ANTENOR PEREIRA BONFIM NETO

O papel do 17β -estradiol no processo luteolítico de cadelas não prenhes

São Paulo

2014

ANTENOR PEREIRA BONFIM NETO

O papel do 17 β -estradiol no processo luteolítico de cadelas não prenhes

Dissertação apresentada ao Programa de Pós-Graduação em Anatomia dos Animais Domésticos e Silvestres da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do Título de Mestre em Ciências

Departamento:

Cirurgia

Área de concentração:

Anatomia dos Animais Domésticos e Silvestres

Orientador (a):

Profa. Dra. Paula de Carvalho Papa

São Paulo

2014

Autorizo a reprodução parcial ou total desta obra, para fins acadêmicos, desde que citada a fonte.

DADOS INTERNACIONAIS DE CATALOGAÇÃO-NA-PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da
Universidade de São Paulo)

T.3012
FMVZ

Bonfim Neto, Antenor Pereira
O papel do 17β -estradiol no processo luteolítico de cadelas não prenhes / Antenor Pereira Bonfim
Neto. -- 2014.
52 f. : il.

Dissertação (Mestrado) - Universidade de São Paulo. Faculdade de Medicina Veterinária e
Zootecnia. Departamento de Cirurgia, São Paulo, 2014.

Programa de Pós-Graduação: Anatomia dos Animais Domésticos e Silvestres.

Área de concentração: Anatomia dos Animais Domésticos e Silvestres.

Orientador: Prof^ª. Dr^ª. Paula de Carvalho Papa.

1. Corpo lúteo. 2. Diestro. 3. Estradiol. 4. Luteólise. 5. Cadela. I. Título.

CERTIFICADO DA COMISSÃO DE ÉTICA

Comissão de Ética no uso de animais

CERTIFICADO

Certificamos que o Projeto intitulado "O papel do 17 β -estradiol no processo luteolítico de cadelas não prenhes", protocolado sob o nº 2592/2012, utilizando 28 (vinte e oito) cães, sob a responsabilidade do(a) Profa. Dra. Paula de Carvalho Papa, está de acordo com os princípios éticos de experimentação animal da "Comissão de Ética no uso de animais" da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião de 11/4/2012.

We certify that the Research "The role of 17 β -estradiol in the luteolytic process of non-pregnant bitches", protocol number 2592/2012, utilizing 28 (twenty eighth) dogs, under the responsibility Profa. Dra. Paula de Carvalho Papa, agree with Ethical Principles in Animal Research adopted by "Ethic Committee in the use of animals" of the School of Veterinary Medicine and Animal Science of University of São Paulo and was approved in the meeting of day 4/11/2012.

São Paulo, 12 de abril de 2012.

Denise Tabacchi Fantoni
Presidente

Av. Prof. Dr. Orlando Marques de Paiva, nº87
Cidade Universitária "Armando de Salles Oliveira"
São Paulo/SP – Brasil
05508-270

Fone: +55 11 30917671 /7676
Fax: + 55 11 3032-2224
E-mail: fmvz@usp.br
<http://www.fmvz.usp.br>

FOLHA DE AVALIAÇÃO

Autor: BONFIM NETO, Antenor Pereira

Título: O papel do 17 β -estradiol no processo luteolítico de cadelas não prenhes.

Dissertação apresentada ao Programa de Pós-Graduação
em Anatomia dos Animais Domésticos e Silvestres da
Faculdade de Medicina Veterinária e Zootecnia da
Universidade de São Paulo para obtenção do Título de
Mestre em Ciências

Data: ____ / ____ / ____

Banca Examinadora

Prof(a). Dr(a): _____

Instituição: _____ Julgamento: _____

Prof(a). Dr(a): _____

Instituição: _____ Julgamento: _____

Prof(a). Dr(a): _____

Instituição: _____ Julgamento: _____

DEDICATÓRIA

*A Deus,
Aos meus pais Gilda e Jackson
À minha irmã Francimary
e aos meus sobrinhos Rafael e Vinícius
firmando-me com orgulho e alegria no caminho
que escolhi.*

AGRADECIMENTOS

Durante essa jornada, muitas pessoas entraram e passaram a fazer parte de minha vida, colaborando direta ou indiretamente com minha formação pessoal e com a conclusão deste trabalho. Por esta razão, deixo aqui registrada minha sincera gratidão.

Agradeço a FAPESP pela bolsa fornecida durante todo este período, que foi essencial para desenvolvimento desta dissertação.

Agradeço à minha orientada Dra. Paula de Carvalho Papa, não só pela paciência, orientação, aprendizagem e contribuição em meu desenvolvimento profissional, mas principalmente pela confiança depositada e oportunidade oferecida desde o início do mestrado.

Aos meus queridos companheiros e ex-companheiros do LEME, com os quais acabei passando mais tempo que com minha própria família e que alguns acabaram se tornando verdadeiros amigos. Cada qual com seu jeito peculiar com os quais sempre tive o que aprender. Agradeço pelo convívio, pelas risadas e momentos de descontração, pelos apuros, sofrimentos, preocupações, apoio científico e emocional.

Ao Rafael Magdanelo Leandro e ao Thiago Senna Di Vincenzo por nos ajudarem com as castrações, o que foi primordial para o término deste trabalho. E a Ana Beatriz Isola Fonseca por ter disponibilizado o espaço em sua clínica para a realização das cirurgias.

Agradeço a Anabela e toda a sua equipe do CCZ de Guarulhos por colaborarem com este trabalho nos cedendo o

material biológico para os testes, nos ajudando na coleta de sangue e pela alegria e bom humor com que sempre nos receberam.

A Pós-Graduação em Anatomia dos Animais Domésticos e Silvestres, da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, sob Coordenação da Profa. Dra. Maria Angélica Miglino, por ter disponibilizado a oportunidade de participar como aluno, desenvolvendo meu projeto com todo apoio.

A todos os meus amigos que fiz dentro da pós-graduação, que ouviram desabaços, compartilharam risadas e participaram dessa jornada.

Aos meus inúmeros outros amigos, pelo enorme apoio ao ver meu esforço, me incentivando a sempre continuar nesse caminho.

Ao meu melhor amigo e companheiro Fernando, pela companhia de todos os dias, pela força, pelo incentivo, por fazer essa jornada mais leve, e por todo o carinho que me deu mesmo quando eu não merecia.

A toda minha família pelo incentivo e apoio nessa etapa da minha vida. Vocês são muito importantes para mim.

E especialmente aos meus pais, que mesmo não entendendo muito bem o que faço e a carreira científica que decidi seguir, nunca deixaram de me incentivar e apoiar. Obrigado por me fazerem ser o que sou hoje, pelas palavras, carinho, conselhos, amor e pelo colo que sempre estava a minha espera quando eu precisava. Minha admiração por vocês é imensa, obrigado por serem meu porto seguro! Amo vocês!

AGRADECIMENTOS ESPECIAIS

À minha eterna orientadora Renata de Britto Mari por ter me mostrado o caminho da pesquisa ainda na graduação, por ter me ensinado os valores que hoje eu prezo tanto, sei que sem a participação dela eu não conseguiria ter realizado esse sonho, você é o meu exemplo, quero ser uma “Renata” quando crescer.

As minhas amigas Gabriela Pacheco Mendes e Luciana Alves, vocês sabem o papel de vocês nesse trabalho, obrigado por toda a ajuda e aprendizado. Nenhum parágrafo pode agradecer vocês da forma que vocês me ajudaram, fica aqui meu eterno agradecimento, o papel de vocês nessa pesquisa foi essencial.

“Se você quer praticar o mal, a ciência pode lhe prover as mais poderosas armas; mas igualmente, se você deseja fazer o bem, a ciência também lhe põe nas mãos as mais poderosas ferramentas.”

Richard Dawkins

RESUMO

BONFIM NETO, A. P. **O papel do 17 β -estradiol no processo luteolítico de cadelas não prenhes.** [The role of 17 β -estradiol in the luteolytic process of non-pregnant bitches]. 2014. 52 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2014.

O 17 β -estradiol (E2) desempenha um papel importante na função reprodutora e na fertilidade feminina, porém, sua ação é estendida para a maioria dos tecidos. Sabendo que o E2 tem funções pleiotrópicas em diferentes tecidos e órgãos, e que pode estar envolvido tanto na proliferação como na morte celular, nossa hipótese é que o E2 seja um dos iniciadores de regressão luteínica em cadelas não prenhes. Para testar nossa hipótese, foram usados corpos lúteos (CL) provenientes de 28 cadelas nos dias 10, 20, 30, 40, 50, 60, 70 e >70 após a ovulação (n = 4/grupo) para os experimentos ex vivo. Nesta etapa foram analisadas as proteínas pró-apoptóticas (CASPASES, 3, 8, 9 e BAX) por imuno-histoquímica e western blotting, além da expressão dos genes *CASP3*, 8, 9, *BAX*, *FAS*, *MKI67*, *ESR1*, *ESR2* por PCR em tempo real. Na segunda etapa deste trabalho, utilizamos CL de 12 cadelas nos dias 20, 40 e 60 após a ovulação (n=4 por grupo), cujas células foram cultivadas e divididas em seis tratamentos: Controle, E2 (tratado com E2), bloqueador de ER α (tratado com MPP), bloqueador de ER β (tratado com PHTPP), E2 + bloqueador de ER α (tratado com E2 + MPP) e E2 + bloqueador de ER β (tratado com E2 + PHTPP). Foram avaliados os mesmos genes do experimento ex vivo, bem como os genes *CYP19A1*, *CYP11A1*, *HSD3B1* e *SLC2A4*. De modo geral a expressão dos fatores pró-apoptóticos foi mais alta a partir do dia 40 e atingiu valores máximos nos dias 60 e 70 após a ovulação, assim como a expressão de *ERS2*. Essa correlação foi observada também nas células do grupo E2 + bloqueador de ER α , que também apresentaram regulação negativa de *HSD3B1*. Quando do bloqueio do ER β , as células luteínicas responderam com aumento dos genes relacionados à esteroidogênese e à proliferação celular, principalmente quando oriundas dos dias 20 e 40 p.o. Dessa forma, conclui-se que o bloqueio do ER α levou ao aumento dos genes pró-apoptóticos, e o bloqueio do ER β possibilitou aumento dos genes luteotróficos. Estes achados confirmam o papel pleiotrópico do estradiol no CL canino e incluem este hormônio, assim como o balanço entre seus receptores, dentre os atores principais da regulação da meia vida do CL canino.

