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ПРОСТОРОВО-ЧАСОВА ДИНАМІКА УГРУПОВАНЬ ЛІТОРАЛЬНОГО ЗООПЛАНКТОНУ ОЛЕКСАНДРІВСЬКОГО ВОДОСХОВИЩА

Представлено результати аналізу просторово-часової динаміки угруповань зоопланктону літоралі Олександрівського водосховища. Виявлено особливості сезонних змін видового складу, фауністичного та екологічного спектрів, кількісних показників (щільності та біомаси) та домінуючих комплексів видів літорального зоопланктону. Здійснено аналіз сезонної динаміки якісних і кількісних показників розвитку зоопланктону в межах літоралі верхньої, середньої та нижньої частин Олександрівського водосховища.

Ключові слова: екологія, Олександрівське водосховище, літораль, угруповання зоопланктону.

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ПРОСТРАНСТВЕННО-ВРЕМЕННАЯ ДИНАМИКА СООБЩЕСТВ ЛИТОРАЛЬНОГО ЗООПЛАНКТОНА АЛЕКСАНДРОВСКОГО ВОДОХРАНИЛИЩА

Представлено результаты анализа пространственно-временной динамики сообществ зоопланктона литорали Александровского водохранилища. Выявлено особенности сезонных изменений видового состава, фаунистического и экологического спектров, количественных показателей (плотности и биомассы) и доминирующих комплексов видов литорального зоопланктона. Осуществлено анализ сезонной динамики качественных и количественных показателей развития зоопланктона в пределах литорали верхней, средней и нижней частей Александровского водохранилища.

Ключевые слова: экология, Александровское водохранилище, литораль, сообщества зоопланктона.

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COMPARATIVE INVESTIGATION OF ANTI-TUBERCULOSIS DRUGS EFFECTS ON TESTICULAR CYP2E1 EXPRESSION AND MALE REPRODUCTIVE PARAMETERS UNDER SEPARATE AND COMBINED ADMINISTRATION IN MALE RATS

Comparative study of anti-tuberculosis drugs anti-androgenic effects and effects on testicular CYP2E1 has been performed. Testicular CYP2E1 mRNA and protein expression, serum total testosterone level, fertility and spermatogenesis parameters in male rats under simultaneous and separate administration of ethambutol, isoniazid, rifampin and pyrazinamide have been investigated. Analysis of the obtained data has proved the prominent role of ethambutol and isoniazid in gonadal toxicity of anti-tuberculosis drugs combination. Activation of CYP2E1-dependent metabolizing systems in testicular steroidogenic cells could stipulate at least a part of ethambutol, isoniazid and anti-tuberculosis drugs combination negative effects on testosterone level and spermatogenesis processes. Mechanisms of spermatogenesis alteration by rifampin and pyrazinamide need to be explored more extensively, but in the light of our observations they do not depend from testicular CYP2E1.

Key words: anti-androgenic effects, anti-tuberculosis drugs, protein expression, ethambutol, isoniazid.

Introduction. The epidemiological situation of tuberculosis in the world keeps worsening [1]. In general, all patients from countries with a known high incidence of resistant *M. tuberculosis* strains, all patients who had been treated previously, and all patients with life-threatening tuberculosis, receive as initial anti-tuberculosis therapy the same combination of isoniazid (INH), rifampin (RMP) and pyrazinamide (PZA), together with at least one additional medicine (ethambutol (EMB) and/or streptomycin) [2].

In such situation investigation of these compounds adverse effects becomes of vitally importance. We have previously shown the antifertility effects of anti-tuberculosis medicines combination in male rats with simultaneous increase in cytochrome P-450 2E1 (CYP2E1) mRNA in their testes [3, 4]. It is important to note that series of **studies clearly demonstrated inducibility of** CYP2E1 in testis, suggesting its possible role in chemicals bioactivation to their toxic metabolites directly in male gonads [4, 5, 6, 7]. Among this, it is well known that both toxic intermediates (which are able to interact with vitally important cells structures) and reactive oxygen species (ROS) overproduction (with the further development of oxidative stress) take place during CYP2E1-mediated xenobiotics metabolism [8].

