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EXPERIMENTAL PAPERS

Marine Green Alga *Codium fragile* Lipid Extract Promotes Erythrocyte Membrane Repair in Stress-Exposed Mice

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Abstract—*Codium fragile* (Suringar) Hariot, the green seaweed species belonging to the family Codiaceae, is one of the mass macrophyte species in the Pacific Region of the Russian Federation. The *C. fragile* total lipid content amounts 13.92 ± 0.22 mg per g of dry tissue, of which 44% are glycolipids, 40% come from neutral lipids, while phospholipids account for 16%. The content of polyunsaturated fatty acids (PUFAs) in *C. fragile* lipid extract is over 50% of the total fatty acids, of which ω-3 (36.2%) and ω-6 (17.8%) are predominant. Here, we studied the effects of the *C. fragile* lipid extract and Omega-3 reference drug on biochemical and physiological parameters of erythrocytes in mice exposed to stress (vertical fixation by the scruff skinfold). Under the impact of stress, erythrocytes underwent alterations in both their dimensional characteristics and membrane phospholipid profiles, leading to changes in their permeability and lability, as well as impeding their circulation through the capillary bed. The endogenous antioxidant defense system in stress-exposed mice experienced a considerable strain, as evidenced by an increase in the malonic dialdehyde level, with a simultaneous decrease in plasma superoxide dismutase and antiradical activities. The administration of the *C. fragile* lipid extract to stress-exposed mice entailed the restoration of the erythrocyte membrane lipid content, a decrease in the amount of lysophospholipids, and the normalization of the sphingomyelin/phosphatidylcholine ratio, which contributed to the recovery of erythrocyte dimensional characteristics and osmotic resistance, as well as blood antioxidant system parameters. The pronounced stress- and membrane-protective effect of the *C. fragile* lipid extract was due to the presence in its composition of a wide range of neutral and polar lipids containing ω-3 and ω-6 PUFAs, which ensured a higher anti-stress efficacy of the algal extract compared to the Omega-3 reference preparation.

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Keywords: lipid extract, *Codium fragile*, omega-3, stress, erythrocytes, phospholipids, mice

INTRODUCTION

Seaweeds are a valuable source of bioactive compounds and functional foods with health benefits. Numerous studies report that they have important biological properties that include anti-inflammatory, antioxidant, antimicrobial, antitumor, and some other beneficial effects [1–3]. Here, we focus on the study of *Codium fragile* (Suringar) Hariot, one of the mass species of marine green algae. *C. fragile* refers to the family Codiaceae and is a commonly recognized cosmopolitan, as it is widely distributed in temperate regions around the globe [4], originating from its native Asia-Pacific region (China, Taiwan, Japan, Korea, Russia). In coastal waters of the Peter the Great Bay (Sea of Japan), *C. fragile* vegetates in

sheltered and semi-sheltered coves near the lower boundary of the littoral zone to a depth of 3 m on rocky, stony, and silty/sandy soils [5]. The alga particularly flourishes in summer and fall.

In Asia, seaweeds have been used for food since time immemorial. In modern Japan and Korea, *C. fragile* is often used as a cooking ingredient, as well as an oriental remedy to treat intestinal and urological disorders [6]. The literature data on the *C. fragile* chemical composition differ significantly, which may be associated with regional climatic conditions, season, and geographical patterns of algal communities. According to some authors [7], the *C. fragile* composition includes significant amounts of carbohydrates (20.47%), proteins (6.13%), and lipids (2.53%), as well as a high content of polyphenols, flavonoids, mineral elements (Mg, Ca, Fe, Cu, Zn). Due to the presence of such a considerable amount of carbohydrates, most *C. fragile* studies focus on the isolation of polysaccharides and the investigation of their biological activity. Sulfated polysaccharides isolated from *C. fragile* are finding wide use in preventing and treating dyslipidemia and obesity [8], as well as immunomodulatory and anti-inflammatory agents [9, 10].

At the same time, no less valuable seaweed components are lipids. Our previous studies of extracts derived from marine macrophyte species referring to different taxa [11] revealed clear differences in the composition and content of lipids and their fatty acids, which determines significant differences in the biological activity of these algal extracts.

Goecke et al. [12] noted that lipid extracts obtained from different Codium species exhibit antibacterial, antiviral, antifungal, and cytotoxic activity which is underlain by various representatives of a vast class of lipids, such as sterols, fatty acids, glycolipids and phospholipids, terpenes, etc. [13]. Due to the ability of seaweeds to synthesize C:18 and C:20 polyunsaturated fatty acids (PUFAs), they have received much research attention worldwide [14]. In addition to having a high nutritional value, essential fatty acids are important constituents of cell membrane phospholipid fractions, participate in the synthesis of a number of hormones, and play an important role in the processes of cellular activity and gene expression. Long-chain fatty acids of marine origin can reduce the risk of thrombosis and atherosclerotic plaques in blood vessels, lower triglyceride blood and cholesterol blood levels, relieve blood pressure [15], and also have hypocholesterolemic, immunostimulatory, and antioxidant effects [16, 17].

