



Original Full Paper

# Developmental changes in the gubernaculum and anogenital distance of male rat offspring exposed in utero to WIN 55,212-2 as a candidate of the endocannabinoid system

Younes Kamali<sup>1</sup>, Zabihollah Khaksar<sup>1\*</sup>, Soudabeh Pourhashemi<sup>2</sup>

<sup>1</sup>Department of Anatomic Sciences, Division of Anatomy and Embryology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.

<sup>2</sup>Department of Biostatic Sciences, Shiraz University of Medical Sciences, Shiraz, Iran

\* Corresponding author: Zabihollah Khaksar, Prof. of Anatomy and Embryology, Department of Anatomical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. Tel: +989171117225; E-mail: khaksar@shirazu.ac.ir

Submitted January 26<sup>th</sup> 2016, Accepted April 22<sup>th</sup> 2016

## Abstract

Cannabinoids can inhibit the release of androgens locally or centrally. For this reason, this study was designed to assess the effect of WIN 55, 212-2, a CB1 receptor agonist, on gubernacular development histologically. Sprague-Dawley female rats were time-mated and divided into treatment and control groups. For prenatal exposures, the groups received injections subcutaneously of 0.5 mg/kg WIN 55, 212-2 (WIN) or vehicle on gestational days 13.5–20.5. Five to 13 male offspring were collected at time points E19 (embryonic), P0 (postnatal), P2, and P8. The body weight and anogenital distance (AGD) of postnatal male pups were recorded at birth. The inguino-perineal region of all the samples after fixing in 4% paraformaldehyde were sectioned oblique-sagittally and stained with Hematoxylin and Eosin (HE) or Masson's trichrome. Measured Factors in this study were based on previous studies and included gubernacular cone height (GCH), gubernacular cone width (GCW), distance from gubernaculum to scrotum (G-S), and pubic symphysis-to-anus distance (PS-A). The former two factors were measured bilaterally and the latter two only on the left side. The gubernaculum at P0 appeared more bulky than that of controls. Failure of eversion at P2 and remaining bulb of gubernaculum at P8 were important findings in WIN-treated group. The mean distance from gubernaculum to scrotum increased significantly only at P2 compared to controls. AGD as a bioassay of fetal androgen action also showed a significant 16% reduction as compared with the control group at birth. These data propose that prenatal exposure to WIN can affect gubernacular development probably due to androgen-disruptive action.

**Key words:** cannabinoids, gubernaculum, anogenital distance, testicular descent.

## Introduction

In most mammals, according to a biphasic model, i.e. transabdominal phase and inguinoscrotal phase involving various anatomical and hormonal factors, the testes migrate to the scrotum from their early intra-abdominal site. The main controlling hormone during the transabdominal stage is Insulin-like 3 peptide, which causes the gubernacular swelling reaction to occur, and thereby allows the developing testis to anchor adjacent to the future inguinal region during fetal abdominal

enlargement (21, 36, 23, 39). Afterwards, the descent of the testes will be followed in a more complex phase which is dependent on androgens and is called inguinoscrotal phase, during which the gubernaculum migrates from the inguinal canal to the scrotum. It is proposed that androgens may indirectly regulate the second phase via masculinizing the genitofemoral nerve (GFN) and as a result by releasing the calcitonin gene-related peptide (CGRP) from its sensory branch terminals (38).

Increasing of our knowledge from prenatal studies shows that a harmful agent to the mother during pregnancy

is also potentially harmful for the fetus. One of the most popular agents in this regard is marijuana (*Cannabis Sativa*) whose Intemperate use has greatly increased particularly among pregnant women in western societies (11).

D-9-tetrahydrocannabinol (D-9-THC) as the main psychoactive component of cannabis and two responding receptors to it (which are called cannabinoid CB1 and CB2 and belong to the superfamily of G-protein coupled receptors) can exert various symptomatic and behavioral effects in our body (3). In some researches, acute administration of THC in pregnant mothers was associated with various changes in sexual hormone levels (4, 29, 30). The ability of cannabinoids to pass through the placental barriers and their possible effects on embryo development has been established (12). Endocannabinoid system has a pivotal role in testis development and in the functioning of the male genital system both centrally and locally (5, 17, 41). Studies in animal models suggest that prenatal exposure to cannabinoid agonists can result in some behavioral and neuroendocrine disturbances in adulthood. Indeed, the brain of the fetus can receive high amounts of cannabinoids due to the incomplete blood-brain barrier (6). On the other hand, in rodent and human embryo tissues, cannabinoid CB1 and CB2 receptors are widely expressed during different stages of development. Following prenatal prescription of THC in mice, death before birth and fetal weight were influenced (18). In addition, testosterone serum levels and testes weight in male offspring born from these mice significantly decreased (8). In adult male rats, short- and long-term use of THC probably due to its effects on hypothalamo-hypophyseal axis leads to significant suppression of testosterone formation and reduced testicular weight (27, 19). In general, a comprehensive review of the literature shows that there is no data concerning the effects of prenatal cannabinoid exposure on the descent of the testes. Hence, the present study aimed to evaluate whether exposure to WIN 55,212-2 (WIN) as a selective CB1 receptor agonist during late gestation of the rat could affect the anatomical development of the gubernaculum which is a main structure involved in testicular descent.

