THE EFFECT OF 1,2,4-TRIOXOLANES WITH BETULIN IN FISH OIL ON OXIDATIVE AND ENERGY METABOLISM UNDER HYPOXIA AND IMMOBILIZATION STRESS IN RATS

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Abstract. 1,2,4-trioxolanes were obtained by the ozonation of fish oil with a mixture of oxygen and ozone to study their physicochemical properties. The biological activity of 1,2,4-trioxolanes with betulin from birch bark extract in fish oil was evaluated under hypoxia and immobilization stress in rats. 1,2,4-trioxolanes composition led to LPO indexes normalization (malondialdehyde, Schiff bases, diene and triene conjugates), the activation of NADP/H and NAD/H-dependent enzymes (GR, G6PDH, LDH, AlDH), as well as SOD and catalase, under stress in rats. Thus, we estimated 1,2,4-trioxolanes with betulin in fish oil to regulate oxidative and energy metabolism under hypoxia and immobilization stress in rats. The findings show that studied composition can be useful to prevent and treat the diseases caused by oxidative stress.

Keywords: antioxidant enzyme defense, hypoxia, immobilization stress, 1,2,4-trioxolanes, botulin, fish oil.

Introduction

Oxidative stress (OS) is one of the main processes related to many diseases (e.g., atherosclerosis, cancer, cardiovascular diseases, neurological diseases, infertility, renal disease, diabetes and obesity, burns, etc.) (Alahmar, 2018; Honda *et al.*, 2019; Kattoor *et al.*, 2017; Madamanchi *et al.*, 2005; Pizzino *et al.*, 2017; Sies *et al.*, 2017; Sinha & Dabla, 2015). OS can be due to great physical stress, emotional stress and physical inactivity too. OS is defined as a ROS level increase and the damage caused by ROS. It is due to the imbalance between ROS production and removal. Two main strategies were used to treat the diseases associated with OS: the approaches of OS increase using prooxidants and those of OS decrease using antioxidants. An initial strategy of therapeutic prooxidants combats the viruses and bacteria by increasing oxidative challengers through host defense. At a later stage the activation of antioxidant enzyme system minimizes tissue damage. Similarly, in cancer there is tumor initiation, promotion and progression, with vastly different roles of oxidants and antioxidants. In all cases, the antioxidant enzymes especially NADP/H and NAD/H-dependent enzymes (GH, G6PDH, LDH, AlDH), as well as SOD and catalase, play an important role in regulating OS level. Hypoxia also induces oxidative stress through increased ROS production by oxygen-dependent enzymes such as cytochrome *c* oxidase, nicotinamide adenine dinucleotide phosphate oxidase, and uncoupled endothelial nitric oxide synthase (Farías *et al.*, 2016). Thus,

modern pharmacological approaches to treat diseases associated with OS consider the therapy which normalize the balance and imbalance of endogenous and exogenous antioxidants and oxidants. The latter ones include hydrogen peroxide, singlet molecular oxygen, or ozonides, which act as second messengers in physiological redox signaling (Sies *et al.*, 2017; Vassalle *et al.*, 2020).

Ozonides are produced by the ozonation of alkenes (Criegee, 1975). The production of a 1,2,4-trioxolane compound is highly dependent on both the structure of alkene being ozonized, and on the reaction condition. 1,2,4-trioxolanes are the compounds with five-membered ring fragments containing three atoms of oxygen in positions 1, 2, and 4. Many alkenes will not produce 1,2,4-trioxolanes as a major product, regardless of reaction conditions. The term ozonide has some ambiguity, as the term ozonide can be referred to either the 1,2,4-trioxolane structure, or to extremely complicated mixture of compounds produced by the ozonation of natural oils waxes, or rubbers. These complicated mixtures are typically hydro-peroxides and their polymers, with some ozonides and diperoxides (Fig. 1). 1,2,4-trioxolanes are commonly synthesized by ozonolysis of unsaturated compounds, as well as by ozonolysis of O-methyl ketone oximes in the presence of carbonyl compounds, which was proposed by Griesbaum (Griesbaum, 1997). In this paper the term 1,2,4-trioxolane will be referred to the secondary ozonides only.

1,2,4-trioxolanes show different kinds of pharmacological activity: anti-HIV, burn repairing, scar inhibitor, anticancer, antiprotozoal, anti-leishmaniasis, antimalarial, antibacterial, antifungal, anti-arthritis, and many other effects (Koech, 2008; Ugazio *et al.*, 2020).

Ozonation of vegetable oils and fats containing unsaturated fat acids proceeds in the same way. Initially, primary ozonides (1,2,3-trioxolanes) are formed. Then, 1,2,3-trioxolanes change into more stable secondary ozonides – 1,2,4-trioxolanes. The ozonation products of vegetable oils are the subject of intensive research as a potential active pharmaceutical ingredient (API) for creating broad-spectrum drugs (Ugazio *et al.*, 2020). Historically, 1,2,4-trioxolanes of oils and fats have not been used. The attention of researchers has been focused on the use of gaseous ozone or ozone solutions in saline or dimethyl sulfoxide, which exhibit bactericidal, antiviral, antifungal properties in clinical practice when applied topically (Smith *et al.*, 2017). These effects are due to strong oxidizing properties of ozone causing the destruction of bacterial cell walls and the cytoplasmic membrane, and the increase in permeability and penetration of ozone into bacterial cells. In addition, ozone is also able to stimulate the innate immune system to counteract microorganisms. In fact, in the presence of ozone, lipoproteins of microorganisms produce lipid oxidation products able to induce higher amounts of H_2O_2 by phagocytes, resulting in better bacteriostatic and bactericidal activity. Unlike short-lived

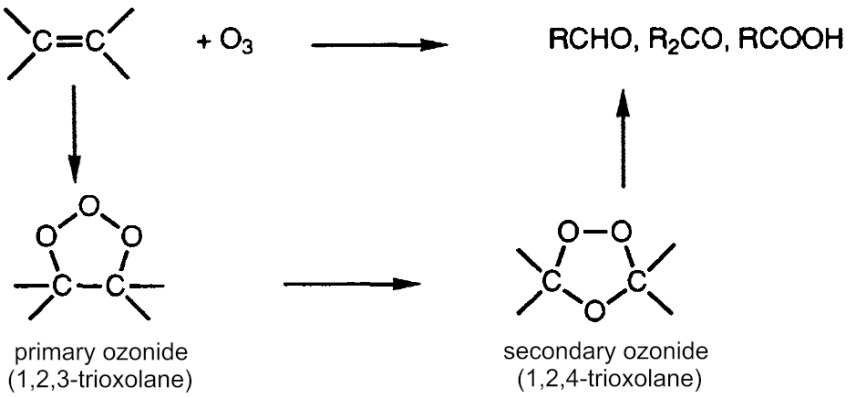


Fig. 1. Scheme of alkene ozonation (Criegee, 1975)

gaseous ozone and its solutions in water or DMSO, lipophilic membrane-soluble 1,2,4-trioxolanes as the ozonation products of oils have a wider pharmacological spectrum of activity including a systemic action. Moreover, inflammatory, restorative effects, the stimulation of the immune system, and a positive effect on the gastrointestinal tract have been proven (Martínez-Sanchez, 2021; Zamora Rodríguez *et al.*, 2007).

