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FREE RADICAL OXIDATION IN LIVER MITOCHONDRIA OF TUMOR-BEARING RATS AND ITS CORRECTION BY ESSENTIAL LIPOPHILIC NUTRIENTS

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The role of free radical oxidation in the increase of mitochondrial membranes permeability in organs which are not involved in oncogenesis and the development of the methods for preventing mitochondria dysfunction remain topical problems. In this work, the interconnection of lipid peroxidation (LPO) in liver mitochondrial fraction with the processes of mitochondrial swelling and cytochrome c release to the cytosol under separate and combined administration of ω-3 polyunsaturated fatty acids (PUFAs) and retinol acetate (vitamin A acetate) to rats with transplanted Guerin's carcinoma was studied. During the intensive tumor growth (14 days) the increase of superoxide radical generation and the content of primary (triene conjugates, TC), secondary (ketodienes and coupled trienes, CD+CT) and terminal (Schiff bases) lipid peroxidation products in the mitochondrial fraction of tumor-bearing rats was detected, which contributed to the mitochondrial swelling and cytochrome c release to the cytosol. Separate administration of ω-3 PUFAs to tumor-bearing rats decreased both free radical processes in mitochondrial fraction and mitochondrial swelling. Separate administration of retinol acetate in a high dose (3000 IU/kg of body weight) intensified free radical processes in the mitochondrial fraction of tumor-bearing rats, while administration of retinol acetate in a physiological dose (30 IU/kg of body weight) did not lead to changes compared to tumor-bearing rats that did not receive the drug. The prooxidant effects of retinoid were partially eliminated in the case of combined administration with ω-3 PUFA.

K e y w o r d s: liver mitochondrial fraction, lipid peroxidation, cytochrome c, mitochondrial swelling, ω-3 polyunsaturated fatty acids, retinol acetate, Guerin's carcinoma.

Mitochondria are intracellular organelles that control vital physiological processes in the body. Being the main consumers of oxygen (they use about 98% of the total amount of oxygen coming from outside), the mitochondria effectively generate the energy necessary for the functioning of the cells [1] and the maintenance of ionic gradients in the plasma membrane [2]. More than 90% of the oxygen consumed by mammals is reduced by mitochondrial cytochrome oxidase to water and only 1-2% is converted into partially reduced products, which are called reactive oxygen species (ROS). It should be noted that the mitochondria of different tissues differ greatly in the relative and absolute activities of enzymes involved in the metabolism of ROS, as well as in the local oxygen concentration (liver, kidney, vascular endothelium, lungs) [3, 4].

Dysfunction of mitochondria not only leads to impaired cellular respiration and ROS overproduction, but also initiates free radical oxidation of macromolecules in the cell, which underlies many pathological processes, including cancerogenesis. The cytotoxic effect of ROS is to initiate free radical oxidation (FRO) of mitochondrial membrane lipids, which leads to an increase in their permeability [5]. It is known [6] that the induction of the mitochondrial pore (MPTP – mitochondrial permeability transition pore) plays a key role in cellular disorders under conditions of oxidative stress and is

one of the links in the pathogenesis of oncological diseases. Therefore, in recent years, the study of the role of FRO in increasing the permeability of mitochondrial membranes during oncogenesis in organs that are not involved in the tumor process is becoming increasingly relevant, since mitochondria play an important role in the initiation of programmed cell death [7].

The basis of the lipid matrix of intracellular membranes are polyunsaturated fatty acids (ω-3 PUFAs) that make up phospholipids and determine the structural and functional properties of membranes. Phospholipids of mitochondrial membranes are an ideal target for the action of ROS. However, the stability of the membranes largely depends on the type of PUFAs that make up their composition. It is known that ω-3 PUFAs exhibit an antioxidant and membrane-stabilizing effect, while ω-6 PUFAs have opposite properties [8]. In addition, the effect of exogenous PUFAs on intracellular processes can manifest itself through PPAR (peroxisome proliferator-activated receptor) nuclear receptors. All PPARs form a heterodimer with a liver X receptor, which in the next step forms a heterodimer with a retinoid X receptor (RXR) [8]. In ligand activation and heterodimerization with RXR, PPAR interact with PPRE (peroxisome-proliferator response element) in the promoter of the target gene. Accession of PPAR can both decrease and increase the intensity of transcription of the corresponding genes. The identification of PPRE in the promoter regions of the catalase and superoxide dismutase genes indicates the involvement of these nuclear receptors in reducing the production of ROS and lipid peroxidation (LPO) products [9].

