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

C. Rosario Tarragó-Castellanos, C. Mario García-Lorenzana, Vicente Díaz-Sánchez & Javier Velázquez-Moctezuma

1 Deptartment of Biology of Reproduction, Universidad Autónoma Metropolitana-Iztapalapa, C P 09340 México City, Mexico.
2 Deptartment of Biology of Reproduction, National Institute of Medical Sciences and Nutrition “Dr. Salvador Zubiran”, México City, Mexico.

Correspondence to: Dr. Javier Velázquez Moctezuma
Departamento de Biología de la Reproducción.
Universidad Autónoma Metropolitana-Iztapalapa
San Rafael Atlixco 186. Col. Leyes de Reforma
C.P. 09340. Iztapalapa. D.F. MÉXICO.
PHONE: (525)7244701; FAX: (525) 7244704
EMAIL: jvm@xanum.uam.mx

Submitted: April 14, 2006 Accepted: May 1, 2006

Key words: phytoestrogens; coumestrol; testicles

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.
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 phytoestrogens (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 ovarietomized 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 spermatogenic motility which was restored when their diet was modified [16].

Thus, it is possible that coumestrol has an influence on the hypothalamic-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) (C18H8O5; 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 testes 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: crano-caudal (a), anterior-posterior (r) and medio-lateral (b). Therefore, testicular volume was calculated with the following formula: \(v = \frac{1}{3} \pi \text{rab}\). 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
represents the number of cells per square millimeter 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 three 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 spermatids. 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...
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-β-hydroxysteroid-dehydrogenase (17β-HSD), which is a key enzyme in the metabolism of androgens and estrogens, can be inhibited by several phytoestrogens like zeralene, 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.

Acknowledgments

The authors wish to express their gratitude to Edith Monroy for their helpful advice on the manuscript. The present study is part of the Doctoral Degree in Biological Sciences of the Universidad Autonoma Metropolitana.of RTC.

REFERENCES

1 Aceitoro J, Llanero M, Parrado E, López-Beltrán A. Neonatal exposure of male rats to estradiol benzoate causes rete testis dilatation and selective backflow impairment of spermatogenesis. The Anatomical Record 1998; 235:17–33.


