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# Modulation of sexual behavior and indicators of oxidative stress in the testes of adult rats as a consequence of chronic stress during puberty

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**Abstract.** Children are often subjected to psychological or physical violence, experience strong emotional distress. Early life stress may lead to disorders of somato-sexual development, to delayed (in boys) or accelerated (in girls) puberty, delayed growth, immune system disorders, alteration of mental health. The sexual performance of adult men who experienced chronic stress during adolescence is not reflected in the scientific medical literature. Deferred effect of stress at adolescence on the androgen profile and sexual behavior of laboratory male animals has been studied very poorly. There are no data on long-term oxidative effects of stress experienced by human and animal adolescents in relation to the organs of the reproductive system. **The aims** of this work were to explore the sexual behavior, testosterone levels in blood and lipid peroxidation (LPO) in the testes of adult male rats in a distant period of time after chronic immobilization stress during puberty. White male rats were subjected to immobilization in plastic tubes for 1 hour a day during postnatal days 30-45. The non-stressed animals were used as controls. Some rats were decapitated at the age of 6 months, and the trunk blood samples were collected for hormone assay. Testosterone levels in blood serum were determined by immunoassay. The testicles were isolated to determine the content of the LPO products. At 7 months of age, the males were tested for the exhibition of male-type sexual behavior, and at 8 months for female-type sexual behavior after orchietomy and priming with estradiol and progesterone. In stressed at puberty adult rats, testosterone levels did not differ from those of controls. Pubertal stress significantly reduced latency of the first and second mounts, that is increased the motivational component of male sexual behavior without increasing the number of mounts and intromissions. Stressed rats did not exhibit lordosis reaction in the presence of active male. As a result of stress in rats during puberty, the content of malonic dialdehyde and diene conjugates increased in the testicles of adult animals, which indicates the presence of oxidative stress.

**Keywords:** stress, puberty, sexual behavior, male rats, testosterone, lipid peroxidation.

Interest of scientists in the problems of stress and related diseases has never faded. The modern way of life, especially in developed countries, creates enormous psychological, emotional and phys-

ical burden on a person. When the war came to the land of Ukraine, interest in stress and its health consequences was renewed even more. And as in any emergency situation, the most vulnerable segment of the population turned out to be children. Today, in many regions of Ukraine, they live under the

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howl of sirens and the roar of bombings; many are forced to move with their families to a foreign environment, including to other countries, where they are forced to adapt to new living conditions. According to an online survey of internally displaced school-aged children and adolescents in Ukraine in connection with Russian aggression, a quarter of the children experienced panic attacks, feelings of fear, danger and defenselessness, about half experienced anger, nightmares, *etc.* [1]. But even in peacetime, children are often subjected to psychological or physical violence, experience strong emotional distress.

Early life stress may lead to disorders of somatosexual development, to delayed (in boys) or accelerated (in girls) puberty, delayed growth, immune system disorders, alteration of mental health, neuropsychiatric disorders, *etc.* Frequent consequences of chronic stress in adolescence are increased anxiety and depression due to stress-induced imbalance of hormones and neurotransmitters in the central nervous system, which is confirmed by clinical and experimental studies [1, 2-4].

One of the negative long-term consequences of chronic stress is disorders of the reproductive system, as evidenced by numerous clinical observations and results of animal research [5]. Common manifestations of post-traumatic stress disorders in men are weakened libido, erectile dysfunction, deterioration in sperm quality and its fertilizing ability. Early life stress is one of the reasons for decreased fertility. In adolescent girls, stress is a common cause of infertility [6]. It may cause primary hypothalamic amenorrhea because of stress-induced suppression of the secretion of GnRH, LH, FSH, and disorders of pulsatile secretion of gonadotropins [7], and accompanied by polycystic ovaries syndrome in almost 60% of patients [8]. There are many reports of risky sexual behavior in young men as a result of early stress associated with sexual abuse. However, the sexual performance of adult men who experienced chronic stress during adolescence is not reflected in the scientific medical literature.

Maternal separation of newborn male and female rats from 2 to 11 days of life did not affect their sexual behavior in adulthood [9]. Decreased sexual motivation a few months after chronic stress during puberty [10], and increased sexual activity [11] as an immediate result of stress in male rats during adolescence have been reported. Therefore, one of

the objectives of this work was to study the sexual behavior and testosterone levels in adult rats in a distant period of time after chronic immobilization stress during puberty.

In information databases, we have not found any studies of long-term oxidative effects of stress experienced by human and animal adolescents in relation to the organs of the reproductive system. For this reason, another aim of the study was to determine the content of LPO products in the testes of adult rats exposed to immobilization stress during the pubertal period of individual development.

