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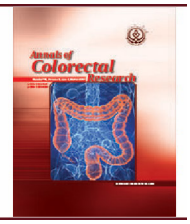

Oral Intake of Semi-refined Carrageenan by Rats Affects Apoptosis of Lymphocytes

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Abstract

Introduction: The safety of generally recognized as safe food additives E407 and E407a is under rigorous debate. Our research aimed to evaluate the effects of the orally administered E407a (semi-refined carrageenan) on the viability of lymphocytes and their cell death modes.
Methods: In total, 16 adult WAG rats were divided into two equal groups (experimental – oral intake of 140 mg/kg of E407a for two weeks; control – oral consumption of drinking water instead). Blood samples were used to obtain leukocyte suspensions stained with Annexin V-FITC and 7-aminoactinomycin D (7-AAD). The region of lymphocytes was analyzed after collecting data using a BD FACSCanto™ II flow cytometer.
Results: Oral administration of E407a led to a decrease in the number of viable (Annexin V^-, 7-AAD^-) circulating lymphocytes. Furthermore, exposure to semi-refined carrageenan resulted in an elevated number of early apoptotic (Annexin V^+, 7-AAD^-) lymphocytes; their percentage was approximately four times higher in rats exposed to E407a compared with the control group.
Conclusion: Our findings indicate that dietary intake of E407a promotes apoptosis of circulating lymphocytes.

Keywords: Processed *Eucheuma* seaweed, Annexin V, 7-aminoactinomycin D, Flow cytometry

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Introduction

Colorectal cancer (CRC) is known to be the second leading cancer-related cause of death (1). Despite some recent advances in its treatment, CRC remains a serious medical and social burden. According to some estimates, CRC was responsible for approximately 2 million deaths globally in 2018 (2). CRC is a heterogeneous disease with numerous modifiable factors, including dietary habits, physical activity, obesity, cigarette smoking, etc., which can contribute to its etiology (3). Among the abovementioned environmental factors that play an important role in the development of CRC, unhealthy dietary patterns are of crucial importance. In particular, a diet based on fruits and vegetables with limited consumption of red meat and confectionary is associated with a lower risk of CRC (4). In addition, there is some

evidence that food additives, even those that are officially approved, have carcinogenic properties (5). One such food additive is carrageenan, registered as E407 (refined carrageenan) and E407a (semi-refined carrageenan). Carrageenans are galactans composed of repeating disaccharides, namely β-1,3- and α-1,4-linked derivatives of galactose with sulfate groups (6). Three major commercially available types of carrageenans (lambda, kappa, and iota) are widely used to improve the texture of processed meat and provide creaminess in dairy products. They are also added to food products as emulsifiers and thickeners (7). It is widely accepted and demonstrated in numerous animal studies that degraded and low-molecular-weight carrageenans (poligeenans), officially prohibited from use as food additives, can promote ulceration and tumor development in the gut upon oral exposure (8). However, undegraded or native food-grade carrageenans have also raised carcinogenicity concerns. Nonetheless, it has been suggested that carrageenans don't act as direct carcinogens or mutagens. Instead, they stimulate the development of intestinal neoplasms via inducing inflammation, which contributes to cancer (9). Indeed, there is strong evidence that food-grade carrageenans ingested by laboratory animals (mice, rats, and guinea pigs) induce intestinal inflammation (7, 8, 10-15). Furthermore, the long-term oral consumption of carrageenans in animal experiments promotes the development of intestinal polyps, which can be precancerous (8, 16). Despite lacking mutagenic and carcinogenic properties, carrageenan creates a favorable inflammatory microenvironment for tumor development. In particular, inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis can be considered an important risk factor for the development of gastrointestinal malignancies (17). Accumulating evidence indicates that consumption of carrageenans contributes to IBD pathophysiology (16, 18). Furthermore, a randomized trial showed that dietary restriction of carrageenans prevents the relapses of ulcerative colitis (19). Therefore, carrageenan-induced intestinal inflammation might be a risk factor for CRC development, and more studies are needed to elucidate both the local and systemic effects of ingested carrageenans, shedding light on the mechanisms underlying their contribution to IBD and CRC.

The purpose of our study was to evaluate the effects of orally administered E407a on the viability of lymphocytes and their cell death modes.

