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Original Article

Impact of Hydroalcoholic Extract of *Humulus Lupulus* L. on Sperm Quality, Reproductive Organs and Hormones in Male Rats*

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ABSTRACT **Objective**: To investigate the impact of *Humulus Lupulus* L. hydroalcoholic extract on the body weights, reproductive organs, sperm quality and hormone levels in male rats. **Methods**: By simple random sampling method, seventy male Sprague-Dawley rats were randomly assigned to 7 groups including control group [distilled water, 1 mL/(kg•d)], Tween 80 group [25% Tween 80 solution, 1 mL/(kg•d)], olive oil group [olive oil, 1 mL/(kg•d)], diethyl stilbestrol (DES) group [DES, 100 μg/(kg•body weight)], H50, H150 and H450 [50, 150 and 400 mg/(kg•d) of *Humulus Lupulus* L extract, respectively]. The administration was performed via gavage once daily for 7 weeks. Body and reproductive organs weights including testes, seminal vesicles, epididymis and prostate were weighted and epididymal sperm quality were determined by digital balance. Blood samples were collected and serum free testosterone (T), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estrogen (E_2) levels were measured by rat specific enzyme-linked immunosorbent assay. **Results**: The percentage increase in mean body weights of rats in the DES and H50, H150 and H450 groups decreased significantly compared to olive oil and Tween 80 groups (all P<0.05). The weights of seminal vesicle, epididymis and testes in rats receiving H50 were significantly higher than the control group (P<0.05 or P<0.01). The sperm count in the rats receiving H50 was significantly lower than the control group (P<0.05). The sperm motile characteristics of the rats receiving hydroalcoholic extract and DES were significantly lower than those of the control or rats receiving vehicles (all P<0.05). In H50, H150, H450 and DES groups, T and LH levels were decreased, and E_2 was significantly increased compared to the control (P<0.05 or P<0.01). The FSH level did not change in all groups (P>0.05). **Conclusion**: *Humulus Lupulus* L. extract significantly increased the seminal vesicle and testes weights and reduced the sperm motility.

KEYWORDS sex organ, phytoestrogen, *Humulus Lupulus* L., sperm quality, male rat

Humulus Lupulus L. is a plant from cannabinaceac family[(1)] which is grown in Iran as well as most regions all over the world. The flowers (cones) of this plant have been used for centuries as a preservative and flavoring agent in beer[(2)]. It has been suggested that *Humulus Lupulus* L. has a powerful estrogenic activity.[(3)] Also, menstrual disturbances were reported among female workers who used to pick the plant *Humulus Lupulus* L. manually.[(4)] Moreover, *Humulus Lupulus* L. baths have been used for the treatment of gynecological disorders[(4,5)] and reducing hot flushes in menopausal women.[(5)]

The putative estrogenic activity of *Humulus Lupulus* L. might be due to phytoestrogen component of the plant. It has recently been shown that the *Humulus Lupulus* L. had 8-prenylnaringenin (8-PN), as a very potent phytoestrogen with equal or greater estrogenic activity than other established plant estrogens[(6,7)] and may be used to prevent cancer via reduction of the estrogen metabolism.[(8)] It has shown that the most abundant source of the content of 8-PN was lupulin within in *Humulus Lupulus* L. products.[(9)] Moreover, *Humulus Lupulus* L. had a high affinity for α and β estrogen receptors (ER).[(10)] Despite preferentially activating estrogen receptor α, 8-PN is only slightly

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uterotrophic,[11] and it also elucidates estrogenic effects on the male reproductive system.

Early developmental exposure to synthetic estrogenic chemicals such as diethylstilbestrol (DES) causes abnormalities, reduction of reproductive organ weights, sperm production, quality and fertility in experimental animals.[12,13] It has also been proven that genistein (GEN), a kind of phytoestrogen, suppressed the *ex vivo* steroidogenic response of Leydig cells to human chorionic growth (hCG) or dibutyryl cyclic adenosine monophosphate (cAMP) by down-regulating their expression of P450 side chain cleavage (P450 scc) without altering the serum level of either lutein hormone (LH) or testosterone (T).[14]

Previous studies showed that estrogen and 17β-estradiol (E_2) reduced the weights of body sex-related organs and prostate.[13,15] Moreover, body and prostate weights decreased in animals feeding with phytoestrogen.[16] Serum levels of LH were shown to reduce by oral estradiol.[17] Similarly, serum levels of T decreased by phytoestrogen[16] or β-estradiol.[15] Also, hemoglobin, hematocrite and erythropoietic activity decreased by β-estradiol.[15,18]

To the best of our knowledge, the impact of *Humulus lupulus* L., which contains phytoestrogen, on reproductive system development have not been investigated. Therefore, the present study aimed at examining the effects of hydroalcoholic extract of the *Humulus Lupulus* L. on the weights of the body and reproductive organs, spermatogenesis in the male rats.

