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ВІТРИФІКАЦІЯ ЕМБРІОНІВ МИШЕЙ НА СТАДІЇ ПРОНУКЛЕУСІВ ПРИ ВИКОРИСТАННІ РІЗНИХ КРІОПРОТЕКТОРІВ ТА ЇХ ЗДАТНІСТЬ ДО ПОДАЛЬШОГО РОЗВИТКУ НА КУЛЬТУРІ КЛІТИН ЯЙЦЕПРОВОДІВ ДО БЛАСТОЦИСТИ

Наведено результати досліджень з кріоконсервування ембріонів мишей на стадії пронуклеусів методом вітрифікації на твердій поверхні із використанням різних кріопротекторів та оцінено їх життєздатність і подальший розвиток in vitro після деконсервування.

Ключові слова: вітрифікація, ембріонів мишей на стадії пронуклеусів, клітини яйцепроводів

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ВИТРИФИКАЦИЯ ЭМБРИОНОВ МЫШЕЙ НА СТАДИИ ПРОНУКЛЕУСОВ ПРИ ИСПОЛЬЗОВАНИИ РАЗЛИЧНЫХ КРИОПРОТЕКТОРОВ И ИХ СПОСОБНОСТЬ К ДАЛЬНЕЙШЕГО РАЗВИТИЯ НА КУЛЬТУРЕ КЛЕТОК ЯЙЦЕПРОВОДОВ К БЛАСТОЦИСТЫ

Приведены результаты исследований по криоконсервировании эмбрионов мышей на стадии пронуклеусов методом витрификации на твердой поверхности с использованием различных криопротекторов и оценена их жизнеспособность и дальнейшее развитие in vitro после деконсервирования.

Ключевые слова: витрификация, эмбрионов мышей на стадии пронуклеусов, клетки яйцепроводов.

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DISORDERS OF ANTENATAL AND POSTNATAL DEVELOPMENT IN LONG-TERM ALCOHOL EXPOSED MALE WISTAR RATS' OFFSPRING

It was established increase of CYP2E1 mRNA expression in rats' testes under chronic 15% ethanol solution administration. In addition, study results suggest significant alteration of locomotor and emotional reactivity in offspring of chronically alcohol-treated males as compared with control. Decrease of horizontal, vertical and exploration activities were recorded. Thus, a direct relationship between paternal alcohol exposure and offspring health and behaviour exists.

Keywords: CYP2E1 mRNA expression, rats' testes, ethanol.

It is well-known that chronic alcohol dependence causes testicular abnormalities and sexual dysfunctions both in humans and animals [1, 2]. Studies that included alcohol as a subject of investigation point to alcohol negative effect on all three factors that influence the male reproductive function: hypothalamus-hypophysis-gonads system, endocrine glands, and hormones [3]. The less attention has the paternal effects of alcohol-addicted male on the posterity. Relatively early epidemiological studies indicate an association between lowered birth weight in offspring and paternal alcoholism [4]. Adolescent sons of alcoholics and non-alcoholics were compared on a battery of intellectual, neuropsychological, personality, and behavioral measures. The former group demonstrated certain neuropsychological deficits in perceptual-motor ability, memory, and language processing [5]. Moreover, adoption studies suggest positive associations between hyperactivity and lowered cognitive abilities in offspring and alcoholism in the biological father but not the adoptive father [6].

It is evident that the human data on preconceptional alcohol consumption paternal effects are limited. And, what about the rodents' data, they are insufficient and contradictory due to great diversity of animal species/strains and models of alcoholism [7]. Due to these facts findings from studies in which males consume alcohol chronically are difficult to interpret, and they are potentially confounded. Moreover, multiple causal mechanisms for a paternal effect (including mutagenicity and epigenetic changes) have been suggested, but none seems satisfactory to explain all findings [7].

Based on this information and considering that alcoholism is chronic disease highly prevalent in the world population, the present work reports the effects of long-term preconceptional 15% alcohol consumption by male Wistar rats on the testicular *CYP2E1* gene expression level, as well as antenatal and postnatal development of these males' offspring. It should be noted that testicular CYP2E1 has attracted your attention, first of all, because this isoform could be involved in production of metabolites with genotoxic properties inducing germ cell mutations and having adverse effects on offspring [8].

Material and methods.

Wistar albino male rats, initial body weight of 150 g to 170 g, were used in the study. 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 study was performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and approved by the Institutional Animal Care and Use Committee.