## Materials and methods

### *Experimental Animals*

Sprague-Dawley female rats were time-mated at the Laboratory Animal Research Center of the Veterinary College of Shiraz University. The morning day when sperm was found in the vagina by vaginal smear was considered as embryonic day (E) 0. Then, pregnant animals were randomly divided into two groups (at least 12 rats in each group) and housed individually in shoebox rat cages under controlled conditions of lighting (12/12-h light/dark cycle) and temperature (22°C). Commercial rat pellets and tap water were provided ad libitum. This

experiment was accomplished under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986, were used regarding the standards in the protection of animals used for experimental purposes. The two groups received WIN 55, 212-2 (Sigma Aldrich) (0.5 mg/kg, dissolved in 0.3% Tween 80-saline) or WIN vehicle (control) at a volume of 1 ml/kg, from E13.5, i.e. developmentally after sex determination to E20.5. Dose selection was based on previous studies in which neither structural malformations nor maternal toxicity were observed (31, 2). The route of administration was subcutaneously in the dorsal neck skin fold. Male offspring from both control and WIN treated groups were obtained at ages E19 (n=5, 7), postnatal day 0 (n=6, 13), postnatal day 2 (n=8, 9), and postnatal day 8 (n=10, 8). The timed-pregnant rats were allowed to deliver spontaneously (late on E21= postnatal day 0), except for the 19-day-old embryos that were removed by caesarean section under general anesthesia with a combination of ketamine 10% and xylazine 2%.

### *Tissue preparation*

Postnatal male pups were chosen on the basis of their approximate anogenital distance, when possible and their body weight was recorded on the first day of birth. Anogenital distance (AGD), measured from the anterior edge of the anus to the posterior base of the genital tubercle, was also determined using an ocular micrometer for each neonate in WIN-treated and control groups. After intracardiac perfusion with 4% paraformaldehyde and then decapitating, the posterior half of the bodies were excised from the rest and immersed in 4% buffered formaldehyde overnight for more detailed dissection. Except for the inguinal regions, by using a stereo microscope, the rest of the additional tissues were removed and the position of the testicle in relation to the neck of the bladder, inguinal ring or scrotum was evaluated. Then the trimmed samples were cut in half oblique-sagittally and transferred into an autotechnicon machine for passing routine histological processes. They were then embedded in paraffin wax and sections were prepared to a thickness of 5  $\mu$ m with disposable metal microtome blades. Some sections were stained with hematoxylin and eosin (H & E) and some other with Masson's trichrome. For each specimen, 5 slides among serial sections with equal distance from each other were selected. For measurement of various structural parameters a light microscope (Olympus BX51, Tokyo, Japan) equipped with a graduated ocular lens was used. Each unit of calibrated line of the lens with magnification of  $\times 40$ ,  $\times 10$  and  $\times 4$  was 2.5, 10 and 23.8 micrometer, respectively. Factors considered in this study are based on previous study and include gubernacular cone height (GCH), gubernacular cone width (GCW), distance from gubernaculum to scrotum (G-S), and pubic symphysis-to-

anus distance (PS-A) (25). Except for the latter two factors, the other measurements in each specimen were taken bilaterally.

### *Statistical Analysis*

Statistical analysis was performed using SPSS (version 22) and a non-parametrical Mann–Whitney test.  $P < 0.05$  was considered statistically significant. All the dimensions were recorded in micrometer ( $\mu\text{m}$ ) and the weights in gram (g).