The function of PPARs is regulated by the exact form of the ligand-binding domain, which is due to the attachment of the ligand and coactivator proteins or corepressor proteins. The endogenous PPARs ligands include free fatty acids and eicosanoids, which are synthesized from PUFAs [10, 11]. As follows from the above, the effects of ω-3 PUFA and retinoids can be interrelated through their effects on PPAR and RXR.

Separate and combined administration of ω-3 PUFAs and various doses of retinoids may have different effects on free radical processes in the mitochondria of liver cells under conditions of growth in the body of a malignant neoplasm.

The aim of the work was to elucidate the role of the separate and combined use of ω-3 PUFA and retinoids in regulating free radical processes in the mitochondria of the liver of rats with transplanted Guerin's carcinoma.

Materials and Methods

The studies were carried out on white outbred female rats weighing 130-150 g. The animals were kept on a standard balanced diet of vivarium.

As a model of malignant neoplasm, Guerin's carcinoma was used. Transplantation of carcinoma was carried out according to the method, by subcutaneous injection of 0.5 ml of a 30% suspension of cancer cells in physiological saline into the upper area of the thigh of the right rat limb [12]. Experiments on animals were carried out in accordance with the International requirements for the humane treatment of animals and compliance with the requirements of Directive 86/609 / EEC on the protection of animals.

The animals were divided into the following groups (12 animals in each group): I – control (intact animals); II – rats with transplanted Guerin's carcinoma; III – tumor-bearing rats, administered daily ω-3 PUFA (120 mg/kg body weight, *per os*) in the form of a commercial drug Vitrum Cardio (manufactured by Unipharm, Inc., USA); IV – tumor-bearing rats, which were daily administered retinol acetate *per os* as an oily suspension in a dose of 30 IU/kg body weight (1 ml of 34.4 mg retinol acetate (100000 IU of vitamin A) (manufactured by Vitamins, Ukraine); V – tumor-bearing rats, daily administered retinol acetate *per os* in a high dose (3000 IU/kg body weight); VI – tumor-bearing rats, to which ω-3 PUFA was administered in combination with retinol acetate (30 IU/kg body weight); VII – tumor-bearing rats, in which ω-3 PUFA was administered in combination with retinol acetate (3000 IU/kg body weight).

ω-3 PUFAs, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and retinol acetate were pre-obtained for 28 days before the transplantation of Guerin's carcinoma and after transplantation during the entire period of tumor growth in the body (14 days).

Animals were decapitated under light ether anesthesia on the fourteenth day of growth in Guerin's carcinoma, which corresponds to the logarithmic phase of oncogenesis. The intensity of tumor growth was estimated on the basis of measuring its parameters calculated by the formula: $V = \pi/6 \cdot (h \cdot l \cdot w)$, where h is the height of the tumor, l is the length of the tumor, w is the width of the tumor.

The mitochondrial fraction of the liver was isolated by the method of differential centrifugation in a cooling medium of the following composition: 250 mM sucrose, 10 mM Tris-HCl and 2 mM EGTA (pH 7.4) [13]. The purity of the mitochondrial fraction was controlled by comparative determination of succinate dehydrogenase activity as a specific marker of the mitochondrial inner membrane [14], Na^+,K^+-ATPase activity [15], as a specific marker of the plasma membrane and glucose-6-phosphatase activity [16], as a specific marker of the endoplasmic reticulum membrane.

The rate of superoxide radical ($O_2^{\bullet-}$) generation was recorded in a test with nitro-blue tetrazolium [17] and expressed in nmol/min per mg of protein. The protein content in the samples was determined by the method of Lowry [18]. The intensity of the mitochondrial phospholipid peroxidation processes was judged by the content of primary, secondary and terminal products in isopropanol extracts. The level of the primary molecular products of lipid peroxidation (triene conjugates, TC) was recorded in the UV range at a wavelength of 268 nm. The value of optical density at a wavelength of 278 nm reflected the content of secondary lipid peroxidation products (ketodienes and conjugated trienes, KD + CT) and at a wavelength of 400 nm the terminal products of LPO (Schiff bases) [19, 20].