### Material and methods

**Ethical approval.** The experiments were carried out in accordance to European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, March 18, 1986), and Recommendations of the First National Congress on Bioethics Issues (Kyiv, Ukraine, September 20, 2001). The experimental design and procedures were approved by the Bioethics Commission of the Institute (the Protocol No 43-KE from June 06, 2022).

**Design of the study.** White female rats of the local Institute vivarium breeding with a regular 4-5 estrous cycle were recruited for fertilization. The animals were housed and treated under standard conditions. They were placed in the cages one by one together with sexually active male, and the day of appearance of spermatozoa in the vaginal smear was considered the first day of conception. Experimental and control male offspring groups were formed by randomization and allowed to be kept in the institutional vivarium until 6-8 months of age.

During postnatal days 30-45, the male offsprings were undergone to everyday strict restriction in plastic tubes for 1 hour a day. Taking into account the daily rhythm of fluctuations in the level of corticosterone in the blood, stress was carried out in the interval of 9-12 am. The non-stressed animals were used as controls.

Some males were decapitated at the age of 6 months and the testes were isolated to determine the content of the LPO products.

At 7 months of age, the males were tested for the exhibition of male-type sexual behavior, and at 8 months for female-type sexual behavior.

**Hormone assay.** After prompt decapitation, the trunk blood samples were collected, and blood

serum was obtained by centrifugation and kept at  $-20\text{ }^{\circ}\text{C}$  prior to be analyzed for testosterone levels. Hormone immunoassays were carried out with Testosterone ELISA kit (LDN, Germany) followed by measuring at the immunoenzymatic Stat Fax analyzer (USA).

**Male-type sexual behavior.** To test male-type sexual behavior using the traditional method [12, 13], 6 animals delivered by different mothers were included into each animal group. The males were kept in the darkness for 4 hours, then they were moved to an empty cage for a 5-minute adaptation. One week before testing, the partner female was ovariectomized and injected intramuscularly with 0.1 mg estradiol diacetate (Sigma, USA) 48 hours before the test. 0.5 mg progesterone oil solution (Biopharma, Ukraine) was introduced 4 hours prior to the test. Then the female was placed in the cage under dim red light with the male. Over the course of 15 min, the following indices of copulative behavior were recorded: duration of latent periods of the first mount, and the first and second intromissions, the first ejaculation, post-ejaculatory refractory period, the numbers of ejaculations, mounts without intromission, and the total number of intromissions. Male-type behavior was tested twice at one-week interval taking into account that after the first test they gained some sexual experience.

**Female-type sexual behavior.** Because of female-type sexual behavior (in particular, lordotic reactions) depends of the sensitivity of the neuroendocrine behavior centers to estrogens, it was tested in previously castrated males, which were administered steroids at a way similarly to that of females. The males were introduced to sexually experienced males that were kept in the test cage under red dim light for at least 5 min. Testing lasted 10 min or up to 10 mounts of an active male. The number of lordotic reactions to approaches or mounts of the normal male was recorded, and lordosis index was calculated as percentage of the number of lordosis reactions referred to the total number of approaches and mounts of an active male.

**Determination of LPO products.** The contents of LPO products, diene conjugates (DC) [14] and malonic dialdehyde (MDA) [15] were determined in tissue homogenates of testicles and calculated per mg of protein, the content of which was measured by the Lowry method.

**Statistical analysis.** The results were averaged and compared with those of appropriate controls. They were presented as mean ( $M\pm m$ ) and processed with Excel computer program by one-way analysis of independent experiments using the *t*-Student's criterion in a case of normal distribution of variants which was tested according to the Shapiro-Wilk criterion. In the absence of a normal statistical distribution, the Wilcoxon-Mann-Whitney non-parametric *U* criterion was used. The difference was set as significant at  $p\leq 0.05$ .

## Results

**Hormone levels.** At the end of the experiment, testosterone levels in blood serum of stressed at puberty 6-month-old rats ( $n=5$ ) did not differ from those of controls ( $n=7$ ): ( $4.89\pm 0.77$  ng/mL *vs.*  $4.35\pm 0.58$  ng/mL correspondingly,  $p>0.05$ ).

**Male-type sexual behavior.** Male-type behavior was tested twice at one-week interval taking into account that after the first test they usually gain some sexual experience.

Temporal and quantitative characteristics of sexual behavior by male type were analyzed separately for each of the two tests, because for some parameters the presence of statistically significant difference between the results of the first and second tests was noted, which is associated with the acquisition of sexual experience and the formation of stereotypical behavioral reactions.

The results of the study of sexual behavior by male type in 7-month-old male rats are shown in **Table 1**. The distribution of quantitative parameters in the control and experimental groups was non-parametric, so they are presented as medians and the scope values in the groups.

