THE CONSEQUENCES OF PHYTOESTROGENIZATION OF THE FATHER AND THE EFFECTS OF PHYTOESTROGENS DURING PUBERTY FOR MALE OFFSPRING

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Exposure to phytoestrogens (PE) during prepuberty and puberty can modulate the functioning of the reproductive axis, causing irreversible damage to reproductive programming.

The aim of the study was to investigate the state of the reproductive system of male offspring of adult rats that were exposed to phytoestrogens in the pubertal period of ontogenesis.

Materials and methods. The work was performed on adult male and female Wistar rats and their male offspring. In the experiment, the biological effect of PE was studied when applying a dose of 20 mg/kg of body weight for 30 days to the father and/or offspring of puberty age starting from the 45th day of postnatal life. Upon reaching the age of six months, male offspring of all studied groups were examined for reproductive function.

Results. The effect of estrogen-like substances on male reproductive function is manifested not only under the conditions of their intake in the critical periods of the embryonic and postnatal periods, but also, even when acting on the germ cells of parents. In male offspring, androgen secretion is disturbed, the hormonal status changes in the direction of hyperestrogenization, fertility decreases due to the reduced quality of germ cells against the background of a normal spermogram.

Conclusion. The reproductive function of sexually mature male offspring of a phytoestrogenized father who received a mixture of phytoestrogens during puberty is characterized by differences in sexual behavior, a decrease in the reproductive potential of males, which occurs due to a decrease in the share of effective fertilization, which indicates negative changes in spermatozoa, the development of which took place in conditions of absolute and relative hyperestrogeny. This indicates that phytoestrogens, as an environmental factor, have adverse consequences not only for individuals who directly use them, but also for their male offspring.

Keywords: phytoestrogens, reproductive system, male offspring, spermogram, sexual behavior, fertility, sex hormones

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1. Introduction

Dietary phytoestrogens (PE) are bioactive compounds with estrogenic activity. As the popularity of plant-based diets has grown, so has the consumption of PE-rich foods, derived from them. Phytoestrogens are polyphenolic molecules with structural similarities to endogenous human hormones, hence their estrogenic activity. The main dietary source of these plant secondary metabolites is legumes (especially soybeans), and to a lesser extent fruits, vegetables, and cereals [1]. Preclinical evidence suggests that these compounds may affect hormones and health, although the results of human trials are unclear. The effect of dietary PEs depends on exposure (type of PE, concentration and bioavailability), ethnic origin, hormone level (related to age, sex and physiological state) and health status of the consumer [2].

According to their chemical structure, phytoestrogens are divided into three classes: isoflavones (genistein, daidzein, alicetein), lignans (enterolactone, enterodiolene), coumestans.

The probable mechanism of PE action is binding to the estrogen receptor (ER). The effect of isoflavones, which have five times greater affinity for β-ER than α-ER [3], on the endocrine system may occur through modulation of the hypothalamic-pituitary axis [4]. However, not all biological effects of PE involve estrogen receptors. They can also activate serotonergic and insulin-like growth factor 1 receptors, induce free radical scavenging and modify tyrosine kinases, cyclic adenosine monophosphate, phosphatidylinositol-3-kinase/Akt, mitogen-activated protein kinase, nuclear factor kappa β transcription, and promote DNA methylation and affect the expression of histones and RNA. In addition, PE can act as intracellular regulators of the cell cycle and apoptosis.

Thus, due to their antioxidant, antiproliferative, antimutagenic and antiangiogenic roles, PEs can improve health [5]. In addition, some authors have observed that estrogen and androgen appear to be involved in breast and prostate cancer by regulating proliferative and migratory signaling, such as Src/PI3K. The response to hor-
mone therapy may vary depending on the interaction between estrogen or androgen receptors and proteins according to hormone levels [6, 7].

Despite the weak estrogenic effect compared to estradiol [8], with long-term use, the effect can be both estrogenic and antiestrogenic - depending on the dose and the presence of endogenous estradiol. The higher the dose of PEs, the greater their anti-estrogenic effect [9].

