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Administration of Coumestrol and/or Dimethyl Sulfoxide Affects Vaginal Epithelium and Sex Hormones in Female Dogs

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ABSTRACT

Background: The overpopulation of free dogs represents a risk to public health in some countries. However, current strategies for its control are insufficient; it is convenient to devise new alternatives.

Methods: To determine the effects of a single oral administration of coumestrol (COU) on vaginal epithelial cells, serum progesterone and estradiol levels and clinical parameters: periovulatory bitches received a biscuit that contained 600 mg COU/kg diluted in DMSO or with DMSO alone or without additives. Each group was subdivided into animals that had ovulated on day 0 and those that had not.

Result: In animals that ovulated, COU did not affect hormonal profiles. In contrast, animals that did not ovulate showed lower circulating P4 concentrations on days 21 and 28 after treatment than the control group. Furthermore, COU reduced parabasal cells and increased superficial anucleate cells. COU and DMSO are estrogenic and their actions depend on the ovulatory status of the animals.

Key words: Coumestrol, Dimethyl sulfoxide, Estradiol, Periovulatory stage, Progesterone.

INTRODUCTION

Dogs perform valuable functions in human society. However, in some underdeveloped countries, the overpopulation of dogs in freedom has become a severe public health problem due to the absence of government policies to control it (Trevejo *et al.* 2005). Furthermore, current strategies for canine birth control (euthanasia, surgical sterilization) have been ineffective. They require trained people and specialized facilities (Chaithra *et al.* 2022; Jha *et al.* 2022) and hormonal treatments can affect dogs' health (Kumar *et al.* 2022) and require repeated applications to control a single fertile period.

Therefore, it is attractive to devise new alternatives for canine control. Natural phytoestrogens, such as coumestrol (COU), participate in hormonal regulation processes (Cederroth *et al.* 2012); COU induces abortion in cows, anovulation in mares, sheep and rats and persistent cornification of vaginal epithelial cells in mice (Kouki *et al.* 2005). To our knowledge, the effects of COU have not been studied in dogs in the periovulatory phase. However, it is known that in anoestrus, bitches alter the hormonal profile (Peña-Corona *et al.* 2019). Therefore, this study evaluates the effects of a single oral administration of COU on vaginal epithelial cells, peripheral concentrations of progesterone (P4) and estradiol (E2), duration of anoestrus and chemical and hematological parameters in bitches.

MATERIALS AND METHODS

The experiment was carried out at the Metropolitan Autonomous University-Iztapalapa, Mexico City, during September 2016 to January 2018.

Ethical review

The Institutional Subcommittee approved all procedures of this study (protocol number DC-2015/2-10) and according to the guidelines of the European Union Directive (2010/63/EU) for animal experimentation.

Animals

Sixteen dewormed and vaccinated intact female dogs participated in the study, which remained in their owners' homes and continued with their usual diet (croquettes of different brands). Small to medium breed (poodle, 5; cocker, 1; schnauzer, 3; pug, 2; undefined, 5), age: 3.75±2.6 years,

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weight: 9.06±4.8 kg and having shown estrus at least once before the study. Vaginal Exfoliating Cytology (VEC) was performed to confirm the stage of the estrous cycle. When we confirmed proestrus or estrus, we gave the treatment (day 0). Duration of diestrus was when serum P4 concentrations were ≥2.5 ng/mL. Anestrus was evaluated by counting the months from the end of diestrus and the first day that apparent signs of estrus were observed for the subsequent cycle.

Treatments

Animals were randomly assigned to one of the following treatments: 1) Control group (n=5): bitches received a single biscuit orally (Pedigree®) with no additives at the beginning of the study (day 0); 2) COU-treated group (n=6) that received a single biscuit impregnated with COU (600 mg/kg of body weight; Sigma Chemical Co. St. Louis, Mo, USA) diluted in DMSO (20 µL); and 3) DMSO-treated group (n=5) that received a biscuit impregnated with DMSO (20 µL/kg of body weight; Sigma Chemical Co. St. Louis, Mo, USA). The dogs were clinically examined at the start and throughout the experiment to confirm their health status.

