ОТТЕГІ ТҮРЛЕРІН (АОТ) ҚАЛЫПТАСТЫРУЫ

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ТҰЖЫРЫМДАМА

Бұл зерттеу Е407а (жартылай тазартылған каррагинан) қоюлатқышы мен эмульгаторын ауыз қуысы арқылы қолданған егеуқұйрық лимфоциттеріндегі активті оттегі түрлерін (АОТ) генерациялау қарқындылығын бағалауға арналған. Кейбір зерттеулер Е407а ішектің қабынуын тудыруы мүмкін екенін көрсетті. Лимфоциттерде АОТ түзілуін екі апта бойы және дене салмағының әр килограмына 140 мг жартылай тазартылған каррагинаны қабылдайтын 8 егеуқұйрықта 2′, 7′-дихлордигидрофлуоресцеин диацетаты (H2DCFDA) бояуын пайдалана отырып, ағымдық цитометрия көмегімен және 8 бақылау жануарларында бағаланды.

Бақылаумен салыстырғанда Е407а әсеріне ұшыраған жануарлар тобында жоғары мөлшерде АОТ мөлшері бар өміршең лимфоциттер санының көбеюі анықталды. Сонымен қатар, АОТ деңгейі жоғары лимфоциттердегі флуоресценттік орташа қарқындылық (MFI) жоғарылады, бұл Е407а препаратын ауыз қуысы арқылы қабылдауға жауап ретінде лимфоциттер арқылы АОТ өндірісінің жоғарылауын көрсетеді.

Негізгі сөздер: каррагинан, тағамдық қоспалар, активті оттегі түрлері, лимфоциттер, ішектің қабынуы

ГЕНЕРАЦИЯ АКТИВНЫХ ФОРМ КИСЛОРОДА (АФК) ЛИМФОЦИТАМИ КРЫС НА ФОНЕ УПОТРЕБЛЕНИЯ ПИЩЕВОЙ ДОБАВКИ Е407А

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РЕЗЮМЕ

Данное исследование посвящено оценке интенсивности генерации активных форм кислорода (АФК) в лимфоцитах крыс, которые перорально употребляли загуститель и эмульгатор Е407а (полуочищенный каррагинан). В некоторых работах показано, что Е407а может индуцировать воспаление кишечника. Образование АФК в лимфоцитах оценивали с помощью проточной цитометрии с использованием красителя 2',7'-дихлордигидрофлуоресцеина диацетата (H2DCFDA) у 8 крыс, получавших 140 мг полуочищенного каррагинана на килограмм веса ежедневно в течение двух недель и у 8 контрольных животных. Увеличение числа жизнеспособных лимфоцитов с высоким содержанием АФК было обнаружено в группе животных, подвергшихся воздействию Е407а, по сравнению с контролем. Кроме того, средняя интенсивность флуоресценции (MFI) в лимфоцитах с высоким уровнем АФК была увеличена, что свидетельствует об усилении продукции АФК лимфоцитами в ответ на пероральное употребление Е407а.

Ключевые слова: каррагинан, пищевая добавка, активные формы кислорода, лимфоциты, воспаление кишечника

Introduction

Marine algae have been used for decades as a source of carbohydrates such as agar, alginates and carrageenans for food industry [1]. In particular, carrageenans, which are anionic sulfated hydrocolloids of heteropolysaccharide nature made up of repeating D-galactose and 3,6-anhydrogalactose monosaccharides, are of huge importance for the market of meat and dairy products, since they show gelling, emulsifying and thickening properties [2]. Carrageenans are registered as food additives E407 and E407a and their share on the food market has been growing for years [3]. These carbohydrates obtained from seaweeds can be divided into three major types (λ, κ, and ι) based on the percentage of sulfated monosaccharide units and distribution of the sulfate groups [4].

