Original Article

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The Effect of Alcoholic Extract of Humulus Lupulus During Pregnancy and Lactation on Sexual Maturation and Some Reproductive Indices in Male Rats

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ABSTRACT

BACKGROUND AND OBJECTIVE: *Humulus lupulus* is known for its estrogenic properties as a raw material in the beverage industry. Exposure to exogenous estrogenic compounds during embryonic and neonatal periods are now of public health concern, since it may cause reproductive impairment. Therefore, this study was conducted to evaluate treatment with *humulus lupulus* during early stages of life on the onset of puberty and some reproductive indices in male rats.

METHODS: In this experimental study, 20 pregnant mice were divided into five groups of four, including control (without treatment), control (saline) and three groups treated with alcoholic extract of *humulus lupulus* at 50, 100, 150 mg/kg/bw concentrations. They were treated daily from the seventh day of pregnancy to seven days after birth by gavage. Then, the effect of alcoholic extract of *humulus lupulus* on the onset of puberty, testicular weight and epididymis, sperm count, viability, and motility, testosterone concentration and the fertility of male children were evaluated.

FINDINGS: The onset of puberty in the 150 mg/kg *humulus lupulus* group (43.6±0.83) occurred later than the control (39.8±0.49) ($p<0.01$). Testicular and epididymis weight decreased in 100 and 150 *humulus lupulus* groups ($p<0.01$). The sperm count, viability, and motility and testosterone concentrations in the 100 and 150 mg/kg *humulus lupulus* group were significantly lower than the control group ($p<0.01$). In addition, the percentage of fertility in the 150 mg/kg *humulus lupulus* group (76.4±2.84) was lower than the control group (89.43±3.31) ($p<0.01$).

CONCLUSION: The results of this study showed that exposure of male rats to *humulus lupulus* in early stages of life causes late puberty and has negative effects on reproductive performance.

KEY WORDS: *Humulus lupulus, Rat, Puberty, Fertility*.

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Introduction

Endocrine disrupting chemicals (EDCs) are a group of natural or synthetic compounds that are molecularly similar in nature to some endogenous hormones. If they enter the body, these compounds can be transmitted in the tissues of the body and interfere with the synthesis, secretion and function of the natural hormones (1, 2). Throughout their lives, humans are exposed to thousands of EDCs in food, air and drinks. So far, the biggest concern with the effects of EDCs on the body was regarding their adverse effects on reproductive system in both males and females (1–3). In recent years, there has been increasing evidence indicating that exposure to EDCs has led to a change in the onset of puberty, constant decrease in the volume of semen, and an increase in male reproductive disorders (4, 5).

Evidence has shown that excessive exposure to estrogens and estrogenic EDCs in embryonic and neonatal periods, which are critical hormonal periods, may disrupt the male reproductive system and reduce fertility. Studies have also shown that testicles are extremely sensitive and vulnerable to estrogen and estrogenic EDCs during embryonic and neonatal periods (3, 6–8). Phytoestrogens are herbal compounds that are structurally similar to estradiol hormone and are able to react with estrogen receptors (3, 9). Laboratory studies have shown that exposure to certain phytoestrogens, such as genistein, especially during embryonic and neonatal periods, can interfere with the development and function of the male reproductive system (10 – 12).

Humulus lupulus is a plant belonging to the Cannabaceae family, which is widely used as a raw material in the production of non-alcoholic beverages, and has industrial and medical uses (13–15). Studies have shown that *humulus lupulus* is a phytoestrogen containing various chemical compounds such as resins, β-myrcene, humulene, tannin, hematinic acid, pectin products, potassium salts and various flavonoids, including 8-Prenylnaringenin and xanthohumol (15). Due to having compounds similar to female reproductive hormones, *humulus lupulus* has been used in regulating menstruation, treating swelling and stiffness of the uterus and menopausal disorders (15, 16). The results of studies have shown that *humulus lupulus* causes significant increase in estrogen, progesterone and testosterone levels in adulthood and increases the number of spermatogonial stem cells, spermatocytes and spermatids in rats (15,17). However, no study has ever been carried out regarding the administration of *humulus lupulus* during embryonic and neonatal periods and their effects on puberty and reproduction. Considering the estrogen-like properties of *humulus lupulus* and the vulnerability of male reproductive tissues to estrogenic compounds in hormone-sensitive period, the present study aims to administer the alcoholic extract of *humulus lupulus* in embryonic and neonatal period on puberty and some sperm parameters and fertility percent in male laboratory mice.

Methods

Extraction: After powdering the dried flowers of *humulus lupulus*, 40 grams of powder was percolated and then, 350 ml of 96% alcohol was added and stored at the laboratory temperature for 72 hours. The extract was then passed through a separatory funnel drop by drop, and meanwhile, the alcohol solvent was added drop by drop until the color of solution containing the extract changed. The extract was placed in bain-marie at a temperature of 50 ° C to evaporate the alcohol and completely condense it and then, it was left to dry (15).