The new delivery routes (not only topical, but also oral or rectal) of 1,2,4-trioxolanes enable to achieve new effects due to other mechanisms of their action. The anti-inflammatory effect of trioxolanes in ozonized krill oil is due to the inhibition of nitric oxide (NO) production and the suppression of mRNA and protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in RAW 264.7 macrophages stimulated with lipopolysaccharides. Ozonized krill oil also reduced mRNA expression of inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in macrophages RAW 264.7, suppressed the phosphorylation of p38 MAPK and N-terminal c-Jun kinases (JNK) (Kim *et al.*, 2018).The oils with dissolved 1,2,4-trioxolanes also have an anti-inflammatory effect (Zamora Rodríguez *et al.*, 2007). In this regard, fish oil being a source of phospholipids, eicosapentaenoic acid (EPA) and decosahexaenoic acid (DHA) is of interest. EPA and DHA have an array of health benefits such as antioxidant, anti-inflammatory, neuroprotective and lipid metabolism correction. An important effect of oral or rectal administration of 1,2,4-trioxolanes in oils is the activation of antioxidant defense enzymes. A significant increase in the activity of SOD, glutathione peroxidase and catalase made it possible to provide gastroprotective effects in the treatment of ethanol-induced ulcers and other gastric and intestinal mucosa damage (Zamora Rodríguez *et al.*, 2007).

In this work we characterized 1,2,4-trioxolanes obtained from fish oil as potential API, and designed the composition formulation. We studied the effect of pharmaceutical compositions of 1,2,4-trioxolanes and betulin in fish oil on the activation of antioxidant defense enzymes and the normalization of energy and lipid metabolism under hypoxia and immobilization stress in rats.

Betulin contained in birch bark extract was chosen as an antioxidant protecting fish oil against oxidation (Fig. 2). Moreover, betulin exhibits well-pronounced anti-inflammatory, wound healing, hepatoprotective, hypolipidemic and other properties (Tuli *et al.*, 2021). In a lipophilic medium, it exhibits the properties of a fish oil antioxidant preventing it from oxidation (Melnikova *et al.*, 2022).

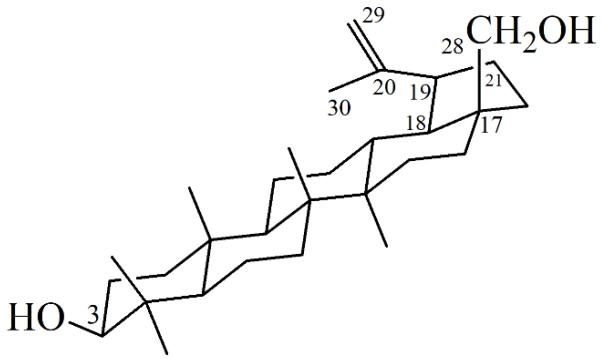


Fig. 2. Structural formula of betulin

Materials and Methods

Materials and reagents

The characteristics of fish oil (MEG-3 1812 TG, Series No: PC00004320, Peru): color according to Gardner – 5; free fatty acid – 0.10%; acid value – 0.2 mg KOH/g; p-anisidine value – 6; peroxide value (at the time of issue) – 0 meq/kg; humidity 0.0%; cold test: stays clean at 0 °C for 3 h; the overall oxidation value is 6; unsaponifiable matter – 1.1%; absorption (233 nm) – 0.3; oligomers – 0.9%.

Betulin ($C_{30}H_{50}O_2$), 99.5% purity (HPLC), was obtained from Sigma-Aldrich (CAS 473-98-3).

Deionized water obtained at the Elix 3 water purification system with a Progard cartridge (Millipore, France), resistivity less than 0.2 μS with pH 5.5 at 20 ± 10 °C, was used without prior cleaning and any processing.

Sodium hydroxide (analytical grade, GOST 4328-77), iodine monochloride, 95+%, 100g (Sigma-Aldrich 208221), potassium iodide (chemically pure, GOST 4232-74), sodium thi-

osulfate (analytical grade, GOST 27068-86), starch (analytical grade, GOST 10163-76), glacial acetic acid (chemically pure, GOST 61-75), phenolphthalein, p-anisidine (IMP GOST, TU 104-94-9), diethyl ether (analytical grade, TU 2600-001-43852015-10), chloroform (GOST 20015-88), alcohol 96%, trimethylpentane (GOST 12433-83).

Synthesis of 1,2,4-trioxolanes

1,2,4-trioxolanes were obtained by ozonizing sesame oil and fish oil using an oxygen concentrator «HG5» (Shenyang Canta Medical TECH. Co., Ltd, China, in the mode of 5 L/min, 0.04-0.08 MPa, 220V, 50 Hz), an ozone generator UOTA-60-01 (Medozon LLC, Moscow, Russia). The range of ozone concentration values in the oxygen-ozone mixture at the unit outlet was from 0 to 80 mg/L, the flow rate of the ozone-oxygen mixture being 0.0-1.0 L/min. We used an ozonation column 1 meter high, 0.1 m in diameter, equipped with a glass filter bed at the base. The volume of fish oil or sesame oil did not exceed 300 ml (Fig. 3). The ozonation product was a viscous light-yellow gel.

Equipment

FTIR Analysis. FTIR spectra in the range of 400–4000 cm^{-1} were registered by an IR Prestige-21 FTIR spectrometer (Shimadzu, Kyoto, Japan) equipped with a KBr beam splitter. A pellet from a well-dried KBr was prepared by standard cold pressing. The resolution was 0.5 cm^{-1}, and the number of scans was 45.

UV Analysis

UV spectra were obtained by UV-1800 (Shimadzu, Kyoto, Japan). PBS was used as a solvent. The wavelength range depended on the analyzed substance.

NMR

^{13}C and ^{1}H NMR spectra were recorded at 100 and 400 MHz, respectively, on a Jeol JNM ECX-400 spectrometer (Jeol Ltd., Tokyo, Japan) using $CDCl_3$ (50 mg/0.6 mL) as the solvent at 25 °C. As reference signals for the scale correction of chemical shifts, we used the signals of residual protons of the solvent (δ = = 7.26 ppm) and carbon atoms of $CDCl_3$ (δ = = 77.16 ppm) for the ^{1}H and ^{13}C nuclei, respectively.

