

FREE RADICAL PROCESSES IN THE LIVER MITOCHONDRIA OF RATS EXPOSED TO DIETHYL PHTHALATE

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Mechanisms of diethyl phthalate (DEF) influence on free radical processes in liver cells, in which it is not only metabolized but can also have a prooxidant effect, remain poorly studied. The aim of our research was to determine ROS formation, the intensity of oxidative modification of proteins (OMP) and proteolytic activity in the mitochondrial fraction of liver tissue of rats exposed to diethyl phthalate. The experimental white outbred rats were divided into three groups: I – intact animals (control), II and III – rats administered DEF orally for three weeks at doses 2.5 and 5.4 mg/kg b.w. respectively. The animals were euthanized on the 14th and 21st day after xenobiotic administration. The mitochondrial fraction from the rat liver was isolated and the level of superoxide and hydroxyl radicals, protein carbonyl derivatives, SH-groups and Schiff bases was determined. Proteolytic activity was assessed in the test of hemoglobin cleavage. It was shown that administration of DEF in a dose of 2.5 mg/kg initiated ROS generation and OMP intensification in the rat liver mitochondria only with prolonged administration for 21 days, whereas its administration in a dose of 5.4 mg/kg led to intensification of these processes already on day 14th followed by further amplification on day 21st. The activity of proteolytic enzymes in the mitochondrial fraction was found to be dependent on the degree of DEF-induced OMP and was increased with the minor OMP intensification but decreased with the significant intensification of protein free radical oxidation.

Key words: diethyl phthalate, liver, mitochondrial fraction, ROS, SH-groups, carbonyl derivatives, proteolytic enzymes.

Intensifying free radical processes in the body can contribute to mitochondrial dysfunction, cells' main generator of reactive oxygen species (ROS) [1]. Mitochondrial dysfunctions are accompanied by several changes resulting in metabolic disorders and the subsequent development of various pathological conditions in the body [2]. Mitochondrial dysfunction may be facilitated by the intake of exogenous xenobiotics, widely used in various industries today. Phthalates are synthetic chemicals that are present in the environment due to their widespread use as plasticizers and additives in personal care and pharmaceutical products [3].

Among phthalates, the most widely used is diethyl phthalate (DEP), which, after entering the body through inhalation, ingestion, and skin contact, is metabolized to the corresponding monoesters – monoethyl phthalate (MEP), which is also likely to have a toxic effect on the functioning of cellular organelles [4]. There is evidence of high doses of MEP in human urine samples, indicating a greater impact

of DEF on the body than other phthalates [5]. The US Environmental Protection Agency and the International Programs on Chemical Safety suggest an acceptable daily intake of DEF at 800 µg per kg of body weight per day. However, due to the constant impact of phthalates on the body, there is continuous low-dose exposure over a long period, which contributes to the intake of much higher doses of DEF into the human body [5].

To date, studies of the effect of this xenobiotic on the intensity of free radical processes in liver cells, in which DEF is not only metabolized but can also have a prooxidant effect, remain limited [6]. In this case, the work of the main energy producers in cells – mitochondria – may be disrupted due to the generation of ROS and the accumulation of oxidative modification of proteins (OMP) [7]. Since numerous metabolic pathways occur in the organelle, which are closely interrelated, free radical oxidation of mitochondrial proteins can often have very heterogeneous biochemical consequences [8]. At the same time,

the mechanisms and direction of DEF action on free radical processes in mitochondria remain unexplored, and their understanding will help prevent the development of pathological processes in the future.

Since mitochondria contain more than 1000 different proteins that are closely related to cell development, and OMP disrupts mitochondrial function and contributes to the development of various pathologies, mitochondrial proteases are critically important for maintaining the stability of mitochondrial proteins [9].

Hence, taking into account the above, the study aimed to assess the rate of ROS formation and the intensity of ROS in the mitochondrial fraction of rat liver under the action of DEF.

Materials and Methods

The study was performed on white outbred rats weighing 120-160 g. The animals were kept, and all manipulations were carried out following the provisions of the European Convention for the Protection of Vertebrate Animals Used for Research and Scientific Purposes (Strasbourg, 1986) and the VII National Congress on Bioethics "General Ethical Principles for Animal Experiment" (Kyiv, 2019) [10] (the Minutes of the meeting of bioethics commission of ES Institute of Biology, Chemistry and Bioresources, Yuriy Fedkovych Chernivtsi National University No 2 dated September 29, 2023).

