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COMMON FOOD ADDITIVE E407a AFFECTS BCL-2 EXPRESSION IN LYMPHOCYTES *IN VITRO*

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Abstract: The aim of this study was to assess the apoptosis rate and expression of antiapoptotic bcl-2 in lymphocytes exposed to food grade kappa-carrageenan-containing processed *Eucheuma* seaweed (PES; food additive E407a) *in vitro*. Lymphocytes from blood untreated with E407a (controls), treated with 2% and 5% PES solutions during 1 and 2 hours were stained with Alexa Fluor® 647 rabbit anti-active caspase-3 and FITC mouse anti-Bcl-2. The results were assessed by flow cytometry. Incubation of rat WBCs with 2% and 5% PES solutions did not affect the amount of active caspase-3^+ lymphocytes. MFI of active caspase-3 was unchanged in lymphocytes treated with E407a compared with the untreated cells. The percentage of bcl-2^+ lymphocytes was not affected as a result of incubation. However, bcl-2 expression was higher in lymphocytes exposed to PES, evidenced by higher values of MFI of bcl-2 in lymphocytes treated with both 2% and 5% E407a solution compared with the control group.
Incubation of rat blood with PES doesn't stimulate apoptosis of lymphocytes. However, overexpression of bcl-2 is found in lymphocytes.

Key words: carrageenan, food additive, flow cytometry, apoptosis, active caspase-3, bcl-2.

INTRODUCTION

Carrageenans are sulfated galactans of polysaccharide nature made up of repeating galactose derivatives linked with galactose-α-1,3-galactose and galactose-β-1,4-galactose bonds extracted from red seaweeds (Calvo et al., 2019; Bhattacharyya et al., 2010). Nowadays they are widely used in food industry due to their gel-forming properties as thickeners (Martino et al., 2017; Necas and Bartosikova, 2013). There are three major commercially available types of CGN: kappa; lambda and iota (Besednova et al., 2019). Structurally, they differ from each other in the amount and location of ester sulfate bonds and, functionally, different types of CGNs form hydrocolloids with diverse properties, e.g. λ-CGN can be dissolved at low temperate making it suitable for dairy products (Blaszak et al., 2018).

CGNs are authorized as food additives in the European Union and are known as E407 (food-grade CGNs) and E407a (processed *Eucheuma* seaweed; PES). The latter is obtained from microalgae *Eucheuma cottonii* and contains κ-carrageenan with impurities of algal cellulose (15%) and up to 5 mg/kg of formaldehyde (Commission Regulation (EU) No 231 / 2012 (E407a)).

According to the Food and Drug Administration, CGNs are generally recognized as safe (GRAS status). Furthermore, their safety was confirmed in a WHO Joint Expert Committee on Food Additives (JECFA) 2015 report and CGNs were allowed to be used even in infant formulas without exceeding the concentration of 1 g/L (David et al., 2018). This food additive is also considered safe by the European Food Safety Authority. However, the safety of CGN oral consumption has been challenged and it has been recently suggested that the safety of E407 and E407a and their regulation should be re-evaluated (David et al., 2018; Younes et al., 2018). Earlier studies have shown that CGNs can cause intestinal inflammation in animals when administering orally (Martino et al., 2017; Necas and Bartosikova, 2013; Tkachenko et al, 2018a; Tkachenko et al., 2018b; Tkachenko et al, 2018c; Gubina-Vakyulyk et al., 2015; Tobacman et al., 2001) Moreover, kappa-CGN can aggravate *C. freundii* DBS100-induced colitis in mice, thus, intensifying the already existing inflammation in the colon (Wu et al., 2017). However, some researchers claim CGNs to be safe (Weiner and Mckim, 2019; McKim et al., 2018; Weiner, 2014).

In addition to experimental works, CGNs have been studied at the cell level *in vitro*. CGNs have been demonstrated to promote innate immune response via activating TLR-4 and stimulate synthesis of IL-8 in human intestinal epithelia (Bhattacharyya et al., 2007; Borthakur et al., 2007). Bhattacharyya et al showed that CGN promoted necrotic cell death and decreased cell proliferation of human intestinal epithelial cells even at low concentrations (Bhattacharyya et al., 2013). Nevertheless it has been also reported that CGNs neither affect the survival of intestinal epithelia nor stimulate expression of cytokines *in vitro* (McKim Jr et al., 2016).

