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Membrane-modifying properties of hydrated fullerene C_{60} in combination with *Spirulina platensis* cell culture

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The study is devoted to the development of new promising nanocomponent compositions with membrane-modulating properties. The effect of a solution of hydrated fullerene C_{60} and a cell suspension of blue-green algae *Spirulina platensis* on the activity of free radical lipid peroxidation and oxidative phosphorylation in mitochondria of rat liver was studied. Fullerene C_{60} and suspension of *Spirulina platensis* cells were administered to Wistar rats for 30 days, once a day, intragastrically, both jointly and singly. The rate of oxygen consumption of phosphorylating and non-phosphorylating mitochondria (in V_3 and V_4 states), the rate and efficiency of ADP phosphorylation (ADP/Δt and ADP/O), respiratory control coefficient (RC) were changed in rat liver mitochondria. In the same samples, the concentration of diene, triene, oxodiene, and tetraene fatty acids was measured. The results of our studies have shown that the solution of hydrated fullerene C_{60} serves as a structural modifier of the lipid bilayer of cell membranes, the effect of which can be corrected by using an antioxidant component such as a suspension of *Spirulina platensis* cells.

Keywords: hydrated fullerene C_{60}, *Spirulina platensis*, lipid peroxidation, mitochondria of liver cells, oxidative phosphorylation

INTRODUCTION

Recently the presence of reactive oxygen species (ROS) in the body is no longer associated exclusively with the development of various pathological processes. ROS has been shown to participate in a variety of physiological and metabolic events, such as intercellular transduction, gene expression and formation of biologically active compounds (Panieri, Santoro, 2015; Green et al., 2014; Kobayashi et al., 2014; Chelombitko et al., 2016; Bieberich, 2012).

In this regard, the study into the formation and metabolism of ROS in the body is an important theoretical scientific task, and the development of methods for the chemical regulation of the ROS generation is its practical component.

Fullerenes are a poorly studied allotropic form of carbon (Piotrovsky, 2006). The prospect of using C_{60} fullerene is of interest worldwide. Its molecule is a truncated icosahedron consisting of 20 hexagons and 12 pentagons. There is a cavity of about 0.5 nm in diameter inside of the icosahedron, (Popov et al., 2013). Fullerenes are highly reactive molecules, wherein the carbon atoms in the spherical carcass are in sp2 hybridization, thus

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achieving a high steric intensity of the molecule and a strong propensity to join the nucleophilic addition reactions (Hirsch, 1999).

To date, information on the antioxidant properties of fullerenes is quite contradictory (Yang et al., 2014). At the same time, a number of authors, using various living objects, proved the involvement of these substances in the inhibition of free radical processes (Andrievsky et al., 2005; Hu et al., 2007; Lin et al., 1999; Tsai et al., 1997). There is a variety of mechanisms of their antioxidant action. For example, their ability to act as traps for free radicals of superoxide anion and hydroxyl (Xiao et al., 2005) has been shown to cause an increase in catalase activity and glutathione-S-transferase in the body of *Daphnia rulex* crustaceans when administered *in vivo* (Klaper et al., 2009).

It is known that unlike fullerenes, the blue-green alga *Spirulina platensis* in the form of powders, tablets, capsules, and the like has antioxidant properties that enables it to be used for the correction of pro- and antioxidant balance in the body (Ovsyannikova et al., 1998; Mazokopakis et al., 2014; Wu et al., 2016). We believe that the use of modulators of free radical processes of fullerenes in combination with the cell culture of *Spirulina platensis*, having an undoubted antioxidant effect, is promising for the development of effective and safe drugs.

The bulk of the injected fullerene is probably metabolized in liver (Gharbi et al., 2005), however, a terminal degradation of xenobiotics (including the *Spirulina platensis* chemical components) occurs in liver. It is also known that mitochondria are among the main producers of reactive oxygen species in cells (Chelombitko et al., 2016; Czarna, Jarmuszkiewicz, 2006). In connection with the foregoing, the aim of our work was to study the effect of the solution of hydrated fullerene C_{60} (C60FWS) and cell suspension of *Spirulina platensis*, separately and in combination *in vivo*, on the activity of free radical lipid peroxidation (LPO) and oxidative phosphorylation processes in mitochondria of rat liver.

