Fluorescence and inmunohistological detection of estrogen receptors in dog testis and epididymis after oral coumestrol administration

Hector Serrano [1], Juan José Pérez-Rivero [2], José Juán Martínez-Maya [2], Alvaro Aguilar-Setién [3], Mario Pérez-Martinez [4], Maria-Dolores García-Suárez [5]

1. Department of Health Sciences, Universidad Autónoma Metropolitana, Iztapalapa campus;
2. Department of Preventive Medicine and Public Health, Universidad Nacional Autonoma de Mexico, School of Veterinary Medicine;
3. Medical Research Unit in Immunology, Children Hospital, Centro Medico Nacional Siglo XXI;
4. Department of Morphology, Universidad Nacional Autonoma de Mexico, School of Veterinary Medicine;
5. Department of Biology, Universidad Autónoma Metropolitana, Iztapalapa campus; Mexico.

Correspondence to: Dr. Hector Serrano
Dept. Health Sciences, Universidad Autonoma Metropolitana-Iztapalapa,
Ave. San Rafael Atlixco 186, Mexico City, 09340, DF, Mexico
phone: (55)5804 4733; fax : (55) 5804 4727
e-mail: hser@xanum.uam.mx; hectorserrano@hotmail.com

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Abstract

BACKGROUND: Estrogens are well recognized as important hormones in male reproduction and act as ligands to α and β estrogen receptors. Both estrogen receptors could interact with estrogen-mimicking compounds such as the fluorescent phytoestrogen coumestrol, which acts both in an agonist or antagonist fashion. **OBJECTIVE**: To investigate the presence of Coumestrol-Estrogen Receptor complexes by fluorescence in testis and epididymis, its effect in the ER expression by immunostain in the same tissues and the effect of this binding in the testis histological characteristics. **DESIGN**: Adult healthy and sexually active dogs were assigned to either the experimental or control group .Coumestrol impregnated dog biscuits were given to each animal from the experimental group once a week for a 4 week period. The control group received a biscuit with no Coumestrol, also once a week and for the same period. Testis morphology, ER immunodetection, and coumestrol-receptor binding were evaluated. **SETTING**: The experiment was done in the facilities of the Mexico City canine shelter. Animals were caged individually with food and water ad libitum and having at least two daily hours for exercise. **RESULTS**: Morphological alterations in testis after oral administration of coumestrol were detected. The main alterations include decreased germinal epithelium in tubule, and the loss of a continuous proliferation and differentiation gamete layer. Fluorescence signals in testis interstitial Leydig cells and epididymus indicating ER-coumestrol complexes were detected at the same points to those Immunohystochemically detected ER. **CONCLUSIONS**: Coumestrol administration induces testis alterations and coumestrol-ER complexes can be co-localized by binding-enhanced fluorescence and immunoprecipitation.

ORIGINAL ARTICLE

INTRODUCTION

Estrogens are now well recognized as important hormones in male reproduction, synthesized by at least three different cell types; Sertoli, Leydig, and germ cells (Hess *et al*. 2001). Estrogen receptors (ER) are members of the nuclear receptor super family sharing similar characteristics (Weihua *et al*. 2003) including a separate binding ligand domain (BLD) and a high affinity DNA binding domain (DBD).

Two ER subtypes, Estrogen Receptor alpha (αER) and Estrogen Receptor beta (βER), have been cloned (Kiuper *et al*. 1996). Both subtypes are widely distributed throughout the body and have been demonstrated in the prostate, osteoblasts, cartilage, adipose tissue, brain, including the pituitary, and testis (de Ronde *et al*. 2003).

Immunostaining for αER in dog testis are moderate in peritubular tissue, interstitium, rete testis, epididymis, and a strong staining has been observed in efferent ductules. For βER, moderate immunostain is detected in germ cells, efferent ductules, and in epididymis a strong staining is detected. (Nie *et al*. 2002).

