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INTAKE OF SEMI-REFINED CARRAGEENAN CAUSES LOW-GRADE COLONIC INFLAMMATION AND ALTERS EXPRESSION OF EPITHELIAL-MESENCHYMAL TRANSITION MARKERS

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Abstract

The aim of this research was to evaluate the effects of semi-refined carrageenan consumed orally on the amount of CD3 and CD68 positive cells residing in the colon and the expression of endothelial-mesenchymal transition (EMT) markers.

Materials and methods. To assess colonic expression of $CD3^+$, $CD68^+$, fascin, vimentin and E-cadherin, sections obtained from 8 rats orally exposed to semi-refined carrageenan (E407a) at a dose of 140 mg / kg of weight during 14 consecutive days and 8 control animals were immunostained with the corresponding antibodies. Levels of expression were assessed quantitatively.

Results. Oral exposure to semi-refined carrageenan resulted in an increase in CD3 and CD68 positive cells in the colonic lamina propria. Quantitative analysis of fascin, vimentin and E-cadherin immunostaining revealed changes in expression of these EMT markers both in the colonic stroma and epithelial cells. Vimentin and fascin were overexpressed in stromal and epithelial cells, while E-cadherin was upregulated in the stroma and downregulated in epithelia.

Conclusions. Our observations suggest that oral intake of semi-refined carrageenan results in the development of low-grade colonic inflammation accompanied by infiltration with CD3 and CD68 positive cells and changes in the expression of EMT markers.

INTRODUCTION

Inflammatory bowel disease (IBD) is defined as a group of chronic intestinal inflammatory disorders and comprises Crohn's disease (CD) and ulcerative colitis (UC) [1]. In UC, inflammation is mainly limited to colonic mucosa and submucosa, while in CD it is transmural and affects mucosal, submucosal, muscular and serosal layers of either/both small or/and large bowels [2]. Both incidence and prevalence of CD and UC have been increasing for decades and approximately 6.8 million cases of IBD were registered worldwide by 2017. This trend is also typical for the pediatric population. Despite the implementation of novel therapeutic strategies, IBD cannot be easily suppressed and,

hence, significantly affects the quality of life of patients. Thus, IBD remains a huge public health problem [3].

The etiology of IBD is still not fully understood. However, it is believed to develop due to the impact of certain environmental factors against the background of genetic predisposition. Recent studies have significantly improved our understating of IBD etiology and pathogenesis. In particular, IBD-associated genetic risk loci have been identified. It has been shown that nucleotide-binding oligomerization domain 2 (NOD2) gene, which encodes a cytosolic protein expressed primarily in macrophages and dendritic cells, is often mutated in IBD [4]. NOD2 plays a crucial role in the regulation of interactions between intestinal microbiota and the host immune system and, thus, gut homeostasis [5]. This corroborates the fact that both CD and UC are characterized by impaired host immune responses to the gut microbiome [6]. Moreover, recent genome-wide association study (GWAS)-based data indicate that there are over 200 risk loci for IBD, including those implicated in autophagy and the IL-17/IL-23 axis [7; 8].

Of interest, most environmental factors contribute to the IBD development via affecting the intestinal microbiota. Diet remains a major such factor. It is well established that the prevalence of IBD is higher in industrialized Western countries. In such societies, diet is different from traditional nutritional habits and is characterized by the switch from plant-based to animal-based sources, high calorie intake, consumption of processed, ultra-processed food and food additives [9]. Among other food additives, carrageenan (CGNs) have been reported to induce intestinal inflammation and to promote morphological changes in the gut similar to those observed in IBD [10; 11]. CGNs are sulfated linear heteropolysaccharides extracted from marine algae, primarily *Kappaphycus alvarezii*, and made up of D-galactose and 3,6-anhydrogalacose monomers linked with either α1,4- or α1,3-glycosidic bonds [12]. CGN is manufactured in two basic grades: refined (E407) and semi-refined (E407a). Both of them are used as gelling agents and thickeners in food industry. However, there is compelling evidence that CGNs have pro-inflammatory and immunogenic properties [13, 14]. CGNs may activate the innate immunity pathways via stimulation of toll-like receptor 4 (TLR4) signaling and B-cell lymphoma/leukemia 10 (BCL10)-dependent activation of a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pro-inflammatory transcription factor [15, 16], modify the gut microbiota [17], stimulate apoptosis [18] promote ROS generation [19-21]. Data from a recently published randomized trial support the view of CGN contribution to IBD. Its findings indicate that CGN-free diet is effective in the management of UC [22]. However, further research is needed to clarify the role of CGN in IBD.