It remains unclear which one of the four co-administered (ATD) plays a crucial role in testicular CYP2E1 expression modulation and the development of antifertility effects. Thus, in terms of *o*ur above mentioned results [3, 4] analysis of potential effects on male gonads of each component of the combination are urgently required. Such data could substantially contribute to our general understanding of causes of the man subfertility. To get the answer on this question we have decided to compare testicular CYP2E1 mRNA and protein expression, serum total testosterone (TS) level, fertility and spermatogenesis parameters in male rats under combined and separate administration of EMB, INH, RMP and PZA.

Materials and methods. Substances of EMB, INH, RMP and PZA were supplied by the SIC "Borzhagovsky Chemical-Pharmaceutical Plant" CJSC, Ukraine.

Wistar albino male with initial body weight (b.w.) 150–170 g (8-9 weeks old) and female rats 150-170 b.w. (9-10 weeks old), were purchased from Biomodel Service (Kyiv, Ukraine). They were kept under a controlled temperature (from 22 °C to 24 °C), relative humidity of 40 % to 70 %, lighting (12 h light-dark cycle), and on a standard pellet feed diet ("Phoenix" Ltd., Ukraine).

The male rats were divided randomly into 6 groups: 1-control (n=12); 2 – EMB administration (n=12); 3 – INH administration (n=12); 4 – RMP administration (n=12); 5 – PZA administration (n=12); 6 – simultaneous ATD administration (n=12). All ATD were suspended in 1% starch gel and was administered intragastrically by gavage in doses used in clinic [9], which for rats (with the coefficient for conversion of human doses to animal equivalent doses based on body surface area) were following: EMB – 155 mg/kg b.w./day, RMP – 74.4 mg/kg b.w./day, INH – 62 mg/kg b.w./day, PZA – 217 mg/kg b.w./day [10]. ATD were administered during entire spermatogenesis cycle, which (with

time of germ cell maturation in epididymis) is 60 days for rats. The control group received only starch gel in corresponding volumes (5 ml/kg b.w.).

After 46 days of repeated administrations, the males from both groups were mated with intact females at the ratio 1 male: 2 females during 14 days (3 oestrous cycles). During this period administrations of ATD to male rats were continued.

According to generally accepted guidelines for the fertility study in laboratory rats [11] the first day of pregnancy was established by vaginal cytology (the first day of sperm detection in vagina). Most males were mated within the first 5 days of cohabitation (i.e. at the females first available oestrus), but part of them demonstrated infertility. This fact was taken into account for evaluation of effects on male fertilizing capacity, which was determined by the index:

$$\frac{number\ of\ pregnant\ females}{number\ of\ females\ mated\ with\ males} \times 10$$

The pregnancy was confirmed by necropsy. The females were sacrificed under mild ether anaesthesia via cervical dislocation on day 20 of pregnancy.

Males were sacrificed on the morning under mild diethyl ether anaesthesia by decapitation after 60 days of experiment. The study was carried out according to the national and international guidelines and the law on animal protection was observed. All animal studies were performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee.

The expression of CYP2E1 mRNA in testes was determined by a reversed transcriptase polymerase chain reaction (RT-PCR). After collection of the testes samples (25 mg), they were quickly frozen in liquid nitrogen, and stored at -80 °C before RNA extraction. The isolation of total mRNA was carried out with a TRI-Reagent (Sigma-Aldrich, Inc., USA). The integrity and concentration of RNA was analysed in a 2 % agarose gel. First-strand complementary DNA (cDNA) was synthesized using a First-Strand cDNA Synthesis Kit (Fermentas, Germany) according to the manufacturer's protocol. The reaction mixture contents for PCR, amplification protocol, and specific primers for the CYP2E1 gene were chosen according to Lankford et al. (2000). The primer sequences were: sense, 5'-CTTCGGGCCAGTGTTCAC-3' and anti-sense, 5'-CCCATATCTCAGAGTTGTGC-3'. RT-PCR with primers of β – actin sense, 5' –GCTCGTCGTCGACAACGGCTC – 3' and antisense 5' – CAAACATGAT CTGGGTCATCTTCT –3') was carried out for internal control. All of the primers were synthesized by "Metabion" (Germany). The MyCycler termocycler (BioRaD, USA) was used for amplification. PCR products (CYP2E1-744 bp and β-actin-353 bp) were separated in a 2 % agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator (BIORAD, USA). Data analysis was carried out with Quantity One Software (USA) and presented in relative units as CYP2E1 mRNA contents / β-actin mRNA ratio.