GC-MS

Low-boiling products were identified and quantitative analyses were performed through GC-MS using a Shimadzu GC-2010 instrument equipped with a GCMS-QP2020 mass spectrometer, FID and VB-1701 capillary column (length, 30 m, inner diameter, 0.32 mm, film thickness, 0.5 μm; VICI, Poulsbo, WA, USA).

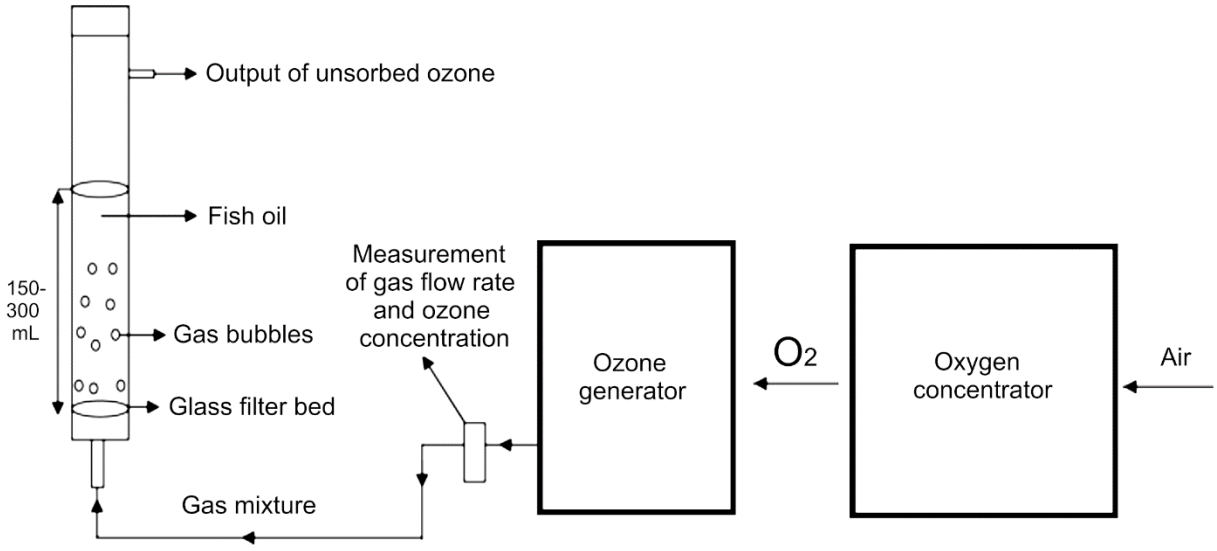


Fig. 3. Principal scheme of fish oil ozonation

Helium was used as a carrier gas. Injector and detector temperatures were 250 °C, the oven temperature was programmed from 100 °C (5 min hold) to 250°C (50 min hold) at a rate of 10 °C/min.

Gel permeation chromatography

Quantitative analysis of high-boiling components of ozonized fish oil and its molecular weights were determined by gel permeation chromatography (GPC) on a Khromos LC-310 liquid chromatograph (Khromos, Russia) equipped with a special HPLC pump, a system of chromatographic column placed in a thermostat, a refractometric detector, and a hardware–software module. For analysis, there was used a system consisting of two consecutive Phenomenex Phenogel 00H-0441-K0 columns (300 × 7.8 mm, Phenomenex, United States) packed with styrene-divinyl benzene (particle size 5 μm, pore diameter 50 A). To protect the columns from impurities, we used a Phenomenex Guards 03B-2088-K0 precolumn (with particle sizes of 5 μm). The columns were preliminarily thermostated at 25 °C. The elution was performed with tetrahydrofuran supplied at a rate of 1.0 mL/min. The quantitative determination of the components was carried out by absolute calibration method using glycerol trioleate as a standard. Polystyrene standards with fixed molecular weights were used to determine the molecular weights of the components of ozonized fish oil.

The fatty acid composition was determined using a method based on the conversion of fatty acid glycerides into methyl esters (FAME) and its subsequent gas chromatographic analysis. The glycerides were converted into FAMEs in accordance with ISO 5509. Chromatographic analysis was carried out using the method described earlier. The concentration of FAMEs was determined by absolute calibration of methyl stearate.

Iodometric titration

Ozonides were determined by iodometric titration using starch as an indicator. A sample of the analyte was placed in two flasks with a capacity of 250 ml. The sample mass was determined by the difference between the weights of flasks with and without the sample. Analytical scales with an accuracy of 0.0002 g were used for weighing. 10 ml of glacial acetic acid and 2 ml of a 50% aqueous solution of potassium iodide were added to the samples. The samples were kept in the dark for 20 min and then titrated with a standard aqueous solution of sodium thiosulfate (0.1 N) using a freshly prepared starch solution. In parallel, a blank experiment was carried out.

Values (acid, peroxide, iodine) assay (European Pharmacopoeia 10th edition)

Acid value. The test portion (1.5 g) was placed in a reaction flask and dissolved in 1:1 (v/v) mixture of 95% ethanol (25 ml) and ethoxyethane (25 mL) previously neutralized with 0.1 M sodium hydroxide, unless otherwise specified, using 0.5 mL of phenolphthalein solution as an indicator. The solution was titrated with standard 0.1 M sodium hydroxide solution, using a phenolphthalein indicator. The determinations were performed thrice. The acid value (I_A) was calculated using the formula (1):

$$I_A = \frac{5.611 \cdot V}{a}, \quad (1)$$

where V is volume of standard 0.1 M sodium hydroxide solution used for titration, mL; *a* is the mass in grams of the oil portion taken, g.

Peroxide value. A mixture of 2 volumes of chloroform and 3 volumes of glacial acetic acid (50 mL) was placed in a conical flask, and the stopper was replaced. The flask should be swirled until the fish oil to be examined (0.5 g, m) has dissolved. Using a suitable volumetric pipette, 0.5 mL of saturated potassium iodide solution was added to the flask, and the stopper was replaced. The solution stood for 60±1 s, it was shaken continuously, and then 30 mL of water was added. The solution was titrated with 0.01 M sodium thiosulfate (V_1 mL), it was added gradually and with constant, vigorous shaking, until the yellow iodine color has almost disappeared. About 0.5 mL of starch solution R1 was added. The titration was continued, with constant shaking especially

near the end-point, to liberate all of the iodine from the solvent layer. The sodium thiosulfate solution was added dropwise until the blue color just disappears. A blank determination was carried out (V_0 mL). If the result of the blank determination exceeds 0.1 mL of titration reagent, the determination should be repeated. The peroxide value (I_P) was calculated using the formula (2):

$$I_p = \frac{1000(V_1 - V_0)c}{m}, \quad (2)$$

where *c* is the concentration of the sodium thiosulfate solution, in moles per liter.