Materials and Methods

Animals and Groups

This research was performed using sixteen adult WAG rats of the female sex. Their weight varied from 160 to 200 g. The rats were used to form two groups on a random basis: experimental and control. Each group consisted of 8 rats. Acclimatization of the rats in the vivarium lasted for two weeks prior to the commencement of the experiment. The rats that formed the experimental group were administered 140 mg of semi-refined carrageenan per kg of weight daily for two weeks. Drinking water was used as a vehicle. The animals from the control group consumed equal volumes of drinking water with no added carrageenan.

The standards for care and housing of laboratory animals (EU Directive 2010/63/EU) were followed. The institutional ethics committee approved the study design.

Preparation of Leukocyte Suspensions

To analyze viability and cell death modes of lymphocytes in rats exposed to the E407a food additive, leukocyte suspensions were obtained from blood samples collected from rats of both groups. Initially, blood was collected in K_2EDTA Vacutainers™ (IMPROVACUTER® Evacuated EDTA K_2 Spray Dried PET Tubes, Guangzhou, China). Then, specimens whose volume was 100 µl were transferred to 12 x 75 mm polystyrene tubes. Subsequently, 2 ml of an ammonium chloride-based lysing reagent (BD Pharmlyse™ Lysing Buffer, lot 0070764, San Jose, USA) was added to lyse erythrocytes. Incubation with 1x Pharmlyse buffer lasted for 15 minutes at 24 °C. Solutions were centrifuged at 500g for 5 minutes. Phosphate buffered saline (PBS; pH 7.4; BD™ Cell Wash, Poland) was used to wash the leukocytes twice.

Staining Protocol

After the resuspension of cells in 1 ml of 1x Annexin-binding buffer (BD Pharmingen™ Annexin V Binding Buffer, lot 8145742, BD Biosciences, San Jose, USA), 100 µl aliquots were incubated with 5 µl of fluorescein isothiocyanate (FITC)-labeled Annexin V (BD Pharmingen™ FITC-Annexin V, lot 8311824, BD Biosciences, San Jose, USA) and 5 µl of 7-aminoactinomycin D (7-AAD, BD Pharmingen™, lot 8263992, BD Biosciences, San Jose, USA). After a 20-minute-long incubation, 400 µl of 1x Annexin-binding buffer was added.

Flow Cytometric Analysis

The BD FACSCanto™ II flow cytometer (Becton Dickinson, USA) was employed for flow cytometric analysis. To collect and process the results, BD FACSDiva™ software was used. Identification of the region of lymphocytes was based on forward versus side scatter (FSC vs. SSC). Annexin V-FITC is optimally excited at 494 nm and has a peak emission at 519 nm. Its fluorescence was registered in the standard FITC (FL1 detector) channel. 7-AAD is excited at 488 nm and emits at approximately 670 nm. Its fluorescence was detected in the FL3 channel. Annexin V and 7-AAD staining is used to identify four populations: 1 - viable cells (Annexin

V$^-$, 7-AAD$^-$); 2 – early apoptotic cells (Annexin V$^+$, 7-AAD$^-$); 3 - late apoptotic/necrotic cells (Annexin V$^+$, 7-AAD$^+$); and 4 - dead necrotic cells (Annexin V$^-$, 7-AAD$^+$).

Statistical Analysis

The Shapiro-Wilk test was used to assess the normality of the data. The outcome of the Shapiro-Wilk test substantiated the use of the non-parametric Mann-Whitney U test. Thus, non-normally distributed data were reported as median and interquartile range. Differences were considered as statistically significant if P-values were below 0.05. Numerical data were analyzed by GraphPad Prism 7.05 (GraphPad Software, USA).

Results

Annexin V/7-AAD staining is a convenient, widely recognized, and accepted approach to discriminate viable, early apoptotic, late apoptotic/necrotic, and dead necrotic cells. In this study, we assessed the way oral exposure to E407a affects the viability and cell death modes of circulating lymphocytes.

Our findings are summarized in Table 1 and Figures 1-3.

