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CYTOCHROME P450 DEPENDENT FREE RADICAL PROCESSES IN THE LIVER MICROSOMES OF RATS ADMINISTERED DIETHYL PHTHALATE

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Diethyl phthalate (DEP) is widely used as a plasticizer and aromatic additive in various consumer products. Biotransformation of this xenobiotic occurs through the cytochrome P450 (CYP) -hydroxylating system, the catalytic cycle of which is accompanied by ROS generation in uncoupling reactions. The present study investigated the effects of DEP administration on the CYP-dependent ROS generation and lipid peroxidation in the rat liver microsomes. The experiment was conducted on three groups of purebred white rats: control (intact animals); rats orally administered with DEP at a dose of 2.5 or 5.4 mg/kg b.w per day for 21 days. CYP-mediated ROS generation was initiated by adding 0.24 μmol/l NADPH to the incubation mixture. It was found that daily administration of DEP at a dose of 2.5 mg/kg led to an increase in the rate of $O_2^{\bullet-}$ formation, H_2O_2 content, and intensification of lipid peroxidation in the liver microsomes only on the 21st day of the experiment. In contrast, administration of DEP at a dose of 5.4 mg/kg resulted in increased content of primary, secondary and final lipid peroxidation products as early as on the 14th day of xenobiotic exposure, indicating a dose- and time-dependent effect of DEP on the oxidative stress intensity in liver microsomes.

K e y w o r d s: diethyl phthalate, liver microsomes, superoxide radical, hydrogen peroxide, lipid peroxidation.

The influence of xenobiotics on the body leads to an increase in the formation of reactive oxygen species (ROS), the majority of which are produced in the endoplasmic reticulum (ER) [1]. Components of the cytochrome P450-hydroxylating system generate ROS, which can interact with vital cellular molecules, disrupting the cellular redox balance and leading to various pathological consequences [2].

In the catalytic cycle of cytochrome P450 (CYP), there are two "shunt" pathways in which ROS are generated without the completion of substrate oxidation. These are known as uncoupling reactions [3]. The first release of ROS involves the formation of the superoxide radical ($O_2^{\bullet-}$), which rapidly dismutates to form hydrogen peroxide (H_2O_2). A second possible release of ROS occurs after protonation of the reduced oxygen complex, resulting directly in the formation of H_2O_2 rather than water. Many factors determine the coupling efficiency of a given CYP reaction, with the substrate playing a significant role, as different CYP isoforms exhibit varying substrate-dependent rates of uncoupling [3, 4].

One of the most important biomolecules directly affected by ROS generated in the monooxygenase system (MOS) are polyunsaturated fatty acids (PUFAs), which are integral components of biomembranes where MOS enzymes are embedded [5]. PUFA oxidation leads to the formation of toxic lipid hydroperoxides and lipid aldehydes, which act as secondary intermediates in the propagation of oxidative stress signals [6].

Xenobiotics can induce oxidative stress in the body, which is itself a significant factor in toxicity. One such xenobiotic is diethyl phthalate (DEP), widely used as a plasticizer and aromatic additive in various consumer products (e.g., food packaging, pharmaceuticals, medical devices, and the perfume industry) [7]. The toxic effects of this compound depend largely on the dose and duration of exposure, which can influence the progression of free radical processes in cells and help elucidate the mechanisms of DEP toxicity.

The biotransformation of DEP occurs through the action of the cellular xenobiotic detoxification system. In the first phase of biotransformation,

DEP is converted into a more toxic intermediate metabolite, monoethyl phthalate (MEP), which is subsequently conjugated in the second phase to form a hydrophilic phthalate glucuronide via UDP-glucuronosyltransferase (UGT) [8]. Since UGT is a membrane-bound enzyme and its structural and functional conformation depends on the lipid environment of ER membranes, it is relevant to study the intensity of lipid peroxidation (LPO) in the liver microsomal fraction, where ROS are produced by MOS components [9]. Increased LPO markers include various products generated during this process, particularly diene conjugates, ketodienes, aldehydes, and Schiff bases [10]. Their quantification allows us to assess the direction and intensity of LPO in liver cells, the primary detoxification site for xenobiotics.

The study aim to evaluate the intensity of reactive oxygen species (ROS) formation and lipid peroxidation in rat liver microsomes under the influence of DEP.