Palavras-chave: Corpo lúteo. Diestro. Estradiol. Luteólise. Cadela.

ABSTRACT

BONFIM NETO, A. P. **The role of 17 β -estradiol in the luteolytic process of non-pregnant bitches.** [O papel do 17 β -estradiol no processo luteolítico de cadelas não prenhes]. 2014. 52 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2014.

The 17 β -estradiol (E2) plays an important role in the reproductive function and female fertility, however, its action is extended to most tissues. Knowing that E2 has pleiotropic roles in different organs and tissues, and that might be involved in both cell death and proliferation, our hypothesis is that E2 is one of the triggers of luteal regression in non-pregnant bitches. To test our hypothesis, corpora lutea (CL) from 28 dogs on days 10, 20, 30, 40, 50, 60, 70 and >70 after ovulation (n = 4/group) were used for ex vivo experiments. Pro-apoptotic proteins expression (CASPASES, 3, 8, 9 e BAX) were analyzed by immunohistochemistry and western blotting, and the gene expression of *CASP3*, 8, 9, *BAX*, *FAS*, *MKI67*, *ESR1* and *ESR2* were analyzed by real-time PCR. In the second step of this study, CL from 12 bitches on days 20, 40 and 60 (n = 4 per group) were used. Luteal cells were isolated and divided into six treatments: Control, E2 (treated with E2), ER α Blocker (treated with MPP), ER β Blocker (treated with PHTPP), ER α + E2 Blocker (treated with E2 + MPP) and E2 + ER β Blocker (treated with E2 + PHTPP). The expression of the same genes from the in vivo experiment was evaluated, as well as that of *CYP19A1*, *CYP11A1*, *HSD3B1* and *SLC2A4*. In general, the expression of pro-apoptotic factors was higher from day 40 and reached highest expression on days 60 and 70 after ovulation, coinciding with increasing in expression of *ERS2*. This correlation was also observed in the cells from the group E2 + ER α blockade, which also showed *HSD3B1* down-regulation. When ER β was blocked, cells of days 20 and 40 post ovulation responded increasing the expression of steroidogenesis and proliferation related genes. Thus, we deduce that the ER α blockade promotes the increase of pro-apoptotic and that o ER β of luteotrophic genes expression. Our findings confirm the pleiotropic role of estradiol in canine CL and include this hormone, as well as the balance between its two receptors, among the factors controlling canine CL lifespan.

Keywords: Corpus luteum. Diestrus. Estradiol. Luteolysis. Bitch.

SUMÁRIO

1	INTRODUÇÃO GERAL	16
2	17β-ESTRADIOL IS A PIVOTAL MEDIATOR OF APOPTOSIS DURING CORPUS LUTEUM REGRESSION IN NON-PREGNANT BITCHES	19
2.1	ABSTRACT.....	19
2.2	INTRODUCTION.....	20
2.3	MATERIALS AND METHODS	22
2.3.1	<i>Dogs and Experimental Design</i>	22
2.3.2.	<i>Immunohistochemistry for CASPASE 3, CASPASE 8, CASPASE 9 and BAX</i>	23
2.3.3	<i>Western blotting</i>	24
2.3.4	<i>Real time PCR for CASP3, CASP8, CASP 9, FAS, BAX, MKI67, ESR1, ESR2, CYP19A1 CYP11A, HSD3B1, and SLC2A4</i>	24
2.3.5	<i>Cell culture</i>	26
2.3.6	<i>17β-estradiol, MPP and PHTPP treatment</i>	26
2.3.7	<i>Statistical analysis</i>	27
2.4	RESULTS.....	27
2.4.1	<i>CASPASE 3, CASPASE 8, CASPASE 9 and BAX protein localization in the canine CL during diestrus.</i>	27
2.4.2	<i>Caspase 3, caspase 8, caspase 9 and BAX protein expression in the canine CL during diestrus.</i>	29
2.4.3	<i>ERS1, ESR2, CASP3, CASP8, CASP9, BAX, FAS, and MKI67, gene expression in the canine CL during diestrus.</i>	30
2.4.4	<i>CASP3, CASP8, CASP9, BAX, FAS, MKI67, CYP19A1, CYP11A1, HSB3B1, and SLC2A4 gene expression in the canine luteal cells in culture after blockade of ERα and ERβ.</i>	32
2.5	DISCUSSION	35
	REFERENCES	39
3	CONSIDERAÇÕES	49
	REFERÊNCIAS	51

INTRODUÇÃO GERAL

1 INTRODUÇÃO GERAL

O cão doméstico (*Canis familiaris*) é uma espécie monoéstrica não sazonal, de ovulação espontânea, cuja atividade reprodutiva compreende um período de pró-estro (13-16 dias), estro (4-12 dias) e diestro (60-90 dias), seguido de um período de anestro que varia de 15 a 165 dias (CONCANNON, 2009). O corpo lúteo (CL) é uma glândula endócrina temporária, que passa por um processo de desenvolvimento, manutenção e regressão, atingindo atividade secretória plena quando sua formação está completa (STOCCO; TELLERIA; GIBORI, 2007).

No entanto, os mecanismos envolvidos na regulação da função e da vida útil do corpo lúteo canino cíclico não foram, até o momento, completamente elucidados, e existem algumas particularidades entre as espécies. Neste contexto, sabe-se que em grandes animais como bovinos, suínos e equinos e, em roedores, como ratos e camundongos, a função do CL é cessada em animais não gestantes pela liberação da prostaglandina $F2\alpha$ ($PGF2\alpha$) uterina, molécula que desencadeia a fase de regressão denominada luteólise (NISWENDER et al., 2000). Em primatas, por sua vez, a luteólise não é mediada pela $PGF2\alpha$ de origem uterina e sim, pela $PGF2\alpha$ produzida localmente no CL (PELUFFO; STOUFFER; TESONE, 2007), enquanto, em cadelas não prenhes, o CL não sofre influência da $PGF2\alpha$ uterina, uma vez que a histerectomia não tem impacto sobre a função do corpo lúteo (DAVIS; RUEDA, 2002) e também não apresenta a capacidade de produzi-la (KOWALEWSKI; MUTEMBEI; HOFFMANN, 2008).

Para que a luteólise ocorra, as células entram em apoptose (SKARZYNSKI et al., 2013), cuja morte programada pode ocorrer por duas principais vias, extrínseca e intrínseca. Na via extrínseca, o acoplamento do ligante FAS ao seu receptor ativa a clivagem de caspase 8, que imediatamente inicia a clivagem dos efetores caspase 3 e 7. Na via intrínseca, a caspase 8 cliva BID, um membro pró-apoptótico da família de linfoma de células B2 (BCL2), que estimula a ligação de outros membros desta família (por exemplo, BAX e BAK) à mitocôndria e inibe a associação de fatores antiapoptóticos constituintes também da família BCL2. Este processo leva a quebra do citocromo C da mitocôndria promovendo a formação do apoptossomo, que por sua vez leva à ativação do efector caspase 3 e consequente fragmentação do DNA (SCAFFIDI et al., 1998).

Uma das funções do 17β -estradiol (E_2) é estimular o crescimento e inibir a apoptose em vários tipos celulares. O E_2 é, por exemplo, capaz de induzir a proliferação de células

cancerígenas de tireóide (KUMAR; KLINGE; GOLDSTEIN, 2010) e células cancerígenas endometriais (FILIGHEDDU et al., 2011). De maneira paradoxal, existem fortes evidências que seja também um hormônio pró-apoptótico, como foi observado em células cancerígenas de mama (LEWIS-WAMBI; JORDAN, 2009) nas quais se verificaram mudanças na relação entre pró e antiapoptóticos da família BCL2, além da ativação via FAS em células da hipófise, tais como lactótrofos (ZALDIVAR et al., 2009), maior indução de apoptose de células germinativas no epitélio seminífero (CHIMENTO et al., 2010) e células intestinais (SCHLEIPEN et al., 2011).

No ovário, este hormônio também demonstrou função pro-apoptótica. O estradiol utilizado em várias concentrações diretamente no meio de incubação de células do ovário de ratas levou a redução da síntese de progesterona e da relação BAX/BCL-2 (CASAIS et al., 2012). Da mesma forma em macacos, o CL foi sensível ao E2 produzido localmente em associação com a regressão do corpo lúteo (DUFFY; CHAFFIN; STOUFFER, 2000), o que também foi verificado em CL humano (VASKIVUO; TAPANAINEN, 2003).

Com base nas evidências relatadas e nos dados obtidos pelo nosso grupo de pesquisa referentes à síntese de 17β -estradiol na segunda metade da fase luteínica em cadelas não prenhes, a hipótese do presente estudo é a de que este hormônio tenha um papel fundamental no processo de regressão do CL nesta espécie.

2 17 β -ESTRADIOL IS A PIVOTAL MEDIATOR OF APOPTOSIS DURING CORPUS LUTEUM REGRESSION IN NON-PREGNANT BITCHES

2.1 ABSTRACT

The pro- and anti-apoptotic effects of estrogens during the luteal cycle are controversial. For example, 17 β -estradiol (E2) enhances apoptosis leading to luteolysis in rats; however, in rabbits, E2 protects the corpus luteum (CL) from cell death. In this study, we evaluated how genes and pro-apoptotic proteins were expressed during canine diestrus and how direct treatment with E2, as dissected by estrogen receptor blockade, affects expression of pro-apoptotic, steroidogenic, and glucose uptake genes. We found that CL showed different responses to estradiol stimulation depending on the particular receptor that was blocked. ER α blockade led to apoptotic and anti-proliferative action, stimulating the expression of genes involved in the extrinsic (CASP8 and FAS) and intrinsic (CASP9, BAX, and CASP3) pathways of apoptosis. ER α blockade also decreased the expression of genes involved in proliferation (MKI67), metabolism (SLC2A4), and synthesis of progesterone from pregnenolone (HSB3B1), which may be indicative of functional luteolysis. The dynamics of caspase expression with plasma E2 levels suggests that E2 binding to ER β mediates luteal regression in bitches. ER β blockade, on the other hand, stimulated genes involved in proliferation, steroidogenesis, and energy uptake, emphasizing the role of ER α , which was up regulated in early diestrus. Thus, in the non-pregnant bitch, our results indicate a pro-survival role for E2 in the first half and a pro-apoptotic role in the second half of diestrus as differentially modulated by ER α and ER β , respectively.

Keywords: Corpus luteum. Diestrus. Estradiol. Luteolysis. Bitch.