IBD pathogenesis is multifactorial and includes a dysregulated immune response. There is strong evidence that lamina propria macrophages and lymphocytes contribute to the IBD-related colonic inflammation [23-25]. Multiple studies have provided scientifically substantiated evidence that inflamed colonic tissues of IBD patients are characterized by an increase in the amount of lamina propria CD3 positive and CD68 positive cells [25-27]. CD68 is a panmonocytic/macrophagic glycoprotein, while CD3 is recognized as a T-lymphocytic marker. Moreover, T cells and macrophages are reported to drive inflammation and tissue injury in IBD [24, 28]. Thus, it can be assumed that CGNs may affect the number of CD3 positive T-lymphocytes and CD68 positive macrophages in the large intestine.

Intestinal fibrosis, which develops on the basis of chronic inflammation, is a common complication of IBD. There is well documented evidence that epithelial-mesenchymal transition (EMT) contributes to the development of IBD-associated fibrotic lesions, including strictures and fistulae [26, 29]. EMT is a process observed in epithelial cells when epithelial markers are downregulated, while mesenchymal markers are upregulated, and, hence, epithelial cells acquire mesenchymal phenotype and fibroblast-like features, including motility [30]. This process plays an important role during ontogenesis and is involved in embryogenesis and organ development [26]. Given the role of EMT in IBD, it is of interest to analyze the impact of CGNs on expression of EMT-associated markers in the large bowel.

The aim of our study was to assess colonic morphology with the emphasis on formation of leukocyte infiltration, its cellular content, as well as expression of EMT-associated markers in the colon of rats orally exposed to semi-refined CGN.

MATERIALS AND METHODS

Animals

Sixteen female WAG rats (175 ± 15 g) were bred in a vivarium of Kharkiv National Medical University (Kharkiv, Ukraine). Rodents were subdivided into two groups in a random order. Group 1 (n=8) included animals consumed the food additive E407a. Group 2 (n=8) consisted of intact rats. The sample size was calculated using a G*Power 3 application with the values of alpha error equal to 0.05 and power equal to 0.8. Rats were housed in cages. Temperature, humidity and lighting conditions were standard and uniform. Access to water and chow was free. The study was approved by the Ethics and Bioethics Committee of Kharkiv National Medical University (Kharkiv, Ukraine, minutes #5, September 17, 2019). 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) were followed.

Semi-refined Carrageenan and its Administration

Semi-refined carrageenan purchased from Foodchem International Corporation (China) was administered orally on a daily basis for 14 consecutive days to rats from group 1 at a dose of 140 mg/kg of weight. Drinking water was used as a vehicle. The food additive was added a drinking water forming a 1% solution. Control animals were treated with water instead of semi-refined carrageenan solution. Rodents from both groups were sacrificed on day 15. Fragments of colon of equal size were collected and cleaned with saline.

Routine Stain Methods and Immunostaining

Formalin-fixed and paraffin-embedded samples of large intestine collected from rats of both groups were used to obtain 4μm-thick sections, which were stained with hematoxylin and eosin in accordance with the standard protocol. In addition, periodic acid–Schiff (PAS) staining was used to detect mucins in colonic goblet cells. For immunostaining, the sections were placed on 3-aminopropyltriethoxysilane-coated slides. Antigen retrieval was performed in a 95–97°C water bath in accordance with a standard protocol. Slides were incubated with antibodies to CD3, CD68, vimentin, fascin, and E-cadherin, respectively. Visualization was performed using UltraVision^{TM} Quanto Detection System HPR DAB (*Thermo Fischer Scientific*, USA). Brown staining was considered positive.

Images were captured using Axiostar-plus" (Zeiss, Germany) microscope.

Description of Scoring Systems

The amount of immunoreactive cells in the lamina propria of large bowel was determined in areas of a fixed size (250x250 μm). In each sample, five such random regions were used to count macrophages ($CD68^+$), T-lymphocytes ($CD3^+$), fascin-expressing, vimentin-positive and E-cadherin-labelled cells. Cells showing any reactivity were considered positive for expression of the corresponding markers.