### **Results**

Based on the histological results in control rats, the gubernaculum was thickened and lengthened due to completion of the swelling reaction at E19. The bulb of gubernaculum consists of a segregate central core of mesenchyme wrapped by two layers of future cremaster muscle (Fig. 1-A). By the day of delivery, which is considered to be P0, the extracellular matrix of the bulb is widely reabsorbed causing the external layers of the cremaster muscle to come into close contact. In this way, the central core in the form of a small proliferative zone is only limited to the cranial end of the gubernacular cone (Fig. 1-B). By day P2, during remodeling and eversion of the bulb, its structure from an initial solid cone becomes a hollow cone, thereby producing the processus vaginalis (PV) and inguinal canal (Fig. 1-C). Up to 8 days after birth, with the continued growth and elongation of the everted gubernaculum towards scrotum, a fibrous attachment is established between them (Fig. 1-D). There were no abnormalities of the gubernaculum in any of the male offspring in control groups. By contrast, some abnormalities in male neonates born from WIN-treated dams were seen in each group. Three out of 13 males (23%) at P0 showed unilateral (2 males) or bilateral (1 male) abnormality in the gubernaculum. Two out of nine males (22%) at P2 exhibited unilateral gubernacular abnormality (only on the left side). Finally, three out of eight males (37.5%) at P8 had abnormality in the gubernaculum unilaterally. The postnatal gubernaculum at P0 was not regressed and due to the failure of matrix reabsorption seemed short and voluminous. However, the central mesenchymal core was tended to the tip of the bulb in one specimen (Fig. 1-B'). The normal eversion and remodeling of the gubernaculum at P2 had not occurred and was intra-abdominal site (Fig. 1-C'). The gubernaculum at P8 appeared shorter than normal (while gubernacular bulb remained), was not everted and seemed to be bulky (Fig. 1-D'). The thickness of gubernacular muscle was also greater than that of the control group (Fig. 2).

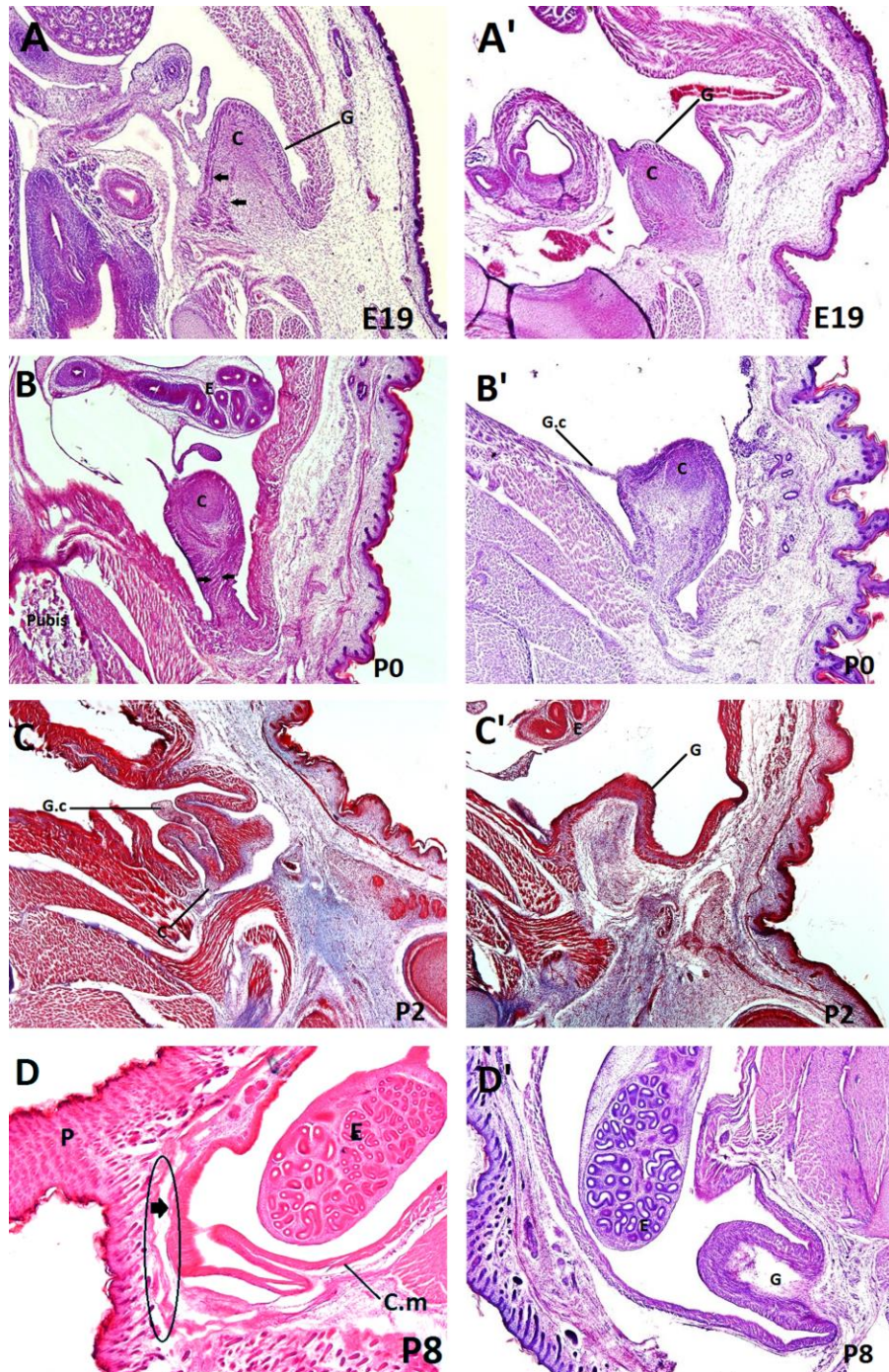
Differences between the two groups in the dimensions of the parameters studied have been evaluated,