Besides their nutritional application, carrageenans have been studied as antiviral, antitumor, and immunomodulatory agents [5, 6]. Growing body of evidence suggests that these marine carbohydrates can prevent virus attachment, entry and replication [7, 8]. In addition to their efficiency demonstrated in many studies, carrageenans are characterized by cost-effectiveness and low toxicity [9, 10]. However, it has been reported that both food-grade carrageenan and its oligosaccharide fragments used as antiviral agents are cytotoxic [11-14]. One of the mechanisms by which carrageenans may promote cytotoxicity is reactive oxygen species (ROS) generation. Bhattacharyya et al showed that carrageenan could stimulate ROS production in cell cultures of human colonic epithelial cells [15]. Furthermore, evidence from another study indicated that low-molecular-weight λ-carrageenan oligosaccharides promote ROS formation in human umbilical vein endothelial cells [16]. Barth et al demonstrated that carrageenans induced ROS generation in peritoneal neutrophils in a model of carrageenan-induced peritonitis [17]. However, the hypothesis on the pro-oxidant properties of carrageenans does not corroborate with the findings of other studies, which deny the effects of carrageenans on the cellular oxidative stress [18, 19]. Such controversial data may be explained by misinterpretation of the results due to the confusion in terms, since different types of carrageenans (food grade, degraded carrageenan and poligeenan) have various toxicity profiles. Degraded carrageenan and poligeenan are widely recognized as toxic compounds and their use in food industry is prohibited [10].

Abe et al stated that not only phagocytic cells could act as targets for carrageenan. The food additive can also promote activation of lymphocytes [20]. Frossard et al demonstrated that orally administered λ-carrageenan caused lymphocyte anergy in mice [21]. It has been reported that semi-refined carrageenan does not promote lymphocyte apoptosis in vitro, but upregulates anti-apoptotic bcl-2 protein [22]. Thus, little information concerning the features of carrageenan impact on lymphocytes both in vivo and in vitro is available.

The aim of this study was to assess ROS production by lymphocytes of rats treated with a thickener and emulsifier E407a (semi-refined carrageenan) during two weeks.

Material and methods

Animals

The animal study was approved by the Committee of Bioethics at Kharkiv National Medical University (Kharkiv, Ukraine). All procedures were performed in accordance with international and institutional guidelines, including the EU Directive 2010/63/EU on the protection of animals used for scientific purposes and recommendations of the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123).

Sixteen female WAG rats weighing 160-190 g were provided by the vivarium. They were kept in standard conditions starting from 2 weeks prior to the beginning of the experiment. This was required for their acclimatization. The animals from the experimental group (n=8) were treated with E407a (140 mg/kg of weight) during 14 days on a daily basis. The control group received no semi-refined carrageenan, i.e. E407a. E407a (processed Eucheuma seaweed, PES or semi-refined carrageenan) is obtained from algae Eucheuma cottonii and Eucheuma spinosum, when they are treated at high temperature with potassium hydroxide solution. This is followed by removal of impurities by washing with alcohols. Besides carrageenan, which is the major component of the food additive, PES may contain some amounts of cellulose (EU Commission regulation No 231/2012 d.d. March 9, 2012).

Measurement of spontaneous ROS in lymphocytes

Determination of ROS production in lymphocytes was carried out by flow cytometry using a cell-permeable probe 2' 7'-dichlorodihydrofluorescein diacetate (H2DCFDA). This dye can diffuse into the cells with the subsequent deacetylation by cellular esterases and its conversion into dichlorofluorescein (DCF) whose fluorescence is proportional to the amount of intracellular ROS.

Blood obtained from the animals of both groups was lysed by FACSLyse solution (Becton Dickinson, San Jose, USA) and washed twice with phosphate-buffered saline (PBS, BD, USA). Then leukocyte pellets were resuspended in PBS and stained with H2DCFDA (InvitrogenTM, USA) for 30 minutes at 37ºC. The concentration of H2DCFDA in working solutions was 5 μM. Prior to the experiment, H2DCFDA solutions in PBS were prepared from its stock solution in DMSO (10mM). Incubation of samples with H2DCFDA occurred in the dark to avoid photooxidation of the probe. Moreover, leukocyte suspensions were incubated during 15 minutes with 7-aminoactinomycin D (7-AAD), a fluorescent dye that can bind to DNA. It is used to evaluate the integrity of cell membranes and, thus, the viability of cells. The fluorescence of DCF and 7-AAD was detected in a FACS Canto II flow cytometer (BD, USA) using the FL-1 and FL-3 channels, respectively.

