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EFECTO DEL COUMESTROL, DEL DIMETIL SULFÓXIDO Y SU COMBINACIÓN EN LA REPRODUCCIÓN DE LA PERRA.

TESIS

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Resumen

La sobrepoblación canina es un problema de salud pública. Los fármacos en uso tienen efectos secundarios negativos. El coumestrol (COU) es un disruptor endocrino y podría actuar como potencial anticonceptivo, usualmente se solubiliza en dimetil sulfóxido (DMSO), pero hay reportes de acciones estrogénicas y adversas del DMSO. Se evaluaron los efectos de una administración oral de COU diluido en DMSO y de DMSO sólo, sobre los niveles séricos de progesterona (P4), estradiol (E2) y el tipo de células vaginales de perras en anestro (AN) y etapa periovular (PO), mediante dos experimentos (EXP) completamente aleatorizados con medidas repetidas en tiempo. EXP1: perras en PO recibieron un biscuit solo (Pedigree®; Control,n=5), o con 600µg de COU/kg diluido en 20µL de DMSO (COU,n=6), o con 20µL de DMSO (DMSO,n=5). Considerando las concentraciones de P4 inicial, los animales se subdividieron en P4>1ng/mL y P4≤1ng/mL. Se efectuaron pruebas de citología vaginal exfoliativa (CVE) en los días: -3, 0 (administración), 7,14 y 21. EXP2: los tratamientos descritos se aplicaron en perras en AN (Control,n=5; COU,n=5 y DMSO,n=4), la CVE se realizó los días 0,14,21 y 28. Para ambos EXP la evaluación hormonal se realizó los días 0,14,21 y 28, y después mensualmente por seis meses. Los datos se analizaron mediante GLM o Kruskal Wallis (P≤0.05). EXP1: en animales P4>1ng/mL, ninguna variable evaluada difirió entre el grupo control y los grupos tratados. En animales P4≤1ng/mL tratados, la P4 fue menor los días 21 y 28 respecto al Control. El E2 fue mayor en el grupo DMSO (día 21) con respecto a los demás. Hubo menos células parabasales y más superficiales anucleadas en el grupo COU respecto al Control. EXP2: no hubo diferencias en E2, en el grupo DMSO: la P4 (día 21), y células nucleadas fueron mayores con respecto al Control. Las concentraciones mensuales de hormonas entre grupos no difirieron. DMSO y COU tienen efecto estrogénico, por tanto, los efectos de COU diluido en DMSO deberán de ser reevaluados en mamíferos.

Palabras clave: Coumestrol, Dimetil sulfóxido, Progesterona, Estradiol, Células Vaginales, Perras.

Abstract

Free-roaming dogs are a public health problem. Available drugs prevent reproduction in bitches but have negative side effects. Coumestrol (COU) is an endocrine disruptor, and it could act as a contraceptive potential, usually is solubilized in dimethyl sulfoxide (DMSO), but reports show that DMSO is estrogenic and harmful in mammals. Objectives were to determine effects of a single oral administration of COU diluted in DMSO and DMSO alone on periferal progesterone (P4) and estradiol (E2) serum levels, and vaginal cells in bitches in periovulatory (PO) or anestrus (AN) stages. Two completely randomized experiments (EXP) with repeated measures over time were conducted. EXP1: Bitches in PO received either a single biscuit (Pedigree®; Control,n=5), or containing 600µg of COU/kg diluted in 20µl of DMSO (COU,n=6), or containing 20µl DMSO (DMSO,n=5). Based on initial P4, groups were subdivided into animals with P4>1ng/mL and P4≤1ng/m. Vaginal exfoliative citology (VEC) was performed in days: -3, 0 (administration), 7, 14 and 21. EXP2: treatments previously described were administered in AN bitches (Control,n=5; COU,n=5 and DMSO,n=4). CVE was analyzed on days 0,14,21 and 28. For both EXP hormonal quantification was on days 0,14,21 and 28 and then monthly for six months. Data were analyzed using GLM or Kruskal Wallis (P≤0.05). EXP1: In P4>1ng/mL bitches, none of the variables differed among groups, but in P4≤1ng/mL treated bitches, P4 was lower on days 21 and 28 than in Controls. E2 was higher in DMSO animals on day 21 than in other groups. There were less parabasal and more superficial anucleated cells in COU bitches than in Contro animals. EXP2: No differences in E2 were found among groups, but in DMSO bitches, P4 on day 21, was higher, and superficial anucleated cells were more than in Controls. In both experiments, no differences in monthly hormonal evaluations were found. DMSO and COU are estrogenic, thus data of COU diluted in DMSO, should be reevaluated in mammals.

Key words: Coumestrol, Dimethyl sulfoxide, Progesterone, Estradiol, Vaginal cells, Bitches.

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Capítulo I. Introducción

La sobrepoblación de perros domiciliados o no, ha cobrado importancia en México por ser un problema que afecta a la sociedad humana de varias maneras: desde agresiones llevadas a cabo por perros a personas, hasta la transmisión de enfermedades zoonóticas (Acha y Szyfres, 2001; Knobel et al, 2005; Pérez-Martínez, 2009). El abordaje de este problema por las comisiones gubernamentales, se basa en dos estrategias: la eutanasia y la esterilización quirúrgica. Dichas estrategias no han tenido el éxito esperado ya que la población de perros ha seguido aumentando (Gaceta Parlamentaria, 2011b). Además, el uso de tratamientos hormonales, vacunas anticonceptivas, disruptores endocrinos tóxicos, y sistemas mecánicos de barrera, usados para el control de la natalidad y/o el manejo de ciclos estrales tienen efectos secundarios en la salud, en el comportamiento de los animales o en ambos, especialmente si se utilizan por tiempos prolongados (Dyer et al, 2013; Kutzler y Wood, 2006; Shrestha et al, 2015; Volpe et al, 2001). En el caso de tratamientos farmacológicos se necesitan dosis repetidas o bien, un excipiente que permita liberación sostenida a largo plazo para lograr el efecto de anticoncepción (Frank et al, 1979; Gobello, 2006). Lo anterior hace que estos métodos anticonceptivos sean de difícil administración, especialmente en perros no domiciliados. Las causas de la sobrepoblación canina son diversas, entre las que resaltan, la falta de regulación de venta de animales, ausencia de un sistema oficial de registro de posesión de canes, censos no existentes, estimaciones no actualizadas de población canina, así como también la falta de información y/o sensibilización de la sociedad humana en cuanto a la responsabilidad de mantener a sus perros domiciliados (Gaceta Parlamentaria, 2011a; OPS, 2013; Pérez-Martínez, 2009). La regulación del ciclo estral en la perra es un tema muy extenso y diverso, la fisiología reproductiva de dicho animal se distingue de la de otras especies domésticas en varios aspectos, por ejemplo: presentan prolongados intervalos de proestro y estro, los cuales son seguidos por el diestro y no el metaestro, además de tener un extendido periodo de anestro; presentan una temprana liberación ovocitaria la cuál ocurre una o dos veces por año con el ovocito liberado en estado de vesícula germinativa, para luego completar la maduración citoplasmática y nuclear en el oviducto, bajo la influencia de niveles crecientes de progesterona (P4) (Köning y Liebich, 2011; Songsasen y Wildt, 2007).

Por añadidura, la maduración meiótica *in vivo* en la perra es finalizada de 48 a 72 horas pos ovulación, periodo más prolongado que el intervalo (12 a 36 horas) requerido en la mayoría de mamíferos estudiados (Songsasen y Wildt, 2007).

El seguimiento de los ciclos estrales de las perras suele ser individual y no grupal como en los animales de granja, lo que complica la identificación del inicio del estro; además de la gran variedad de factores que pueden intervenir en la ciclicidad estral, como la cercanía con otras perras, herencia, entre otros (Rangel et al, 2009; Sánchez, 1999). Desde los años 40 del siglo pasado se ha visto que compuestos vegetales como el coumestrol (COU) tienen impacto en la reproducción, pues ejercen su efecto en la fertilidad de animales silvestres y domésticos, a través de la regulación de los receptores estrogénicos (RE) α y β (Bennetts et al, 1946; Kirkpatrick et al, 2011; Romero-R et al, 1997)**.** Por otro lado, el COU, puede ser probado como una herramienta para el control de la reproducción en perras sin la interferencia de su potencial presencia en alimentos comerciales, pues no se encontró COU en un estudio en donde se evaluó la presencia y cantidad de fitoestrógenos (FE) (Cerundolo et al, 2004). Por la naturaleza lipofílica del COU, es común utilizar un disolvente para su administración, por lo tanto, el fabricante recomienda el uso de dimetil sulfóxido (DMSO), sin embargo, hay reportes de que éste solvente también puede tener efectos en la reproducción (Burroughs et al, 1985; Sigma Aldrich, 2017a). A pesar de lo anterior, no se tienen establecidas las dosis, los efectos, y el mecanismo de acción que ejerce COU y/o el DMSO sobre la fisiología reproductiva de la perra. Lo anteriormente mencionado, indica que es conveniente realizar investigaciones que permitan hacer aproximaciones en el mecanismo de acción del COU y DMSO sobre la reproducción en la perra para poder desarrollar nuevas estrategias para el estudio de su control reproductivo. Por lo anterior, el tema central del presente trabajo es evaluar los efectos del COU diluido en DMSO, y del DMSO sólo sobre las concentraciones séricas de E2 y P4, así como su acción sobre el epitelio vaginal en perras.

Capítulo II. Revisión de la literatura y planteamiento del problema

Sobrepoblación canina

La sobrepoblación canina es un riesgo potencial de salud pública en países en vías de desarrollo (Pérez-Martínez, 2009). Se han reportado hasta 53 enfermedades zoonóticas transmitidas por perros causando morbilidad y mortalidad en la población humana de todo el mundo, en México, algunas de las más comunes son: rabia, brucelosis, toxoplasmosis, leptospirosis, tularemia, dermatomicosis, amibiasis, coccidiosis, leishmaniasis, tripanosomiasis, toxocariasis, giardiasis, y sarna (Gaceta Parlamentaria, 2011a).

A través de un estudio de meta-análisis, se calcularon en el mundo más de 700 millones de perros en el año 2014, la Organización Panamericana de la Salud en el 2001, reportó una población canina en América Latina de 65 millones de perros, y particularmente en México de 18 millones (Hughes y Macdonald, 2013; OPS, 2013). Las instituciones gubernamentales correspondientes no han realizado censos o aproximaciones oficiales con base estadística sólida acerca de la cantidad de perros existentes en México. Sin embargo, la Secretaría de Salud estimó en el 2013 alrededor de 1.5 millones de perros en la Ciudad de México (CDMX), asimismo, reportaron que las delegaciones con más caninos son: Gustavo A. Madero, Iztapalapa, Milpa Alta y Xochimilco, además, que al año nacen aproximadamente 128 mil canes (Gaceta Parlamentaria, 2013).

Con el fin de tener una aproximación de la cantidad de perros en México, se utilizaron datos oficiales provenientes de una encuesta de “bienestar subjetivo en México”, realizada en el 2014 en las 32 entidades federativas por el Instituto Nacional de Estadística y Geografía (INEGI), en la cuál se encuestó a un sólo adulto por hogar asignado. El tamaño de muestra garantiza la representatividad nacional y por entidad federativa de las respuestas para la población adulta. La muestra está constituida sobre la base de cuatro estratos socioeconómicos (bajo, medio bajo, medio alto y alto), supone tres etapas de selección: 1) La o las Unidades Primarias de Muestreo (UPM) seleccionada(s) del resto de UPM en el estrato (cada UPM urbana es un área conformada por un conjunto de manzanas); 2) La vivienda dentro de la UPM (ambas selecciones son aleatorias) y 3) La persona dentro de la vivienda. Para garantizar la aleatoriedad de esta última etapa, el adulto que se seleccionó aquel en el hogar más próximo a cumplir años.

Con base en la información anterior, se estimó la población canina a través de dos maneras:

1) Se realizó una correlación entre la cantidad de perros domiciliados y de personas encuestadas, se obtuvo la ecuación: y=0.4978x–14215 (dónde: -14215 es la ordenada al origen y 0.4978 es la pendiente de la recta), con un coheficiente de correlación de (r) de 0.96. Por lo tanto, hay un incremento de 49% en la población de perros domiciliados por cada millón de habitantes. Con dicha ecuación, utilizando 131,725,676 como valor de x, correspondiente a la población en México a inicios del 2018 (Countrymeters, 2018), se estimaron **65,557,430** perros domiciliados (teniendo en cuenta al menos un perro en cada casa), con una proporción humano-perro de 2:1 (Anexo 1). Cabe mencionar que se ha estimado una proporción humano-perro no domiciliado de 4.3-1 para Baja California y Puebla (Fishbein et al, 1992; Flores-Ibarra y Estrella-Valenzuela, 2004).

2) Se utilizó la proporción de perros por estado en la población encuestada en el 2014, y se extrapoló a la cantidad de humanos en el 2018 (utilizando la tasa de crecimiento poblacional anual), se obtuvieron **61, 579, 539** perros en total para México, y de **2, 784, 570** caninos para la Ciudad de México, con esta información se realizó un mapa de calor en donde se representa la distribución canina por intensidad de color (Anexo 2).

Métodos de control para la sobrepoblación canina

Muerte

La norma NOM-033-SAG/ZOO-2014 “métodos para dar muerte a los animales domésticos y silvestres”, establece que la matanza y eutanasia en perros, debe estar basada en la utilización de sobredosis de anestésicos, previa tranquilización o sedación (SAGARPA, 2014). En la CDMX durante 2010 se sacrificaron 26 mil 839 animales de compañía (Gaceta Parlamentaria, 2011b).

Esterilización quirúrgica

La esterilización quirúrgica a través de ovario-histerectomía por la línea media tradicional o por el flanco lateral, y la ovariectomía por laparoscopia o por medios quirúrgicos tradicionales, es uno de los procedimientos más utilizados en la práctica veterinaria para controlar a la población canina, requiere el uso de anestésicos, analgésicos, antibióticos, desinfectantes, personal especializado, instalaciones quirúrgicas y alojamientos destinados a la recuperación pos operatoria (Levy et al, 2008; Massei y Miller, 2013; Shariati et al, 2014).

Esta estrategia también se utiliza con el fin de disminuir o eliminar el comportamiento sexual y para prevenir enfermedades relacionadas con el tracto genital como piometra o tumor de glándula mamaria en las perras (Shariati et al, 2014; Smith, 2006). Sin embargo, comunmente se dan casos de complicaciones postquirúrgicas como síndrome de ovario remanente, hemorragias, incontinencia urinaria, respuestas adversas a ligaduras, estros recurrentes, y adhesiones intestinales (Miller, 1995; Pearson, 1973). Las técnicas descritas previamente han sido ineficaces para controlar el incremento de la población canina, además tienen limitaciones éticas y sociales, como actualmente hay medidas alternativas, las cuales, se exponen a continuación.

Tratamientos hormonales

Hormonas esteroides como progestinas, estrógenos y andrógenos se han utilizado como inhibidores reproductivos en perras con resultados variables.

Progestinas

Las progestinas actúan suprimiendo eficazmente la ciclicidad ovárica mediante la prevención de aumentos de la pulsatilidad de hormona luteinizante (LH), en un estudio en el que se administró 2,200 µg/kg de acetato de megestrol por ocho días a perras en proestro temprano y 550 µg/Kg a perras en anestro por 32 días, se suprimieron los estros en un 92% y 98% respectivamente (Burke y Reynolds, 1975). A pesar del eficaz funcionamiento de fármacos con P4 sintética para la inhibición de la fertilidad, se han reportado tumores de glándula mamaria en perras tratadas durante 45 meses con acetato de medroxiprogesterona (68.4 a 85% de los animales) y/o P4 (65 a 100% de las hembras) (Frank et al, 1979).

De hecho, las perras son particularmente sensibles a dicha progestina, ya que se ha demostrado que el mencionado progestágeno no induce tumores mamarios en roedores (FDA, 2015). El acetato de megelestrol está asociado con patologías uterinas y no se recomienda su uso en perras (Kutzler y Wood, 2006).

La dosificación frecuente y por tiempo prolongado de las moléculas antes citadas, cuando se encuentran en formatos farmacológicos de acción a corto plazo, junto con los efectos secundarios mencionados hacen impráctico su uso en perras (Misdorp, 1991); sin embargo, existen progestágenos en preparaciones farmacológicas parenterales de lenta liberación (inyecciones depot), cuyos efectos duran de 2 semanas a 6 meses, lo que hace factible su utilización con una efectividad mayor al 95% para suprimir la presentación del estro en caninos (Wiebe y Howard, 2009).

Por ejemplo, la proligestona COVINAN® fue evaluada en hospitales veterinarios de Holanda y del Reino Unido habiendo obteniendo un 97% de efectividad en cuanto a la supresión del estro con una inyección subcutánea cuyo efecto dura 5 meses, en ese estudio, de 1608 tratamientos con dicho fármaco (776 perras con dos o más aplicaciones de 3 a 5 meses cada uno), solo se presentaron 5 casos (0.03%) del complejo hiperplasia quística-piometra (Vanos y Oldenkamp, 1978).

De acuerdo con la información citada, se puede asumir que no existe un tipo de progestina, una presentación farmacológica o una dosis que sea 100% efectiva o universalmente segura en perras, en caso de requerir su uso para prevenir la concepción, se debe elegir a la proligestona en su presentación depot.

Sin embargo, el uso de éstos fármacos (de liberación continua) son poco recomendables, debido a que si se presentan efectos colaterales nocivos, no es posible dar por terminada su acción (Wiebe y Howard, 2009).

Agonistas y Antagonistas de la Hormona Liberadora de Gonadotropinas (GnRH)

Los agonistas de GnRH como nafarelin, o deslorelin, imitan su acción, cuando se usan en aplicaciones sostenidas, pues estimulan la producción y la liberación de gonadotropinas (McRae et al, 1985; Rubion et al, 2006; Trigg et al, 2001; Wright et al, 2001). La aplicación de éstos fármacos en perras en anestro induce un ciclo estral fértil (efecto flare-up), y posteriormente inhibe el eje gonadal seguido de quiescencia ovárica por la regulación a la baja de los receptores para GnRH (Gobello, 2006).

Los antagonistas de GnRH como acyline, bloquean competitivamente los receptores para GnRH en hipófisis y se detiene la actividad del eje gonadal, además, ejercen una acción inmediata, en comparación con los agonistas (Valiente et al, 2009). En un estudio en donde se probó la eficacia de este fármaco en la interrupción del ciclo estral, se les administró 110 µg/Kg y/o 330 µg/Kg a perras en proestro por 60 días y se indujo anovulación en todos los animales tratados, además que no se observaron efectos secundarios, por lo cual el tratamiento se consideró eficiente, seguro, y reversible, si se administra en etapas iniciales del ciclo estral (Valiente et al, 2009). En un experimento realizado en los años 80, se observó que la disociación del antagonista con su receptor en la membrana de la glándula pituitaria fue cuatro veces más lenta que la del agonista (Heber et al, 1982).

Debido a que los compuestos antes mencionados para ejercer sus efectos requieren ser aplicados frecuentemente y durante intervalos prolongados; la administración suele ser a través de implantes subcutáneos, o inyecciones diarias, lo que dificulta el éxito de los tratamientos y, en el caso de los implantes subcutáneos, no es posible retirar el tratamiento aún cuando se presenten efectos colaterales no deseados.

Inmunoanticoncepción

La inmuno anticoncepción actúa mediante la inducción de la producción de anticuerpos contra proteínas u hormonas esenciales en la reproducción. En las perras, los más comunes son contra la GnRH (prevención de la ovulación evitando la liberación de gonadotropinas) y zona pelúcida (ZP) (inhiben la fertilización y foliculogénesis) los cuales se discutirán a continuación.