listed, averaged, and summarized in Tables. Following in utero exposure to WIN, the mean distance from gubernaculum to scrotum was greater than that of the control group at P0, P2, and P8. But it was statistically significant only at P2 ( $P < 0.05$ ,  $4802.78 \pm 139.44 \mu\text{m}$  vs  $4615.63 \pm 150.56 \mu\text{m}$ ) (Table 1). In the latter group the mean height of the left gubernaculum was also significantly decreased compared to the control group ( $P < 0.05$ ,  $1121.67 \pm 125.90 \mu\text{m}$  vs  $1225 \pm 59.77 \mu\text{m}$ ). Although, the gubernacula of the WIN-treated group compared to the control group at P0 and P8 on the left side showed a slight reduction in height and an increase in width, the differences did not reach a significance level. No significant difference was observed at parameters measured in the two groups at E19.

Table 2 compares the dimensions of AGD and body weight in male pups on P0 between the WIN-treated and control groups. The mean AGD in male pups born to WIN-treated dams on P0 was  $2090.63 \pm 384.50 \mu\text{m}$  showing a significant ( $P < 0.05$ ) 16% reduction as compared with the control group. There was no significant difference between the mean body weight of the two experimental groups on P0 ( $P > 0.05$ ).

### **Discussion**

The influence of cannabinoids on androgens with more consistent effects on animal models than human ones has been documented in several earlier publications. Testosterone production was significantly suppressed by putting both whole decapsulated mouse testis and rat isolated Leydig cells in the media containing THC (8, 9, 22). Chronic exposure to THC in male mice could induce Leydig cells degeneration and spermatogenesis arrest (10). Short-term THC exposure resulted in the decrease of serum testosterone and inhibited the ability of testosterone to recover castration-induced changes in accessory sex structures in male rats (24, 14). It has been suggested that the effects of cannabinoids on androgens are not limited to testosterone, because dihydrotestosterone binding to androgen receptors of rat prostate cells was blocked by cannabinoids *in vitro* (37). The ability of Leydig cells to express CB1 receptors and the interaction of these receptors with endocannabinoids has a significant role in postnatal development of adult Leydig cells (5). Wenger et al. (40) showed that serum testosterone levels declined in CB1 knockout mice probably due to disrupted Leydig cells function induced by deregulation in endocannabinoid system during development (40). In addition to the direct reported effects on testis, endocannabinoid system regulates gonadal androgens centrally via down-regulating the release of LH from the anterior pituitary gland and GnRH from the hypothalamus (13, 32).



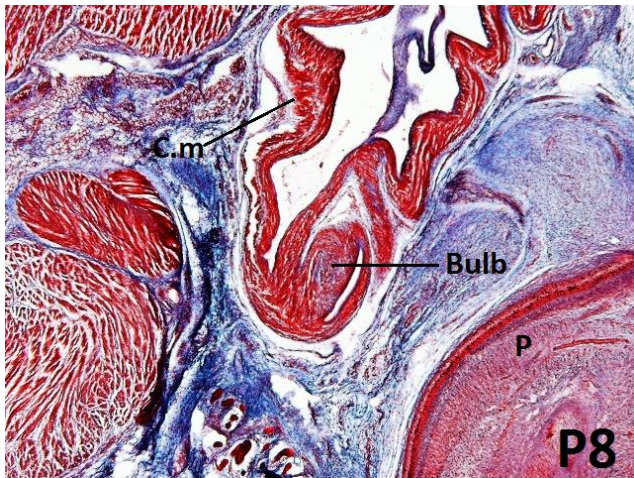
**Figure 1.** A, B, C, and D show oblique-sagittal sections of the inguinal region of the control male rats on embryonic day 19 (E19), postnatal day 0 (P0), P2, and P8, respectively. A', B', C', and D' depict the same sections in WIN-treated group. A and A': No differences are observed in gubernacula between the two groups at E19. (C) Central core of mesenchyme surrounded by (arrowheads) two layers of developing cremaster muscle. By day P0 (B), the gubernaculum is regressed and mesenchymal core (C) reduced to a proliferative zone at the cranial end of the gubernacular bulb, therefore the outer layers of the cremaster muscle are drawn towards each other (arrows). B' shows a bulky gubernacular bulb without any resorption of extracellular matrix. C: Complete remodeling and eversion of the gubernaculum on postnatal day 2 which has not occurred for WIN-treated group (C') and is similar to the prenatal gubernaculum. Note the mesenchymal core (C) at the tip of the everted gubernaculum. D: At P8, the bulb of gubernaculum has nearly disappeared and the cremaster muscle (C.m) is thin at the rim of the gubernaculum (arrowhead). Note the secondary attachment of gubernacula to the skin of scrotum (encircled by oval). D': remaining and uneverted gubernaculum (G) in WIN-treated group at P8. (Panels C, C' were stained with Masson's trichrome and panels A, A', B, B', D, D' by H&E, 40X).