Biological samples

On days-3, 0, 7, 14 and 21, a sterile vaginal swab was taken for VEC. On the same days and every 30 days until the 6th month of the experiment, blood samples were taken from the cephalic vein; with the serum, P4 and E2 were quantified. Additionally, to determine whether ovarian status altered responses to treatments, each group was subdivided into animals that had already ovulated on day 0 (P4>1 ng/mL) and bitches that had not ovulated on day 0 (P4≤1 ng/mL). / mL). Animals P4>1 ng/mL per group were Control (n=3), COU (n=3) and DMSO (n=3); Bitches P4≤1 ng/mL per group were: Control (n=2), COU (n=3) and DMSO (n=2). In addition, the animals underwent a complete blood count and a biochemical blood test on day 0 and at the end of the experiment.

Laboratory analysis

VEC's vaginal cell smears were fixed on slides (Citospray, CTR Scientific, Mexico) and stained using a modified Papanicolaou procedure. The parabasal, intermediate and superficial vaginal cells were counted during the first 28 days after the application of the treatments by light microscopy (ten fields per animal, chosen at random/smear, 40x objective, Axio Scope.A1 microscope, Zeiss, Mexico). In addition, serum concentrations of P4 and E2 were determined by enzyme-linked immunosorbent assays (DGR Instruments, GmbH, Germany). The interassay range was 0.01 to 40 ng/mL for P4 and 10.6 to 2000 pg/mL for E2.

Design and statistical analysis

The sample size was estimated by using published data of circulating P4 in female dogs determined on days 3 and 4 post LH peak (Hase *et al.* 2000), considering an α and β values of 0.05 and 0.80, respectively and with the online calculator: The Survey System Software (Creative Research Systems). A completely randomized design for mixed models with repeated measures over time was used. Data relative to E2 and P4 during the first 28 days and from the second to the sixth month after application of treatments in Control, COU and/or DMSO treated bitches, were analyzed with analysis of variance, using PROC GLM of SAS (version 9.3; SAS Ins. Inc., Cary, NC). The model included treatment, sample, animal, sample/animal and interactions. The Tukey-Kramer test was used for specific contrasts. The x^2 test analyzed the vaginal cells number in SPSS (version 24; IBM, IL, USA). The criteria for statistical significance was $P<0.05$.

RESULTS AND DISCUSSION

Coumestrol (COU) is a phytoestrogen that negatively affects mammalian reproduction. This study determines the effects of a single oral administration of COU on vaginal epithelial cells on dogs' serum levels of P4 and E2. COU has low solubility in aqueous and lipophilic media; it is recommended to dissolve it in dimethyl sulfoxide (DMSO) before administration to animals. However, there is evidence that DMSO is not safe for dogs and modulates the receptor-mediated and non-receptor-mediated estrogenic responses and significantly induces the expression of StAR and P450scc proteins in both salmon brain and kidney (Lyssimachou and Arukwe, 2007).

For these reasons, the health and reproductive effects of DMSO in bitches were determined. We also investigated whether DMSO interferes with or enhances the estrogenic actions of COU when administered to female dogs.

Hormones and vaginal cells during the first 28 days

Unstratified female dogs by the initial concentration of progesterone

Relative to values of the Control group, both COU- and DMSO-treated animals showed a reduction in serum concentrations of P4 on days 21 and 28 post-treatment (Fig 1). In contrast, serum levels of E2 on day 21 post-treatment in bitches treated with DMSO were higher than in Control and COU-treated animals.

Stratified bitches by the initial concentration of progesterone

In animals that had ovulated on day 0 (P>1 ng/mL), COU and DMSO did not affect the profiles of P4 and E2 (Fig 2a and 2b). In contrast, relative to Control bitches, animals treated with COU or DMSO that had not ovulated by day 0 (P≤1 ng/mL) showed lower circulating of P4 on days 21 and 28 post-treatment (Fig 2c). Bitches treated with DMSO had higher E2 levels than Control and COU-treated bitches on day 21 post-treatment (Fig 2d).

