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# 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

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## 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 Hematoxilin 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 (22ºC). 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 authotechnicon machine for passing routine histological processes. They were then embedded in paraffin wax and sections were prepared to a thickness of 5 µm 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 ×40, ×10 and ×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 (µm) and the weights in gram (g).

## Results

Based on the histological results in control rats, the gubernaculam 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±139.44 µm vs 4615.63±150.56 µm) (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±125.90 µm vs 1225±59.77 µm). 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±384.50 µm 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).

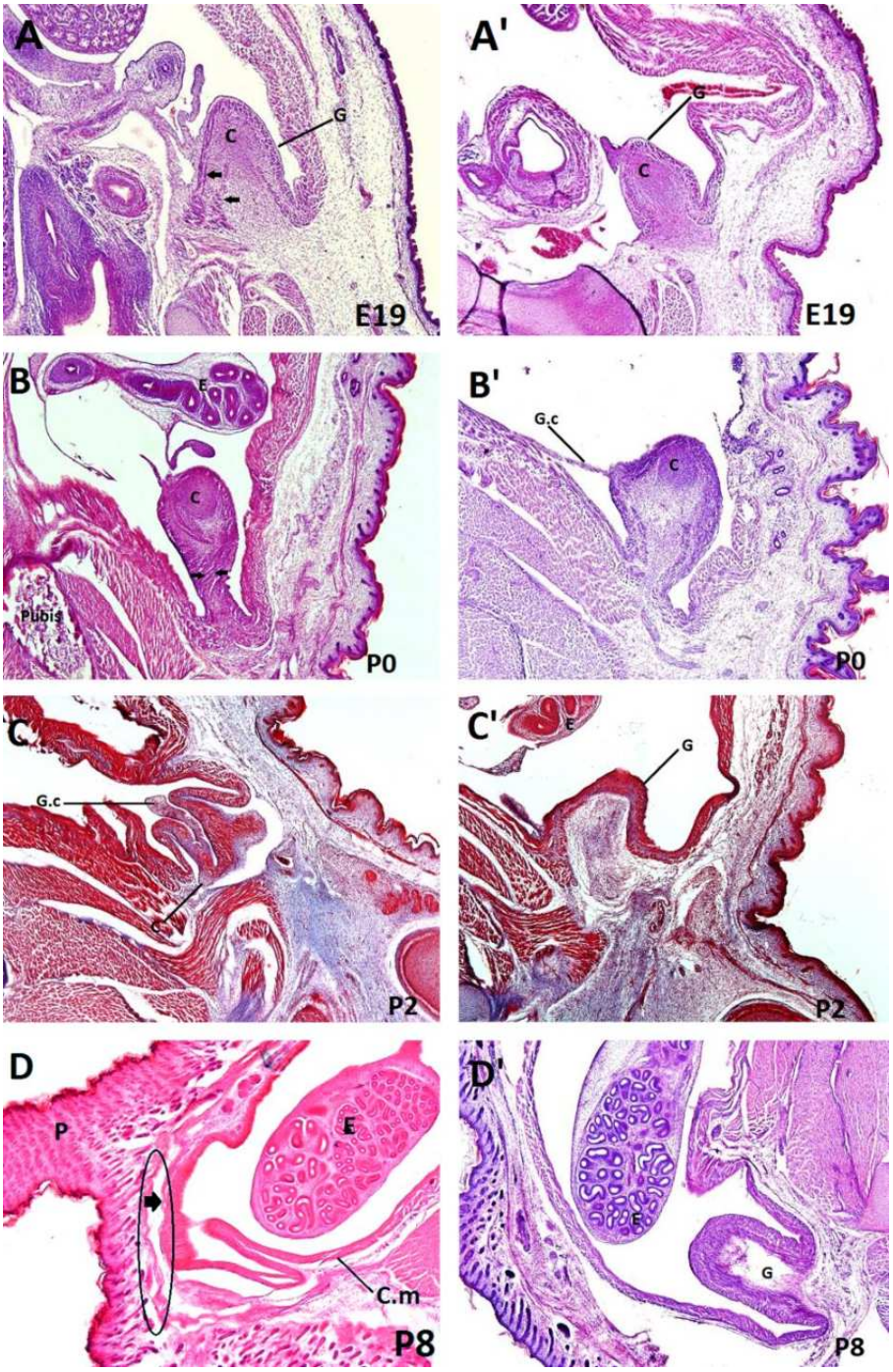



**Figure 1.** A, B, C, and D show oblique-sagittal sections of the inguinal region of the control male rats on embryonic day 19 (E19), postnatal day 0 (P0), P2, and P8, respectively. A', B', C', and D' depict the same sections in WIN-treated group. A and A': No differences are observed in gubernacula 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 gubernaculums 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 | Control (n=8) | | WIN-Treated (n=9) | | |
|---|---|---|---|---|---|
| **Parameter** | **Min-Max** | **Mean±SD** | **Min-Max** | **Mean±SD** | **P Value** |
| 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 | Control (n=24) | | WIN-Treated (n=30) | | |
|---|---|---|---|---|---|
| **Parameter** | **Min-Max** | **Mean±SD** | **Min-Max** | **Mean±SD** | **P Value** |
| Body Weight (g) | 4.5-7.2 | 6.05±1.15 | 4.1-7.5 | 6.00±1.21 | 0.057[c] |
| AGD (µm) | 2225-2675 | 2500±134.37 | 1500-2625 | 2090.63±384.50 | 0.016 |

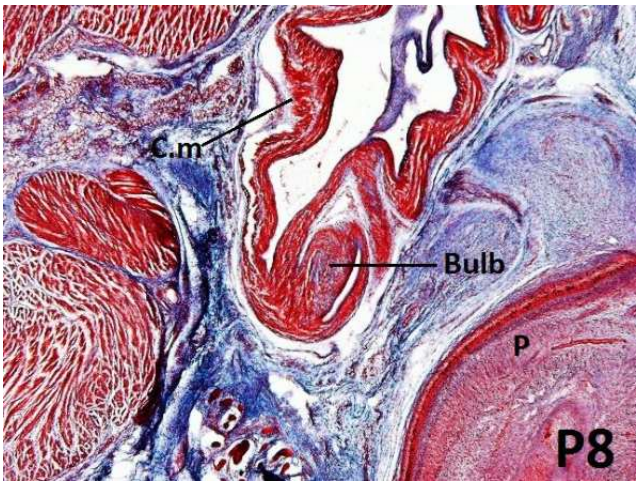



**Figure 2.** The gubernaculum in WIN-treated group has a thick cremaster muscle (C.m) and a remaining solid bulb. P, Penis. (Trichrom staining, 40X).

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.

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