The swelling of mitochondria, as an indicator of MPTP in the presence or absence of Ca^{2+}, was assessed by monitoring the reduction in light scattering at a wavelength of 525 nm [21]. For this, mitochondria were placed in an incubation medium containing 125 mM KCl, 20 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 2 mM KH_2PO_4, 2 mM malate, 5 mM glutamate and 1 μM EGTA. A decrease in the optical density of the mitochondrial suspension was recorded for 60 min of their swelling in the presence of an inducer of the mitochondrial pore, calcium. Mitochondrial swelling was induced by the addition of $CaCl_2$ at a final concentration of 10^{-4} M. The content of cytochrome *c* in the mitochondrial and cytosolic fractions was determined by the method [22]. The results were processed by the method of variation statistics using Student's *t*-criterion.

Results and Discussion

The results of the studies showed that under conditions of intensive growth in the body of Guerin's carcinoma the rate of formation of the superoxide radical increases in the liver mitochondrial fraction, which is 3.3 times higher than the control value (Fig. 1).

The generation of superoxide radical can occur mainly due to the I, II and III complexes of the mitochondrial respiratory chain, which are considered the main generators of ROS. In the process of oxidative phosphorylation, odd electrons are formed, which interact with O_2, as a result of which $O_2^{\bullet-}$ is formed, which is later converted to other ROS, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) [23].

Increased $O_2^{\bullet-}$ generation leads to the initiation of lipid peroxidation processes, as evidenced by elevated levels of primary (Fig. 2, *A*), secondary (Fig. 2, *B*) and final (Fig. 2, *C*) lipid peroxidation products in the mitochondrial fraction of the liver of tumor-

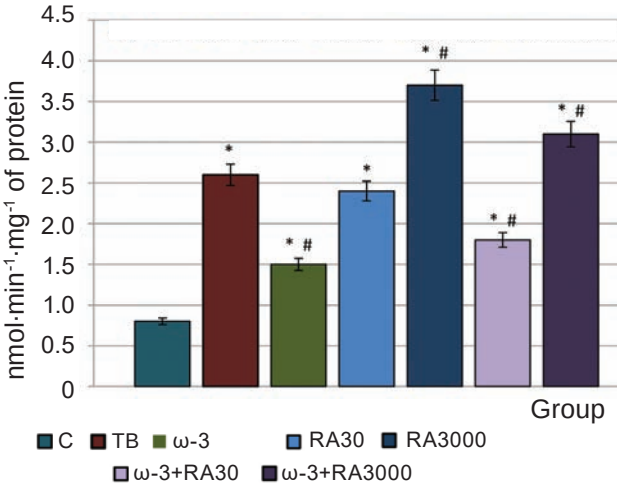

*Fig. 1. The rate of superoxide radical formation in the liver mitochondria of tumor-bearing rats under the influence of ω-3 polyunsaturated fatty acids and retinol acetate. C – intact animals; TB – tumor-bearing rats in the period of intensive growth of the Guerin's carcinoma; ω-3 – tumor-bearing rats that were administered with ω-3 PUFAs; RA30 – tumor-bearing rats that were administered with retinol acetate in a dose of 30 IU/kg body weight; RA3000 – tumor-bearing rats that were administered with retinol acetate in a dose of 3000 IU/kg body weight; ω-3+RA30 – tumor-bearing rats that were administered with ω-3 PUFAs and retinol acetate in a dose of 30 IU/kg body weight; ω-3+RA3000 – tumor-bearing rats that were administered with ω-3 PUFAs and retinol acetate in a dose of 3000 IU/kg body weight; *statistically significant difference vs. control (P ≤ 0.05); #statistically significant difference vs. tumor-bearing rats (P ≤ 0.05)*

