Gonadotrophin levels and morphological testicular features in rats after different doses of the phytoestrogen coumestrol

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Abstract

INTRODUCTION: Phytoestrogens are a wide variety of chemical compounds, mainly isoflavoniods, from a vegetable source. Their name makes reference to their ability to induce estrogenic responses in mammals. Coumestrol is a phytoestrogen that can be found in high concentrations in the dietary elements of cattle. Some endocrine alterations have been reported for cows and ewes after ingestion of vegetables with high concentrations of coumestrol. However, these studies have been made mainly in females.

OBJECTIVE: To analyze some features of the masculine endocrine response in rats after several doses of the phytoestrogen coumestrol.

DESIGN: Adult male rats were injected with several doses of coumestrol. Plasma gonadotrophins (LH/FSH) and testosterone levels were assessed. In addition, morphology of the testicles was analyzed.

SETTING: Experiments were done in the facilities of the neurosciences area at the University.

RESULTS: No significant changes were observed in gonadotrophin levels after the administration of coumestrol. Testosterone levels showed a significant decrease with the higher doses. Morphological analysis showed an inhibitory effect on spermatogenesis expressed mainly in the right testicle. Testicular volume decreased and the tubular area increased significantly after coumestrol treatment.

CONCLUSIONS: These results suggest that the endocrine effect of coumestrol is mainly expressed in peripheral targets in male rats. In addition, the possible mediation of estrogen beta receptors is discussed.

ORIGINAL ARTICLE

Introduction

Phytoestrogens are a wide variety of chemical compounds of vegetable origin that are capable of inducing estrogenic responses [15]. They have been chemically characterized as isoflavonoids and they have been subdivided into: isoflavones, pterocarpens and coumestanes [20]. It has been shown that phytoestyrogens (isoflavones and coumestanes) are able to couple with estrogen receptors and modify growing breast cancer cells [15]. Among coumestanes, coumestrol can be found widely distributed in the vegetables of the dietary elements of cattle. It has been reported that the amount of coumestrol in alfalfa increases from 1.5–2.5 mg /kg in healthy plants to 50–200 mg/kg in plants with a micotic disease caused by *pseudopesisa medicaginis* and, the ingestion of alfalfa with high levels of coumestrol [20–50 mg of coumestrol/kg of alfalfa) induces reproductive impairments [17]. Administration of coumestrol (7.5–25 mg/ 24 hours) induces a long-lasting estrogenization in ovariectomized sheep [23]. A similar diet in cows results in alterations of the estrous cycle and in a lower fertility rate [18] also inducing an increase of sexual behavior and hyperplasia of the udder and uterus [27]. In 1995, Adams et al reported that a diet with high levels of coumestrol elicits a total loss of fertility that can be transient or irreversible [2, 3]. In addition, bulls fed with alfalfa containing an average concentration of 80 mg/kg of coumestrol, showed a decrease of spermatic motility which was restored when their diet was modified [16].

Thus, it is possible that coumestrol has an influence on the hyphothalamic-pituitary-gonadal axis and/or on peripheral organs linked to reproduction. The goal of the present study was to analyze the effects of different doses of coumestrol on gonadotrophin and testosterone levels in male rats as well as to assess the effects on the morphological features of the testis.

Methods

Adult male Wistar rats (weight: 300–350 g: age: 90 ± 2 days) were used in this study. Subjects (Ss) came from our own vivarium and were kept in a room with a normal light cycle (12/12) with food and water available ad lib. All housing and behavioral procedures conform to the Principles of Laboratory Animal Care issued by the National Institutes of Health. Ss were randomly assigned to one of seven groups (N = 10 per group) with the following treatment with coumestrol (Cou) ($C_{18}H_8O_5$; Mw: 268.22; Acros Lab, Belgium):

A: Total dose: 12.5 μg. (weight range: 41.7–37.9 ug/kgbw)
B: Total dose: 25 μg (weight range: 77–70.8 ug/kgbw)
C: Total dose: 50 μg (weight range: 161.3–143.7 ug/kgbw)
D: Total dose: 100 μg (weight range: 335.6–306.7 ug/kgbw)
E: Total dose: 200 μg (weight range: 658–602.4 ug/kgbw)
F: Total dose: 400 μg (weight range 1460–1315.8 ug/kgbw) and
G: A control group injected with vehicle.