2.2 INTRODUCTION

17 β -Estradiol (E2) is a steroid hormone involved in reproduction. However, during the last decade it was discovered that practically every animal cell/tissue/organ system responds to E2 in some way. The effects of E2 are now known to be much broader, with roles in differentiation of several tissues and organs, bone metabolism, and modulation of inflammation in brain, liver, nervous, and cardiovascular systems (MENDELSON; KARAS, 1999; GRUBER et al., 2002; PEARCE; JORDAN, 2004). Other non-conventional actions of E2 have been described based on the identification of estrogen (ER) receptors in non-classical tissues such as periodontium (NEBEL, 2012), scalp skin (THORNTON et al., 2003), pancreas (ACKERMANN et al., 2009), and heart (LIOU et al., 2010) in addition to non-classical intracellular localization.

Knowledge of the molecular mechanisms by which estrogens exert pleiotropic functions in different tissues and organs has evolved rapidly during the past two decades. The effects of E2 on cell proliferation (ACKERMANN et al., 2009; CHIMENTO et al., 2010; ATTIA; EDERVEEN, 2012; DIOTEL et al., 2012; CHEN, F. P. et al., 2013), as well cell death (ACKERMANN et al., 2009; ADAMS, S. M. et al., 2010; CHIMENTO et al., 2010; ATTIA; EDERVEEN, 2012; QU et al., 2014) have been described in extensive studies. In addition, previous studies have suggested that in the rat, E2 is a key hormone regulating reproductive processes. When acting on the corpus luteum, high levels of E2 were associated with a decline in the progesterone biosynthetic capacity marked by reduced 3 β -HSD mRNA expression. E2 also modified the expression of anti-apoptotic BCL-2 and pro-apoptotic BAX (BCL2-associated X protein) genes, which are critical regulators of cell survival and death (CASAIS et al., 2012). During mid-pregnancy in rats, E2 is required for luteal survival, whereas at the end of pregnancy, high serum concentrations are responsible for increased synthesis of prostaglandin F2 α and luteal expression of prostaglandin F2 α receptors in association with functional regression of the corpora lutea (GIBORI et al., 1984; BUSSMANN, 1989; MCLEAN et al., 1989; CASAIS et al., 2012). The findings support the premise that this E2 plays important roles in female reproduction.

The corpus luteum (CL) is a transient but dynamic endocrine gland that develops from the wall of an ovarian follicle after ovulation. The ovary produces progesterone, the hormone necessary for the establishment and maintenance of intrauterine pregnancy in mammals

(STOCCO; TELLERIA; GIBORI, 2007), and in bitches it also produces E2 (PAPA, P. E. C. et al., 2014).

At the end of each ovarian cycle in the absence of pregnancy, or when it is no longer required for the maintenance of pregnancy, the CL ceases to produce progesterone (i.e., functional regression) and regresses (i.e., structural regression), which ultimately leads to the formation of the corpus albicans. Recent studies have suggested that the major event that causes the regression of the CL is luteal cell death by apoptosis (type I programmed cell death) (STOCCO; TELLERIA; GIBORI, 2007). Indeed, apoptosis takes place in the ovaries of cows (JUENGEL et al., 1993; RUEDA et al., 1997), rats (BOWEN et al., 1996; GAYTÁN et al., 2000; TELLERIA et al., 2001), sheep (RUEDA et al., 1995), and humans (SHIKONE et al., 1996) during both spontaneous and induced CL regression.

Although not yet fully understood, the regulatory mechanisms underlying these processes show distinct species specificities. For example, in farm animals such as cattle, pigs, and horses, and in rodents such as rats and mice, luteolysis in non-pregnant animals is triggered by a release of endometrial prostaglandin F2a (PGF2a) (NISWENDER et al., 2000). In contrast, in dogs and cats (HOFFMANN et al., 1992; HOFFMANN et al., 2004), as well as primates (PELUFFO; STOUFFER; TESONE, 2007), luteal regression in non-pregnant animals is independent of a uterine luteolysin.

There is emerging evidence of distinct pathways by which E2 and ERs may regulate biological processes (HALL; COUSE; KORACH, 2001), including the induction of apoptosis in various cell types like breast cancer cells (LEWIS et al., 2005), pituitary gland (ZALDIVAR et al., 2009), germ cells of the seminiferous epithelium (CHIMENTO et al., 2010), intestinal cells (SCHLEIPEN et al., 2011), pancreatic B cells (ACKERMANN et al., 2009), and rat luteal cells (CASAIS et al., 2012). During diestrus in bitches, the circulating concentration of E2 is highest on day 40 post-ovulation (p.o.) (PAPA, P. E. C. et al., 2014), while ER α expression in the vagina of the bitch varied throughout proestrus, estrus, and early diestrus according to the histological compartment involved (superficial, intermediate, and deep epithelia and superficial and deep stroma) (ITHURRALDE et al., 2013) and the ratio of ER α / ER β varies over diestrus (PAPA, P. C.; HOFFMANN, 2011).

Estradiol, at various concentrations utilized directly in the rat ovarian incubation medium, led to reduced progesterone release (CASAIS et al., 2012). Likewise, monkey corpora lutea were sensitive to E2 locally produced in association with the regression of the corpus luteum (DUFFY; CHAFFIN; STOUFFER, 2000), whereas E2 was shown to promote apoptosis in human luteal cells (VASKIVUO; TAPANAINEN, 2003).

In the ovary, E2 modifies the expression of anti-apoptotic BCL-2 and pro-apoptotic Bax genes that are essential regulators of cell survival and death, respectively (YIN; OLTVAI; KORSMEYER, 1994; CASAIS et al., 2012). Moreover, we have assessed the BCL-2-to-BAX ratio in dogs, which is critically associated with luteal apoptosis as well as survival (ANTONSSON, 2001; AIUDI et al., 2006). When added to ovarian cell cultures, E2 caused a decline in both Bcl-2 and Bax expression, as well as in the Bcl-2-to-Bax ratio, suggesting that the equilibrium between these two molecules favors Bax abundance and apoptosis (CASAIS et al., 2012). These findings are in agreement with a previous report indicating that these genes can be regulated by sex steroids (GOMPEL et al., 2000).

When E2 was administered to pregnant rats between days 7 and 14 of pregnancy, serum progesterone declined on day 15, likely as a consequence of a uterine-mediated effect via PGF2 α (TAMURA, 1983). Furthermore, in another study, when E2 was given to rats postpartum, it accelerated luteal regression through a mechanism that possibly involves the secretion of pituitary prolactin (GOYENECHE; TELLERIA, 2005). However, the effect of E2 on luteal function appears to be species-dependent, as in rabbits the steroid protects the corpus luteum from apoptosis rather than promoting apoptosis (GOODMAN et al., 1998).

We hypothesized, therefore, that E2 might be involved in cyclic apoptosis of the canine corpus luteum contributing to the already well-established regression process described for the non-pregnant bitch.

2.3 MATERIALS AND METHODS

2.3.1 Dogs and Experimental Design

Canine CL from 28 crossbred bitches were harvested during ovariosalpingohysterectomy (OSH) on days 10, 20, 30, 40, 50, 60, 70, and >70 p.o. (n = 4/group) for *in vivo* experiments and from 12 bitches for cell culture. The ovulation date (d0) was considered the day when plasma progesterone (P4) concentrations reached 5 ng/ml (CONCANNON, 2009). The experimental protocol, including premedication (Acepran 0.05 mg/ Kg + Tramadol 3 mg/ kg), induction (Propofol 3 mg/ Kg + ketamine 1 mg/ Kg), maintenance (isoflurane), and surgery, was approved by the Committee of Ethics on the Use

of Animals of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil (protocol number 2592/2012).

After collection, CL were dissected from the surrounding ovarian tissue and immediately frozen in liquid nitrogen for total RNA and protein extraction for use in real time PCR and western blotting, respectively, or fixed in 4% buffered formalin for 24 h for immunohistochemistry. For cell culture, CL were dissected and immediately washed with fresh phosphate buffered saline (PBS) containing 1% antibiotic-antimycotic solution (A5955, Sigma-Aldrich, St. Louis, MO, USA) and then prepared as described below.

2.3.2. Immunohistochemistry for CASPASE 3, CASPASE 8, CASPASE 9 and BAX

Expression of caspase 3, caspase 8, caspase 9 and BAX proteins was detected by an immunoperoxidase method on 2 µm tissue sections prepared from four CL per dog, using one section per CL and four dogs per group to assure accuracy (MARIANI et al., 2006). The primary antibodies used were: polyclonal anti rabbit for caspase 3, caspase 8, caspase 9 and BAX (Table 1). Negative controls were prepared using IgG isotype control antibodies (Normal rabbit IgG; Santa Cruz Biotechnologies, Dallas, TX, USA). Positive controls were mouse lymph node sections prepared according to the manufacturer's protocol.

Table1 - List of antibodies for immunohistochemistry and western blotting

Antibody	Isotype	Immunogen	Dilution	Catalog No:
Caspase 3	Polyclonal Rabbit IgG	Recombinant catalytically active human caspase-3	1:2500	IMGENEX (IMG-5700)
Caspase 8	Polyclonal Rabbit IgG	Recombinant catalytically active human caspase-8	1:2500	IMGENEX (IMG-5703)
Caspase 9	Polyclonal Rabbit IgG	Recombinant catalytically active human caspase-9	1:2500	IMGENEX (IMG-5705)
BAX	Polyclonal Rabbit IgG	Full length recombinant mouse Bax	1:2500	IMGENEX (IMG-5684)

Source: (BONFIM NETO, A. P., 2014).

2.3.3 Western blotting

CL samples were homogenized in buffer containing 50 mM potassium phosphate (pH 7.0), 0.3 M sucrose, 0.5 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, and phosphatase inhibitor cocktail (1:100; Sigma-Aldrich). Total protein content was determined using the Bradford method (BRADFORD, 1976), and values were extrapolated against a standard curve of albumin read at 595 nm. Fifty micrograms of protein were resolved on 15% SDS-PAGE minigels and electrophoretically transferred onto polyvinylidene difluoride membranes (PVDF, Bio-Rad Laboratories, Hercules, CA, USA). CASP3, CASP8, CASP9, and BAX were detected with specific antibodies (Table 1) and visualized using an Enhanced Chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ, USA). Images were captured by ChemiDoc MP Image system (Bio-Rad Laboratories) and normalized to actin-beta (ACTB; 42 kDa) using ImageJ Software (Bio-Rad Laboratories).