Iodine value. 1.0 g of the substance (m) was introduced into a 250 mL flask fitted with a ground-glass stopper and dissolved in 15 mL of chloroform. 25.0 mL of iodine bromide solution was added very slowly. The flask was closed and kept in the dark for 30 min, it was shaken frequently. 10 mL of a 100 g/L solution of potassium iodide R and 100 mL of water were added. The mixture was titrated with 0.1 M sodium thiosulfate, it was shaken vigorously until the yellow color was almost discharged. 5 mL of starch solution was added. The titration was continued adding the 0.1 M sodium thiosulfate dropwise until the color was discharged (n_1 mL of 0.1 M sodium thiosulfate). A blank test was carried out under the same conditions (n_2 mL of 0.1 M sodium thiosulfate). The iodine value (I_I) was calculated using the formula (3):

$$I_I = \frac{1.269(n_2 - n_1)}{m} \quad (3)$$

Biological study

The study involved male Wistar rats (200-250 g) at the age of 5-7 months. 15 groups of 5 animals were formed. The animals were purchased from the Animal Breeding Facilities «Andreevka», Federal State Budgetary Institution of Science «Scientific Center for Biomedical Technologies», Federal Medical and Biological Agency (Andreevka, Moscow region, Russia). All procedures for maintenance and sacrifice (care and use) of animals were carried out according to the criteria outlined by European Convention ET/S 129, 1986 and directives 86/609 ESC. The animals were handled humanely, kept in plastic suspended cages, and placed in a well-ventilated and hygienic rat house under suitable conditions of room tem perature (27 ± 2 °C) and humidity. They were given food and water ad libitum and subjected to a natural photoperiod cycle of 12 h light and 12 h dark. The animals were allowed two weeks of acclimatization before the commencement of all animal model experiments in the study.

All blood collection from animals for the experiment was performed under anesthesia, with efforts made to minimize suffering.

The study, as presented, was approved by the Local Ethics Committee of Privolzhsky Research Medical University, Russian Federation (Protocol No. 1, January 18, 2021).

Stress models

To simulate chronic immobilization stress (IS), long-term restriction of mobility was used: for 10 days, daily from 9:00 to 15:00, animals were placed in small-volume chambers that limited their mobility, without access to food and water (Solin & Lyashev, 2015). On day 10, all rats were sacrificed under anesthesia (Zoletil 60 μg·kg^{-1} «Virbac Sante Animale», Vauvert, France; Xyla 6 mg·kg^{-1}, Interchemie, Venray, The Netherlands).

Acute hypoxic hypoxia was caused by a six-hour exposure of the experimental animals in a ventilated pressure chamber with a residual pressure corresponding to an ascent to a height of 8000 m (Ryabkov, 2014). On day 10, all rats were sacrificed under anesthesia (Zoletil 60 μg·kg^{-1} «Virbac Sante Animale», Vauvert, France; Xyla 6 mg·kg^{-1}, Interchemie, Venray, The Netherlands).

Compositions for in vitro biological analysis

The compositions were administered to rats daily for 10 days, 2 ml orally by gavage.

The fraction of ozonized fish oil containing about 70% 1,2,4-trioxolanes (1,2,4-T) with peroxide value 7000 was used for the compositions (Table 1).

Body weight of rats on Day 10 of in vivo experiments

The animals were weighted on Day 0 (intact group) and Day 10 (all groups, Table 2).

Table 1

Studied compositions[a]

Group	Composition	Daily dose
Betulin	Betulin suspension in water (0.1%)	0.002 g of betulin
FO	Fish oil	2 g of fish oil
1,2,4-T	1,2,4-T (10.0%) in fish oil	0.14 g of 1,2,4-trioxolanes in 1.86 g of fish oil
FO+1,2,4-T+Betulin	The pharmaceutical composition consisted of 1,2,4-T (10.0%), betulin (0.1%), ascorbyl palmitate (0.01%) and non-ozonized fish oil (up to 100%).	0.002 g of betulin, 0.14 g of 1,2,4-trioxolanes in 1.86 g of fish oil

[a] FO – Fish oil; 1,2,4-T – 1,2,4-trioxolanes.

Table 2

Body weight of rats on Day 10 of *in vivo* experiments (*n* = 5; mean ± SD)[a]

Animal state	Treatment				
	Control (without treatment)	Betulin	FO	1,2,4-T	FO+1,2,4-T +Betulin
Intact	223.0 ± 9.4	219.7 ± 10.5	221.6 ± 8.3	214.7 ± 11.3	215.4 ± 10.2
Hypoxia	203.6 ± 12.3	211.2 ± 13.3	209.6 ± 12.0	207.5 ± 6.2	215.4 ± 10.2
IS	205.8 ± 13.0	209.8 ± 7.2	206.4 ± 10.8	205.7 ± 9.4	207.2 ± 8.9

[a] Body weight of intact rats on Day 0 was equal to 228.1 ± 7.9 g; FO – Fish oil; 1,2,4-T – 1,2,4-trioxolanes; IS – immobilization stress.

In vitro biological analysis

In vitro biological analysis was performed using blood stabilized with sodium citrate (1:9). Erythrocytes were washed twice with 0.9% NaCl by centrifugating for 10 min at 1600×g. Enzyme activity and LPO metabolites level were evaluated using methods as described early (Melnikova *et al.*, 2022). The intensity of lipid peroxidation (LPO) was estimated using the malondialdehyde (MDA) level in erythrocytes according to Uchiyama and Mihara methods. Superoxide dismutase SOD activity (EC 1.15.1.1) was measured in erythrocytes using the inhibition of adrenaline auto-oxidation. Catalase activity (EC 1.11.1.6) was determined via spectrophotometry based on the decomposition of hydrogen peroxide by the catalase. Glutathione reductase GR activity (EC 1.8.1.7) was studied via spectrophotometry based on the oxidized glutathione reduction. The activity of glucose-6-phosphate dehydrogenase G6PDH (EC 1.1.1.49) was determined in the hemolysate of erythrocytes via spectrophotometry based on glucose-6-phosphate oxidation to phosphoglucolactone with the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The energy metabolism in erythrocytes was analyzed using the catalytic activity of lactate dehydrogenase LDH (EC 1.1.1.27) in direct (LDH_{direct}, substrate – 50 mM of sodium lactate) and in reverse ($LDH_{reverse}$, substrate – 23 mM of sodium pyruvate) reactions. The activity of aldehyde dehydrogenase (EC 1.2.1.3) was estimated spectrophotometrically in accordance with the previous methods. The specific activity of the enzymes was calculated from the protein concentration analyzed via the modified Lowry method.

Statistical Analysis

The data were statistically processed using Statistica 6.0 software (StatSoft Inc., Tulsa, OK, USA). The results are presented as the mean ± standard deviation (SD). Shapiro–Wilk test was used for normal distribution analysis. We performed Kruskal–Wallis nonparametric ANOVA. Difference was considered to be statistically significant if $p < 0.05$.

