Endocrinological and histological changes induced by flutamide treatment on the hypothalamo-hypophyseal testicular axis of the adult male rat and their incidences on fertility

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Abstract. Adult male Wistar rats were treated with flutamide from 90 to 105 days of age. In a first experiment, testis and accessory sex organs were weighed. In the same animals, hypothalamic LRH content, pituitary gonadotrophin concentrations, plasma LH, FSH, prolactin and testosterone levels, and testicular gonadotrophin receptors were evaluated. In a second experiment, fertility was tested at the end of the treatment, and histology of the testis was performed. All the results were compared to those obtained in control animals of the same age. Accessory glands of genital tract were significantly lower in flutamide-treated animals (P < 0.01). Hypothalamic LRH, pituitary and plasma FSH, and prolactin concentrations were unchanged, while pituitary and plasma LH level and especially plasma testosterone concentration were increased (P < 0.001).

Flutamidé therefore exerted a strong inhibition on testosterone-dependent organs, and blocked the negative feedback of testosterone on the hypothalamo-pituitary axis, increasing the LH levels. Testis weight, intertubular tissue volume, total number and total volume of Leydig cells/testis, as well as total length and diameter of seminiferous tubules were unchanged in flutamide treated rats. However number of LH receptors/Leydig cell, nuclear area of Sertoli cells, number of FSH receptors/Sertoli cell, number of leptotene spermatocytes and of round spermatids per cross section, and yield of spermatogonial divisions were decreased after treatment. Flutamide treatment also decreased fertility by 48% (P < 0.05). This lowered fertility is likely the result of impaired spermatogenesis and/or a dysfunction of accessory sex organs.

Flutamide (4'-nitro-3'-trifluoro-methylisobutyramide, Sch 13521), is a potent, non-steroidal anti-androgen with neither progestational nor oestrogenic effects (Neri et al. 1972; Neumann et al. 1977). As shown previously (Viguier-Martinez et al. 1983), flutamide induces important changes in growing male rats on the hypothalamo-pituitary axis as well as on testicular physiology. The present work was designed to study the effects of flutamide treatment on the hypothalamo-pituitary testicular axis in the adult and to compare to those already obtained in the prepubertal rat. This experiment performed in adult rats could moreover allow to test the incidence of flutamide on the fertility, at the end of a short-term treatment. Such a model could be used to investigate the possible differences in the feedback control of LH and FSH, as well as to estimate the direct or indirect actions of a pure anti-androgen on the testicular physiology of the adult rat.

Materials and Methods

a. Animals

Two groups of 15 male Wistar rats each received a daily sc injection from 90 to 105 days of age. In the control group, rats received solvent (0.2 ml arachis oil/benzyl alcohol 9:1 v/v); in the flutamide group, rats received 10 mg/kg/day of flutamide (Schering Corp. Bloomfield,
N. J., USA) dissolved in 0.2 ml solvent. Rats were housed in group cages under controlled light (14:10 d) and temperature (22°C). In Experiment I, 10 animals from the control group and 10 animals from the flutamide group were slaughtered by decapitation on the morning after the last injection. Testes, epididymes, ventral prostate and seminal vesicles were immediately dissected out and weighed. Testes were used for testicular LH and FSH receptors assay. Blood samples were collected after decapitation, centrifuged and frozen until radioimmunoassay. Hypothalami and pituitaries were dissected out, homogenized in 0.1 n-HCl for hypothalami, or distilled water for pituitaries, and frozen until radioimmunoassay. In Experiment II, the remaining 5 animals from each group were tested for fertility during the last 5 days of the treatment (from 100 to 105 days of age), then slaughtered by decapitation on the morning after the last injection. In this experiment, testes were dissected out, weighed and used for histological analysis.

b. Radioimmunoassays
Pituitaries and plasma samples were analysed for FSH content using a specific radioimmunoassay kit for the rat FSH (NIAMDD) and the LH content using a specific double antibody radioimmunoassay previously described (Viguier-Martinez 1976). The results were expressed in terms of NIAMDD rat FSH-RP1 for FSH, and purified rat LH SX1-1 for LH. One unit of LH SX1-1 was equivalent to 1.58 units of NIH-LH S 11. Plasma levels of prolactin were measured using a specific radioimmunoassay method described by Martinat et al. (1979). The potency of the standard (Prl, I.N.R.A.) was about twice that of the NIAMDD rat Prl RP1. Plasma testosterone was extracted with 2 ml of ethyl acetate/cyclohexane mixture (1:1, v/v) per 0.3 ml plasma and then measured by radioimmunoassay (Viguier-Martinez et al. 1983). The intra-assay variation of this method was 6% and the detection limit 50 pg testosterone/ml plasma. LH content was estimated by a specific radioimmunoassay (Caraty et al. 1980). All samples of each hormone were processed in duplicate. The hormone concentrations were determined using logit-log transformations.