It appears that it is the significant content of ω-3 and ω-6 PUFAs, which are the main constituents of the polar fraction of the *C. fragile* lipid complex [18], which accounts for its high pharmacological activity. It follows from the published data by S. Khotimchenko [19] that the lipid content in the thallus of *C. fragile* growing in the Far Eastern seas can reach 4.4–5.3 mg/g of wet weight. At the same time, one of the important members of the *C. fragile* lipid component is represented by phospholipids referring to the category of structure-forming and functional components of biomembranes. However, the study of the *C. fragile* lipid extract as a possible membrane protector under the impact of stress exposure on the organism has not received due development thus far.

Vertical fixation by the scruff skinfold is used as a model of stress in laboratory studies on small (murine) rodents [20]. Regardless of the nature of stress (physical, immobilization, cold, emotional), the organism responds to its exposure with an invariable set of biochemical and physiological reactions, such as adrenal hypertrophy, thymolymphatic atrophy and gastrointestinal ulceration, making up a classic "triad of stress", first described by Hans Selye. The impact of stress factors (stressors) leads to the release of the so-called stress hormones (corticosteroids, catecholamines) into blood, which regulate all metabolic processes in the organism. In addition, intense stress entails an increase in the production of reactive oxygen species (ROS), accompanied by lipid peroxidation in cell membranes [21]. As a result, the ensuing formation of polar lipid hydroperoxides and the imbalance in the ratio of membrane phospholipid fractions alter membrane permeability and cause possible damage [22]. Importantly, erythrocytes are the first target of stressors. The lipid fraction of erythrocyte membranes is one of the important indicators characterizing the state of cell membranes in organs and tissues of the whole organism under pathology [23]. At the same time, erythrocyte membranes represent a classic model to explore the protective effect of drugs, as they are the only plasma membranes that can be isolated in pure form, being uncontaminated by alien membranes.

This work was aimed to study the composition of

the lipid extract isolated from the thallus of the marine green alga *C. fragile*, as well as its effect on erythrocyte membranes in mice exposed to acute stress.

MATERIALS AND METHODS

The marine green alga *Codium fragile* (Suringar) Hariot served as the object of study. All *C. fragile* specimens were collected manually during the summer months in the Peter the Great Bay of the Sea of Japan at a depth of no more than 2 m. The algal sample amounted to 100 thallomes. The collected material was pre-processed at the research station. To remove all contaminants, the algae were thoroughly washed, first with seawater and then with freshwater. Next, the purified macrophyte specimens were submerged in boiling water for 2 min to inhibit their own enzyme activity, after which they were squeezed out and dried under natural conditions to a ~30–40% residual moisture. The dried algal specimens were ground in a laboratory mill and stored at –20°C until further analysis. Lipid extraction from the dried materials was carried out according to the method proposed by Bligh and Dyer [24]. To do this, one kilogram of the algal powder was extracted with 1.5 L of a chloroform–methanol mixture (1 : 2 v/v) and left overnight. For phase separation, the mixture was added with 500 mL of chloroform and distilled water and gently stirred afterward. The upper water-methanol layer was pipetted out and discarded, while the lower (chloroform) layer containing the lipid fraction was concentrated in a vacuum evaporator (Type 349/2, Unipan, Poland) at a temperature not exceeding 37°C. Total lipids were determined in the extract by weighing the aliquots dried to a constant weight in 5 replicates.

Chromatographic distribution of lipids was accomplished by micro-thin-layer chromatography (TLC) on glass plates coated with a layer of KSK silica gel (LLC Labhimos, Russia). An acetone–benzene–water (91 : 30 : 8 v/v) solvent system was used to separate plant glycolipids [25]. Glycolipids were detected on chromatograms using the anthrone reagent [26]. Total phospholipids in the algal extract were determined by the method of Vaskovsky et al. [27]. Phospholipids were fractionated by a two-dimensional TLC solvent system: in the first direction—chloroform–methanol–28% ammonia (65 : 35 : 5 v/v), in the second direction—chloroform–acetone–methanol–glacial acetic acid–water (50 : 20 : 10 : 10 : 5 v/v). Separated phospholipid fractions were detected on chromatograms by a 10% sulfuric acid/methanol solution followed by heating the plates on a closed electric stove. The content of individual phospholipid fractions was calculated as a percentage of their total amount.

Chromatographic distribution of neutral lipids was carried out using a one-dimensional TLC [28] in the hexane–sulfuric ether–glacial acetic acid solvent system (80 : 20 : 1 v/v or 90 : 10 : 1 v/v). After chromatography, the samples were visualized by iodine vapor. The content of individual fractions was expressed as a percentage of total neutral lipids.