Despite numerous *in vitro* and *in vivo* findings on the ability of CGNs to induce inflammation in the gut, some authors believe that controversial data on E407 safety may be due to the confusion between food-grade CGN, degraded CGN and poligeenan and, thus, misinterpretation of experimental results. In particular, it has been reported that the toxicity is characteristic of degraded CGNs (20-40 kDa) and poligeenan (10-20 kDa), but not for high-molecular-weight food-grade CGNs (200-800 kDa) (Weiner and Mckim et al., 2019; McKim et al., 2018). However, food-grade CGN, especially semi-refined E407a, is contaminated with poligeenan and degraded CGN (Younes et al., 2018). Furthermore, there is some evidence that food-grade

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high-molecular-weight CGN can undergo acidic hydrolysis in the stomach with the formation of average- and small-molecular-weight products (Ekstrom, 1985).

Thus, novel both *in vivo* and *in vitro* studies aimed at evaluating biological effects of CGNs are required. Since leukocytes are of vital importance for the inflammatory response, it is important to shed light on the features of their interaction with CGNs.

The aim of our study was to evaluate *in vitro* the ability of food grade kappa-carrageenan-containing PES (food additive E407a) to promote apoptosis in lymphocytes and the changes in expression of antiapoptotic bcl-2 molecule in lymphocytes exposed to E407a.

MATERIALS AND METHODS

Description of animals, groups and conditions of incubation

Eight female adult WAG rats weighing 160-190 g were used in our study. They were provided by the vivarium of Kharkiv National Medical University. The animals were housed in two cages (4 rats in each cage) in standard laboratory conditions at room temperature (24 ± 2 °C) and relative humidity of 50-60 %. Water and food were provided *ad libitum*. When the animals were sacrificed, their blood was collected into sterile EDTA VACUTAINER tubes. Then 1 ml of blood was taken from each sample and added to 2 capped polysterene test tubes (500 µl of blood in each). This was followed by the addition of 100 µl of 2% and 5% E407a solution (group 1 and group 2, respectively). Blood treated with 2% and 5% E407a solutions was incubated for 1 and 2 h at 37 ºC. The control samples were incubated for the same periods of time in the same conditions untreated with E407a solutions.

Carrageenan description and preparation of solutions

Food grade k-carrageenan-containing 2% and 5% PES stock solutions were prepared a day prior to the experiment. In addition to k-carrageenan, PES may contain up to 15% of algal cellulose (Cohen and Ito, 2002). Stock solutions of E407a were prepared by adding E407a to sterile calcium- and magnesium-containing phosphate buffered saline (PBS, pH 7.4; BD, USA). This was done to provide magnesium and calcium cations necessary for maintaining a random conformation of carrageenan in the solutions. One hundred µl of 2% and 5% stock solutions were added to 500 µl of blood for incubation during 1 and 2 h. Solutions were pre-warmed to 37 °C and vortexed prior to their addition to blood samples.

Lyse/wash protocol

After incubation, blood samples untreated and treated with carrageenan were used to obtain leukocyte suspensions in accordance with the lyse/wash procedure (Becton Dickinson Technical Support Protocol, 2002). Briefly, 100 µl of blood samples untreated with carrageenan (controls) and incubated with 2% and 5% PES solution were added to 12 x 75 mm capped polysterene test tubes. Then 2 ml of 1x FACSLyse solution (Becton Dickinson, San Jose, USA) was added. Solutions were vortexed and incubated for 15 minutes at room temperature in the dark. Then the solutions were centrifuged at 500g during 5 minutes. The supernatant was discarded. Two ml of PBS was added. It was followed by centrifugation at 500g during 5 minutes. The supernatant was removed.

Flow cytometry staining protocol

Washed leukocytes were stained with Alexa Fluor® 647 rabbit anti-active caspase-3 (BD Pharmingen™, USA) and FITC mouse anti-Bcl-2 (BD Pharmingen™, USA). Initially, cells were fixed and permeabilized using Fixation/Permeabilization Solution Kit (BD Cytofix/Cytoperm™, USA). Cell pellets obtained as a result of the lyse/wash procedure were resuspended in 0.5 ml of BD Cytofix/Cytoperm™ fixation/permeabilization solution. Suspensions were incubated at 4 °C during 20 minutes. After centrifugation during 5 minutes at 500 g, the supernatant was discarded. Then leukocytes were washed twice in 0.5 ml of BD Perm/Wash™ buffer (1x) without the complete removal of buffer. After double washing procedure, leukocytes were resuspended in 100 µl of BD Perm/Wash™ buffer (1x). Then 20 µl of Alexa Fluor® 647 rabbit anti-active caspase-3 and FITC mouse anti-Bcl-2 were added. This was followed by incubation for half an hour at room temperature. Then each sample was washed in 1 ml of BD Perm/Wash™ buffer (1x) with the subsequent resuspension of WBCs in 0.5 ml of BD Perm/Wash™ buffer (1x).