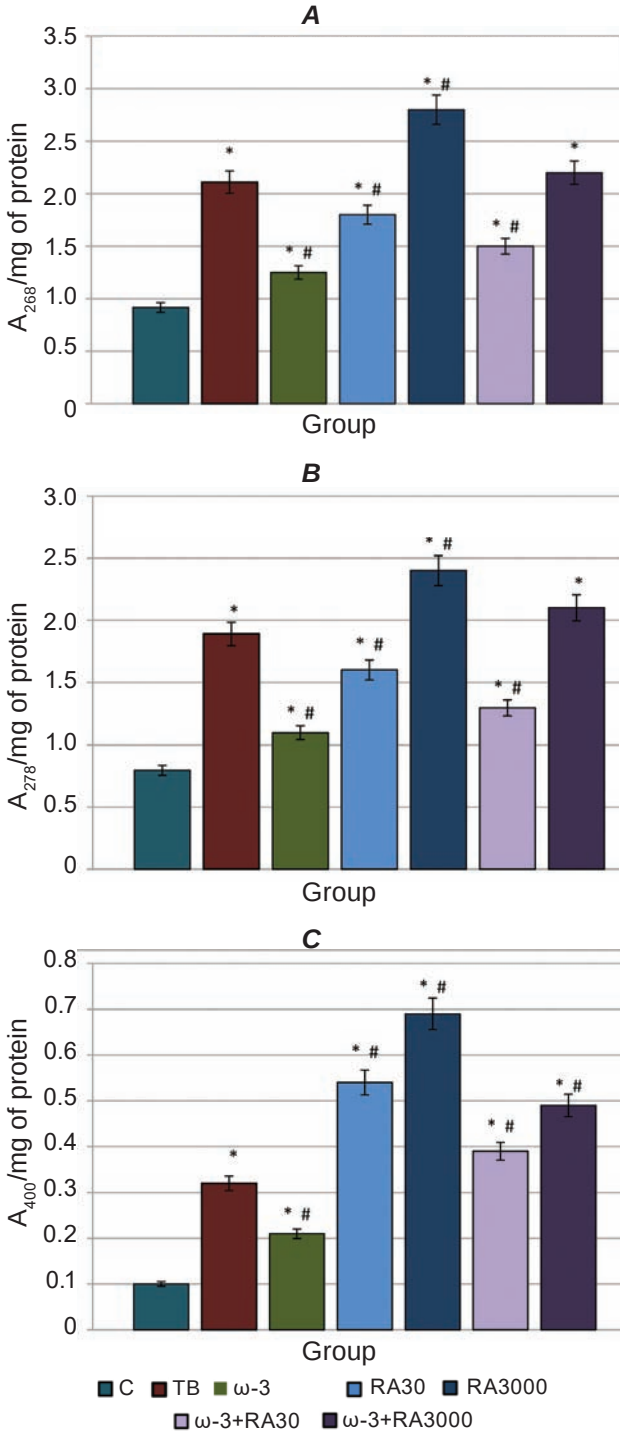

*Fig. 2. The content of primary (**A**), secondary (**B**) and terminal (**C**) lipid peroxidation products in the mitochondria of the liver cells of tumor-bearing rats under the influence of ω-3 polyunsaturated fatty acids and retinol acetate*

bearing rats compared to control. Increased FRO of the phospholipid mitochondrial membranes may be one of the causes of changes in the permeability of the inner membrane of the liver mitochondria, which leads to swelling of the mitochondria (Fig. 3) and, as a consequence, depolarization of the mitochondrial membranes [1, 24]. One of the consequences of this is the hydrolysis of membrane phospholipids by endogenous phospholipase A_2, whose activity is increased due to an increase in the concentration of Ca^{2+} ions. An increase in the permeability of the inner membrane for cations and anions leads to the entry of water into the matrix as a result of the osmotic pressure of proteins. Another reason for mitochondrial swelling may be the opening of the mitochondrial pore, as a consequence of the action of ROS on the protein structure of the pore [25].

Disorders in the functioning of the liver mitochondria that occur during oncogenesis can be prevented by introducing into the body ω-3 PUFAs, which are components of membrane phospholipids. Analysis of the results of our studies showed that the administration of ω-3 PUFA to animals before and after tumor grafting results in a 1.7-fold reduction in the rate of $O_2^{\bullet-}$ formation in the mitochondrial fraction of the liver of Guerin's transplanted rats compared with tumor-bearers that did not receive ω-3 PUFA (Fig. 1).

At the same time, due to the action of ω-3 PUFA, the intensity of LPO is reduced, which is confirmed by a decrease in the primary (Fig. 2, *A*), secondary (Fig. 2, *B*) and final (Fig. 2, *C*) products of LPO in comparison with tumor-bearing rats. Probably, ω-3 PUFAs are incorporated into the phospholipids of mitochondrial membranes and replace ω-6 PUFAs in their composition, which explains the membrane-protective effect of ω-3 PUFAs. On the other hand, ω-3 PUFAs can activate the antioxidant cell protection link [26] in the liver of tumor-bearers.