The fatty acid profile of the algal lipid extract was analyzed by gas-liquid chromatography (GLC). For this purpose, fatty acid methyl esters (FAMEs) were obtained by lipid transesterification using the method of Carreau and Dubacq [29]. The resultant FAMEs were purified by TLC, using benzene in the solvent system, and eluted from silica gel by hexane; finally, the isolated eluate was evaporated. FAMEs was re-dissolved in a certain volume of hexane and analyzed by GLC in a LHM-2000 chromatograph (Chromatograph, Russia) with a flame ionization detector. Fatty acids were identified by comparing retention time (Rt) with the standards and quantum numbers of carbon [30]. The results were calculated as a percentage of total fatty acids.

Forty outbred albino male mice aged 8 weeks with a body weight of 25–30 g were used in the experiment on stress exposure modeling. During the 7-day adaptation period, the animals were kept in a vivarium at a room temperature of 22 ± 2°C, housed by 5 animals per cage, on a basic diet with ad libitum access to water. Then, the mice were randomly divided into control (n = 10) and experimental (n = 30) groups. Experimental animals were exposed to stress modelled through a vertical fixation by the scruff skinfold for 24 h. Immediately before the experiment, 10 mice received the *C. fragile* extract, 10 mice received Omega-3 reference preparation (for specification see below), and 10 more mice received a normal saline solution (NSS); 6 h later, the same agents were re-administered. To create equal conditions, both control and stressed animals were administered with an equivoluminous amount of 0.9% NaCl solution. NSS does not affect the

results of the experiment but rules out errors in research, because any external stimulation is stressful.

The *C. fragile* lipid extract was standardized by the sum of total lipids. Animals of one of the experimental groups were administered with a pharmaceutical Omega-3/Fish Oils formula (Now Foods, USA), which was used as a reference preparation (hereinafter called Omega-3). The Omega-3 dietary supplement represents a concentrate of natural anchovy oils. One gram of Omega-3 contains 0.25 g of saturated fatty acids, 0.25 g of monounsaturated fatty acids, and 0.5 g of PUFAs represented by eicosapentaenoic (EPA, 180 mg) and docosahexaenoic (DHA, 120 mg) acids. The *C. fragile* lipid extract and the Omega-3 lipid complex were administered at a dose of 1g/kg animal weight. The dose selection was based on the data by Novgorodtseva et al. [31], as well as our own findings. As a result, the following groups were formed: group 1—control; group 2—stress (vertical fixation) + NSS; group 3—stress + *C. fragile* lipid extract; group 4—stress + Omega-3.

At the end of the experiment, the animals were decapitated under light ether anesthesia. After the separation of plasma from heparinized blood, the erythrocyte fraction was washed three times with an isotonic NaCl solution cooled to 4°C, followed by centrifugation, each time removing the supernatant. An Abacus hematology analyzer (Diatron, Austria) was used to determine the following erythrocyte dimensional characteristics: mean corpuscular volume (MCV) and mean corpuscular diameter (MCD). Physicochemical properties of erythrocytes were assessed uisng the method of determining the osmotic resistance of erythrocytes (ORE) to various exposures, in this case, to changes in NaCl concentration [32].

Lipid extract was obtained from the erythrocyte fraction by the traditional method [24]. To fractionate and quantify phospholipids, the two-dimensional TLC method was used as described above. The content of individual phospholipid fractions was calculated as a percentage of their total amount.

To assess the antioxidant potential of the organism, the following parameters were used: antiradical activity (ARA), superoxide dismutase (SOD) activity, and malonic dialdehyde (MDA) plasma level. All biochemical measurements were performed on a Shimadzu UV-2550 spectrophotometer (Shimadzu, Japan).

Blood antiradical activity was assayed by the method proposed by Bartosz et al. [33]. Ten μL of plasma sample, 2.6 mL of 0.1 M phosphate buffer, and 90 μL of 5mM ABTS [2,2'-azinobis-(3-ethylbenzothiozoline-6-sulfonate)] solution were mixed in a thermostated cuvette and incubated for 5 min at 37°C. The reaction was triggered by adding 300 μL of 200 mM ABAP [(2,2'-azobis(2-amidopropane) hydrochloride)] solution (Sigma, USA) and recorded at $\lambda = 414$ nm. ABAP thermal decomposition was accompanied by the formation of alkyl peroxyl radicals, which, oxidizing ABTS, yield colored staining. Trolox (water-soluble vitamin E analog) was used as a comparison standard. Antiradical activity was expressed in μM Trolox per mL of plasma.