**Table 1.** Dimensions of various parameters between the control and WIN-treated groups at P2 (µm). G-S: distance from gubernaculum to scrotum; PSA: distance from pubic symphysis to anus; GCH: gubernacular cone height; GCW: gubernacular cone wide; SD: standard deviation; ns-non significant.

P2 Parameter	Control (n=8)		WIN-Treated (n=9)		P Value
	Min-Max	Mean±SD	Min-Max	Mean±SD	
G-S	4375-4875	4615.63±150.56	4625-5050	4802.78±139.44	0.02
PS-A	4925-5300	5140.63±130.88	4950-5300	5141.67±118.59	ns
Left GCH	1125-1300	1225±59.77	900-1250	1121.67±125.90	0.047
Left GCW	440-600	538.13±61.40	450-710	578.33±79.77	ns
Right GCH	1175-1350	1245±55.36	1150-1375	1252.78±72.98	ns
Right GCW	440-590	521.25±47.65	460-570	524.44±35.75	ns

**Table 2.** Body weight and anogenital distance of male rat offspring at birth.

P0 Parameter	Control (n=24)		WIN-Treated (n=30)		P Value
	Min-Max	Mean±SD	Min-Max	Mean±SD	
Body Weight (g)	4.5-7.2	6.05±1.15	4.1-7.5	6.00±1.21	0.057 <sup>c</sup>
AGD (µm)	2225-2675	2500±134.37	1500-2625	2090.63±384.50	0.016



**Figure 2.** The gubernaculum in WIN-treated group has a thick cremaster muscle (C.m) and a remaining solid bulb. P, Penis. (Trichrom staining, 40X).

The first important finding of the present study is that prenatal administration of WIN 55,212-2 in rats, at a dose that is not related to unconcealed signs of toxicity, from E13.5 to E20.5 can induce some developmental changes in the gubernacula of male offspring. The findings of the control group are consistent with previous observations in rodents. Based upon these observations, development of the gubernaculum as a fundamental structure in testicular descent divides into two sequential phases: (1) an initial growth and swelling of the bulb of the gubernaculum along with shortening of the gubernacular cord from E16 to E19 in the abdomen, and (2) postnatal shrinkage, remodeling and eversion of the gubernaculum out of the abdominal muscles and its elongation towards

the scrotum (35). Gubernacular responsiveness to androgens in the latter phase through two proposed subpopulations of androgen receptors (AR), located at the inguinal fat pad (IFP) and the gubernaculum itself, has long been documented. By recruiting AR in the IFP, androgens may indirectly control the development of the gubernaculum with an effect on masculinizing the GFN (1, 42). Histologically, gubernacula of the control group at different time points depict normal changes in progress with migrating from intra-abdominal to inguinoscrotal site, whereas gubernacula in WIN-treated rats were retarded in postnatal development and showed failure of regression or eversion. The bulb of gubernaculum based on its appearance suggests a persistence of the embryonic swelling reaction. It has been shown that in utero exposure to the antiandrogen flutamid from E16 to E19 can lead to impairment in differentiation of gubernacular mesenchymal cells and also to gubernacular eversion in the inguinoscrotal phase without any effect on transabdominal phase which occurs prenatally (34).

Also, the cremaster muscle derived from gubernaculum in flutamid-treated rats was thicker than that of controls. Some authors have hypothesized that increasing the thickness of the muscle layers of the gubernaculum in cryptorchid testis is because of the failure of gubernacular eversion or prolonged cell proliferation (28, 34). In our study, although the muscle layers by day P8 were seemingly thicker than those of the control, for confirming this claim we need to detect the muscle fibers using antibody against desmin.