MATERIALS AND METHODS

The experiments were carried out in sexually mature Wistar white rats. The animals were divided into four groups. Control group (Group 1) was represented by intact animals. The experimental groups included: Group 2, which consisted of the rats that were intragastrically administered with 500 mg/kg suspension of *Spirulina platensis* cells; Group 3 included the animals which were intragastrically administered with 1.0 ml of a solution of hydrated fullerene C_{60} at a concentration of 10^{-9} M; Group 4 was made up of the rats co-administered with a suspension of *Spirulina platensis* cells and a C_{60} fullerene solution in the above doses. All the manipulations with animals were performed prior to feeding in the morning, daily for 30 days. The animals were kept at the standard Animal House conditions under natural illumination and nutritional regimen recommended for these animals. The studies were conducted according to the national "General Ethical Principles of Experiments on Animals" (Reznikov, 2003), which are consistent with the provisions of the "European Convention for the Protection of Vertebrates Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986). At the end of the experiment, the animals were removed from the experiment by decapitation and the liver was isolated. Mitochondria were isolated from the hepatocytes, in which the indices of their functional activity and concentration of LPO primary products were determined.

Mitochondria from the animals' liver were isolated by the standard method of differential centrifugation (Severin, Solovyova, 1989). The isolation medium contained 250 mM sucrose, 1 mM EGTA, 5 mM Tris-HCl (pH 7.4). To remove endogenous fatty acids, mitochondria were pre-incubated with fatty acid-free BSA. A mitochondrial suspension (60–70 mg of mitochondrial protein in 1 ml of isolation medium) was stored on ice. The protein was determined by the Bradford method, BSA was used as the standard (Severin, Solovyova, 1989).

Recording the respiration of a mitochondrial suspension. Mitochondrial respiration was recorded at a temperature of 25°C by means of Clark electrode and a polarograph (Severin, Solovyova, 1989). The concentration of the mitochondrial protein in an oxygen cell was 0.7–1.3 mg/ml. The incubation medium contained 200 mM sucrose, 5 mM KH-2PO, 20 mM KCl, 5 mM potassium succinate, 2 mM $MgCl_2$, 0.5 mM EGTA, 10 mM Tris-HCl (pH 7.4). Immediately after the mitochondrial 2 μM rotenone was added to the oxygen cell. In determining the respiration rate during the oxidative synthesis of ATP (V_3 state), in addition, 200 μM ADP were added to the mitochondria additionally 2–3 min after the rotenone.

Tris, potassium succinate, fatty acid-free BSA ("Sigma", USA), rotenone, EGTA ("Serva", Germany), sucrose ("Fluka" Germany), KCl, $MgCl_2$ ("Merck", Germany) were used. Other reagents of "chemically pure" and "extra pure" grades were produced in the CIS.

The concentrations of LPO products were spectrophotometrically measured determining their absorption spectra at different wavelengths in the ultraviolet region of the spectrum, after extraction of LPO products with the heptane-isopropanol mixture. Accordingly, the following were measured: diene conjugates (DC) at 233 nm, molar extinction coefficient $\varepsilon_0 = 2.2 \times 105\ cm^{-1} \cdot M^{-1}$; triene (TC) at 268 nm, molar extinction coefficient $\varepsilon_0 = 4.34 \times 10\ cm^{-1} \cdot M^{-1}$ oxodiene (ODC) at 276 nm, molar extinction coefficient $\varepsilon_0 = 2.7 \times 10^{-5}\ cm^{-1} \cdot M^{-1}$ tetraene (TET) – at 287 nm, the molar extinction coefficient for TET was not determined. The calculation was carried out using the molar extinction coefficients. The data were expressed in nmol per 1 mg of protein for DC, TC, UDC, and in extinction units per 1 mg of protein for TET (Shvedova, 1992).