Con la inoculación mensual de preparados de ZP canina (cZP) y ZP porcina (pZP) por seis meses, se documentó infertilidad de las perras tratadas con pZP, sin embargo, una hembra tratada con cZP resultó preñada, lo cual se asoció posteriormente con la falta de pureza proteica del preparado ya que la administración de una fracción purificada canina fue efectiva para inhibir la concepción, cabe mencionar que se encontraron quistes ováricos en perras tratadas (Mahi-Brown et al, 1982; Mahi-Brown et al, 1985; Mahi-Brown et al, 1988). En otro estudio, se evaluó la eficacia inmuno-anticonceptiva de proteínas recombinantes ZP2 y ZP3 conjugadas con toxoide de difteria, y se observó que el 100% de las perras tratadas con ZP2 se preñaron, en contraste al 25% que se inmunizaron contra ZP3; también se observó inhibición de desarrollo folicular en la histoarquitectura ovárica (Srivastava et al, 2002).

En México, se documentó que con una vacuna contra GnRH (GonaCon®) se disminuyeron las concentraciones de P4, y la cantidad de anticuerpos circulantes contra GnRH (Vargas-Pino et al, 2013). Con base en la información anterior, los tratamientos experimentales para probar la eficacia anticonceptiva de dichas vacunas no muestran resultados satisfactoriamente concluyentes, por lo que hasta el momento no es posible utilizar este método para el control de la población canina.

Diepóxido

Se ha utilizado la combinación de 4-vinylcyclohexano diepóxido y triptolide para combatir la sobrepoblación de ratas en los Estados Unidos de América, este químico es altamente específico, actúa en el ovario causando aceleración de falla ovárica y atresia de folículos, la inhibición de la progresión folicular provoca esterilización permanente, e irreversible. Este tratamiento actualmente no se ha probado en perras, sin embargo, se contempla probar en un futuro su efectividad en dichos animales (Dyer et al, 2013).

Conjugados mecánicos

Comercialmente hay espermicidas y dispositivos intrauterinos para perras, sin embargo, no se recomiendan, pues se ha reportado que estos sistemas de barrera, no son prácticos debido a la dificultad de la canulación transcervical requerida para su aplicación (Kutzler y Wood, 2006; Volpe et al, 2001).

Interrupción de la gestación

En perras, básicamente se han estudiado cuatro grupos de fármacos cuya acción es finalizar la preñez: los estrógenos, los antiestrógenos, las antiprogestinas y los inhibidores de la prolactina. A pesar de que este tema escapa del enfoque de la presente tesis, se discutirán brevemente, debido a que la inducción de aborto también es una estrategia del control de la población canina.

Estrógenos y anti-estrógenos

El mecanismo predominante mediante el cual los agentes estrogénicos finalizan la preñez es por la inducción de retraso de tránsito del ovocito a través del oviducto; en un estudio se reportó que la eficacia abortifaciente de dietilestilbestrol y cipionato de E2, administrado por siete días, es cuando mucho de 50% (Bowen et al, 1985).

En otro experimento se determinó que el tamoxifen es efectivo para terminar la preñez en perras en proestro temprano, estro, e inicios de diestro (día 2); sin embargo, también se reportaron efectos secundarios como endometritis, piometra, y ovarios quísticos en el 25% de las hembras, por lo que no se recomienda su uso (Bowen et al, 1988).

Antiprogestinas

Para finalizar la preñez en las perras, se han utilizado antiprogestinas como la mifepristona (RU 486) y la aglepristona (RU 534), las cuales provocan abortos en el 100% de las perras tratadas pocos días después de la administración; sin embargo, en algunos casos, se necesitó más de una aplicación para obtenerlos, pues con una dosis no se logró aborto (Concannon et al, 1990; Galac et al, 2000; Linde-Forsberg et al, 1992).

Inhibidores de la Prolactina

Al menos dos compuestos que inhiben la secreción de prolactina han sido utilizados en perras con el fin de interrumpir la gestación. Por un lado, la bromocriptina, un agonista de la dopamina disminuye las concentraciones de P4 en caninos gestantes o que se encuentran ciclando durante la fase de diestro; no obstante, sus efectos son irregulares, ya que mientras en un estudio no indujo el aborto en los animales tratados dentro de 8-22 días de gestación, en otro se indujo la expulsión fetal en el 100% de las perras tratadas en el día 42 post-ovulación (Concannon et al, 1987). Por otro lado, se ha reportado que la administración de 1.65 µg/Kg de carbergolina por 5 o 6 días, es efectiva para finalizar con la preñez al 100% cuando se administra después de 40 días con respecto a la oleada de LH, y dicha sustancia, es efectiva al 50% administrada antes de los 40 días (Eilts, 2002).

La necesidad de mas de una dosis de los compuestos antes descritos, y la exactitud de los dias de administración con respecto al inicio de la preñez, hacen dificil el uso de abortifacientes como método de control poblacional.

Sumario

Los temas discutidos en los párrafos previos, indican que la administración de compuestos que interfieren con la fertilidad de las perras, está restringida a animales que se encuentran bajo la atención de los propietarios, esto debido a los cuidados, tiempo, elevados costos de la asesoría veterinaria, y los medicamentos requeridos en caso de la esterilización quirúrgica. En cuanto a las otras opciones discutidas, la necesidad de proporcionar repetidas dosis para lograr la inhibición o interrupción de la preñez. Se debe añadir el aspecto de los efectos secundarios nocivos que pueden presentarse. Por lo tanto, hasta el momento no se han logrado establecer procedimientos que permitan un control efectivo de la reproducción de los cánidos.

Ciclo estral de la perra.

Los ciclos estrales comprenden una serie de eventos ováricos endocrinos y conductuales recurrentes con el objetivo de conseguir una gestación (Galina y Valencia, 2008). La perra, es un animal politoco, monoestrico, de ovulación espontánea, típicamente no estacional, aunque se ha reportado que el periodo de reproducción se concentra entre verano y otoño, y varían dependiendo de la raza (Christie y Bell, 1971; Concannon, 2011; Kooistra y Okkens, 2001; Tani et al, 1996).

El ciclo canino está dividido en proestro, estro, diestro, y anestro (Figura 1). Los niveles de P4 y E2 son muy diversos durante el ciclo estral, varían dependiendo del estudio y de la raza de en las que se cuantifican, por lo tanto, su manejo reproductivo es individual y no grupal como en las demás especies de animales domésticos (Christie y Bell, 1971; Rangel et al, 2009).

Una técnica usada para determinar la etapa del ciclo estral de las perras es la citología vaginal exfoliativa (CVE), se basa en que al aproximarse el celo, la concentración de E2 aumenta, induciendo proliferación y diferenciación de las células vaginales, representando estadíos de muerte celular relacionados positivamente con el tamaño, forma y picnosis nuclear (de Buen, 2001; Galina y Valencia, 2008; Hernández et al, 1999; Lacruz y Fariña, 2008; Wright y Parry, 1989). Estas células, se clasifican según su morfología en parabasales, intermedias, superficiales nucleares y superficiales anucleares (Anexo 3).

A continuación, se dará una breve y general revisión de la endocrinología y aspectos clínicos de cada fase estral.

Anestro

La perra es la única especie doméstica que tiene un anestro (2 a 3 meses) que forma parte del ciclo estral, comprende desde el final del diestro y el inicio del proestro (Galina y Valencia, 2008). Los factores que intervienen en la duración del anestro son: la estacionalidad, la herencia, las feromonas de perras en estro, interacciones sociales, y ciclos circanuales endógenos (Sánchez, 1999). El anestro inicia cuando termina el parto en hembras gestantes o cuando la P4 cae por debajo de 1 ng/mL, después del diestro en perras no preñadas (Galina y Valencia, 2008; Okkens y Kooistra, 2006).

Durante la etapa temprana y media de dicha fase, las hormonas se mantienen en niveles basales, hacia el final, hay aumento en la hormona folículo estimulante (FSH), en la capacidad de respuesta hipofisaria a la GnRH, en la expresión de aromatasa hipotalámica y RE en hipotálamo e hipófisis, y de E2 (Concannon, 1986; 2011; de Gier et al, 2008; Tani et al, 1996).

Clínicamente la perra no manifiesta atracción hacia el macho, ni cambios físicos característicos, no hay diferencias clínicas entre las perras en anestro, diestro y ovariectomizadas (Martí, 2011; Sánchez, 1999; Tani et al, 1996).

Proestro

Es la fase que sigue del anestro, tiene una duración de 5 a 20 días. En etapas tempranas, del proestro hay una cohorte folicular, los folículos en crecimiento, sobresalen a la superficie ovárica y secretan E2 de manera semi-autónoma y auto limitante. Los estrógenos inducen una oleada de LH, que endocrinológicamente marca el término del proestro y el inicio del estro (Concannon, 2011; de Gier et al, 2008). El incremento progresivo de E2, provoca fragilidad capilar, permeabilidad de los vasos sanguíneos, y afluencia de eritrocitos hacia el lumen uterino (Concannon, 1986). La P4 empieza a producirse en los folículos en desarrollo aún antes de la ovulación, se comienza a elevar al final del proestro y la relación hormonal E2/P4 ocasionan el inicio de la receptividad sexual (Galina y Valencia, 2008). Clínicamente, se observa sangrado a través de la vagina (fluido seroso que contiene eritrocitos/hemoglobina), vulva aumentada de tamaño y enrojecida, durante toda esta fase la hembra atrae al macho, aunque aún no se encuentra receptiva (Martí, 2011).

Ovulación y periodo periovular (PO)

La ovulación se da en respuesta a un brusco final de una oleada de LH, de 48 a 60 horas, y/o dos días después del primer aumento de P4 de 0.5 ng/mL a ≥ 0.9 ng/mL (Las dos hormonas aumentan concomitantemente en el 95% de los ciclos) (Concannon, 2011). En las perras, en contraste con la mayoría de los mamíferos, los ovocitos se liberan en fase de ovocito primario al interior del infundíbulo en el cuerno uterino en donde se maduran, la fecundación se lleva a cabo de 60 a 108 horas posteriores con respecto a la liberación del ovocito.

La pared de la cavidad folicular se pliega por la caída de presión, y se forma el cuerpo amarillo en el cuál, se reconocen dos estadíos; uno temprano de proliferación; y otro tardío, o de vascularización, cabe mencionar que sólo el 15% de los folículos maduros se llegan a convertir en cuerpos lúteos (Köning y Liebich, 2011; Reynaud et al, 2012; Tsutsui, 1989). El PO comprende el final del proestro y el inicio del estro, basado principalmente en aumento de LH, en ciclos fértiles, su incremento va de 3 a 40 ng/mL en promedio 13 ng/mL (Concannon, 2011).

Estro

Es el periodo de receptividad sexual, dura de 5 a 15 días, su comienzo está marcado además de la oleada de LH que rompe el estigma folicular por un aumento progresivo de P4 sérico y disminución progresiva de estrógenos, en esta etapa la perra es fértil, el intervalo inter-estro va de 5 a 12 meses (Concannon, 2011). Clínicamente, la vulva de la perra se observa turgente con textura suave y flácida, se caracteriza por receptividad proactiva para ser montada por machos, el estro finaliza en el momento que la hembra no acepta el apareamiento, o cuando la P4 llega a más de 10 pg/mL y puede haber o no, sangrado vaginal, al inicio del estro (Beaver, 2009; Christie y Bell, 1971; Martí, 2011).

Diestro

Es una fase de predominancia progestagénica, llega a su máxima concentración en el día 25 para ir declinando lentamente, su longitud es similar en perras vacías y preñadas de 64 ± 1 y 63 ± 5 días respectivamente, aunque en la perra gestante suele ser más duradero, pues hay lisis del cuerpo lúteo provocado por prostaglandinas, las cuales llevan a la caída rápida de P4 (Christie y Bell, 1971; Concannon, 2011; Galina y Valencia, 2008; Luz et al, 2006). Al final del proceso de involución del cuerpo amarillo queda una cicatriz que se conoce como cuerpo blanco (Köning y Liebich, 2011). Al inicio del diestro hay aumento de prolactina, el cuerpo lúteo canino es independiente del soporte gonadotrópico (primeros 25 días), posteriormente la LH y la prolactina toman el control del cuerpo lúteo, el cual se torna sensible a la acción de las prostaglandinas (Concannon, 2011; Hoffmann et al, 2004; Kooistra y Okkens, 2001). En el comienzo del diestro, la vulva puede seguir turgente, no hay flujo sanguíneo, ni atracción al macho, en esta etapa es probable que se presente pseudogestación, o piometra (Concannon, 2011).

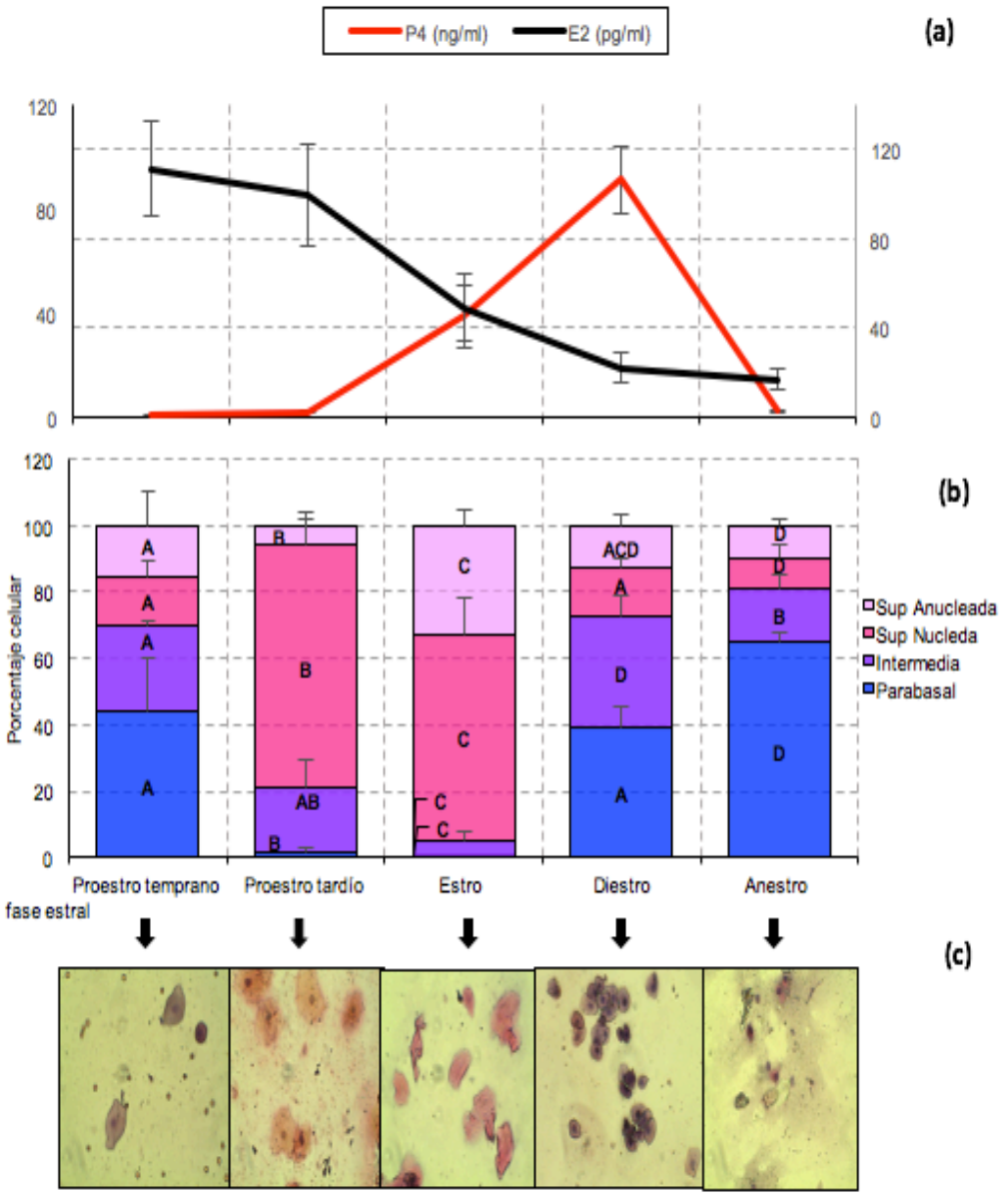

Figura 1. Cambios de los niveles hormonales y de las células vaginales a través del ciclo estral de la perra. a) Concentraciones periféricas de progesterona (P4) y estradiol (E2); b) Cantidad de cada tipo celular obtenida obtenidas a través de la realización de citología vaginal exfoliativa; c) Imágenes características de cada fase estral tomadas en microscopio óptico a 40 x. ^{ABC}Distintas letras denotan diferencias significativas entre la cantidad del tipo celular por fase estral, ($P<0.05$). Los datos de la figura provienen de las hembras del grupo Control utilizadas en los experimentos reportados en la presente tesis.

Sumario

Las etapas del ciclo estral de la perra revisadas anteriormente, dan un panorama general de la gran variabilidad y dependencia de factores exógenos (raza, edad, cercanía con otras hembras, disponibilidad de alimento), endógenos (mecanismos de regulación hormonal, herencia), y aspectos peculiares de la especie en cuanto a la duración de las fases que comprenden el ciclo reproductivo, lo cual hace complejo el manejo grupal en este ámbito.

Estrógenos y disruptores endócrinos reguladores de la reproducción

Como ya se revisó, los aspectos endocrinos y fisiológicos que permiten la sucesión de las fases del ciclo estral en la perra están regulados por hormonas, en particular el estradiol, cuya actividad depende entre otras cosas, de la afinidad a sus receptores y la saturación de los mismos. En las últimas décadas se ha observado que moléculas similares estructuralmente al estradiol como el coumestrol tienen actividad en la regulación del ciclo estral, pues se unen a los RE, su actividad depende de la concentración estrogénica endógena, además, regulan indirectamente a la P4. Con base en lo anterior, es factible tenerlos en cuenta para el control reproductivo canino. Dado que comúnmente se utiliza el dimetil sulfóxido como vehículo para la administración de COU, es necesario evaluar si provoca alteraciones en la reproducción, especialmente porque dicho vehículo ha mostrado tener efecto sobre moléculas reproductivas. A continuación se discutirán brevemente el mecanismo de acción de los estrógenos, la clasificación de los fitoestrógenos, el potencial estrógenico del coumestrol, así como sus efectos la reproducción de mamíferos.

Estrógenos

Los estrógenos son esteroides que favorecen la diferenciación celular, el crecimiento de las glándulas mamarias, útero, vagina, ovarios, sistema vascular y sistema nervioso (Lenis et al, 2010). Se han identificado dos tipos de RE; α y β, con tres dominios funcionales (NH_2 terminal o A/B, el de unión ADN o C, y el de unión a ligando o D/E/F), pertenecen a la superfamilia de receptores nucleares, tienen 95% de homología en la región de unión a ADN y 55% en el de unión a ligando (Nilsson et al, 2001; Whitten y Patisaul, 2001). Los estrógenos pueden actuar a través de dos maneras: la vía clásica o genómica y la no clásica. En la primera, dichas hormonas, se unen y activan a los RE, provocando su dimerización y su internalización al núcleo celular para interactuar con elementos de respuesta a estrógenos, a receptores hormonales, o factores de transcripción en forma de interacciones proteína-proteína (Nilsson et al, 2001).

La vía no clásica, implica activación de respuestas celulares rápidas (liberación de óxido nítrico, flujo de calcio, proteína cinasa activada por mitógeno y/o por AMP, vías de estrés oxidante, el factor nuclear kappa B, y cinasas reguladas por señales extracelulares) a través de unión de ligando a RE localizados en membrana plasmática (clásicos y no clásicos), citosol, retículo endoplásmico, mitocondria y/o receptores GPR30 (Björnström y Sjöberg, 2005; Cederroth et al, 2012; D'Eon et al, 2005; Ropero et al, 2006; Thomas et al, 2005; Watson et al, 2007). En ausencia de estrógenos, los ER se asocian con los co-represores que inhiben la actividad de transcripción (Korach et al, 2003).