Selection of the sectioning plane was done according to a study conducted by Lam et al. 1998 (25). In this type of sectioning plane, the position of gubernaculum relative to the bottom of the scrotum, and therefore, the

dimensions are more accurate and real. The results of the control group agree with the latter study, showing that for gubernaculum to reach the floor of the scrotum, eversion along with active elongation is essential for migration to occur completely. The mean gubernacular distance to the bottom of the scrotum increased at time points P0, P2, and P8 in WIN-treated group and it was significant only at P0. On the one hand, shortening of the gubernaculum and on the other disruption in eversion could be considered as reasons for increasing the distance by day P2.

The other interesting finding in this study were changes in the anogenital distance at birth. In some neonates born to dams exposed to WIN due to a short anogenital distance, the gender determination by external observation was misleading. As perineal growth in rodents is controlled by dihydrotestosterone, hence the AGD as a standard indicator for assessment of in utero androgen status in this study was measured. Exposure to various agents with the endocrine-disruptive action in pregnant rats has resulted in alterations in the AGD of offspring. Among the most important of such compounds that can be named are androgens such as testosterone and androstenedione (16), the antiandrogens vinclozolin (15), flutamide (7) (20), and DBP (33), and estrogens, including the isoflavonoid phytoestrogen genistein, as well as estradiol benzoate and diethylstilbestrol (DES) (26). As already mentioned, cannabinoids can inhibit dihydrotestosterone binding sites in rat prostate cells (37). For this reason, we hypothesize that a significant reduction in the AGD succeeding WIN treatment as an exogenous cannabinoid is not unexpected. Also, following up the exposed offspring until adulthood will be required to determine whether the decrease of AGD is permanent or transitory.

In conclusion, the present study showed that prenatal exposure to WIN 55, 212-2 after sex determination and between E13.5 and E20.5 (encompassing the onset of testosterone production by the fetal testis and the critical window of male reproductive development) could cause developmental changes in gubernacula at three time points in the postnatal period and reduce AGD at P0 which is in accordance with the anti-androgenic role of WIN. However, whether the effects are associated with the direct interaction of WIN to the AR on the primary Leydig cells or centrally through hypothalamus- hypophysis axis needs to be investigated further with immunohistochemical techniques or other pharmacological methods.

#### Acknowledgements

We thank Bahman Moghissi and Ghassem Yousefi for technical assistance. We are also grateful to Dr. J. Jalaei for drug preparation. This research was financially supported by grants of Shiraz University Research Council, as part of the first author's PhD thesis.

#### References

1. ALLNUTT B., BURAUNDI S., FARMER P., SOUTHWELL BR., HUTSON JM, BALIC A. The common fetal development of the mammary fat pad and gubernaculum. **J. Pediatr. Surg.**, 2011, 46, 378-383.
2. ANTONELLI T., TOMASINI MC., TATTOLI M., CASSANO T., TANGANELLI S., FINETTI S., MAZZONI E., TRABACE L., STEARDO L., CUOMO V. Prenatal exposure to the CB1 receptor agonist WIN 55,212-2 causes learning disruption associated with impaired cortical NMDA receptor function and emotional reactivity changes in rat offspring. **Cereb. Cortex.**, 2005, 15, 2013-2020.
3. BHATTACHARYYA S., MORRISON PD., FUSARPOLI P., MARTIN-SANTOS R., BORGWARDT S., WINTON-BROWN T., NOSARTI C., MO'CARROLL C., SEAL M., ALLEN P. Opposite effects of delta-9-tetrahydrocannabinol and cannabidiol on human brain function and psychopathology. **Neuropsychopharmacology**, 2010, 35, 764-774.
4. BRAUNSTEIN G., ASCH R. Predictive value analysis of measurements of human chorionic gonadotropin, pregnancy specific beta 1-glycoprotein, placental lactogen, and cystine aminopeptidase for the diagnosis of ectopic pregnancy. **Fertil. Steril.**, 1983, 39, 62-67.
5. CACCIOLA G., CHIOCCARELLI T., MACKIE K., MECCARIELLO R., LEDENT C., FASANO S., PIERANTONI R., COBELLIS G. Expression of type-1 cannabinoid receptor during rat postnatal testicular development: possible involvement in adult Leydig cell differentiation. **Biol. Reprod.**, 2008, 79, 758-765.
6. CAMPOLONGO P., TREZZA V., RATANO P., PALMERY M., CUOMO V. Developmental consequences of perinatal cannabis exposure: behavioral and neuroendocrine effects in adult rodents. **Psychopharmacology**, 2011, 214, 5-15.
7. CLEMENS LG., GLADUE BA., CONIGLIO LP. Prenatal endogenous androgenic influences on masculine sexual behavior and genital morphology in male and female rats. **Horm. Behav.**, 1978, 10, 40-53.
8. DALTERIO S., BARTKE A. Perinatal exposure to cannabinoids alters male reproductive function in mice. **Science**, 1979, 205, 1420-1422.
9. DALTERIO S., BARTKE A., BURSTEIN S. Cannabinoids inhibit testosterone secretion by mouse testes in vitro. **Science**, 1977, 196, 1472-1473.
10. DIXIT V., SHARMA V., LOHIYA N. The effect of chronically administered cannabis extract on the testicular function of mice. **Eur. J. Pharmacol.**, 1974, 26, 111-114.
11. EL MARROUN H., TIEMEIER H., JADDOE VW., HOFMAN A., MACKENBACH JP., STEEGERS EA., VERHULST FC., VAN DEN BRINK W.,