The experimental animals were divided into three groups: Group I – intact animals (control) ($n = 12$), Group II – rats administered DEF at a dose of 2.5 mg/kg body weight ($n = 18$), and Group III – rats administered DEF at a dose of 5.4 mg/kg body weight ($n = 18$). DEF was administered orally for three weeks in doses that reflect the dose levels received by humans [11].

The animals were euthanized under light ether anesthesia on the 14th and 21st day after the start of the xenobiotic administration.

Differential centrifugation isolated the mitochondrial fraction from rat liver [12]. The rate of ROS formation, namely the formation of superoxide radical ($O_2^{\cdot-}$) [13] and hydroxyl radical ($\cdot OH$) [14, 15], was determined in the mitochondrial suspension. The rate of $O_2^{\cdot-}$ formation was recorded in the nitroblue tetrazolium test [13] and expressed as nmol/min per mg of protein. The $\cdot OH$ -radical was determined using the classical method of measuring the amount of reactive thiobarbituric acid products formed during $HO\cdot$ -mediated degradation of deoxyribose [15]. The incubation medium for determining

hydroxyl radical formation contained 20 mM sodium phosphate buffer (pH 7.4), 20 mM deoxyribose, 1 mM H_2O_2 , and mitochondrial fraction. After incubation for 30 min at 37°C, 0.5 ml of 1% thiobarbituric acid solution in 50 mM NaOH and 0.5 ml of 2.8% trichloroacetic acid solution were added to the mixture, incubated again for 20 min in a boiling water bath, cooled, and the absorbance was measured at $\lambda = 532$ nm. $\cdot OH$ generation was judged by the rate of TBC-active products formation (nmol) per minute per mg of protein [14, 15].

OMP was evaluated by determining the level of carbonyl derivatives [16], SH-groups [17], and Schiff bases [18]. The proteolytic activity in the mitochondrial fraction was determined by the modified Anson method [19], the principle of which is the cleavage of hemoglobin (casein) by mitochondrial proteases to products that are not precipitated by trichloroacetic acid. The proteolytic activity was expressed in micromoles of tyrosine formed in 1 min per mg of protein at 37°C. The protein content was determined by the Lowry method [20].

The results were statistically analyzed using analysis of variance (ANOVA). Differences between groups were considered significant at $P \leq 0.05\%$.

Results and Discussion

Phthalates in elevated concentrations and prolonged exposure to the body can cause severe cellular damage to the liver through the metabolic activation of highly reactive substances such as free radicals. The mechanisms of DEF damage may be based on the generation of ROS and subsequent initiation of free radical oxidation of mitochondrial proteins.

The results of the studies showed that under the influence of DEF at a dose of 2.5 mg/kg body weight for 14 days, there were no changes in the rate of $O_2^{\cdot-}$ formation in the mitochondrial fraction of the rat liver since the studied indicator was at the level of intact animals (Fig. 1). In animals treated with DEF at a dose of 5.4 mg/kg body weight for 14 days, an increase in the generation of $O_2^{\cdot-}$ in the mitochondrial fraction of the rat liver was observed by 1.7 times compared with that of intact animals (Fig. 1).

When DEF was administered during the 21st day, an increase in $O_2^{\cdot-}$ generation was detected in the mitochondrial fraction of the rat liver both under conditions of administration of the studied xenobiotic at a dose of 2.5 mg/kg animal weight and at a dose of 5.4 mg/kg animal weight, since the

studied index was 1,3 times and 2,6 times higher than the control index, respectively (Fig. 1). The increased generation of $O_2^{\cdot-}$ may be associated with a violation of the metabolic mechanisms of regulation of oxygen homeostasis in cells under the influence of DEF, which disrupts electron transport in the mitochondrial respiratory chain with the generation of $O_2^{\cdot-}$ [21]. The generated $O_2^{\cdot-}$ can be a source of the formation of more reactive $\cdot OH$ [22].