Fitoestrógenos

Los FE son compuestos polifenólicos no esteroides derivados del metabolismo de las plantas por estrés ambiental (falta de nutrientes, infecciones, enfermedades foliares) (Howitz y Sinclair, 2008; Lenis et al, 2010). Se han reconocido cerca de 100 FE, se categorizan por su estructura química que es parecida al E2, en 4 clases: isoflavonoides (genisteína, daidzeína, formononetina); flavonoides (naringenina, kaemferol); coumestanos (COU, sativol, diacetato de coumestrol, 4-metoxicoumestrol), y lignanos (enterolactona y enterodiol) (Nilsson et al, 2001; Woclawek-Potocka et al, 2013)

Además de los cuatro anillos de carbono que comparten con el E2, los FE tienen dos grupos hidroxilo que les confieren la capacidad de unirse a RE interfierendo en la síntesis, secreción, transporte, y metabolismo de hormonas reproductivas, en el desarrollo sexual, pubertad, funciones ováricas y comportamiento, actuando como agonistas (totales o parciales) y/o antagonistas dependiendo de la proporción FE/estrógenos, por lo que también son considerados disruptores endocrinos (Adams, 1995; Almstrup et al, 2002; Cederroth et al, 2012; Ropero et al, 2006; Shanle y Xu, 2011; Whitten y Patisaul, 2001). Los efectos de los FE en la reproducción, se detectaron desde los años 40, cuando se describió en ovejas el “síndrome del trébol” el cual consiste en infertilidad, prolapso de útero y distocia (Bennetts et al, 1946).

Coumestrol

El COU (3,9-dihidroxi-6h-benzofuro[3,2-c][1]benzo-piran-6-ona) es el coumestano mejor conocido, se produce en gran cantidad en la alfalfa (11-118 µg/g), soya (71.1 µg/g) (Knuckles et al, 1976). Basado en la capacidad para afectar el peso uterino en ratón, el COU es de 30 a 100 veces mas activo que otros fitoestrógenos (Bickoff et al.,1972)

El COU es ingerido como 4´metoxi coumestrol (forma inactiva), después de ser desmetilado dentro del organismo animal, tiene alta actividad estrogenica, la cual se ha calculado de 1/1000 con referencia al E2, además, compite con este esteroide por la unión con sus receptores, tiene siete veces más afinidad por el β que el α; se une en un 33% a REα y 100% REβ con respecto a E2; estimula la actividad transcripcional de ambos, puede generar una respuesta de la misma magnitud que el E2 a concentraciones de 10-100nM y es agonista de los dos receptores (Knuckles et al, 1976; Kuiper et al, 1998; Lenis et al, 2010). Los efectos puntuales del COU sobre los RE se pueden observar a nivel fisiológico en la reproducción de mamíferos, y varían dependiendo de la especie, dosis y tiempo de exposición (Cuadro 1). Para una correcta administración del COU, se requiere un vehículo que facilite el manejo de dicha sustancia, su distribuidor sugiere que sea disuelto en DMSO (Sigma Aldrich, 2017a).

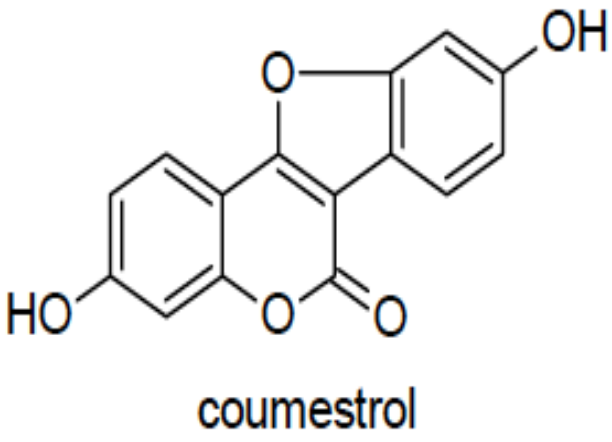

Figura 2. Estructura quimica del coumestrol

Dimetil sulfóxido

El DMSO es un líquido incoloro, con olor característico de los compuestos de azufre y sabor amargo; comercialmente se encuentra al 99.5% de pureza, es altamente higroscópico, se usa como vehículo de fármacos, como esteroides, agentes antifúngicos, y anestésicos en piel, pues atraviesa con facilidad las membranas celulares (Brown et al, 1963; Kligman, 1965).

Está documentado que el DMSO tiene efectos en la reproducción, a nivel fisiológico se ha observado que provoca, apertura vaginal prematura en ratonas y efectos oculares negativos en el ojo de perros (Burroughs et al, 1985; Noel et al, 1975). A nivel molecular, provoca alteraciones en genes, y proteínas relacionadas con la reproducción en salmones (aromatasa, StAR, P450scc ERα y ERβ) e Induce diferenciación en células de glándula mamaria (Costlow, 1984; Lyssimachou y Arukwe, 2007; Lyssimachou et al, 2006; Mortensen y Arukwe, 2006; Zucchi et al, 2002).

Sumario

Además de la regulación endógena, el sistema reproductivo puede regularse por disruptores endocrinos como el COU y el DMSO. Hasta donde la literatura disponible nos lo permite, no hay estudios en los que se evalúe la influencia de dichos disruptores en la reproducción de perras, por lo que la presente tesis está enfocada en determinar los efectos que el COU diluido en DMSO y el DMSO solo ejercen sobre los perfiles de estradiol y progesterona, así como en las células vaginales.

Cuadro 1. Antecedentes de los efectos del COU en variables reproductivas de mamíferos

Vehículo	Dosis de COU	n	Exposición	Vía	Animal	Efectos de COU	Referencia
Pastura*	25-100 mg COU/kg de pastura	1750	26 días	VO	Oveja	Reduce tasa de ovulación.	Smith et al, 1979
Aceite de maíz	132 mg COU/cría de oveja	24	12 días	VO	Oveja	Disminuye peso ovárico, aumenta peso uterino (efecto de COU similar a 2.5mg de E2)	Newsome y Kitts, 1980
Etanol 95%	100 mg COU/Kg de alimento	60	25 días	VO	Ratona	Sin efecto en tamaño y peso de camada, disminuye ingesta de alimento.	Elias y Kincaid, 1984
Pastura*	4, 18 o 40 mg COU/Kg de peso	3	15 días	IV	Oveja	Disminuye la amplitud de pulsos de LH.	Montgomery et al, 1985
DMSO	.100 mg de COU/Kg de peso	5	5 días	VSc	Ratona	Induce apertura vaginal precoz, cornificación vaginal persistente.	Burroughs, 1985
DMSO	001, .005, .025, .05, y .10 mg COU/Kg de peso	10	5 días	VSc	Ratona	Induce apertura vaginal precoz, cornificación vaginal persistente, hiperplasia glandular quística y folículos hemorrágicos.	Burroughs et al, 1990
Pastura*	66.8 mg de COU/Kg de alfalfa, 500 mg de COU diarios	608	Un año	VO	Vaca	Induce sindrome estrogénico (aborto, útero turgente, ninfomanía).	Romero-R et al, 1997
-	0.02% de COU mezclado en comida	6	10 días	VO	Ratona	Aumenta LH en hipófisis, actúa a través de REα.	Jacob et al, 2001
Aceite	60 mg/kg/día	6-8	3 días	VO	Ratona	Incremento en peso de útero, vagina y cérvix. Aumento en fase S celular comparables con benzoato de estradiol.	Tinwell et al, 2000
DMSO con ciclodextrina	0.4 o 1.6 mg	6	8.5 horas	IV	Rata	Inhibición de pulsos de LH, reducción de la respuesta a GnRH	McGarvey et al, 2001
Tween 80	.1 mg/mL	5	3 días	VO	Ratona	Respuesta similar al estradiol (100 ng/mL). Incrementa peso uterino.	Pocock et al, 2002
Aceite	1, 3 mg de COU	11	Una vez	VSc	Rata	Provoca estro prolongado, disminuye peso ovárico, suprime mecanismos de inducción de ovulación.	Kouki et al, 2005
DMSO y sangre	.2 mg de COU	5	Una vez	VO	Murciélago	Induce crecimiento folicular anómalo.	Serrano et al, 2007
DMSO	.300 mg de COU/Kg de peso	5	1 dosis semanal/4	VO	Perro	Anormalidades en espermatozoides, en histoarquitectura testicular y alteraciones en el comportamiento olfatorio.	Pérez-Rivero et al, 2009a
DMSO	.600 mg de COU/Kg de peso	8	1 dosis	VO	Perro	Anormalidades en espermatozoides, y en histoarquitectura testicular.	Gualo, 2010
Pastura*	5000 a 8000 mg de alfalfa por yegua	16	5 meses	VO	Yegua	Provoca edema uterino, anovulación, descarga excesiva de moco cervical (reducción de efectos entre 2 y 3 semanas después de retiro de pastura).	Ferreira-Dias et al, 2013
Pastura*	2500-7500 mg de alfalfa	4	14 días	VO	Yegua	Aumento de estradiol y disminución de P4 en sangre.	Szóstek et al, 2016

*Pastura con infección fúngica.
Vía de administración: VO: oral; IV: intravenosa; VSc: subcutánea.

III. Hipótesis

Ya que se ha reportado que el coumestrol tiene un efecto estrógenico, una aplicación oral de coumestrol diluido en dimetil sulfóxido:

1: Perras en etapa periovular aumenta las concentraciones de E2, disminuirá las de P4 e incrementará la cantidad de celulas vaginales superficiales.

2: Perras en anestro, los niveles séricos de P4, de células vaginales superficiales y la duración de anestro y diestro pos tratamiento aumentarán.

3: Perras en etapa periovular y en anestro no se alterarán los parámetros clinicos.

Con base en los reportes existentes del efecto estrógenico de dimetil sulfóxido sobre moleculas involucradas con la reproducción, una administración oral de dicho compuesto en:

1: Perras en etapa periovular y en anestro aumentrá las concentraciones de E2 y la cantidad de células vaginales superficiales.

2: Perras en etapa periovular y en anestro no se alterarán los parámetros clinicos.

IV. Objetivos

Objetivo general: Evaluar los efectos de una administración por vía oral de coumestrol diluido en dimetil sulfóxido o de dimetil sulfóxido sólo, aplicados en perras durante las fases periovular y de anestro, en algunas variables reproductivas.

Objetivos específicos: Determinar los efectos de una aplicación oral de coumestrol diluido en dimetil sulfóxido o de dimetil sulfóxido solo administrados a perras en las etapas periovular y de anestro en :

1: Las concentraciones circulantes de estradiol y progesterona.

2: En las células vaginales.

3: En la duración del diestro y del anestro.

4: Parámetros clínicos de perras en fases periovular y en anestro.

Capítulo V. Efecto del coumestrol y dimetil sulfóxido sobre los niveles circulantes de estradiol, progesterona y los tipos de células epiteliales vaginales en perras en etapa periovular.

Administration of coumestrol and/or dimethyl sulfoxide affects vaginal epithelium and sex hormones in bitches

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Abstract

To determine the effects of a single oral administration of coumestrol (COU) diluted in dimethyl-sulfoxide (DMSO) and DMSO-alone on vaginal epithelial cells, on serum progesterone (P4) and estradiol (E2) levels, and on clinical parameters (CPs), bitches in the periovulatory stage received either a biscuit (Control, n=5), a biscuit containing 600µg COU/kg diluted in 20µL DMSO (COU, n=6), or a biscuit containing 20µL DMSO/kg (DMSO, n=5). Vaginal cytology and hormones levels were evaluated within the first 21 and 28 days respectively. Hormones were determined monthly from the 2nd to the 6th month. CPs were evaluated on day 0 and on the 6th month. Based on P4 levels on day 0, groups were divided into animals that had ovulated (P4>1ng/mL) or not (P4≤1ng/mL). The ovulated animals were not affected by treatments. Contrastingly, relative to Controls, in non-ovulated bitches, COU and DMSO decreased P4 levels ($P<0.05$), but DMSO increased E2. In the COU-treated bitches, anucleated cells increased while in COU- and DMSO-group the parabasal cells decline. However, from 2nd to 6th months, treatments did not affect P4 or E2. All CPs were within reference values and no health alterations were detected during clinical examinations, but some bitches treated with COU and/or DMSO presented irregular vulva bleeding and mammary gland growth and galactorrhea. Therefore, treatments affect P4, E2, and vaginal cells only the first 28 days but their effects depend on the ovulatory stage. Finally, neither COU nor DMSO altered animal's health but some treated bitches presented side effects.

Key words: Coumestrol, dimethyl sulfoxide, bitch, estradiol, progesterone, periovulatory stage.

Introduction

Dogs serve valuable roles in human society but in some countries the great number of owned and unowned free-roaming dogs has become a serious health problem for the human population, because of inadequate or absent policies of dog ownership, accumulation of garbage due to poor waste disposal management, and the absence of governmental programs for reducing the canine population, the number of dogs is increasing in many of the underdeveloped countries (Elliot et al. 1985; Hsu et al. 2003; Trevejo et al. 2005).

Currently, strategies that have been devised to ameliorate this issue are available. For example catching dogs, and subjecting them to euthanasia or surgical sterilization, as well as the use of hormones for controlling the estrous cycle; however, trained people and specialized facilities are required in the case of euthanasia or surgery, and hormone procedures are either inefficient, affect negatively the health of bitches, and/or require repeated applications to control a single fertile period (Kutzler and Wood 2006; Wiebe and Howard 2009). Thus, it is attractive to devise new alternatives to control canine overpopulation. An ideal method would be to have a substance that were non-invasive, easy to be administered by untrained people, and that a single application would avoid reproduction, independent of the estrous cycle stage of bitches.

Naturally occurring phytoestrogens like coumestrol (COU), participate in hormonal regulation processes (Almstrup et al. 2002; Cederroth et al. 2012). COU could be a potential alternative to avoid unwanted pregnancies, because in some experiments this phytoestrogen affects negatively the reproduction of mammals; for example, COU induces abortion in cows, anovulation in mares, ovarian weight loss in sheep and rats, and persistent vaginal epithelial cell cornification in mice (Burroughs et al. 1990; Ferreira-Dias et al. 2013; Kouki et al. 2005; Newsome and Kitts 1980; Romero-R et al. 1997). To the best of our knowledge, the effects of COU have not been studied in female dogs but in male dogs cause abnormalities in spermatozoids (Pérez-Rivero et al. 2009). Thus, COU may be tested as a potential tool for controlling reproduction in bitches. However, it is known that COU has low solubility in aqueous and lipophilic media thus the manufacturer recommends dissolving COU in dimethyl sulfoxide (DMSO) before administering to animals (Franco et al. 2009; Sigma Aldrich, 2017). Accordingly, DMSO was used as a vehicle for oral administration of COU in female bats, mice, and male dogs (Burroughs et al. 1985; Burroughs et al. 1990; Pérez-Rivero et al. 2009; Serrano et al. 2007).

However, there is evidence that DMSO is not innocuous because it may induce adverse effects on the health of dogs and modulate the estrogenic responses in fish (Lyssimachou and Arukwe 2007; Lyssimachou et al. 2006; Mortensen and Arukwe 2006; Noel et al. 1975). Thus, it is convenient to determine the effects of DMSO on the health and reproduction of bitches. Similarly, it should be convenient to elucidate whether DMSO used as a diluter of COU masks, interferes, or enhances the estrogenic actions of COU when given to bitches.

In mammals, estrogenic compounds induce vaginal epithelial proliferation, which can be observed through counting of the exfoliated vaginal cells (EVC); in the bitch these cells have been classified, morphologically described, and their approximate variations throughout the stages of an estrous cycle were documented (Antonov 2017; Post 1985; Root 2012; Schutte 1967). However, to our knowledge, an organized, statistical study of variations in numbers and measures of vaginal cell types has not been performed in bitches as it has been reported for ruminants and laboratory rodents (Cora et al. 2015; Ola et al. 2006; Pérez-Martínez et al. 2009; Siregar et al. 2016). This void in knowledge potentially is a source of error when determining the stage of an estrous cycle in bitches.

Due to the non-availability of experimental dog packs, before attempting to know if COU and/or DMSO are effective contraceptive drugs, we decide to determine the effects of those substances in some reproductive variables of bitches in the periovulatory stage, having in mind that our hypothesis is that both DMSO and COU act as estrogenic compounds when are administered to bitches estrogenically susceptible. Our main objective was to evaluate the effects of a single oral administration of COU diluted in DMSO and DMSO-alone on variations in vaginal epithelial cells, concentrations of peripheral progesterone (P4) and estradiol (E2), duration of diestrus and anestrus, as well as clinical chemistry and hematologic parameters in bitches. A collateral objective was to provide a detailed characterization of morphometry and quantity of vaginal cell types throughout the estrous cycle in the bitch.

Materials and methods

Compliance with Ethical Standards

All procedures in this study were approved by The Institutional Subcommittee for Care and Usage of Experimental Animals of the Graduate Program of The College of Veterinary Medicine and Animal Husbandry, National Autonomous University of Mexico, under protocol number DC-2015 / 2-10 and according to the guidelines of the European Union Directive (2010/63/EU) for animal experimentation. None of the authors has a personal or financial relationships with other organizations or people that could influence or bias the content of the paper.

Animals, general management, and treatments

Sixteen female dogs that remained throughout the study at their owner's house were used. Animals were fed with commercial dry dog food (kibbles) of different brands, complemented in some cases with leftovers from the owner's meals. In all cases, housing was adequate and the owners personally took care of animals. All bitches had a health card with the history of periodic visits to a veterinary center, had been treated against internal and external parasites, and vaccinated against leptospira, distemper, adenovirus, parvovirus, parainfluenza, and rabies. Bitches were intact, small-to-medium size breeds (poodle, 5; cocker, 1; schnauzer, 3; pug, 2; indefinite, 5), aged (mean ± SD) 3.75 ± 2.6 years old, weighed 9.06 ± 4.8 kg, and had displayed estrus at least once before the study. At the beginning of the experiment, bitches were in the periovulatory stage (swollen vulva and blood discharge through vulva). We explained to the owners, characteristic signs of estrus and asked to report any abnormal signs, as swelling of the vulva, vaginal discharge, and mammary gland growth. The duration of diestrus was the number of days between the first sample with serum concentrations of P4 at or above 2.5 ng/mL and the sample when P4 declined below 2.5 ng/mL and then remained at basal levels. Anestrus was evaluated by counting days from the end of diestrus and the first day when evident signs of estrus of the subsequent cycle were observed. Animals were randomly assigned to one of the following treatments: 1) Control group (n=5), in which bitches received orally a single biscuit (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 µg/kg of body weight; Sigma Chemical Co. St. Louis, Mo, USA) diluted in DMSO (20 µL per 600 µg of COU); 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).

At the beginning of the study and during each visit to the animals, all dogs were clinically examined by an accredited veterinarian to confirm their health status.

Biological samples

On days -3, 0, 7, 14, and 21 a sterile vaginal smear was taken as previously reported (Aydin et al. 2011). These samples were used for VEC. On days 0, 14, 21, and 28, and thereafter every 30 days to the 6th month of the experiment, blood samples were taken by venipuncture from the cephalic vein; serum was obtained and used for quantification of P4 and E2. Furthermore, another blood sample collected from animals on day 0 and at the end of the 6th month was used to perform a blood chemistry test and blood cell count.

Laboratory analysis

For VEC, cotton swabs containing vaginal cells were rolled over a slide, fixed (Citospray, CTR Scientific, Mexico), and stained using a modified Papanicolaou procedure (Pérez et al. 2005); the modification consisted on the substitution of saline solution by distilled water to preserve erythrocytes. Parabasal, intermediate, and superficial vaginal cells were counted using optical microscopy (ten fields per animal, randomly chosen/smear, 40× objective, microscope Axio Scope.A1, Zeiss, Mexico). The microscope was equipped with a photographic digital camera (Motic China Group, CO., China) and photographs were taken using Motic Images Plus 2.0. Photographs were used for the morphometric analyses by determining cells and nuclei area. Serum concentrations of P4 and E2 were determined using immune-enzymatic assays (Enzyme-Linked Immune-Absorbent Assay, DGR Instruments, GmbH, Germany). The inter-assay rank was 10.6 to 2000 pg/mL for E2 and 0.01 to 40 ng/mL for P4.