The samples stained with Alexa Fluor® 647 rabbit anti-active caspase-3 and FITC mouse anti-Bcl-2 were analyzed by flow cytometry using a BD FACSCanto™ II flow cytometer (Becton Dickinson, USA).

In each sample, 10,000 events were collected with the threshold of 20,000.

Evaluation of flow cytometry results

Evaluation of results was carried out using BD FACSDiva™ software. The corresponding regions of lymphocytes was gated and the percentage of active caspase-3^+ and Bcl-2^+ cells was analyzed. The example of gating strategy is available in Figure 1. Median fluorescence intensity (MFI) of Alexa Fluor® 647 active caspase-3 and FITC Bcl-2 was evaluated in positively stained lymphocytes.

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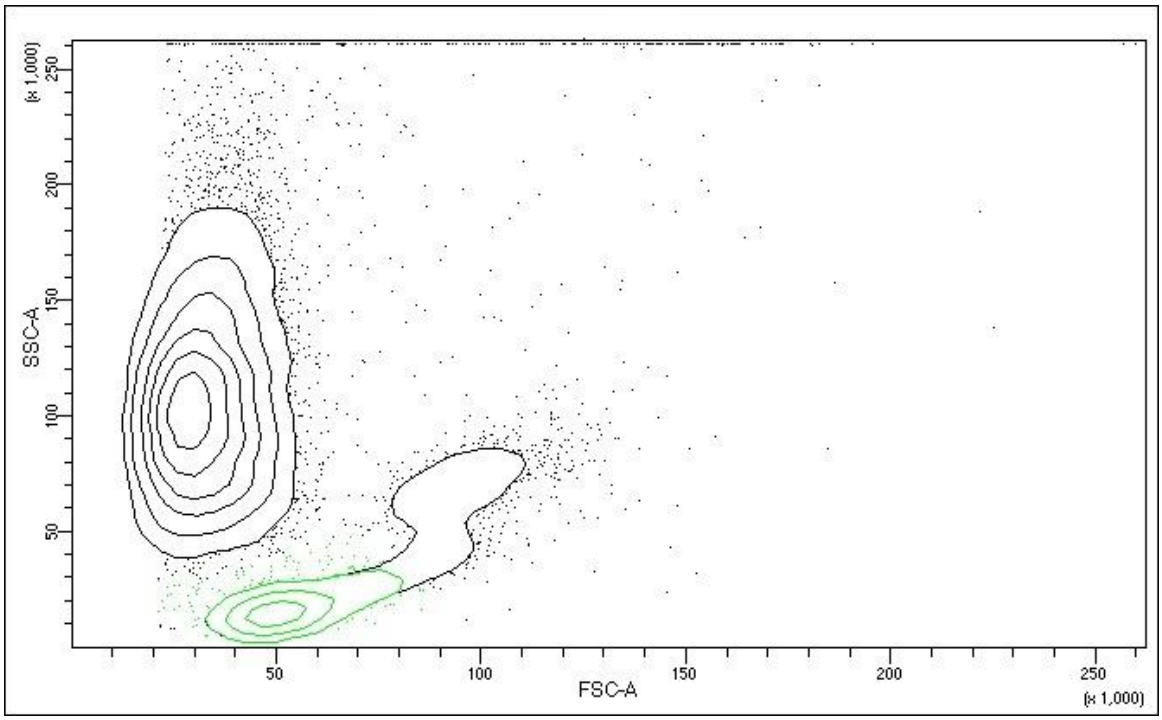

Fig. 1. Representative FSC / SSC dotplot showing the gating strategy. The region of lymphocytes is highlighted in green.

Bioethics

All institutional and national guidelines for the care and use of laboratory animals were strictly followed.