Immunohistochemical staining for testicular CYP2E1 was performed using 4 μm thick sections of BS-fixed, paraffin-embedded sections. Briefly, tissue sections were dewaxed in xylene and were placed in water through graded alcohols. Antigen retrieval has been performed by microwaving slides in 10 mM citrate buffer (pH 6.2) for 30 min at high power, according to the manufacturer's instructions. Human polyclonal antibody against CYP2E1 (Thermo scientific, USA) were used as primary antibodies. To remove the endogenous peroxidase activity, the sections have been treated with freshly prepared 1.0 % hydrogen peroxide in the dark for 30 min at 37 °C temperature. Non-specific antibody binding was blocked by means of blocking serum. The sections were incubated for 30 min, at 37 °C temperature, with the primary antibodies against CYP2E1 diluted 1:100 in phosphate buffered saline (PBS) pH 7.2 then a triple washing with PBS follows. Anti-(rabbit IgG)–horseradish peroxidase conjugate (1:40000 dilution) has been fulfilled for the detection of the CYP2E1 primary antibodies, then the sections were incubated for 20 min, at 37 °C temperature. The reaction products were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB). Immunohistochemical study of testicular CYP2E1 was performed in EMB-treated and vehicle-treated rats, respectively. Any intensity of reactivity for CYP2E1 in testes was considered positive. The proportion of testes staining positive was scored semiquantitatively as positive, focal/weakly positive, or negative.

The sperm count in epididymal suspensions was estimated as described Chitra et al. [12] using Goryaev's counting chamber and light microscope (200 x).

The determination of the spermatogenic index in testicles was carried out according to four points system. It was based on the estimation of number of cell layers, types of cells, and the presence of late spermatids in the seminiferous tubules. The criteria were as follows: 1 – only spermatogonia present; 2 – spermatogonia and spermatocytes present; 3 – spermatogonia, spermatocytes and round (early) spermatids present with < 5 late spermatids per tubule; 4 – spermatogonia, spermatocytes, and round spermatids present with up to 25 late spermatids per tubule [11]. Spermatogenic index was calculated as a ratio of stages of spermatogenesis total to number of examined tubules. Two hundred seminiferous tubules per testis of each animal were observed by microscopy.

Blood samples from femoral vein were collected. Serum samples were separated and kept frozen at −70°C. Serum total testosterone levels were measured using DRC testosterone ELISA kit (Germany) according to manufacturer's instruction.

The obtained data were calculated by one-way analysis of variance (ANOVA) and compared using the Tukey test. Differences were considered statistically significant at $p<0.05$. Numerical data are represented as means ± SEM.

Results and discussion. We have shown that combined administration of ATD caused significant decrease in serum TS (Fig.1). At this group it was lower 2.8 folds as compared with control. Separate administration of EMB and INH also significantly influenced TS level, lowering it in average 1.6 folds as compared with control (Fig.1). At the same time RMP and PZA did not alter this parameter (Fig.1).

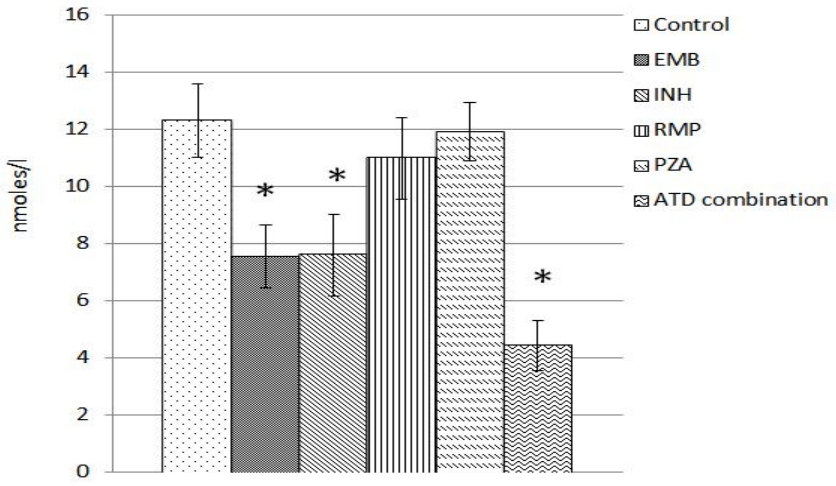

Fig. 1. Total testosterone level in blood serum of male rats following separate and combined ATD treatment