SOD (EC 1.15.1.1) activity was determined spectrophotometrically at 340 nm by the method of Paoletti et al. [34] based on measuring the decrease in optical density during superoxide anion-induced NADH oxidation. The reaction mixture contained 80 mM Tris-HCl buffer (pH 7.4), 100 μM NADH, 80 μM/40 μM EDTA/$MnCl_2$, and 0.1 mL of plasma. After incubating the mixture at 25°C, the reaction was triggered by adding 0.1 mL of 10 mM mercaptoethanol. The decrease in optical density absorbance was recorded for 10 min. SOD activity was expressed in conventional units.

The MDA content was determined by the method [35] based on the capacity of resulting low-molecular-weight aldehydes to interact with thiobarbituric acid to form a colored complex with an absorption maximum at $\lambda = 535$ nm. The initial mixture containing 1 mL of plasma and 2 mL of reagent mixture (15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.25% hydrochloric acid) was thoroughly stirred up and heated on a water bath for 15 min to be followed by centrifugation at 1000 *g* to remove the precipitate. Optical density was determined against a blank sample containing all the ingredients except the plasma sample. MDA concentration was expressed as μmol/mL of plasma.

All biochemical studies (control and experimental groups) were performed in at least three replicates. The obtained quantitative data were expressed as $M \pm m$. Data were processed using the Instat 3.0 statistical package (GraphPad Software Inc. USA). Statistical significance of differences between mean

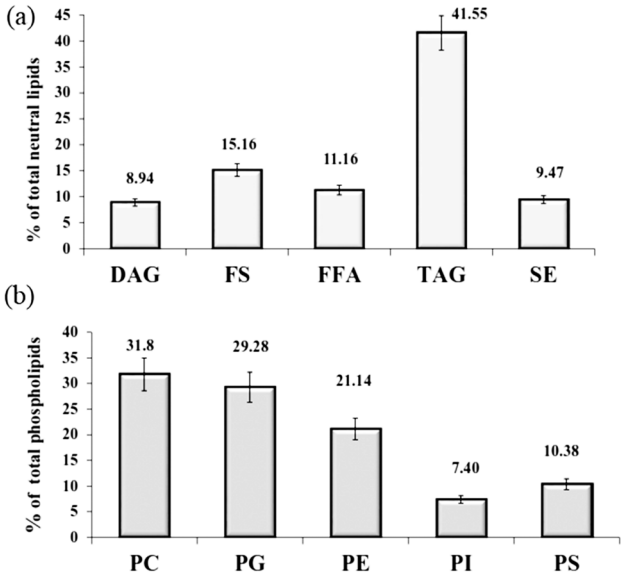

Fig. 1. The content of fractions of neutral lipids (a) and phospholipids (b) in the *Codium fragile* lipid extract. DAG—diacylglycerols, FS—free sterols, FFA—free fatty acids, TAG—triacylglycerols, SE—sterol esters, PC—phosphatidylcholine, PG—phosphatidylglycerol, PE—phosphatidylethanolamine, PI—phosphatidylinositol, PS—phosphatidylserine.

values was assessed by the nonparametric Kruskal–Wallis *H*-test followed by the Dunn's multiple comparisons test when comparing several independent sample. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The study of the composition of the lipid extract isolated from the green alga *C. fragile* showed that the total lipid content amounted to 13.92 ± 0.22 mg per g of dry tissue. Among them, glycolipids accounted for 44% of total lipids (6.12 mg/g dry tissue), neutral lipids—40% (5.57 mg/g dry tissue), while phospholipids—16% (2.23 mg/g dry tissue). The composition of phospholipids and glycolipids comprises essential PUFAs, which, along with other biologically active compounds, determine the value of marine organisms.

Figure 1 shows the content of neutral lipids and phospholipids fractionated in the *C. fragile* lipid extract. Among the neutral lipids, triacylglycerols (41.55 ± 2.15%) and sterols (15.16 ± 0.74%) were predominant, while the content of other minor fractions averaged 9–11%, including diacylglycerols, sterol esters, and free fatty acids. Among the isolated polar fractions, five phospholipids were identified: phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). At the same time, the major phospholipids (PH, FG, PE), referring to structure-forming and functional components of biological membranes, were characterized by a relatively high content compared to other phospholipids. Their amount was in the range of 21–31% of total phospholipids. The obtained quantitative indicators of the *C. fragile* lipid extract composition are consistent with the data reported previously by Khotimchenko [19].

Marine green algae are similar to terrestrial plants in their fatty acid composition, but can differ greatly from them by the content of individual acids. The study of the *C. fragile* fatty acid composition (Table 1) showed that the content of PUFAs prevailed among other identified fatty acids, amounting to 54% of their total sum. At the same time, the proportion of saturated fatty acids (SFAs) in the lipid extract was 34%, while that of monounsaturated fatty acids (MUFAs) was 12%. Out of the SFAs, palmitic acid (16:0) was predominant, with its amount in the *C. fragile* lipid extract being more than 28%. Among the MUFAs, the largest amount (10.72%) fell on oleic acid (18:1 ω-9). Among PUFAs, α-linolenic acid (18:3 ω-3) (19.7%) and hexadecatrienoic acid (16:3 ω-3) (12.2%) proved to be predominant. At the same time, the content of oleic and α-linolenic acids in the green algae significantly exceeded that in terrestrial plants.