RESULTS AND DISCUSSION

In the first part of the experiments, bioenergetic parameters of rat liver mitochondria were studied. The oxygen absorption rate in the third phosphorylation state (V_3) and the fourth non-phosphorylation (V_4) was calculated using polarograms. The respiration rate was expressed as nanogram atoms per minute per milligram protein. The ratio of the rate of phosphorylating respiration to the rate of non-phosphorylating respiration characterizes the coefficient of respiratory control (RC) according to Chance-Williams. Its value is indicative of the degree of conjugation of oxidation and phosphorylation, as well as the degree of intactness of mitochondrial preparations. The ADP/O coefficient characterizing the efficiency of the phosphorylation system was calculated as the ratio of the molar amounts of added ADP and atomic oxygen absorbed by the mitochondrial suspension during the phosphorylation of the added amount of ADP. The rate of phosphorylation of ADP/Δt was expressed in nmol of ADP per 1 min per 1 mg protein. The results are shown in Figs. 1 and 2. In the second part of the experiment, the activity of free radical LPO processes in rat liver mitochondria was studied, which was evaluated by the formation of LPO primary products, i.e., LPO-diene, triene, oxodienic, and tetraene fatty acid conjugates. The data are presented in Fig. 3.

The data in Fig. 1 demonstrate that the rate of oxygen uptake by mitochondria in state V_4 (respiration of non-phosphorylation mitochondria) did not have significant differences between the groups, although there is a tendency to an increase of this index in Group 3. But already with phosphorylation (V_3), the mitochondrial respiration of rats receiving Spirulina and fullerenes solely was more intense compared to the control and the group with the mixture.

This was accompanied by a faster conversion of ADP to ATP if compared to the control (ADP/Δt). At the same time, the ADP/O phosphorylation efficiency decreased in the groups of the animals that received the C_{60} fullerene solution and suspension of *Spirulina platensis* cells if compared to the control and group of the rats treated with the mixture of C_{60} fullerene solution and suspension of *Spirulina platensis* cells, as shown in Fig. 2. That is, the rate of respiration during phosphorylation in these groups increased, but more oxygen was also consumed to form ATP.

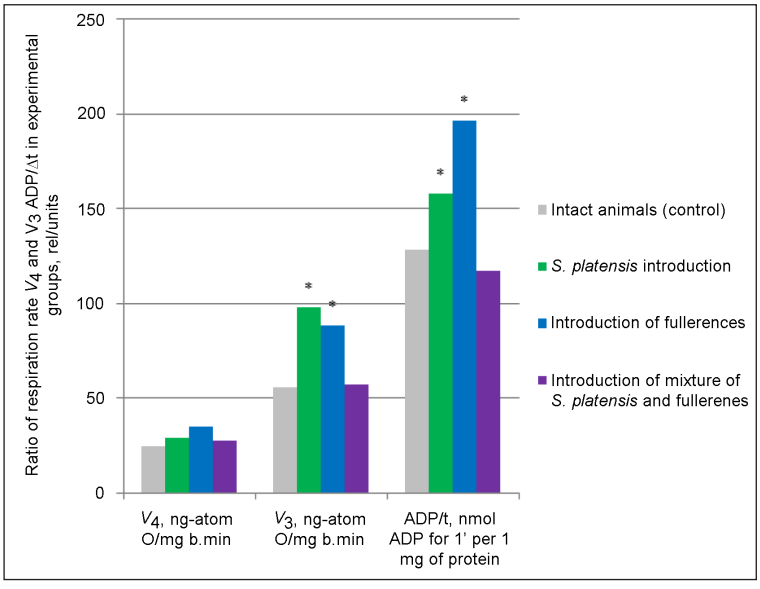

Fig. 1. Respiration rate in states of non-phosphorylation (V_4) and phosphorylation (V_3) rat liver mitochondria and ADP/Δt phosphorylation rate (* – significance versus groups 1 and 4, $p < 0.05$)