Materials and Methods

This study used two-month-old outbred white rats (both males and females) weighing 120–160 g. The animals were maintained on a standard vivarium diet balanced for all essential nutrients. Housing and handling of animals were carried out by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and the guidelines of the VII National Congress on Bioethics "General Ethical Principles for Experiments on Animals" (Kyiv, 2019).

During the experiment, the animals were divided into three groups: group I – control group (intact animals) (n = 12), group II – rats orally administered DEP at a dose of 2.5 mg/kg (n = 9), group III – rats orally administered DEP at a dose of 5.4 mg/kg (n = 9).

The choice of doses was based on literature data, which state that the maximum effective dose of DEP is 5 mg/kg per day [11], and the average dose is 2.5 mg/kg per day. DEP administration was performed for 21 days. The animals were euthanized under light ether anesthesia on days 14 and 21 from the beginning of xenobiotic administration.

The liver microsomes were isolated using differential centrifugation [12]. The reaction was initiated to determine CYP-mediated ROS formation, by adding 0.24 μmol/l NADPH to the incubation mixture [13]. The incubation mixture contained 0.55 nmol of CYP, 1 mg of protein, and potassium phosphate buffer (pH 7.4). CYP content was determined of using of Omura and Sato methods [14]. The rate of $O_2^{\bullet-}$ formation in the liver microsomal fraction was determined by the reduction of nitroblue tetrazolium (NBT) by superoxide, forming a colored diformazan measured at 540 nm [15].

The H_2O_2 content in microsomes were were detected using the method with xylenol orange [16]. The working solution consisted of reagent A ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ в 2.5 M H_2SO_4) and reagent B (100 mmol/l sorbitol and 125 μmol/l xylenol orange) in a 1:100 ratio. An aliquot of the sample was added to test tubes containing 50 mM phosphate buffer (pH 6.0) to a final volume of 400 μl. The control sample contained 400 μl of buffer alone. Then, 2.0 ml of the working solution was added to each sample, mixed, and incubated for 15–20 min at room temperature. Absorbance was measured relative to the control at λ = 540 nm. Hydrogen peroxide concentration was calculated from a calibration curve and expressed per mg of protein.

Heptane-isopropanol lipid extracts were isolated from the liver microsomes to determine LPO products. The LPO products were assessed in the heptane phase, where fatty acid peroxidation metabolites are extracted. The intensity of LPO in microsomes were evaluated by measuring primary, secondary, and tertiary products. Primary LPO products, including diene conjugates (DC) and ketodienes plus conjugated trienes (KD + CT), were measured at 268 and 278 nm in the UV spectrum, respectively. Secondary products (TBA-active products) were determined at 532 nm [17]. Tertiary products (Schiff bases) were measured at 400 nm [18].

Statistical analysis was performed using Microsoft Excel. All data are presented as M ± SEM. Two-way ANOVA was used to assess statistical significance, and differences were considered statistically significant at $P < 0.05$.

Results and Discussion

The MOS of ER is one of the key cellular systems responsible for generating free radicals. ROS formed during cytochrome P450-dependent reactions initiate free radical processes within cells. Analysis of the results on cytochrome P450-mediated ROS formation showed that, after a two-week administration of DEP at a dose of 2.5 mg/kg, there was no significant increase in the rate of $O_2^{\bullet-}$ forma-

tion (Fig. 1, *A*) or in hydrogen peroxide (H_2O_2) levels (Fig. 1, *B*) in liver microsomes, compared to the control (intact animals). However, prolonged exposure to DEP at the same dose (21 days) led to an increase in the rate of $O_2^{\bullet-}$ generation and H_2O_2 content by 1.3-fold and 1.4-fold, respectively, compared to the control group (Fig. 1).

A more pronounced pro-oxidant effect of DEP was observed at a dose of 5.4 mg/kg, as a slight activation of ROS production was already evident on 14th day. In particular, the intensity of superoxide anion radical generation and the level of hydrogen peroxide exceeded those of the control group by 30 and 40%, respectively (Fig. 1).