2.3.4 Real time PCR for CASP3, CASP8, CASP 9, FAS, BAX, MKI67, ESR1, ESR2, CYP19A1 CYP11A, HSD3B1, and SLC2A4

Total RNA was isolated from CL in different stages by Trizol[®] reagent (Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. Unless otherwise stated, all reagents and equipment were from Life Technologies. Concentration and quality of RNA were determined using a BioPhotometer (Eppendorf, Hamburg, Germany), and integrity was analyzed by electrophoresis through a 2% agarose gel. Following DNase treatment, 1 µg of total RNA (for *in vivo* experiments) and 0.5 µg of total RNA per sample (for *in vitro* experiments) was reverse transcribed using Superscript III reverse transcriptase according to the manufacturer's instructions. PCR reactions were performed with an automated fluorometer (ABI Prism[®] 7500), using 96-well optical plates. Each sample (25 ng of total RNA) was analyzed in duplicate. Primers used are listed in Table 2. Relative quantification of CASP3, CASP8, and CASP9, Fas cell surface death receptor (FAS), BAX, marker of proliferation Ki-67 (MKI67), estrogen receptor 1 (ESR1), and estrogen receptor 2

(ESR2) was performed by normalizing their signals against Cyclophilin A and GAPDH expression (reference genes), as determined by NormFinder (ANDERSEN; JENSEN; ØRNTOFT, 2004).

Ratios were calculated using the Linear Regression PCR program (LinRegPCR 7.0) (RAMAKERS et al., 2003; ROUSSEL et al., 2007), followed by the Pfaffl method (PFAFFL, 2001).

Table 2 - List of primers for RT-PCR

GENE	PRIMERS	SEQUENCES	PROBES	GENBANK Nº
<i>CASP8</i>		ID cf02627553_m1		DQ223013.1
<i>CASP9</i>		ID cf02627331_m1		DQ116956.1
<i>CASP3</i>		ID cf02622232_m1		AB085580.1
<i>FAS</i>		ID cf02651136_m1		XM_543595.2
<i>BAX</i>		ID cf02622186_g1		AB080230.1
<i>MEI67</i>		ID cf02642424_m1		XM_533319.2
<i>CYP11A1</i>		ID CF02635588_g1		XM_535539.2
<i>HSD3B1</i>	Forward	5' TCCCCAGTGTTCCTGATTCC 3'		AY739720.1
	Reverse	5' CACCAACAAATGCACGATTTC 3'		
<i>SLC2A4</i>	Forward	5' GCCTGCCAGAAAGAGTCTGAAG 3'	CAGTCCCCAGATACAT	NM_001159327
	Reverse	5' GCTTCCGCTTCTCCTCCTT 3'		
<i>PPLA</i>		ID CF03986523_gH		XM_843327.1
<i>ESR1</i>	Forward	5' CCTGCAAAGCCTTCTTCAAGAG 3'	TCAATGCTCCCCTGGATGG	AJ313195.1
	Reverse	5' GGAAGCCGGACAGCTGTAC 3'		
<i>ESR2</i>	Forward	5' CCTGCAAGCCTTCTTCAAGA 3'	CATCCAAGGGAACATC	AJ313196.1
	Reverse	5' GGCTGGGCAGCTGTACTC 3'		
<i>CYP19A1</i>	Forward	5' GTACCGGCCTGACCAGTT 3'	CATGCCAGAGCGCTTC	NM_001008715.1
	Reverse	5' ACTTAATGATGGAGAGATGAGCTGACT 3'		
<i>GADPH</i>	Forward	5' ACGGGAAGTCCATCTCCATCTT 3'	ATGTTGGCAGGATCTC	XM_549736.3
	Reverse	5' CACCAGCATCACCCCATTTG 3'		

Source: (BONFIM NETO, A. P., 2014).

2.3.5 Cell culture

Canine luteal cells were isolated from donor tissues obtained from twelve healthy mongrel female dogs at early (day 20 p.o.), mid (day 40 p.o.), and late diestrus (day 60 p.o.; n = 4 animals / group). After washing with fresh phosphate buffered saline (PBS) containing 1% antibiotic-antimycotic solution (A5955, Sigma-Aldrich), CLs were cut into small pieces. These pieces were transferred to 1 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich.), 20 mM HEPES (Sigma-Aldrich), 1% antibiotic-antimycotic solution (A5955, Sigma-Aldrich), and 1 mg/ml collagenase type 1 (C0130; Sigma-Aldrich). Samples were incubated for 1 h with shaking (60 shakes/ min) at 37°C. The suspension was centrifuged at 200 ×g for 10 min, re-suspended in DMEM, and filtered through a cell strainer (70 µm; BD Falcon; BD Biosciences, Durham, NC, USA). The filtrate was centrifuged at 200 ×g for 10 min, re-suspended in DMEM (v/v) for 10 min, centrifuged at 200 ×g for 10 min, and re-suspended in DMEM. Subsequently, the cells were seeded in 24-well plates and incubated (5% CO₂) at 37°C until cultures reached 90% confluence.

2.3.6 17β-estradiol, MPP and PHTPP treatment

After cultures reached 90% confluence, culture medium was removed and replaced by serum-free medium for 24 h to achieve serum starvation. Cultures were divided into six groups: Control, E2 (treated with E2), ERα block (treated with methyl-piperidino-pyrazole [MPP]), ERβ block (treated with pyrazolo (1,5-a) pyrimidine [PHTPP]), E2 + ERα block (treated with E2 + MPP), and E2 + ERβ block (treated with E2 + PHTPP). Concentrations of ERα and ERβ antagonists, determined by a concentration curve for 24 h, were 10 nM MPP (TOCRIS Biosciences, Bristol, UK) and 10 µM PHTPP (TOCRIS Biosciences), and 100 nM 17β-Estradiol (Sigma-Aldrich). For RNA preparations, 1 ml of TRIzol[®] was added to the cells, followed by scraping of the cell layer, freezing in liquid nitrogen, and storage at -80°C until further processing.

2.3.7 Statistical analysis

The *ex vivo* experiments were performed with a minimum of four replicates for each of the eight diestrus periods analyzed. *In vitro* experiments analyzed three periods, with six treatments per period and four replicates per treatment. Data were tested for homogeneity and normality, and presented as mean $\pm$ SEM. Data showing uneven distributions were compared by Kruskal-Wallis test followed by Dunn's multiple comparisons test. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA).

2.4 RESULTS

2.4.1 Caspase 3, caspase 8, caspase 9 and BAX protein localization in the canine CL during diestrus.

To test our hypothesis, we verified the localization of apoptotic proteins (caspase 3, caspase 8, caspase 9, and BAX) in the canine corpus luteum over diestrus. Caspase 3 staining could be observed in the cytoplasm and nucleus of luteal, endothelial, and stromal cells from day 10 until day > 70 p.o. (Figure 1). Caspase 8, caspase 9, and BAX followed the same expression pattern as caspase 3; however, the nuclear staining was evident only in CLs of the > 70 p.o. group (Figures 2 and 3, >70). We observed an increase in cells expressing these proteins over time.

Figure 1 - Immunolocalization of CASPASE 3, CASPASE 8, CASPASE 9 and BAX in CL during diestrus. Lines 10, 20, 30, 40, 50, 60, 70, and > 70 days after ovulation. NC, negative control. Black arrows indicate the cytoplasmic and white arrows indicate nuclear staining. Scale bar 50 μ m

Source: (BONFIM NETO, A. P., 2014).

2.4.2 Caspase 3, caspase 8, caspase 9 and BAX protein expression in the canine CL during diestrus.

Western blotting analysis revealed that caspase 3 expression was increased at the end of diestrus, with highest expression on days 60 and 70 p.o. (Figure 2A). Caspase 8 expression was highest on day 70 p.o. (Figure 2B), although a significant increase could be observed beginning on day 60 p.o. Caspase 9, however, started increasing on day 50 p.o. (Figure 2C). BAX protein expression followed the same pattern as Caspase 9 (Figure 2D).

Figure 2 - Protein expression of proteins Caspase 3 (A), Caspase 8 (B), Caspase 9 (C) and BAX (D) in canine corpus luteum. Representative blots on top of each column, which represent mean $\pm$ standard error (n = 4/group). Different letters indicate significant differences among groups ($P \leq 0.05$)

Source: (BONFIM NETO, A. P., 2014).

2.4.3 *ERS1*, *ESR2*, *CASP3*, *CASP8*, *CASP9*, *BAX*, *FAS*, and *MKI67*, gene expression in the canine CL during diestrus.

ESR1 (Figure 3A) and *ESR2* (Figure 3B) mRNA expression changed significantly during diestrus ($P < 0.0001$); *ESR1* expression was highest on day 20 p.o. ($P < 0.0001$) decreasing gradually until the end of diestrus, whereas *ESR2* decreased from day 20 to 30 p.o. and peaked on day 70 p.o.

The mRNA expression of *CASP3*, *CASP8*, *CASP9*, *FAS*, *BAX*, and *MKI67* could be detected throughout diestrus. *CASP3* expression increased from day 40 p.o., reaching maximum values in the > 70 group ($P < 0.05$) (Figure 3C). There was an effect of time ($P < 0.0001$) on *CASP8*, which increased from day 20, reached the highest levels on days 60 and 70, followed by decrease after day 70. (Figure 3D). *CASP9* expression increased gradually ($P < 0.0001$) throughout diestrus, reaching maximum values on day >70 p.o. (Figure 3E).

BAX expression, similar to *CASP9*, also increased gradually throughout diestrus ($P < 0.0001$), being highest on days 70 and > 70 p.o. (Figure 3F). *FAS* expression showed a decrease at day 20 p.o., but was increased beginning on day 40; we observed the highest expression of *FAS* on day 70 p.o. ($P = 0.0002$) (Figure 3G).

To confirm that cells in the tissue are undergoing apoptosis, rather than turnover, we analyzed the expression of proliferation marker Ki-67. Compared to pro-apoptotic genes, *MKI67* expression was highest in early diestrus (days 10–20 p.o.), declining gradually thereafter ($P < 0.05$) (Figure 3H).

Figure 3 - Gene expression of *ESR1* (A), *ESR2* (B), *CASP8* (C), *CASP9* (D), *CASP3* (E), *FAS* (F), *BAX* (G), and *MKI67* (H) in canine CL during diestrus (10 to > 70 days p.o.). Data are presented as mean $\pm$ standard error of relative gene expression (n = 4 animals / group). Bars with different letters indicate significant differences among groups (P < 0.05)

Source: (BONFIM NETO, A. P., 2014).

2.4.4 *CASP3*, *CASP8*, *CASP9*, *BAX*, *FAS*, *MKI67*, *CYP19A1*, *CYP11A1*, *HSB3B1*, and *SLC2A4* gene expression in the canine luteal cells in culture after blockade of ER α and ER β .