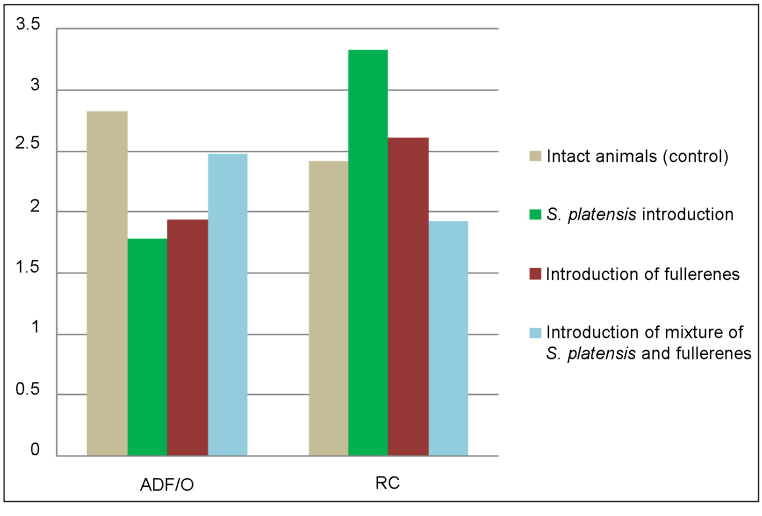

Fig. 2. Phosphorylation efficiency (ADP/O) and respiration control coefficient in rat liver mitochondria and ADP/Δt phosphorylation rate (* – significance versus groups 1 and 4, $p < 0.05$; ** – significance versus groups 1–3, $p < 0.05$)

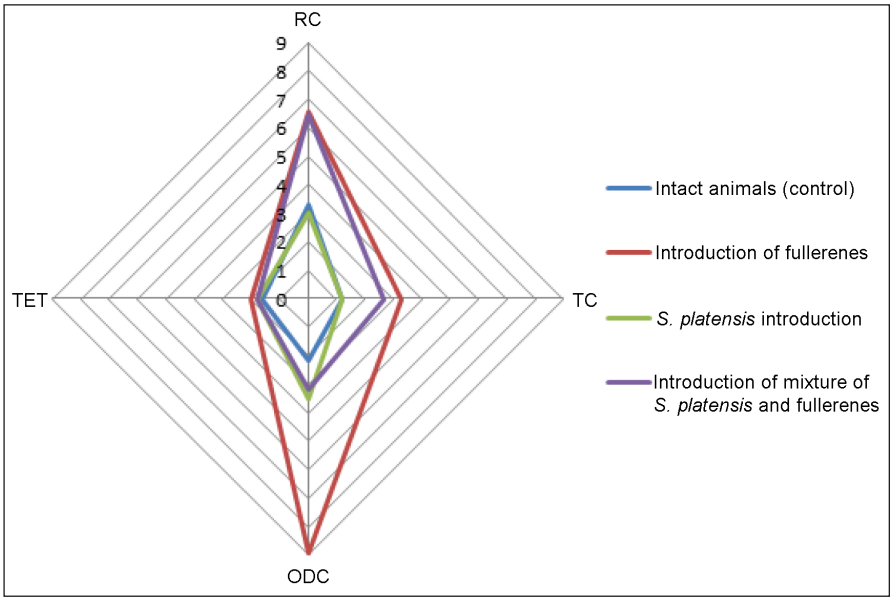

Fig. 3. Concentration of LPO products in rat liver mitochondria and ADP/Δt phosphorylation rate (scales of RC, TC, ODC – nmol per 1 g protein, and TE – 20 × one extinction unit per 1 mg protein)

In the group of the rats that received both the C_{60} fullerene solution and a suspension of *Spirulina platensis* cells, the rate of ATP formation did not increase statistically significantly when compared to the control and the groups that received the substances separately. It is logical to assume that the fullerene and the substances containing *Spirulina platensis* successfully compete for protons and oxygen with the process of oxidative phosphorylation. In this case, fullerene C_{60} interacts with protons, and *Spirulina platensis* does with oxygen. This hypothesis is supported by the fact that fullerene is characterized by quite a good solubility in fatty acid esters (Cataldo, 2008). The result of the extraction of oxygen and protons from the process of oxidative phosphorylation is the change in the DC value, which is lower in the group of the rats receiving the mixture of substances as compared to other three groups (Fig. 2).