The analysis of the results showed that a fourteen-day exposure to DEF at a dose of 2.5 mg/kg body weight leads to a slight increase in the rate of TBA-active products formation from deoxyribose (1.2 times) compared to the control (Fig. 2). At the same time, in rats administered DEF for 14 days at a dose of 5.4 mg/kg body weight, the rate of TBA-active products formation increased by 1.8 times compared to the control (Fig. 2).

As follows from the results, the rate of TBA-active products formation from deoxyribose can indicate the formation $\cdot OH$ that is expressed to a greater extent than the rate of formation of $O_2^{\cdot-}$. Presumably, $\cdot OH$ is formed from $O_2^{\cdot-}$ and H_2O_2 in the Haber-Weiss reaction, which will cause oxidative stress in the mitochondria. Since $\cdot OH$ is a highly reactive radical, its formation locally will lead to mitochondrial OMP [23].

The administration of DEF during the 21st day contributed to a more significant increase in the rate of TBA-active products formation from deoxyribose. Thus, in animals treated with a dose of 2.5 mg/kg, the rate of TBA-active products formation was 1.6 times higher than in intact animals, whereas DEF administration at a dose of 5.4 mg/kg increased the formation of TBA-active products by 3.1 times compared to the control (Fig. 2).

The revealed results point out an increase in the intensity of $\cdot OH$ formation that can lead to mitochondrial damage with subsequent cell damage. With severe or prolonged stress, the concentration of ROS in the cell may increase, and, from a certain threshold level, the cell defense systems weaken, leading to liver cell death [23].

Mitoptosis can result from increased ROS production, depletion of the bioantioxidant pool, and increased local reactions [24]. These disorders may be the result of oxidative modification of mitochondrial proteins. To test this assumption, we determined the level of carbonyl derivatives as one of the markers of OMP.

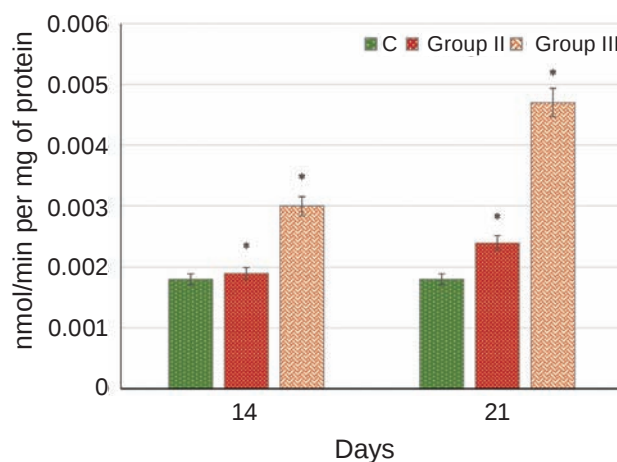


Fig. 1. The rate of superoxide radical formation in the mitochondrial fraction of rat liver under the influence of diethyl phthalate: C – intact animals (control); Group II – rats administered diethyl phthalate at a dose of 2.5 mg/kg; Group III – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group, $P < 0.05$

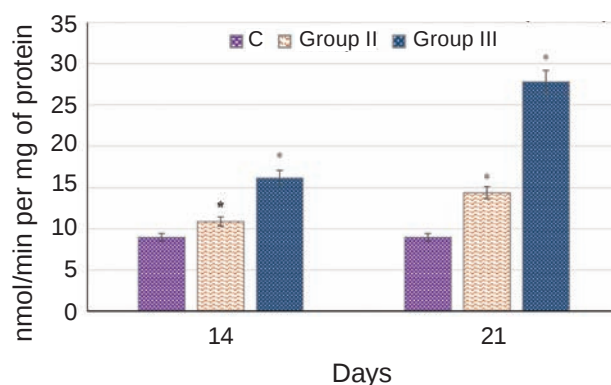


Fig. 2. The rate of TBA-active products formation from deoxyribose in the mitochondrial fraction of rat liver under the influence of diethyl phthalate: C – intact animals (control); Group II – rats administered diethyl phthalate at a dose of 2.5 mg/kg; Group III – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group, $P < 0.05$

The results of the studies showed that in the liver mitochondria of rats under the conditions of fourteen-day exposure to DEF at a dose of 2.5 mg/kg of animal body weight, no increase in the level of carbonyl derivatives was observed compared to intact animals (Fig. 3). At the same time, DEF ad-

ministration at a dose of 5.4 mg/kg body weight was accompanied by a 2-fold increase in the content of carbonyl derivatives in the mitochondrial fraction of the rat liver compared to the control values (Fig. 3).