We measured the mRNA expression of genes associated with apoptosis in luteal cells after treatment with E2 (Figure 4). *CASP3* gene expression was increased when canine luteal cells were treated with 17 β -estradiol plus ER α block. However, cells from CLs collected on day 60 p.o. also showed an increase in *CASP3* when treated with E2 alone. *CASP8*, *CASP9*, *BAX*, and *FAS* expression followed the same pattern of response to ER α and ER β blockade; however, *CASP9* did not show a significant increase on day 40, as was observed with the other apoptotic genes.

mRNA expression of *MKI67*, steroidogenesis-associated genes, and *SLC2A4* is shown in Figure 5. *MKI67* gene expression was increased when canine luteal cells were treated with 17 β -estradiol plus ER β blockade; however, it was down-regulated under 17 β -estradiol plus ER α blockade. *CYP19A1* and *CYP11A1* followed the same pattern of *MKI67* response to ER α and ER β blockade.

There was a significant decrease in expression of *HSD3B1* when canine luteal cells collected on days 20 and 40 were treated with E2, whereas CL cells treated with E2 plus ER β block showed a significant increase in the relative expression of *HSD3B1*. The expression of *SLC2A4* was identified only in cells collected on day 20 p.o. The glucose transporter gene was up-regulated in cells treated with E2 + ER β block relative to the E2 + ER α block group.

Figure 4 - Gene expression of *CASP3*, *CASP8*, *CASP9*, *BAX*, and *FAS* in canine luteal cells collected on days 20, 40, and 60 after ovulation. Bars indicate six different groups: Control, E2 (treated with E2), ER α block (treated with methyl-piperidino-pyrazole [MPP]), ER β block (treated with pyrazolo (1,5-a) pyrimidine [PHTPP]), E2 + ER α block (treated with E2 + MPP), and E2 + ER β block (treated with E2 + PHTPP). Data represent mean $\pm$ standard error of relative gene expression (n = 4 animals / group). Bars with different letters indicate significant differences among groups (P < 0.05)

Source: (BONFIM NETO, A. P., 2014).

Figure 5 - Gene expression *MKI67*, cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1*), cytochrome P450, family 11, subfamily A, polypeptide 1 (*CYP11A1*), 3- β -hydroxysteroid dehydrogenase/ Δ -5-4 (*HSD3B1*), solute carrier family 2 (facilitated glucose transporter), and member 4 (*SLC2A4*) in CL at 20, 40, and 60 days after ovulation in diestrus bitches. Bars indicate six different groups: Control, E2 (treated with E2), ER α block (treated with methyl-piperidino-pyrazole [MPP]), ER β block (treated with pyrazolo (1,5-a) pyrimidine [PHTPP]), E2 + ER α block (treated with E2 + MPP), and E2 + ER β block (treated with E2 + PHTPP). Data represent mean $\pm$ standard error of relative gene expression (n = 4 animals / group). Bars with different letters indicate significant differences among groups (P < 0.05)

Source: (BONFIM NETO, A. P., 2014).

2.5 DISCUSSION

This is the first report of canine corpus luteum showing a biphasic response to 17 β -estradiol. We described opposite responses of luteal cells to estradiol stimulation depending on the day of the estrous cycle and the receptor through which E2 acts. *ESR2* was up-regulated from day 60 after ovulation, which matched the pattern of expression of the pro-apoptotic genes and proteins. ER α blockade with E2 treatment resulted in increased expression of pro-apoptotic genes, whereas ER β blockade had a positive effect on genes responsible for proliferation, metabolism and steroidogenesis.

The objective of this study was to characterize the role of E2 in the luteal regression of non-pregnant bitches. According to our hypothesis, E2 treatment should induce apoptosis in the canine CL. However, our data point toward a luteotrophic role for E2 when acting via ER α , and a pro-apoptotic and anti-proliferative role when binding to ER β .

The expression of *ESR1* and *ESR2* (genes encoding ER α and ER β , respectively) in CL followed different patterns: *ESR1* has its highest expression in early estrus, whereas *ESR2* increases beginning on day 60 p.o. with highest expression on day 70. These findings corroborate the report of Paech et al. (PAECH et al., 1997) where ER subtypes have differential expression and responses to specific ligands, suggesting that the two receptors promote different regulatory functions. In general, *ESR1* is associated with cell proliferation, while *ESR2* has anti-proliferative effects, and in some tissues, such as ovary and breast cancer cells, it acts as a pro-apoptotic factor (WEIHUA et al., 2003; RIZZA et al., 2014).

Expression of the pro-apoptotic genes and proteins followed mostly the same pattern observed for *ESR2*. Since the identification of ER β (KUIPER et al., 1996), several investigations have shown that a loss of ER β expression in ovary is associated with down-regulation of apoptosis (PUJOL et al., 1998; BARDIN et al., 2004a). A similar down-regulation of ER β has also been noted in prostate, breast, and colon tumors (CAMPBELL-THOMPSON; LYNCH; BHARDWAJ, 2001; ROGER et al., 2001; CHENG et al., 2004). It has been reported that ER β up-regulates apoptosis in ovarian cancer cells (BARDIN et al., 2004b) and enhances FasL expression, a major extrinsic apoptotic regulator, in normal ovarian cells (SAPI et al., 2002). Our study found similar results: *FAS* and *CASP8* (genes involved in the extrinsic pathway of apoptosis) showed the highest expression on day 70 p.o., accompanying expression of *ESR2*, although *FAS* showed the first increase on day 40 p.o.

and CASP8 on day 20 p.o. In the CL, apoptosis via the intrinsic cascade is thought to play an important role in controlling the rate of cell death (ADAMS; CORY, 1998), and our results showed the expression of *BAX* and *CASP9* (genes involved in the intrinsic pathway of apoptosis) to be highest on day 70 p.o.; although, similar to FAS, BAX showed an initial increase on day 40 p.o. As already discussed, similar results were found in breast (CHEN et al., 2004), colon (CAMPBELL-THOMPSON; LYNCH; BHARDWAJ, 2001), and prostate (CHENG et al., 2004) cancer cells. Thus, similar to the cow, luteolysis in the dog is multifactorial and can occur via both apoptotic pathways (SKARZYNSKI et al., 2013). Moreover, our results indicate that ER β is able to stimulate the extrinsic as well as the intrinsic pathways of apoptosis.

To confirm the *in vivo* results, we treated the luteal cells with E2 and blocked its two receptors, each separately or both together, with commonly used antagonists (KIM et al., 2005; LAREEF et al., 2005; LIN, J. et al., 2014; MARTINEZ et al., 2014; WATANABE et al., 2014). We found opposite responses after the blockade of these two receptors, as described for other various cell types. When we treated luteal cells with E2 plus MPP (ER α blockade), there was an increased expression of extrinsic and intrinsic pro-apoptotic genes. Activated ESR1 binds to and activates phosphoinositide-3-kinase (PI3K) (SIMONCINI et al., 2000; MANNELLA; BRINTON, 2006). PI3K, via phosphatidylinositol (3,4,5) triphosphate (PtdIns(3,4,5)P3) and 3-phosphoinositide-dependent protein kinase-1 (PDK(PDPK1)) activates V-akt murine thymoma viral oncogene homologs (AKT(PKB)) (SCHEID; WOODGETT, 2003). Upon E2 exposure, activated AKT (PKB) promotes several anti-apoptotic pathways. Thus, the blockade of this receptor can block anti-apoptotic cellular processes. With the blockade of ER α , E2 binds to ER β . The pro-apoptotic role of ER β has been seen in other tissues, especially in breast and ovarian cancer cells treated with E2 or SERMs (Selective Estrogen Receptor Modulators) (CHEN; CHIEN, 2013; CHEN et al., 2013; IVANOVA et al., 2013), which was corroborated by our findings.

The same apoptotic actions of ER β also were found in rat prostate cancer cells (MIRÓ et al., 2011; ATTIA; EDERVEEN, 2012), neurons (ADAMS et al., 2010) renal carcinoma (YU et al., 2013), breast cancer (PAN et al., 2013), sertoli cells (SIMÕES et al., 2013), liver (LIN et al., 2012), heart (LIOU et al., 2010), and umbilical cord cells (POWAZNIAK et al., 2009); all of these studies reported the correlation of ER β binding with increased expression of the pro-apoptotic genes, suggesting that the role of this receptor is to induce apoptosis.

Treatment with E2 plus PHTPP (ER β blockade) resulted in up-regulation of *MKI67*. The negative correlation between ER β and MKI67 has also been reported in different types of

cancers (ROGER et al., 2001; LINDGREN et al., 2004; HARTMAN et al., 2009) and reinforces the opposing role of this receptor relative to that of ER α . When the availability of E2 is similar, one can infer that ER α /ER β ratio determines the fate of the tissue (PAECH et al., 1997; ROA et al., 2008; MIRÓ et al., 2011).

Assuming that *MKI67* expression increases parallel with CL maturation so that the full hormone production capacity is reached (HOFFMANN et al., 2004; PAPA; HOFFMANN, 2011), it would be expected that the expression profile of steroidogenic enzymes parallels that of *MKI67*. We showed that *CYP19A1* (encodes P450aromatease) and *CYP11A1* (encodes P450scc) also increased when cells were treated with ER β blockade, which points towards a direct influence of the action of E2 on ER α on days 20 and 40 p.o. Estradiol has been reported to alternately increase or decrease aromatase expression and enzyme activity (ROSELLI; RESKO, 1997; NEGRI-CESI et al., 2001; ZHAO et al., 2007). These reports are consistent with our current findings that E2 showed a biphasic stimulatory effect on aromatase mRNA levels in luteal cells. Moreover, our observation that E2-induced aromatase expression is dependent on the presence of ESR1 is also consistent with the findings of other groups employing similar E2 concentrations under various *in vivo* and *in vitro* settings (RUNE et al., 2002; HOJO et al., 2004; IIVONEN et al., 2006). E2 also can stimulate P450 cholesterol side-chain cleavage (P450scc) activity in cultures of human placental syncytiotrophoblasts (BABISCHKIN et al., 1997). The relationship between P450scc and ER α has been described in chondrosarcoma tumors, bovine theca cells, and rabbit CL cells (ROBERTS; SKINNER, 1990; TOWNSON et al., 1996; MEIJER et al., 2011), which showed a positive correlation of enzyme and receptor expression, indicating that ER α has steroidogenic effects at the beginning of diestrus.