Other lipophilic nutrients that may have a corrective effect on mitochondrial membranes are retinoids. The ambiguity of reports on the use of retinoids in oncogenesis can be associated with both the different reaction of the transformed and normal tissues of the body of the tumor-bearer to these essential nutrients, and with the doses that the body receives. In addition, through a complex mechanism of realization of their biological functions by retinoids (interaction with more than 500 genes is possible), there is no unambiguous answer about the molecular aspects of their influence on free radical processes under oncogenesis [12].

The results of the studies showed that administration of retinol acetate (vitamin A acetate) in a

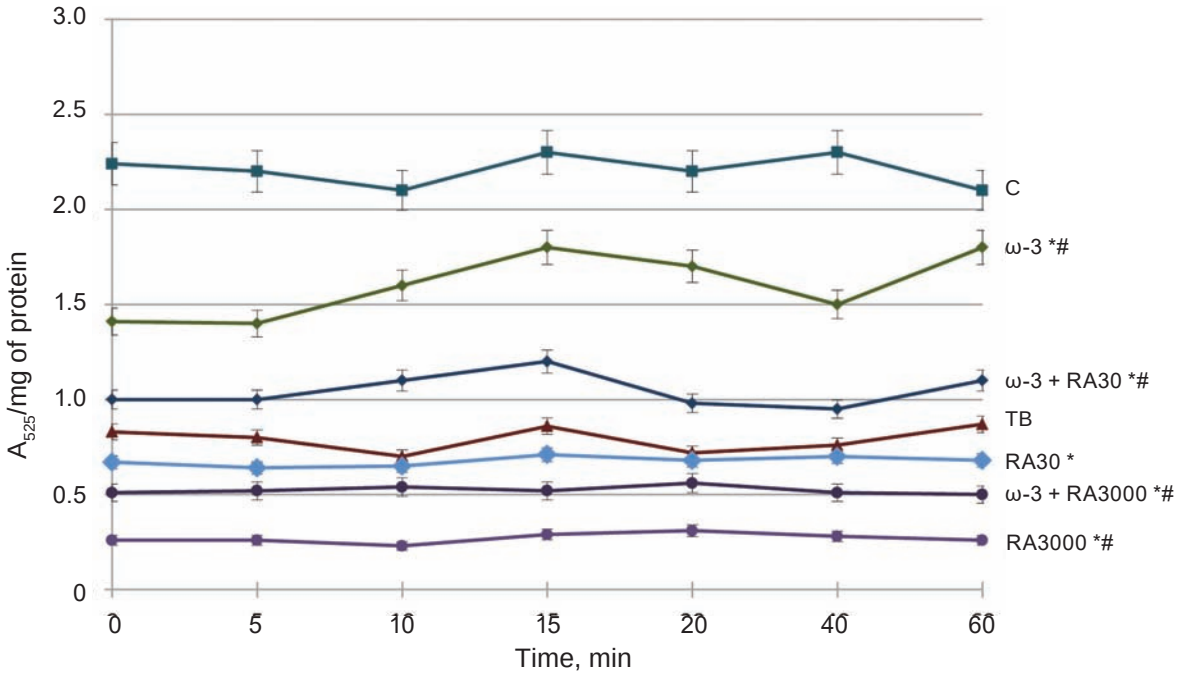

Fig. 3. The dynamics of mitochondrial swelling of the liver cells of tumor-bearing rats under the influence of ω-3 polyunsaturated fatty acids and retinol acetate

low dose (30 IU/kg body weight) before and after tumor transplantation does not lead to changes in the rate of $O_2^{\bullet-}$ formation in the mitochondrial fraction of the liver of tumor-bearing rats compared to control tumor bearers that did not receive the drugs under study (Fig. 1). As for the content of the LPO products, as a result of research, a slight decrease in the primary (Fig. 2, *A*) and secondary (Fig. 2, *B*) LPO products in the mitochondrial fraction of rat liver was found compared with the control tumor-bearers. The decrease in these LPO products is likely due to the formation of covalent bonds between the amino groups of mitochondrial membrane proteins and secondary LPO products with the formation of Schiff bases, the content of which increases (Fig. 2, *C*). Schiff bases have high reactivity to create intermolecular "crosslinks", as well as participation in polymerization and polycondensation reactions [27].