Coumestrol (COU) is a naturally occurring phytoestrogen present in alfalfa (Medicago sativa) sprouts and growing soy (Glycine sp.) plants, that acts in a dose dependent fashion as either an agonist or antagonist estrogen. In mammals, COU binds to the αER and βER (Roselli *et al*. 2000). In cattle, COU consumption induces reproductive alterations, such as estrogenic syndrome in females, and poor libido and oligospermic bulls (Romero *et al*. 1997). Hemorrhagic follicles in mice (Burroughs 1995), low weight and low libido in male rats (Whiten *et al*. 1995), and loss of testicular histological architecture in vampire bats (Serrano *et al*. 2007) have also been associated to COU ingestion. In dogs, COU induce a drastic reduction in ejaculated sperm concentration and count, and increases sperm abnormalities (Pérez-Rivero *et al*, in press).

COU displays a high affinity to αER and βER LBD and an increase in fluorescence was detected when COU binds to αER and βER in cultured cells (Miksicek 1993). COU binding can be followed by fluorescence microscopy methods and used as a non-isotopic method for αER and βER characterization (Miksicek 1993).

Dog exhibit a worldwide overpopulation as stray and feral animals (Slater 2001), which raises the importance of developing a male contraceptive method, to complement the accepted surgical neutering. αER and βER are potential targets to disrupt reproduction in male dogs. Therefore, the objective of the present work was to investigate the presence of COU-ER binding, by fluorescence in testis and epididymis, the effect in the ER expression, by immunostain in the same tissues, and the effect of this binding in testis histological characteristics.

MATERIALS AND METHODS

Animals

Ten adult, healthy, and sexually active dogs (3 Coker, 2 York Shire, 5 Mongrel) weighing 12.7 ± 6.5 kg were evaluated for normal libido and ejaculate parameters (Kawakami *et al*. 2005) prior to the experiment and were randomly assigned to either the experimental or the control group and kept in our animal facility, fed with commercial food (Purina Mexico) and water ad libitum. Coumestrol (300 μg/kg, Fluka St. Louis, MO) was dissolved in 40 μl dimethyl sulfoxide (DMSO, Sigma Chem. Co, St. Louis, MO), impregnated into a commercial dog biscuit and given to each animal from the experimental group once a week for a 4 week period. Control treatment was offered with a DMSO-impregnated biscuit also once a week for a four week period.

After that, on week number five, testes were obtained after routine surgical castration. Animal experiments were approved by the Animal Care and Use Committee of the participating institutions.

Morphological evaluation

Immediately after castration, testis and associated epididymis were fixed with 10% buffered formalin for 12 hours at 4°C, the tissues were embedded in paraffin. Sections of 5μm thickness were cut and stained with metylpironine green for morphological evaluation; light microscopy evaluation was made for gross morphology alterations under a Zeiss Axioplan microscope. Digital images were captured and evaluated by at least three independent observers by analyzing 20 tubules per slide.

Estrogen receptor immunostaining and coumestrol detection

Paraffin embedded tissue slides were deparaffinized, rehydrated, placed in Coplin jars with 0.1M citrate buffer solution (pH 6.0), heated in a pressure cooker for 15 minutes, and then allowed to cool at room temperature. Immunostaining was performed with a ER Poly Detector Peroxidase kit (BioSB, Santa Barbara CA) and handled according to the manufacturer's instructions. In short, slides were blocked for 5 minutes, covered with HER primary antibody and incubated for 30 minutes. After excess antibody removal, the tissue was covered with DAB substrate-chromogen and incubated for 10 minutes. Counterstaining was made with hematoxilin and observed under light microscope.

For COU fluorescence detection, the immunostained slides from control and coumestrol-treated animals were observed in a Zeiss Axioplan 2 microscopy, under ultraviolet fluorescence light; digital images were captured and analyzed with AxioVision 4.6 software (Zeiss Germany).

Statistical analysis of fluorescence intensity between coumestrol treatment and control group was made by using the t-test facility of Microstat II software (Microsoft, v. 5).