The expression of *HSD3B1* did not show the same expression pattern as other genes linked to hormone production that decreased under E2 treatment. The inhibition of HSD3B1 expression by E2 was reported previously (DEPP et al., 1973; CASAIS et al., 2012). However, when we blocked ER β , this negative effect on P4 production was neutralized; our data suggest that E2 via ER β impairs luteal progesterone production by decreasing its synthesis. Luteal regression in bitches begins with a decline in the progesterone-producing capacity of the corpora lutea, followed by structural regression of the gland (HOFFMANN et al., 2004), which also occurs via apoptosis, as we demonstrated in the present work. This finding suggests that the link between E2/ER β can be directly involved in the process of functional luteolysis.

For steroidogenesis to occur, energy support is needed (SKARZYNSKI et al., 2013); thus, glucose uptake is an important process for the corpus luteum during its lifespan. SLC2A4 (encodes GLUT4) could be detected in cell culture after E2 treatment only on day 20 p.o. when the canine corpus luteum is producing high levels of P4 (HOFFMANN et al., 2004). There was no significant difference in SLC2A4 expression after E2 treatment compared to the control group, but we observed that SLC2A4 reacts differently to stimulation with E2 depending on the receptor that was blocked. This observation can be explained because the activation of ER α increases protein expression through SP1 (specificity protein 1), a stimulator of GLUT4 expression. ER α also inhibits the binding of NF- κ B (nuclear factor- κ B) to its response element. NF- κ B is a potent repressor of GLUT4 expression, and its inhibition might lead to increased GLUT4 expression. In contrast, activation of ER β opposes ER α action and inhibits SP1-induced gene expression, reducing GLUT4 content (BARROS; MACHADO; GUSTAFSSON, 2006).

In summary, the corpus luteum showed different responses to stimulation with estradiol depending on the receptor that was utilized. ER α binding stimulated genes involved in proliferation, steroidogenesis, and energy uptake, whereas *ex vivo* experiments showed up-regulated of these genes in early diestrus. ER β had apoptotic and anti-proliferative activities, stimulating the expression of genes involved in the extrinsic and intrinsic pathway of apoptosis, and decreasing expression of genes involved in proliferation and metabolism. Thus, the dynamics of these proteases together with plasma estrogen levels and different amounts of ER expression suggests an important role for this hormone during the formation and regression of the CL in bitches.

REFERENCES

- ACKERMANN, S.; HILLER, S.; OSSWALD, H.; LÖSLE, M.; GRENZ, A.; HAMBROCK, A. 17beta-Estradiol modulates apoptosis in pancreatic beta-cells by specific involvement of the sulfonylurea receptor (SUR) isoform SUR1. **J Biol Chem**, v. 284, n. 8, p. 4905-4913, 2009.
- ADAMS, J. M.; CORY, S. The Bcl-2 protein family: arbiters of cell survival. **Science**, v. 281, n. 5381, p. 1322-1326, 1998.
- ADAMS, S. M.; AKSENOVA, M. V.; AKSENOV, M. Y.; MACTUTUS, C. F.; BOOZE, R. M. ER- β mediates 17 β -estradiol attenuation of HIV-1 Tat-induced apoptotic signaling. **Synapse**, v. 64, n. 11, p. 829-838, 2010.
- AIUDI, G.; ALBRIZIO, M.; CAIRA, M.; CINONE, M. Apoptosis in canine corpus luteum during spontaneous and prostaglandin-induced luteal regression. **Theriogenology**, v. 66, n. 6-7, p. 1454-1461, 2006.
- ANDERSEN, C. L.; JENSEN, J. L.; ØRNTØFT, T. F. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. **Cancer Res**, v. 64, n. 15, p. 5245-5250, 2004.
- ANTONSSON, B. Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. **Cell Tissue Res**, v. 306, n. 3, p. 347-361, 2001.
- ATTIA, D. M.; EDERVEEN, A. G. Opposing roles of ER α and ER β in the genesis and progression of adenocarcinoma in the rat ventral prostate. **Prostate**, v. 72, n. 9, p. 1013-1022, 2012.
- BABISCHKIN, J. S.; GRIMES, R. W.; PEPE, G. J.; ALBRECHT, E. D. Estrogen stimulation of P450 cholesterol side-chain cleavage activity in cultures of human placental syncytiotrophoblasts. **Biol Reprod**, v. 56, n. 1, p. 272-278, 1997.
- BARDIN, A.; BOULLE, N.; LAZENNEC, G.; VIGNON, F.; PUJOL, P. Loss of ERbeta expression as a common step in estrogen-dependent tumor progression. **Endocr Relat Cancer**, v. 11, n. 3, p. 537-551, 2004a.
- BARDIN, A.; HOFFMANN, P.; BOULLE, N.; KATSAROS, D.; VIGNON, F.; PUJOL, P.; LAZENNEC, G. Involvement of estrogen receptor beta in ovarian carcinogenesis. **Cancer Res**, v. 64, n. 16, p. 5861-5869, 2004b.

BARROS, R. P.; MACHADO, U. F.; GUSTAFSSON, J. A. Estrogen receptors: new players in diabetes mellitus. **Trends Mol Med**, v. 12, n. 9, p. 425-431, 2006.

BOWEN, J. M.; KEYES, P. L.; WARREN, J. S.; TOWNSON, D. H. Prolactin-induced regression of the rat corpus luteum: expression of monocyte chemoattractant protein-1 and invasion of macrophages. **Biol Reprod**, v. 54, n. 5, p. 1120-1127, 1996.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal Biochem**, v. 72, p. 248-254, 1976.

BUSSMANN, L. E. Prostaglandin F-2 alpha receptors in corpora lutea of pregnant rats and relationship with induction of 20 alpha-hydroxysteroid dehydrogenase. **J Reprod Fertil**, v. 85, n. 2, p. 331-341, 1989.

CAMPBELL-THOMPSON, M.; LYNCH, I. J.; BHARDWAJ, B. Expression of estrogen receptor (ER) subtypes and ERbeta isoforms in colon cancer. **Cancer Res**, v. 61, n. 2, p. 632-640, 2001.

CASAI, M.; VALLCANERAS, S. S.; CAMPO VERDE ARBOCCO, F.; DELGADO, S. M.; HAPON, M. B.; SOSA, Z.; TELLERIA, C. M.; RASTRILLA, A. M. Estradiol promotes luteal regression through a direct effect on the ovary and an indirect effect from the celiac ganglion via the superior ovarian nerve. **Reprod Sci**, v. 19, n. 4, p. 416-422, 2012.

CHEN, F. P.; CHIEN, M. H. Phytoestrogens induce apoptosis through a mitochondria/caspase pathway in human breast cancer cells. **Climacteric**, v. 17, n. 4, p. 385-392, 2013.

CHEN, F. P.; CHIEN, M. H.; CHEN, H. Y.; HUANG, T. S.; NG, Y. T. Effects of estradiol and progestogens on human breast cells: regulation of sex steroid receptors. **Taiwan J Obstet Gynecol**, v. 52, n. 3, p. 365-373, 2013.

CHEN, J. Q.; DELANNOY, M.; COOKE, C.; YAGER, J. D. Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells. **Am J Physiol Endocrinol Metab**, v. 286, n. 6, p. E1011-1022, 2004.

CHENG, J.; LEE, E. J.; MADISON, L. D.; LAZENNEC, G. Expression of estrogen receptor beta in prostate carcinoma cells inhibits invasion and proliferation and triggers apoptosis. **FEBS Lett**, v. 566, n. 1-3, p. 169-172, 2004.

CHIMENTO, A.; SIRIANNI, R.; DELALANDE, C.; SILANDRE, D.; BOIS, C.; ANDÒ, S.; MAGGIOLINI, M.; CARREAU, S.; PEZZI, V. 17 beta-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ER alpha. **Mol Cell Endocrinol**, v. 320, n. 1-2, p. 136-144, 2010.

CONCANNON, P. W. Endocrinologic control of normal canine ovarian function. **Reprod Domest Anim**, v. 44, p. 3-15, 2009. Supplement 2.

DEPP, R.; COX, D. W.; PION, R. J.; CONRAD, S. H.; HEINRICHS, W. L. Inhibition of the pregnenolone delta 5-3 beta hydroxysteroid dehydrogenase-delta 5-4 isomerase systems of human placenta and corpus luteum of pregnancy. **Gynecol Invest**, v. 4, n. 2, p. 106-120, 1973.

DIOTEL, N.; VAILLANT, C.; GABBERO, C.; MIRONOV, S.; FOSTIER, A.; GUEGUEN, M. M.; ANGLADE, I.; KAH, O.; PELLEGRINI, E. Effects of estradiol in adult neurogenesis and brain repair in zebrafish. **Horm Behav**, v. 63, p. 193-207, 2012.

DUFFY, D. M.; CHAFFIN, C. L.; STOUFFER, R. L. Expression of estrogen receptor alpha and beta in the rhesus monkey corpus luteum during the menstrual cycle: regulation by luteinizing hormone and progesterone. **Endocrinology**, v. 141, n. 5, p. 1711-1717, 2000.

GAYTÁN, F.; MORALES, C.; BELLIDO, C.; AGUILAR, R.; MILLÁN, Y.; MARTÍN DE LAS MULAS, J.; SÁNCHEZ-CRIADO, J. E. Progesterone on an oestrogen background enhances prolactin-induced apoptosis in regressing corpora lutea in the cyclic rat: possible involvement of luteal endothelial cell progesterone receptors. **J Endocrinol**, v. 165, n. 3, p. 715-724, 2000.

GIBORI, G.; CHEN, Y. D.; KHAN, I.; AZHAR, S.; REAVEN, G. M. Regulation of luteal cell lipoprotein receptors, sterol contents, and steroidogenesis by estradiol in the pregnant rat. **Endocrinology**, v. 114, n. 2, p. 609-617, 1984.

GOMPEL, A.; SOMAÏ, S.; CHAOUAT, M.; KAZEM, A.; KLOOSTERBOER, H. J.; BEUSMAN, I.; FORGEZ, P.; MIMOUN, M.; ROSTÈNE, W. Hormonal regulation of apoptosis in breast cells and tissues. **Steroids**, v. 65, n. 10-11, p. 593-598, 2000.

GOODMAN, S. B.; KUGU, K.; CHEN, S. H.; PREUTTHIPAN, S.; TILLY, K. I.; TILLY, J. L.; DHARMARAJAN, A. M. Estradiol-mediated suppression of apoptosis in the rabbit corpus luteum is associated with a shift in expression of bcl-2 family members favoring cellular survival. **Biol Reprod**, v. 59, n. 4, p. 820-827, 1998.