It is noteworthy that algae of the division Chlorophyta, including *C. fragile*, are distinguished by the presence of significant amounts of C_{16} and C_{18} PUFAs. The algae of the genus Codium sp. (family Codiacea) are characterized by a high 16:3 PUFA content, which is a taxonomic hallmark of this genus [12]. At the same time, Codium sp. algae are also capable of synthesizing long-chain C_{20} and C_{22} PUFAs. Our results on the fatty acid content in the *C. fragile* lipid fraction agree with the data reported elsewhere [12, 14, 19].

The next stage of the experimental study consisted in comparative analyzing the effects of the *C. fragile* lipid extract and Omega-3 on biochemical and physiological parameters of mouse erythrocytes under stress conditions.

Table 1. Fatty acid composition in the lipid fraction of the *Codium fragile* Suringar (Hariot) thallome

Fatty acids (% of the sum of all fractions)	
Myristic acid (14:0)	1.7 ± 0.02
Palmitoleic acid (16:0)	28.38 ± 1.45
Stearic acid (18:0)	0.9 ± 0.03
Palmitoleic acid (16:1 ω-7)	1.6 ± 0.01
Oleic acid (18:1 ω-9)	10.72 ± 0.46
16:2 ω-6	2.6 ± 0.12
Linoleic acid (18:2 ω-6)	9.0 ± 0.36
Hexadecatrienoic acid (16:3 ω-3)	12.2 ± 0.56
α-Linolenic acid ((18:3 ω-3)	19.7 ± 0.64
Arachidonic acid (20:4 ω-6)	6.2 ± 0.23
Eicosapentaenoic acid (20:5 ω-3)	4.3 ± 0.32
Behenic acid (22:0)	2.7 ± 0.04
Σ SFA	33.68
Σ MUFA	12.32
Σ PUFA	54.0

SFA—saturated fatty acids, MUFA—monounsaturated fatty acids, PUFA—polyunsaturated fatty acids.

Table 2. Effect of stress (vertical fixation) on dimensional characteristics and osmotic resistance of mouse erythrocytes and their correction by the *Codium fragile* (codium) lipid extract and the Omega-3 reference preparation (Omega-3); $M \pm m$

Indices of erythrocytes	Group 1 Control	Group 2 Stress	Group 3 Stress + Codium	Group 4 Stress + Omega-3
Mean corpuscular diameter (μm)	6.50 ± 0.12	8.21 ± 0.18***	6.56 ± 0.12[3]	7.00 ± 0.14
Mean corpuscular volume (μm^3)	56.20 ± 1.9	110.66 ± 2.8***	56.46 ± 1.86[3,a]	70.08 ±2.50*
Osmotic resistance (% NaCl)	0.45 ± 0.01 0.35 ± 0.01	0.50 ± 0.02 0.45 ± 0.02**	0.40 ± 0.02[1] 0.30 ± 0.01[1]	0.40 ± 0.01[1] 0.30 ± 0.01[1]

Changes are statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$—vs. control; [1] $p < 0.05$, [2] $p < 0.01$, [3] $p < 0.001$—vs. group 2 (stress); [a] $p < 0.05$—in group 3 (*C. fragile* extract) vs. group 4 (Omega-3). Osmotic resistance: upper value—hemolysis onset; lower value—complete hemolysis.

Under the effect of stressful vertical fixation, group 2 animals demonstrated significant differences both in the erythrocyte dimensional parameters and in their osmotic resistance vs. control group (Table 2). Namely, MCD exceeded the control parameters by 25% ($p < 0.001$), while MCV significantly increased twofold.

Quantification of the degree of erythrocyte hemolysis in stress-exposed animals revealed a decrease in the range of erythrocyte osmotic resistance. In stressed animals, erythrocytes began degrading earlier, namely at a NaCl concentration of 0.50 ± 0.02% (0.45 ± 0.01% in the control group), while complete hemolysis was already observed at a NaCl

Table 3. Effect of the *Codium fragile* (codium) lipid extract and the Omega-3 reference preparation (Omega-3) on phospholipid content in erythrocyte membranes of stress-exposed mice (% of the sum of fractions, $M \pm m$)