Design and statistical analysis

A completely randomized design for mixed models with repeated measures over time was used. Data relative to E2, P4 during the first 28 days and hormone levels from the second to the sixth month after application of treatments in Control, COU, and/or DMSO, 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. Additionally, once P4 data were obtained, to determine whether the ovarian status alters the responses to treatments, each group was subdivided according to a previous study (Concannon 2011) 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).

The P4>1 ng/mL animals by group were: Control (n=3), COU (n=3), and DMSO (n=3); P4≤1 ng/mL bitches by group were: Control (n=2), COU (n=3), and DMSO (n=2). The statistical approach previously mentioned was applied to analyze the new set of data. In order to examine the variations in morphometry of vaginal cells in Control animals, the surface area of cells and their nuclei were analyzed using analyses of variance. The Tukey-Kramer test was used for specific contrasts in all analyses mentioned above. When data were examined for normality, both hormonal and vaginal cells of Control animals and vaginal cells of treated bitches were not normally distributed. Therefore, to contrast mean values between estrous cycle phases, P4 and E2 data in Control animals, and vaginal cells in treated bitches were examined using the Kruskal-Wallis test in SPSS (version 24; IBM, IL, USA) whereas their vaginal cells number were analyzed by the $\chi 2$ test. Criteria for statistical significance was $P<0.05$.

Author contributors

Alejandro Villa-Godoy: Re-design of the study, critical revision of different drafts of the article, writing, and approval of the final version of the article.

Héctor Serrano: Conception and initial design of the study, critical evaluation of the first draft of the article.

Sheila I. Peña-Corona: Contribution to the original design, conduction, and coordination of the trial, collection of data and biological samples as well as laboratory analysis of hormones and vaginal cells, statistical analysis of data, and writing the first draft of the article.

Pablo León-Ortiz: Help to collection and handling animals.

Salvador M. Villanueva: Periodical clinical examination of animals, coordination and interpretation of blood chemistry tests and blood cells counts.

Adriana Mendoza-Rodríguez and José J. Martínez-Maya: Statistical consultants and critical evaluation of the experiment and article.

Results

Animal health

All animals, Control and treated, remained in adequate health throughout the study, as evidenced by clinical periodic examinations, as well as blood chemistry tests and blood cell counts performed at the beginning and end of the study (Table 1), in which all health-related variables were within the reference values in all groups.

Table 1. Clinical chemistry and hematologic parameters in bitches that received no treatment (Control) or that were treated with oral coumestrol (COU)*, and dimethyl sulfoxide (DMSO). Analyses were performed at the beginning (application of treatment) and at the end (6 months after treatment) of the study (mean ± SD). *Diluted in dimethyl sulfoxide.

Issue (reference values)	Control		COU		DMSO	
	Beginning n=5	End n=3	Beginning n=6	End n=6	Beginning n=5	End n=4
Glucose (3.38–6.88 mmol/L)	3.1±1.3	4.11±1.3	4.9±1.3	4.0±1.7	3.4±2.0	5.5±1.0
Urea (2.1-7.91 mmol/L)	4.6±1.4	6.78±1.4	7.1±3.5	5.7±1.9	5.4± 2.7	3.6±0.1
Creatinine (60-126 µmol/L)	76.4±10.7	104±19.3	85.5±12.2	76.7±17.0	97.6±12.9	84±16.6
Cholesterol (2.85-7.76 mmol/L)	6.4±0.9	6±0.4	5.5±0.7	4.5±1.3	5.4±0.6	6.3±1.7
Total bilirubin (<5.2 µmol/L)	0.9±0.8	1.9±0.9	1.7±0.6	1.8±1.1	1.8±0.8	2.9±1.3
Alanine aminotransferase (4.0-70 UI/L)	53.8±8.8	77±12.3	52.0±10.8	53.0±10.6	55.6±30.9	45.8±20.3
Alkaline phosphatase (6-189 UI/L)	68.4±37.5	53±18.0	76.0±56.7	71.2±18.5	57.0±25.4	88.3±18.7
Total protein (56-75 g/L)	77.0±7.9	68±9.5	69.3±14.3	64.3±4.7	66.2±1.3	67.0±3.6
Albumin (29-40 g/L)	38.6±1.5	36±1	35.2±9.5	35.8±3.5	33.8±4.0	35.3±1.0
Globulin (24-39 g/L)	38.4± 7.4	32±8.7	34.2±6.4	28.7±4.3	32.4±3.6	29.3±2.5
Phosphorus (0.75-1.70 mmol/L)	1.4±0.2	1.46±0.2	1.1±0.2	1.5± 0.2	1.5±0.0	1.3±0.2
Bicarbonate (17-25 mmol/L)	20.6±2.6	20±2.0	20.5± 3.4	19.0±2.1	16.8±3.4	21.0±2.6
Hematocrit (.37-.55 L/L)	0.5±0.1	0.47±0	0.4±0.1	0.4±0	0.4±0.0	0.5±0.1
Hemoglobin (120-180 g/L)	168.6±23.5	156±2.1	141.3±19.9	129.0±8	148.4±15.1	145.3±21.7
Red cells (5.5-8.5 $X10^{12}$/L)	7.7±1.1	7.8±0.4	6.4±1.0	5.9±0.6	7.0±0.7	7.6±0.4
Mean corpuscular volume (60-77 fL)	65.2±1.1	60±3.5	66±1.7	66.2±3.3	63.2±3.3	69.0±5.7
Mean corpuscular hemoglobin (320-360 g/L)	333.2±0.4	331±1.5	332.7±1.5	331.8±0.4	332.2±1.3	331.8±1.3
Reticulocyte count (>60 × 10^9/L)	0±0	0±0	0±0	0±0	0±0	0±0
Platelet count (200-900 ×10^9/L)	433.6±96.3	450±100.3	385.3±137.7	340.0±0	378.4± 147.9	469.3±51.7
Total protein (60-75 g/L)	75.2±5.0	72±4.6	69.5±8.9	65.8±4.7	70.2±3.0	69.0±5.4
Leukocyte count (6.0-17.0 ×10^9/L)	16.3±12.3	12.6±10.2	9.8±3.9	11.3±2.7	15±7.8	13.7±5.2
Neutrophils (3.0-11.5 × 10^9/L)	12.3±9.6	9.4±7.8	5.9± 2.6	7.1±1.4	8.4± 4.1	11.7±4.2
Neutrophils on band (0-0.3 ×10^9/L)	0±0	0±0	0±0	0±0	0±0	0±0
Metamyelocytes (0 × 10^9/L)	0±0	0±0	0±0	0±0	0±0	0±0
Myelocytes (0 × 10^9/L)	0±0	0±0	0±0	0±0	0±0	0±0
Lymphocytes (1.0-4.8 × 10^9/L)	2.2±1.8	2±1.7	2.4±0.9	2.6±0.9	3.3±1.4	2.5±1.5
Monocytes (0.1-1.4 × 10^9/L)	1.0±0.7	1.2±0.6	1.0±0.5	0.8±0.3	1.7±0.9	2.0±1.0
Eosinophils (0-0.9 × 10^9/L)	0.9±0.9	0±1.2	0.5±0.3	0.8±0.4	1.8±1	0.3±0.2
Basophils (Rare)	0±0	0±0	0±0	0±0	0±0	0±0

Hormones and vaginal cytology in an estrous cycle of Control bitches

Variations of P4 and E2 in the Control animals throughout the estrous cycle (Table 2) were similar to results reported previously (Concannon 2011). All types of vaginal cells in Control bitches (Table 2) varied throughout the estrous cycle. The mean number of parabasal cells was high in anestrus, and declined in diestrus and proestrus, to become practically non-existent in estrus. For intermediate cells, numbers were relatively high in diestrus, became lower in proestrus and anestrus, and were the lowest during estrus. A distinct pattern was observed for total superficial cells that were highest in number during estrus, followed by proestrus to decrease in diestrus and anestrus. When superficial cells were classified as nucleated and anucleated, it was observed that they did not show a similar pattern where the anucleated superficial cells were more numerous in estrus and less numerous in late proestrus whereas the nucleated cells were abundant in late proestrus and estrus and were in the lowest number during anestrus. Cell surface area (Fig.1a) did not vary in intermediate cells throughout stages of the estrous cycle. However, parabasal cells had a smaller surface area during anestrus than in proestrus and diestrus. Superficial cells had a smaller area during diestrus and anestrus than in proestrus and estrus. The nuclear surface area of parabasal cells was similar in all stages of the estrus cycle in which it was measured. In intermediate cells, nuclear surface area was smaller during proestrus relative to diestrus while in estrus and anestrus the nuclear area did not differ between them or with proestrus and estrus (Fig.1b). The nuclear area of superficial cells was greatest during proestrus and diestrus, and was lower in estrus and became smaller during anestrus. In Control bitches, nucleated and anucleated superficial cells had a similar variation throughout the estrous cycle. The greatest surface area was observed during late proestrus and estrus and the smallest during anestrus and early proestrus.

Table 2. Number of the different types of vaginal cells (mean ± SE; maximum and minimum values) and peripheral concentrations of progesterone and estradiol in Control bitches (mean ± SE). [a,b,c,d,e,f] Unequal letters within a row, indicate difference (P<0.05) between means of a cell type or hormone between phases of an estrous cycle.

	Phase of an estrous cycle					
Cell types and hormones	Proestrus	Early Proestrus	Late Proestrus	Estrus	Diestrus	Anestrus
Parabasal:	22.8±14.0[a]	54.5±16.5[b]	1.7±1.7[c]	0.2±0.2[cd]	47.3 ±6.4[b]	76.7±3.2[f]
Maximum	71	71	5	1	80	82
Minimum	0	38	0	0	13	71
Intermediate:	28.2±5.2[a]	32.5±1.5[ab]	25.3±8.9[abc]	6.0±2.8[d]	40.1±6.3[ae]	19.3±4.4[ac]
Maximum	43	34	43	17	74	28
Minimum	15	31	15	1	21	14
Superficial total:						
Maximum	76.4±17.2[a]	37.5±5.0[b]	102.3±11.6[c]	110.6±9.5[c]	33.3±3.0[b]	22.3±3.8[d]
Minimum						
	125	43	125	148	45	28
	32	32	87	95	18	15
Superficial nucleate:						
Maximum	64.0±19.6[a]	18.0±5.0[b]	94.7±9.7[c]	72.1±10.8[ad]	18.1±2.8[b]	10.3±4.5[b]
Minimum	114	23	114	109	30	19
	13	13	83	50	5	4
Superficial anucleate:						
Maximum	12.4±4.5[a]	19.5±10.5[b]	7.7±2[ac]	38.4±5[d]	15.2±3.4[ade]	12.0±2.1[ace]
Minimum	30	30	11	47	34	16
	4	9	4	19	3	9
Progesterone (ng/mL)	1.5±0.3[a]	1.0±0[a]	1.9±0.3[a]	38.8±12.0[b]	91.4±12.9[c]	2.3±0.4[a]
estradiol (pg/mL)	104.3±14.5[a]	111.45±21.3[a]	99.5±22.9[a]	49.0±15.3[b]	21.8±6.8[b]	17.0±4.9[b]

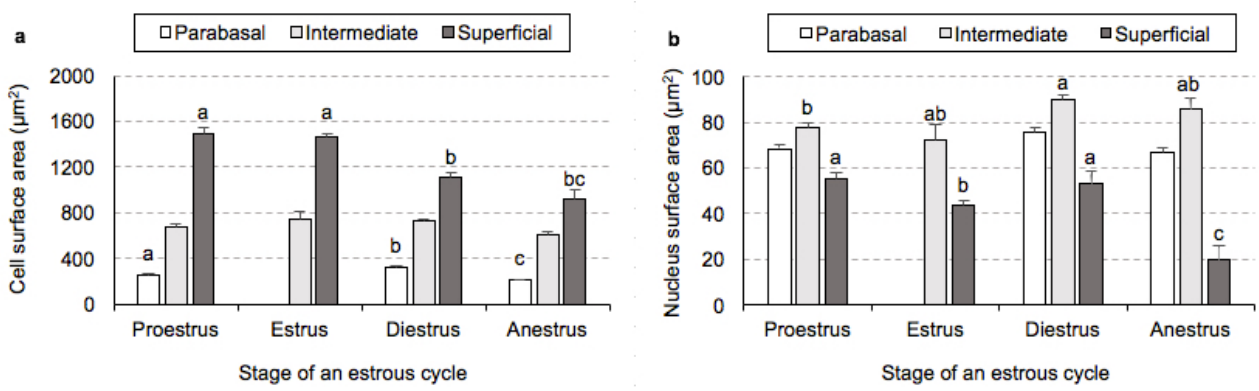

Figure 1. Surface area of cells and nuclei (mean ± SE) of the different types of vaginal cells obtained from Control bitches during an estrous cycle. In estrus, the number of parabasal cells was insufficient to determine cells and nuclei area. [a,b,c] Unequal letters indicate difference between the type of cells within the stage of the estrous cycle (P<0.05).

Hormones and vaginal cells during the first 28 days

Unstratified bitches by the initial concentration of progesterone

Relative to values recorded in Control bitches, both COU- and DMSO-treated animals showed a reduction in serum concentrations of P4 on days 21 and 28 post-treatment (Fig.2). In contrast, serum levels of E2 on day 21 post-treatment in bitches treated with DMSO were higher in comparison with Control and COU-treated animals.

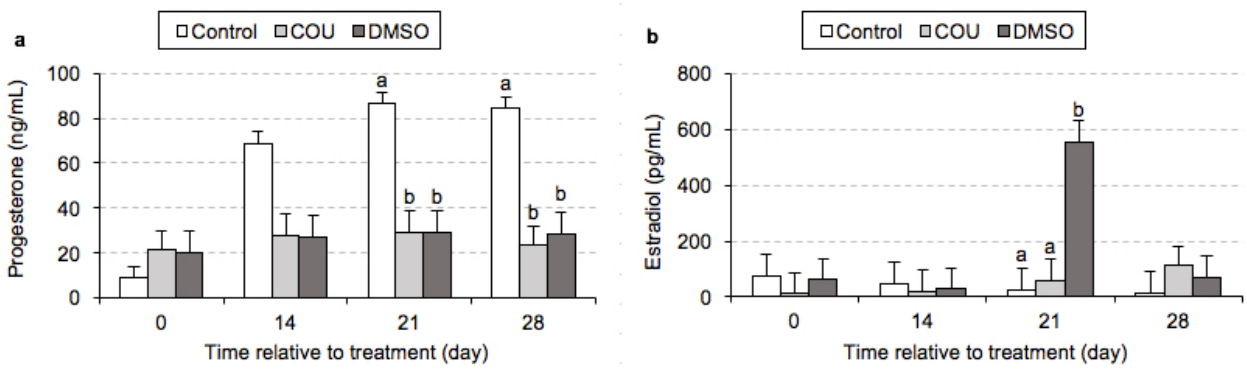

Figure 2. Least square means ± SE of progesterone and estradiol in serum taken from bitches that received an oral administration of a biscuit (Control; n=5); a biscuit containing coumestrol (COU, n=6)*, or a biscuit containing dimethyl sulfoxide (DMSO, n=5); [a,b] Unequal letters indicate difference between treatments within sampling day ($P<0.05$). * Diluted in dimethyl sulfoxide.

Stratified bitches by the initial concentration of progesterone

In bitches that had ovulated on day 0 (P>1 ng/mL), COU and DMSO did not affect the profiles of P4 and E2 (Fig.3a and 3b). 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 concentrations of P4 on days 21 and 28 post-treatment (Fig.3c), and bitches treated with DMSO had higher E2 levels than Control and COU-treated bitches on day 21 post-treatment (Fig.3d).

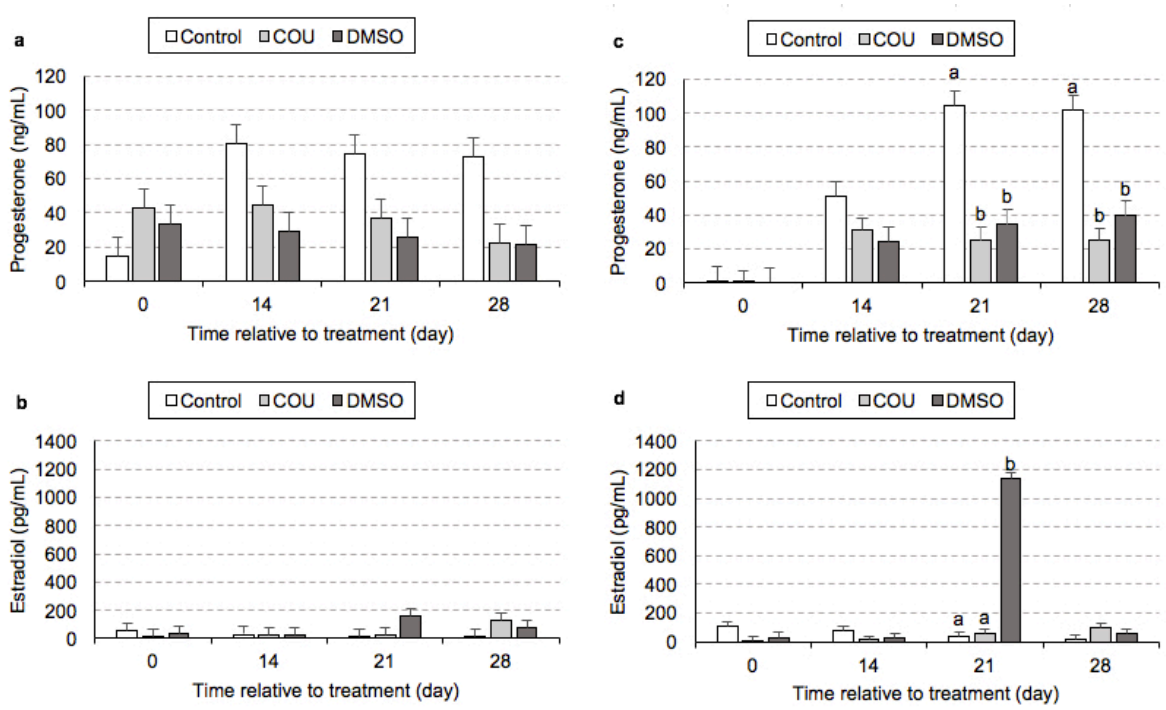

Figure 3. Least square means ± SE of progesterone and estradiol in serum from bitches that had ovulated (A and B) or not (C and D) at the beginning of the study. Bitches were treated (day 0) with coumestrol (COU)*, dimethyl sulfoxide (DMSO), or remained without treatment (Control). [a,b] Unequal letters between treatments and within day denote difference between treatment means ($P<0.05$). * Diluted in dimethyl sulfoxide.

Similar to hormone results, in bitches that had ovulated, COU and DMSO did not have any effects on vaginal cells of any type (Fig.4a). In contrast, in animals that had not ovulated, COU and DMSO reduced the numbers of parabasal cells relative to the controls (Fig.4b). Additionally, COU but not DMSO increased anucleated superficial cells (Fig.4b).