Results

Physicochemical properties of ozonized fish oil

We compared the physicochemical properties of ozonized fish oil with literature data of 1,2,4-trioxolanes from ozonized sesame oil (Sega *et al.*, 2010; Zanardi *et al.*, 2008).

The 1,2,4-trioxolanes of fish oil and sesame oil showed similar physicochemical values analyzed according to pharmacopoeia (European Pharmacopoeia 10th edition). The kinematic viscosity of ozonized fish oil was 5044 $mm^2 \cdot s^{-1}$; acid value was 7.5 mg $KOH \cdot g^{-1}$; iodine value was 22.0 mg iodine·100 g^{-1}; ozonide value was 7101 meq $O_2 \cdot kg^{-1}$. The kinematic viscosity of ozonized sesame oil was 3454 $mm^2 \cdot s^{-1}$; acid value was 15.9 mg $KOH \cdot g^{-1}$; iodine value was 20.5 mg iodine·100 g^{-1}; ozonide value was 6690 meq $O_2 \cdot kg^{-1}$.

Table 3 presents the data of ^{1}H- and ^{13}C-NMR spectra of ozonized fractions containing 1,2,4-trioxolanes. Table 3 data show a variety of structures of 1,2,4-trioxolane fragments as ozonation products. They are similar to those of the secondary ozonides of sesame oil (Sega *et al.*, 2010; Zanardi *et al.*, 2008).

The structures of 1,2,4-trioxolanes fragments are shown in Table 3. In contrast to the initial sesame oil and fish oil, the FTIR spectra of ozonized samples contain a band at 1105 cm^{-1}, which is the characteristic of the stretching vibrations of the trioxolane fragment (Table 4, Fig. 4).

In accordance with GC-MS spectra, the initial fish oil contained 16.03% and 9.09% of eicosa-5,8,11,14,17-pentaenoic acid (EPA) and 4,7,10,13,16,19-docosahexaenoic acid (DHA), respectively. The concentration of EPA and DHA in ozonized fish oil was minimal. Table 5 presents the percentage of other fatty acids according to GC-MS.

The total mass fraction of low molecular weight products of oxidative cleavage (mainly aldehydes and ketones) did not exceed 0.66%. The mass fractions of substances with different molecular weights were estimated according to gel permeation chromatography (GPC). The mass fraction of the substances with molecular weight of 200 $g \cdot mol^{-1}$ was equal to 0.62%. The mass fractions of the substances with molecular weight of 800–1000 $g \cdot mol^{-1}$ was 29.32%. The mass fraction of trioxolanes with molecular weight of about 3000 $g \cdot mol^{-1}$ was 26.39%. The mass fraction of trioxolanes with a molecular weight of more than 4500 $g \cdot mol^{-1}$ was 43.68%. The mass fraction of the substances with molecular weight of about 800-1000 $g \cdot mol^{-1}$ was taken as 100% (initial fish oil).

The mass fraction ratio of the substances with molecular weight of about 3000 $g \cdot mol^{-1}$ and 4500 $g \cdot mol^{-1}$ was 1 to 1.66.

Thus, we obtained 1,2,4-trioxolanes, their content being about 70% (with molecular weight of more than 3000 $g \cdot mol^{-1}$) resulted from fish oil ozonation. The structure and composition of 1,2,4-trioxolanes were estimated by FTIR and NMR spectra, GPC, GC-MS and physicochemical properties.

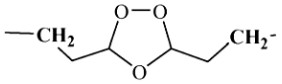
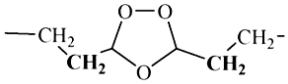
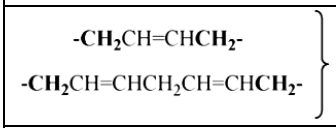
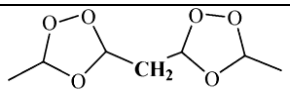
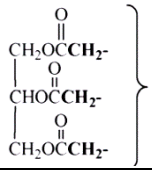
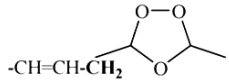
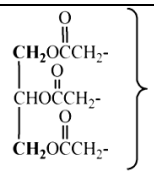
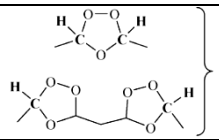
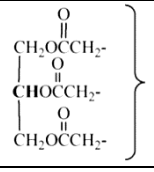
We used the fraction of ozonized fish oil containing about 70% 1,2,4-trioxolanes (1,2,4-T). Peroxide value of this fraction was equal to 7000. The pharmaceutical composition consisted of 1,2,4-T (10.0%), betulin (0.1%), ascorbyl palmitate (0.01%) and non-ozonized fish oil (up to 100%).

The effect of 1,2,4-trioxolanes and their pharmaceutical composition with betulin in fish oil on the activity of antioxidant defense enzymes and energy metabolism in rat models of chronic immobilization stress and acute hypoxic hypoxia

As the indicators of the initial phase of lipid-peroxidation, the concentrations of diene conjugates (DC), triene conjugates (TC) and Schiff's bases (SB) were measured in the work. The MDA level depends on the lipid saturation level since peroxidation occurs only when li pids are in an unsaturated form (Sies *et al.*, 2017; Vassalle *et al.*, 2020). The activation of antioxidant defense enzymes was studied by evaluating the specific activity of SOD, catalase, glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH), and aldehyde dehydrogenase (AlDH) without treatment and under the effect of the studied compositions. Energy metabolism was studied by the ratio of lactate dehydrogenase (LDH) activity in direct and reverse reactions. The effect of the components of the composition was studied under stress both without and under treatment.

Table 3

^{1}H- and ^{13}C-NMR data of ozonized fish oil and ozonized sesame oil[a]

Group	^{1}H-NMR, δ, ppm		^{13}C-NMR, δ, ppm	
	OFO	OSO	OFO	OFO
–CH_3 **CH_3**–(CH_2)–	[0.82, 0.84, 0.85, 0.86, **0.87**, 0.88] (m); 0.98 (m)	0.82 (m)	7.86, 8.11, 14.16, 14.25	13.8, 14.0
–$(CH_2)_2$–	[**1.24**, 1.27, 1.29] (bp)	1.20–1.25 (bp)	22.77, 22.82, 29.15, 29.20, 29.24, 29.40, 29.49, 29.58, 29.61, 29.78, 29. 83, 32.05	22.3, 22.5, 28.8, 28.9, 29.1, 29.3, 29.4, 29.6, 31.2, 31.4, 31.7, 31.8, 32.3
	1.40 (bp)	1.35 (bp)	–	23.3, 23.4, 23.6, 23.7, 23.8
	–	1.55 (bp)	24.99	24.7
	[**1.60**, 1.64] (bp)	1.63 (bp)	30.86	30.6
	1.96-2.20 (bp)	1.96–2.03 (bp)	–	27.1, 27.3
	2.16 (s)	2.04–2.12 (m)	–	35.6
	2.20, 2.19 (weak)	2.25 (t)	34.18	33.8, 34.0
	[2.28, **2.30**, 2.32] two triplets	2.36–2.42 (m)	–	41.5
–CH=CH**CH_2**CH=CH–	2.51 (bp), 2.82 (bp)	2.72 (t)	25.03	25.6
	4.14 (dd), 4.28 (dd)	4.10 (dd), 4.26 (dd)	62.23	62.0
	5.12, 5.13, 5.14 (m)	5.08–5.11 (m)	103.39, 104.29, 104.34, 104.39, 104.47	103.4, 104.4, 104.1, 104.3
	[5.16, 5.18, 5.19] (m)	5.22 (m)	69.00	68.9

[a] OFO – ozonized fish oil, OSO – ozonized sesame oil; s – singlet; dd – double doublet; t – triplet; m – multiplet; bp – broad peak.