In the present study, the effects of COU diluted in DMSO and DMSO given alone were observed exclusively in animals that had not ovulated at the time of treatment administration. It has been documented that COU binds to oestrogen receptor (ER) β 7-fold higher in comparison to ERα (Kuiper *et al.* 1998). In addition, evidence from mammals other than

dogs suggests that COU exerts effects in the hypothalamus, adenohypophysis and ovary, as well as other tissues containing receptors to E2, such as the uterus and vaginal epithelium (Serrano *et al.* 2007).

Like hormone results, in dogs that had ovulated, COU and DMSO did not have any effects on vaginal cells (Fig 3a). In contrast, in animals that had not ovulated, COU and DMSO reduced the numbers of parabasal cells relative to the controls (Fig 3b). Additionally, COU but not DMSO increased anucleated superficial cells (Fig 3b).

DMSO initiates the differentiation of granulosa cells from chicken preovulatory follicles (Morley *et al.* 1993). Therefore, it is convenient to reassess the influence of DMSO on the effects attributed to COU. Our study exerted estrogenic actions on vaginal cells and the ovary by decreasing the number of parabasal cells, increasing circulating concentrations of E2 and reducing peripheral levels of E2 and P4. To our knowledge, this is the first time that estrogenic effects of DMSO have been reported in periovulatory bitches. Regardless of their mechanism of action, COU and DMSO, this study indicates that the results of both substances depend on the ovulatory condition of the animal.

Hormone levels and duration of diestrus and anestrus from second to sixth months

Concentrations of P4 and E2 from the second to the sixth month post-treatment did not differ between groups regardless of whether female dogs had ovulated or not by day 0 of the study (Fig 4a and 4b).

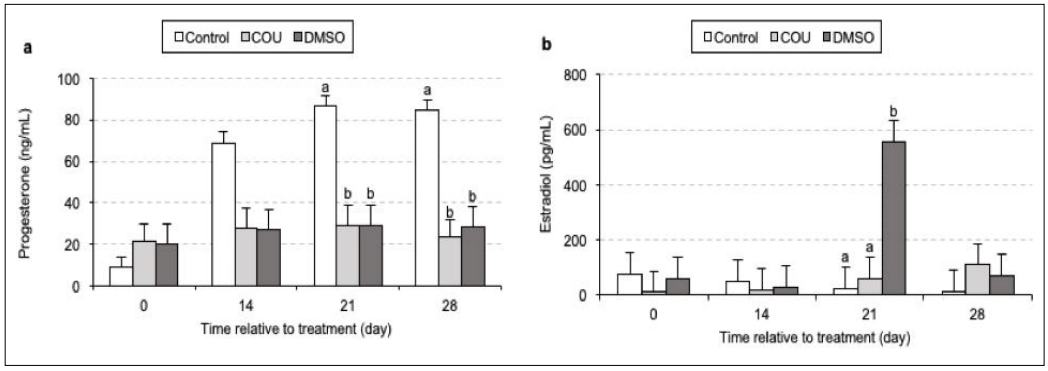

Fig 1: Least square means ± S.E.M. of progesterone and estradiol in serum taken from bitches that received an oral administration of a biscuit (Control, n=5); a biscuit containing coumestrol (COU, n=6)*, or a biscuit containing dimethyl sulfoxide (DMSO, n=5); [a,b] Unequal letters indicate difference between treatments within sampling day (P<0.05), assessed by ANOVA for repeated measures (error type III) and Tukey-Kramer test for specific contrasts. * Diluted in dimethyl sulfoxide.