METHODS

Ethical Considerations

The protocol of the study was approved by the ethics committee from Shiraz University of Medical Sciences, and the study was conducted according to the university's guidelines for the care and use of laboratory animals (ethical No. 3001).

Chemical Reagents

DES, a synthetic nonsteroidal estrogen, was purchased from Iran Hormone Company (Tehran, Iran, lot No.G477); rat specific enzyme linked immunosorbent assay (ELISA) kits including rat T, LH, follicle-stimulating hormone(FSH), E_2 (Cat No.CSB-E05100r, CSB-E06869r, CSB-E12654r, CSB-05110, respectively) were purchased from Cusabio Biotech Co., Ltd (Japan).

Preparation of *Humulus Lupulus* L. Extract

Flowers of *Humulus Lupulus* L. were obtained from Zarband Pharmacy Firm[19] and were ground to powder. Hydroalcoholic extract was prepared through percolation method.[20]

Animals

Seventy male Sprague-Dawley (SD) rats (22-day-old, 220–240 g) were obtained from Animal Breeding Center of Shiraz University of Medical Sciences. Every rat was housed in a cage for 7–8 weeks in a light (12 h light/12 h dark), temperature (25 ± 1 ℃) condition with standard rat chow and tap water *ad libitum*.

Animal Grouping and Administration

By simple random sampling method, seventy SD rats were assigned to 7 groups (n=10 in each group) and treated as follows. The rats in control, Tween 80 or olive oil groups received distilled water, 25% Tween 80 solution (as the hydroalcholic extract solvent) or olive oil (as the DES solvent) at the dose of 1 mL/(kg•d) via gavage, respectively. The rats in H50, H150 and H450 groups received hydroalcoholic extract of *Humulus Lupulus* L. at 50, 150 or 450 mg/(kg•d) via gavage, respectively. The DES group received DES (100 μg/kg body weight) via gavage.[19,21,22] All rats were adminstrated orally by gavage once daily for 7 weeks.

Measurement of Body and Reproductive Organs Weights

Animals were weighted on a weekly basis, and the doses of *Humulus Lupulus* L. extract were adjusted accordingly of weight gain. At the end of 7 weeks, animals were anesthetized with overdose ether and sacrificed by cervical dislocation, and the weights of the testes, seminal vesicles, epididymis and prostate were determined by digital balance as previously reported.[23] Also, volume of the testis and prostate was measured by Arashmidos method.[24] The percentage increase of body weight was calculated according to the following formula: increase in body weight %= (7-week body weight–initial body weight)/initial body weight × 100%.

Sperm Count

The head of epididymis was chopped with a sharp scissor, 20 μL of the semen was removed and minced in 10 mL 0.9% saline for 90 s at laboratory temperature. Afterward, the dilution was charged into neubauer counting chambers (white blood cell squares), then the number of spermatozoa was counted in 4 squares. Finally, the average count for 4 separate regions was calculated and multiplied by 5×10^4 as a dilution coefficient.(12-14)

Analysis of Sperm Kinematics

Sperm suspension used for sperm counts was also used to assess the sperm morphology. The sperm suspension was further diluted to allow the individual sperms to be clearly assessed. Ten microliter of the sperm suspension was placed on a glass slide and covered with a glass cover ship. The slide was left at laboratory temperature for 1 min to allow the saline and sperm to settle. Under the 40 magnification objective lens, the sperms were classified as normal morphology or a head or tail defect. Head defects included a sideways, misshapen or double head. Examples of tail defects are the tail being significantly curled or bent up towards the head and sperm having a double tail. The sperms with normal morphology were classified as being motile and immotile. After that the motile sperms were classified into full (with normal kinetic), sluggish (with abnormal kinetic) and slow (with less than normal kinetic). Finally, the percentage of motile/ total number of the sperms was determined for each animal.(12-14)

Hormone Measurement

After separating the blood serum, the concentrations of LH, FSH, free T and E_2 were measured by rat specific ELISA kits. Intra-assay coefficients of variations for all hormones were less than 8%.

Statistics Analysis

All data were expressed as mean ± standard error of mean ($\bar{x}\pm$SEM) and analyzed with GraphPad Prism software (version 6.0, Graphpad Software, La Jolla, CA, USA). Data were analyzed using one way analysis of variance and comparisons were performed by LSD *post hoc* test. $P<0.05$ were considered statistically significant.