On the 21st day of the experiment, high doses of DEP further intensified the formation of ROS in the MOS of the rat liver. The formation of ROS may be associated with changes in the structural and functional conformation of enzymes of the cytochrome P450-hydroxylating system and disruption of the monooxygenase cycle of CYP [3]. Such changes in MOS function largely depend on the lipid environment. The formed ROS can attack PUFAs in phospholipid membranes with sufficient energy to form organic radicals, particularly peroxyl radicals.

Analysis of the content of primary LPO products showed an increase by 1.3 times in rat liver microsomes on the 14th day under the influence of DEP at a dose of 5.4 mg/kg compared to the control ($P < 0.05$) (Fig. 2).

On the 21st day of the experiment, the content of primary lipid products exceeded the control value by 1.5 times when DEP was administered at a dose of 2.5 mg/kg and by 2.4 times when DEP was administered at a dose of 5.4 mg/kg ($P < 0.05$) (Fig. 2).

The pro-oxidant effect of DEP observed in our study may be associated with the formation of a more reactive intermediate metabolite, MEP. The formation of DC, one of the main lipid peroxidation products, results from free radical damage to PUFAs caused by the xenobiotic. This damage leads to the formation of a lipid radical ($L^{\bullet}$), which is subsequently oxidized to a lipid peroxyl radical ($LOO^{\bullet}$). The lipid peroxyl radical removes a hydrogen atom from another lipid molecule, forming lipid hydroperoxide (LOOH), thereby propagating the process and