For the experimental (chronic alcoholism) model, reproducing male rats were selected according to the method for measuring voluntary alcohol self-administration in rats, which provides a continuous choice between an alcohol solution and water (two-bottle preference test) [9].

Ten selected rats were used for chronic alcoholism modelling by replacing water with a 15 % ethanol solution during 150 days. The consumption of 15 % ethanol was measured as ml and was calculated as g x kg^{-1} x day^{-1} of pure ethanol. On this regimen daily ethanol consumption was in average 10 g x kg^{-1} x day^{-1}. Ten intact male rats (of the same age and weight) were used as controls. From the beginning of the experiment, they were kept in the same conditions as experimental animals, but were given only water *ad libitum*.

Following 150 days of 15% ethanol solution repeated self-administrations, the males from both groups were mated with virgin females in proestrus at the ratio 1 male : 2 females. According to generally accepted guidelines for the fertility study in laboratory rats [10] first day of pregnancy was established by vaginal cytology (first day of sperm detection in vagina). Most males were mated within the first 5 days of cohabitation (i.e. at the first available oestrus), but some of them demonstrated infertility. This fact was taken into account for evaluation of effect of ethanol on male fertilizing capacity, which was determined by the index:

$$\frac{\textit{number of pregnant females}}{\textit{number of females mated with males}} \times 100\%$$

After mating, both the experimental and control male rats were sacrificed under a mild diethyl ether anaesthesia by decapitation.

Testes were used for investigation of the expression of CYP2E1 mRNA by a reversed transcriptase polymerase chain reaction (RT-PCR). Testes samples (25 mg) were collected, 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, Ink., 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.] [11]. 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.

Part of females was sacrificed under mild ether anaesthesia via decapitation on day 20 of gestation for determination of embrional/fetal development indices. The numbers of corpora lutea in ovaries, of implantation sites and of live and dead fetuses in each uterine horn were counted after laparotomy of pregnant females. Indices of embryonic death at pre- and postimplantation periods of development were calculated according to standard procedures [12, 13].

Part of females was kept until natural delivery for examination of offspring postnatal development according to standard procedures [12, 13]. Litter size was evaluated as the total number of pups in the litter, dead or alive. Sex ratio was determined as the number of male pups divided by the number of females in the litter.

The behavioural of offspring was studied in the open-field and in hole board tests [14]. In the open-field during a 2-min trial the number of sections crossed, vertical standing positions, defecations (fecal boli) and urinations were recorded. The open-field was cleaned after every trial. 3 daily open-field trials were considered appropriate to test the emotional reactivity.

Numerical data are represented as means ± S.E.M. and were compared using unpaired Student's t-test to find out the level of significance between control and experimental data. Differences were considered statistically significant at $p<0.05$.

Results and discussion.

Many reviews describe an essential role of cytochrome P-450 2E1 (CYP2E1) in ethanol-induced toxic effects [15, 16], but interrelations between ethanol-caused testicular CYP2E1 induction and male reproduction remain out of sight of most researchers. It must be stressed, that series of studies clearly demonstrated presence of CYP2E1 in testis, suggesting its possible role in bioactivation of chemicals to their toxic metabolites directly in male gonads [17-19]. In keeping with this idea we performed RT-PCR to evaluate the effect of chronic alcohol intoxication on CYP2E1 transcriptional activation in testes.

It was demonstrated that the mRNA expression of CYP2E1 in alcohol-treated rats' testes was three times as much as compared with control group (Fig.).