Treatments were applied in one daily subcutaneous injection in a volume of 0.1 ml of corn oil during three consecutive days. Four days later, two hours after the onset of the light period (11 AM), Ss were sacrificed by decapitation, their trunk blood was obtained and the testicles were removed for histological analysis.

Hormonal assessment:

Blood samples were centrifuged; serum was separated within 15 minutes and kept frozen until analysis.

Luteinizing hormone (LH) was determined by radioimmunoassay (RIA) using a commercial kit (Amerlex-M LH-RIA) with a sensitivity of 0.62 mlU/ml. Standard curve concentration range was from 0 to 144 mlU/ml with a cross-reaction of < 4% for thyroid-stimulating hormone (TSH), < 0.1% for follicle stimulating hormone (FSH) and < 49.7% for corionic-gonadotrophin hormone (CGH).

Follicle stimulating hormone (FSH) was determined by RIA using a commercial kit (Amerlex-M FSH-RIA) with a sensitivity of 0.54 mlU/ml. Standard curve concentration range was from 0 to 83.8 mlU/ml, with a cross-reaction of < 0.09% for TSH, < 0.08% for LH and < 0.006% for CGH.

Testosterone (T) was also determined by RIA using a commercial kit (Amerlex Testosterone RIA DSL-4000) with a sensitivity of 0.08 ng/ml. Standard curve concentration range was from 0 to 25 ng/ml with a cross-reaction of < 5.9% for 5 alpha DHT, < 2.3% for androstenedione and < 0.14% for androstenediol.

Histological analysis

Testicles were weighed and testicular/somatic index (TSI = testicular weight/body weight X 100) was obtained. Testicles were fixed in a Bouin-Duboscq solution (9], and were gradually dehydrated with increasing concentrations of ethanol (70%, 80%, 96% and 100%), cleared with Xylene and included in Paraplast (Oxford, labwere, St. Louis). The slices (5 μm) were stained using Hematoxyline/eosine and periodic acid-Schiff (PAS) and were analyzed using an Image Analyzer (KS300 Zeiss).

The following testicular diameters were obtained: craneo-caudal (a), anterior-posterior (r) and medio-lateral (b). Therefore, testicular volume was calculated with the following formula: $v = 1/3\ \pi$ rab. Using an Image Analyzer, the tubular area was assessed and the number of spermatogonies, spermatocytes and spermatides was determined. Total number of cells represents the sum of all the cellular types present in the tubule. Cell density

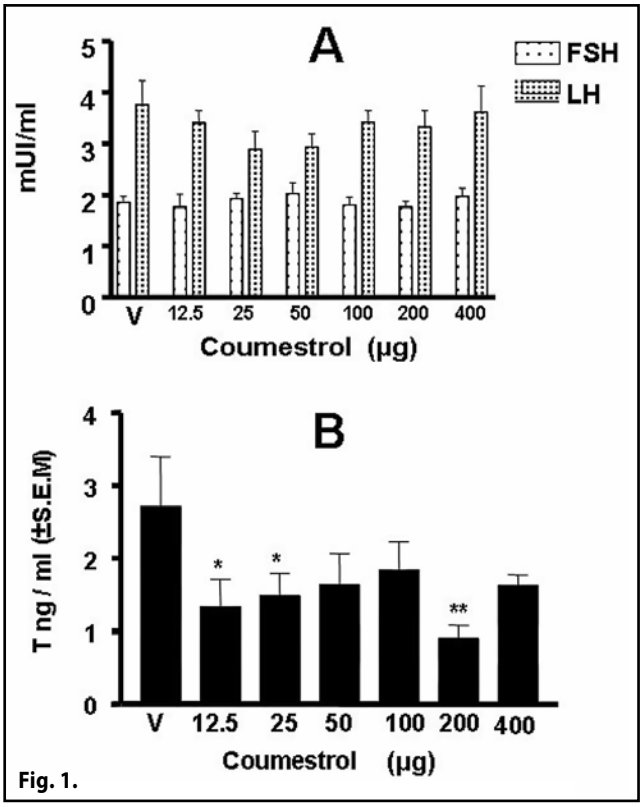

Fig. 1.