c. Testicular LH and FSH receptors
The numbers of LH and FSH receptor sites were measured in Experiment I from a pool of testicular membranes (10 testes per pool) as the protein content per rat testis was not sufficient to perform individual assays. Crude plasma membranes from rat testes were prepared as previously described in the ram (Barenton & Pelletier 1980). Testicular LH and FSH receptors were assayed according to Viguier-Martinez et al. (1983).

d. Testicular histology
Testes of control and flutamide-treated rats of Experiment II were fixed in Bouin Hollande solution. Intertubular and tubular tissue were analysed as described by Hochereau-de-Reviers et al. (1976, 1979).

\[ \text{Fig. 1.} \]

**Effect of flutamide treatment on the body weight and the weight of genital tract of adult male rats (m ± SEM).**

For testicular weight, left and right testis have been weighed separately.
e. Study of fertility
In Experiment II, each rat was placed with three cyclic females for 5 consecutive days. Number of pregnant females and number of pups in each litter were determined at parturition.

f. Statistics
In the figures and the tables, data are presented as mean ± standard error of the mean. Student’s t-test was used in the analysis of differences between means.

Results

a. Genital tract
Flutamide treatment did not affect body weight, nor testicular weight of adult rats, but caused a significant decrease in the weight of the epididymis, seminal vesicles and ventral prostate by 19%, 46% and 35%, respectively (Fig. 1, P < 0.001).

b. Endocrinological data
Hypothalamic LH content was appreciably increased (28%) after flutamide treatment (Fig. 2), but this increase was not significant. Pituitary weight and pituitary FSH concentration remained unchanged after flutamide treatment, but pituitary LH concentration was significantly increased by about 81% (P < 0.01). In flutamide-treated rats, plasma FSH and prolactin levels remained unchanged as compared to control animals, but plasma LH level was significantly (P < 0.01) increased by 60% after flutamide treatment (Fig. 3). Plasma levels of testosterone were greatly increased after flutamide treatment (700%, P < 0.001).

c. Testicular gonadotrophin receptors
In assays of both LH and FSH receptors, Scatchard plots showed only slope, indicating a single class of binding sites for both types of receptors. As shown in Table 1, the binding affinity constant (Ka) of LH and FSH to their respective receptor sites did not change after flutamide treatment. The number of LH receptors was slightly decreased (about 5%) when expressed per mg protein, but this decrease is more apparent (22% and 23%, respectively) when expressed either per testis, or per Leydig cell in flutamide treated rats. The number of FSH receptors expressed per mg protein, per testis or per Sertoli cell was decreased in all cases (23%, 37% and 44%, respectively) in flutamide treated rats.

d. Testicular histology
Flutamide treatment induced few modifications on histological testicular parameters (Table 2). Intertubular tissue volume, total volume and total num-
Effect of flutamide treatment on plasma levels of gonadotrophins, prolactin and testosterone of adult male rats (m ± SEM).

(During treatment, the number of Leydig cells/testis, cytoplasmic and nuclear area of Leydig cells, as well as total length and diameter of seminiferous tubules were unchanged in flutamide treated rats, as compared to control rats. Total number of Sertoli cells was not significantly different, but their nuclear area was decreased by about 17% (P < 0.05). Spermatogenesis was affected by the treatment, since the numbers of leptotene spermatocytes and of round spermatids per cross section were decreased by 10% (P < 0.05), as well as yield of spermatogonial divisions (20% decrease).

c. Fertility
In all the flutamide-treated rats, the percentage of pregnant females was significantly decreased by 48% (P < 0.05) at the end of the treatment, as compared to control rats. Conversely, the number of pups in each litter was not affected by the flutamide treatment.

Table 1.
Effect of flutamide treatment on testicular gonadotrophic hormones receptors in adult male rats.