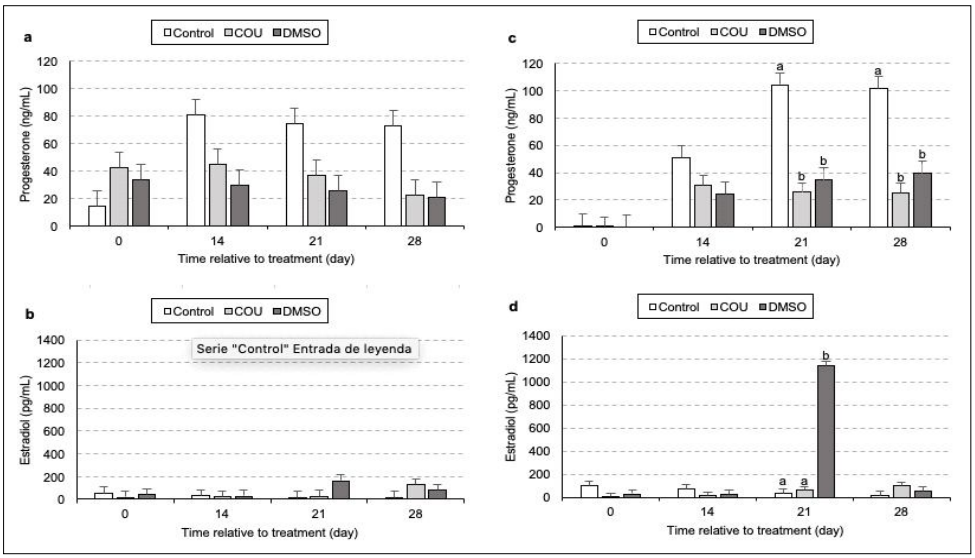

Fig 2: Least square means ± S.E.M. of progesterone and estradiol in serum from bitches that had ovulated (a and b) or not (c and d) at the beginning of the study. Bitches were treated (day 0) with coumestrol (COU)*, dimethyl sulfoxide (DMSO), or remained without treatment (Control). [a,b] Unequal letters between treatments and within day denote difference between treatment means (P<0.05), assessed by ANOVA for repeated measures (error type III) and Tukey-Kramer test for specific contrasts.
* Diluted in dimethyl sulfoxide.

In comparison with the control animals, neither COU nor DMSO altered the duration (mean±SE) of diestrus (Control: 2.40±0.31; COU: 2.73±0.29 and DMSO: 3.14±0.46 months) or anestrus (Control: 3.40±0.22; COU: 3.00±0.40 and DMSO: 4.29±0.47 months).

Animal health

Based on clinical examinations monthly, information of owners and chemical and blood cell tests realized six months after treatments; our results indicate that a single oral application of either COU diluted in DMSO or DMSO-alone did not affect the clinical parameters of dogs (supplementary data).

Regarding the safety of DMSO, there are conflicting results. For example, four oral applications at weekly intervals of COU diluted in DMSO induced no adverse effects in male dogs (Pérez-Rivero *et al.* 2009). Similarly, one or two intratesticular applications of DMSO with zinc gluconate did not harm dogs' health (Vannucchi *et al.* 2015). In addition, in laboratory species, the beneficial use of DMSO in treating ischemic heart disease and inflammation has been reported (Lapuente *et al.* 2013). Therefore, it is fair to say that COU and DMSO are safe for dogs at the doses and application frequencies used here and, in the documents cited above. However, caution should be exercised when administering a higher or more frequent administration DMSO or COU, as it may induce rodent neural and behavioral disorders or eye injuries in dogs (Hanslick *et al.* 2009). Therefore, although more comprehensive studies on the safety of COU and DMSO are needed, our data support those experiments indicating that cautious use of both substances poses a relatively low risk to an animal's health.

Collateral effects

Relative to the Control group (Fig 5), where all bitches ended their diestrus by the third month and usually behaved, four out of six COU-treated animals showed vaginal bleeding in the second month; another bitch exhibited abnormal mammary gland growth and galactorrhea that lasted 15 days during the third month.

In the DMSO group, three out of five animals showed vaginal bleeding in the second month, two of which had mammary gland growth and galactorrhea (15 days). Furthermore, two animals had serum concentrations of P4≥2.7 ng/mL for at least six months. The observed abnormalities were independent of concentrations of P4 on day 0 or the duration of diestrus and anestrus. Besides, dogs bled through the vulva did not show signs of oestrus.