Animals, groupings and treatments: In this experimental study, after being approved by the University's Ethics Committee, BALB/c mice were prepared from Razi Research Institute, Hesarak, Karaj. The male and female rats were separately kept in standard plastic cages, subjected to 12 L: 12 D (12 hours of light alternating with 12 hours of darkness) and 21±1 °C temperature with free access to water and food. Twenty pregnant mice were required to conduct this study.

For this purpose, each male mice was mated with two female mice in a cage at 7 P.M. and after about 12 hours, the vaginal plug detection method was used to determine the first day of pregnancy. Twenty mice with vaginal plug were selected. Pregnant rats were randomly divided into five groups of four, including control (without treatment), control (saline) and three groups treated with alcoholic extract of *humulus lupulus* at 50, 100, 150 mg/kg/bw concentrations. Administrations were done daily from the seventh day of pregnancy until seven days after birth by gavage. The infants were deprived of breastfeeding on the 21st day after birth. Then, two or three male children were randomly selected from each mother, and ten male rats were placed in each of the five subgroups, and were used to determine the onset of puberty and other

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reproductive indices. It should be noted that, due to the necessary care, none of the mice died during the study.

Examination of the onset of puberty: Balano-preputial separation (BPS) is suggested as an appropriate external index indicating the activation of the hypothalamic–pituitary–gonadal axis (HPG axis) and the onset of puberty in male rodents. In this method, after separating the male mice from mother on day 30 after birth, genital prominence of male rats was monitored from 8:00 A.M. to 11:00 A.M. using an Ocular Loupe. Date of BPS occurred by causing contraction in the glans, as a result of the cornification of the cells covering this area after increasing testosterone at about 40 days after birth (18 – 20).

Fertility percent: On the 80th day after birth, five mice were randomly selected from each group, and each were mated with two healthy mice of the same race for ten days, the fertility of which was already proven. After observing the presence of sperm on the vagina of the mouse and determining this day as the first day of pregnancy, the mice were deeply anesthetized with chloroform on the 16th day of gestation and after opening the abdomen, the number of embryos in the uterus was counted. The ovaries were also isolated and after washing in sodium chloride 9%, the number of their corpus luteum was counted using the Loupe. The fertility index was calculated according to the method of Oberlander et al. by dividing the number of embryos by the number of corpus luteum multiplying by 100 (21).

The weight of the reproductive organs: Male rats were deeply anesthetized through intraperitoneal injection of Ketamine/Xylazine on the 90th day after birth, and after opening the animal's abdomen, blood was collected from the animal's heart for hormonal measurements. Then, the testicles and epididymis were isolated, and were weighed for later testing.

Sperm count: The left tail of the epididymis was completely crushed using a glass stirrer to form a homogeneous mixture. A small amount of this suspension was transferred to a white blood cell count pipette (up to 0.5 mL pipette). Then, sodium bicarbonate 5% solution was added to this suspension until it reached 11 mL pipettes. Then, one drop of this new solution was slowly placed on a NeoBar slide and was covered with a clean lamella. Sperm were counted in 64 small squares for white blood cell count using a 100X optical microscope. The total sperm count was calculated in millions/mL using the following formula (22, 23):

$$Sperm\ count\ (million/ml) = \frac{\mathrm{N} \times 20 \times 1000}{0.4}$$

N = number of counted sperm

Motility percentage and sperm viability: A portion of the right tail of the epididymis was crushed in and homogenized 0.2 ml physiologic serum. One drop of this mixture was transferred to the NeoBar slide and the sperm motility percentage was determined by counting the number of motile and immotile sperm in 20 specified squares and was expressed as percentages. To assess the sperm viability, one drop of the above mixture was placed on a slide and stained with one drop of Eosin-Nigrosin and examined under a microscope. Viable sperm do not absorb Eosin-Nigrosin stain but dead sperm absorb the stain. After counting 100 sperm randomly under microscope field of view, the ratio was expressed as percentage, and this was repeated for three times (22, 23).

The testosterone level test: The collected blood samples were centrifuged at 3000 rpm for 15 minutes and the serum was separated and was kept in a freezer until the hormones were measured. The serum testosterone levels were measured by ELISA and by the RIA reagent kits obtained from Radium (Pomezia, Italy) based on the kit's manual. Data were analyzed using SPSS 19 software, ANOVA and Tukey post hoc tests. $P<0.05$ was considered significant.