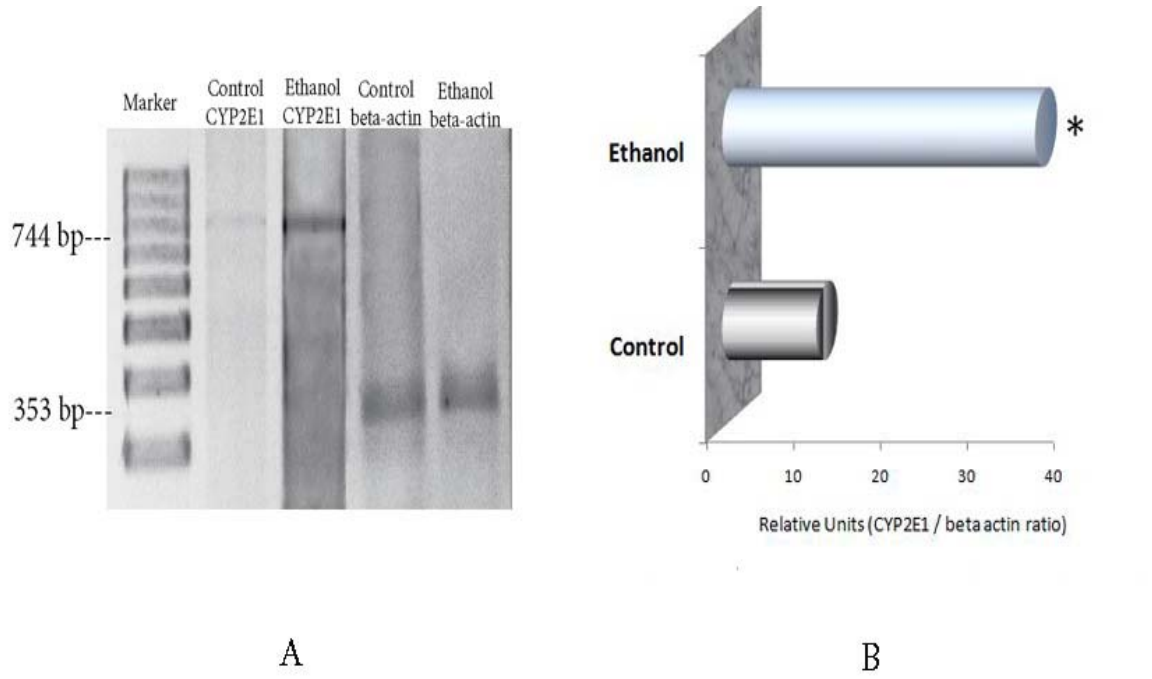

Figure. CYP2E1 mRNA in alcohol-treated rats'.
Representative electrophoregrams of CYP2E1 (744 b.p) and reference-gene β-actin (353 b.p.) RT-PCR products are shown in panel A. Average rate of CYP2E1 mRNA expression is shown in panel B, M ± S.E.M., n=6

*– P<0.05 in comparison with control

In our opinion activation of CYP2E1-dependent metabolizing systems in testes, at least partially, could determine gonadal toxicity of alcohol. Possibly induction of CYP2E1 also and may contribute to late paternal effects on offspring.

It should be noted that in our experiment we have fixed only slight decrease (15%) of male fertility (table 1). In experimental group of 20 mating females 16 became pregnant, while in the control group – 19.

Table 1 Rats male fertility index following 150 days alcohol consumption

Groups	Number of mated females	Number of pregnant females	Fertility index, %
Control	20	19	95
Chronic alcoholism	20	16	80

Thirteen females from control group and ten – from experimental group were sacrificed on day 20 of gestation for determination of embrional/fetal development indices. Six females from each group were kept until natural delivery for examination of offspring postnatal development.

The comparison of embryogenesis indices in experimental and control groups demonstrated great negative impact of long-term alcohol treatment on preimplantational lethality level (table 2), it was 32%, while in control group – 9.6%. The level of postimplantational embryonal and fetal death had no differences between groups.

The total embryonal/fetal death in alcoholic group increased 22% in comparison with control (table 2).

We found that female fecundity was not significantly reduced by mating with alcohol-treated males (Table 2). Group differences in litter size for foetuses also were not significant (Table 2).

***Table* 2. Effects of paternal alcohol exposure on intact females' fecundity and embryogenesis/fetogenesis parameters on day 20 of gestation**

Indices	Groups	
	Control	*Chronic alcoholism*
Number of pregnant females	13	10
Total number of corpora lutea	146	128
Number of corpora lutea per one female, mean ± S.E.M	11.23±0.73	12.80±0.90
Total number of implantational sites	132	87
Number of implantational sites per one female, mean ± S.E.M	10.15±0.55	8.70±0.87
Preimplantational loss, abs / %	17 / 9.58	41 / 32.03
Preimplantational loss per one female, mean ± S.E.M	1.31±0.41	4.10±0.88*
Postimplantational loss, abs / %	10 / 7.58	8 / 9.19
Postimplantational loss per one female, mean ± S.E.M	0.76±0.32	0.80±0.36
Total number of living foetuses, abs / %	125 / 94.69	81 / 93.10
Number of living foetuses per one female, mean ± S.E.M	9.62±0.40	8.10±0.82
Total embryonal/fetal death, %	14.38	36.72

*– *P*<0.05 statistically significant in comparison with control

It should be noted that these results seem to contradict those of Randall [et al.], who have reported no effects on prenatal mortality observed on day 19 of gestation, following chronic ethanol treatment of male mice for a month [20]. But our findings are in good accordance with another study demonstrating significant increase of total embryonic deaths following preconceptional alcohol consumption by male Long-Evans rats [21].