There were no alterations in the clinical parameters and the dogs did not present disease; however, the adverse

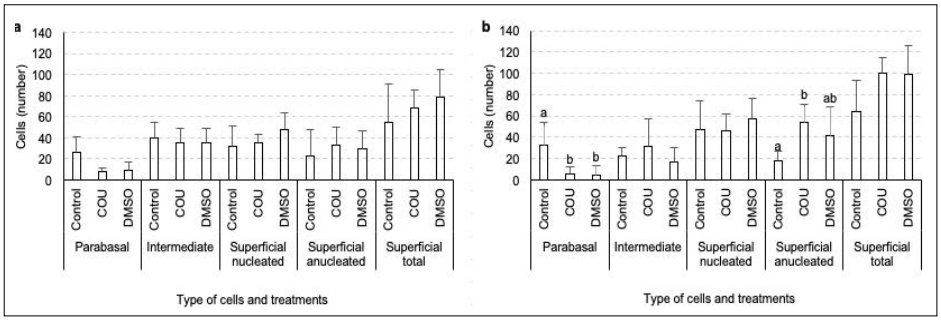

Fig 3: Mean ± S.E.M. of the different types of vaginal cells during the first 28 days after application of treatments in untreated bitches (Control) and in coumestrol (COU)*, or dimethyl sulfoxide (DMSO) treated animals. Vaginal cells in bitches that had ovulated (a) or that had not ovulated (b) at treatment time (day 0). Within the type of cell, unequal letters indicate difference between treatment means [a, b] ($P<0.05$), assessed by x^2 test. * Diluted in dimethyl sulfoxide.

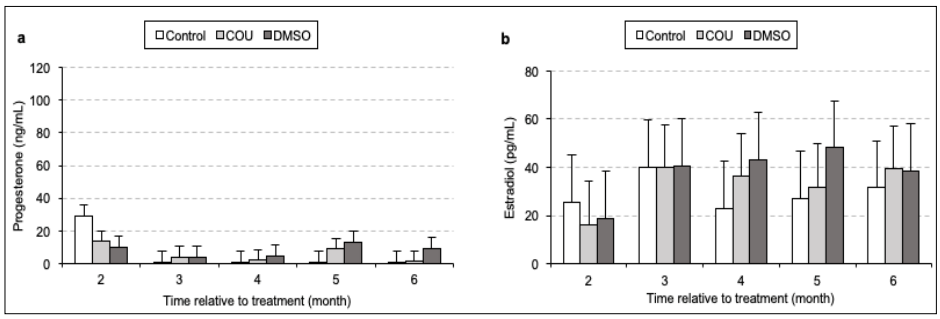

Fig 4: Least Square Means ± S.E.M. of concentrations of progesterone and estradiol from the second to the sixth month after treatments in bitches that were not treated (Control) or that received coumestrol (COU)*, dimethyl sulfoxide (DMSO). Data were analyzed by ANOVA for repeated measures (error type III) and Tukey-Kramer test was used for specific contrasts. * Diluted in dimethyl sulfoxide.