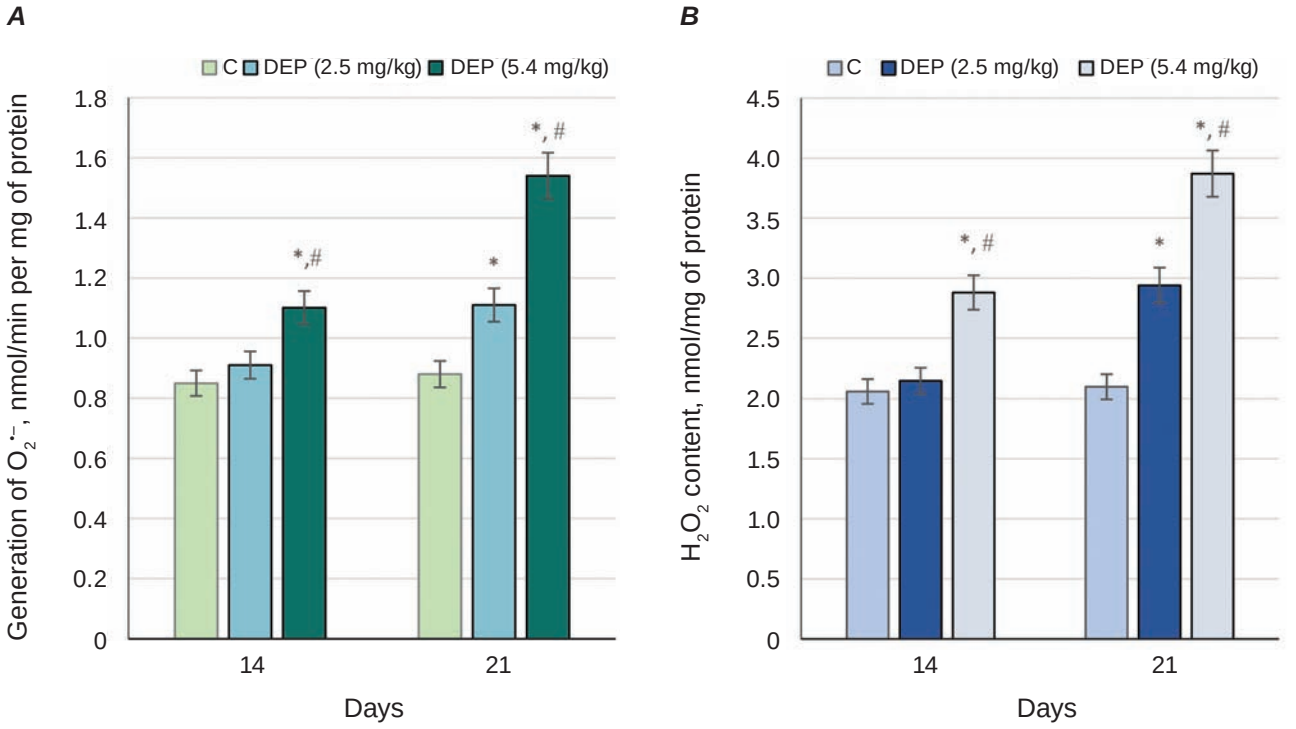

*Fig. 1. Rate of superoxide radical (**A**) formation and hydrogen peroxide (**B**) content in rat liver microsomes under the influence of diethyl phthalate. C – control group (intact animals); DEP 2.5 mg/kg – rats administered diethyl phthalate at a dose of 2.5 mg/kg; DEP 5.4 mg/kg – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group ($P < 0.05$); #statistically significant difference compared to rats administered DEP at a dose of 2.5 mg/kg ($P < 0.05$)*

promoting the accumulation of lipid peroxidation (LPO) products [18]. Additionally, the induction of oxidative stress in liver cells may be associated with a decrease in the activity of antioxidant enzymes under the influence of the studied xenobiotic [19].

Along with primary LPO products, an increase in secondary LPO products was observed in the microsomal fraction of the liver as the xenobiotic was introduced into the body. Thus, the highest levels of ketodienes and conjugated trienes in rat liver microsomes were observed on the 21st day of the experiment, when their content was 1.6 and 3.1 times higher than the control levels in rats administered DEP at doses of 2.5 and 5.4 mg/kg, respectively (Fig. 3).

The accumulation of primary LPO products can result from a decrease in the activity of antioxidant enzymes. DEP is known to inhibit the expression and activity of superoxide dismutase (SOD), while its metabolite MEP induces oxidative stress by inhibiting the expression and activity of glutathione peroxidase (GPX). It can be assumed that the accumulation of superoxide detected in our study results from a decrease in SOD activity under the direct action of DEP, and the accumulation of hydrogen peroxide is associated with a decrease in GPX activity due to the action of MEP, the main metabolite of DEP [8, 19].

As a result of the continued progression of the LPO process after the addition of free radicals, fatty acid chains break into aldehyde fragments, which are highly reactive. If the break occurs on both sides of the chain, a secondary product, malondialdehyde (MDA), is formed, which constitutes the main part of TBA-active products [10, 20].

The results of the study showed that under the administration of DEP at a dose of 2.5 mg/kg, an increase in rat liver microsomes of TBA-active products by 1.4 times was observed compared to the corresponding indicator in intact animals only on the 21st day of the experiment (Fig. 4).

When DEP was administered at a dose of 5.4 mg/kg, the content of TBA-active products was 1.8 times higher than the control value in rat liver microsomes on the 14th day of the experiment (Fig. 4). With prolonged administration of DEP at a dose of 5.4 mg/kg, there was no further increase in the content of TBA-active products, as their level

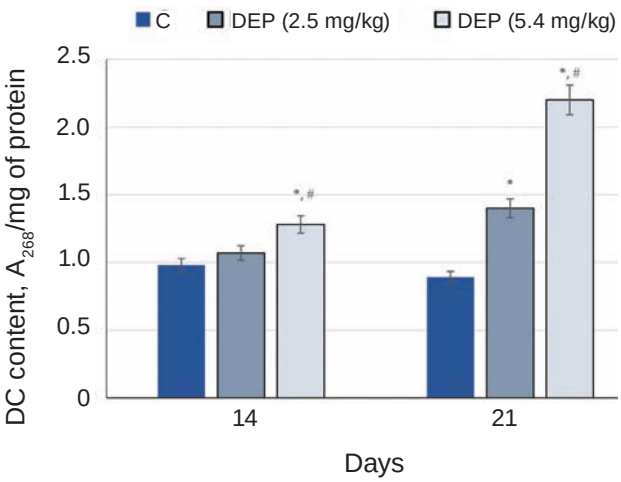

*Fig. 2. Content of diene conjugates as lipid peroxidation products in rat liver microsomes under the influence of diethyl phthalate. C – control group (intact animals); DEP 2.5 mg/kg – rats administered diethyl phthalate at a dose of 2.5 mg/kg; DEP 5.4 mg/kg – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group (P < 0.05); #statistically significant difference compared to rats administered DEP at a dose of 2.5 mg/kg (P < 0.05)*