The administration of DEF during the 21st day showed that in animals administered a dose of 2.4 mg/kg, the level of carbonyl derivatives increased 1.4 times, and at a dose of 5.4 mg/kg – 3.2 times compared to the control (Fig. 3).

Since not only free amino groups but sulfhydryl groups are subjected to oxidative modification in protein molecules, we also determined the content of SH-groups as a marker of OMP.

The analysis of the results of the studies showed a decrease in the level of protein SH-groups in the mitochondrial fraction of the rat liver, which increased with an increase in the dose and duration of DEF administration, which confirms the intensification of the OMP under the action of this xenobiotic. Thus, under the conditions of administration of medium doses of DEF (2.5 mg/kg) for 14 days, the level of SH-groups in the mitochondrial fraction of the rat liver did not differ from the control value (Fig. 4). In rats administered the maximum dose of DEF, a 1,6-fold decrease in the content of SH-groups was observed compared to the control (Fig. 4).

At the same time, the effect of DEF on day 21 showed that the level of SH-groups in the mitochondrial fraction of the rat liver decreased by 1.7 times compared to the control in animals receiving a dose of 2.5 mg/kg and by two times in animals receiving a dose of 5.4 mg/kg (Fig. 4).

Presumably, the generation of $O_2^{\cdot-}$ and $\cdot OH$ leads to OMP, as indicated by the oxidation markers of protein amino and sulfhydryl groups. Since one of the functions of the SH group is to reduce the cytotoxic and destructive effects of ROS, a decrease in their content may indicate changes in the redox potential of the body's cells. SH-groups located on the surface of a protein molecule can inactivate ROS by oxidizing to disulfides, thus protecting the internal parts of proteins from damage [25]. The decrease in the content of this indicator may be due to both the increased intensification of free radical processes and the increased use of these compounds.

Thus, an increase in the content of carbonyl derivatives and a decrease in the content of SH groups of proteins indicates an increase in the intensity of OMP in mitochondria, which results from ROS generation in response to the intake of DEF. Oxidized proteins can interact with aldehydes and form

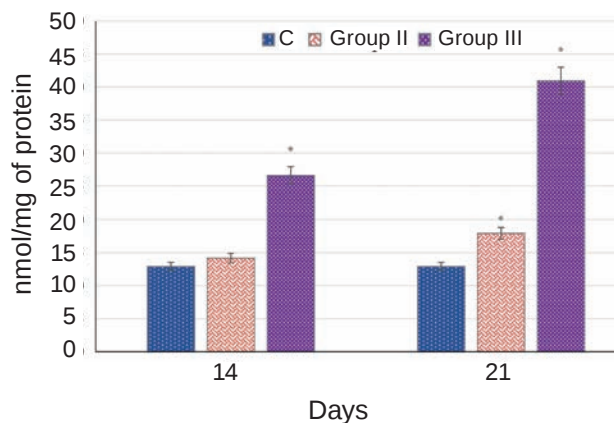


Fig. 3. The content of carbonyl derivatives in the mitochondrial fraction of rat liver under the influence of diethyl phthalate: C – intact animals (control); Group II – rats administered diethyl phthalate at a dose of 2.5 mg/kg; Group III – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group, $P < 0.05$