The number of CD3 positive and CD68 positive cells per area was assessed and compared. Moreover, the $CD68^+/CD68^-$ and $CD3^+/CD3^-$ ratios were calculated. The number of cells that express EMT markers in the colonic lamina propria was analyzed in the same way.

Furthermore, expression of fascin, vimentin and E-cadherin in the stromal cells and epithelial layer was analyzed quantitatively. With the help of light microscopy, the relative intensity of brown DAB coloration was determined by a trained pathologist. An immunostaining scoring system based on the determination of luminosity of areas formed by several positively stained epithelial cells and single immunoreactive stromal cells was applied [31]. The background luminosity-to-immunoreactive cell luminosity ratio was calculated. Then the common logarithms of the numerical values obtained were found. The results were expressed in arbitrary units (a.u.). Five random areas of the epithelial lining and stroma were analyzed in each slide to avoid biases.

Statistical Analysis

The Kolmogorov-Smirnov and Shapiro-Wilk tests showed the normal distribution of variables. Thus, analysis of scoring results was performed by conducting Student's t test for comparing two independent groups. Numerical values were presented as the mean ± standard error of mean (SEM). The statistically significant difference between two groups of variables was observed when p values were below 0.05. GraphPad Prism 5.0 application (GraphPad software, USA) was used to process the data of immunohistochemical scoring.

RESULTS AND DISCUSSIONS

Microscopically, the colon of rats from the control group has the typical histological structure. In contrast to the control group, the strong expansion of the colonic lumen against the background of thinner walls was observed in the experimental group of animals. At the same time, the mucous membrane was so thinned that crypts looked much shorter than in controls. Small areas where crypts are absent could be noticed. Visually, the cryptal epithelium had a reduced number of goblet cells. Moreover, numerous foci with densely placed and elongated epithelial cells were found. In some regions, the epithelial layer had 2-3 rows. Of note, the surface epithelium lacked the basement membrane in such areas.

The mucosal lamina propria between the crypts was much wider than in the control group. Signs of edema were revealed. Leukocyte infiltrate was found to be more abundant in rats orally exposed to semi-refined carrageenan. In addition, the amount of fibroblasts and fibrocytes was visually higher in the experimental group. The data from scoring is provided in Tables 1-3.

Analysis of CD3 immunoreactivity revealed that CD3 positive cells, i.e. T-lymphocytes, were visualized in the colonic lamina propria in both groups. Their distribution was predominantly diffuse. To assess CD3 expression quantitatively, the total amount of CD3 positive cells per area and CD3 positive cells-to-CD3-negative cells ratio were compared between experimental and control groups. Oral exposure to E407a was found to be associated with an increase in the absolute number of CD3 positive cells within the lamina propria (Figure 1). The difference was statistically significant and exceeded 40% compared with controls. The proportion of CD3 positive cells was also almost three times higher (Table 1).

Table 1. CD3 and CD68 staining parameters in the lamina propria of large bowel in rats exposed to semi-refined carrageenan (Mean±SEM)

Groups Parameters	Control group (n=8)	Rats orally exposed to semi-refined carrageenan (n=8)
CD3 positive cells	2.3±1.0	3.2±1.1 p<0.0001
CD3 positive cells-to- CD3 negative cells ratio	0.16±0.07	0.45±0.16 p<0.0001
CD68 positive cells	2.3±1.3	3.6±1.1 p<0.0001
CD68 positive cells-to- CD68 negative cells ratio	0.14±0.08	0.43±0.15 p<0.0001