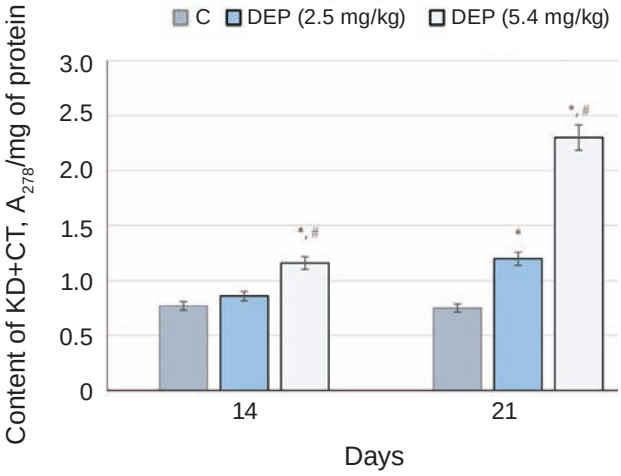

*Fig. 3. Content of ketodienes and conjugated trienes as lipid peroxidation products in rat liver microsomes under the influence of diethyl phthalate. C – control group (intact animals); DEP 2.5 mg/kg – rats administered diethyl phthalate at a dose of 2.5 mg/kg; DEP 5.4 mg/kg – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group (P < 0.05); #statistically significant difference compared to rats administered DEP at a dose of 2.5 mg/kg (P < 0.05)*

in liver microsomes remained consistently high and was 1.9 times higher than the control value (Fig. 4).

Thus, under the influence of high doses of DEP, there was no further increase in the level of TBA-active products. The detected changes may be related to the interaction of MDA with amino groups of proteins, resulting in the formation and accumulation of Schiff bases, which act as markers of LPO [21].

To test this assumption, we investigated the level of Schiff bases. The results showed an increase in the final products of lipid peroxidation in rat liver microsomes exposed to DEP compared to intact animals, which depended on the dose and duration of xenobiotic administration. Thus, two weeks of DEP administration at a dose of 2.5 mg/kg did not lead to an increase in the level of Schiff bases, but after three weeks of DEP administration, their level increased by 2.4 times compared to the control (Fig. 5).

Under the conditions of DEP administration at a dose of 5.4 mg/kg, an increase in the content of Schiff bases was observed already on the 14th day of the experiment, when the studied indicator was 2.5 times higher than the control ($P < 0.05$) (Fig. 5). Prolonged administration of the xenobiotic (up to the 21st day) led to an increase in the content of Schiff bases by 3.4 times compared to the control ($P < 0.05$) (Fig. 5).

Probably, the absence of a tendency to increase the content of TBA-active products as the xenobiotic is introduced is the basis for the increase in the level of Schiff bases. This fact is indicated by the Schiff formation index (the ratio of Schiff bases to the content of TBA-active products), which increases as DEP is introduced into the body. The consequences of LPO can be structural damage to membranes and the formation of oxidized products, some chemically reactive and covalently modify macromolecules, which are the main effectors of tissue damage [6].

Therefore, the toxicity of DEP consists in its ability to trigger free radical oxidation processes in the body, one of the mechanisms of which is cytochrome P450-mediated generation of ROS, leading to the initiation of LPO with the formation of primary, secondary, and final LPO products.

Conclusions. The intensity of free radical processes in rat liver microsomes is mediated by the components of the MOS. As DEP enters the body, the cytochrome P450-mediated rate of $O_2^{\bullet-}$ generation and the content of H_2O_2 increase, which is accompanied by an intensification of LPO processes.

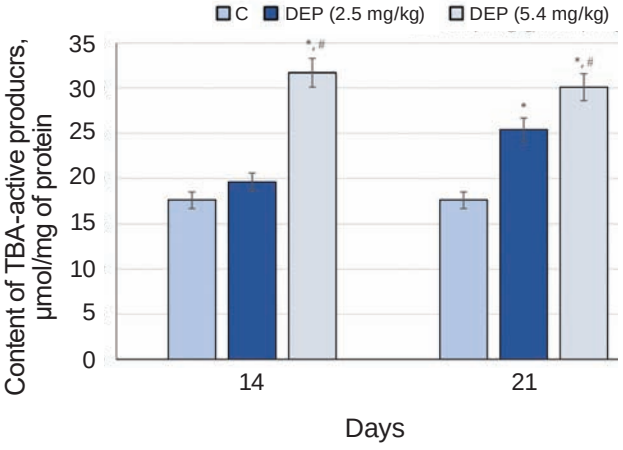

*Fig. 4. Content of TBA-active products in rat liver microsomes under the influence of diethyl phthalate. C – control group (intact animals); DEP 2.5 mg/kg – rats administered diethyl phthalate at a dose of 2.5 mg/kg; DEP 5.4 mg/kg – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group (P < 0.05); #statistically significant difference compared to rats administered DEP at a dose of 2.5 mg/kg (P < 0.05)*