The experiment was designed and all manipulations with animals were performed according to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes and the Council of Europe Convection for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123).

7. Statistical analysis.

Kruskal-Wallis ANOVA test was used to compare three independent parameters. Differences were considered statistically significant at $p < 0.05$. The data obtained in this study were analyzed using GraphPad Prism 5.0 (GraphPad software, USA).

RESULTS

Flow cytometry detection of apoptotic active caspase-3 in lymphocytes of blood untreated with CGN-containing PES and treated with 2% and 5% PES solutions did not demonstrate any statistically significant differences in the amount of active caspase-3^+ cells (Figures 2; Table 1). Examination of caspase-3 activation in lymphocytes revealed a low rate of apoptosis. In order to assess the amount of active-caspase 3 in active caspase-3^+ lymphocytes, we evaluated MFI of active caspase-3 enzyme. We observed statistically significant changes in MFI of active caspase-3 after neither 1-hour incubation nor 2-hour incubation with PES solutions (Table 2). Neither 2% nor 5% PES solutions affected MFI of active caspase-3 in lymphocytes (Table 2).

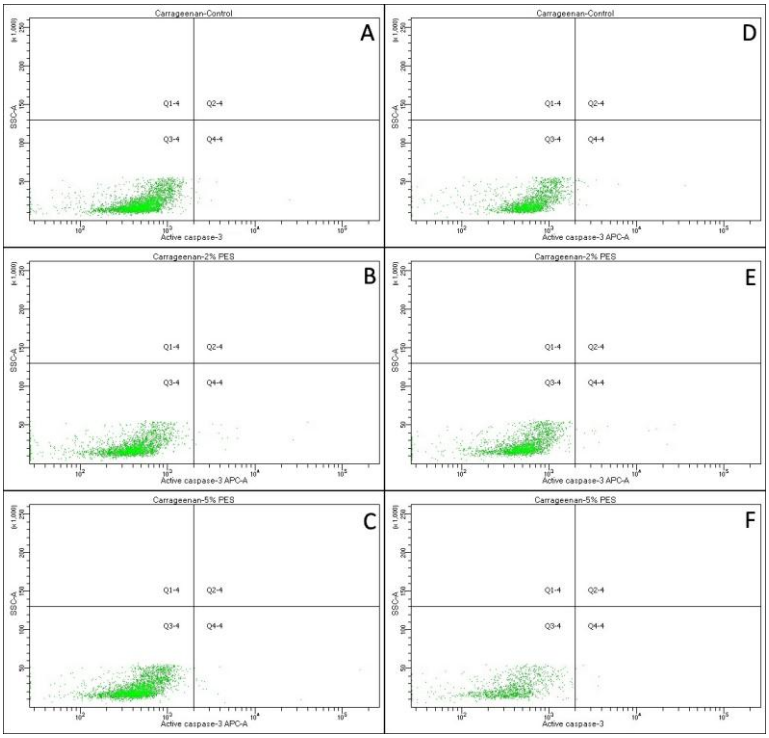

Fig. 2. Representative SSC / Alexa Fluor® 647 active caspase-3 plots of gated lymphocytes from blood untreated with carrageenan (control group) and treated with 2% and 5% carrageenan solutions (group 1 and group 2, respectively) during 1 h (A, B, C) and during 2 h (D, E, F).

Table 1.

The percentage of active caspase-3$^+$ and Bcl-2$^+$ lymphocytes of blood untreated, treated with 2% and 5% E407a solution during 1 and 2 hours (Me [IQR])

Cells, time of incubation		**The percentage of active caspase-3$^+$ cells**			**The percentage of active Bcl-2$^+$**		
		Control group	Group 1 (2% carrageenan solution)	Group 2 (5% carrageenan solution)	Control group	Group 1 (2% carrageenan solution)	Group 2 (5% carrageenan solution)
Lymphocytes	1 h	0.70 [0.35; 0.98]	0.50 [0.25; 0.50] p > 0.05	0.70 [0.43; 1.05] p > 0.05	95.15 [93.52; 96.90]	95.45 [93.90; 97.18] p > 0.05	94.80 [92.48; 97.78] p > 0.05
	2 h	0.85 [0.60; 1.20]	0.65 [0.53; 0.88] p > 0.05	0.70 [0.55; 1.35] p > 0.05	95.80 [92.93; 95.85]	96.45 [92.77; 97.53] p=0.75	94.30 [92.65; 98.52] p > 0.05

Note: Differences were considered statistically significant at $p < 0.05$

Table 2.