Table 4

FTIR data of initial and ozonized fish oil[a]

Sample	ν, cm^{-1}				
	3010–3500	**1700–1750**	**1654**	**1100–1105**	**900–1100**
FO	3013 (O-H)	1746 (C=O)	1654 (C=C)	–	914 (C-O), 968 (C-O), 1098 (C-OH)
OFO	3014 (O-H)	1743 (C=O)	–	1105 (trioxolane fragment)	–

[a] OFO – ozonized fish oil, OSO – ozonized sesame oil.

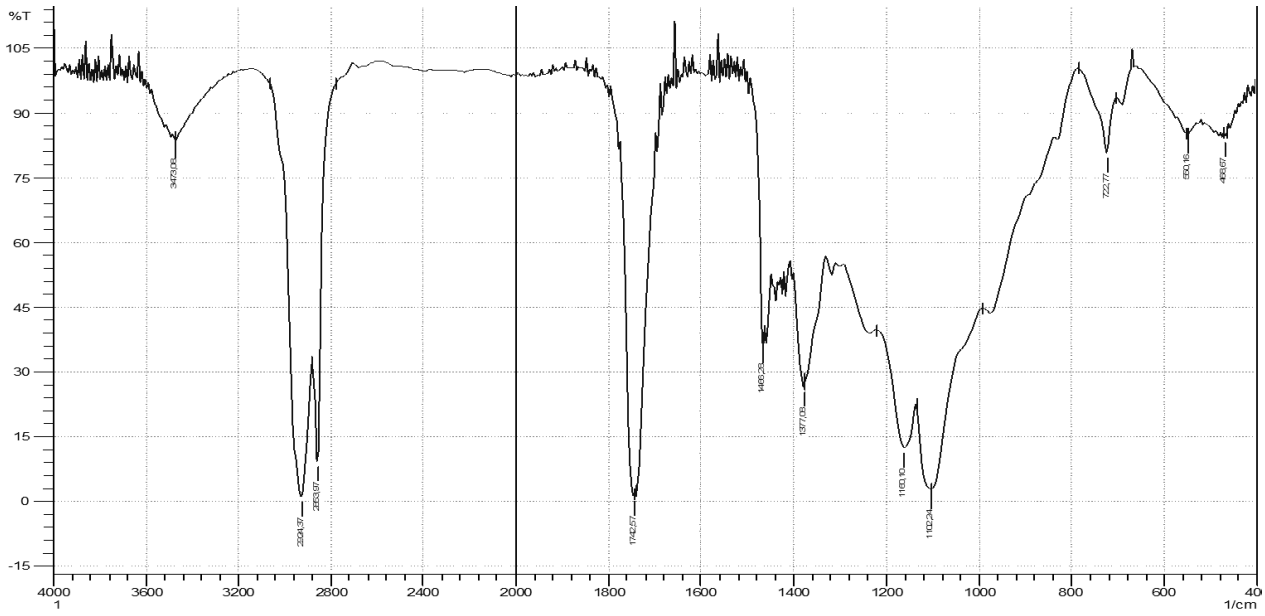


Fig. 4. FTIR-spectrum of ozonized fish oil

Table 5

The composition of theinitial and ozonized fish oil samples (GC-MS)

Component	Content, %	
	Initial fish oil	**Ozonated fish oil**
5-oxo-Pentanoic acid	-	0.16
Octanoic acid	0.03	0.22
Nonanoic acid	0.01	–
Decanoic acid	0.03	0.10
9-oxo-Nonanoic acid	–	1.72
Dodecanoic acid	0.11	–
Tridecanoic acid	0.06	–
Myristoleic acid	0.04	–
Myristic acid	7.59	5.93
Pentadecanoic acid	0.49	0.37
9-Hexadecenoic acid	9.15	1.59
Hexadecanoic acid	18.88	12.42
7,10-Hexadecadienoic acid	1.91	0.21
10-Heptadecenoic acid	0.24	0.05

End of table 5

Component	Content, %	
	Initial fish oil	Ozonated fish oil
Heptadecanoic acid	0.41	0.33
9-Octadecenoic acid	6.69	1.36
9,12-Octadecadienoic acid	3.45	0.55
Stearic acid	2.74	2.39
octadeca-6,9,12,15-tetraenoic acid	3.17	0.16
9,12,15-Octadecatrienoic acid	0.60	–
Nonadecanoic acid	0.11	0.07
5,8,11,14-Eicosatetraenoic acid	0.66	–
11-Eicosenoic acid	1.24	0.18
Eicosa-5,8,11,14,17-pentaenoic acid	16.03	–
8,11,14,17-eicosatetraenoic acid	1.17	–
11-(3,4-Dimethyl-5-propyl-2-furyl)-undecanoic acid	0.09	–
4,7,10,13,16-docosapentaenoic acid	0.28	–
13-Docosenoic acid	1.30	0.27
4,7,10,13,16,19-Docosahexaenoic acid	9.09	0.08
Tricosanoic acid,	0.03	–
15-Tetracosenoic acid	0.41	–

Enzyme activity under hypoxia and immobilization stress without treatment

A statistically significant increase in DC level under hypoxia and immobilization stress (IS) was by 33% and 29%, respectively. Under the same conditions, the level of Schiff bases increased by 22% ($p < 0.05$) and MDA level in erythrocytes decreased by 17–18% in both: during hypoxia and IS (Table 6). The level of TC in all cases remained practically normal.

There was a decrease in the specific activity of SOD by 39% in hypoxia and by 13% in IS. The catalase activity depended on the stress model. Catalase activity decreased by 37% in hypoxia, while catalase activity increased by 8% in IS.

A compensatory increase in the activity of NADPH/NADP-dependent enzymes was shown as follows: GH by 117% (hypoxia) and 49% (IS); G6PDH by 15% (hypoxia) and 102% (IS) (Table 7).