Traditionally, more attention was paid to the pathogenetic role of PEs, particularly in infertility, in the female body. It was this direction that laid the foundation for future research in 1946 [10]. Currently, there are many experiments and clinical studies that prove that, under certain circumstances, PEs can play a provoking or pathogenetic role in the occurrence of reproductive disorders. In addition, the presence of estrogen-like effects in the absence of complications inherent to natural female hormones determined the therapeutic use of PE mainly in women of climacteric and menopausal age as hormone replacement therapy [2, 11, 12].

The period of development, during which people are exposed to endocrine disruptors, is a very important factor [13, 14]. This fact encompasses the concept of the origin of health and the development of disease, which is increasingly discussed in the scientific community and is nothing more than the relationship between environmental influences during the stages of development that lead to diseases in adulthood. The concept is that environmental stressors, including malnutrition and exposure to these deregulators during critical periods of development, cause changes in gene expression that result in permanent changes in an organ, tissue, or structure. These changes lead to greater health and disease risks later in life [15, 16].

The developing organism is considered being in a period of extreme plasticity, in which gene expression and cellular signaling pathways are sensitive to environmental cues and able to adapt as a result of these stimuli. These adaptations can negatively impact health, leading to higher disease risk, as this plasticity is more prevalent during key developmental windows, i.e., the perinatal gestation period and prepubertal period [17, 18]. The normal development of the reproductive system of males and females in different species goes through different phases. They are sensitive to deregulation, as they have a plastic characteristic and can adapt to external stimuli [19]. Thus, germ cells (oocytes and sperm) in the developing gonads are also vulnerable to early life events, as the development and maturation of these cells is also regulated by the secretion of gonadal hormones and the activity of the hypothalamic-pituitary-gonadal axis (HPGA) [17].

The onset of puberty can be characterized as a process of maturation of the HPG axis, a transitional period between juvenile and adult states, which leads to secondary sexual characteristics and fertility [20]. In fact, it is during the prepubertal period that the hypothalamus receives more powerful stimuli for the secretion of gonadotropin-releasing hormone, which stimulates the production of gonadotropins in the pituitary gland with a subsequent effect on the cells present in the gonads [21].

Due to these developmental characteristics, exposure to isoflavones during prepuberty and puberty can modulate the functioning of the reproductive axis, causing irreversible damage to HPGA programming and persistent impairment of reproductive function [22].

Therefore, the aim of our study was to investigate the state of the reproductive system of male offspring of adult rats that were exposed to phytoestrogens in the pubertal period of ontogenesis.

2. Materials and methods

The research was conducted in accordance with the national "General ethical principles of animal experiments" (Ukraine, 2001), which are consistent with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1985) [23]. And in accordance with the principles of working on animals, approved by the Bioethics Committee of the State University "Institute of Endocrine Pathology Problems named after V. Y. Danylevskyi, NASof Ukraine" (protocol No. 6 dated 12/15/2019).

Experimental animals were kept in the vivarium of the State University "Institute of Problems of Endocrine Pathology named after V. Y. Danylevskyi of the NAS of Ukraine" under natural lighting and a diet, recommended for this species of animals, and drinking regime ad libitum [24].

The work was performed on 70 adult male and female Wistar rats weighing 220-320 g and their male offspring in the amount of 36 heads. Animals were euthanized by quick decapitation without the use of anesthesia to prevent effects on the level of sex hormones.

The experimental animals were divided into three groups of 12 heads each: 1) intact father/intact offspring – F(I)/O(I); 2) father received PE/offspring also received PE – F(PE)/O(PE); 3) father is intact/offspring received PE – F(I)/O(PE).