* – P<0.05 in comparison with control

Thus, our results indicate considerable TS level impairment in EMB, INH and ATD-combination-treated male rats, which, in turn, caused lowering of sperm count in these groups (Fig.2).

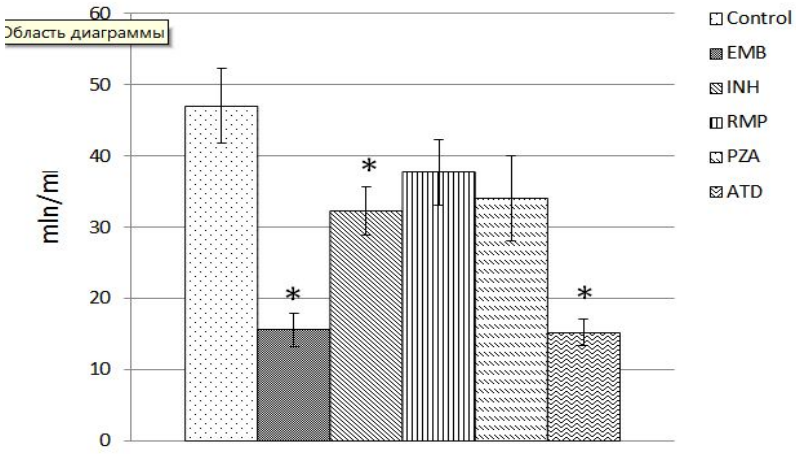

Fig. 2. Epididymal suspension sperm count following separate and combined ATD treatment

* – P<0.05 in comparison with control

Interestingly that administration (separate and combined) of all ATD caused a development of destructive changes in spermatogenic epithelium. It is seen from the data summarized in Table 1 that spermatogenic index at all experimental groups was *significantly* decreased in comparison with control group. Primary spermatogenesis cell population also was affected, namely mitotic activity was inhibited and number of spermatogonia in testes tubules sections was decreased. In addition, the number of cells at XII stage of spermatogenesis (characterizing primary spermatocytes meiotic division processes) in EMB and ATD combination-groups was lower than in control. This parameter was not significantly changed by RMP, INH, and PZA, but it demonstrated the tendency to decreasing. At ATD combination-treated group we detected epithelium exfoliation into the lumen of seminiferous tubules in substantial quantity too.

***Table* 1. Parameters of spermatogenic epithelium in testes following separate and combined ATD treatment**

Groups of males	Spermatogenic index (stages of spermatogenesis total / number of examined tubules)	Number of spermatogonia (per tubular cross section)	Cells at XII stage of spermatogenesis, %	Exfoliation of epithelium, %
Control	3.615±0.011	69.393±0.742	3.563±0.365	0.313±0.120
EMB	3.484±0.008*	57.840±0.465*	2.000±0.316*	1.000±0.316
RMP	3.530±0.012*	59.540±0.901*	2.200±0.510	0.600±0.400
INH	3.494±0.007*	62.110±0.936*	2.200±0.663	0.800±0.374
PZA	3.552±0.007*	61.060±1.016*	2.400±0.245	1.000±0.316
ATD combination	3.535±0.014*	59.573±0.861*	2.412±0.508	1.882±0.363*

* – P<0.05 in comparison with control

Our data on fertilizing capacity of experimental males was in accordance with TS level and sperm count results (table 2).