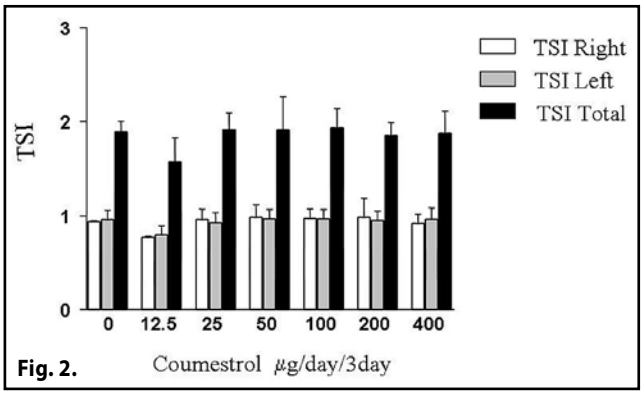

Fig. 2.

Figure 1. Panel A. Plasma levels of gonadotrophins after several doses of coumestrol. No significant changes were observed after coumestrol when compared to control group. Panel B shows plasma levels of testosterone after coumestrol. Compared to control, there is a decrease from the lower doses, (* = $p < 0.05$). ANOVA followed by Dunnet test**).**

Figure 2. Testicular/somatic index (TSI) after different doses of coumestrol. No differences were observed when the right testicle was compared to the left testicle. Total TSI decreased significantly only after the lower dose of coumestrol. (Anova followed by Tukey test. * = $p < 0.05$ compared to control).

represents the number of cells per square milimeter of tubular area.

Statistical Analysis

Hormonal levels were analyzed using ANOVA followed by a Dunnet test. TSI data were analyzed using an ANOVA followed, when significant, by a Duncan Test. Morphology was analyzed using a one-way ANOVA followed, when significant, by the Bonferroni Control Test.

Results

No behavioral or weight changes were observed during the threee days of treatment. As can be seen in Figure 1 (Panel A), no changes were observed in the concentration of either FSH or LH after the administration of different dose of coumestrol.On the other hand, plasma levels of T (Panel B) showed a statistical significant decrease at all coumestrol doses tested.

Figure 2 shows the results obtained concerning the TSI. As can be seen, only the group treated with 12.5 µg of coumestrol showed significantly lower values when compared to both the control and the other experimental groups.

Histological and Morphometric Analysis of the Testicles:

Coumestrol treatment induced morphological changes in the testicles, however, the changes were observed mainly in the right testicle. In some of the analyzed parameters, no changes were observed in the left testicle at any of the doses tested. Concerning Leydig cells a clear enlargement was observed at all the doses tested. (Figure 3).

Significant differences were found in the vehicle control group when the right testicles were compared to the left testicles. Left testicles showed a significantly smaller volume, a lower total cell number, cell density, spermatocytes and later spematids. In addition, higher values in the left testicle were observed in tubular area, spermatogonies and early spermatids.

Right and left testicles were analyzed after coumestrol administration by comparing each one with the vehicle control of the same side. Testicular volume decreases significantly when compared to the vehicle control group and at all the doses of coumestrol. However, this effect was observed only in the testicles of the right side. The tubular area displayed significant changes in the right testicle at all the doses tested, however, there was no dose-response pattern.

Discussion

It is well known that estradiol exerts an inhibitory effect on the hypothalamus-pituitary-gonadal axis (HPG) [For review see: 12]. This inhibitory action of estradiol results in the decrease of testosterone plasmatic levels, which can be due to the inhibition of gonadotrophin release or to a direct effect on the testicles. It has been repeatedly shown that estradiol, even at small concentrations, induces a suppression of both LH and FSH [14, 22, 25]. Thus, it was expected that the estrogenic effect of coumestrol would result in an inhibition