Median fluorescence intensity (MFI) for active caspase-3$^+$ and bcl-2$^+$ lymphocytes of blood untreated, treated with 2% and 5% E407a solution during 1 and 3 hours (Me [IQR])

Cells, groups, and time of incubation			Active caspase-3	Bcl-2
Lymphocytes	Control group	1 h	324 [253; 439]	252 [223; 267]
		2 h	331 [228; 461]	236 [221; 261]
	Group 1 (2% carrageenan solution)	1 h	325 [147; 539] p > 0.05	288 [268; 314] p < 0.05*
		2 h	303 [176; 495] p > 0.05	291 [274; 306] p < 0.05*
	Group 2 (5% carrageenan solution)	1 h	286 [170; 340] p > 0.05	289 [262; 327] p < 0.05*
		2 h	277 [218; 393] p > 0.05	296 [280; 311] p < 0.05*

Note: *Differences were considered statistically significant at $p < 0.05$.

Expression of bcl-2 was examined by flow cytometry in lymphocytes of rat blood untreated with PES and treated with 2% and 5% PES solutions during 1 and 2 h. No statistically significant changes were found between the percentage of bcl-2$^+$ lymphocytes in group 1 and group 2 compared with the control group (Figures 3; Table 1). However, while evaluating MFI of bcl-2 anti-apoptotic protein, it was revealed that its expression was statistically significantly higher ($p < 0.05$) in bcl-2$^+$ lymphocytes treated both with 2% and 5% PES solutions (Table 2). In group 1, MFI of bcl-2 in bcl-2$^+$ lymphocytes was 14.3% higher compared with controls, while in group 2, it was 14.7% after 1 h incubation. The further incubation resulted in 23.3% and 25.4% elevation of MFI of bcl-2 protein, respectively (Table 2). Thus, the bcl-2 expression was time-dependent but dose-independent.

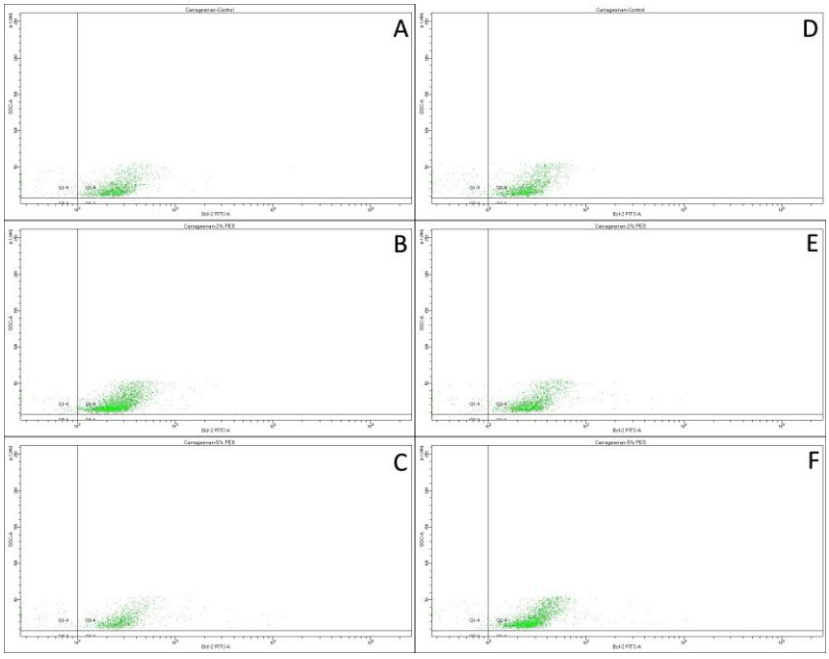

Fig. 3. Representative SSC / FITC Bcl-2 plots of gated lymphocytes from blood untreated with carrageenan (control group) and treated with 2% and 5% carrageenan solutions (group 1 and group 2, respectively) during 1 h (A, B, C) and during 2 h (D, E, F).