***Table* 2. Male rats' fertility index following separate and combined ATD treatment**

Groups of males	Number of mated females	Number of pregnant females	Fertility index, %
Control	12	11	91.66
EMB	12	4	33.33
RMP	12	10	83.33
INH	12	8	66.67
PZA	12	10	83.33
ATD combination	12	1	8.33

Counting of the number of pregnant intact females which were mated with experimental males demonstrated a significant reduction in the fertility of males receiving ATD combination. Of the 12 mated females only one became pregnant, while in the control fertility index was 92%. Evaluation of fertility index in animals separately treated with ATD found that all four drugs reduced this parameter to some extent, but in case of INH (index – 66.67%) and EMB (index – 33.33%) – most definitely.

In addition, we have recorded fatal decrease of the number of live fetuses in EMB (6.3 folds) or ATD combination-treated (36.6 folds) groups (table 3).

***Table* 3. Number of live fetuses in offspring of males following separate and combined ATD treatment**

Groups of males	Parameter	
	total number of live fetuses, abs.	number of live fetuses per one female, abs.
Control	110	9.16±1.14
EMB	16	1.33±0.62 *
RMP	86	7.17±1.55 #
INH	111	9.25±1.30 #
PZA	112	9.33±1.30 #
ATD combination	3	0.25±0.25 *

* – P<0.05 in comparison with control
# – P<0.05 in comparison with ATD combination-treated animals

RT-PCR was performed to evaluate the effect of separate and combined ATD administration on CYP2E1 mRNA expression in testes.

We have not found statistically significant increase in testicular CYP2E1 mRNA expression following administration of RMP and PZA (Fig. 3). At the same time, there was a significant rise in CYP2E1 mRNA expression after administration of INH (28 folds), EMB (8.7 folds), and ATD combinations (19 folds). Slightly lower CYP2E1 gene expression level in ATD-treated probably could be due to the competitive antagonistic relationship of EMB and INH.

The evaluation of the testicular CYP2E1 protein level was performed using immunohistochemical analysis. The immunoexpression of CYP2E1 in testes was confirmed by the presence of brown staining. Figure 4 illustrates broad expression of CYP2E1 in EMB, INH and ATD-combination treated rats' testes that were significantly higher than in PZA, RMP, and control groups (Fig.6).

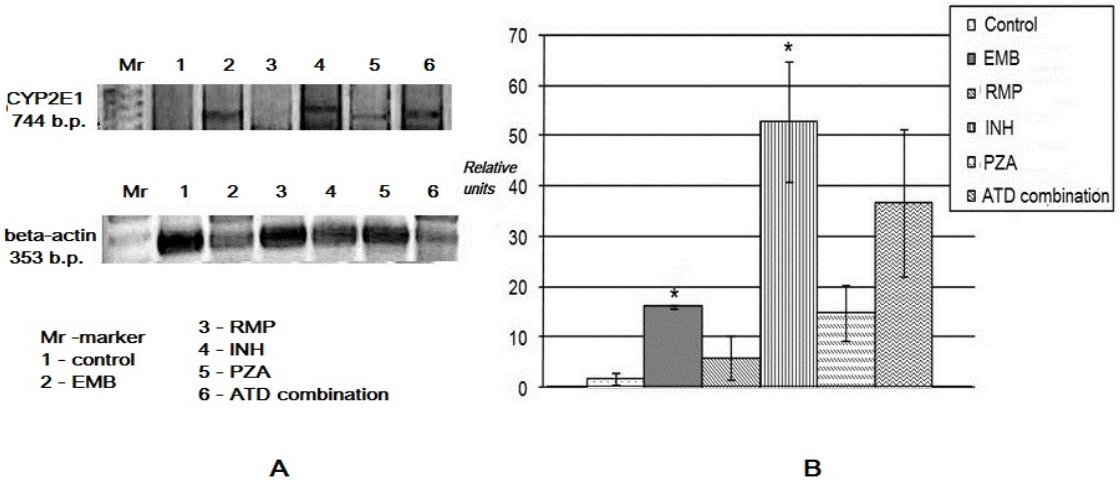

Fig 3. Representative electrophoregrams of CYP2E1 (744 b.p) and reference-gene β-actin (353 b.p.) RT-PCR (panel A); average rate of CYP2E1 mRNA expression (panel B) in rats testes following separate and combined ATD treatment

*- P<0.05 in comparison with control.