Results

Results of puberty at baseline: The mean date of BPS was 39.8±0.49, 39.4±0.56, 40.1±0.81, 40.7±0.62 and 43.6±0.83 days after birth in control (without treatment), control (saline), and 50, 100, and 150 mg/kg *humulus lupulus* groups, respectively. Date of BPS was significantly higher in the 150 mg/kg *humulus lupulus* group ($p < 0.05$) compared to the control group (without treatment) (late puberty). There was no significant difference between different groups and the control group (without treatment) in the mean body weight on the date of BPS (Fig. 1).

Results of examining the weight of reproductive organs: The weight of the testicles and the epididymis significantly decreased in the 100 and 150 *humulus lupulus* group compared to the control group (without treatment) ($p<0.05$) (Table 1).

The results of examining the sperm parameters: Sperm motility, viability and count in groups 100 and 150 *humulus lupulus* group showed a significant

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decrease compared to the control group (without treatment) (p<0.05) (Table 2).

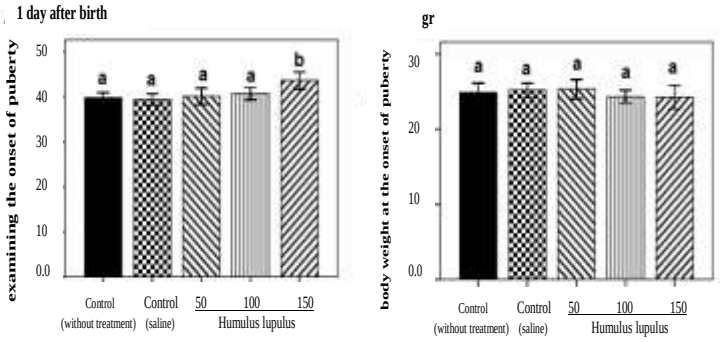

Figure 1. The same letters indicate lack of significant difference using the Tukey test at a probability level of 5%, n = 10, (a) examining the onset of puberty, (b) body weight at the onset of puberty

Table 1. Comparison of the effects of *humulus lupulus* treatments on the weight of reproductive organs in the studied groups

Group	Testicular weight (mg)	Weight of epididymis (mg)
Control (without treatment)	186.2±2.81 [a]	45.5±0.85 [a]
Control (saline)	181.8±2.53 [a]	46±0.71 [a]
Humulus lupulus		
50 mg/kg	178.90±2.38 [ac]	44.1±0.89 [a]
100 mg/kg	156±4.23 [b]	34.8±1.64 [b]
150 mg/kg	170.7±2.79 [bc]	35.7±1 [b]

The same letters in each column indicate lack of significant difference using the Tukey test at a probability level of 5%, n=10

Table 2. Comparison of the effect of *humulus lupulus* treatments on sperm motility, viability and count in the studied groups

Group	Sperm motility (%)	Sperm viability (%)	Sperm count (million/ml)
Control (without treatment)	83.4±2.14 [a]	84.2±1.79 [a]	41.8±0.71[ab]
Control (saline)	86.5±1.28 [a]	81.9±2.04 [a]	42.7±1 [a]
Humulus lupulus			
50 mg/kg	73.5±2.64 [b]	83.4±2.39 [a]	38.3±0.82 [bc]
100 mg/kg	73.5±2.64 [b]	71.6±3.65 [b]	36.1±1.03 [c]
150 mg/kg	66.5±1.93 [b]	70.3±2.82 [b]	35.1±1.34 [c]

The same letters in each column indicate lack of significant difference using the Tukey test at a probability level of 5%, n=10

Results of testosterone concentration test: Testosterone concentration significantly decreased in 100 and 150 *humulus lupulus* groups, compared to control group (without treatment) (p<0.05) (Fig 2).

Results of fertility percentage: 90% of females in the control group and 100% of them in the control group were pregnant and this amount was 80, 70 and 70% in the 50, 100 and 150 *humulus lupulus* groups, respectively. In addition, the fertility percentage in 150 *humulus lupulus* groups significantly decreased compared to control group (without treatment) (p<0.05) (Table 3).

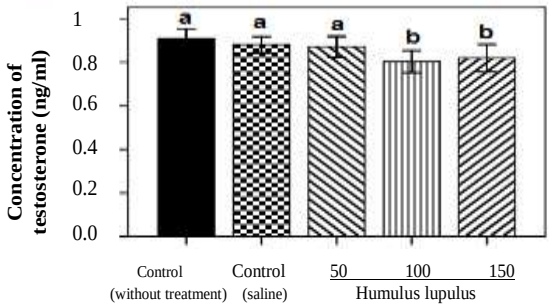

Figure 2. The same letters in each column indicate lack of significant difference using the Tukey test at a probability level of 5%, n = 10

Table 3. Comparison of effect of *humulus lupulus* treatments on fertility percentage in the studied groups