GOYENECHE, A. A.; TELLERIA, C. M. Exogenous estradiol enhances apoptosis in regressing post-partum rat corpora lutea possibly mediated by prolactin. **Reprod Biol Endocrinol**, v. 3, n., p. 40, 2005.

GRUBER, C. J.; TSCHUGGUEL, W.; SCHNEEBERGER, C.; HUBER, J. C. Production and actions of estrogens. **N Engl J Med**, v. 346, n. 5, p. 340-352, 2002.

HALL, J. M.; COUSE, J. F.; KORACH, K. S. The multifaceted mechanisms of estradiol and estrogen receptor signaling. **J Biol Chem**, v. 276, n. 40, p. 36869-36872, 2001.

HARTMAN, J.; EDVARDSSON, K.; LINDBERG, K.; ZHAO, C.; WILLIAMS, C.; STRÖM, A.; GUSTAFSSON, J. A. Tumor repressive functions of estrogen receptor beta in SW480 colon cancer cells. **Cancer Res**, v. 69, n. 15, p. 6100-6106, 2009.

HOFFMANN, B.; HÖVELER, R.; HASAN, S. H.; FAILING, K. Ovarian and pituitary function in dogs after hysterectomy. **J Reprod Fertil**, v. 96, n. 2, p. 837-845, 1992.

HOFFMANN, B.; BÜSGES, F.; ENGEL, E.; KOWALEWSKI, M. P.; PAPA, P. Regulation of corpus luteum-function in the bitch. **Reprod Domest Anim**, v. 39, n. 4, p. 232-240, 2004.

HOJO, Y.; HATTORI, T. A.; ENAMI, T.; FURUKAWA, A.; SUZUKI, K.; ISHII, H. T.; MUKAI, H.; MORRISON, J. H.; JANSSEN, W. G.; KOMINAMI, S.; HARADA, N.; KIMOTO, T.; KAWATO, S. Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. **Proc Natl Acad Sci U S A**, v. 101, n. 3, p. 865-870, 2004.

IIVONEN, S.; HEIKKINEN, T.; PUOLIVÄLI, J.; HELISALMI, S.; HILTUNEN, M.; SOININEN, H.; TANILA, H. Effects of estradiol on spatial learning, hippocampal cytochrome P450 19, and estrogen alpha and beta mRNA levels in ovariectomized female mice. **Neuroscience**, v. 137, n. 4, p. 1143-1152, 2006.

ITHURRALDE, J.; COSTAS, A. L.; PESSINA, P.; CUETO, E.; FILA, D.; MEIKLE, A. Immunohistochemical determination of estrogen receptor- α in canine vaginal biopsies throughout proestrus, estrus, and early diestrus. **Theriogenology**, v. 80, n. 7, p. 805-811, 2013.

IVANOVA, M. M.; RADDE, B. N.; SON, J.; MEHTA, F. F.; CHUNG, S. H.; KLINGE, C. M. Estradiol and tamoxifen regulate NRF-1 and mitochondrial function in mouse mammary gland and uterus. **J Mol Endocrinol**, v. 51, n. 2, p. 233-246, 2013.

JUENGEL, J. L.; GARVERICK, H. A.; JOHNSON, A. L.; YOUNGQUIST, R. S.; SMITH, M. F. Apoptosis during luteal regression in cattle. **Endocrinology**, v. 132, n. 1, p. 249-254, 1993.

KIM, S.; WU, J. Y.; ZHANG, Z.; TANG, W.; DOSS, G. A.; DEAN, B. J.; DININNO, F.; HAMMOND, M. L. Estrogen receptor ligands. 12. Synthesis of the major metabolites of an ERalpha-selective, dihydrobenzoxathiin antagonist for osteoporosis. **Org Lett**, v. 7, n. 3, p. 411-414, 2005.

KUIPER, G. G.; ENMARK, E.; PELTO-HUIKKO, M.; NILSSON, S.; GUSTAFSSON, J. A. Cloning of a novel receptor expressed in rat prostate and ovary. **Proc Natl Acad Sci U S A**, v. 93, n. 12, p. 5925-5930, 1996.

LAREEF, M. H.; GARBER, J.; RUSSO, P. A.; RUSSO, I. H.; HEULINGS, R.; RUSSO, J. The estrogen antagonist ICI-182-780 does not inhibit the transformation phenotypes induced by 17-beta-estradiol and 4-OH estradiol in human breast epithelial cells. **Int J Oncol**, v. 26, n. 2, p. 423-429, 2005.

LEWIS, J. S.; MEEKE, K.; OSIPO, C.; ROSS, E. A.; KIDAWI, N.; LI, T.; BELL, E.; CHANDEL, N. S.; JORDAN, V. C. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. **J Natl Cancer Inst**, v. 97, n. 23, p. 1746-1759, 2005.

LIN, F. S.; SHEN, S. Q.; CHEN, Z. B.; YAN, R. C. 17 β -estradiol attenuates reduced-size hepatic ischemia/reperfusion injury by inhibition apoptosis via mitochondrial pathway in rats. **Shock**, v. 37, n. 2, p. 183-190, 2012.

LIN, J.; ZHU, J.; LI, X.; LI, S.; LAN, Z.; KO, J.; LEI, Z. Expression of Genomic Functional Estrogen Receptor 1 in Mouse Sertoli Cells. **Reprod Sci**, p. 1-12, 2014.

LINDGREN, P. R.; CAJANDER, S.; BÄCKSTRÖM, T.; GUSTAFSSON, J. A.; MÄKELÄ, S.; OLOFSSON, J. I. Estrogen and progesterone receptors in ovarian epithelial tumors. **Mol Cell Endocrinol**, v. 221, n. 1-2, p. 97-104, 2004.

LIOU, C. M.; YANG, A. L.; KUO, C. H.; TIN, H.; HUANG, C. Y.; LEE, S. D. Effects of 17 β -estradiol on cardiac apoptosis in ovariectomized rats. **Cell Biochem Funct**, v. 28, n. 6, p. 521-528, 2010.

MANNELLA, P.; BRINTON, R. D. Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. **J Neurosci**, v. 26, n. 37, p. 9439-9447, 2006.

MARIANI, T. C.; DO PRADO, C.; SILVA, L. G.; PAARMANN, F. A.; LIMA, M. C.; CARVALHO, I.; CAMPOS, D. B.; ARTONI, L. P.; HERNANDEZ-BLAZQUEZ, F. J.; PAPA, P. C. Immunohistochemical localization of VEGF and its receptors in the corpus luteum of the bitch during diestrus and anestrus. **Theriogenology**, v. 66, n. 6-7, p. 1715-1720, 2006.

MARTINEZ, L. A.; PETERSON, B. M.; MEISEL, R. L.; MERMELSTEIN, P. G. Estradiol facilitation of cocaine-induced locomotor sensitization in female rats requires activation of mGluR5. **Behav Brain Res**, v. 271, p. 39-42, 2014.

MCLEAN, M. P.; PURYEAR, T. K.; KHAN, I.; AZHAR, S.; BILLHEIMER, J. T.; ORLY, J.; GIBORI, G. Estradiol regulation of sterol carrier protein-2 independent of cytochrome P450 side-chain cleavage expression in the rat corpus luteum. **Endocrinology**, v. 125, n. 3, p. 1337-1344, 1989.

MEIJER, D.; GELDERBLOM, H.; KARPERIEN, M.; CLETON-JANSEN, A. M.; HOGENDOORN, P. C.; BOVEE, J. V. Expression of aromatase and estrogen receptor alpha in chondrosarcoma, but no beneficial effect of inhibiting estrogen signaling both in vitro and in vivo. **Clin Sarcoma Res**, v. 1, n. 1, p. 5, 2011.

MENDELSON, M. E.; KARAS, R. H. The protective effects of estrogen on the cardiovascular system. **N Engl J Med**, v. 340, n. 23, p. 1801-1811, 1999.

MIRÓ, A. M.; SASTRE-SERRA, J.; PONS, D. G.; VALLE, A.; ROCA, P.; OLIVER, J. 17 β -Estradiol regulates oxidative stress in prostate cancer cell lines according to ERalpha/ERbeta ratio. **J Steroid Biochem Mol Biol**, v. 123, n. 3-5, p. 133-139, 2011.

NEBEL, D. Functional importance of estrogen receptors in the periodontium. **Swed Dent J Suppl**, n. 221, p. 11-66, 2012.

NEGRI-CESI, P.; COLCIAGO, A.; MOTTA, M.; MARTINI, L.; CELOTTI, F. Aromatase expression and activity in male and female cultured rat hypothalamic neurons: effect of androgens. **Mol Cell Endocrinol**, v. 178, n. 1-2, p. 1-10, 2001.

NISWENDER, G. D.; JUENGEL, J. L.; SILVA, P. J.; ROLLYSON, M. K.; MCINTUSH, E. W. Mechanisms controlling the function and life span of the corpus luteum. **Physiol Rev**, v. 80, n. 1, p. 1-29, 2000.

PAECH, K.; WEBB, P.; KUIPER, G. G.; NILSSON, S.; GUSTAFSSON, J.; KUSHNER, P. J.; SCANLAN, T. S. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. **Science**, v. 277, n. 5331, p. 1508-1510, 1997.

PAN, W. L.; WONG, J. H.; FANG, E. F.; CHAN, Y. S.; YE, X. J.; NG, T. B. Differential inhibitory potencies and mechanisms of the type I ribosome inactivating protein marmorin on estrogen receptor (ER)-positive and ER-negative breast cancer cells. **Biochim Biophys Acta**, v. 1833, n. 5, p. 987-996, 2013.

PAPA, P. C.; HOFFMANN, B. The corpus luteum of the dog: source and target of steroid hormones? **Reprod Domest Anim**, v. 46, n. 4, p. 750-756, 2011.

PAPA, P. E. C.; SOUSA, L. M.; SILVA, R. O. S.; DE FÁTIMA, L. A.; DA FONSECA, V. U.; DO AMARAL, V. C.; HOFFMANN, B.; ALVES-WAGNER, A. B.; MACHADO, U. F.; KOWALEWSKI, M. P. Glucose transporter 1 expression accompanies hypoxia sensing in the cyclic canine corpus luteum. **Reproduction**, v. 147, n. 1, p. 81-89, 2014.

PEARCE, S. T.; JORDAN, V. C. The biological role of estrogen receptors alpha and beta in cancer. **Crit Rev Oncol Hematol**, v. 50, n. 1, p. 3-22, 2004.