RESULTS

Body Weight Measurement

The data obtained from the mean body weights in 7 groups are shown in Table 1. Only 64 of 70 rats completed the experiment. Six rats died due to respiratory arrest during oral gavage. Initial body weight did not differ among the 7 groups ($P>0.05$). During 7-week experimental period, the body weights were increased in all groups and percentage increase in mean body weight (weight gain) in rats receiving DES and *Humulus Lupulus* L. hydroalcoholic extract (H50, H150, H450) were significantly lower than the olive oil and Tween 80 groups (all $P<0.05$).

Table 1. Body Weight and Percentage of Increase in Body Weight among 7 Groups ($\bar{x}\pm$SEM)

Group	*n*	Time	Body weight (g)	increase (%)
Control	10	Initial	221.6 ± 5.6	42.4 ± 3.7
		7 weeks	314.1 ± 4.5	
Olive oil	9	Initial	221.2 ± 5.0	40.3 ± 2.4
		7 weeks	311.3 ± 7.4	
Tween 80	8	Initial	215.3 ± 2.6	39.7 ± 4.4
		7 weeks	298.0 ± 6.1	
H50	10	Initial	221.8 ± 4.4	30.0 ± 2.6*
		7 weeks	287.8 ± 5.5	
H150	9	Initial	222.3 ± 5.0	32.1 ± 2.5*
		7 weeks	295.0 ± 10.8	
H450	9	Initial	239.7 ± 5.4	21.5 ± 3.2*
		7 weeks	291.2 ± 1.6	
DES	9	Initial	212.7 ± 6.0	29.8 ± 1.5*
		7 weeks	275.0 ± 7.1	

Note: *$P<0.05$, *vs*. olive oil and Tween 80 group

Sex Organ Weight

There was no significant difference in prostate and testes weights (normalized to body weight) among the 7 experimental groups ($P>0.05$). There was no significant difference between the seminal vesicle or epididymis weight of control rats and those receiving Tween 80 or olive oil. The weights of seminal vesicle, epididymis and testes in rats receiving H50 were significantly higher than the control group ($P<0.05$ or $P<0.01$, Figure 1).

Sperm Count and Motility

As shown in Figure 2, there was no significant difference in the sperm count and motility between the control and rats receiving vehicle ($P>0.05$). Among groups receiving *Humulus Lupulus* extract, only sperm count of H50 rats significantly lowered than that of control ($P<0.05$). The sperm motile characteristics of the rats receiving hydroalcoholic extract at 150, 450 mg/(kg•d) and DES were significantly lower than those of the control or rats receiving vehicles (all $P<0.05$).

Hormone Levels

Figures 3 illustrates the hormonal profiles of male rats of the experimental and control groups. In the rats

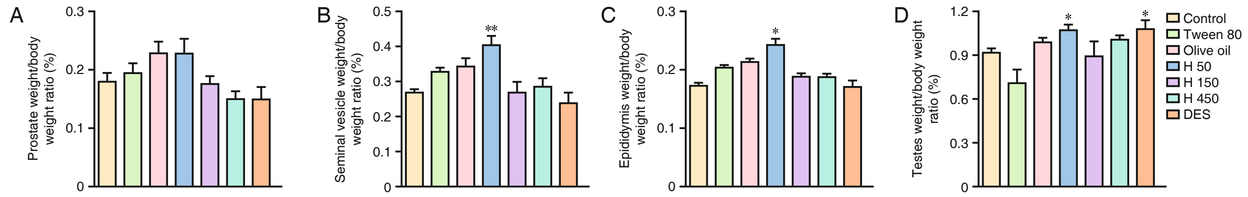

Figure 1. Percentage of Reproductive Organ Weight/ Body Weight Ratio among Different Groups ($\bar{x}\pm$SEM)

Notes: A: prostate weight/body weight ratio; B: seminal vesicle weight/body weight ratio; C: epididymis weight / body weight ratio; D: testes weight/body weight ratio; $^{*}P$<0.05, $^{**}P$<0.01 *vs.* control group

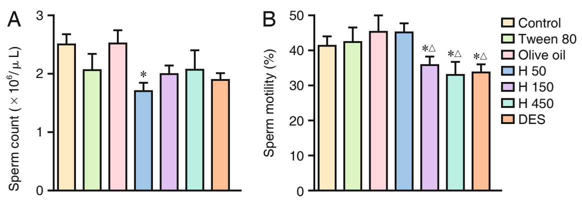

Figure 2. Sperm Count (A) and Motility (B) in Male Rats from Different Groups ($\bar{x}\pm$SEM)