Analysis of the data obtained shows a significant reduction in the time to the first mounting and a noticeable tendency to reduce the latent period of the first and second intromissions in the first testing. In the second testing, the latent period of the second intromission was significantly reduced.

**Female-type sexual behavior.** When testing female sexual behavior, no lordosis reactions were detected in males of both groups. In the group of stressed males, homosexual behavior was detected in one male, *i.e.* matings were noted on a normally active male (**Table 2**).

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**Table 1.** Male sexual behavior parameters of 7-month-old pubertal-stressed male rats and control animals

Feature	First test		Second test	
	Control	Stressed	Control	Stressed
<b>Latency period, sec</b>				
first mount	74 (8-279)	8.5 (2-15)*	2 (1-355) <sup>+</sup>	1 (1-3) <sup>+</sup>
first intromission	89 (28-519)	27 (9-548)	10 (2-464) <sup>+</sup>	9 (3-10) <sup>+</sup>
second intromission	161 (45-611)	37 (11-675)	29 (4-576)	13 (7-22) ** <sup>+</sup>
first ejaculation	-	-	-	550
postejaculatory refractory period	-	-	-	881
<b>Number</b>				
mounts without intromission	4.5 (4-9)	3,5 (1-6)	3 (2-6)	3 (1-5)
mounts with intromission	8 (2-17)	22(2-22)	12 (4-26)	16 (14-21)
ejaculations	0	0	0	1

Notes: 6 rats in each group. The test lasted 15 min. The data are presented as medians and minimum, and maximum values (in the brackets). Statistical analysis by Wilcoxon-Mann-Whitney U criterion. \* –  $p \leq 0.05$  compared to controls; <sup>+</sup> –  $p \leq 0.05$  compared to the first test.

**Table 2.** Parameters of female sexual behavior of 8-month-old pubertal-stressed male rats

Parameter	Intact males (control)	Stressed males
Number of lordosis	0	0
Lordosis index	0	0
Number of mounts of a receptive male on active male	0	10
Number of males with:		
- receptive female behavior	0/6	0/6
- homosexual behavior	0/6	1/6
- bisexual behavior	0/6	0/6

Note: 6 rats in each group. The duration of the test was 10 minutes.

**LPO products in testes**

The results of measuring the content of LPO products in the testicles of normal and stressed at puberty rats are shown in **Table 3**. The obtained data indicate the intensification of LPO in the testicles, which is a sign of oxidative stress.

**Table 3.** The content of LPO products in the testes of 6-month-old rats (nmol/mg protein)

Animal group	MDA	DC
Controls (n=8)	2.40±0.04	0.73±0.01
Stressed (n=6)	3.68±0.10*	1.27±0.04*

Note: \* -  $p < 0.05$  compared to controls.

**Discussion**

Immobilization of early pubertal rats for two weeks is an experimental model of chronic stress, because, unlike adults, adolescents did not demonstrate habituation to chronic stress and responded more strongly to repeated stress [16].

It is known that male sexual behavior consists of two main components – motivational (*i.e.*, heterosexual orientation and sexual desire) and copulatory one. In studies of male rat sexual behavior, the motivational component is quantitatively characterized by the length of time from the mating of a receptive female to the first mounting, *i.e.*, the corresponding latent period. In this work, the study of sexual behavior revealed a reliable dramatic reduction of the latent periods of the first mount: the median value in adult stressed males in the first testing decreased by 8.7 times in comparison with the control group of intact males. The latent time indicators of the first and second intromission in the first testing had a clear tendency to decrease. In the second testing, the latent period of the second intromission was more than halved.

There is a report on a weakening of sexual motivation in 3-month-old males who experienced stress from 25<sup>th</sup> to 50<sup>th</sup> postnatal day [10]. Contrary, the data obtained in our study definitely indicate a significant increase in sexual motivation against the background of a normal level of testosterone in the blood of adult animals five and a half months after prolonged stress during puberty.

Perhaps this difference is explained not so much by more duration of stress as by a different method of stressing: the rats were kept in social isolation, one by one in the cage, which caused psychoemotional stress.

In another study [3], immobilization of male rats for 6 hours daily for 15 or 60 days, starting from the 40<sup>th</sup> day of life, led to a prolongation of the latent period of the first mating, however, the authors concluded that stress had a stimulating effect on copulatory sexual behavior, because the frequency of intromissions increased by 2.5 times. In the meantime, they tested sexual behavior at the end of stressing period, that does not allow to compare their results with our data.

Thus, on the basis of findings of this work, it is possible to draw a conclusion about a certain stimulating effect of a prolonged state of immobilization stress during puberty on the male sexual behavior of adult male rats. This effect did not depend on the testosterone blood levels, therefore it was associated with modulation of neuroendocrine control of sexual behavior.