Number Groups	Male mice	Female mice	Pregnant females N(%)	Fertility percent Mean±SD
Control (without treatment)	5	10	9 (90)	89.43±3.31[a]
Control (saline)	5	10	10 (100)	88.30±2.03[a]
Humulus lupulus				
50 mg/kg	5	10	8 (80)	84.5±2.1 [ab]
100 mg/kg	5	10	7 (70)	84±2.81 [ab]
150 mg/kg	5	10	7 (70)	76.4±2.84 [b]

The same letters in each column indicate lack of significant difference using the Tukey test at a probability level of 5%, n=10

Discussion

The results of this study indicated delayed puberty and significant reduction in sperm motility, viability and count, testicular weight and epididymis, testosterone concentration and fertility percentage after administration of alcoholic extract of *humulus lupulus* during embryonic and neonatal periods. Puberty in the 150 *humulus lupulus* group occurred significantly later. Decline in testosterone concentration was observed in this study. Some previous reports have

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shown that BPS can be affected by estrogen and estrogenic EDCs. These studies have shown that exposure to estrogenic compounds in male rodent models leads to delays in puberty (18,24). Exposure of mice to diethylstilbestrol (DES, a non-steroidal estrogenic compound) during pregnancy and infancy leads to a delay in the onset of puberty and delayed testicular descent, which is consistent with the present study (18,24). Recent studies have shown that the exposure of rodents to steroids and estrogen-like compounds in the early stages of life is essential in regulating the proper expression of the kisspeptin gene in the hypothalamus, which plays an important role in regulating the onset of puberty. Exposure of infant rodents to synthetic estrogen and estrogen-like compounds has reduced the expression of kisspeptin in puberty (18, 25, 26).

Due to the estrogenic properties of *humulus lupulus*, this may lead to a delay in puberty. Some studies suggest that puberty may be due to changes in body weight. Slimness and weight loss delay puberty (27–29). According to the results of this study, there was no significant difference in body weight at the onset of puberty. In this regard, some other studies have shown that body weight is not necessary to regulate the onset of puberty in humans or lab animals (30). The weight of the testicles and epididymis, and sperm motility, viability and count were significantly decreased in 100 and 150 *humulus lupulus* groups. Studies have shown that the weight of testicle and epididymis, as well as the sperm function, are mainly regulated by testosterone (31,32).

Therefore, administration of *humulus lupulus* in the initial period of fetal development may have side effects, directly on the testes or indirectly on higher levels that control testicle, i.e. the hypothalamus and the hypophysis, due to its estrogenic compounds. Studies during embryonic and neonatal periods have shown the distribution of estrogen receptors in the germ cells, leydig and sertoli in the testicles of mammals.

These studies have shown that the presence of the least amount of estrogen is necessary for the development and the function of the testicles, but the increase in estrogen (or estrogen-like compounds) during critical periods may have adverse effects on reproductive tissues through various mechanisms including activation of apoptosis pathways (6–8). A study showed that the use of genistein may reduce the number of leydig cells and decrease the concentration of testosterone by interfering with the expression of steroid regulating genes, including cytochrome P450 (33). The results have shown that genistein is capable of messaging through estrogen receptors due to structural similarity to estrogens (34). Other studies have shown that resveratrol, an estrogen-like compound found in grapes and wine, has adverse effects on the steroidogenesis of leydig cells and the production of testosterone by inhibiting the expression of StAR and cytochrome P450 genes (35).

Another study has shown that the exposure of male mice to DES during embryonic development has led to cryptosporidiosis and hypospadias and lack of proper development of the reproductive organs (36). Similarly, neonatal treatment of male rats with DES resulted in a wide range of reproductive disorders, including delayed testicular descent, delayed onset of spermatogenesis in puberty, testicular weight loss, morphological changes in testicles and the attached glands, decreased leydig, sertoli and sex cell count, and decreased testosterone and FSH concentration (5,37). Observing the decrease in the fertility rate of female mice can also be explained by the decline in sperm motility, viability and count of male rats. Studies have shown that 8-Prenylnaringenin in *humulus lupulus* is found to be one of the strongest known phytoestrogen compounds.

Some reports also indicate that 8-Prenylnaringenin has the highest tendency to estrogen receptor among all phytoestrogens (38, 39). In addition to the 8-Prenylnaringenin, other compounds with estrogenic properties such as β-acids, β-sitosterol, o-hydroxy dibenzoyl methane, xanthohumol and desmethyl xanthohumol have been identified in *humulus lupulus* (16, 38–40). Future studies investigating the effects of estrogenic compounds in *humulus lupulus* may be fruitful. According to the results of this study, it was determined that embryonic and neonatal treatment of *humulus lupulus* alcoholic extract causes delayed puberty and has adverse effects on reproductive ability of mice, which may be caused by its estrogenic and permanent effects during embryonic and neonatal development on various components of the HPG axis.

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