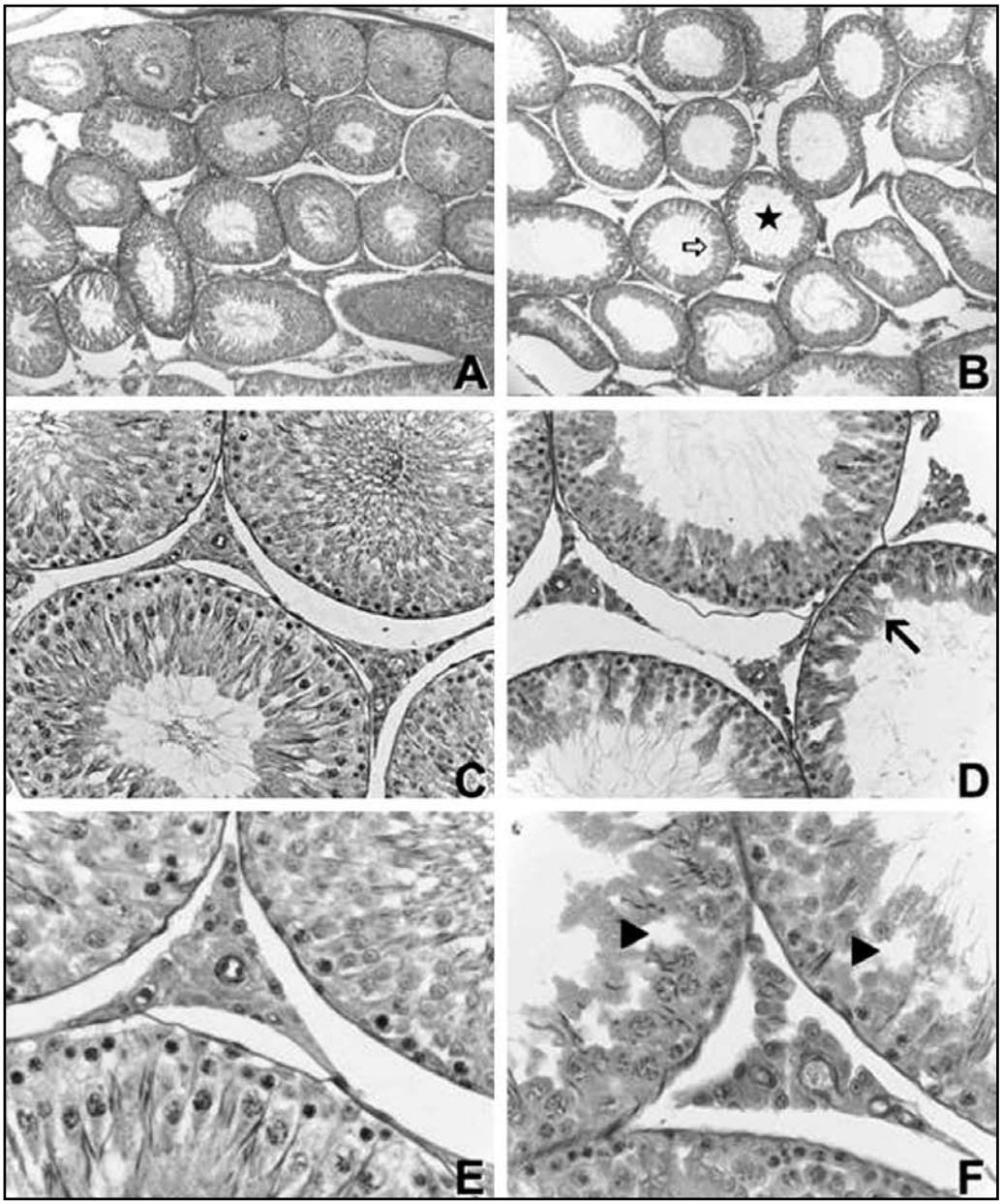

Figure 3. Photomicrographs showing transverse sections of seminiferous tubules of rat testis. Left side: (A.C and E): oil treated control. As no significant differences were detected between left and right testicles, control images are similar in both testicles. Right side (B, D and F) shows images of right testicles treated with coumestrol. At the first level of amplification (A, B. 100x) coumestrol treated testis show an increase in the luminal area with poor development of the epithelium. At a larger amplification (C, D. 200 x) diminished cellular stratification is noticeable with less heterogeneity of the diverse types of cellular associations. At the highest amplification, the experimental group shows an inhibition of the seminiferous epithelium phases, with a high proportion of cellular associations corresponding to phases VII and VIII of the epithelial seminiferous cycle. In addition, a clear increase of the intercellular space can be observed between the tubules of the experimental group.

of gonadotrophin release, however, the present results indicate that the systemic administration of coumestrol has no effects on plasma levels of gonadotrophins. This raises the possibility that this phytoestrogen is unable to cross the blood brain barrier. These results are similar to those reported for Sprague-Dawley rats submitted to a diet of phytoestrogens from soy [29], where there was a decrease in the plasma levels of testosterone with no changes in gonadotrophin levels.