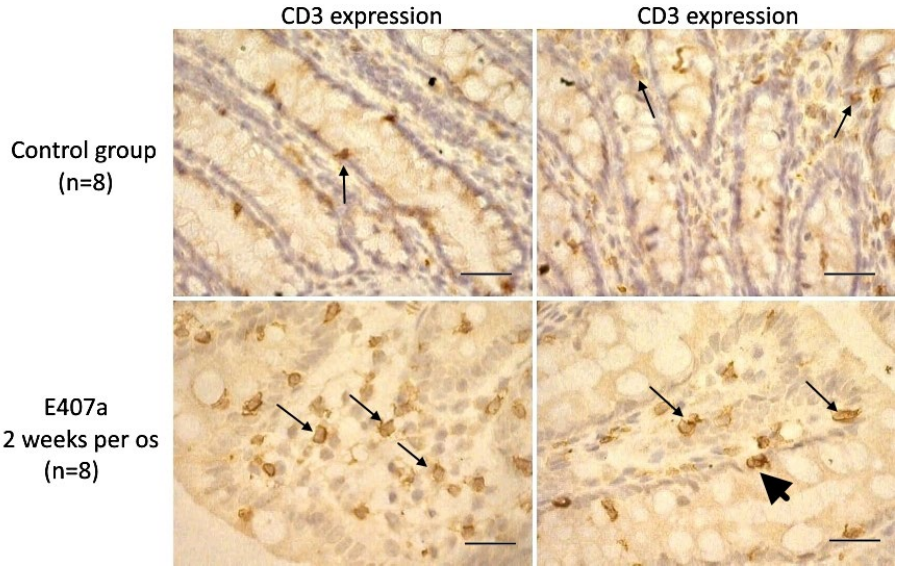

Figure 1. The colonic mucous membrane of control rats and rodents orally exposed to semi-refined carrageenan were immunostained for T-lymphocyte-specific marker CD3 A, B). Control rats. CD3 positive cells are diffusely distributed in the lamina propria. 400x. C, D) Animals exposed to E407a. The number of CD3 positive cells in the lamina propria is higher compared with the control group. 400x. Scale bar: 25 μm. Expression of the marker is shown with arrows.

Similar changes in CD68 immunostaining within the colonic mucosa were revealed (Figure 2). The amount of cells that express this macrophage-specific antigen in the large intestinal lamina propria was approximately 54% higher is rats treated with E407a in relation to controls. The difference between groups was statistically significant. Meanwhile, the CD68 positive cells-to-CD68 negative cells ratio was significantly more than 3-fold higher in animals exposed to refined carrageenan than in the control group (Table 1). It is worth mentioning that in contrast to T-lymphocytes distributed predominantly diffusively, macrophages tended to form groups composed of several cells.

Immunohistochemical analysis of fascin expression in the colonic lamina propria showed that oral exposure to E407a was associated with an increase in the number of fascin-expressing cells (3.3-fold higher compared with control, $p<0.0001$). The difference was even more pronounced when comparing the ratio of fascin-positive cells to fascin-negative cells. In the experimental group, this parameter was statistically significantly ($p<0.0001$) 6.8 times higher than in the control group (Table 2; Figure 3).