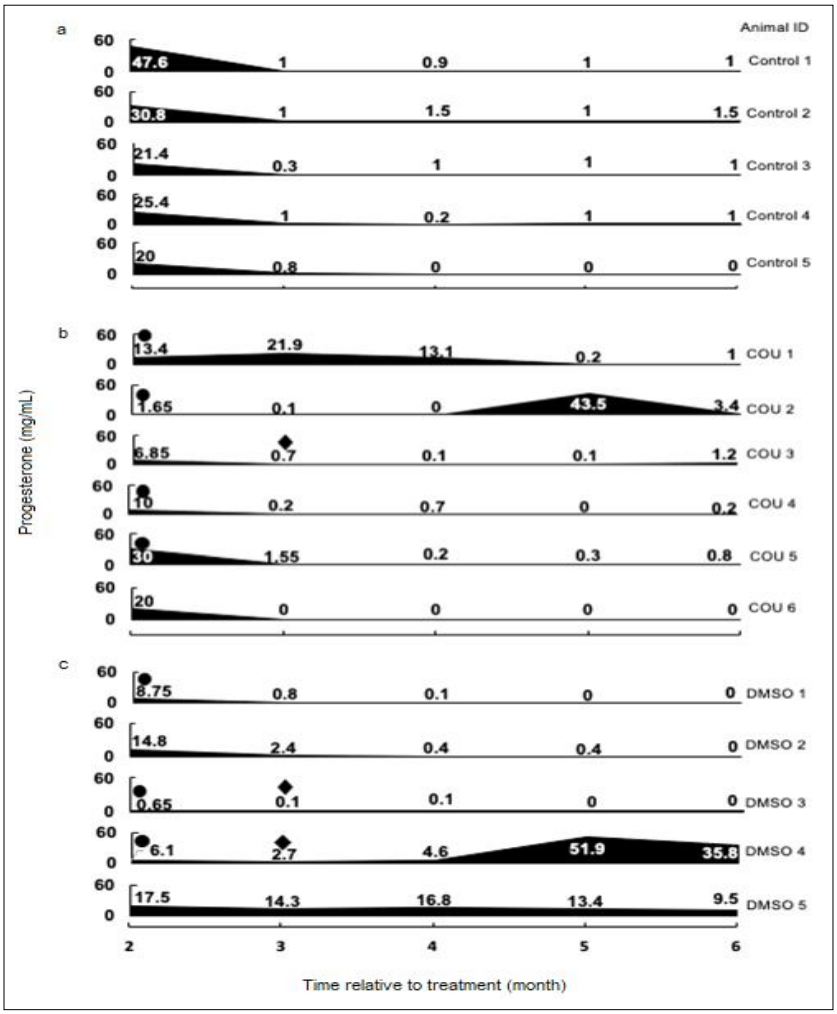

Fig 5: Profiles of progesterone, abnormal vaginal bleeding (•) and mammary gland growth followed by galactorrhea (♦), in individual bitches that remained untreated (a: Control) or were treated with coumestrol (b: COU)* or dimethyl sulfoxide (c: DMSO). Animals were monitored from the second through the sixth month after treatment.*Diluted in dimethyl sulfoxide.

effects of some animals already mentioned in our study are consistent with other studies in female monkeys (Foth and Cline, 1998) so must take these side effects in dogs into account during use.

One deficiency of our study was the inability to control the feeding of the dogs. However, we assume that diet did not influence the results because it is not common for commercial dog food brands to contain COU (Cerundolo *et al*. 2004) nor phytoestrogens (isoflavones) confirmed by normal levels of P4 and E2 in the control group. This study indicates that both COU and DMSO are estrogenic in bitches during an estrogen-sensitive stage and that both compounds act within the first month after administration. These findings are promising and warrant exploring the effects of COU- or DMSO-impregnated biscuits on fertility.

CONCLUSION

Periodical clinical examinations did not show any abnormality in the experimental animals; thus, a single oral administration of COU and/or DMSO does not affect the health condition of bitches. Most bitches treated with COU and/or DMSO showed irregular vaginal bleeding, displayed mammary gland growth and galactorrhea; thus, both substances induce a shifted differentiation of the vaginal epithelium and may induce mammary gland growth and differentiation. Within the first month after a single oral administration, in bitches that had ovulated when treatments were given, neither COU nor DMSO altered circulating P4 and E2 levels or variations in vaginal cells. In contrast, in non-ovulated bitches, COU increased the number of vaginal anucleated superficial cells and reduced parabasal cells as well as circulating concentrations of P4. During the same interval, DMSO reduced numbers of parabasal cells and peripheral concentrations of P4 but increased circulating E2. Therefore, our last conclusion is that COU and DMSO are estrogenic with respect to vaginal tissue and the ovarian functions studied here; however, their actions depend on the ovulatory condition of animals.

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Conflict of interest: None.

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