As mentioned above, in our study testosterone levels remained normal in the experimental males. Interestingly, other investigators have found out an increase in testosterone level in male rats exposed to pubertal stress by everyday 6 hours immobilization since 40<sup>th</sup> to 55<sup>th</sup> days [17] or a decrease when stressing was prolonged up to 100<sup>th</sup> day of life [18]. However, hormone assay has been done immediately after end of stressing. Possibly, experiment timing has a crucial role in those controversial results.

The results of testing female sexual behavior in stressed males, castrated and treated with sex hormones, indicate the preservation of refractoriness to the stimulating effect of estrogens on the hypothalamic secretion of LH-releasing hormone, which is characteristic of the female sex. Therefore, androgen-dependent sexual differentiation of the brain remains normal, which is consistent with modern ideas about the critical period of this process in the perinatal period of individual development of rats [19].

All types of stress are accompanied by a violation of pro-antioxidant status, which is an important factor in the pathogenesis of stress-induced disorders, including reproductive disorders [20, 21]. On the other hand, oxidative stress may be one of the long-term negative consequences of chronic stress. Unfortunately, the few retrospective studies of this problem in humans leave the exact age at which they were exposed to early life stress un-

known [22-24]. For the first time, it was found that chronic pubertal stress causes a state of oxidative stress in the testes in adult rats, which is confirmed by a 50% increase in the content of malonic dialdehyde and diene conjugates in them.

There are reports on delay of testicular maturation and decreased spermatogenic and androgenic testicular functions in adult rats submitted to immobilization-induced stress from prepuberty [11, 17, 18]. It is likely that the damaging effect of oxidative stress may cause disturbances in the generative and steroid-synthesizing function of the testicles.

## Conclusions

1. Chronic pubertal stress increased the motivational component of male sexual behavior in adult male rats without increasing the number of mounts and intromissions.
2. Pubertal immobilization stress does not cause feminization of the sexual behavior in adult males.
3. As a result of stress in rats during puberty, the content of LPO products increases in the testicles of adult animals, which indicates the presence of oxidative stress.

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## Abbreviation

LPO – lipid peroxidation

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### Модуляція статевої поведінки і показники оксидативного стресу в сім'яниках дорослих щурів як наслідок хронічного стресу в пубертатному віці

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**Резюме.** Діти часто піддаються психологічному чи фізичному насильству, відчувають сильні емоційні переживання. Стрес у ранньому віці може призвести до порушень сомато-статевого розвитку, до затримки (у хлопчиків) або прискореного (у дівчаток) статевого дозрівання, затримки росту, розладів імунної системи, зміни психічного здоров'я. Статева активність дорослих чоловіків, які пережили хронічний стрес у підлітковому віці, не відображена в науковій медичній літературі. Відтермінований вплив пубертатного стресу на андрогенний профіль і статево поведінку самців лабораторних тварин вкрай слабо досліджені. Немає даних щодо оксидантних ефектів тривалого впливу стресу, який відчувають підлітки людини і тварин, на органи репродуктивної системи. Цілі цієї роботи полягали в дослідженні сексуальної поведінки, рівня тестостерону в крові та перекисного окислення ліпідів (ПОЛ) у статевих залозах дорослих самців щурів у віддалений період часу після хронічного іммобілізаційного стресу під час статевого дозрівання. Білих самців щурів піддавали іммобілізації в пластикових тубах протягом 1 год на добу з 30 по 45 день постнатального життя. Тварини без стресу були використані як контроль. Деякі щури були декапітовані у віці 6 місяців, а зразки крові були зібрані для гормонального аналізу. Рівень тестостерону в сироватці крові визначали імуноферментним методом. Яєчка виділяли для визначення вмісту продуктів ПОЛ. У віці 7 місяців самців перевіряли на статево поведінку чоловічого типу, а у 8 місяців – статево поведінку жіночого типу після орхіектомії та попередньої обробки естрадіолом і прогестероном. У дорослих щурів, підданих стресу під час статевого дозрівання, рівень тестостерону не відрізнявся від такого в контрольних тварин. Пубертатний стрес достовірно зменшував латентність першої та другої садок, тобто посилював мотиваційний компонент статевої поведінки самців без збільшення кількості садок і інтромісії. Стресовані щури не виявляли реакції лордозу в присутності активного самця. У результаті стресу в період статевого дозрівання в сім'яниках дорослих тварин підвищувався вміст малонового діальдегіду та дієнових кон'югатів, що свідчить про наявність окисного стресу.

**Ключові слова:** стрес, пубертація, статева поведінка, самці щурів, тестостерон, перекисне окислювання ліпідів.

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