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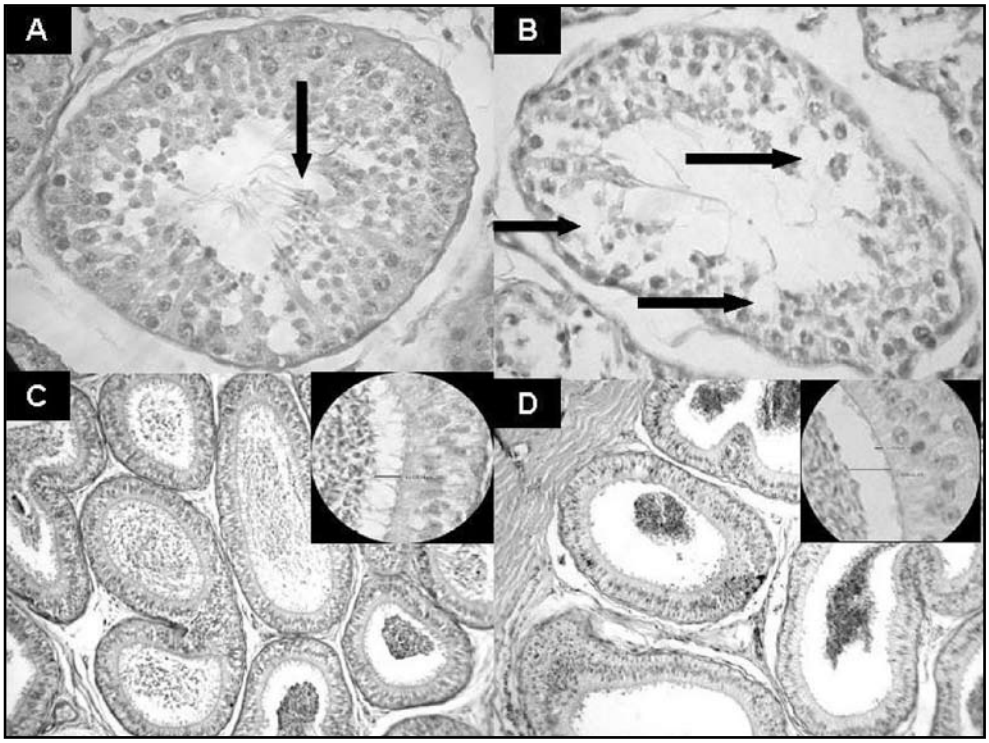

Figure 1. – Histological alterations induced by coumestrol treatment. Adult healthy animals were treated with COU or DMSO (control) as described under material and methods. Tubules in control testis (A) have a structured germinal epithelium and mature sperm cells in their lumen (arrow), whereas those from coumestrol treated dogs (B) show a decrease in germinal epithelium length, and no sperm cells in their lumen. Control epididymis (C) show full lumen covered sperm cells, normal ciliated cells were observed (insert); epididymis from coumestrol treated animals (D) show reduced sperm cells in lumen and decreased ciliated cells (A and B 400X, C and D 100X). (*Publisher's note*: 86% of original size)