Notes: $^{*}P$<0.05 *vs.* control group; $^{\triangle}P$<0.05 *vs.* vehicles

receiving vehicles (Tween 80 or olive oil), hormonal profiles did not differ compared to control, whereas in the groups receiving hydroalcoholic extract of *Humulus Lupulus* L.at 50, 150 or 450 mg/(kg•d) or DES, the levels of T and LH were significantly decreased, and E_2 level was significantly increased compared to the control group (P<0.05 or P<0.01). The FSH level did not change in all groups.

DISCUSSION

Our study indicated a significant decrease of body weight in animals treated with DES and phytoestrogen diet compared to the controls. In addition, animal's weight significantly changed regarding their age at the end of the exposures, which indicated normal growth during the experiment. We showed that only 50 mg/(kg•d) *Humulus Lupulus* extract caused a significant increase in relation to the weight of the epididymis, testes, and seminal vesicle in male rats for 7 weeks compared to the control group. However, the prostate weight ratio was not significantly changed. In our study, treatment with *Humulus lupulus* L. extract (H150, H450) and DES caused a significant decrease in sperm motility approximately 15%–25%, but only H50 had a reduction effect on sperm count. In addition, the level of LH and T decreased with DES and *Humulus lupulus* L. extract (H50, H150 and H450) treatment, but the level of E_2 increased. The mentioned pretreatments had no effect on the FSH level.

Recently, It has been identified that *Humulus Lupulus* L. extract is considered a potent phytoestrogen in *Humulus Lupulus* L. since the plant includes 8-PN which is a very potent phytoestrogen.(6) It is reported that *Humulus Lupulus* L. extract can reduce hot flushes in postmenopausal women.(3) According to another study, exposure to estrogenic compounds, particularly during critical periods of development, has been associated with impaired prostate development, function,(13,25) and modulation some responses to environmental estrogens.(13)

In this study, there was an attempt to understand the possible effects of 8-PN as a kind of phytoestrogen in *Humulus Lupulus* L. on reproductive development. We also compared the effects of phytoestrogens with DES on reproductive system that compete with phytoestrogens in ER.

17β-estradiol at low- and high-doses has caused a significant decrease in the body weight.(17) Also the significant differences in absorption/metabolism may account for the differences in body weight where alterations in leptin and adipose tissue deposition

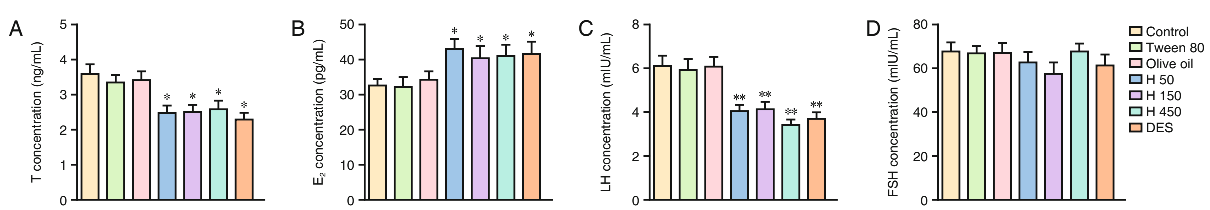

Figure 3. Comparison of T, E_2, LH and FSH Concentrations among the Experimental and Controls Groups ($\bar{x}\pm$SEM)

Note: $^{*}P$<0.05, $^{**}P$<0.01, *vs.* control group were seen between phytoestrogen and its free fed animals.[16,26,27] In agreement with our result, Weber, et al,[16] showed that rats fed with phytoestrogen diet displayed a significant decrease in body weight after 5 weeks compared with rats feeding with free diet.[16] It has also been shown that genistein as a phytoestrogen at low- and high doses resulted in a significant increase in body weight in ovarectomized rats during 3 months.[17] But Jung, et al[13] showed that there were no significant differences in body weight between control and genistein or estradiol-treated male mice during 5 weeks. These contradictions may have two reasons, firstly, it is probably because of some differences in animal sex, species, strains and races or route, timing, duration, type of phytoestrogen and dose of the application chosen. Secondly, it can be due to metabolism or feeding behavioral change. This idea was supported by Weber, et al[16] which showed that estrogens were known to alter the feeding behavior, body weight, and significantly increase locomotion in phytoestrogen fed rats.