The enhancement of free radical processes in the mitochondrial fraction of the liver of tumor-bearing rats is observed under conditions of administration of a high dose of retinol acetate (3000 IU/kg body weight). So, in this group of animals, the rate of $O_2^{\bullet-}$ formation increases by 1.4 times (Fig. 1) and the processes of LPO are intensified (Fig. 2) compared with those in control tumor-bearers. Since liver is an organ capable of accumulating retinoids, the accumulation of high amounts of retinol acetate in liver cells can contribute to its conversion into toxic hydroxylated metabolites by the cytochrome P450 system [12], which later have a prooxidant effect on mitochondrial membranes. An increase in the permeability of mitochondrial membranes leads to swelling of the liver mitochondria, most pronounced in the group of tumor-bearing rats that received high doses of retinoids (Fig. 3). Since the inner membrane of mitochondria is larger in area than the outer membrane, the latter is broken, as a result of which cytochrome *c* is released into the cytosol. Thus, with a high dose of retinol acetate, the level of cytochrome *c* in the mitochondrial fraction decreases by 2 times (Fig. 4, *A*), while at the same time, its level increases by 1.8 times in the cytosol (Fig. 4, *B*) compared to the control rat tumor carriers.

So, excess retinol acetate accumulates in the liver of tumor-bearing rats on the background of enhanced ROS generation and free radical processes in the mitochondrial fraction, which suppress the energy supply of the cells of this organ. At the same time, the release of cytochrome c into the cytosol of liver cells can trigger the mitochondrial pathway of apoptosis, which will negatively affect the state of the tumor-bearing organism.

Since ω-3 PUFAs and retinol acetate have the opposite effect on free radical processes in the mitochondrial fraction of the liver of rats with transplan-

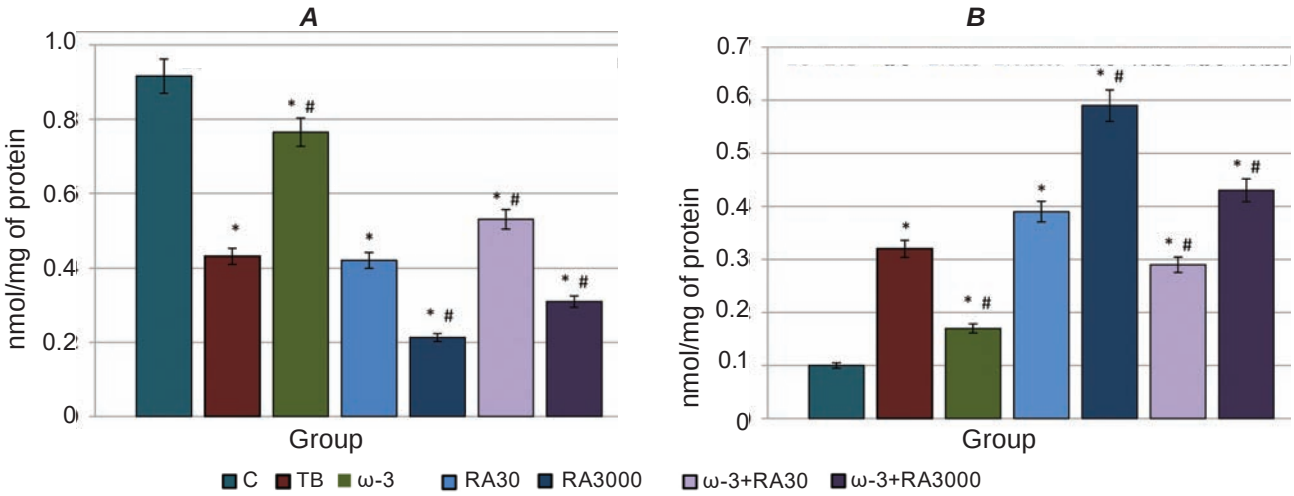

*Fig. 4. Cytochrome c content in the mitochondria (**A**) and cytosol (**B**) of the liver cells of tumor-bearing rats under the influence of ω-3 polyunsaturated fatty acids and retinol acetate*

ted Guerin's carcinoma during the period of intensive tumor growth, it would be advisable to check their combined effect on the processes under study.