Hypoxia and IS were accompanied by an imbalance of NAD/NADH-dependent enzyme LDH in direct and reverse reactions (Table 8). The activity ratio of LDH_{rev}/LDH_{dir} was 4.15 in intact rats, but it was 9.37 and 4.99, under hypoxia and IS, respectively. This fact characterizes lactate accumulation that can result in lactic acidosis (Table 8). The AlDH activity during hypoxia increased by about 5% in hypoxia and by 20% in IS.

Therefore, under hypoxia and IS the activity of SOD as the main enzyme of antioxidant defense decreased, the activation of NAD/NADH- and NADP/NADPH-dependent enzymes decreased as well. The activation of catalase was found under IS only.

The effects of the pharmaceutical composition components on the enzymes of antioxidant defense and energy metabolism

Fish oil (FO) being the main component of the pharmaceutical composition and betulin (B) had a minor effect on the activity of SOD and catalase, while fish oil and betulin activated GR and AlDH, and increased LDH activity both in direct and reverse reactions in intact rats (Tables 7 and 8). In contrast, trioxolanes (1,2,4-T) significantly affected all enzymes, increasing their activity in normal conditions and in various pathologies (Tables 7 and 8).

Under the action of the pharmaceutical composition with 1,2,4-trioxolanes and betulin in fish oil (FO+1,2,4-T+B), the activation of antioxidant defense enzymes was observed in intact rats (Tables 7 and 8). The activity of SOD, cat-

Table 6

The biochemical indexes of oxidative stress products under hypoxia and IS (n = 5; mean ± SD)[a]

Biochemical index	Group	Treatment				
		Control (without treatment)	Betulin	FO	1,2,4-T	FO+1,2,4-T+Betulin
MDA_{er}, μmol·L^{-1}	Intact	7.28 ± 0.25	7.97 ± 0.06	8.91 ± 0.07	8.36 ± 0.07	9.93 ± 0.17
	Hypoxia	5.97 ± 0.21	6.76 ± 0.24	6.86 ± 0.13	7.93 ± 0.10	7.50 ± 0.23
	IS	6.04 ± 0.01	4.50 ± 0.21	4.79 ± 0.10	5.48 ± 0.13	6.71 ± 0.17
DC_{plasma}, a.u.	Intact	0.66 ± 0.01	0.67 ± 0.01	0.76 ± 0.00	0.74 ± 0.01	0.74 ± 0.01
	Hypoxia	0.88 ± 0.06	0.93 ± 0.01	0.92 ± 0.02	0.94 ± 0.02	0.93 ± 0.01
	IS	0.85 ± 0.03	0.89 ± 0.01	0.91 ± 0.00	0.90 ± 0.00	0.91 ± 0.00
TC_{plasma}, a.u.	Intact	0.26 ± 0.00****	0.26 ± 0.01****	0.28 ± 0.01****	0.28 ± 0.00****	0.29 ± 0.01****
	Hypoxia	0.25 ± 0.01****	0.24 ± 0.00****	0.25 ± 0.01****	0.24 ± 0.00****	0.22 ± 0.01****
	IS	0.27 ± 0.00****	0.27 ± 0.00****	0.28 ± 0.00****	0.27 ± 0.01****	0.27 ± 0.00****
SB_{plasma}, a.u.	Intact	0.14 ± 0.01****	0.16 ± 0.01****	0.18 ± 0.00****	0.19 ± 0.00****	0.20 ± 0.00****
	Hypoxia	0.18 ± 0.00****	0.18 ± 0.00****	0.19 ± 0.00****	0.19 ± 0.00****	0.15 ± 0.00****
	IS	0.18 ± 0.00****	0.18 ± 0.00****	0.20 ± 0.01****	0.19 ± 0.01****	0.17 ± 0.00****

[a] FO – Fish oil; 1,2,4-T – 1,2,4-trioxolanes; IS – immobilization stress; MDA_{er} - malondialdehyde level in erythrocytes; SB – Schiff bases; DC – diene conjugates; TC – triene conjugates. **** $p < 0.0001$ indicates statistical difference when compared to corresponded control group (one-way ANOVA).

Table 7

Specific activity of oxidoreductases (SOD, catalase, GR, G6PDH) under hypoxia and IS (n = 5; mean ± SD)[a]

Enzyme	Group	Treatment				
		Control (without treatment)	Betulin	FO	1,2,4-T	FO+1,2,4-T +Betulin
SOD, % inh·min^{-1}·mg of protein^{-1}	Intact	959.22 ± 11.27	995.79 ± 8.22	992.71 ± 10.49	1085.89 ± 16.08	1233.42 ± 15.13
	Hypoxia	585.66 ± 8.76	666.10 ± 9.52	665.41 ± 11.60	903.92 ± 3.71	992.69 ± 8.20
	IS	831.50 ± 1.51	1002.41 ± 9.43	997.15 ± 12.09	999.39 ± 9.54	998.99 ± 7.05
Catalase, μmolH_2O_2·min^{-1}·mg of protein^{-1}	Intact	35.75 ± 1.25	38.99 ± 0.58	36.58 ± 0.51	45.81 ± 1.04	50.89 ± 0.51
	Hypoxia	22.63 ± 0.42	27.08 ± 0.66	26.93 ± 0.22	38.12 ± 0.70	45.29 ± 0.26*
	IS	38.71 ± 0.19	38.22 ± 0.44	42.70 ± 0.41	53.86 ± 0.44	54.96 ± 0.31
GR, NADPH·min^{-1}·mg of protein^{-1}	Intact	90.20 ± 1.49	279.85 ± 5.81***	118.43 ± 1.93	317.07 ± 2.02****	269.20 ± 5.07***
	Hypoxia	195.77 ± 4.24	242.94 ± 3.24	207.32 ± 0.73	229.42 ± 5.82	158.94 ± 0.95
	IS	134.26 ± 3.13	176.34 ± 1.86	162.97 ± 1.19	218.13 ±1.81	161.18 ± 1.53
G6PDH, NADPH·min^{-1}·mg of protein^{-1}	Intact	40.85 ± 1.17	55.77 ± 0.26	44.65 ± 0.19	75.58 ± 3.48	72.90 ± 0.34
	Hypoxia	46.82 ± 0.53	45.72 ± 0.14	39.54 ± 0.34	103.52 ± 1.00	97.08 ± 1.94
	IS	82.62 ± 0.49	112.09 ± 2.78	62.59 ± 0.90	83.58 ± 0.26	114.01 ± 0.21

[a] FO – Fish oil; 1,2,4-T – 1,2,4-trioxolanes; IS – immobilization stress; SOD – superoxide dismutase; GR – glutathione reductase; G6PDH – glucose-6-phosphate dehydrogenase. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$ indicates statistical difference when compared to corresponded control group (one-way ANOVA).