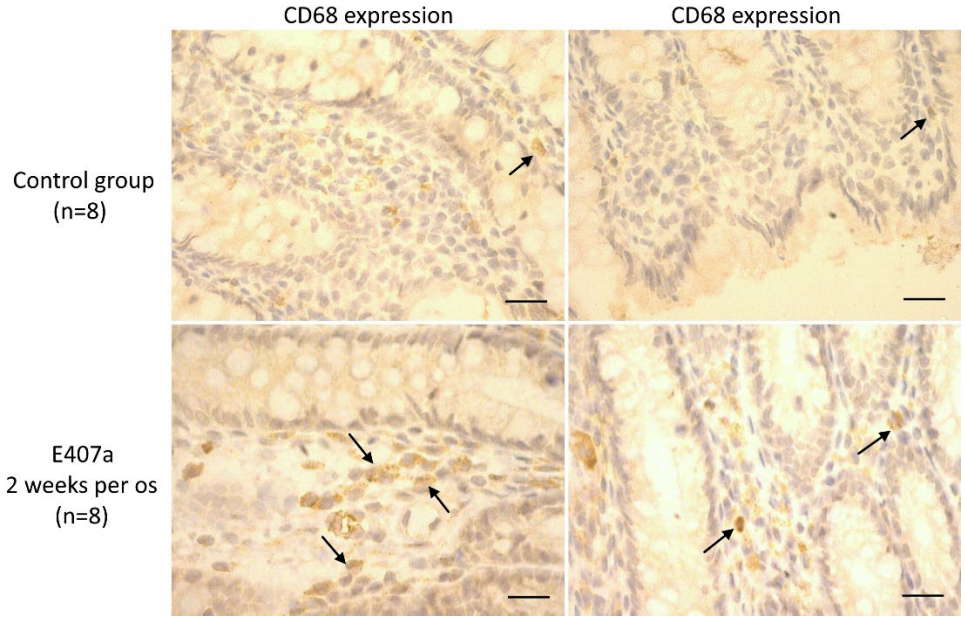

Figure 2. Images of macrophage-specific CD68 staining of the large bowel in control rats and animals administered semi-refined carrageenan. A, B). Control animals. Normal colonic mucosa shows a moderate amount of macrophages located in the lamina propria. 400x. C, D). Rats orally administered semi-refined carrageenan. Regions of the lamina propria contain more CD68 positive cells. Note that macrophages tend to form groups of cells. 400x. Scale bar: 25 µm. Expression of the marker is shown with arrows.

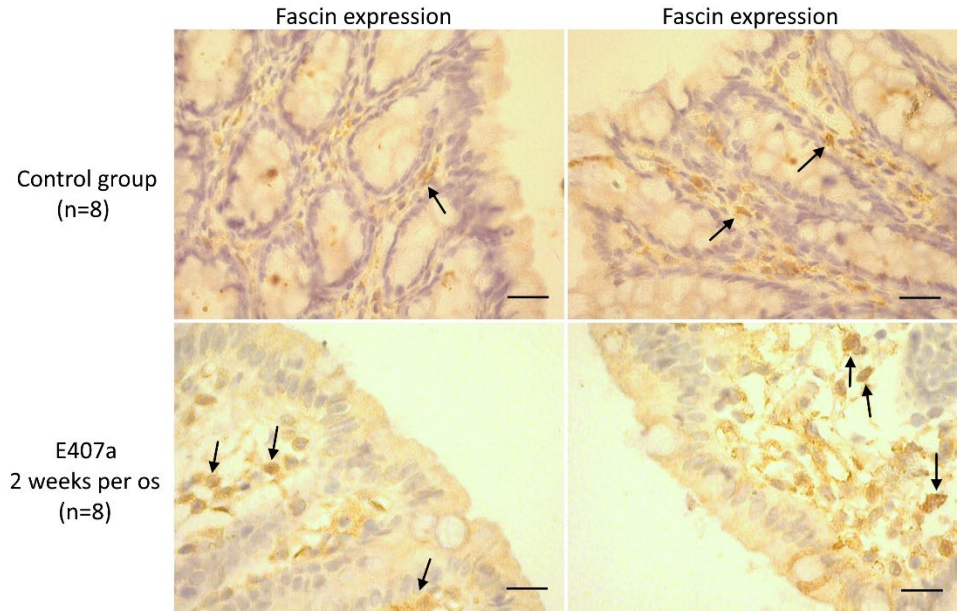

Figure 3. Representative images showing fascin immunoreactivity in the colonic mucosa of control rats and animals exposed to E407a. A, B). Control rats. Fascin-expressing cells of mesenchymal origin are observed in the lamina propria. 400x C, D). Intake of semi refined carrageenan by rats. The proportion of positively stained cells in the lamina propria is higher than in controls. 400x. Scale bar: 25 µm. Expression of the marker is shown with arrows.

Table 2. Evaluation of immunostaining for EMT markers in the colonic stroma in rats treated with semi-refined carrageenan (Mean±SEM)

Groups Parameters	Control group (n=8)	Rats orally exposed to semi-refined carrageenan (n=8)
Fascin positive cells	4.1±0.3	13.7±0.6 p<0.0001
Fascin positive cells-to- fascin negative cells ratio	0.39±0.03	2.64±0.32 p<0.0001
Vimentin positive cells	4.6±0.4	11.3±0.6 p<0.0001
Vimentin positive cells-to-vimentin negative cells ratio	0.81±0.21	2.50±0.31 p<0.0001
E-cadherin positive cells	4.0 ±0.3	9.6±0.6 p<0.0001
E-cadherin positive cells-to-E-cadherin negative cells ratio	0.55±0.06	2.22±0.43 p=0.0002

Changes in expression of another mesenchymal marker, i.e. vimentin, in response to semi-refined carrageenan in the colonic stroma were similar to those observed for fascin (Figure 4). Both parameters of vimentin immunostaining analyzed in this study were higher in rodents exposed to semi-refined carrageenan compared to controls. In particular, the total amount of vimentin-expressing cells was approximately 2.5–fold as high in the experimental group as in animals not treated with E407a, whereas the proportion of vimentin-expressing cells to vimentin-negative cells in the large intestinal lamina propria was almost 3.1 times higher, respectively (Table 2).