We didn't indicate negative effects of alcohol consumption on litter size (table 3). Accidents of dead born were not fixed in the control group, while in experimental group two accidents were recorded (table 3).

The percentage of males in litters sired by alcohol-treated males was slightly higher than the percentage sired by intact fathers, as well as sex ratio index (table 3).

***Table* 3. Effects of paternal alcohol exposure on some indices of foetuses state in the first day of postnatal development**

Indices	Groups	
	Control	*Chronic alcoholism*
Number of pregnant females, abs.	6	6
Total number of fetuses, abs	56	78
Alive fetuses, abs.	56	76
Dead born fetuses, abs.	0	2
Females/males, %	59 / 41	56 / 44
Sex ratio	0.69	0.78

Important indicator of the newborn is their survival during the first three weeks of postnatal life, and key terms for these observations is the 4^{th}, 7^{th}, 14^{th} and 21th days after birth. The data of table 4 evidence increase of newborns mortality at 4^{th} and 14^{th} days in alcoholic offspring. The total mortality in this group within 21 days of postnatal development was 10% (table 4).

***Table* 4. Effects of paternal alcohol exposure on newborns mortality (abs./ %)**

Groups	Number of new-borns	Terms of observation, days after delivery				Total mortality, abs. (%)
		4^{th}, abs. (%)	*7^{th} abs. (%)*	*14^{th} abs. (%)*	*21th abs. (%)*	
Control	56	- (0 %)	- (0 %)	- (0 %)	- (0 %)	- (0 %)
Chronic alcoholism	76	2 (2.6 %)	- (0 %)	6 (8,0 %)	- (0 %)	7 (10 %)

Newborn body weight is important indicator of its state. These data are presented in table 5.

***Table* 5. Effects of paternal alcohol exposure on newborns body weight**

Groups	Number of new-borns	The weight of one litter, g				
		1^{st} day	*4^{th} day*	*7^{th} day*	*14^{th} day*	*21th day*
Control	56	5.98±0.15	9.38±0.67	11.61±0.99	21.89±2.20	35.05±4.93
Chronic alcoholism	76	6.56±0.16*	9.71±0.45	14.67±0.96	24.56±0,89	42.50±4.23

It was recorded, that on the first postnatal day the weight of one litter in alcoholic group significantly increased as compared with control. In the further terms of observations the litter weight in this group has no significant difference with control (table 5).

Normal physical development of offspring is characterized by timely appearance of certain external features, which inherent in each individual during postnatal life. Results on offspring physical development are presented in table 6. And according to it, prolonged paternal consumption of alcohol has no significant effects on terms of appearance of all investigated external features.

***Table* 6. Effects of paternal alcohol exposure on offspring physical development**

Parameters	Groups	
	Control	Chronic alcoholism
Pinna detachment appearance day	2.18±0.06	2.1±0.04
Hair growth appearance day	4.74±0.07	4.78±0.06
Teeth eruption appearance day	6.0±0	6.0±0
Eye opening appearance day	13.63±0,08	14.0±0
Testes descent day	23.0±0	23.0±0
Vaginal opening day	31.21±0,12	32.0±0.15

Offspring, which was obtained from intact females and alcohol-addicted males, were checked for the essential reflexes and locomotor development on the first postnatal day (surface righting, cling, tail pull, rotation, linear movement, climb, exploration). It was shown that all reflexes in both males and females pups were within normal limits.

The evaluation of behavioural effects is an important component in developmental toxicity studies. In the table 7 we represent the results of alcohol-addicted males' offspring behaviour in the open field in comparison with control group.

***Table* 7. Effects of paternal alcohol exposure on offspring behaviour in the open field (mean ± S.E.M.; n=24)**

Parameters		Groups	
		Control	Chronic alcoholism
Latent period, sec		1,29±0,20	1,03±0,30
Horizontal activity, number of sections crossed within 3 min	peripheral sections	92,83±4,60	62,09±6,90*
	central sections	20,50±2,60	12,00±1,40*
Vertical activity, number of rearing within 3 min		11,41±0,94	8,40±1,01*
Exploration activity, number of looking into holes within 3 min		9,95±0,78	6,37±0,82*
Grooming behavior, number of reactions within 3 min		1,25±0,17	1,68±0,37
Urinations, number within 3 min		1,0±0,18	0,90±0,13
Defecations, number within 3 min		1,25±0,25	1,12±0,19

The present findings suggest significant alteration of locomotor and emotional reactivity in offspring of chronically alcohol-treated males as compared with control. Particularly, in experimental group we recorded decrease of horizontal (number of crossed peripheral and central sections were less than in control 32% and 44% respectively), vertical (26%) and exploration activities (46%). Thus, our data provide support for relationship between offspring's open field activity and paternal alcohol intake.