Table 8

Specific activity of AlDH and LDH under hypoxia and IS (n = 5; mean ± SD)[a]

Enzyme	Group	Treatment				
		Control (without treatment)	Betulin	FO	1,2,4-T	FO+1,2,4-T +Betulin
AlDH, nmol NADH·min^{-1}·mg of protein^{-1}	Intact	40.06 ± 1.75	44.84 ± 0.44	52.14 ± 0.27	40.91 ± 0.66	42.65 ± 0.83
	Hypoxia	42.07 ± 2.19	59.06 ± 0.64	75.86 ± 0.28*	89.14 ± 0.82**	56.91 ± 2.98
	IS	48.26 ± 0.11	52.54 ± 0.31	46.37 ± 0.24	42.03 ± 0.43	41.64 ± 0.25
LDH_{dir}, nmol NADH·min^{-1}·mg of protein^{-1}	Intact	42.48 ± 1.14****	43.76 ± 0.12****	43.56 ± 0.28****	45.98 ± 1.17****	47.19 ± 0.21****
	Hypoxia	18.84 ± 0.32****	25.99 ± 0.58****	38.01 ± 0.29****	32.02 ± 0.37****	40.42 ± 0.40****
	IS	47.77 ± 0.63****	43.82 ± 0.16****	41.07 ± 0.63****	45.37 ± 0.40****	51.70 ± 0.18****
LDH_{rev}, nmol NADH·min^{-1}·mg of protein^{-1}	Intact	176.47 ± 3.65****	183.09 ± 2.51****	356.95 ± 5.19****	377.73 ± 3.92****	354.70 ± 4.90****
	Hypoxia	176.46 ± 2.03****	198.77 ± 2.52****	288.88 ± 1.31****	251.78 ± 0.41****	256.15 ± 0.54****
	IS	238.31 ± 4.02****	195.73 ± 2.69****	182.47 ± 4.02****	246.50 ± 1.89****	270.04 ± 2.95****

[a] FO – Fish oil; 1,2,4-T – 1,2,4-trioxolanes; IS – immobilization stress; AlDH – aldehyde dehydrogenase; LDH_{dir} – lactate dehydrogenase in direct reaction; LDHrev – lactate dehydrogenase in reverse reaction. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$ indicates statistical difference when compared to corresponded control group (one-way ANOVA).

alase, GR and G6PDH increased significantly in intact animals under the action of the composition FO+1,2,4-T+B by 29%, 42%, 198% and 26%, respectively. At the same time, under the action of the studied composition, the MDA concentration in erythrocytes, the level of DC, TC, SB increased significantly in intact rats by 36%, 12%, 16%, 43%, respectively.

The effect of the FO+1,2,4-T+B composition on the energy metabolism of erythrocytes led to an increase in LDH_{dir} and LDH_{rev} activity by 11% and 101%, respectively. Under the action of FO+1,2,4-T+B, the ratio of LDH_{rev}/LDH_{dir} in normal conditions increased from 4.15 (intact rats) to 7.52. Under hypoxia, the action of the composition resulted in a decrease in LDH_{rev}/LDH_{dir} from 9.37 (hypoxia without treatment) to 6.34. Under IS condition, the ratio changed slightly from 4.99 (IS without treatment) to 5.22.

The findings characterize the accumulation of lactic acid in the studied pathologies (hypoxia and IS) both in normal conditions and under the action of the pharmaceutical composition components.

Discussion

We showed 1,2,4-trioxolanes to be able to activate the enzymatic defense under oxidative stress in the models of hypoxia and immobilization stress in rats. We estimated the increase in the specific activity of NADP/H and NAD/H dependent enzymes (GR, G6PDH, LDH, AlDH), as well as SOD and catalase under the treatment by the compositions with an oxidizing agent (1,2,4-trioxolanes) and betulin. As a result, the balance and imbalance of oxidative and antioxidant factors were probably normalized under the action of 1,2,4-trioxolanes. The findings are generally consistent with the discussion in literature about pharmacological strategies for treating diseases with antioxidants and/or oxidants caused by oxidative stress (Sies *et al.*, 2017; Vassalle *et al.*, 2020). The clear conclusions were demonstrated on the example of oncological, neurodegenerative, cardiovascular diseases treatment.

The so-called «oxidative therapy», using the toxic properties of ROS, might be promising for the abovementioned diseases, despite the fact that excessive generation of ROS caused cellular damage. The action of 1,2,4-trioxolanes can be similar to ozone effects in clinical ozone therapy of the musculoskeletal system, where muscle oxygenation under the action of ozone was indicated to lead to patients` state improvement (Seyam *et al.*, 2018). Ozone has been shown to contribute to the maintenance and increase of endogenous antioxidant systems, that in turn increases the activity of nuclear factor kappa B, an important inflammation immunomodulator (Re *et al.*, 2008).

In addition, a positive effect on the balance and imbalance of oxidants and antioxidants in oxidative stress is likely to be exerted by the components of fish oil (ω-3-fatty acids EPA and DHA). During the oxidation of EPA and DHA in the human body, they are able to form endoperoxide cyclopentane fragments, from which, in turn, prostaglandins, prostacyclins, thromboxanes, isothromboxanes, leukotrienes, isoleukotrienes, lipoxins, hepoxylins (epoxy derivatives) and other biologically active substances can be formed. Oxidized metabolites, especially short-chain ones, are more rapidly absorbed and more effectively reduce the oxidative stress effect (Bazan, 2007; Mori *et al.*, 2000; Nakamoto *et al.*, 2010; Wojenski *et al.*, 1991).

Thus, the experiment showed the antioxidant and antihypoxic effect of pharmaceutical composition to be based on ozonized fish oil with a high content of EPA and DHA and betulin from birch bark extract. The composition FO+1,2,4-T+B had a normalizing effect on most indexes of oxidative and energy metabolism under hypoxia and immobilization stress.

Conclusions

In this work we obtained 1,2,4-trioxolanes by ozonating fish oil with a mixture of oxygen and ozone. The physicochemical properties of 1,2,4-trioxolanes correspond to the literature data on sesame oil 1,2,4-trioxolanes. A new pharmaceutical composition was developed, including 1,2,4-trioxolanes of fish oil 1–3%, betulin (0.1%) in fish oil.

1,2,4-trioxolanes of fish oil can be the elements in «oxidative» pharmacotherapy to treat

and prevent the diseases associated with oxidative stress (cancers, cardiovascular and neurological diseases, etc.) by correcting the balance and imbalance of endogenous and exogenous antioxidants and oxidants. The pharmaceutical composition of 1,2,4-trioxolanes in a fish oil medium with betulin can be useful for a large number of population groups experiencing hypoxic and hypodynamic stress in modern society: students, office workers, the elderly, the disabled and others. In addition, taken into account the favorable effect of oxygen-ozone therapy, the precursor of «oxidative» pharmacotherapy, on the musculoskeletal system, the state of muscles and muscle mass, the proposed composition can be used in sports medicine.

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