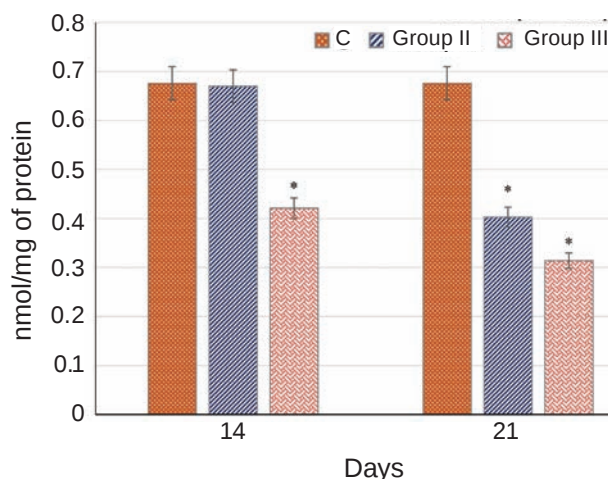


Fig. 4. The content of sulfhydryl groups in the mitochondrial fraction of rat liver under the influence of diethyl phthalate: C – intact animals (control); Group II – rats administered diethyl phthalate at a dose of 2.5 mg/kg; Group III – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group, $P < 0.05$

Schiff bases, the final products of biomolecular oxidation in the cell.

The analysis of the results showed that DEF administration for 14 days led to an increase in the level of Schiff bases in the mitochondrial fraction

of the liver only in the group of animals receiving a high dose of xenobiotic (5.4 mg/kg) since the studied index was 2.8 times higher than that of intact animals (Fig. 5). In animals treated with DEF during the 21st day, the level of Schiff bases increased by 3.7 times – at a dose of 2.5 mg/kg and by 4.6 times – at a dose of 5.4 mg/kg compared to intact animals (Fig. 5).

The accumulation of Schiff bases may indicate destabilization of the structural and functional state of mitochondrial membranes since this indicator is also the end product of the peroxidation of lipids that form membranes.

Changes in mitochondrial OMP may be reflected in changes in the activity of mitochondrial proteases since, on the one hand, they may be activated to hydrolyze oxidized proteins, and on the other hand, they may be inactivated due to oxidation. These changes will affect mitochondrial function since mitochondrial proteases form a complex system that performs limited and final proteolysis to form the mitochondrial proteome.

The results of our studies showed that after fourteen days of exposure to DEF at a dose of 2.5 mg/kg, no changes in proteolytic activity were observed compared to the control, while further administration of DEF revealed a 1.6-fold increase in the studied index compared to the control (Fig. 6). The activation of mitochondrial proteases is likely to occur in response to the formation of oxidized proteins that need to be utilized from the cell.

When DEF was administered at a dose of 5.4 mg/kg, the level of proteolytic activity increased 1.8-fold compared to the control after fourteen days of xenobiotic administration, presumably for the degradation of oxidized proteins (Fig. 6). On day 21 of the experiment, a decrease in protease activity was detected in this group of animals compared to the control, which is obviously due to their oxidation (Fig. 6).

The results obtained indicate that there is a correlation between the intensity of OMP and increased proteolytic activity. Thus, the enhancement of proteolysis by DEF administration may be due to both the activation of proteolytic enzymes and the modification of substrate proteins, increasing their sensitivity to these enzymes' action. Due to the increase in carbonyl derivatives, insoluble aggregates can form in the mitochondria that proteases, damaging cells and tissues, cannot neutralize. The high intensity of OMP leads to a decrease in the activity of proteases, presumably due to their oxidation [26].

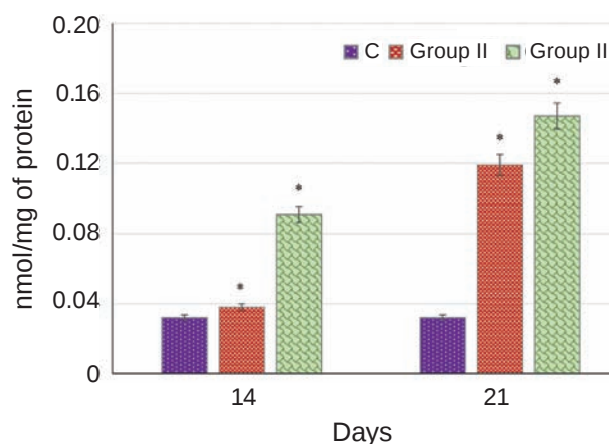


Fig. 5. The content of Schiff bases in the mitochondrial fraction of rat liver under the influence of diethyl phthalate: C – intact animals (control); Group II – rats administered diethyl phthalate at a dose of 2.5 mg/kg; Group III – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group, $P < 0.05$