The specifics of the electron-deficient fullerene nucleus, which manifests itself as an oxidizer in the aqueous medium due to the transition to a superoxide radical, has been reported. In the excited state, fullerene C_{60} is able to generate singlet oxygen and can interact with electron donors (Panteleev, 2012). Mitochondria are the main source of free radicals in the cell. It is believed that approximately 2–5% of electrons passing through the electron transport chain participate in the one-electron reduction of oxygen to superoxide (Inoue et al., 1999). In this connection, it was of interest to find out how the situation with LPO is changing, in particular, with the accumulation of its primary and later products, in mitochondria of the liver. Data on the concentration of LPO products in mitochondria are shown in Fig. 3.

In mitochondria of the liver of rats treated with fullerenes, the level of both the early products of LPO-DC as compared with the control ($p_{1,3} < 0.05$) and later products, i.e., TC ($p_{1,3} < 0.05$) increases, which allows thinking of the ability of fullerenes to loosen the membrane lipid bilayer. Moreover, this indirectly corresponds to the reports on the ability of fullerenes to be accumulated and metabolized in the liver (Yamago et al., 1995) and to be dissolved in fatty acids (Cataldo, 2008). Secondary products, ketodienes (ODCs), increased compared with the control ($p_{1,3} < 0.02$). All this indicates an intensive development of peroxide processes in mitochondria of animal liver, fed with fullerenes. Thus, fullerenes in mitochondria exhibit pro-oxidant properties.

In the group of rats receiving a suspension of *Spirulina platensis* cells, the changes in the level of LPO products in mitochondria were observed only in the form of a trend for ODC ($p_{1,2} < 0.1$) in comparison with the control. Spirulina, being used in a cocktail with fullerenes, suppressed the pro-oxidant effect of the latter. The level of DC and TC in Group 4 increased in comparison with the control ($p_{1,4} < 0.05$, in both cases) to the same extent as in the use of fullerenes solely, but the ODC decreased more than 2 times if compared with Group 3 ($p_{3,4} < 0.02$), i.e., approached the control level ($p_{1,4} < 0.1$).

CONCLUSIONS

The results of our studies show that fullerene C_{60} acts as a structural modifier of the lipid bilayer of membranes, and some chemical components of *Spirulina platensis* reveal an antioxidant effect. When self-administered, Spirulina cell culture does not contribute to the antioxidant status of mitochondria, being a common nutritional factor. In this connection, the use of the *Spirulina platensis* (500 mg/kg) and C_{60} fullerene during the subchronic period, both jointly and individually, may be useful for initiating membrane-modifying effects, both anti- and pro-oxidant.

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HIDRATUOTO FULERENO C_{60} SAVYBĖS, MODIFIKUOJANČIOS MEMBRANĄ KARTU SU *SPIRULINA PLATENSIS* LĄSTELIŲ KULTŪRA

Santrauka

Tyrimas skirtas naujų, perspektyvių nanokomponentų kompozicijų, turinčių membraną, koreguojančioms savybėms sukurti. Tirtas hidratuoto fulereno C_{60} ir mėlynai žalių dumblių *Spirulina platensis* ląstelių suspensijos poveikis laisvųjų radikalų lipidų peroksidacijai ir oksidaciniam fosforilinimui žiurkių kepenų mitochondrijose. Fulerenas C_{60} ir *Spirulina platensis* ląstelių suspensija 'Wistar' žiurkėms buvo duodama 30 dienų kartą per dieną ir kartu, ir atskirai. Buvo stebimas deguonies suvartojimo pokytis fosforilinančiose ir nefosforilinančiose žiurkių kepenų mitochondrijose (V_3 ir V_4 stadijose), stebėtas ADP fosforilinimo (ADP / Δt ir ADP / O) greičio ir efektyvumo bei kvėpavimo kontrolės koeficiento (RC) pokytis. Tuose pačiuose mėginiuose matuota dieno, trieno, oksodieno ir tetraeno riebalų rūgščių koncentracijos. Tyrimo rezultatai rodo, kad hidratuotas fulerenas C_{60} veikia kaip struktūrinis ląstelių membranų lipidų dvigubo sluoksnio modifikatorius. Jo poveikį galima modifikuoti naudojant antioksidantų komponentą, pvz., *Spirulina platensis* ląstelių kultūrą.

Raktažodžiai: hidratuotas fulerenas C_{60}, *Spirulina platensis*, lipidų peroksidacija, kepenų ląstelių mitochondrijos, oksidacinis fosforilinimas