<table>
<thead>
<tr>
<th></th>
<th>Ka for LH receptors (10^{10} M^{-1})</th>
<th>Number of LH receptor sites</th>
<th>Ka for FSH receptors (10^{10} M^{-1})</th>
<th>Number of FSH receptor sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fmoles/mg protein</td>
<td>fmoles/testis</td>
<td>binding sites/Leydig cell</td>
<td>fmoles/mg protein</td>
</tr>
<tr>
<td>Control* n = 10</td>
<td>6.11</td>
<td>8.13</td>
<td>186.00</td>
<td>11 544</td>
</tr>
<tr>
<td>Flutamide* n = 10</td>
<td>5.67</td>
<td>7.69</td>
<td>144.37</td>
<td>8868</td>
</tr>
</tbody>
</table>

* Numbers of LH and FSH receptor sites were measured for a pool of testicular membranes (10 testes per group) as the protein content per rat testis was not sufficient to perform individual assay.
Discrimination

Flutamide treatment in the adult rat exerted a strong inhibition on the epididymis, seminal vesicles and ventral prostate, but did not modify testis weight. Flutamide treatment also caused large increases in pituitary LH and plasma LH and in plasma testosterone. There were no effects on pituitary or plasma FSH or on hypothalamic LRH. The effect of flutamide on accessory sex organs has also been shown in the dog (Neri & Monahan 1972) and in the mature rat (Neumann et al. 1977). This involution of accessory organs of the genital tract is a direct consequence of the ability of flutamide to bind androgen receptors (Liao et al. 1974; Mainwarine et al. 1974). However, the decrease in accessory sex organs weight is more marked if rats are treated before puberty (Viguier-Martinez et al. 1983), as growing sex organs are more sensitive to the anabolizing action of testosterone.

In our study, hypothalamic LRH content was unchanged after 15 days of flutamide treatment. This result is not inconsistent with the increase in the hypothalamic secretion of LRH observed by Reznikov & Volkova (1979). The decrease in LRH content observed after castration in the rat (Caraty et al. 1981) shows that testosterone feedback inhibits the release rather than the synthesis of LRH in the hypothalamus. During the first days of administration flutamide, which blocks testosterone binding both at hypothalamic and pituitary levels (Reznikov et al. 1978), increases LRH secretion and above all LRH release in portal blood, as well as pituitary sensitivity to LRH (Södersten et al. 1975).

Our data also show a difference between the LH and FSH responses to flutamide treatment. The large increase in pituitary and plasma LH resulting from the central antiandrogen action of flutamide, shows that LH synthesis and secretion is mainly regulated by the negative androgen feedback. On the other hand, pituitary and plasma FSH are unchanged after a short-term flutamide treatment in the adult, while pituitary and serum FSH are increased after 6 weeks of treatment with 10 mg/day of flutamide in the adult rat (Schacher & Neumann 1988). The difference between LH and FSH responses to 15 days treatment with flutamide suggests that FSH is not very sensitive to the negative feedback of androgens in adults rats. In contrast, a short-term flutamide treatment in-

### Table 2.

Effect of flutamide treatment on testicular histology, spermatogenesis and fertility of adult male rats (m ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>Flutamide (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular weight</td>
<td>g</td>
<td>1.607 ± 0.019</td>
</tr>
<tr>
<td>Intertubular tissue volume</td>
<td>mm³</td>
<td>320 ± 10</td>
</tr>
<tr>
<td>Total volume/testis</td>
<td>mm³</td>
<td>62.7 ± 0.6</td>
</tr>
<tr>
<td>Total number/testis</td>
<td>× 10⁶</td>
<td>9.7 ± 0.5</td>
</tr>
<tr>
<td>Leydig cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic area</td>
<td>µm²</td>
<td>84 ± 2</td>
</tr>
<tr>
<td>Nuclear area</td>
<td>µm²</td>
<td>31 ± 1</td>
</tr>
<tr>
<td>Total length</td>
<td>m</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Total number/testis</td>
<td>× 10⁶</td>
<td></td>
</tr>
<tr>
<td>Seminiferous tubules</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter</td>
<td>µm</td>
<td>277 ± 1</td>
</tr>
<tr>
<td>Sertoli cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>corrected for nuclear size</td>
<td></td>
<td>19.4 ± 0.5</td>
</tr>
<tr>
<td>Nuclear area</td>
<td>µm²</td>
<td>69 ± 3</td>
</tr>
<tr>
<td>A Spermatogonia/cross section</td>
<td></td>
<td>2.38 ± 0.35</td>
</tr>
<tr>
<td>Leptotene spermatocytes/cross section</td>
<td></td>
<td>51 ± 1</td>
</tr>
<tr>
<td>Round spermatids/cross section</td>
<td></td>
<td>131.2 ± 4.6</td>
</tr>
<tr>
<td>Elongated spermatids/cross section</td>
<td></td>
<td>173.4 ± 5.1</td>
</tr>
<tr>
<td>Yield of spermatogonial divisions</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>Yield of meiosis and spermatogenesis</td>
<td></td>
<td>2.57</td>
</tr>
<tr>
<td>Fertility test: percentage of pregnant female</td>
<td></td>
<td>73 ± 9</td>
</tr>
</tbody>
</table>