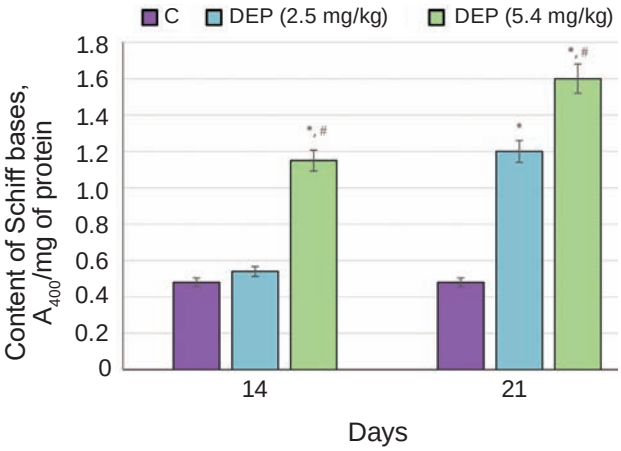

*Fig. 5. Content of Schiff bases in rat liver microsomes under the influence of diethyl phthalate. C – control group (intact animals); DEP 2.5 mg/kg – rats administered diethyl phthalate at a dose of 2.5 mg/kg; DEP 5.4 mg/kg – rats administered diethyl phthalate at a dose of 5.4 mg/kg; *statistically significant difference compared to the control group (P < 0.05); #statistically significant difference compared to rats administered DEP at a dose of 2.5 mg/kg (P < 0.05)*

Oxidative destruction of ER phospholipids, with the accumulation of primary, secondary, and final products of LPO, leads to changes in the structural and functional conformation of the components of the cytochrome P450-hydroxylating system, which further enhances free radical processes in the cell.

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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ЦИТОХРОМ Р450-ЗАЛЕЖНІ ВІЛЬНОРАДИКАЛЬНІ ПРОЦЕСИ В МІКРОСОМАХ ПЕЧІНКИ ЩУРІВ ЗА УМОВ ВВЕДЕННЯ ДІЕТИЛФТАЛАТУ

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Діетилфталат (ДЕФ) широко використовується як пластифікатор та ароматична добавка в різних споживчих товарах. Біотрансформація цього ксенобіотика відбувається цитохром Р450 (CYP)-гідроксилюючою системою, каталітичний цикл якої супроводжується утворенням активних форм кисню (АФК) у реакціях роз'єднання. У цьому дослідженні вивчено вплив введення ДЕФ на CYP-залежне утворення АФК та пероксидне окислення ліпідів у мікросомах печінки щурів. Експеримент проводили на трьох групах білих безпородних щурів: контрольна (інтактні тварини); щури, яким перорально вводили ДЕФ у дозі 2,5 або 5,4 мг/кг маси тіла щодня протягом 21 дня. CYP-опосередковане утворення АФК ініціювали додаванням до інкубаційної суміші 0,24 мкмоль/л NADPH. Встановлено, що щоденне введення ДЕФ у дозі 2,5 мг/кг призвело до збільшення швидкості утворення $O_2^{\bullet-}$, вмісту H_2O_2 та посилення пероксидного окислення ліпідів у мікросомах печінки лише на 21-й день експерименту. Натомість, введення ДЕФ у дозі 5,4 мг/кг призвело до збільшення вмісту первинних, вторинних та кінцевих продуктів перекисного окислення ліпідів вже на 14-й день впливу ксенобіотика, що вказує на дозо- та часозалежне посилення оксидативного стресу в мікросомах печінки.

Ключові слова: диетилфталат, мікросоми печінки, супероксидний радикал, пероксид водню, пероксидне окислення ліпідів.

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