In addition, it has been reported that phytoestrogens bind with both alpha and beta estrogen receptors, but preferentially with the beta receptor subtype [5]. Localization studies of estrogen receptor subtypes have shown that the secretory cells in the anterior pituitary exhibit the alpha receptor subtype while the beta receptor subtype has not been located in the pituitary [21]. These data could explain the lack of effect of coumes-

trol on gonadotrophin plasma levels observed in the present study.

Concerning the effect of coumestrol administration on testosterone levels, it is possible that the phytoestrogen is acting through the same mechanism that has been reported for estradiol. Kalla et al [10] reported that estradiol inhibits the activity of the 17-20-desmolase in Leydig cells that results in the arrest of steroid hormones synthesis and in the increase of 17-α-hydroxyprogesterone levels. It is also possible that in rats coumestrol has effects similar to those reported in humans [11], where the activity of 17-beta-hydroxysteroid-dehydrogense (17beta-HSD), which is a key enzyme in the metabolism of androgens and estrogens, can be inhibited by several phytoestrogens like zeralenone, coumestrol, quercetin and biochanina A.

On the other hand, it has been reported that estradiol administration induces a significant decrease in the size and weight of the testicles in adult [10; 19] and in neonate rats [4]. In the present study, only the smaller dose tested induced a significant decrease in both size and weight. Nevertheless, the morphological analysis showed a clear lateralization effect of coumestrol. The left testicles showed no differences when compared to control, while the right testicles displayed morphological changes that closely resemble the changes reported after the administration of estradiol [24, 28]. A similar lateralization effect has been reported after administration of estradiol benzoate to neonate rats that results in an impairment of the development, mainly, of the right testicle [1]. It has been suggested that the right testicle is more sensitive to pharmacological stimulation due to differences either of irrigation [6] and/or of innervation [7]. Recent reports indicate the existence of a clear asymmetric innervation of the testicles by the vagus nerve influencing the action of gonadotrophins on the synthesis of steroids [8]. Furthermore, it has been shown that in men, the administration of LH increases testosterone concentration mainly in the right testicular vein [26]. Thus, it is possible that coumestrol is exerting its estrogenic effect on plasma levels of testosterone acting mainly on the right testicle.

The present results suggest that coumestrol has an estrogenic effect that seems limited to peripheral targets, without affecting the central regulation of gonadotrophin release. The full picture of the influence of coumestrol on male reproductive mechanisms remains to be elucidated.