Besides epithelial lining, an epithelial marker E-cadherin was found to be expressed in the colonic lamina propria (Figure 5). The amount of E-cadherin positive cells in the stroma of large bowel was 2.4 times increased in rats administered E407a compared with controls. At the same time, the E-cadherin positive cells-to-E-cadherin negative cells ratio was statistically significantly (p=0.0002) 4-fold higher after exposure to semi-refined carrageenan than in controls (Table 2).

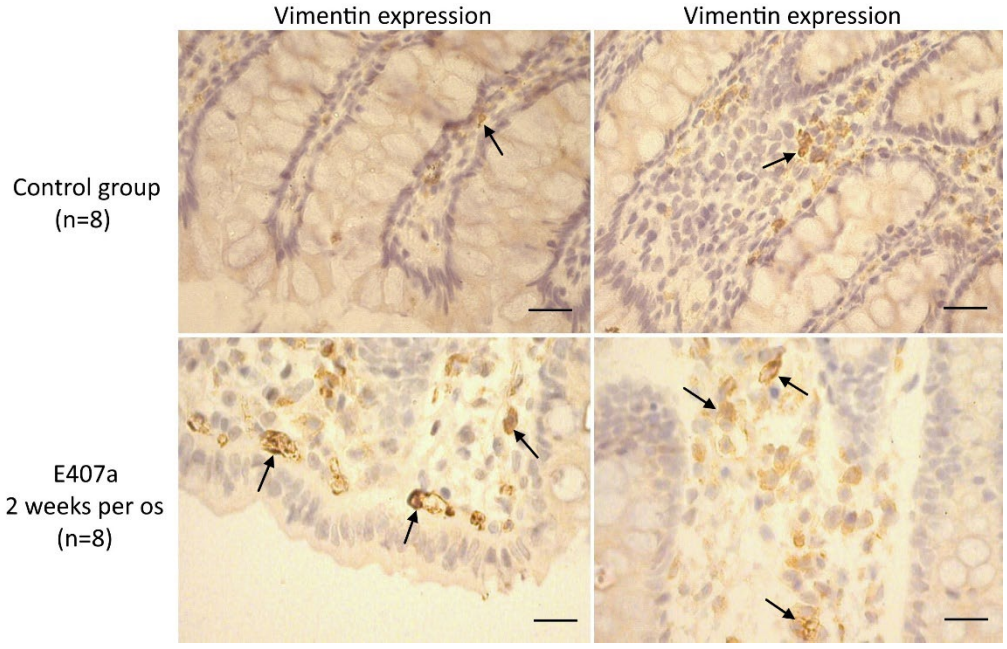

Figure 4. Representative examples of vimentin staining in the colonic mucosa of control animals and rats orally exposed to E407a. A,B). Control group. Vimentin expression is cytosolic and limited to the stroma. 400x. C, D). Rats orally treated with E407a. Vimentin immunoreactivity is stronger in the colonic lamina propria than in controls. 400x. Scale bar: 25 µm. Expression of the marker is shown with arrows.