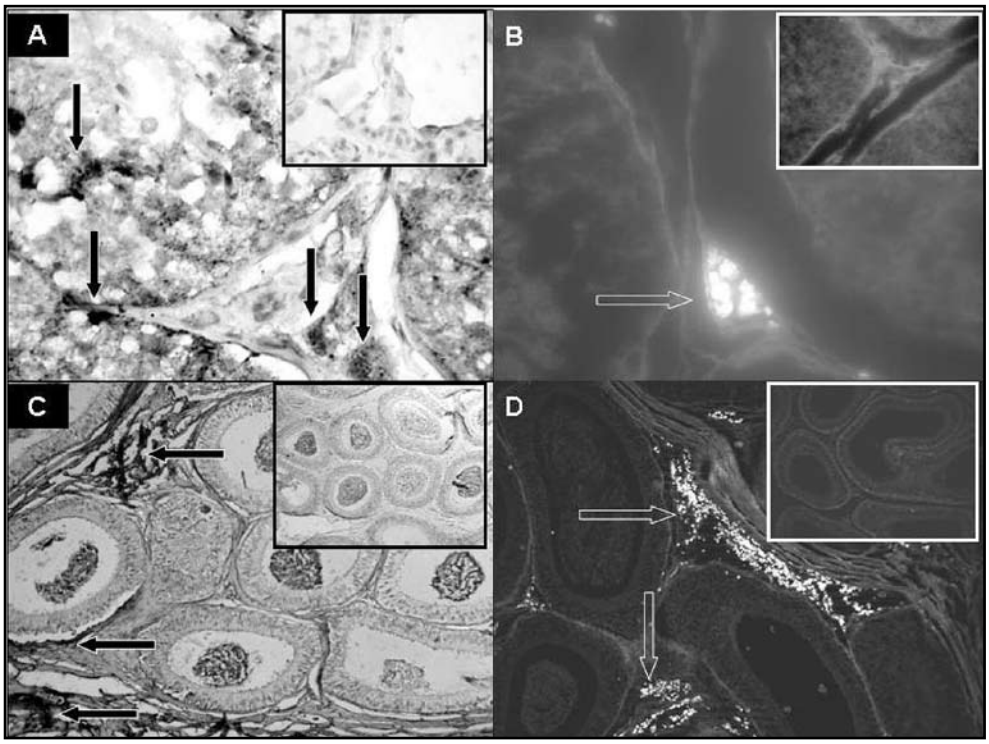

Figure 2.- Inmuno- and fluorescence detection of ER in testis and epididymis of control and treated animals. A COU-containing dog biscuit was offered weekly to treated dogs or vehicle-containing biscuit (controls) for 4 weeks. Treated testis (A,B) and epididymis (C,D) show increased immunostainig (A,C) and fluorescent signals (C,D) than their respective controls (inserts). Arrows indicate equivalent sections of immunostained and fluorescence signals. All views were at 4000 × magnification. (*Publisher's note*: 86% of original size)

RESULTS

Oral COU treatment had a negative effect in the histoarchitecture of the dog testis. As it can be seen in Figure 1, seminiferous tubules from coumestrol treated dogs were severely altered. Even when there was a decrease in the total germinal epithelium, no spermatozoa could be distinguished in the lumen, but an increased interstitial space. The epididymis of treated animals showed a decrease in the number of luminal sperm cells, and the length and amount of cillia were reduced.

Treated dogs ER immunostaining was strongly localized in interstitial Leydig cells, round spermatids, spermatogonia and spermatocytes (Figure 2A), and connective epidydimal tissue (Figure 2C), compared with weak immunostaining of control dogs, as can be seen in inserts (Figure 2A,C).

A strong fluorescence signal was detected in the interstitial Leydig cells (Figure 2B) and connective tissue cells of epididymis of coumestrol treated dogs (Figure 2D). In control dogs, no fluorescent signals were detected, as shown in the respective insert in Figure 2B and 2D. Green fluorescence difference expressed in pixel intensity between treated (177±41) and control (58±13) groups were significantly different ($p<0.005$).

DISCUSSION

Estrogen Receptor disruption induce infertility in male mice (Eddy *et al*. 1996). In αER gene-deficient knock out mice, sperm low count, subnormal motility, atrophic and degenerative seminiferous tubule in testis were described (Ogawa 1999). COU binds to both ER types (Morito *et al*. 2002) and can act as receptor antagonist (Whitten *et al*. 2002). Coumestrol fluorescent signal in the interstitial tissue of the dogs' testes and epidymis (Figure 2B,D) is associated to the location of the αER and βER detected by immunohistochemistry (Nie *et al*. 2002). As in COU-treated male vampire bats (Serrano *et al*. 2007), dogs also show altered histological architecture of testis and tubules (Figure 1B). Recently, it has been shown that oral COU treatment drastically impairs sperm cell production in dogs decreasing the ejaculated sperm number, concentration and an increase in abnormal sperm morphology (Perez-Rivero *et al*. in press). However, no data concerning altered ER receptor distribution or COU binding sites have been attempted. As has been shown in Figures 1 and 2, ER and COU-binding sites are almost the same. We assume that the COU can act as an ER antagonist ligand in dogs, disrupting them and reducing their reproductive performance.

An interesting finding in treated dogs is the induction of reduced fertility by orally-administered COU. These findings open the possibility to develop an oral reproductive control strategy for stray and feral dog populations. Further studies are needed to assess the reproductive impact of COU on the biochemical and endocrine physiology looking for any negative side effects.

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