* P < 0.05.
increased both plasma LH and FSH levels in growing rats (Viguier-Martinez et al. 1983). This would indicate that the negative feedback of testosterone on FSH secretion is more potent in growing than in adult rats, or that other factors controlling FSH secretion, such as inhibin, are more affected by short-term flutamide treatment in prepubertal than in adult rats. Comparable results have been obtained in rats which were passively immunized against testosterone: a high dose of antiserum increased both plasma LH and FSH concentrations (as flutamide in growing rats) while a low dose of antiserum increased only serum LH concentration (as flutamide in adult rats) (Main et al. 1980).

Plasma testosterone levels have been shown to be extensively increased by flutamide treatment in adult rats (Södersten et al. 1975; Reznikov et al. 1978) as well as in prepubertal rats (Viguier-Martinez et al. 1983). In contrast to the growing rat no increase in Leydig cell populations was obtained in adult rats despite the very high level of LH. This may indicate that the Leydig cell population is no longer multiplying, or is multiplying at a very low level in adult animals (Bergh & Damber 1978), and that variations in the number of Leydig cells are not related to plasma LH levels. This increase in testosterone levels may be accounted for that of LH. It is likely that flutamide increases the responsiveness of Leydig cells to LH despite the decrease in their sensitivity as judged by the decrease in the number of LH receptors. Such dissociation between Leydig cell sensitivity and responsiveness has been previously reported (Haour et al. 1978). It was also observed in growing rats (Viguier-Martinez et al. 1983) as well as in cryptorchid rats (Barenton et al. 1982). The level of testosterone increased 8-fold as compared to the normal concentration of testosterone. This very high level of testosterone may result from a passive accumulation of a non-active steroid, marked by an increase in the half-life and a decrease in the metabolic clearance rate of testosterone, as suggested by Reznikov et al. (1978). This has been demonstrated by Wickings et al. (1976) after active immunization of male rabbit against testosterone.

In our adult rats, flutamide treatment induced few changes in somatic and germinal cells of seminiferous tubules. However in the adult as in the young rat the number of FSH receptors per Sertoli cells was decreased after flutamide treatment, indicating that the sensitivity of Sertoli cells to FSH depends on factors other than FSH, possibly androgens. The A spermatogonia were not affected by flutamide treatment, but there was a slight inhibition of leptotene primary spermatocytes and round spermatids. Elongated spermatids were not affected. The fact that the inhibition of spermatogenesis was so drastic in comparison with that observed in male mice by Vojtiskova et al. (1978) is possibly due to the shorter duration of treatment. The low rate of fertility that we observed at the end of the treatment has been previously described after one week of flutamide treatment in male adult rats (Neumann et al. 1977). These investigators demonstrated that the inhibition of fertility is temporary, fertility being partly or totally restored after the third week of treatment. These results are in agreement with those obtained after a long-term treatment in rats (Neri et al. 1972) or in dogs (Neri & Monahan 1972). Furthermore our decrease in fertility cannot be related to a decrease in sexual behaviour since Södersten et al. (1975) and Gray (1977) have demonstrated that flutamide does not impair the sexual behaviour of the male rat. Nor is this partial loss of fertility likely to be a consequence of a lower production of sperm, as elongated spermatids were not affected, but related to a dysfunction of accessory sex organs, specially epididymis (Dhar & Setty 1976) and ventral prostate (Mainwaring et al. 1974). In this case, a 15 day treatment with flutamide could reveal this temporary decrease in fertility, while after a prolonged administration, spermatogenesis and the normal activity of accessory sex organs could be restored by the high level of testosterone induced by the treatment.

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