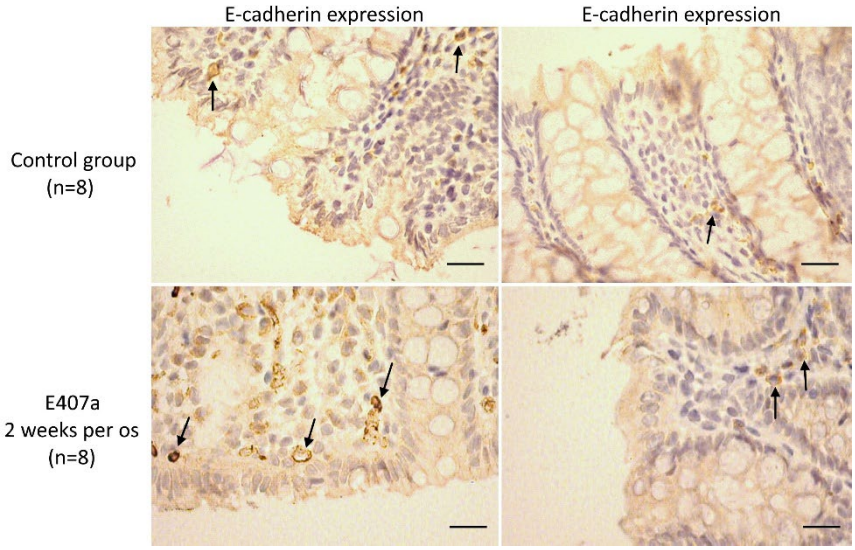

Figure 5. Distribution of E-cadherin positive staining in the mucous membrane of large bowel of control animals and rats treated with semi-refined carrageenan. A, B). In the control group, strong E-cadherin immunoreactivity is observed in the epithelial cells. The amount of E-cadherin cells in the lamina propria is low. 400x. C, D). In rats administered E407a, the degree of E-cadherin expression in epithelia decreases, while a higher number of E-cadherin-expressing cells in the stroma is noted. 400x. Scale bar: 25 μm. Expression of the marker is shown with arrows.

Quantitative evaluation of expression of EMT markers is summarized in Table 3. Fascin and vimentin were found to be statistically significantly upregulated both in the stromal cells and epithelial layer in rats treated with E407a compared with controls (Table 3). E-cadherin was revealed to be overexpressed in cells of the colonic lamina propria in the experimental group. However, oral consumption of semi-refined carrageenan resulted in downregulation of this epithelial marker in colonocytes (Table 3).

Table 3. Quantitative assessment of EMT markers in the large intestinal stroma and epithelial cells in rats exposed to E407a (Mean±SEM)

Groups / **Parameters**	**Control group (n=8)**	**Rats orally exposed to semi-refined carrageenan (n=8)**
Fascin staining in the stromal cells, a.u.	0.051±0.004	0.081±0.003 p<0.0001
Fascin staining in the colonic epithelial lining, a.u.	0.023±0.002	0.044±0.003 p<0.0001
Vimentin staining in the stromal cells, a.u.	0.065±0.005	0.101±0.004 p<0.0001
Vimentin staining in the colonic epithelial lining, a.u.	0.026±0.002	0.034±0.002 p=0.0071
E-cadherin staining in the stromal cells, a.u.	0.049±0.004	0.085±0.004 p<0.0001
E-cadherin staining in the colonic epithelial lining, a.u.	0.055±0.004	0.031±0.002 p<0.0001

Due to the fact that approximately 70% of immune cells of the body reside in the gut, the intestinal mucosa can be considered to be "physiologically inflamed." Thus, quantitative evaluation of immune cells in the intestine is a valuable approach to detect low grade inflammation [32]. Our observations indicate that oral exposure to food-grade semi-refined carrageenan is accompanied by infiltration of colonic lamina propria with macrophages and T-lymphocytes suggesting the development of low-grade colonic inflammation. Furthermore, our findings that demonstrate the accelerated cellular regeneration of the colonic surface epithelium in animals treated with semi-refined carrageenan *per os* indicate the damage to epithelial cells with their subsequent death. It is interesting to note that our previous study showed that the small intestinal epithelium suffered to a greater extent in response to the

same exposure to E407a, since regenerative capabilities were not sufficient to completely restore the epithelial layer [33].

Our findings concerning expression of EMT-related markers, including fascin, vimentin and E-cadherin, support the conclusion of the development of low-level colonic inflammation in response to oral semi-refined carrageenan exposure by rats. Fascin is a 55kDa evolutionary conservative actin-bundling protein that participates in the formation of spikes and filopodia, which are crucial for cellular motility [34]. Vimentin is a 57kDa cytoskeletal intermediate filament protein found in migratory cells [35]. Both proteins are expressed in cells of mesenchymal origin and those cells that underwent EMT. Both EMT markers mentioned above are implicated in IBD pathogenesis [36, 37]. Fascin is upregulated in epithelial lining of inflamed colonic tissue in IBD [36]. Furthermore, it has been suggested that fascin is involved in tissue repair and closure of gaps in the epithelial barrier, since its expression is stronger in damaged regions with active repair. It is interesting to mention that fascin expression in colonic epithelia is associated with low-grade inflammation in IBD [36]. Vimentin has been also demonstrated to be overexpressed in intestinal tissues in IBD [37]. Furthermore, it has been assumed that vimentin might mediate experimental colonic inflammation in mice due to its important role in the immune response to intestinal microbiota [38]. Given the altered immune response to colonic microbes in IBD, the reported role of vimentin expression in regulation of intestinal microbial homeostasis provides evidence for its involvement in IBD pathogenesis.