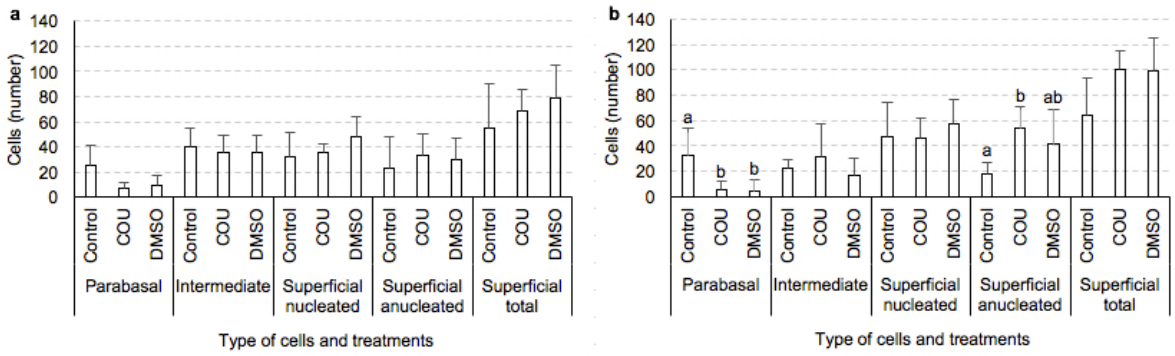

Figure 4. Mean ± SE of the different types of vaginal cells during the first 28 days after application of treatments in untreated bitches (Control) and in coumestrol (COU)*, or dimethyl sulfoxide (DMSO) treated animals. Vaginal cells in bitches that had ovulated (A) or that had not ovulated (B) at treatment time (day 0). Within type of cell, unequal letters indicate difference between treatment means [a, b] ($P<0.05$). * Diluted in dimethyl sulfoxide.

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

Concentrations of P4 and E2 during the interval from the second to the sixth month after treatment did not differ between groups regardless of whether bitches had ovulated or not by day 0 of the study (Fig.5).

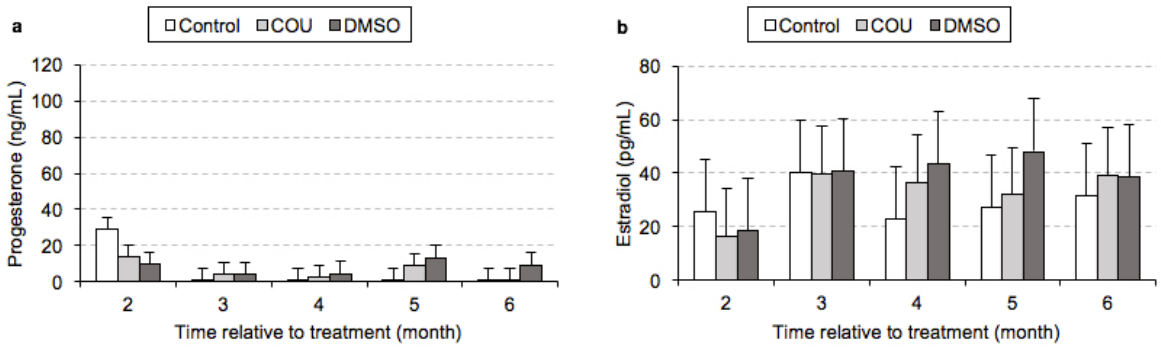

Figure 5. Least square means ± SE of concentrations of progesterone and estradiol from the second to the sixth month after treatments in bitches that were not treated (Control) or that received coumestrol (COU)*, dimethyl sulfoxide (DMSO). * Diluted in dimethyl sulfoxide.

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).

Collateral effects

Relative to the Control group (Fig.6) where all bitches ended their diestrus by the third month and behaved normally, 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 on 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 that bled through vulva did not show signs of estrus.

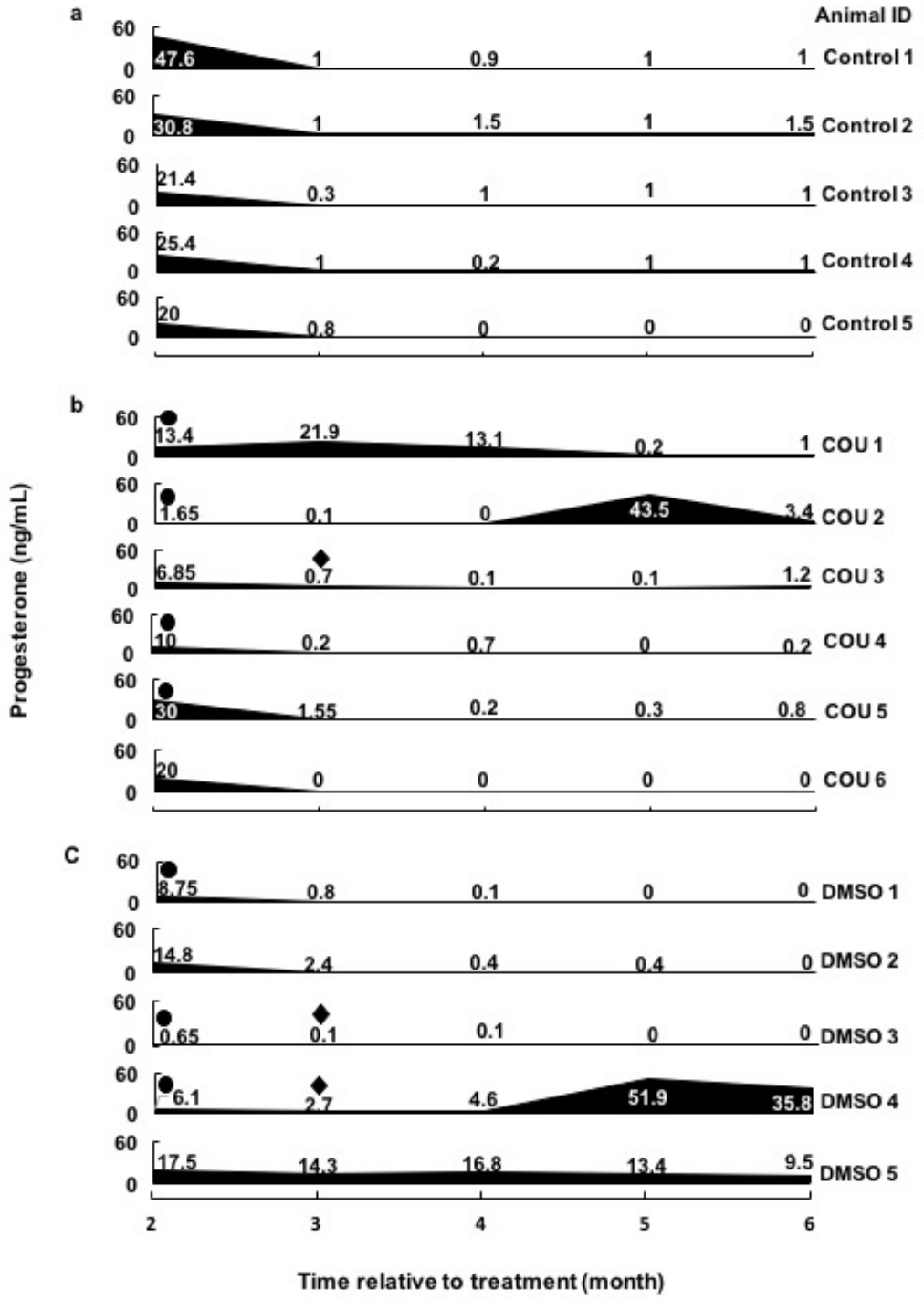

Figure 6. Profiles of progesterone, abnormal vaginal bleeding (•) and mammary gland growth followed by galactorrhea (♦), in individual bitches that remained untreated (a: Control) or were treated with coumestrol (b: COU)* or dimethyl sulfoxide (c: DMSO). Animals were monitored from the second through the sixth month after treatment.* Diluted in dimethyl sulfoxide.

Discussion

Animal health

Based on clinical examinations performed monthly, information of owners, as well as 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 negatively affect the health status of dogs, according to World Health Organization (WHO) “state of complete physical, mental and social well-being and not merely the absence of disease or infirmity” (World Health Organization 2018). To our knowledge, this is the first time that a single oral administration of COU and/or DMSO has been given to bitches; however, four oral applications at weekly intervals of COU diluted in DMSO did not induce any health abnormality in male dogs (Pérez-Rivero et al. 2009). Similarly, one or two intratesticular applications of DMSO with zinc gluconate did not alter body temperature, behavior, or cardiac and respiratory rates of dogs clinically evaluated on a daily basis. (Soto et al. 2009; Vannucchi et al. 2015). Therefore, it is fair to say that COU and DMSO are innocuous to dogs in the doses and frequencies of application used here and the works cited above. In fact, evidence exists that daily ingestion of COU and other phytoestrogens benefits health status in humans (Rietjens et al. 2017; Ungar and Shimoni 2004). Nevertheless, caution must be taken when a higher dose is given and/or a more frequent or prolonged administration of COU is applied, since COU, in addition to reported reproductive adverse consequences, may induce negative side effects such as neural and behavioral alterations in rodents (Whitten and Patisaul 2001). With reference to the influence of DMSO on an animal’s health, there are conflicting results. Several studies in laboratory species indicate the potential beneficial use of DMSO in the treatment of medical disorders such as head and spinal cord injury, stroke, memory dysfunction, ischemic heart disease, nociception, and inflammation (Colucci et al. 2008; Jacob and de la Torre 2009; Lapuente et al. 2013; Nagel et al. 2007). In contrast, other works provide evidence of deleterious effects on animal derived from chronic treatment with DMSO. For example, DMSO causes neural injury in rats and ocular damage in dogs (Hanslick et al. 2009; Noel et al. 1975). Thus, although more thorough studies on the safety of COU and DMSO need to be performed, our data provide evidence in support of those experiments indicating that the cautious use of both substances is of relatively low risk for an animal’s health.

Although no alterations were found in the clinical parameters, and the bitches did not show evidence of disease, in our study some animals treated with COU and/or DMSO displayed an increased mammary gland size during the third month after treatment, independent of the ovarian stage, followed by galactorrhea. Apparently, these effects of COU and DMSO were exerted directly on the mammary gland, because abnormal mammary gland growth has been documented in studies where phytoestrogens were applied to female monkeys (Foth and Cline 1998). Additionally, reports exist that DMSO induces differentiation of cell lines derived from the rat mammary gland (Costlow 1984; Zucchi et al. 2002). Regardless, these undesirable side effects must be taken into consideration because it may preclude the usage of COU, diluted or not in DMSO, as a potential tool to prevent conception in bitches.

Hormones and cytology in Control bitches during the estrous cycle

Variations of P4 and E2 observed in Control bitches throughout the estrous cycle followed patterns previously reported by other authors (Christie and Bell 1971; Concannon 2011; Groppetti et al. 2015; Groppetti et al. 2010; Rota et al. 2007). These data indicate that randomization of animals to different treatments was adequate and that the effects of COU and DMSO could be evaluated without bias. A shortcoming in our study was the impossibility of controlling diets offered to the animals. Because the feeds were extremely variable among bitches and in some cases feeds changed within dogs throughout the duration of the experiment, we were unable to carry out a reliable determination of the phytoestrogen content of the feed. However, samples collected from 24 brands of commercial dog food that contained soy had genistein, daidzein, and glycitein but not COU (Cerundolo et al. 2004). Thus, we assume that COU was not present in the diet of any of the bitches but it is possible that in some cases other phytoestrogens could have been present in feed and influence our results. However, because of the normal variations of P4 and E2 observed in Control bitches, it is unlikely that the assumed presence of phytoestrogens in feeds were high enough to induce abnormalities. The previous statement is possible because only feeds with high contents of isoflavones induced elevated concentrations of E2 in dogs (Cerundolo et al. 2009).

In the available literature, we could not find studies where all types of vaginal cells were quantified and measured in the bitch. In most works, the approach is to facilitate identification of estrus and thus is focused in determining proportions of cell types and changes in superficial cells (Bouchard et al. 1991; Post 1985; Schutte 1967; Wright and Parry 1989).

Morphometric variations of vaginal cells during an estrous cycle have been documented in cats, sheep, and rats (Centola 1978; Clemente et al. 2013; Mills et al. 1979). Thus, the present study recorded the morphometric changes of all types of vaginal cells across an estrous cycle for the first time. However, despite the detailed determination of vaginal cell variations in the present work, when data obtained here are integrated with the VEC and this procedure is used for estrus detection, data are insufficient for a precise estrus determination since some values of any type of vaginal cell found in proestrus overlap with those recorded in estrus. Thus, to confidently identify estrus in the bitch quantification of serum P4 is still required.

Hormones and cytology in Control and treated bitches

In the present study, 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 estrogen receptor (ER) β with the same affinity as E2 and to ERα with 33% lower affinity (Kuiper et al. 1998). Evidence from mammals other than dogs suggests that COU exerts effects in the hypothalamus, adenohypophysis, and ovary, as well as other tissues containing α and β receptors to E2 such as the uterus, and vaginal epithelium (Ferreira-Dias et al. 2013; McGarvey et al. 2001; Montgomery et al. 1985; Muñoz et al. 2002; Newsome and Kitts 1980; Pocock et al. 2002; Smith et al. 1979). Data from the present study agree with the effects of COU cited above, because they show that a single oral administration of COU exerts an estrogenic effect on the vaginal epithelium and the ovary of bitches; those effects included decreased number of parabasal cells, increased number of anucleated superficial cells, and a reduction in the serum concentrations of P4. It was determined that DMSO as vehicle modulates the receptor-mediated and non-receptor-mediated estrogenic responses and significantly induced expression of the StAR protein and P450scc in both the brain and head kidney in salmon (Lyssimachou and Arukwe 2007; Lyssimachou et al. 2006). Moreover, DMSO initiates the differentiation of granulosa cells from chicken pre-ovulatory follicles (Morley and Whitfield 1993).

According to the reported effects of DMSO in fish and birds, DMSO given orally to bitches in our study exerted estrogenic actions on vaginal cells and ovary because it decreased the number of parabasal cells, increased circulating concentrations of E2, and reduced levels of peripheral P4.

To our knowledge, this is the first time that the estrogenic effects of DMSO in mammals are reported. Independently of their mechanism of action of COU and/or DMSO, this study indicates that the effects of both substances depend on the ovulatory condition of the animal.

Considering the results reported here, it is feasible that DMSO used as a vehicle masks or enhances effects of COU. DMSO is a substance often used as an excipient for COU; however, this and a previous study performed in rats demonstrated that DMSO has estrogenic properties (Burroughs et al. 1985). Therefore, it is convenient to reevaluate the influence that DMSO could have had on the effects attributed to COU in studies carried out in female bats, rats, mice and male dogs (Burroughs et al. 1990; McGarvey et al. 2001; Pérez-Rivero et al. 2009; Serrano et al. 2007) in which DMSO was used as an excipient for COU. Similarly, the present data suggest that the effects of COU diluted in an estrogenically inert carrier on the reproduction of mammals in general, and of bitches in particular, need to be reevaluated.

Our long-term purpose is to develop a biscuit or kibble impregnated with a substance capable of preventing pregnancies in bitches. In many countries, owned dogs are allowed to roam freely outside their owner's property for at least a part of the day (Hiby and Hiby 2017), contributing to increase canine overpopulation. At least in this subpopulation of dogs, the administration of a single oral contraceptive by owners could help to maintain under control natality of unwanted dogs.

Estrus in the bitch occurs in response to the decline in circulating concentrations of E2 with a simultaneous increase in P4 (Concannon 2011). Results of this study indicate that both COU and DMSO are estrogenic in bitches during an estrogen susceptible stage and that both compounds act within the first-month after their administration. In addition, between 2 and 6 months both compounds elicited disarrangements of the estrous cycle in some bitches. These findings are promising and we guarantee to explore in a near future the effects of biscuits impregnated by COU or DMSO on the fertility of bitches in different stages of the estrous cycle.

Conclusions

Periodical clinical examinations, as well as chemical and blood cell tests, realized six months after treatments, did not show any abnormality in the experimental animals; thus, our first conclusion is that 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, our second conclusion is that 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 have estrogenic effects in vaginal tissue and the ovarian functions studied here; however, their actions depend on the ovulatory condition of animals.

Conflict of interest statement

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Capítulo VI. Efecto del coumestrol y dimetil sufóxido sobre los niveles circulantes de estradiol, progesterona y los tipos de células epiteliales vaginales en perras en anestro.

Effect of a single application of coumestrol diluted in dimethyl sulfoxide and dimethyl sulfoxide alone on serum sex hormones levels and vaginal cytology of bitches during anestrus.

Running title: Coumestrol and/or DMSO on E2, P4, and vaginal cells in dogs.

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Abstract

Canine overpopulation is a public health problem, despite the existence of strategies for controlling dog populations. Coumestrol (COU) imitates estrogenic actions and alters reproduction in mammals. Commonly, COU is dissolved in dimethyl sulfoxide (DMSO); however, evidence indicates that DMSO is not inert. Our aim was to determine effects of a single oral administration of COU diluted in DMSO or DMSO-alone, on serum progesterone (P4) and estradiol (E2), vaginal cells, and length of anestrus and diestrus in bitches in anestrus. Fifteen bitches in anestrus received either a single biscuit (Control, n=5), a biscuit with 600 µg of COU/kg diluted in 20 µL of DMSO (COU, n=5), or a biscuit with 20 µL of DMSO (DMSO, n=5). Circulating P4, E2 and vaginal cells, were assessed within the first month, and only the hormones from the second to the sixth month post-treatment. Health of animals was monitored by clinical examinations. Statistical analysis was by GLM for repeated measures. COU only increased serum E2, but DMSO increased serum P4, anucleated superficial cells, and diestrus length. All dogs remained healthy, but mammary gland growth occurred in two COU and in one DMSO-treated bitches. In anestrous bitches, COU has estrogenic effect on hormones and inert in vaginal cells. Contrastingly, DMSO is estrogenic regarding vaginal tissue but antiestrogenic in hormones and diestrus length. Health of bitches is not altered, but COU and/or DMSO induce mammary growth and/or galactorrhea. Present data reveal that effects of COU need to be reevaluated in bitches and perhaps in other mammals.

Key words: Coumestrol, dimethyl sulfoxide, estradiol, progesterone, vaginal cells, bitches.

Introduction

During approximately 15,000 years dogs have had a close and beneficial relationship with humans.[1,2] However, numbers of these animals have increase steadily worldwide turned into a public health problem particularly in underdeveloped countries.[3,4]

Strategies available to control and handle canine overpopulation are insufficient to effectively control the canine overpopulation because they have economic, ethical or social constraints, and/or adverse side-effects on dogs' health.[5,6] Thus, it is convenient to use alternatives procedures that help us to control canine reproduction.

The bitch is the only domestic animal that has an anestrus that lasts between two and three months, as part of the estrous cycle. During early and medium anestrus, hypothalamus and ovaries are relatively inactive but in late anestrus, increase the expression of genes encoding for estrogen receptors, and the P450 aromatase that catalyzes estrogen biosynthesis in the canine hypothalamus, in preparation for the beginning of a new estrous cycle.[7] Estradiol (E2) and progesterone (P4) have a fundamental role in the periodicity of the estrous cycles in bitches.[8]

Thus, molecules as coumestrol (COU) that have the capacity to bind and activate estrogenic receptors (ER) α and β, influence or imitate the estrogenic actions, inducing proliferation of cells in uterine, and vaginal epithelia, and reduce fertility in mares, cows, female bats, ewes, and rats.[9-14]

In mice, bitch, and male dogs, the use of COU has been proposed as a tool for controlling reproduction.[15-17] To facilitate the administration of COU is common to use dimethyl sulfoxide (DMSO) as a diluent; however, DMSO may influence reproduction in mammals because it modulates estrogenic responses in hepatocytes of fish,[18,19] and induces differentiation in cell lines of mammary gland from rat.[20] Moreover, in a previous study, we reported that COU and/or DMSO acted as estrogenic compounds when administered to bitches that were in the periovulatory stage,[15] by altering their peripheral levels of P4 and E2, and increasing the cornified vaginal cells. However, to the best of our knowledge, no studies have been done in which COU and/or DMSO were applied to bitches during a non-estrogenically susceptible stage such as anestrus.