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REFERENCES

1 Aceitero J, Llanero M, Parrado E, López-Beltrán A. Neonatal exposure of male rats to estradiol benzoote causes rete testis dilation and selective backflow impairment of spermatogenesis. The Anatomical Record 1998; **252**:17–33.
2 Adams NR. Organizational and activational effects of phytoestrogens on the reproductive tract of the ewe. Proc Soc Exp Biol Med 1995a; **208**:87–91.
3 Adams NR. Detection of the effects of phytoestrogens on sheep and cattle. J Anim Sci 1995b; **73**:1509–1515.
4 Bellido G, Gaytán F, Aguilar R, Pinilla L, Aguilar E. Prepuberal reproductive defects in neonatal estrogenized male rats. Biol Reprod 1985; **33**:381–387.
5 Casanova M, You L, Gaido KW, Archibeque-Engel S, Jansen DB, Heck Hd´A. Developmental effects of dietary phytoestrogens in sprague-dawley rats and interactions of genistein and daidzein with rat estrogen receptors α and β in vitro. Toxicol Sci 1996; **51**:236–244.
6 Gerendai I, Motta M. Effects of unilateral vagotomy on serum gonadotrophin concentration in rats with two testes and in hemicastrates. Endocrinol Exp 1990; **161**:186–187.
7 Gerendai I, Halass B Neuroendocrine asymmetry. Front Neuroendocrinol 1997; **18**:354–381.
8 Gerendai I, Csaba ZS, Cseravas V. Lateralized effects of right and left sided vagotomy on testicular steroidogenesis and serum gonadotaropins levels in hemicastrated peripuberal rats. Neuroendocrinol Lett 1995; **17**:193–198.
9 Humason GL. (Ed.).Animal tissue technique. 1979. WW. Freeman and Co. San Francisco.
10 Kalla NR, Ínsula BC, Menard R, Loriaux DL. The effect of estradiol on testicular testosterone biosynthesis. Endocrinol 1980; **106**: 35–39.
11 Krazein A, Breitling R, Moller G, Admski J. Human 17beta-hydroxysteriod dehydrogenase type 5 is inhibited by dietary flavonoids. Adv Exp Med Biol 2002; **505**:151–161.
12 Krester DM, Burger HG, Hudson B (Eds.). The pituitary and testis. Clinical and Experimental studies. 1983. Springer Verlag. Berlin.
13 Kula K. Induction of precocious maturation of spermatogenesis in infant rats by human menopausal gonadotropin and inhibition by simultaneous administration of gonadotropins and testosterone. Endocrinol 1988; **122**:34–39.
14 Kulin HF, Reiter EO. Gonadotrophin suppression by low dose estrogen in men: Evidence for differential effects upon FSH and LH. J Clin Endocrinol Metab 1972; **35**:836–839.
15 Kurzer MS, Xu X Dietary phytoestrogens. Annu Rev Nutr 1997;**17**:353–381.
16 Le Bars J, Hurard JB, Infertilité mâle bovine et tenuer en coumestrol de la luzaerne. Pharmacol Toxicol Vétérinaires. Colloques de l´INRA 1982; **8**:157–158.
17 Le Bars J, Le Bars P, et Brice G Presence accumulation et devenir du coumestrol dans la Luzerne et ses derives Rec Med Vet 1990; **166**:463–469.
18 Lotane E, Adler JK. Early effects of excessive alfalfa feeding on bovine fertility. Refuah Vet 1966; **23**:102–110.
19 Oko E, Hrudka F. Comparison of the effect of gosypol, estradiol 17β and testosterone compensation on male rat reproductive organs. Biol Rep 1984; **30**:1198–1207.
20 O´Neill MJ, Adisanya SA, Roberts MF, Pantry IR. Inducible isoflavonoids from the lima bean Phaseolus lunatus. Phytochem 1986; **25**:1315–1322.
21 Pelletier G, Labrie C, Labrie F. Localization of oestrogen receptor α, oestrogen receptor β and androgen receptors in the rat reproductive organs. J Endocrinol 2000; **165**:359–370.
22 Sherins RJ, Loriaux DL. Studies on the role of sex steroids in the feedback control of FSH concentration in men. J Clin Metab 1973; **36**:886–893.
23 Shutt DA, Braden WH, Lindner HR. Plasma coumestrol levels in sheep following administration of synthetic coumestrol or ingestion of medi (Medicago littoralis). Aust J Agric Res 1969; **20**:65–89.
24 Steinberger E. Hormonal control of mammalian spermatogenesis. Physiol Rev 1971; **51**:1–22.

25 Stewart-Bentley M, Odell W, Horton R. The feedback control of luteinizing hormone in normal adult men. J Clin Endocrinol Metab 1974; **38**:545–553.
26 Turner TT, Evans WS, López TJ. Gonadotroph and Leydig cell responsiveness in the male rat. Effects of experimental left varicocele. J Androl 1990; **11**:555–562.
27 Valderrabano J. Alteraciones reproductivas asociadas al consumo de Fitoestrógenos. Investigación Agraria 1992; **7**:115–124.
28 Van der Malen HJ, Vrenkmann AO, Jong FH, Rommerts FF. Testicular oestrogens. J Endocrinol 1981; **89**:33–45.
29 Weber KS, Setchell KD, Stocco DM, Lephart ED. Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5alpha-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague Dawley rats. J Endocrinol 2001; **170**:591–599.