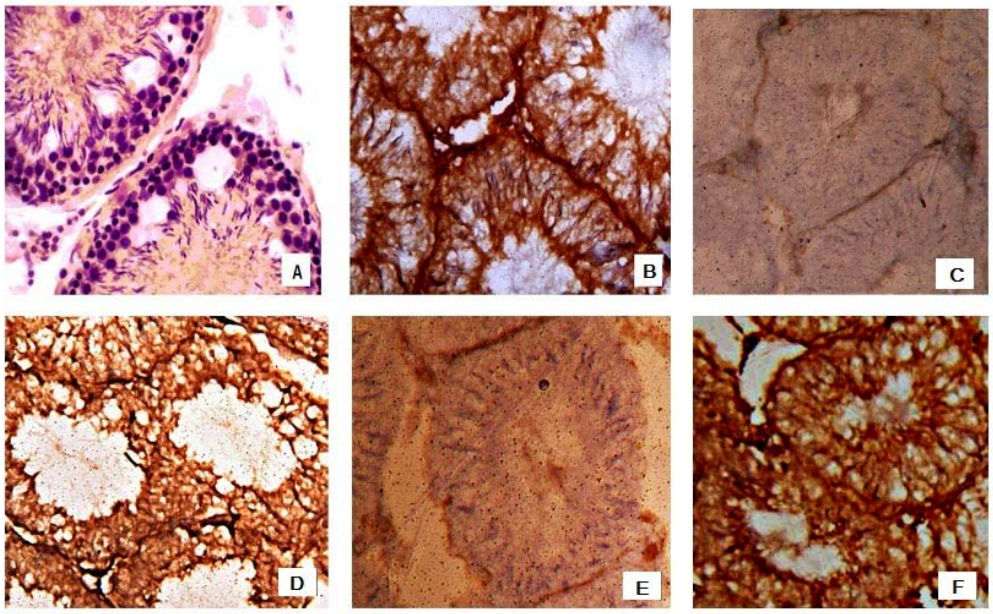

Fig. 4. Immunohistochemistry analysis of CYP2E1:
A – negative reaction in testis of control rat; B, D, and F – strongly positive reaction in testis of EMB, INH, and ATD combination-treated rats; C and E – weakly positive reaction in testis of RMP and PZA-treated rats

It should be noted that adult spermatogenesis consists of three phases: proliferation of spermatogonia, meiosis of spermatocytes, and differentiation of spermatids or spermiogenesis. These events are precisely controlled at each stage to ensure the formation of genetically balanced gametes [13]. Affecting of any step could dysregulate the process of spermatogenesis and the spermatozoa may become defective [14]. After analyzing our results, firstly, it can be seen the definite evidences of the spermatogenesis impairment in separate and combined ATD-treated male rats. For instance, the decrease of spermatogenic index and spermatogonia number confirms depression of spermatogenic cells activity. Moreover we have detected intensification of germ cells exfoliation into the lumen of the seminiferous tubules, as evidence of loss of their adhesion with Sertoli cells or shearing of Sertoli cells cytoplasm [15].

We also demonstrated that following separate EMB and INH, as well as combined ATD administration alterations of spermatogenesis along with low TS level, have led to a reduction in spermatozoids production. As a result the fertility index and number of live fetuses in offspring at these groups was three folds lower than in control. Also at these groups we have demonstrated the significant increase of testicular CYP2E1 mRNA and protein levels. This phenomenon could be an indication of this iso-enzyme induction.

Our results on testicular CYP2E1 induction following EMB, INH and ATD combination administration is of importance because this process occure in Leydig cells [6, 7], which provide the synthesis of androgens necessary for the maintenance of spermatogenesis and extra-gonadal androgen actions in mammals [16]. Consequently, these structures, and their microenvironment damage by free radicals as a result of ATD-mediated CYP2E1 induction could be one of the reasons of steroidogenesis enzymes inhibition and spermatogenesis disruption. It is known that CYP2E1 is an effective generator of hydrogen peroxide [17], and it acts directly on Leydig cells to diminish TS production by inhibiting cytochrome P450 side chain cleavage enzyme (P450scc) activity and steroidogenic acute regulatory (StAR) protein expression [18]. Moreover, recently it has been reported, that ROS signaling-mediated c-Jun upregulation suppresses the expression of steroidogenic enzyme genes by inhibiting Nur77 transactivation (one of the major transcription factors that regulate the expression of steroidogenic enzyme genes), resulting in the reduction of testicular steroidogenesis [19]. The fact that lowered level of TS can affect Sertoli cells function and also negatively influence spermatogenesis is of importance too [20].