Considering the previous statements, our hypothesis was that in bitches in anestrus, COU and DMSO act at ovarian and vaginal levels by increasing concentrations of circulating E2, decreasing peripheral levels of P4, and altering the populations of vaginal cells without affecting the duration of diestrus and anestrus; therefore, our objective was to evaluate the effects of a single oral administration of COU diluted in DMSO and DMSO-alone on concentrations of serum P4 and E2, numbers and proportions of vaginal epithelial cells, as well as the duration of diestrus and anestrus in bitches in anestrus. A collateral objective was to clinically assess the effects of the drugs mentioned above on the health status of the experimental dogs.

In this work we proved for the first time that DMSO alone exerts estrogenic effects in anestrous bitches. Therefore, estrogenic actions attributed to COU here and in earlier reports in which COU was diluted in DMSO, should be reevaluated.

Materials and methods

Ethical review

All the procedures in this study were approved by The Institutional Subcommittee for Care and Usage of Animals in Experimentation of the College of Veterinary Medicine and Animal Husbandry, National Autonomous University of Mexico, according to the Mexican Official Regulation NOM-062-ZOO-1999, under protocol number DC-2015 / 2-10. The owners of the dogs included in this research provided a written consent for their dogs' participation in the study.

Animals and general management

We used fifteen bitches that remained throughout the study at their owner's house. Animals were fed with commercial dry dog food (kibbles) of different brands, complemented in some cases with leftovers from the owner's meals and had free access to water. In all cases, housing was adequate and the owners took care of animals. All bitches had a health card and history of periodic visits to a veterinary center. Animals had been treated against internal and external parasites, and had been vaccinated against leptospira, distemper, adenovirus, parvovirus, parainfluenza, and rabies.

At the beginning of the trial and during each visit to the animals, an accredited veterinarian confirmed the adequate health status of all dogs by clinical examination that included: a) Physiological constants (heart rate, respiratory rate, and rectal temperature); b) Respiratory system (absence of abnormal nasal discharge, coughing, and dyspnoea); c) Integumentary system (absence of: skin lesions, abnormality of hair cover, external parasites, mucous pallor or cyanosis); d) Bones and skeletal muscle system (normal standing and walking); and e) Ocular examination: Tests of hand motion, light perception, palpebral blink reflex, corneal sensitivity, and absence of: Endo or exophthalmoses, signs of nasolacrimal or lacrimal puncta obstruction, strabismus, asymmetry, conjunctivitis, tumors and change of iris color. Additionally, at the beginning of the trial a complete blood cells test[21] was performed in which all dogs had values within normal ranges of: Hematocrit, hemoglobin, red blood cells, mean corpuscular volume, mean corpuscular hemoglobin, reticulocites count, total protein, leukocytes count, neutrophils and neutrophils on bound counts, myelocytes and metamyelocytes counts, monocytes count and basophiles count. At least during the previous six months, illness had not been diagnosed in any of the dogs and they had not received antibacterial medication. Bitches were intact, of small-to-medium size breeds (Poodle, 5; Schnauzer, 3; Pug, 2; Yorkshire, 1; mongrel, 4), were (mean ± SD) 4.53 ± 1.80 years old, weighed 8 ± 4.29 kg, and had been in estrus at least once. At the beginning of the experiment, bitches were in anestrus which was defined as follows: between two and three months after detection of estrus by owners, and exfoliative vaginal cytology (EVC) corresponding to anestrus.[22]

Treatments

Animals were randomly assigned to one of the following treatments: 1) Control group (n=5), in which bitches received a single biscuit (Pedigree®) with no additives at the beginning of the study (day 0); 2) COU-treated group (n=5) that received a single biscuit impregnated with COU (600 µg/kg of body weight; Sigma Chemical Co. St. Louis, Mo, USA) diluted in DMSO (20 µL per 600 µg of COU; Sigma Chemical Co. St. Louis, Mo, USA, 99.9% purity); and 3) DMSO-treated group (n=5), that received a biscuit impregnated with DMSO (20 µL/kg of body weight). After weighting each bitch, we deposited in the center of a biscuit with a micropipette of 10-100 µL, the adequate amount of either coumestrol diluted in DMSO or DMSO-alone, and then we waited (between 10 and 15 min) until compounds were absorbed by each biscuit. We also confirmed that all dogs ate the corresponding biscuit completely.

The dose of COU that we used was previously tested by Gualo in male dogs.[23] The criteria for selecting the dose of DMSO was the minimum quantity of the drug required for diluting 600 µg of COU without the need of vortexing or heating the preparation, and that was within the range approved by Food and Drug Administration (FDA) for dogs.[24] The dose of DMSO that met the cited criteria was 20 µL.

Biological samples

Hormones

On days 0, 14, 21, and 28, and thereafter every 30 days for a 6-month period, blood samples were taken by venipuncture from a cephalic vein; serum was obtained and used for quantification of P4 and E2 by immune-enzymatic assays (Enzyme-Linked Immune-Absorbent Assay, DGR Instruments, GmbH, Germany). The inter-assay rank was 10.6 to 2000 pg/mL for E2 and 0.01 to 40 ng/mL for P4.

EVC

On days 0, 7, 14, 21 and 28 relative to treatments, a vaginal smear was taken from the caudal portion of the vagina; cotton swabs containing vaginal cells were rolled over a slide, fixed (Citospray, CTR Scientific, Mexico), and stained using a modified Papanicolaou procedure.[25] The modification consisted of the substitution of saline solution by distilled water to preserve erythrocytes. These samples were used for EVC. Parabasal, intermediate, and superficial (nucleated and anucleated) vaginal cells were counted using optical microscopy (ten fields per animal, randomly chosen/smear, 40x objective, microscope Axio Scope.A1, Zeiss, Mexico).

Anestrus and diestrus duration

The owners were trained and then asked to report signs of estrous. Based on their observations and concentrations of P4 in serum, we defined diestrus as follows: the dogs did not show signs of estrus and P4 was sustained for at least two months > 2.5 ng/ml. Thus, the beginning of diestrus was the day after estrus when first serum sample contained P4 > 2.5 ng/mL, and was followed by samples with those P4 levels for at least two months. The end of diestrus was the day when P4 was < 2.5 ng/mL in at least two consecutive samples. We considered 2.5 ng/ml as the limit between diestrus and anestrus because 2 ng/mL of P4 was the highest concentration found during anestrus in bitches[26]; besides in the P4 assays performed here, 2.5 ng differed ($P<0.05$, regression analysis) from 2.0 ng of P4 in the standard curves.

Anestrus began one day after the end of diestrus, and was considered as finished when the owners observed bleeding through vulva for the first time after treatments.

Design and statistical analysis

A completely randomized design for repeated measures over time was used. Data relative to E2, P4, and vaginal cells during the first 28 days and hormone levels from the second to the sixth month after application of treatments were analyzed by analysis of variance, using PROC GLM [SAS, version 9.3; SAS Ins. Inc., Cary, NC, USA]. The statistical model included: treatment, animal (treatment), period, and treatment by period; the Tukey-Kramer test was used for specific contrasts. To examine anestrus and diestrus duration, data were analyzed through analysis of variance, using GLM [SPSS Statistics, 24 version IBM, USA]; in this case, the model included treatment and animal, and Bonferroni test was used for specific contrasts.

Results and discussion

Hormones

Relative to the control group, in animals receiving COU, concentrations of circulating P4 did not vary in any of the samples collected during the first 28 days after treatment (Figure 1A). In contrast, in a previous work[15] COU induced a marked reduction of P4 on days 21 and 28 post-treatment in bitches that were in a periovulatory stage. In the present work, P4 was at basal level according to the anestrous phase of the dogs were, thus a further decrease would be undetectable, but anyhow the effects of COU on P4 levels differ between periovulatory and anestrous dogs. Thus, it is possible that COU actions on P4 are dependent on the stage of the estrous cycle when they are given to bitches. To our knowledge, no other experiments have been published in which COU has been given to bitches, however in other animal models, actions of COU are dependent as in our study of the physiological stage; for example, in a works realized *in vitro* with luteal cells from pregnant cows, COU did not affect P4 secretion[27] but increased the secretion of P4 from luteal cells obtained on days [16–19] of the estrous cycle, and decreased P4 secretion from luteal cells collected on days [11–15] of cycle.[28]

In this study COU induced an increment of peripheral E2 on days 21 and 28 after treatment (Figure 1B). A contrasting effect was recorded in bitches treated equally during the periovulatory stage, in which COU was unable to increase E2.[15] As mentioned above, no other works have been published about effects of COU in bitches. However, the dycotomic effect of COU was also observed in different animal models and hormones.

For example, COU given orally to ovariectomized ewes decreased the amplitude of pulses of luteinizing hormone during the breeding but not during the anestrous season.[29] Similarly, in cows and heifers COU increased secretion of E2 from granulosa cells in ovarian follicles <1 cm of diameter but in larger follicles no effects were detected.[28]

In the present work, animals from the DMSO group, showed an increment (P=0.0038) in serum concentrations of P4 on day 21 after treatment (Figure 1A).

Consequently, DMSO exerts a progestogenic effect in anestrus bitches but that action was not observed in periovulatory dogs, in which DMSO given orally in the same dose reported here, reduced significantly the circulating concentrations of P4 from day 21 to 28 post-treatment15; therefore, as in the case of COU, this action of DMSO on P4 seems to depend on the stage of the estrous cycle, phenomenon that to our knowledge has not been reported in any other mammal.

We observed that DMSO did not alter the peripheral concentrations of E2 during the first 28 days post-treatment (Figure 1B). However, in periovulatory bitches, the same dose of DMSO offered orally to bitches,[15] increased circulating E2 on day 21 after treatment. A said above, to our knowledge no other studies have been published about effects of DMSO on E2 in any other mammals; however, evidence exists that E2 receptors (ER) mediate at least some of the effects of DMSO. For example, DMSO stimulates ERα and ERβ in salmon hepatocytes,[19] as well as StAR and P450scc proteins in rat brain and kidney.[30] Apparently, from the present result and others,[15] DMSO seems to affect E2 differently, depending on the reproductive stage of the bitches.

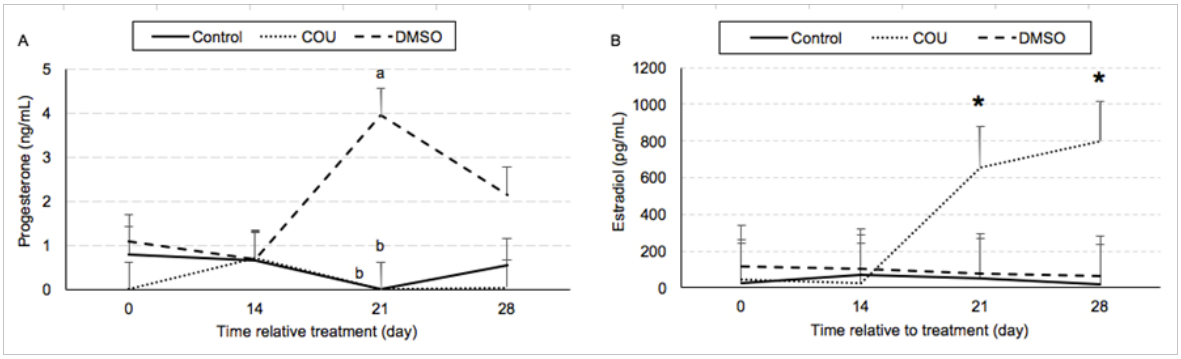

Figure 1. Least square means ± standard error of progesterone (A) and estradiol (B) in serum from bitches that received a biscuit alone (Control; n=5), a biscuit with coumestrol diluted in dimethyl sulfoxide (COU; n=5), or a biscuit with dimethyl sulfoxide alone (DMSO; n=5). [a,b] Within sampling day, unequal letters indicate difference between treatments (P<0.05). *Indicates increment on days 21 (P<0.0048) and 28 (0.0008).

In this work, concentrations of P4 and E2 during the interval from the second to the sixth month after treatment did not differ between groups (Table 1). Because the influence of COU and DMSO treatments on these hormones was first detected on day 21 after their application and no effects were observed afterward, manifestation of COU and DMSO effects is delayed but relatively short, because only were detected on days 21 and/or 28 post application.

This idea is credible because our observations are similar to those recorded in periovulatory dogs[15] that received the same treatments than those applied here, and in rats, in which exogenous E2 induced changes in uterus and vagina 10 day after the administration.[31] In the case of COU, this delayed but short activity is probably due to the interaction between COU and the ER.[32]

There is no evidence about binding of DMSO to ERs but as it was mentioned previously, DMSO stimulates the concentration of ERα and ERβ in salmon hepatocytes,[19] thus it is possible that DMSO acts directly or indirectly through the E2 pathway.

Table 1. Peripheral concentrations of progesterone and estradiol in bitches during the interval between the second and the sixth month after treatment.

		Month after treatment				
Hormone	Group	2	3	4	5	6
P4 (ng/mL)	Control	9.4 ± 4.9	39.7 ± 23.4	50.9 ± 29.2	24.1 ± 12.9	5.3 ± 5.0
	COU	15.5 ± 6.5	6.8 ± 2.8	18.3 ± 11.2	3.3 ± 1.4	0.3 ± 0.1
	DMSO	25.3 ± 19.0	18.8 ± 10.8	3.4 ± 1.2	52.2 ± 24.2	3.1 ± 1.3
E2 (pg/mL)	Control	33.8 ± 15.1	38.5 ± 17.3	45.1 ± 20.2	53.9 ± 24.1	39.1 ± 17.5
	COU	14.8 ± 5.3	52.8 ± 15.5	49.5 ± 5.3	42.8 ± 8.1	34.2 ± 4.4
	DMSO	246.7 ± 196.4	39.5 ± 25.6	12.8 ± 1.5	72.2 ± 34.6	68.6 ± 34.8

Mean ± standard error of progesterone (P4), and estradiol (E2) concentrations of bitches given a biscuit only (Control, n=5); one biscuit with coumestrol dissolved in dimethyl sulfoxide (COU, n=5) and one biscuit with dimethyl sulfoxide alone (DMSO, n=5).

Vaginal cells

In this work, COU did not affect proportions (Table 2) or numbers (Figure 2) of any of the vaginal cells studied here, whereas in a previous experiment carried out in periovulatory animals,[15] COU reduced numbers of parabasal cells and increased superficial anucleated cells. As in the case of hormones, comparison of this and a previous work realized in bitches[15] allow to think that perhaps COU actions on vaginal cells depend on the stage of the estrous cycle.

The different response of the vaginal epithelium to a similar dose of COU due to stage of the estrus cycle detected in dogs here and elsewhere,[15] may not be similar in other species. For example, in rodents COU and other phytoestrogens induce cornification of vaginal epithelium independent of the reproductive status and age; while in women, variations of the vaginal epithelium in response to phytoestrogen consumption are attributed to the dose of phytoestrogens consumed instead of the reproductive condition.[33]

In the present study, DMSO enhanced proportions (P=0.007) of superficial nucleated cells only on day 28 post-treatment (Table 2). Similarly, numbers of superficial nucleated cells during the 28 days that followed the treatment administration were higher (P=0.056) in animals treated with DMSO than in the other groups (Figure 2).

Despite the increments in serum P4 and that circulating E2 did not change, DMSO showed an estrogenic effect because it increased proportions of nucleated superficial cells in bitches that were in anestrus, but in bitches that were in periovulatory stage,[15] DMSO enhanced numbers of parabasal cells; consequently, as in the case of P4 and E2, actions of DMSO in vaginal epithelium vary according to the stage of the estrous cycle when this substance is administered to dogs.

Diestrus and anestrus duration

No difference was found in the duration of anestrus among groups (Figure 3); however, in the same figure is noticed than diestrus was longer in DMSO treated animals relative to the Control (P=0.060) and COU bitches (P=0.018). DMSO but not COU prolonged diestrus by an unknown mechanism. However, because DMSO increased circulating P4 on day 21 post-treatment, and cystic bitches34 show relatively high peripheral concentrations of P4, it may have induce ovarian cysts and prolonged diestrus.

Health and collateral effects

At the beginning of the study all bitches were in normal health status according to the clinical examination; besides all blood parameters from all bitches were within reference values. During the six months after treatments administration, no alterations were recorded with respect to animal health. During the same interval, all animals in the Control group (Figure 4) showed a profile of P4 as should be expected in their reproductive condition, and no abnormalities were detected in their behavior.

Table 2. Proportion of vaginal cells of bitches treated with coumestrol or dimethyl sulfoxide.

Day	Group	Vaginal cells			
		Parabasal	Intermediate	Superficial	
				Nucleated	Anucleated
0	Control	46.4 ± 5.6	21.6 ± 1.4	13.5 ± 2.1	18.5 ± 3.8
	COU	50.1 ± 3.4	24.1 ± 5.6	8.6 ± 1.0	17.6 ± 6.1
	DMSO	45.4 ± 1.6	32.8 ± 6.5	18.4 ± 5.8	4.5 ± 1.7
7	Control	41.1 ± 2.2	32.6 ± 1.5	10.1 ± 0.4	16.2 ± 2.5
	COU	45.8 ± 1.1	20.3 ± 7.0	13.7 ± 1.7	20.6 ± 7.0
	DMSO	49.1 ± 3.1	30.9 ± 5.3	17.4 ± 4.6	5.1 ± 2
14	Control	54.3 ± 5.1	23.7 ± 3.1	9.8 ± 3.5	12.1 ± 2.4
	COU	47.0 ± 1.7	22.5 ± 7.7	13.1 ± 2.2	18.2 ± 9.0
	DMSO	52.8 ± 5.0	29.1 ± 4.8	16.4 ± 3.9	5.7 ± 2.4
21	Control	49.5 ± 5.0	30.4 ± 6.6	12.3 ± 4.3	8.8 ± 7.9
	COU	48.9 ± 1.5	24.6 ± 6.7	1 6.6 ± 2.8	10.9 ± 5.6
	DMSO	50.8 ± 6.8	23.2 ± 6.8	26.8 ± 7.8	5.6 ± 3.5
28	Control	51.1 ± 4.7	28.1 ± 4.0	9.9 ± 4.1[a]	10.8 ± 1.6
	COU	49.5 ± 3.7	20.6 ± 5.9	13.3 ± 4.6[a]	17.1 ± 5.2
	DMSO	45.3 ± 1.3	20.0 ± 6.1	30.4 ± 4.3[b]	6.3 ± 4.5

[a, b] Within sampling day, unequal letters indicate difference between means (P=0.007).
Proportions ± standard error of the different types of vaginal cells of bitches given a biscuit only (Control, n=5); with coumestrol dissolved in dimethyl sulfoxide (COU, n=5), and with dimethyl sulfoxide alone (DMSO, n=5).

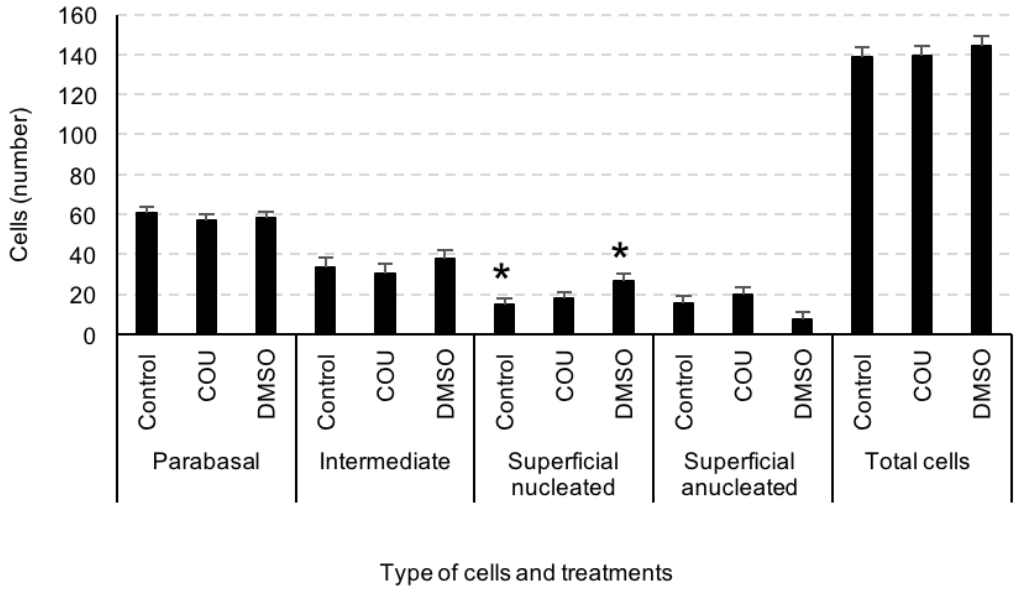

Figure 2. Mean ± standard error of the number of the different types of vaginal cells during the first 28 days after bitches received a biscuit alone (Control, n=5), with coumestrol dissolved in dimethyl sulfoxide (COU, n=5), or with dimethyl sulfoxide alone (DMSO, n=5). Within the type of cell, means sharing an asterisk differ (P=0.056).