- HUIZINK AC. Demographic, emotional and social determinants of cannabis use in early pregnancy: the Generation R study. **Drug Alcohol. Depend.**, 2008, 98, 218-226.
12. EL MARROUN H., TIEMEIER H., STEEGERS EA., ROOS-HESELIN JW., JADDOE VW., HOFMAN A., VERHULST FC., VAN DEN BRINK W., HUIZINK AC. A prospective study on intrauterine cannabis exposure and fetal blood flow. **Early Hum. Dev.**, 2010, 86, 231-236.
  13. GAMMON CM., FREEMAN JR GM., XIE W., PETERSEN SL., WETSEL WC. Regulation of gonadotropin-releasing hormone secretion by cannabinoids. **Endocrinology**, 2005, 146, 4491-4499.
  14. GHOSH S., CHATTERJEE T., GHOSH J. Antiandrogenic effect of delta-9-tetrahydrocannabinol in adult castrated rats. **J. Reprod. Fertil.**, 1981, 62, 513-517.
  15. GRAY L., OSTBY JS., KELCE WR. Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. **Toxicol. Appl. Pharmacol.**, 1994, 129, 46-52.
  16. GREENE R., BURRILL M., IVY A. Experimental intersexuality. The effect of antenatal androgens on sexual development of female rats. **Am. J. Anat.**, 1939, 65, 415-469.
  17. GYE MC., KANG HH., KANG HJ. Expression of cannabinoid receptor 1 in mouse testes. **Arch. Androl.**, 2005, 51, 247-255.
  18. HARBISON RD., MANTILLA-PLATA B. Prenatal toxicity, maternal distribution and placental transfer of tetrahydrocannabinol. **J. Pharmacol. Exp. Ther.**, 1972, 180, 446-453.
  19. HARCLERODE J., NYQUIST S., NAZAR B., LOWE D. Effects of cannabis on sex hormones and testicular enzymes of the rodent. **Adv. Biosci.**, 1978, 22, 395.
  20. HIB J., PONZIO R. The abnormal development of male sex organs in the rat using a pure antiandrogen and a 5 alpha-reductase inhibitor during gestation. **Acta Physiol. Pharmacol. Ther. Latinoam.**, 1995, 45, 27-33.
  21. HUTSON JM., BAKER M., TERADA M., ZHOU B., PAXTON G. Hormonal control of testicular descent and the cause of cryptorchidism. **Reprod. Fertil. Dev.**, 1994, 6, 151-156.
  22. JAKUBOVIC A., MCGEER E., MCGEER P. Effects of cannabinoids on testosterone and protein synthesis in rat testis Leydig cells in vitro. **Mol. Cell. Endocrinol.**, 1979, 15, 41-50.
  23. KUBOTA Y., NEF S., FARMER PJ., TEMELCOS C., PARADA LF., HUTSON JM. Leydig insulin-like hormone, gubernacular development and testicular descent. **J. Urol.**, 2001, 165, 1673-1675.
  24. KUMAR M., CHEN C. Effect of an acute dose of delta 9-THC on hypothalamic luteinizing hormone releasing hormone and met-enkephalin content and serum levels of testosterone and corticosterone in rats. **Subst. Alcohol Actions Misuse**, 1983, 4, 37-43.
  25. LAM SK., CLARNETTE TD., HUTSON JM. Does the gubernaculum migrate during inguinoscrotal testicular descent in the rat? **Anat. Rec.**, 1998, 250, 159-163.
  26. LEVY JR., FABER KA., AYYASH L., HUGHES CL. The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. **Proc. Soc. Exp. Biol. Med.**, 1995, 208, 60-66.
  27. LIST A., NAZAR B., NYQUIST S., HARCLERODE J. The effects of delta-9-tetrahydrocannabinol and cannabidiol on the metabolism of gonadal steroids in the rat. **Drug Metab. Dispos.**, 1977, 5, 268-272.
  28. MATSUNO Y., KOMIYAMA M., TOBE T., TOYOTA N., ADACHI T., MORI C. Association of testicular undescend induced by prenatal flutamide treatment with thickening of the cremaster muscle in rats. **Reprod. Med. Biol.**, 2003, 2, 109-113.
  29. MENDELSON JH., CRISTOFARO P., ELLINGBOE J., BENEDIKT R., MELLO NK. Acute effects of marihuana on luteinizing hormone in menopausal women. **Pharmacol. Biochem. Behav.**, 1985, 23, 765-768.
  30. MENDELSON JH., MELLO NK., ELLINGBOE J., SKUPNY AS., LEX BW., GRIFFIN M. Marihuana smoking suppresses luteinizing hormone in women. **J. Pharmacol. Exp. Ther.**, 1986, 237, 862-866.
  31. MEREU G., FÀ M., FERRARO L., ANTONELLI T., TATTOLIM, GHIGLIERI V., TANGANELLIS, GESSA GL., CUOMO V. Prenatal exposure to a cannabinoid agonist produces memory deficits linked to dysfunction in hippocampal long-term potentiation and glutamate release. **Proc. Natl. Acad. Sci. U S A**, 2003, 100, 4915-4920.
  32. MURPHY L., STEGER R., SMITH M., BARTKE A. Effects of delta-9-tetrahydrocannabinol, cannabidiol and cannabidiol, alone and in combinations, on luteinizing hormone and prolactin release and on hypothalamic neurotransmitters in the male rat. **Neuroendocrinology**, 1990, 52, 316-321.
  33. MYLCHREEST E., CATTLEY RC., FOSTER PM. Male reproductive tract malformations in rats following gestational and lactational exposure to di (n-butyl) phthalate: an antiandrogenic mechanism? **Toxicol. Sci.**, 1998, 43, 47-60.
  34. NATION T., BALIC A., BURAUNDI S., FARMER P., NEWGREEN D., SOUTHWELL B., HUTSON J. The antiandrogen flutamide perturbs inguinoscrotal testicular descent in the rat and suggests a link with mammary development. **J. Pediatr. Surg.**, 2009, 44, 2330-2334.
  35. NATION T., BURAUNDI S., FARMER P., BALIC A., NEWGREEN D., SOUTHWELL B., HUTSON J. Development of the gubernaculum during testicular descent in the rat. **Anat. Rec.**, 2011, 294, 1249-1260.