To simulate the alimentary supply of an excess of PE, the preparation Genistin Soy Complex isoflavone-rich (Soylife, USA) was used, the relative content of isoflavones in which (in terms of individual aglycones) was: 1) daidzein 60 %, 2) glycitein 22 %, 3) genistein 18 %. The dose was calculated according to the so-called "genistein equivalent", which shows the conditional estrogenic activity of various bioflavonoids relative to the indicated activity of genistein. The required dosage of the drug was monitored every Sunday according to the weight of the animal. In the experiment, the biological effect of PE was studied when applying a dose of 20 mg/kg of body weight for 30 days to the father and/or offspring of puberty age, starting from the 45th day of postnatal life.

Infant rats of all groups were weaned from their mothers at the age of 30 days. In the future, the dynamics of the body weight of males was monitored. Upon reaching the age of six months, the sexual behavior, fertility, state of spermatogenesis, hormone level, and mass of the examined organs were studied in the male offspring of all studied groups.

The sexual behavior of the males was studied for 15 min in a paired test with an ovariectomized receptive female at dusk. The test determined the number of approaches of the male to the female, the number of mounts, intromissions and ejaculations. Using a stop-
watch, time indicators were evaluated: the latency of mounts, intromission, ejaculation, the duration of the post-ejaculatory interval, the coefficient of mount/intromission was calculated.

Ovariectomy of the female rats was performed according to the generally accepted method. Receptivity in ovariectomized females was induced by sequential administration of oil solutions of estradiol-dipropionate (10 μg per rat, subcutaneously) for 48 h and progesterone (500 μg per rat, subcutaneously) 4–5 h before the test.

Fertility of the males was determined by the results of mating with intact females. The first day of pregnancy was considered the day when spermatozoa were found in morning vaginal smears. Fertilization index (as the ratio of the number of fertilized females to the number of females in the group) and pregnancy index (as the ratio of the number of pregnant females to the number of fertilized) were calculated. The females were euthanized on the 20th day of pregnancy, the ovaries were removed, in which the number of corpora lutea of pregnancy was counted, and also the number of implantation sites and fetuses in the uterus. The level of pre-implantation, post-implantation and total intrauterine losses in pregnant females was determined.

The state of spermatogenesis was studied according to the generally accepted method, determining the concentration of epididymal sperm, their motility and the percentage of pathological forms. Morphological studies of testes were carried out.

At the end of the experiment, the weight of the body and organs of the males was determined, namely: testicles and their appendages (epididymis), seminal vesicles, ventral lobe of the prostate gland, adrenal glands, pituitary gland, and the suspension of epididymal spermatozoa was examined to determine the state of spermatogenesis. Blood serum samples were collected to determine the concentration of hormones (T and E2), which were stored until analysis at a temperature of ~ 18 °C. The level of sex hormones was determined with the help of test kits "Estradiol-IFA", "Testosterone-IEA" and "Corticosterone-IEA" (HEMA LLC, Kyiv).

The statistical analysis of the obtained data was carried out using Excel 2003 and Statistica 6.0. The normality of the distribution of variables was determined using the Kolmogorov-Smirnov test. To compare indicators, characterized by a normal distribution, the Student's t test was used. Scheffe's method was used to compare several groups with a normal distribution. U-Mann-Whitney tests were used to compare variables with a distribution other than normal. The obtained results are presented in the tables as the arithmetic mean (т) and its error (Sₜ). The statistical hypothesis testing was carried out at the significance level p < 0.05.

### 3. Research results

In the period from the first to the thirtieth day of postnatal life, rat pups undergo rapid somatic growth, transition to self-feeding and existence apart from the mother. Events occur in their reproductive system that determine the future sexual pattern, the release of gonadotropins (tonic or cyclic), that is, the sexual differentiation of the brain according to the male or female type. And the action of factors with the properties of endocrine disruptors can affect, first of all, precisely this process. Later, in the period from 35 to 65 days, there is a transition from sexual immaturity to sexual maturity. This includes the beginning of the secretion of sex hormones and the production of mature spermatozoa or eggs by the gonads, the formation of the estrous cycle, the formation in full of the negative feedback mechanism between the gonads and the neuroendocrine structures of the brain, and many other transformations that lead to the fact that the individual becomes capable of reproduction. Thus, the appropriate conditions for the proper passage of puberty are also very important. That is why the task of this study was to determine the significance of the presence of PE in this period for the further functioning of the reproductive system of male rats, as well as changes in the reactivity of the offspring to the effect of PE in this period under the conditions of the presence or absence of contact of their father with PE before their mating with intact females.