In this study, we observed overexpression of fascin and vimentin in the colonic epithelia and stroma upon exposure to E407a. Such observations are consistent with those observed in IBD [36, 37].

Furthermore, our observations indicate that consumption of semi-refined carrageenan increases the amount of colonic mesenchymal stromal cells. These can be fibroblasts and myofibroblasts that express vimentin and fascin, as well as the fibroblast-like former epithelial cells that acquired the mesenchymal phenotype, migratory capacities and entered the lamina propria through the basement membrane or in regions that lack the basement membrane described above.

Besides fascin and vimentin, expression of another EMT marker, namely E-cadherin, was analyzed in the large bowel of animals treated with E407a. E-cadherin is a key component of epithelial adherent junctions, which are crucial for providing cell-cell adhesions in the colonic epithelial lining [39]. This adhesion molecule is of paramount importance for maintaining the integrity of single-cell mucosal epithelial layer and, hence, protects intestinal subepithelial layers from the luminal microbiota [40]. There is strong evidence that E-cadherin dysregulation in the gut is observed in IBD [40, 41]. Downregulation of E-cadherin in the intestinal epithelia compromises the integrity of epithelial barrier, contributes to the flow of bacterial antigens into the mucosal subepithelial layers, which exacerbates inflammation in IBD. An abundant amount of literature has confirmed that E-cadherin deregulation plays a pivotal role in the progression of IBD [42]. However, the role of E-cadherin in intestinal homeostasis is not limited to the maintenance of epithelial barrier. There is accumulating evidence that E-cadherin acts in the gut as a signaling hub that regulates a complex network of crosstalk between epithelia, immune cells, and colonic microbiota [40].

Our observations confirm that primarily E-cadherin is expressed in the colonic epithelia. However, quantitative analysis of its expression revealed a significantly greater amount of E-cadherin positive cells in the stroma in response to semi-refined carrageenan. Thus, oral intake of E407a by rats is associated with E-cadherin redistribution within the colonic mucosa. It is important to note that E-cadherin is a canonical epithelial marker, which is not expressed in cells of mesenchymal origin [42]. Therefore, no E-cadherin expression is observed in fibroblast-like cells, which are products of EMT. Nevertheless when cells lose their epithelial phenotype and gain the novel mesenchymal one during EMT, they may pass a transient partial EMT state in which such cells express markers of both types [43]. It can be assumed that a stronger E-cadherin immunostaining detected within the colonic lamina propria in this study may be partially explained by this phenomenon.

Thus, oral intake of semi-refined carrageenan promotes the same changes in expression of key EMT markers as those observed in IBD. Patterns of vimentin, fascin and E-cadherin expression in colonic epithelia observed in this study seem to be compensatory, develop in response to tissue damage caused by carrageenan and aim at preserving the epithelial integrity. In this study, we did not reveal significant alterations of large intestinal epithelial layer. However, overexpression of vimentin and fascin against E-cadherin downregulation in the colonic epithelial lining suggests this damage, which is probably compensated for by proliferation. Carrageenan has been reported to affect colonic intestinal cell either directly stimulating pro-inflammatory pathways or in a microbiota-mediated way [15-17, 44]. Furthermore, carrageenan might aggravate bacterial intestinal inflammation [44]. Thus, carrageenan cannot only contribute to manifestations of IBD in predisposed individuals but rather to exacerbate IBD in patients who have this disease. This hypothesis is consistent with earlier relapses observed in patients with IBD remission in response to carrageenan [22].

CONCLUSIONS

Taking all experimental results together, we can assume that oral intake of semi-refined carrageenan by rats causes low-grade colitis associated with infiltration of the colonic lamina propria with macrophages and T-lymphocytes, as well as altered expression of EMT markers in the large bowel. The food additive E407a is toxic and its effects should be re-evaluated.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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