36. NEF S., PARADA LF. Cryptorchidism in mice mutant for *Insl3*. **Nat. Genet.**, 1999, 22, 295-299.
37. PUROHIT V., AHLUWAHLIA BS., VIGERSKY RA. Marijuana inhibits dihydrotestosterone binding to the androgen receptor. **Endocrinology**, 1980, 107, 848-850.
38. SHENKER NS., HUYNH J., FARMER PJ., HUTSON JM. A new role for androgen in testicular descent: permitting gubernacular cell proliferation in response to the neuropeptide, calcitonin gene-related peptide. **J. Pediatr. Surg.**, 2006, 41, 407-412.
39. TOMIYAMA H., SASAKI Y., HUYNH J., YONG E., TING A., HUTSON JM. Testicular descent, cryptorchidism and inguinal hernia: the Melbourne perspective. **J. Pediatr. Urol.**, 2005, 1, 11-25.
40. WENGER T., LEDENT C., CSERNUS V., GERENDAI I. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. **Biochem. Biophys. Res. Commun.**, 2001, 284, 363-368.
41. WENGER T., RETTORI V., SNYDER G., DALTERIO S., MCCANN S. Effects of delta-9-tetrahydrocannabinol on the hypothalamic-pituitary control of luteinizing hormone and follicle-stimulating hormone secretion in adult male rats. **Neuroendocrinology**, 1987, 46, 488-493.
42. YONG EX., HUYNH J., FARMER P., ONG SY., SOURIAL M., DONATH S., TING AY., CHOW Y., HUTSON JM. Calcitonin gene-related peptide stimulates mitosis in the tip of the rat gubernaculum in vitro and provides the chemotactic signals to control gubernacular migration during testicular descent. **J. Pediatr. Surg.**, 2008, 43, 1533-1539.