Upon reaching sexual maturity, the reproductive system of male offspring rats was studied. Thus, Table 1 shows data on the content of steroid hormones in males of different groups. It can be seen, that dietary intake of PE in the pubertal period does not affect the content of E₂, testosterone and their ratio. At the same time, there is a lower concentration of corticosterone.

<table>
<thead>
<tr>
<th>Group</th>
<th>Estradiol, nmol/l</th>
<th>Testosterone, nmol/l</th>
<th>Testosterone/estradiol</th>
<th>Corticosterone, nmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. F(I)/O(I)</td>
<td>n=15</td>
<td>0.41±0.08</td>
<td>32.74±3.12</td>
<td>89.69±13.00</td>
</tr>
<tr>
<td>2. F(I)/O(PE)</td>
<td>n=6</td>
<td>0.32±0.04</td>
<td>25.45±5.41</td>
<td>86.31±22.24</td>
</tr>
<tr>
<td>3. F(PE)/O(PE)</td>
<td>n=8</td>
<td>1.24±0.09</td>
<td>6.19±0.45</td>
<td>6.40±1.12</td>
</tr>
</tbody>
</table>

Note. p – level of significance of the differences between the specified groups

| n₁, n₂ | P₁₂<0.05 |

Under the conditions of receiving PE during this period, the offspring of the phytoestrogenized father (group 3) experience an increase in the content of E₂, a decrease in testosterone, which leads to a significant de-
increase (up to 19 %) in their ratio. These animals also differed from phytoestrogenized rats, whose father did not receive PE. That is, according to the indicators of the hormonal state of the offspring, their reactivity to PE largely depends on the presence or absence of PE use by the father.

When studying sexual behavior in the group of male rats that received PE during puberty, a statistically significant decrease in the frequency of copulatory reactions by 26–29 % of the values of intact animals was noted (Table 2).

### Table 2

**Sexual behavior of the males who received phytoestrogens during puberty and were the offspring of a phytoestrogenized father, (X ± S,

<table>
<thead>
<tr>
<th>Indicator/Group</th>
<th>1. F(I)/O(I)</th>
<th>2. F(I)/O(PE)</th>
<th>3. F(PE)/O(PE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proceptive behavior, c.u.</td>
<td>n=9 3.4±0.3</td>
<td>n=12 2.9±0.4</td>
<td>n=10 5.1±0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>Latency, s</td>
<td>n=8 25.6±8.0</td>
<td>n=11 37.3±3.1</td>
<td>n=10 103.0±21.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>number</td>
<td>n=9 23.7±2.6</td>
<td>n=12 17.6±1.0</td>
<td>n=10 21.3±2.0</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>Intromission</td>
<td>n=9 41.1±15.8</td>
<td>n=12 46.7±14.4</td>
<td>n=10 104.0±21.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>number</td>
<td>n=9 23.3±2.8</td>
<td>n=12 16.6±0.7</td>
<td>n=10 20.7±1.9</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ejaculation</td>
<td>n=9 676.7±60.1</td>
<td>n=12 603.8±47.1</td>
<td>n=6 630.8±73.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>number</td>
<td>n=9 1.0±0</td>
<td>n=12 1.0±0</td>
<td>n=6 1.2±0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postejaculatory interval, s</td>
<td>n=2 297.5±67.5</td>
<td>n=3 276.7±11.7</td>
<td>&gt;900</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of intromissions</td>
<td>n=9 21.9±2.7</td>
<td>n=12 15.7±0.8</td>
<td>n=7 18.0±2.8</td>
</tr>
</tbody>
</table>

*Note: p – level of significance of the differences between the specified groups*

In addition, ejaculation in these animals occurred after fewer intromissions, which indicates the activation of the peripheral mechanism of sexual behavior regulation.