The number of viable lymphocytes that remained unstained (Annexin V$^-$, 7-AAD$^-$) was found to be significantly lower in rats treated with semi-refined carrageenan over two weeks compared with control samples (Table 1). In addition, exposure to E407a resulted in a larger number of circulating early apoptotic cells (Annexin V$^+$, 7-AAD$^-$). Such cells are characterized by phosphatidylserine (PS) externalization, i.e., the translocation of PS (an anionic phospholipid located in the inner leaflet of the cell membrane in normal conditions) to the outer leaflet. Annexin V, in turn, binds to PS. At the same time, the membrane of such cells remains intact, leading to positive 7-AAD staining. The percentage of such early apoptotic lymphocytes in the experimental group of animals was revealed to be almost four-fold higher relative to the control group (Table 1). Of note, oral exposure to E407a did not affect the number of late apoptotic/necrotic (Annexin V$^+$, 7-AAD$^+$) lymphocytes, i.e., cells with externalized PS and compromised cell membrane integrity. No significant difference was found between the percentages of dead necrotic (Annexin V$^-$, 7-AAD$^+$) circulating lymphocytes of rats untreated and treated orally with semi-refined carrageenan (Table 1).

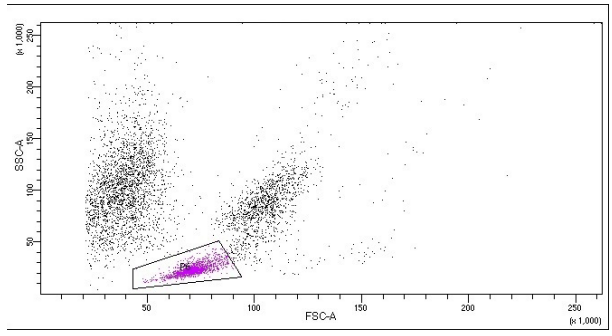

Figure 1: An FSC/SSC dot-plot shows how the region of lymphocytes is identified.

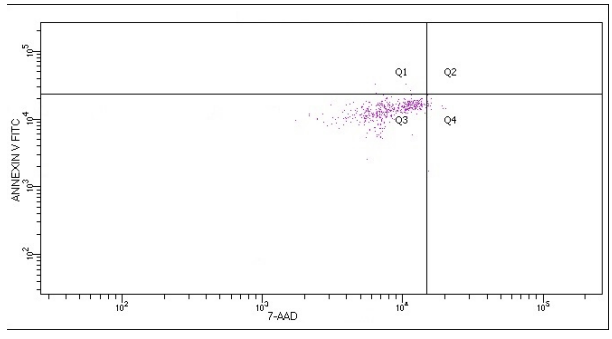

Figure 2: A representative FL-1/FL-3 dot-plot of a control sample. Different populations of lymphocytes are demonstrated: Q1 - early apoptotic lymphocytes (Annexin V$^+$, 7-AAD$^-$ cells); Q2 - late apoptotic/necrotic lymphocytes (Annexin V$^+$, 7-AAD$^+$); Q3 - viable lymphocytes (Annexin V$^-$, 7-AAD$^-$ cells); and dead necrotic CD45$^+$ lymphocytes (Annexin V$^-$, 7-AAD$^+$).

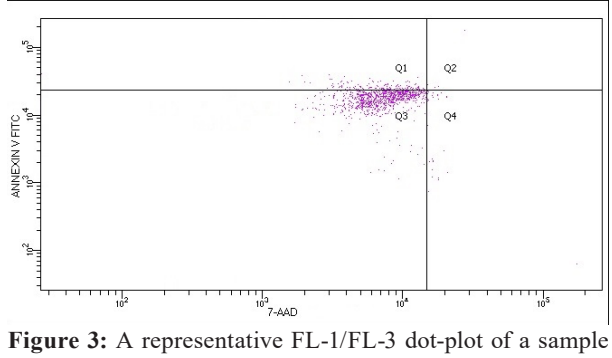

Figure 3: A representative FL-1/FL-3 dot-plot of a sample obtained from a rat exposed to E407a. Four populations of lymphocytes can be seen: Q1 - early apoptotic lymphocytes (Annexin V$^+$, 7-AAD$^-$ cells); Q2 - late apoptotic/necrotic lymphocytes (Annexin V$^+$, 7-AAD$^+$); Q3 - viable lymphocytes (Annexin V$^-$, 7-AAD$^-$ cells); and dead necrotic CD45$^+$ lymphocytes (Annexin V$^-$, 7-AAD$^+$).