DISCUSSION

Little is known about the mechanisms by which CGNs may induce intestinal inflammation. At the moment, it is not agreed whether food-grade CGNs are absorbed in the intestine or not. Nicklin et al demonstrated that some amounts of dietary food-grade CGNs are absorbed (Nicklin and Miller, 1984). This data is supported by Sugita-Konishi et al who believe

that macrophages in the gut can be exposed to quite high concentrations of CGNs absorbed in the intestine (Sugita-Konishi et al., 2013). However, such data are contradictory to recent reports indicating that food-grade CGN is not absorbed in the gut (Weiner and McKim, 2019; McKim et al., 2018; McKim Jr et al., 2016). This issue is of huge importance, since CGNs are able to induce TLR4 expression, nitric oxide (NO) production and TNF-α expression in macrophages[(26)]. Ogata et al demonstrated that pretreatment with CGN stimulates LPS-induced TNF-α expression in leukocytes (Ogata et al., 1999). Thus, features of food-grade CGN interaction with intestinal leukocytes may underlie the mechanisms of carrageenan-induced intestinal inflammation.

Some reports demonstrate no effects of CGNs on the viability of macrophages (McKim Jr et al., 2016; Sugita-Konishi et al, 2003). However, Chen et al reported that CGNs interacted with macrophages, activated pro-inflammatory transcription factor NF-κB via TLR4, which resulted in upregulation of TNF-α (Chen et al., 2014). It is interesting to note that kappa-CGN in single cultures of intestinal epithelial Caco-2 cells and activated macrophage-like THP-1 cells did not show cytotoxic properties. Nor it activated synthesis of cytokines. However, when both types of cells were co-cultured, kappa-CGN promoted apoptosis of Caco-2 cells and upregulated pro-inflammatory TNF-α, IL-1β and IL-6 in both types of cells (Jiang et al., 2013). Furthermore, *in vivo* studies indicate that carrageenan-induced intestinal inflammation is associated with the activation of enterocyte apoptotic processes (Tkachenko et al., 2018a; Gubina-Vakyulyk et al., 2015). Choi et al believe that CGN-induced expression of macrophage inhibitory cytokine 1 (MIC-1) may induce apoptosis of intestinal epithelial cells (Choi et al., 2014).

Even less is reported on the interaction of CGNs with lymphocytes. This fact substantiates out interest to features of the interactions of CGN and lymphocytes. Abe et al reported that CGN can bind NK cells directly and promote production of IFN-γ (Abe et al., 2002). In this study, apoptosis of lymphocytes was not activated under the influence of E407a, evidenced by statistically insignificant differences in the percentage of active caspase-3^+ lymphocytes between groups. However, anti-apoptotic bcl-2 was overexpressed. We believe that this overexpression provided the survival of lymphocytes and prevented their apoptosis.

It is interesting to note that Jazzara et al demonstrated the ability of λ-CGN to upregulate pro-apoptotic bax and downregulate antiapoptotic bcl-2 in human breast cancer cells (Jazzara et al., 2016). According to Chen et al, λ-CGN oligosaccharides at high concentration also downregulated bcl-2 in human umbilical vein endothelial cells (Chen et al., 2009). The data mentioned above contradict our findings. We can presume that our short-term incubation initially leads to upregulation of bcl-2 to prevent cell death, since bcl-2 is known to inhibit caspase-3 and prevent apoptosis. However, the crosstalk between pro-apoptotic and anti-apoptotic factors under the influence of CGNs requires further consideration.

Thus, in our research, incubation of E407a with WBCs had no effect on cell apoptosis and a limited impact on bcl-2 expression. This study may add new insights for elucidation of the mechanisms via which CGN may promote inflammation.

CONCLUSION

Food grade kappa-carrageenan-containing processed *Eucheuma* seaweed (E407a) does not promote apoptosis of lymphocytes *in vitro*. A higher MFI of bcl-2^+ lymphocytes indicates a higher content of this anti-apoptotic protein in lymphocytes exposed to E407a.

AUTHOR CONTRIBUTIONS

All authors contributed to planning and design of the study, data analysis, drafting and critically revising the paper. Each author mainly contributed in the following way: conceptualization (Anton Tkachenko), methodology (Anton Tkachenko, Valeriy Myasoedov), data collection (Anatolii Onishchenko, Anton Tkachenko), data validation (Anatolii Onishchenko); data processing (Anatolii Onishchenko, Anton Tkachenko), writing-original draft preparation (Valeriy Myasoedov, Anton Tkachenko), writing-review and editing (Vladimir Lesovoy, Anton Tkachenko). All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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