Under the conditions of prior phytoestrogenization of the father, courtship behavior was activated in his offspring, phytoestrogenized at puberty, but sexual reactions to a receptive female were slowed down - increased mating latency and intromission compared to intact animals. The nature of the difference in indicators from those in the group of descendants of intact parents was the same. That is, according to the indicators of sexual behavior, phytoestrogenization of the father significantly affected the reactivity to PE of his sexually mature male offspring.

When PE was administered at the age of puberty in the adult animals, the body weight did not differ from that of the control rats, as did the mass coefficients (except for the pituitary gland (2.2±0.2 vs. 3.3±0.3 mg; p<0.05). In the event that the father receives PE before mating with a female, the receipt of PE at puberty leads to disorders of somatic development in his male offspring, which has the form of a decrease in body weight, seminal vesicles, and the ventral lobe of the prostate gland (by 28, 60 and 62 %, respectively), an increase in the relative mass of the adrenal glands and pituitary gland (by 46 and 59 %, respectively). The obtained results cannot be explained by the direct inhibitory effect of isoflavones on protein synthesis due to its absence in the F(I)/O and PE group. It is more likely to be considered a consequence of relative estrogenization of animals due to an increase in the concentration of E2 and a decrease in the testosterone content in animals of this group.

A similar direction of PE action was found when studying the spermogram of epididymal sperm and morphometric indicators of the testicles (Table 3). That is, the alimentary supply of PE to males during puberty (under the conditions of intact parents) did not make any changes in the studied parameters. At the same time, the quality of sperm significantly deteriorates in the offspring of the phytoestrogenized father.
The state of spermatogenesis and the spermogram of the males who received phytoestrogens during puberty and were the offspring of a phytoestrogenized father

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Tubular diameter, μm</th>
<th>Number of spermatagonia, pieces</th>
<th>Index of spermatogenesis, points</th>
<th>Concentration, ml/ml</th>
<th>Motility, %</th>
<th>Pathological forms, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. F(I)/O(I)</td>
<td>n=60 66.4±1.5</td>
<td>n=30 80.6±2.7</td>
<td>n=100 3.5±0.1</td>
<td>n=12 27.5±4.4</td>
<td>n=12 65.3±5.4</td>
<td>n=12 19.0±2.5</td>
</tr>
<tr>
<td>2. F(I)/O(PE)</td>
<td>n=60 57.8±1.1</td>
<td>n=30 80.9±3.3</td>
<td>n=100 3.4±0.1</td>
<td>n=11 30.0±2.4</td>
<td>n=11 53.8±5.2</td>
<td>n=11 23.7±3.0</td>
</tr>
<tr>
<td>3. F(PE)/O(PE)</td>
<td>n=60 51.2±1.1 P2,3&lt;0.05</td>
<td>n=30 84.1±2.8</td>
<td>n=100 3.1±0.1 P2,3&lt;0.05</td>
<td>n=9 18.2±3.1 P1,3&lt;0.10</td>
<td>n=9 24.4±4.5 P1,3&lt;0.001</td>
<td>n=9 48.1±6.0 P1,3&lt;0.002</td>
</tr>
</tbody>
</table>

Note: p – level of significance of the differences between the specified groups

Thus, the concentration and motility of sperm in them were reduced by 54 and 62 %, respectively, which corresponds to a decrease in the diameter of the seminiferous tubules by 13 % and the index of spermatogenesis. At the same time, the increased percentage of pathological forms of spermatozoa also indicates a violation of the last stages of gamete differentiation, which may be a consequence of changes in the content of sex hormones in these animals. These data echo the results of Jiang C. X. and co-authors [25], who showed that daidzein (one of the PEs, included in the mixture) is able to cause underdevelopment of the testes and disruption of spermatogenesis upon reaching sexual maturity in rats, exposed to PEs during puberty.

Further, the fertility of the rat offspring was studied. The fertilizing ability of the females and sperm quality of the experimental males were evaluated based on the results of their mating with the intact females (Table 4).