Discussion

Our studies performed earlier have demonstrated that carrageenan administered orally to laboratory animals promotes the development of enterocolitis, evidenced by both local and systemic alterations,

Table 1: Effects of E407a on cell death modes of circulating lymphocytes

Groups of animals Percentage of cells	Control group (n=8), %	E407a intake during 14 days per os (n=8), %	P value
Viable lymphocytes (Annexin V$^-$, 7-AAD$^-$ cells)	94.95 [94.10; 96.10]	86.90 [85.85; 88.15]	0.0006
Early apoptotic lymphocytes (Annexin V$^+$, 7-AAD$^-$ cells)	1.45[0.88; 1.95]	5.75 [3.88; 7.95]	0.0019
Late apoptotic/necrotic lymphocytes (Annexin V$^+$, 7-AAD$^+$ cells)	0.75 [0.10; 1.28]	1.45 [0.40; 2.90]	0.2664
Dead necrotic CD45$^+$ lymphocytes (Annexin V$^-$, 7-AAD$^+$ cells)	2.60 [1.83; 2.60]	3.83 [2.83; 5.10]	0.1235

including the affected morphology of both small and large bowels, changes in the blood serum cytokine profile with the elevation of circulating pro-inflammatory cytokines, oxidative stress development, and modifications of the phospholipid bilayer of cell membranes (11-13, 20). This study has supplemented conclusions concerning the toxicity of E407a. Our findings support the hypothesis that orally consumed carrageenans can result in extraintestinal effects, in particular, promotion of lymphocyte apoptosis. Our data are consistent with other studies in which food-grade refined carrageenan was reported to induce apoptosis of leukocytes (21). In addition, E407a has been shown to induce reactive oxygen species (ROS) generation by lymphocytes after oral administration (22). This suggests the possible role of ROS-mediated pathways of apoptosis induction in lymphocytes of rats exposed to this food additive. Moreover, there is some evidence that incubation of cells with carrageenans results in ROS overproduction (23), which supports the implication of ROS in lymphocyte apoptosis after the administration of E407a. However, it is interesting to note that while the direct impact of E407a on lymphocytes does not stimulate apoptosis, short-term incubation with E407a leads to upregulation of anti-apoptotic Bcl-2 (24). Furthermore, no direct cytotoxic effects of carrageenans have been revealed in other studies (25, 26). Thus, we believe that apoptosis activation observed in our research can be mediated by indirect mechanisms via an inflammatory response that emerges locally in the intestine. A plethora of animal studies that support the toxicity of E407 and E407a and the controversial data of experiments performed on cell cultures suggest that the effects of carrageenans in the body are complex and involve combined interactions. In particular, interactions of carrageenan with the gut microbiota may mediate the pro-inflammatory response to the food additive. This hypothesis is in agreement with numerous findings that have revealed the role of carrageenan in enhancing bacterial lipopolysaccharide (LPS)-induced upregulation of pro-inflammatory cytokines (27, 28). Furthermore, carrageenan has been shown to worsen bacterial intestinal inflammation in mice. Authors state that κ-carrageenan stimulates the Bcl10-NF-κB-mediated pathway and thereby activates LPS-induced secretion of IL-8 while promoting the expression of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). This transcriptional factor is known to be highly pro-inflammatory (28).

Our study sheds light on the effects of E407a consumption on cell death modes of lymphocytes. However, more well-designed research is needed to study the mechanisms involved in carrageenan-induced activation of apoptosis. In addition, this research reinforces the need for novel studies, primarily clinical ones, to evaluate the role of carrageenans in the development of IBD and CRC.

Conclusion

Our data indicate that the food additive E407a stimulates apoptosis of circulating lymphocytes.

Funding

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Authors Contributions: Study concept and design: Tkachenko. Data analysis and interpretation: Tkachenko and Onishchenko. Drafting: Tkachenko. Critical revision of the manuscript for important intellectual content: Tkachenko and Onishchenko. Statistical analysis: Tkachenko.

Conflicts of interests: None declared.

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