Conclusion. Our findings prove the prominent role of EMB and INH in male reproductive toxicity of ATD combination. In our opinion, activation of CYP2E1-dependent metabolizing systems in steroidogenic cells could stipulate at least a part of EMB, INH and ATD-combination negative effects on testosterone level and spermatogenesis processes. Mechanisms of spermatogenesis alteration by RMP and PZN need to be explored more extensively, but in the light of our observations they do not depend from testicular CYP2E1. It seems warranted to conclude that the critical examination of both laboratory animals' and epidemiological data is required.

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ПОРІВНЯЛЬНЕ ДОСЛІДЖЕННЯ ЕФЕКТІВ НАРІЗНОГО І КОМБІНОВАНОГО ВВЕДЕННЯ ПРОТИТУБЕРКУЛЬОЗНИХ ЛІКАРСЬКИХ ЗАСОБІВ НА ТЕСТИКУЛЯРНУ ЕКСПРЕСІЮ CYP2E1 І РЕПРОДУКТИВНІ ПОКАЗНИКИ САМЦІВ ЩУРІВ

Було проведено порівняльне вивчення анти-андрогенних ефектів протитуберкульозних препаратів, а також їх впливу на тестикулярний CYP2E1. Досліджували експресію мРНК і білка CYP2E1 в сім'яниках, рівень загального тестостерону, фертильність і показники сперматогенезу у самців щурів при окремому і сумісному введенні етамбутолу, ізоніазиду, рифампіцину і піразинаміду. Аналіз отриманих даних доводить важливу роль етамбутолу і ізоніазиду в гонадотоксичної дії комбінації протитуберкульозних препаратів. Активація CYP2E1-залежних метаболізуючих систем в тестикулярних стероїдогенних клітинах може обумовлювати, щонайменше, частину негативних ефектів етамбутолу, ізоніазиду і комбінації протитуберкульозних препаратів на рівень тестостерону і процеси сперматогенезу. Механізми порушень сперматогенезу, викликані рифампіцином і піразинамідом, вимагають більш детального дослідження, але, виходячи з наших спостережень, вони не залежать від тестикулярного CYP2E1.

Ключові слова: анти-андрогенний ефект, протитуберкульозні препарати, експресія мРНК, етамбутол, ізоніазид.

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СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ ЭФФЕКТОВ РАЗДЕЛЬНОГО И КОМБИНИРОВАННОГО ВВЕДЕНИЯ ПРОТИВОТУБЕРКУЛЕЗНЫХ ЛЕКАРСТВЕННЫХ СРЕДСТВ НА ТЕСТИКУЛЯРНУЮ ЭКСПРЕССИЮ CYP2E1 И РЕПРОДУКТИВНЫЕ ПОКАЗАТЕЛИ САМЦОВ КРЫС

Было проведено сравнительное изучение анти-андрогенных эффектов противотуберкулезных препаратов, а также их воздействия на тестикулярный CYP2E1. Исследовали экспрессию мРНК и белка CYP2E1 в семенниках, уровень общего тестостерона, фертильность и показатели сперматогенеза у самцов крыс при раздельном и совместном введении этамбутола, изониазида, рифампицина и пиразинамида. Анализ полученных данных доказывает важную роль этамбутола и изониазида в гонадотоксичного действия комбинации противотуберкулезных препаратов. Активация CYP2E1-зависимых метаболизирующих систем в тестикулярных стероидогенных клетках может обуславливать, по меньшей мере, часть отрицательных эффектов этамбутола, изониазида и комбинации противотуберкулёзных препаратов на уровень тестостерона и процессы сперматогенеза. Механизмы нарушений сперматогенеза, вызванные рифампицином и пиразинамидом, требуют более детального исследования, но, исходя из наших наблюдений, они не зависят от тестикулярного CYP2E1.

Ключевые слова: анти-андрогенные эффекты, противотуберкулезные препараты, экспрессия мРНК, этамбутол, изониазид.