Phospholipids	Group 1 Control	Group 2 Stress	Group 3 Stress + Codium	Group 4 Stress + Omega-3
Phosphatidylethanolamine	24.55 ± 0.36	21.07 ± 0.69**	23.82 ± 0.39[2]	23.12 ± 0.16
Phosphatidylcholine	29.43 ± 0.62	25.22 ± 0.25***	27.93 ± 0.57[1]	27.79 ± 0.39[1]
Sphingomyelin	19.38 ± 0.31	23.22 ± 0.51***	19.90 ± 0.49[2]	20.16 ± 0.42[1]
Lysophosphatidylethanolamine	4.87 ± 0.27	6.05 ± 0.26*	4.53 ± 0.25[2,a]	5.0 ± 0.17
Lysophosphatidylcholine	4.40 ± 0.53	5.87 ± 0.34*	4.71 ± 0.28	4.92 ± 0.17[1]
Phosphatidylinositol	6.60 ± 0.15	7.40 ± 0.31*	6.57 ± 0.42	7.04 ± 0.23
Phosphatidylserine	7.13 ± 0.20	7.62 ± 0.37	7.02 ± 0.44	7.71 ± 0.39
Phosphatic acid	3.64 ± 0.20	3.55 ± 0.16	5.52 ± 0.16***[3,a]	4.26 ± 0.20
Sphingomyelin/ Phosphatidylcholine	0.66	0.92	0.71	0.72

Changes are statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$—vs. control; [1] $p < 0.05$, [2] $p < 0.01$, [3] $p < 0.001$—vs. group 2 (stress); [a] $p < 0.05$—group 3 (codium) vs. group 4 (Omega-3).

concentration of 0.45 ± 0.02% (0.35 ± 0.01% in the control group). Consequently, stress elicited a decrease in osmotic resistance, i.e., hemolyzed erythrocytes began to emerge at a higher than the normal NaCl concentration. Complete erythrocyte hemolysis in stressed animals was detected at a NaCl concentration that was 29% ($p < 0.001$) higher than in the control group.

The effect of stress was also reflected on the content of certain phospholipid fractions which play an important role in the structural and functional integrity of erythrocyte membranes. From Table 3, it follows that stress exposure elicits a significant decrease in the PC and PE levels by an average of 14%, while there was an increase in the concentration of their lysoforms, lysophosphatidylcholine (LPC) by 33% ($p < 0.05$) and lysophosphatidylethanolamine (LPE) by 24% ($p < 0.05$), which may have been due to activation of phospholipases by ROS generated in large amounts under stress [36]. The excess of lysophospholipids produced under the effect of endogenous phospholipases has a membranolytic effect, which leads to membrane structural disorganization, resulting in an increase in their permeability, membrane matrix disintegration and its further degradation [37].

The sphingomyelin (SM) content exerts a considerable impact on the structural and functional properties of membranes. Since it predominantly contains saturated fatty acids, it is less susceptible to peroxidation. The significant stress-induced increase in the SM level (by 20%, $p < 0.001$), as observed in this study, is a protective and adaptive response to the reduction in the amount of PC in the membrane. By Shevchenko and Shishkina [38], SM is an important component of membrane stability and one of the main erythrocyte fractions in rodents of different species. An increase in the SM fraction contributes to the increase in lipid bilayer microviscosity of erythrocyte membranes. The calculated SM/PC ratio was higher by almost 40% (Table 2) compared to the control, which may indicate an increase in the erythrocyte membrane stiffness and a decrease in erythrocyte mobility. The increase in the amount of PI (by 12%, $p < 0.05$), which is characterized by a high metabolic rate compared to other phospholipids, with its bulk being concentrated in the inner layer of membranes, is also worth noting. PI forms an intricate protein–lipid complex and provides information transmission to compounds that control physiological activity of the cell, while maintaining its homeostasis at the molecular level [39]. Under stress exposure, the demand for information transmission rises manyfold, which appears