The region of lymphocytes was gated based on FSC/SSC dotplots. The percentage of ROS^{high}, $7\text{-}AAD^{-}$ lymphocytes was analyzed in both groups. Then the mean fluorescence intensity (MFI) of DCF was registered. BD FACSDivaTM software was used to process the results of flow cytometry.

Statistics

The results obtained in both groups of animals were compared using Mann-Whitney U test. Values were presented as the median (Me) and interquartile range (IQR). We considered the differences statistically significant if p values were below 0.05. To process numerical data, Graph Pad Prism 5.0 was used.

Results

To analyze the redox state of lymphocytes obtained from the animals treated with E407a, we used H2DCFDA. The region of lymphocytes was gated in FSC/SSC dotplots (Figure 1). Then $7\text{-}AAD^{-}$ cells were selected in the region of lymphocytes to analyze the fluorescence of DCF in order to exclude non-viable cells, since 7-AAD is known to be a DNA intercalator that is able to enter the cell only if its membrane integrity is compromised, which is typical for late apoptotic and necrotic cells.

Depending on DCF fluorescence, $7\text{-}AAD^{-}$ lymphocytes, i.e. viable cells, were subdivided into ROS^{high} and ROS^{low} subpopulations. We present data on flow cytometric detection of

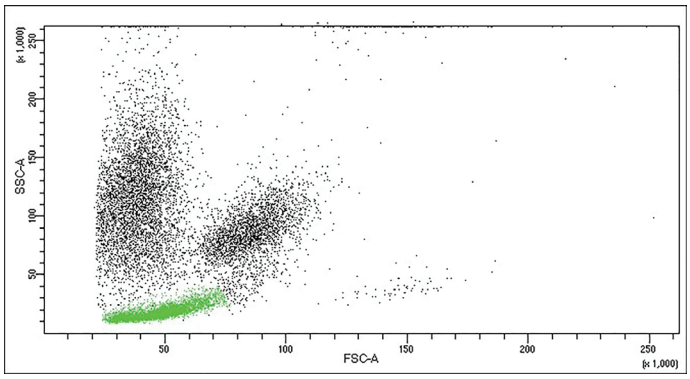

Figure 1 - Representative FSC/SSC dotplot. The region of lymphocytes was gated based on the forward and side scatters. It is highlighted in green.

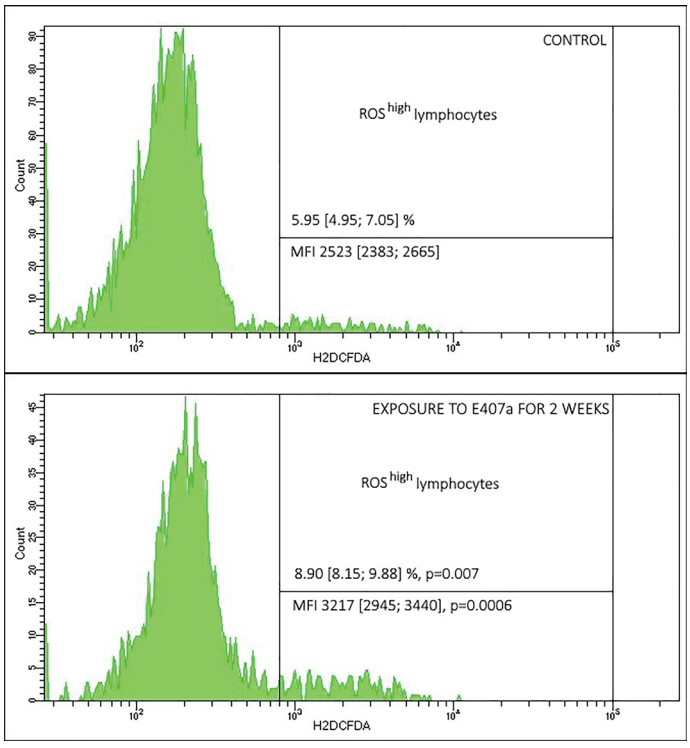

Figure 2 - Representative SSC/H2DCFDA histograms of 7-AAD$^-$ lymphocytes obtained from animals of the control (A) and experimental groups (B). Treatment of rats with E407a resulted in an increase in both the amount of ROS^{high} viable lymphocytes and the mean fluorescence intensity (MFI) of dichlorofluorescein (DCF) in such cells compared with controls.