It has been shown that 8-PN like other phytoesrogens had a strong affinity for estrogen receptor-β (ER-β) as well as ER-α which mediated the typical effects of E_2 in the uterus, vagina and mammary gland.[7,17,28] In addition, Adeoya-Osiguwa, et al[29] proved that E_2 also might be binding with different receptors except ER, so the effective dose of E_2 may be more stronger than 8-PN. Another study revealed that *P.mirifica* (a kind of phytoestrogen) caused an increase in epididymis weight in male rats only at the dose of 1000 mg/kg, whereas the seminal vesicle showed a non-significant weight loss.[30] Also, Weber, et al[16] reported a significant reduction in the ventral prostate weight in phytoestrogen-fed animals. We did not measure the phytoestrogen levels in circulation so this difference was difficult to explain because the prostate was an androgen sensitive tissue[31] and T level was reduced in our study. On the other hand, it has been proven that phytoestrogens inhibited the aromatase enzyme and reduced the formation of local estradiol.[32] It occurd at different doses of this phytoestrogen which were used in our study.

Hypertrophic effects of E_2 can be explained by two mechanisms. Firstly, Fritz, et al[25] have reported that most of the genistein in the circulation is in the conjugated form which is less biologically active. It might have minimized the feedback inhibition of LH unless pharmacological concentrations of genistein [e.g., 100 mg/kg•(body weight)] was used which increased the unconjugated form. Secondly, by increasing of E_2 and occupation of all receptors in gonadotropin-releasing hormone (GnRH) containing neurons in hypothalamus, GnRH and galanin gene expression became blocked,[19] resulting in reduced circulating T and LH levels. In accordance with other studies, phytoestrogens like isoflavonoid-rich diet led to the down regulation in androgen, estrogen α and β receptor mRNA expression.[33] It can probably be the mechanism of significant increase of the organ's weight only in the lowest dose of *Humulus Lupulus* L. extract (50 mg/kg). Therefore, higher doses could not act on the receptors and apply their effects. Glover, et al[12] showed that exposure of adult male rats to a diet with high phytoestrogen content for 3 days transiently reduced their fecundity by up-regulating expression of epididymal androgen receptor (AR), ER-α and reducing steroid-regulated antioxidant protection in the epididymis that led to oxidative damage and loss of sperm function. In addition, another study has found that DES induced reactive oxygen species (ROS) production in sperm cells in a dose-dependent manner even during short time usage (1 week). ROS caused the DNA and proteins oxidation in the spermatocytes and initiated the apoptosis.[34,35] Our results suggested that low-dose of *Humulus Lupulus* L. led to reduced sperm count, but higher doses were effective only on sperm motility. Probably the estrogenic activity of 8-PN had not made enough sperm toxicity via oxidative stress at low-dose, but in contrast with the abovementioned research for sperm count, like organ weight parameters and hormone levels, it was not dose-dependent. It may be related to the use of the hydroalcholic extract in this study, because the crude extract is composed of different substances and the most effective substance is not included similarly at different doses. The role of every substance should be determined in future studies.

The second evidence can be related to phytoestrogen compound bounded in the *Humulus Lupulus* L. extract (phenylninargenin) that might be more potent than genistein and affected the motility or quality of the sperm more than sperm synthesis. In agreement with this evidence, another study showed that sperms differentiate in epididymis and this process depends on the steroid's concentration, when the receptors gene expression changed, they fail to gain fertilizing ability.[36] In addition another study has shown that potency of 8-PN was 10 times more than genistein for ER-α.[3,37,38]

Our study did not show a significant effect on FSH level in either treatment groups fed with phytoestrogen or DES. Previous results revealed that inhibin and estradiol are the main regulators to suppress the secretion of the FSH in rats but only the latter is for LH.[39] Also, *P.mirifica* decreased the FSH and LH levels in a dose-dependent manner in menopausal monkeys.[40] Presumably no changes of FSH might be related to inhibin levels in male rats or the dose of *Humulus lupulus* L. in our study. The inhibin levels should be measured in future studies.

Taken together, we concluded that exposure to DES or *Humulus Lupulus* L. extract significantly increased the E_2 level, but the seminal vesicle, testes, and epididymis growth and development increased only by H50. Also, sperm motility, T and LH reduced by both of DES and *Humulus lupulus* L. extract.

Conflict of Interest

The authors have no conflicts of interest to declare.

Author Contributions

Sadeghi N performed the surgical operation, prepared and submitted the article. Karbalaei N performed the surgical operation and reviewed the article. Malekzadeh A contributed to the data analysis and Nekoeian A completed the final revision.

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