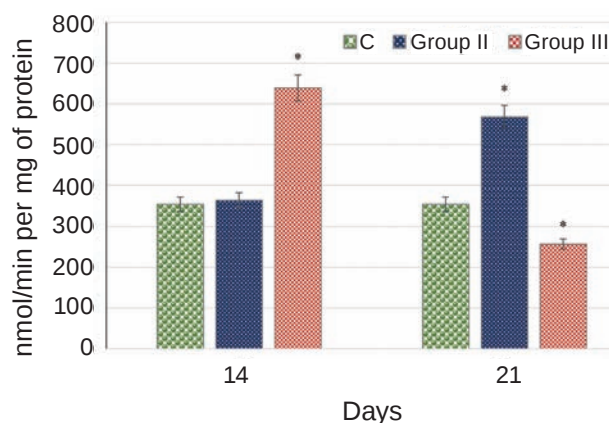


Fig. 6. Proteolytic activity in the mitochondrial fraction of rat liver under the influence of diethyl phthalate: C – intact animals (control); Group II – rats administered diethyl phthalate at a dose of 2.5 mg/kg; Group III – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group, $P < 0.05$

Thus, the administration of DEF causes the development of mitochondrial dysfunction, which can serve as an effector of the final pathology due to impaired energy and redox homeostasis. Administration of the xenobiotic at a dose of 5.4 mg/kg leads to the generation of ROS and intensification of OMP in the mitochondrial fraction of the rat liver on day

14 of its administration, followed by an increase in free radical processes on day 21. The use of medium doses of DEF (2.5 mg/kg) initiates the generation of ROS only with prolonged administration of the xenobiotic, as indicated by an increase in the rate of $O_2^{\cdot-}$ and $\cdot OH$ formation and the intensity of OMP after three weeks of DEF administration. The activity of proteolytic enzymes in the mitochondrial fraction directly depends on the degree of OMP, increasing with minor changes in OMP markers and then inactivating with a significant intensification of free radical oxidation of proteins.

Conflict of interest. The 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.

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ВІЛЬНОРАДИКАЛЬНІ ПРОЦЕСИ В МІТОХОНДРІЯХ ПЕЧІНКИ ЩУРІВ, ЯКІ ЗАЗНАЛИ ВПЛИВУ ДІЕТИЛФТАЛАТУ

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На сьогоднішній день залишаються не зрозумілими механізми впливу ксенобіотика діетилфталату (ДЕФ) на вільнорадикальні процеси в клітинах печінки, в яких він не тільки метаболізується, але й може проявляти прооксидантну дію. Метою наших досліджень було визначення утворення активних форм кисню (АФК), інтенсивності окисної модифікації протеїнів (ОМП) та протеолітичної активності у мітохондріальній фракції печінки щурів за впливу діетилфталату. Білих безпородних щурів розділили на три групи: I – інтактні тварини (контроль), II та III – щури, яким вводили ДЕФ перорально протягом трьох тижнів у дозах 2,5 і 5,4 мг/кг маси тіла, відповідно. На 14 і 21 добу після початку введення ксенобіотиків тварин евтаназували. З печінки щурів виділяли мітохондріальну фрак-

цію та визначали рівні супероксидного та гідроксильного радикалів, карбонільних похідних протеїнів, SH-груп і основ Шиффа. Протеолітичну активність оцінювали в тесті на розщеплення гемоглобіну. Введення ДЕФ щурам у дозі 2,5 мг/кг ініціювало утворення АФК та інтенсифікацію ОМП у мітохондріях печінки лише при введенні протягом 21 дня, тоді як за введення ДЕФ в дозі 5,4 мг/кг спостерігали інтенсифікацію цих процесів вже на 14-й день з наступним посиленням на 21-й день. Встановлено, що активність протеолітичних ензимів у мітохондріальній фракції залежить від ступеня ДЕФ-індукованої ОМП, а саме підвищується за незначної інтенсифікації ОМП та знижується у разі значної інтенсифікації вільнорадикального окислення протеїнів.

Ключові слова: діетилфталат, печінка щурів, мітохондріальна фракція, АФК, SH-групи, карбонільні похідні, протеолітичні ензими.

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