The results obtained on the assession of the influence of the studied lipophilic nutrients on free radical processes in the mitochondrial fraction showed that the combined administration of ω-3 PUFAs and retinol acetate significantly reduced the free radical processes in the mitochondrial fraction of the liver of tumor-bearing rats (Fig. 1, Fig. 2). in comparison with the administration of retinol acetate as administered 30 IU/kg of body weight, and in a dose of 3000 IU/kg of body weight. The mechanism of the membrane-protective action of ω-3 PUFAs may consist in the ability of PUFAs to be incorporated into membrane phospholipids and to replace ω-6 PUFAs in their composition. The location and number of double bonds in the ω-3 PUFA structure (EPA – $C_{20:5}$, DHA – $C_{22:6}$) limits the rotation around the C = C bonds and modifies the membrane fluidity, which is probably the basis of the protective properties of ω-3 PUFAs from the action of free radicals [27].

At the same time, the combined administration of ω-3 PUFAs and various doses of retinol acetate is accompanied by a decrease in mitochondrial swelling (Fig. 3) and release of cytochrome c from the mitochondria into the cytosol (Fig. 4) compared with tumor-bearers, receiving only retinol acetate, which indicates stabilization of mitochondrial membranes. Since ω-3 PUFAs and retinol acetate in the cell are metabolized by the cytochrome P450 system, the combined administration of these drugs can lead to their competitive binding to the cytochrome P450 in the liver. As a result, ω-3 PUFAs can inhibit the hydroxylation of retinol acetate to toxic metabolites [12]. On the other hand, the combined administration of ω-3 PUFAs and retinol acetate facilitates the formation of nuclear receptor heterodimers (PPARγ/RXR i RAR/RXR), which modulate the expression of the corresponding genes [10].

So, in the period of intensive growth of Guerin's carcinoma in the mitochondrial fraction of the liver of tumor-bearing animals, the processes of free radical oxidation of lipids are enhanced, which leads to the swelling of mitochondria and the release of cytochrome *c* into the cytosol. Providing the body with physiological doses of retinol acetate leads to changes in the studied parameters, whereas the administration of high doses of the studied retinoids enhances free radical destruction of mitochondria compared with tumor-bearers who did not receive the specified nutrients. Correction of the established changes in the mitochondrial fraction of tumor-bearing rats occurs both under the conditions of the monoadministration of ω-3 PUFAs, and under the conditions of their combined use with retinol acetate.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

ВІЛЬНОРАДИКАЛЬНІ ПРОЦЕСИ В МІТОХОНДРІЯХ ПЕЧІНКИ ЩУРІВ-ПУХЛИНОНОСІЇВ ТА ЇХ КОРЕКЦІЯ ЕСЕНЦІАЛЬНИМИ ЛІПОФІЛЬНИМИ НУТРІЄНТАМИ

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Вивчення ролі вільнорадикального окислення у підвищенні проникності мітохондріальних мембран за онкогенезу в органах, які не задіяні в пухлинному процесі, та пошук шляхів запобігання порушенням у функціонуванні мітохондрій є актуальними. У роботі оцінено показники пероксидного окислення ліпідів (ПОЛ), процес набухання мітохондрій та вихід цитохрому *с* в цитозоль клітин печінки щурів-пухлиноносіїв за роздільної та поєднаної дії омега-3 поліненасичених жирних кислот (ω-3 ПНЖК) й ацетату ретинолу (вітаміну А-ацетату). У мітохондріальній фракції печінки щурів-пухлиноносіїв у період інтенсивного росту пухлини (14 діб) підвищувалась швидкість генерації супероксидного радикала, зростали рівні первинних (трієнових кон'югатів, ТК), вторинних (кетодієнів і спряжених трієнів, КД+СТ) та кінцевих продуктів ПОЛ (основ Шиффа), що сприяло набуханню мітохондрій та виходу цитохрому *с* в цитозоль. Мононведення ω-3 ПНЖК істотно знижувало вільнорадикальні процеси та набухання мітохондрій печінки щурів із трансплантованою карциномою Герена. Введення високої дози (3000 МО/кг маси тіла) ацетату ретинолу до та після трансплантації пухлини посилювало вільнорадикальні процеси в мітохондріальній фракції печінки щурів-пухлиноносіїв, тоді як введення фізіологічної дози (30 МО/кг маси тіла) ацетату ретинолу не призводило до змін досліджуваних показників у контрольних щурів, які не отримували препарат. Прооксидантні ефекти ретиноїду частково усувалися в умовах його поєднаного введення з ω-3 ПНЖК.

Ключові слова: мітохондріальна фракція печінки, пероксидне окислення ліпідів, цитохром *с*, набухання мітохондрій, ω-3 поліненасичені жирні кислоти, ацетат ретинолу, карцинома Герена.

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