ROS production in Figure 2. ROS^{high} and ROS^{low} populations are demonstrated in the corresponding histograms.

The percentage of cells in ROS^{high} subpopulation of lymphocytes was revealed to be statistically significantly 49.6% higher in the rats exposed to E407a compared with the control group (Figure 2). Moreover, MFI of DCF was determined in ROS^{high} lymphocytes to quantitatively assess ROS generation, since ROS production is proportional to DCF fluorescence in cells. When comparing MFI values, we found to be statistically significantly higher (27.5%) in the experimental group than in controls (Figure 2). Thus, the increase in ROS generation was also reflected when our data were demonstrated as MFI.

Discussion

ROS are known to be produced by many types of immunocompetent cells, including lymphocytes [23,24]. Excessive ROS generation in lymphocytes may promote the development of oxidative stress and immunosenescence [25]. The former is observed when ROS overproduction is not counterbalanced by the adequate activation of antioxidant systems. Despite the fact that canonically ROS have been believed to be cytotoxic agents inducing damage to intracellular molecules, nowadays ROS in lymphocytes are also thought to act as signaling molecules. They are involved in regulation of cytokine production, cell activation, antibody production, apoptosis, and proliferation [26, 27]. It is important to mention that the major sources of ROS in lymphocytes are NADPH oxidase and leakage of electrons from the electron transport chain [23, 26]. Given the complex role of ROS in lymphocytes, they are important players in inflammation.

In this study, oral exposure to semi-refined carrageenan resulted in overproduction of ROS in lymphocytes. However, it is not clear whether this generation was stimulated by E407a directly or lymphocytes produced free radicals in response to pro-inflammatory cytokines. There are many reports concerning different effects of carrageenans on the redox state of cells in the body and cell cultures. There is strong evidence that carrageenan-induced inflammation is accompanied by excessive ROS generation by various types of cells and the development of oxidative stress [17,28,29]. Our current findings corroborate with such data. In addition, it is emphasized that ROS in carrageenan-induced inflammation are produced by neutrophils. In our study, it was shown that lymphocytes also contributed to this process.

Unexpectedly, many studies, including our own researches (unpublished data), have demonstrated that incubation of immune cells with carrageenan doesn't affect ROS generation [18, 19]. Furthermore, it has been shown that carrageenans have no cytototoxic effects in experiments performed using cell cultures [18]. Nor this food additive can promote apoptosis of cells even at relatively high concentrations [22].

However, carrageenans were able not only to induce ROS generation by macrophages but also to upregulate cytokines at high concentration in a dose-dependent manner [30]. ROS were also induced by carrageenans in neutrophils [31, 32]. Thus, data on direct immunotoxic and immunomodulatory effects of carrageenans is controversial and this issue requires further thorough investigation. Our findings suggest that, apart from neutrophils and macrophages, lymphocytes are also involved in ROS generation promoted by carrageenans.

Excessive ROS synthesis by lymphocytes caused by exposure to semi-refined carrageenan may promote the release of pro-inflammatory cytokines, which contributes to the progression of inflammation. However, to shed a light on the role of lymphocyte-derived ROS in the pathogenesis of carrageenan-induced intestinal inflammation, effects of carrageenans on various mechanisms of ROS production by lymphocytes have to be scrutinized. Some evidence on the possible ability of carrageenans to magnify lipopolysaccharide (LPS)-induced ROS generation has been accumulated [32, 33]. Thus, carrageenans may make already existing intestinal inflammation more severe via intensifying bacteria-stimulated reactions.

Conclusion

A common food additive E407a can induce ROS generation by lymphocytes after oral administration. Thus, lymphocytes are involved in the mechanisms of carrageenan-induced inflammation development.

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