In contrast during the third month, two out of five COU-treated animals, showed mammary gland growth and galactorrhea that lasted about 15 days (bitches identified as COU-3 and COU-5; Figure 4). Furthermore, in the DMSO group, one out of five animals (bitch DMSO-4) showed galactorrhea with no apparent mammary growth in the sixth month, and one animal (DMSO-5) had serum concentrations of P4 ≥ 3.7 ng/mL during at least six months. During the periodical clinical examinations, we did not detect health-related abnormalities in any of the animals under study. Our observation coincides with a previous work in which bitches in periovulatory stage treated like the animals studied here, did not show signs of disease based on monthly clinical examinations as well as in chemical and blood cell tests realized six months after treatments.[15] Thus, it is apparent that single oral applications of COU and/or DMSO do not affect negatively the health status of bitches. The side effects of phytoestrogens in mammary gland cited above have been documented before in ovariectomized macaques,[35] and reports indicate that DMSO induces differentiation of cell lines derived from rat mammary gland.[20] Thus it must be expected that some bitches will show mammary gland and/or galactorrhea when treated with COU and/or DMSO.

This undesirable side effect must be considered when using these drugs in an attempt to control reproduction in bitches. We do not consider that development of mammary gland and galactorrhea are signs of disease because the use of estrogenic substances accompanied with P4 is frequent to induce lactations in infertile ruminants,[36-38] in non-human primates[39] and in human surrogate pregnancies.[40] Furthermore, once the first case of galactorrhea was reported by owners, we recommended them to look for behavioral changes typical of pseudopregnancy, such as circling, digging or nesting.[26] However no signs were observed and two bitches of the COU group (bitches COU 3 and 5, Figure 4), and one of the DMSO group (bitch DMSO 4, Figure 4) that showed mammary development and galactorrhea had low serum concentrations of P4, when it was established long ago that this condition occurs in bitches that have relatively high concentrations of peripheral P4;[41] thus it is unlikely that either COU diluted in DMSO, or DMSO-alone in the dose and frequency used here induce pseudopregnancy. Despite that we and others did not observe negative effects of COU and/or DMSO on health, caution must be taken if their application is more frequent, or their dose is higher than here, because evidence shows that prolonged administration of COU in rodents and humans,14 and DMSO in dogs, pigs and rodents[42] induce health alterations.

Integrated discussion

What would be the mechanisms of action of COU and why it shows dichotomic effects? These questions cannot be answered fully, but most of the actions of phytoestrogens are trough the classic mechanisms of E2, which are mediated by two distinct intracellular estrogen receptors (ER): ERα and ERβ which show different distribution in corporal tissues. ERα is predominant in cells of mammary gland, uterus and vagina; whereas ERβ primarily distributes in ovary and hypothalamus.[32] Because phytoestrogens have lower affinity than E2-17β to the ERs, they may exert mild estrogenic actions, but as E2 itself, phytoestrogens may evoke positive or negative responses in some tissues such as the hypothalamus and, in occasions, even act as an anti-estrogenic agent.[32] In addition, phytoestrogens act trough mechanisms that are not mediated by ER,[43] thus the actions of COU, at the cellular and molecular level, are influenced by many factors including but not limited to: COU dose, ER status, degree of exposition of target tissues to endogenous E2, and type of target tissue or cell. Summarizing, stage of the estrus cycle among other factors, determines that COU act as an agonist or antagonist of E2 in biologic models,

both in vivo and in vitro;[44,45] and even COU may induce responses unrelated to the known actions of E2.[32]

A shortcoming in our study was the impossibility of controlling diets offered to the animals. Because the feeds were extremely variable among bitches and, in some cases, feeds changed within dogs throughout the duration of the experiment, we were unable to carry out a reliable determination of the phytoestrogen content in feeds. However, samples collected from 24 brands of commercial dog food that contained soy had genistein, daidzein, and glycitein but not COU.[46]

Thus, we assume that COU was not present in the diet of any of the bitches but it is possible that in some cases other phytoestrogens could have been present in feed and influence our results. However, because of the normal variations of P4 and E2, as well of the dynamic variations of vaginal cells observed in Control bitches, it is unlikely that the assumed presence of phytoestrogens in feeds were high enough to induce abnormalities or bias our data.

DMSO is often used as a diluent for COU but in this and in earlier studies,[15,47] DMSO show that is estrogenic. Therefore, it is convenient to reevaluate the influence that DMSO have on the effects attributed to COU in multiple studies carried out on mammals, such as bats, mice, and dogs, in which DMSO was the diluent for COU,[11,16,47] and to investigate the mechanisms about how DMSO influence reproduction.

The original idea of testing COU as a potential alternative for controlling dogs' population has merit. However, besides the need to determine effects of COU without the interference of DMSO, our findings relative to absence of effects of the phytoestrogen in diestrus duration, along with the negative side effects observed in 40% (2/5) of the bitches that received COU, hinder the potential of developing a product based on this substance for controlling dogs' populations. In relation to DMSO, we produced evidence that is an endocrine disruptor in bitches, but despite that it prolonged diestrus, and showed estrogenic effects in vaginal cells, it was progestogenic and induced galactorrhea in 20% of the dogs; thus it seems not viable as a tool for controlling dogs' population.

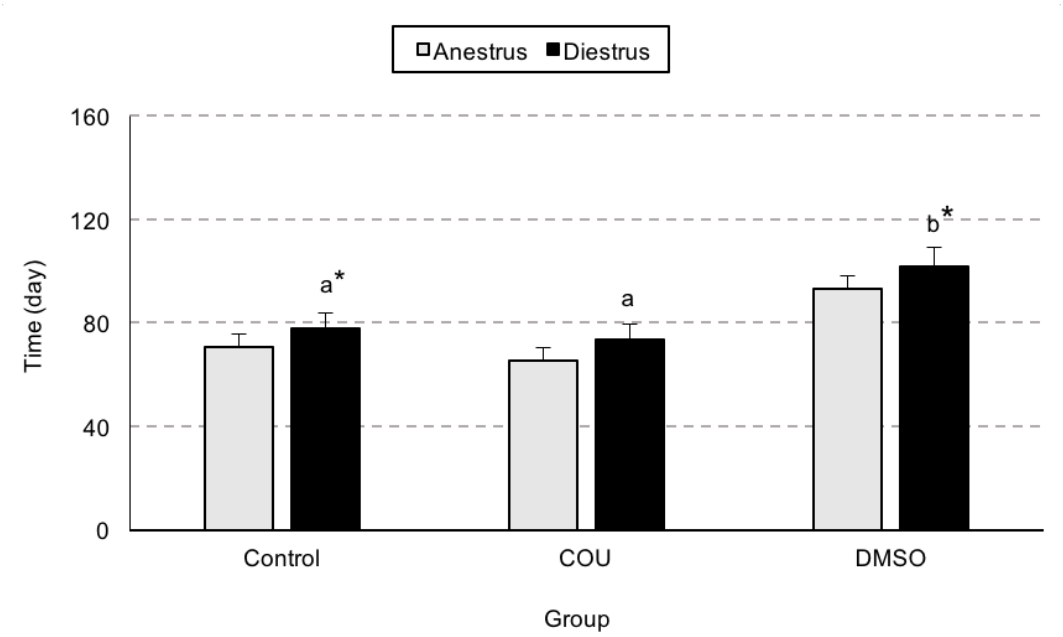

Figure 3. Least square means ± standard error of duration of anestrus and diestrus of bitches that received a biscuit alone (Control, n=5), a biscuit with coumestrol dissolved in dimethyl sulfoxide (COU, n=5), or a biscuit with dimethyl sulfoxide alone (DMSO, n=5). [a, b] Unequal letters indicate difference between treatments (P=0.018), Within groups, means sharing an asterisk differ (P=0.060).

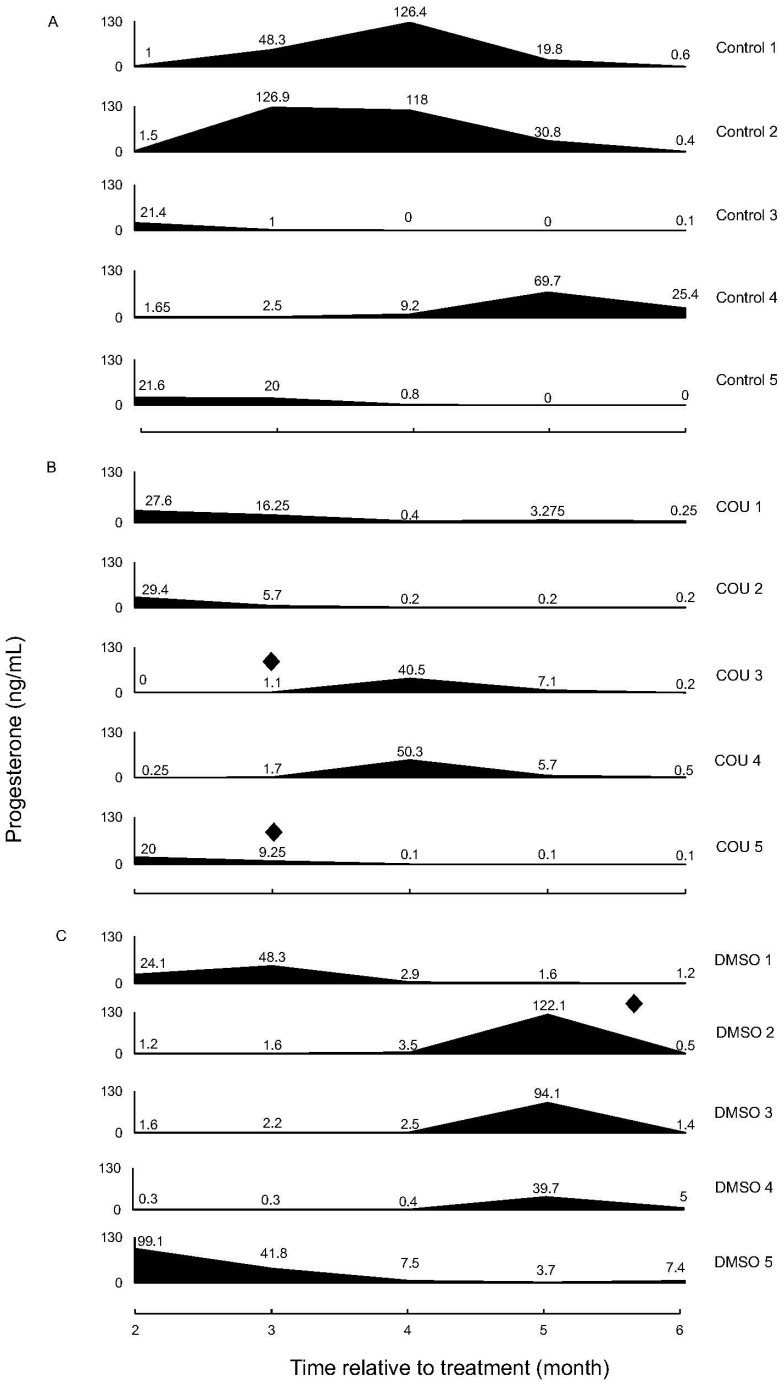

Figure 4. Profiles of serum progesterone and abnormal growth of mammary gland and galactorrhea (♦) in individual bitches that remained untreated (A: Control; n=5) or were treated with coumestrol diluted in dimethyl sulfoxide (B: COU; n=5), or dimethyl sulfoxide alone (C: DMSO; n=5) during the second to the sixth month after administration of treatments.

Conclusions and Repercussions

COU only increased serum E2, but DMSO increased anucleated superficial cells, serum P4, and diestrus length. All dogs remained healthy, but mammary gland growth accompanied or not of milk flow occurred in two COU and in one DMSO-treated bitches; thus, our conclusions are: 1) In anestrous bitches, COU is estrogenic on hormones and inert in vaginal cells; in contrast, DMSO is estrogenic on vaginal tissues but antiestrogenic on the circulating hormones examined here, and on diestrus length. 2) The health of bitches is not altered, but COU and/or DMSO induce mammary growth and/or galactorrhea. The major contribution of this article is the demonstration of the profound effect that a single oral administration of DMSO exerts in reproduction of bitches, indicating the convenience of reevaluating the action attributed to COU diluted in DMSO in this and previous reports on reproduction of bitches and perhaps other mammals.

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Competing interests

None of the authors has a personal or financial relationships with other organizations or people that could influence or bias the content of the paper.

Author contributors

SP-C. Contributed to the original design, conducted and coordinated the trial, collected data and biological samples, and performed the laboratory analysis of hormones and vaginal cells, statistical analysis of data, and writing the first and subsequent drafts of the article. PL, AS, and GM. Handled animals, helped collecting data and biological samples. MV. Periodical clinical examination of animals. EM and DV. Interpreted data and performed a critical evaluation of the first draft of the article. HS. Conceived the initial design of the study. AV-G. Re-designed the study, critical revision of different drafts of the article, writing, and approval of the final version of the article. All authors read and approved the final manuscript.

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Capítulo VII. Discusión general

En la presente tesis, se evaluó el efecto de una aplicación de COU y/o DMSO sobre los posibles efectos secundarios del COU y DMSO sobre las variables clínicas de perras en etapa periovular y en anestro a través de exámenes físicos mensuales, información proporcionada por los propietarios acerca de signos y síntomas de enfermedad (durante y después del experimento), y a través de la valoración de parámetros bioquímicos y hemograma en sangre (estos exámenes clínicos, se realizaron en todas las hembras al inicio del estudio y por falta de presupuesto, sólo en las perras tratadas en etapa PO al finalizar el seguimiento). De acuerdo a los resultados obtenidos, en ningún caso se encontraron alteraciones en las variables mencionadas. De hecho, a casi tres años de la administración de los tratamientos, todas las perras cuentan con buen estado de salud.

La información disponible acerca del efecto de COU y DMSO en la salud de caninos es limitada y contradictoria. En éstos animales, se ha reportado que el DMSO provoca miopía y cambios en el humor vítreo cuando se administra al menos 18 meses vía intragástrica cinco veces a la semana (Noel et al, 1975). En contraste, con una o dos aplicaciones intratesticulares de DMSO con gluconato de zinc no se observaron cambios negativos en temperatura, y tasas cardiaca y respiratoria (Soto et al, 2009; Vannucchi et al, 2015).

Hasta donde la literatura nos permite conocer, no se ha evaluado la toxicidad de COU en perros, sin embargo, se ha visto que la administración semanal de dicho fitoestrógeno por cuatro semanas, no provoca alteraciones en su salud (Pérez-Rivero et al, 2009b).

Dada la información anterior, es necesario realizar estudios exhaustivos sobre la seguridad de COU y DMSO, pues la dosis y la duración de exposición de los mismos, son fundamentales para provocar o no, efectos en la salud animal. Los resultados del presente trabajo, indican que una aplicación de COU, con DMSO como su vehículo, o de DMSO sólo, pueden tener un riesgo relativamente bajo, cuando se administra una vez en perras en etapa periovular o en anestro. Sin embargo, se necesitan otras pruebas farmacológicas especificas para afirmarlo.

Los efectos de COU y/o DMSO sobre los perfiles hormonales y las células del epitelio vaginal, dependieron de la fase estral en que se administraron. En las perras que ya habían ovulado cuando se administró el tratamiento no se observaron alteraciones.

En contraste, en aquellas hembras en las que no se había dado la ovulación al comienzo del experimento, las concentraciones de P4 disminuyeron en los grupos COU y DMSO, y las de estradiol aumentaron en el grupo DMSO dentro del primer mes pos administración. En los animales tratados en anestro, el DMSO provocó aumento de P4 y de la cantidad de células vaginales nucleadas, también, dentro de los primeros 28 días con respecto al inicio del experimento.

Los presentes resultados sugieren que ambos tratamientos son estrogénicos si se administran en perras estrogénicamente susceptibles. En contraste, cuando las hembras no están endocrinamente activas no hay efecto del tratamiento COU en hormonas y células vaginales, por su parte, el DMSO tiene efecto progestagénico con respecto a los niveles hormonales, y estrogénico en el epitelio vaginal. Estos hallazgos apoyan reportes previos en donde se menciona que la actividad estrogénica y/o antiestrogénica de los fitoestroestrógenos es determinada por la proporción FE/estrógenos (Adams, 1995). Así como también aquellos trabajos en donde se ha documentado que el DMSO tiene efecto sobre moléculas clave para la regulación de la esteroidogénesis y parámetros reproductivos (Burroughs et al, 1985; Lyssimachou y Arukwe, 2007; Lyssimachou et al, 2006; Mortensen y Arukwe, 2006; Sigma Aldrich, 2017b).

En cuanto a los resultados observados a largo plazo, no se encontraron alteraciones en las concentraciones hormonales evaluadas mensualmente por grupo, aunque si se observaron alteraciones individuales. Por ejemplo, se reportó crecimiento mamario con lactogénesis y lactopoyesis anormal al tercer mes pos administración independientemente de la etapa estral en la que fueron tratadas (1 y 2 perras del grupo COU y DMSO respectivamente tratadas en etapa PO y 2 animales del grupo COU tratadas en AN). El aumento en glándula mamaria se ha documentado en estudios con fitoestrógenos en primates no humanos y perras (Cline et al, 2001; Foth y Cline, 1998; McClain et al, 2005). Además, se ha reportado el efecto de DMSO sobre la adquisición de diferenciación de glándula mamaria (Costlow, 1984; Zucchi et al, 2002). Debido a que ambos compuestos actúan sobre la mama, no se le puede atribuir únicamente el efecto colateral al COU o al DMSO. Por otra parte, se observó que en dos hembras tratadas en etapa PO (DMSO 4 y DMSO 5), las concentraciones de P4 fueron anormales, pues después de la administración de DMSO se mantuvieron arriba de 2.5 pg/mL por 11 y 6 meses respectivamente (Anexo 4), por lo que la duración de diestro fue muy larga con respecto a los animales Control, además en ese tiempo no se observaron signos de celo.

En las perras tratadas en anestro con DMSO, el periodo de diestro pos administración también fue mayor con respecto a las hembras control. La influencia de DMSO sobre células ováricas ya está documentado, pues se ha reportado que dicho vehículo inicia la diferenciación de células de granulosa de folículos preovulatorios de gallina (Morley y Whitfield, 1993). Lamentablemente fue imposible hacer el seguimiento por al menos 12 meses de todas las hembras tratadas, pues por diversas razones de los propietarios, se tuvo que limitar el tiempo a seis meses.

Los resultados revisados anteriormente con respecto a los efectos colaterales que se documentaron, muestran la necesidad de hacer estudios enfocados a conocer el mecanismo de acción de cada compuesto de manera individual, sobre la glándula mamaria y el ovario de la perra. Además, considerando los resultados en la presente tesis, es posible que con la administración de COU con DMSO como excipiente, algunos efectos del FE sean enmascarados, por lo que es conveniente reevaluar la influencia del DMSO sobre múltiples estudios en los cuales se ha utilizado este vehículo.