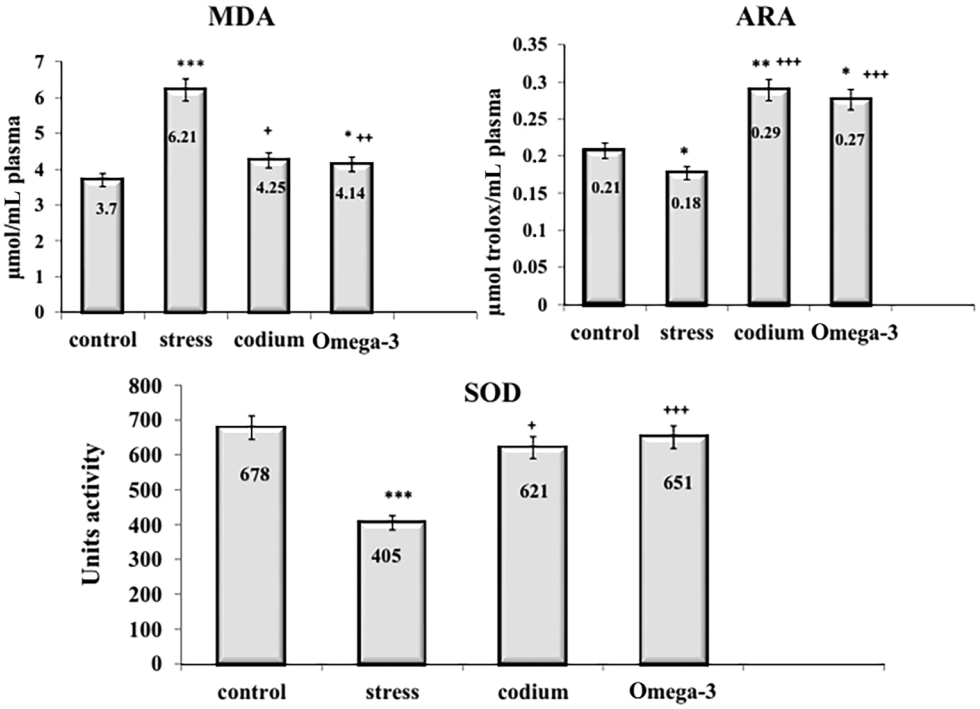

Fig. 2. Effect of the *Codium fragile* (codium) lipid extract and the Omega-3 reference preparation (Omega-3) on parameters of the plasma antioxidant system in stress-exposed mice. MDA—malondialdehyde; ARA—antiradical activity; SOD—superoxide dismutase. Changes are statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$—vs. control; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$—vs. group 2 (stress).

to cause an increase the PI content in the membrane.

As follows from the obtained data, erythrocytes undergo significant alterations under stress both in their dimensional characteristics and in the ratio of membrane phospholipid components, which is probably reflected on their physicochemical properties, leading to changes in permeability and lability, as well as impeding their circulation through the capillary bed.

Stress-induced generation of free radicals [21] is one of the main causes for the changes revealed in the content of membrane phospholipids, which are most vulnerable to their action as being easily oxidized. The resulting oxygen radicals interact with phospholipid PUFAs to yield a fatty acid radical, which leads to lipid peroxidation (LPO) of cell membranes and the formation of oxidized phospholipids.

The accumulation of secondary highly toxic LPO products suggests the activation of phospholipid fatty acid peroxidation, as evidenced by a 68% increase in MDA ($p < 0.001$) with a simultaneous decrease in antiradical plasma activity by 14% ($p < 0.05$) (Fig. 2). The reduction in the activity of SOD, one of the key antioxidant enzymes, by 40% ($p < 0.001$) also indicates an uncontrolled enhancement of LPO processes and oxidative stress development.

The administration of the *C. fragile* lipid complexe and Omega-3 to mice engendered a tendency toward restoration of the stress-disturbed dimensional characteristics and osmotic stability of erythrocytes (Table 2). The impact of the *C. fragile* lipid extract was accompanied by the retention of erythrocyte dimensional values and no significant differences from control values. At the same time, in animals administered with Omega-3, MCV was found to increase by 25% ($p < 0.05$) compared to the control.

With regard to erythrocyte osmotic resistance in animals of both groups administered with lipid preparations under stress conditions, the range of erythrocyte resistance to a hemolytic agent was noted to expand. For example, erythrocyte hemolysis started at a NaCl concentration of 0.40 ± 0.02%, while complete hemolysis proceeded at 0.30 ± 0.01%, which was by 20 and 33% lower than the

same concentrations in group 2 (stress), respectively. Hence, the *C. fragile* lipid extract and Omega-3 promoted the recovery of ORE to a lowering of the sodium chloride concentration under stress conditions.

When studying the content of the main phospholipid fractions in the erythrocyte membranes of experimental animals in groups 3 and 4 administered with *C. fragile* lipid complexes, no significant differences were found compared to the control values (Table 2). A comparison of these parameters with similar values in group 2 (stress) showed an increase in the amount of the main structural membrane phospholipids: PC by an average of 11% ($p < 0.05$) and PE by an average of 13% ($p < 0.01$) and 10%, respectively. Regarding their lysofractions, there was a 25% ($p < 0.01$) decrease in the amount of LPE in group 3 (codium) and by 16% ($p < 0.05$) in group 4 (Omega-3). The decrease in the number of phospholipid lysofractions may indicate phospholipase activity downregulation elicited by the effect of lipid complexes. In both mouse groups, SM levels in the erythrocyte membranes significantly decreased by an average of 13–14%, which caused the SM/PC ratio to decrease by 22–23% compared to group 2 (stress). It is also worth noting a significant 55% increase in the level of phosphatidic acid (PA) ($p < 0.001$) in the erythrocyte membrane of mice administered with the *C. fragile* lipid extract. This fact is of considerable importance for the repair of stress-damaged erythrocyte membranes, as PA is known to be the basis for the synthesis of many phospholipids, including PC and PE. In mice administered with Omega-3, the PA level was also increased, but not that significantly.