Table 4

Pregnancy rates of the intact females, inseminated by males, treated with phytoestrogens during puberty, offspring of phytoestrogenized parents

<table>
<thead>
<tr>
<th>Group</th>
<th>Number (per female)</th>
<th>Embryos loss, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corpora lutea</td>
<td>Implantation sites</td>
</tr>
<tr>
<td>1. F(I)/O(I)</td>
<td>n=20 11.7±0.5</td>
<td>n=20 10.5±0.7</td>
</tr>
<tr>
<td>2. F(I)/O(PE)</td>
<td>n=8 12.8±0.6</td>
<td>n=8 12.4±0.7</td>
</tr>
<tr>
<td>3. F(PE)/O(PE)</td>
<td>n=11 3.1±1.5</td>
<td>n=11 2.9±1.5</td>
</tr>
</tbody>
</table>

Note: p – level of significance of the differences between the specified groups

The data show that when eggs are fertilized with spermatozoa of the rats that were exposed to PE during puberty and were the offspring of a phytoestrogenized father, low-quality zygotes are formed, as evidenced by a significant decrease in the number of implantation sites and live fetuses in the uterus of an intact female. In addition, due to the increase in preimplantation losses, the total intrauterine death of fetuses increases. In the case of phytoestrogenization of a male offspring of intact parents, no changes in his fertility are observed. Other studies have also shown changes in sperm motility and a reduction in litter size, accompanied by signs of embryo loss after implantation [26].

4. Research results discussion

Significant deterioration of reproductive health requires determination of the reasons that cause this phenomenon. The study of the effect of PE on reproductive functions is largely determined by practical everyday life, because, as is known, PE enters the body in rather large quantities in various ways. Phytoestrogens, as a factor of the environment, have adverse consequences not only for individuals who directly use them, but also for their descendants. These circumstances must be taken into account to preserve reproductive health.

Phytoestrogens are present in numerous dietary supplements and are widely marketed as a natural alternative to estrogen replacement therapy. Soy infant formula now accounts for up to a third of the US market, and soy protein is now added to many processed foods [27, 28]. As weak estrogen agonists/antagonists with molecular and cellular properties similar to synthetic endocrine disruptors, PEs are a useful model for comprehensively investigating the biological effects of endocrine disruptors in general.

Thus, the effect of estrogen-like substances on male reproductive function is manifested not only under the conditions of their intake in the critical periods of the embryonic and postnatal periods [29], but also, even when acting on the germ cells of parents [30]. In male offspring, the programming peak of androgen secretion is disturbed, the hormonal status changes in the direction of hyperestrogeniza-
tion, and fertility decreases due to the reduced quality of germ cells against the background of a normal spermatogram.

**Limitations of the research.** The limitation of the study is the experimental model of hyperestrogeny in male rats as a result of the animal's consumption of phytoestrogens and the effect of the latter on the reproductive system of male offspring.

**The prospect of further research** is the study of the reproductive function of female offspring, which were born to phytoestrogenized parents.

**5. Conclusions**

1. The presence of an excess of compounds with estrogenic activity in the diet of pubescent males leads to irreversible damage to the programming of the hypothalamic-pituitary-gonadal axis and persistent violations of reproductive function in sexually mature males.

2. The reproductive function of sexually mature male offspring of a phytoestrogenized father who received a mixture of phytoestrogens during puberty is characterized by differences in sexual behavior, a decrease in the reproductive potential of males, which occurs due to a decrease in the proportion of effective fertilizations, which indicates negative changes in spermatogenesis, the development of which took place in conditions of absolute and relative hyperestrogeny. This indicates that phytoestrogens, as a factor of the environment, have adverse consequences not only for individuals who directly use them, but also for their male offspring.

**Conflict of interests**

The authors declare that they have no conflict of interest in relation to this research, including financial, personal, authorship or other nature, which could affect the research and its results, presented in this article

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**Data availability**

Data will be provided upon reasonable request.

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