El metabolismo de COU y de DMSO en la perra no está estudiado. Sin embargo, está documentado en humanos que genisteína y daidzeína llegan a la concentración sérica máxima de 6 - 8.4 y de 6 - 7.4 horas con una vida media de excreción de 3.8 - 8.4 y de 2.9 - 6 horas respectivamente dependiendo de la fuente de alimentación (Whitten y Patisaul, 2001). Los efectos inhibitorios de COU (0.4 o 1.6 mg) sobre la concentración de LH se reportaron a las 8.5 horas después de su administración intravenosa en ratas (McGarvey et al, 2001). Por su parte, el DMSO se absorbe rápidamente cuando se administra de forma aguda, pues hay reportes en donde la concentración sérica máxima de DMSO se da después de cuatro horas de su administración oral en humanos (700 o 1000 µg/kg), detectándose DMSO y su metabolito dimetilsulfona ($DMSO_2$) hasta 120 y de 48-400 horas pos administración respectivamente (Hucker et al, 1967).

La respuesta relativamente tardía de ambos tratamientos sobre las variables estudiadas en la presente tesis, y el metabolismo rápido de ambos compuestos, hacen suponer que tanto el COU como el DMSO actúan desencadenando a mediano plazo una respuesta de desfasamiento hormonal en los ciclos estrales, el mecanismo de acción sobre el eje hipotálamo-hipófisis-gónada, no está reportado en la perra. Sin embargo en borregas se ha observado que el COU interfiere con la relación entre los ovarios y la hipofisis, provocando una reducción de gonadotropinas (Smith, 1979), y se une a RE en la hipófisis en una manera similar a los RE en útero (Newsome, 1980).

Además de que es necesario dilucidar el mecanismo de acción de COU en la perra, también hay que explorar a fondo la participación de ambos compuestos en las alteraciones inducidas por exposición aguda, ya que la mayoría de los trabajos realizados son por periodos relativamente prolongados, y dosis en rangos de los mg, por lo que es de importancia, evaluar otras dosis y tiempo de exposición con el fin de describir parámetros farmacológicos importantes para el uso de COU como anticonceptivo viable para las perras, específicamente en la fase PO en donde se encontró un mayor efecto de los tratamientos, pues a juzgar por los resultados, inducir un desfase de esta etapa estral, podría ser viable para el uso de un anticonceptivo de corta duración y de efecto inmediato.

Capítulo VIII. Conclusiónes generales y repercusiones

No se documentaron alteraciones en los examenes físicos, análisis clínicos, e información (relacionada con signos reproductivos) proporcionada por los propietarios después de la administración oral de un biscuit impregnado con COU y/o DMSO en todas las perras de ambos experimentos, en consecuencia, la primera conclusión es que una dosis por vía oral de dichos compuestos no afecta la salud de las hembras, independientemente de su estado estral en que sea aplicado el tratamiento.

Con respecto al Control y durante los primeros 28 días relativo a la administración del tratamiento, los niveles de P4 disminuyeron en las hembras de ambos grupos experimentales (COU y DMSO) y los de E2 aumentaron en las perras del grupo DMSO; en cuanto a las células vaginales, en los grupos COU y DMSO la cantidad de parabasales disminuyó, y en el grupo COU aumentó el número de anucleadas. Los resultados anteriores, corresponden a hembras que no habían ovulado cuando se aplicó el tratamiento, pues en las que ya se había dado la ovulación no se observaron cambios en ninguna variable. En las perras tratadas en anestro, la administración de COU con DMSO no afectó los niveles hormonales ni la cantidad de tipos celulares del epitelio vaginal, en contraste, el tratamiento con DMSO, provocó aumento en P4 y de las células nucleadas. Dadas estas evidencias, la segunda conclusión es que el efecto de COU y/o DMSO sobre las concentraciones hormonales y las células del epitelio vaginal está condicionado a la fase estral en que se administran y se da dentro de los primeros 28 días después de su aplicación. El COU con DMSO y el DMSO sólo, tienen efecto estrogénico en los niveles hormonales y en el epitelio vaginal cuando se administra a perras susceptibles a estrógenos (antes de la ovulación), e inertes en un ambiente endocrino rico en P4 (después de ovular). Por otro lado, la administración de COU con DMSO, no provocó alteraciones, y el DMSO tuvo efecto progestagénico con respecto a las concentraciones hormonales y estrogénico relativo al epitelio vaginal, cuando se administra en perras en anestro

Con respecto a la administración del biscuit, e independiente del estado ovárico de las perras, en el tercer mes, algunas de ellas mostraron lactogénesis y lactopoyesis con duración de alrededor de 15 días, regresando las mamas a su tamaño normal después de este tiempo. La duración del diestro postratamiento en las hembras en Anestro tratadas con DMSO, fue más largo con respecto a los grupos COU y Control. Por lo tanto, la tercera conclusión es que el COU y el DMSO pueden producir efectos colaterales en la glándula mamaria a largo plazo, y el DMSO aumenta la duración del diestro, si se administra en periodo de anestro.

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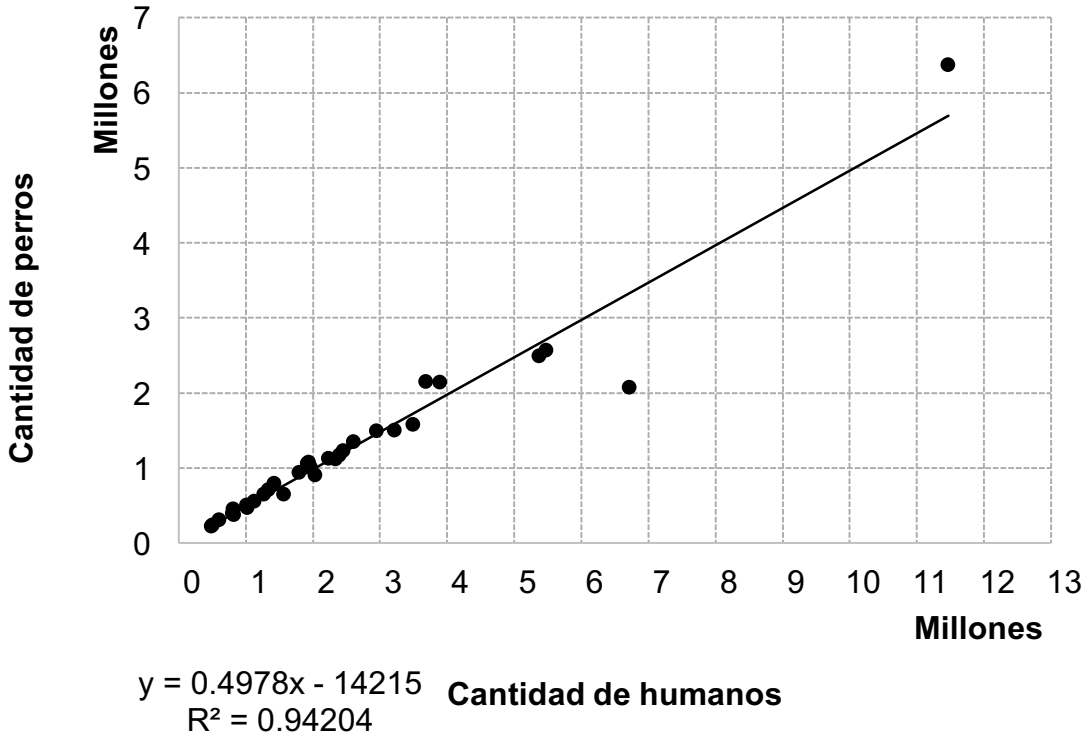
Anexos

Estimación de la cantidad de canes en los estados de la República Mexcana

Entidad	Perros domiciliados[1]	Personas encuestadas[1]	Porcentaje de perros[2]	Tasa de crecimiento poblacional humano (2015)[1]	Población humana		Perros
					2015[1]	2018[3]	2018[4]
Aguascalientes	375, 839	822, 385	45.70	0.022	1, 312, 544	1, 401, 091	640, 314
Baja California	1, 120, 504	2, 339, 725	47.89	0.013	3, 315, 766	3, 446, 769	1, 650, 672
Baja California Sur	241, 278	505, 675	47.71	0.026	712, 029	769, 023	366, 932
Campeche	311, 916	600, 735	51.92	0.020	899, 931	955, 013	495, 866
Ciudad de México	2, 076, 519	6, 710, 885	30.94	0.003	8, 918, 653	8, 999, 161	2, 784, 570
Chihuahua	1, 234, 076	2, 456, 393	50.24	0.010	3, 556, 574	3, 664, 341	1, 840, 942
Chiapas	1, 506, 882	3, 214, 921	46.87	0.018	5, 217, 908	5, 504, 777	2, 580, 172
Coahuila	1, 080, 223	1, 938, 207	55.73	0.016	2, 954, 915	3, 099, 032	1, 727, 187
Colima	221, 572	486, 847	45.51	0.020	711, 235	754, 768	343, 507
Durango	553, 731	1, 125, 135	49.21	0.016	1, 754, 754	1, 840, 337	905, 715
Estado de México	6, 368, 923	11, 465, 861	55.55	0.014	16, 187, 608	16, 877, 050	9, 374, 667
Guerrero	1, 131, 931	2, 236, 791	50.61	0.009	3, 533, 251	3, 629, 509	1, 836, 718
Guanajuato	2, 149, 494	3, 686, 697	58.30	0.014	5, 853, 677	6, 102, 989	3, 558, 291
Hidalgo	1, 061, 449	1, 921, 135	55.25	0.015	2, 858, 359	2, 988, 924	1, 651, 415
Jalisco	2, 493, 686	5, 372, 207	46.42	0.015	7, 844, 830	8, 203, 169	3, 807, 770
Michoacán	1, 493, 062	2, 949, 073	50.63	0.012	4, 584, 471	4, 751, 500	2, 405, 598
Morelos	648, 904	1, 269, 050	51.13	0.016	1, 903, 811	1, 996, 663	1, 020, 955
Nayarit	393, 330	801, 487	49.08	0.019	1, 181, 050	1, 249, 657	613, 270
Nuevo León	1, 575, 650	3, 490, 323	45.14	0.021	5, 119, 504	5, 448, 853	2, 459, 797
Oaxaca	1, 350,219	2, 602, 561	51.88	0.009	3, 967, 889	4, 075, 989	2, 114, 639
Puebla	2, 140, 753	3, 888, 939	55.05	0.014	6, 168, 883	6, 431, 620	3, 540, 429
Querétaro	713, 994	1, 334, 553	53.50	0.024	2, 038, 372	2, 188, 685	1, 170, 960
Quintana Roo	467, 400	1, 022, 566	45.71	0.027	1, 501, 562	1, 626, 501	743, 450
Sinaloa	906, 236	2, 028, 978	44.66	0.015	2, 966,321	3, 101, 817	1, 385, 416
San Luis Potosí	943, 710	1, 790, 748	52.70	0.011	2, 717, 820	2, 808, 498	1, 480, 056
Sonora	1, 004, 647	1, 965, 689	51.11	0.016	2, 850, 330	2, 989, 346	1, 527, 830
Tabasco	651, 810	1, 563, 092	41.70	0.015	2, 395, 272	2, 504, 684	1, 044, 454
Tamaulipas	1, 170, 097	2, 394, 776	48.86	0.012	3, 441, 698	3, 567, 091	1, 742, 895
Tlaxcala	455, 822	815, 926	55.87	0.018	1, 272, 847	1, 342, 825	750, 178
Veracruz	2, 572, 747	5, 474, 401	47.00	0.013	8, 112, 505	8, 433, 023	3, 963, 180
Yucatán	797, 872	1, 419, 824	56.20	0.015	2, 097, 175	2, 192, 970	1, 232, 343
Zacatecas	502, 897	1, 007, 575	49.91	0.013	1, 579, 209	1, 641, 602	819, 350
						Total	61,579,539

[1] INEGI, 2018.
[2] Perros domiciliados/total de personas encuestadas.
[3] 1+tasa de crecimiento(2015)*Población humana en 2017 (obtenida con la misma fórmula respecto al año anterior).
[4] Proporción(^{2}) del año 2018.

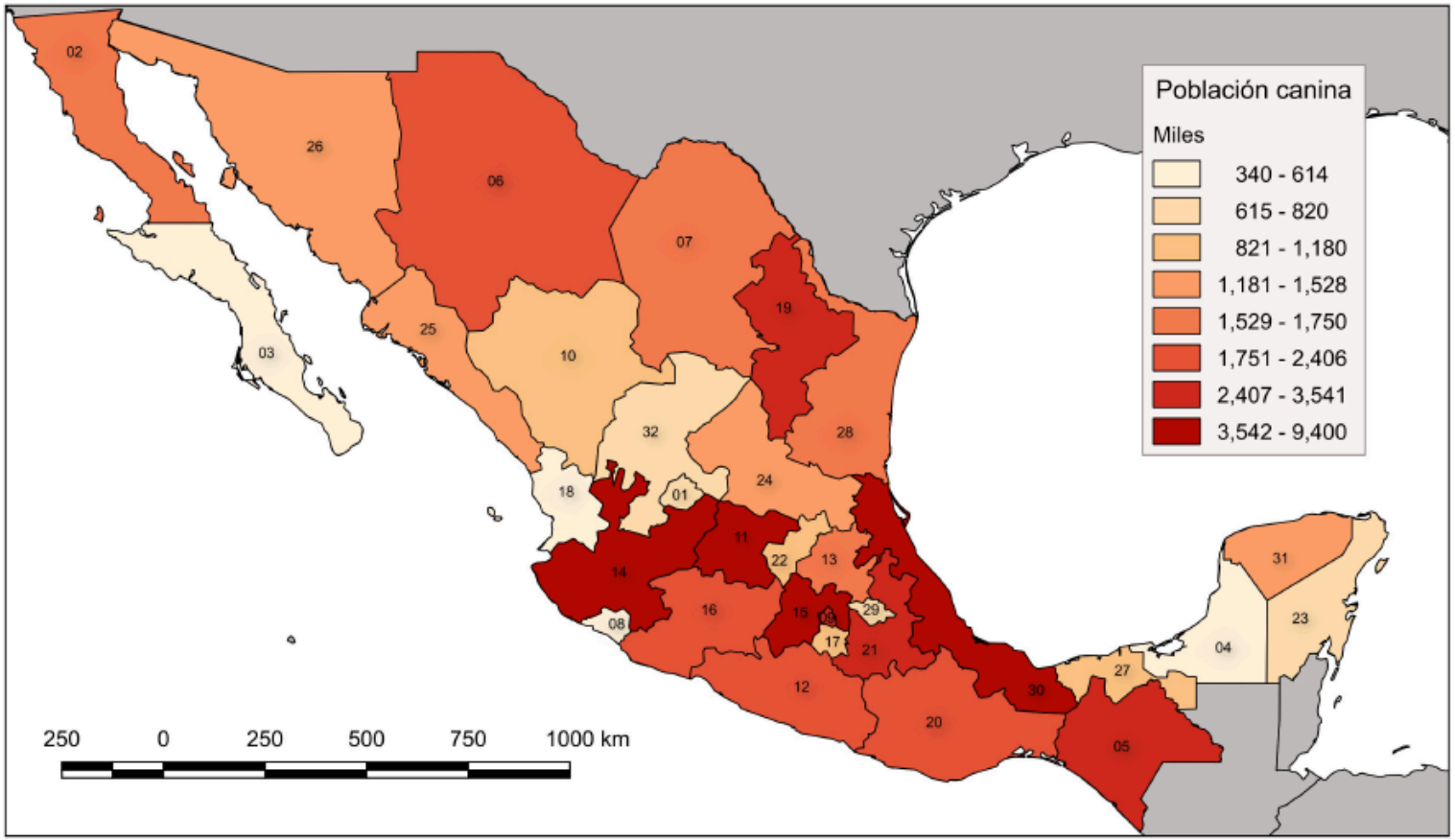
Anexo 1



Correlación entre la cantidad de perros y el total de la población encuestada en el año 2014 por INEGI.

Anexo 2

Estimación de la cantidad de perros domiciliados por estado de la República Mexicana en el 2018



Código, estado y estimación del número de perros domiciliados en México en el 2018

01 Aguascalientes	640,314	08 Colima	343,507	15 Edo. México	9,374,667	22 Querétaro	1,170,960	29 Tlaxcala	750,178
02 Baja California	1,650,672	09 CDMX	2,784,570	16 Michoacán	2,405,598	23 Quintana Roo	743,450	30 Veracruz	3,963,180
03 Baja California Sur	366,932	10 Durango	905,715	17 Morelos	1,020,955	24 San Luis Potosí	1,480,056	31 Yucatán	1,232,343
04 Campeche	495,866	11 Guanajuato	3,558,291	18 Nayarit	613,270	25 Sinaloa	1,385,416	32 Zacatecas	819,350
05 Chiapas	2,580,172	12 Guerrero	1,836,718	19 Nuevo León	2,459,797	26 Sonora	1,527,830		
06 Chihuahua	1,840,942	13 Hidalgo	1,651,415	20 Oaxaca	2,114,639	27 Tabasco	1,044,454	Total: 61,579,539	
07 Coahuila	1,727,187	14 Jalisco	3,807,770	21 Puebla	3,540,429	28 Tamaulipas	1,742,895		

Estimación de la población de perros domiciliados (al menos uno por casa) por estado en México para el 2018, con base en datos la encuesta: “Bienestar subjetivo en México” realizada por INEGI en el año 2014.

Anexo 3

Morfología y fase estral de predominancia de los tipos celulares encontrados a lo largo del ciclo estral de la perra

Tipos de células	Caracteristicas morfólogicas	Fase estral de predominancia
Parabasales	Redondas, núcleo grande y poco citoplasma	**Anestro:** células parabasales e intermedias, no hay eritrocitos, pueden haber o no neutrófilos
Intermedias	Varían en tamaño, núcleo vesicular, bordes celulares redondos y/o poligonales	**Proestro:** en etapas tempranas, se observan parabasales e intermedias, sin neutrófilos y con eritrocitos.
Superficiales nucleadas	Más grandes identificadas, citoplasma angular afilado, núcleo pequeño y picnótico.	**Diestro:** pocas células superficiales, abundantes intermedias, parabasales, y abundantes neutrófilos
Superficiales anucleadas	Grandes, sin núcleo, de bordes irregulares, se les conoce también como cornificadas	**Estro**: se observan células superficiales (98-100%), puede haber o no eritrocitos.

Referencias: Christie y Bell, 1971; Hernández et al, 1999; Lacruz y Fariña, 2008; Post, 1985; Wright y Parry, 1989

Anexo 4

Perfiles individuales de progesterona (ng/mL) de algunas perras en estado periovular (PO) y en anestro (AN), después de seis meses de la administración de COU+DMSO (grupo COU) y/o DMSO (grupo DMSO).

		Mes					
Fase estral	**ID**	**7**	**8**	**9**	**10**	**11**	**12**
PO	COU 1	-	-	-	-	-	-
	COU 2	0.5	0	1.1	44.2	2.7	0.7
	COU 3	27.8	9.9	0.5	0.2	0.2	24.4
	COU 4	2.8	30.7	7.6	0.2	0.3	0.9
	COU 5	0.4	31	20.1	1.3	0.5	0.3
	COU 6	0.1	76.4	19.2	3.4	0.3	0.5
	DMSO 1	53.4	13.1	1.9	-	-	-
	DMSO 2	0	0	58.1	42.1	6.3	2.2
	DMSO 3	0	34.4	36.1	-	-	-
	DMSO 4	**87.7**	**24**	**7.2**	**18**	**13.4**	**17.7**
	DMSO 5	**8.7**	**8.7**	-	-	-	-
AN	COU 1	**-**	**-**	**-**	**-**	**-**	**-**
	COU 2	0.2	38	6.9	0.6	0.2	0.9
	COU 3	0.1	0.5	0.3	11.9	25.9	3.8
	COU 4	0.4	0.2	1.1	11.8	26	0
	COU 5	26.3	5.25	3.9	0.4	0.3	10.2