The data obtained in this study on the effect of *C. fragile* lipid complexes and Omega-3 on the erythrocyte membranes under conditions of stress exposure may attest to the restoration of membrane lipid structures, respectively, their permeability and lability, as well as the reduction in their increased rigidity.

The *C. fragile* lipid extract and Omega-3 exhibited pronounced antioxidant effects, as evidenced by a significant increase in ARA by 63 and 56%, respectively, as compared to the similar values in group 2 (stress) (Fig. 2). There was also observed the recovery of plasma SOD activity up to the control level against the background of a significant decrease in the MDA content by an average of 32–33% compared to group 2 (stress), indicative of a downregulation of the activity of free-radical oxidative processes under the effect of lipid complexes of marine origin. The observed effects of the *C. fragile* lipid extract and the reference drug Omega-3 appear to be due to the impact of PUFA ω-3, capable of activating antioxidant defense enzymes, including SOD [40], thus protecting erythrocyte membranes from damage. According to the study by Richard et al. [41], ω-3 PUFAs are effective antioxidants of directed action, capable of quenching free radicals.

A comparative analysis of the effect of the *C. fragile* lipid extract and Omega-3 under conditions of stress exposure in mice revealed that both lipid complexes demonstrated pronounced protective effects while restoring erythrocyte dimensional parameters, their osmotic resistance to hemolysis, as well as membrane phospholipid and antioxidant system's indicators. In the meantime, however, some significant differences were noted in a number of indicators between groups 3 and 4 when calculating statistical significance. For example, in the stressed mice administered with Omega-3 (group 4), the MCV value was significantly different from the control values and exceeded by 24% ($p < 0.05$) the respective values in group 3 of animals administered with the *C. fragile* lipid extract. With regard to phospholipid fractions of erythrocyte membranes, it was found that the PA level in animals of group 3 (codium) exceeded the respective values in group 4 (Omega-3) by 30% ($p < 0.05$). At the same time, in the lysofractions of membrane phospholipids in mice administered with the algal lipid extract, the erythrocyte LPE level was significantly lower (by 10%) compared to the respective values in animals administered with the comparison Omega-3 drug.

Thus, the *C. fragile* lipid extract under conditions of stress-inducing exposure showed a higher efficacy compared to Omega-3 in restoring both erythrocyte dimensional characteristics and a phospholipid component of their membranes, which may be mainly due to the different composition of the distinct lipid complexes. It is noteworthy that Omega-3 supplements contain ω-3 (EPA) and ω-6 (DHE) PUFAs, as well as SFAs and MUFAs, as the main active substances. In its turn, the *C. fragile* lipid extract is characterized by a more diverse composition. It includes five major phospholipid species containing fatty acids with a predominance of ω-3

(α-linolenic, hexadecatrienoic, eicosopentaenoic) and ω-6 (linoleic, arachidonic) PUFAs, as well as saturated and monounsaturated fatty acids. Apparently, such a multicomponent repertoire of the *C. fragile* lipid extract accounts a higher biological activity compared to Omega-3.

CONCLUSION

The composition analysis of the *C. fragile* lipid extracts showed the presence of nonpolar and polar fractions of lipids containing essential ω-3 and ω-6 PUFAs. From the data on the structural and physiological characteristics of mouse erythrocytes under conditions of stress exposure, it follows that the *C. fragile* lipid extract has a membrane-protective effect, as manifested in the absence of erythrocyte macrocytosis, preservation of their osmotic resistance to a hemolytic agent, as well as in the restoration of membrane phospholipid and antioxidant defense indicators. The *C. fragile* phospholipids, as the major structure-forming and functional components of all biomembranes, appear to provide a membrane-reparative function by incorporating in damaged erythrocyte membranes. The advantage of the protective effect exerted by the *C. fragile* lipid extract over that of the Omega-3 reference preparation is due to the presence in its composition of a wide range of lipid components, such as glycolipids, neutral lipids, phospholipids, and polyunsaturated fatty acids with a predominance of ω-3 and ω-6 PUFAs. The obtained data indicate that the use of lipid complexes isolated from seaweeds, specifically, from *C. fragile*, has great prospects for the creation of prophylactic drugs with membrane-protective properties.

AUTHORS' CONTRIBUTION

S.E.F.—experimental design, data collection and processing, writing the manuscript; N.F.K.—experimental design, data collection and processing; V.G.S.—experimental design, data processing, technical support in animal-related experiments.

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ETHICS APPROVAL

The experiments with animals were conducted in compliance with the NIH Guidelines for the care and use of laboratory animals (http://oacu.od.nih.gov/regs/index.htm). Experimental protocols were approved by the Ethics Committee of Ilyichev Pacific Oceanological Institute (Meeting minutes No. 21 of November 10, 2022).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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