By contrast, E.L. Abel have uncovered opposite behavioural effects of paternal alcohol exposure. Notably, 18-day-old offspring of male rats intubated with 3 or 2 g/kg ethanol twice a day for 7 month were more active in the open field compared with controls, and they took a greater number of trials to complete a passive avoidance learning task [22]. Our data on behaviour are more consistent with another study of the same author demonstrating dose-dependent decreases in physical activity of alcohol addicted mice males' offspring at 20 and 24 days of age [23].

Also our results partially are in accordance with other similar laboratory studies, which have indicated that exposure of male mice and rats to alcohol has numerous effects on their offspring, including reduced litter size, developmental retardation, increased mortality, and compromised immunity as well as behavioural deficiency, such as impaired discrimination on spatial tasks and altered aggressive, risk taking, and anxiety-like behaviour [24-29]. The severity of these effects was related to the duration and dosage of ethanol exposure. There were also interspecies differences. But, in general, our current investigation, as well as studies of other authors, provides evidences that the exposure of males to ethanol can lead to behavioral changes in offspring, likely via the paternal germline.

Here it is important to note that remains difficult to disentangle the effects of mutations from epigenetic modifications in perpetuating paternal effects, but there are some evidences for speculation on the role of epigenetic factors in explaining the effects of paternal alcohol consumption on offspring. For example D.M. Bielawski [et al.] have revealed reductions in DNA-methyltransferase RNA in sperm, as well as reductions in offspring weight after chronic alcohol treatment of male rats. These results suggest that alterations in DNA methylation in the germ line may lead to physical abnormalities in offspring [309]

On the other hand it is known that induction of CYP2E1 leads to prominent epigenetic effects [16]. Probably, in our experiments the activation of testicular CYP2E1 triggered epigenetic events leading to paternal effects on posterity.

In sum, the paternal effects of ethanol described above are consistent with a growing body of evidence for the existence of transgenerational response phenomena (i.e., the transmission of environmentally induced states from exposed generations to unexposed generations The mechanisms are likely to involve alcohol-induced epigenetic changes in the gametes or, alternatively, selection effects within the germ line [7].

Conclusion. Our findings suggest that offspring not directly exposed to alcohol *in utero* may nevertheless be born with developmental abnormalities if their father consumed alcohol prior to conception. Thus, a direct relationship between paternal alcohol exposure and offspring health and behaviour exists.

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ПОРУШЕННЯ АНТЕНАТАЛЬНОГО ТА ПОСТНАТАЛЬНОГО РОЗВИТКУ НАЩАДКІВ ЩУРІВ-САМЦІВ ЛІНІЇ ВІСТАР, ТРИВАЛО СПОЖИВАНОГО АЛКОГОЛЮ

Було встановлено зростання експресії мРНК CYP2E1 в сім'яниках щурів за умов хронічного введення 15% розчину етанолу. У той же час було показано значний негативний вплив довгострокового споживання самцями алкоголю на рівень доімплантаційної смертності їх потомства. Таким чином, існує прямий зв'язок між впливом алкоголю на батька та здоров'ям і поведінкою потомства.

Ключові слова експресії мРНК CYP2E1 сім'яниках щурів, етанол.

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НАРУШЕНИЕ АНТЕНАТАЛЬНОГО И ПОСТНАТАЛЬНОГО РАЗВИТИЯ ПОТОМСТВА КРЫС-САМЦОВ ЛИНИИ ВИСТАР, ДЛИТЕЛЬНО ПОТРЕБЛЯВШИХ АЛКОГОЛЬ

Было установлено рост экспрессии мРНК CYP2E1 в семенниках крыс в условиях хронического введения 15% раствора этанола. В то же время было показано значительное негативное влияние долгосрочного потребления самцами алкоголя на уровень доимплантационной смертности их потомства. Таким образом, существует прямая связь между воздействием алкоголя на отца и здоровьем и поведением потомства.

Ключевые слова экспрессии мРНК CYP2E1 семенниках крыс, этанол.