PELUFFO, M. C.; STOUFFER, R. L.; TESONE, M. Activity and expression of different members of the caspase family in the rat corpus luteum during pregnancy and postpartum. **Am J Physiol Endocrinol Metab**, v. 293, n. 5, p. E1215-1223, 2007.

PFAFFL, M. W. A new mathematical model for relative quantification in real-time RT-PCR. **Nucleic Acids Res**, v. 29, n. 9, p. e45, 2001.

POWAZNIAK, Y.; KEMPFER, A. C.; DE LA PAZ DOMINGUEZ, M.; FARIAS, C.; KELLER, L.; CALDERAZZO, J. C.; LAZZARI, M. A. Effect of estradiol, progesterone and testosterone on apoptosis- and proliferation-induced MAPK signaling in human umbilical vein endothelial cells. **Mol Med Rep**, v. 2, n. 3, p. 441-447, 2009.

PUJOL, P.; REY, J. M.; NIRDE, P.; ROGER, P.; GASTALDI, M.; LAFFARGUE, F.; ROCHEFORT, H.; MAUDELONDE, T. Differential expression of estrogen receptor-alpha and -beta messenger RNAs as a potential marker of ovarian carcinogenesis. **Cancer Res**, v. 58, n. 23, p. 5367-5373, 1998.

QU, J.; LIU, W.; HUANG, C.; XU, C.; DU, G.; GU, A.; WANG, X. Estrogen receptors are involved in polychlorinated biphenyl-induced apoptosis on mouse spermatocyte GC-2 cell line. **Toxicol In Vitro**, v. 28, n. 3, p. 373-380, 2014.

RAMAKERS, C.; RUIJTER, J. M.; DEPREZ, R. H.; MOORMAN, A. F. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. **Neurosci Lett**, v. 339, n. 1, p. 62-66, 2003.

RIZZA, P.; BARONE, I.; ZITO, D.; GIORDANO, F.; LANZINO, M.; DE AMICIS, F.; MAURO, L.; SISCI, D.; CATALANO, S.; WRIGHT, K. D.; GUSTAFSSON, J. A.; ANDÒ, S. Estrogen receptor beta as a novel target of androgen receptor action in breast cancer cell lines. **Breast Cancer Res**, v. 16, n. 1, p. R21, 2014.

ROA, J.; VIGO, E.; CASTELLANO, J. M.; GAYTAN, F.; NAVARRO, V. M.; AGUILAR, E.; DIJCKS, F. A.; EDERVEEN, A. G.; PINILLA, L.; VAN NOORT, P. I.; TENA-SEMPERE, M. Opposite roles of estrogen receptor (ER)-alpha and ERbeta in the modulation of luteinizing hormone responses to kisspeptin in the female rat: implications for the generation of the preovulatory surge. **Endocrinology**, v. 149, n. 4, p. 1627-1637, 2008.

ROBERTS, A. J.; SKINNER, M. K. Estrogen regulation of thecal cell steroidogenesis and differentiation: thecal cell-granulosa cell interactions. **Endocrinology**, v. 127, n. 6, p. 2918-2929, 1990.

ROGER, P.; SAHLA, M. E.; MÄKELÄ, S.; GUSTAFSSON, J. A.; BALDET, P.; ROCHEFORT, H. Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. **Cancer Res**, v. 61, n. 6, p. 2537-2541, 2001.

ROSELLI, C. E.; RESKO, J. A. Sex differences in androgen-regulated expression of cytochrome P450 aromatase in the rat brain. **J Steroid Biochem Mol Biol**, v. 61, n. 3-6, p. 365-374, 1997.

ROUSSEL, Y.; HARRIS, A.; LEE, M. H.; WILKS, M. Novel methods of quantitative real-time PCR data analysis in a murine *Helicobacter pylori* vaccine model. **Vaccine**, v. 25, n. 15, p. 2919-2929, 2007.

RUEDA, B. R.; WEGNER, J. A.; MARION, S. L.; WAHLEN, D. D.; HOYER, P. B. Internucleosomal DNA fragmentation in ovine luteal tissue associated with luteolysis: in vivo and in vitro analyses. **Biol Reprod**, v. 52, n. 2, p. 305-312, 1995.

RUEDA, B. R.; TILLY, K. I.; BOTROS, I. W.; JOLLY, P. D.; HANSEN, T. R.; HOYER, P. B.; TILLY, J. L. Increased bax and interleukin-1 β -converting enzyme messenger ribonucleic acid levels coincide with apoptosis in the bovine corpus luteum during structural regression. **Biol Reprod**, v. 56, n. 1, p. 186-193, 1997.

RUNE, G. M.; WEHRENBURG, U.; PRANGE-KIEL, J.; ZHOU, L.; ADELMANN, G.; FROTSCHER, M. Estrogen up-regulates estrogen receptor α and synaptophysin in slice cultures of rat hippocampus. **Neuroscience**, v. 113, n. 1, p. 167-175, 2002.

SAPI, E.; BROWN, W. D.; ASCHKENAZI, S.; LIM, C.; MUNOZ, A.; KACINSKI, B. M.; RUTHERFORD, T.; MOR, G. Regulation of Fas ligand expression by estrogen in normal ovary. **J Soc Gynecol Investig**, v. 9, n. 4, p. 243-250, 2002.

SCHEID, M. P.; WOODGETT, J. R. Unravelling the activation mechanisms of protein kinase B/Akt. **FEBS Lett**, v. 546, n. 1, p. 108-112, 2003.

SCHLEIPEN, B.; HERTRAMPF, T.; FRITZEMEIER, K. H.; KLUXEN, F. M.; LORENZ, A.; MOLZBERGER, A.; VELDEERS, M.; DIEL, P. ER β -specific agonists and genistein inhibit proliferation and induce apoptosis in the large and small intestine. **Carcinogenesis**, v. 32, n. 11, p. 1675-1683, 2011.

SHIKONE, T.; YAMOTO, M.; KOKAWA, K.; YAMASHITA, K.; NISHIMORI, K.; NAKANO, R. Apoptosis of human corpora lutea during cyclic luteal regression and early pregnancy. **J Clin Endocrinol Metab**, v. 81, n. 6, p. 2376-2380, 1996.

SIMÕES, V. L.; ALVES, M. G.; MARTINS, A. D.; DIAS, T. R.; RATO, L.; SOCORRO, S.; OLIVEIRA, P. F. Regulation of apoptotic signaling pathways by 5 α -dihydrotestosterone and 17 β -estradiol in immature rat Sertoli cells. **J Steroid Biochem Mol Biol**, v. 135, n., p. 15-23, 2013.

SIMONCINI, T.; HAFEZI-MOGHADAM, A.; BRAZIL, D. P.; LEY, K.; CHIN, W. W.; LIAO, J. K. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. **Nature**, v. 407, n. 6803, p. 538-541, 2000.

SKARZYNSKI, D.; PIOTROWSKA-TOMALA, K.; LUKASIK, K.; GALVÃO, A.; FARBEROV, S.; ZALMAN, Y.; MEIDAN, R. Growth and regression in bovine corpora lutea: regulation by local survival and death pathways. **Reprod Domest Anim**, v. 48 Suppl 1, n., p. 25-37, 2013.

STOCCO, C.; TELLERIA, C.; GIBORI, G. The molecular control of corpus luteum formation, function, and regression. **Endocr Rev**, v. 28, n. 1, p. 117-149, 2007.

TAMURA, H. Role of the nongravid part of the uterus in the luteolytic effects of estrogen in pregnant rats. **Endocrinol Jpn**, v. 30, n. 5, p. 615-620, 1983.

TELLERIA, C. M.; GOYENECHE, A. A.; CAVICCHIA, J. C.; STATI, A. O.; DEIS, R. P. Apoptosis induced by antigestagen RU486 in rat corpus luteum of pregnancy. **Endocrine**, v. 15, n. 2, p. 147-155, 2001.

THORNTON, M. J.; TAYLOR, A. H.; MULLIGAN, K.; AL-AZZAWI, F.; LYON, C. C.; O'DRISCOLL, J.; MESSENGER, A. G. Oestrogen receptor beta is the predominant oestrogen receptor in human scalp skin. **Exp Dermatol**, v. 12, n. 2, p. 181-190, 2003.

TOWNSON, D. H.; WANG, X. J.; KEYES, P. L.; KOSTYO, J. L.; STOCCO, D. M. Expression of the steroidogenic acute regulatory protein in the corpus luteum of the rabbit: dependence upon the luteotropic hormone, estradiol-17 beta. **Biol Reprod**, v. 55, n. 4, p. 868-874, 1996.

VASKIVUO, T. E.; TAPANAINEN, J. S. Apoptosis in the human ovary. **Reprod Biomed Online**, v. 6, n. 1, p. 24-35, 2003.

WATANABE, C.; FUKUZAWA, K.; TANAKA, S.; AIDA-HYUGAJI, S. Charge Clamps of Lysines and Hydrogen Bonds Play Key Roles in the Mechanism to Fix Helix 12 in the Agonist and Antagonist Positions of Estrogen Receptor α : Intramolecular Interactions Studied by the Ab Initio Fragment Molecular Orbital Method. **J Phys Chem B**, v. 118, n. 19, p. 4993-5008, 2014.

WEIHUA, Z.; ANDERSSON, S.; CHENG, G.; SIMPSON, E. R.; WARNER, M.; GUSTAFSSON, J. A. Update on estrogen signaling. **FEBS Lett**, v. 546, n. 1, p. 17-24, 2003.

YIN, X. M.; OLTVAI, Z. N.; KORSMEYER, S. J. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. **Nature**, v. 369, n. 6478, p. 321-323, 1994.

YU, C. P.; HO, J. Y.; HUANG, Y. T.; CHA, T. L.; SUN, G. H.; YU, D. S.; CHANG, F. W.; CHEN, S. P.; HSU, R. J. Estrogen inhibits renal cell carcinoma cell progression through estrogen receptor- β activation. **PLoS One**, v. 8, n. 2, p. e56667, 2013.

ZALDIVAR, V.; MAGRI, M. L.; ZÁRATE, S.; JAITA, G.; EIJO, G.; RADL, D.; FERRARIS, J.; PISERA, D.; SEILICOVICH, A. Estradiol increases the Bax/Bcl-2 ratio and induces apoptosis in the anterior pituitary gland. **Neuroendocrinology**, v. 90, n. 3, p. 292-300, 2009.

ZHAO, C.; FUJINAGA, R.; TANAKA, M.; YANAI, A.; NAKAHAMA, K.; SHINODA, K. Region-specific expression and sex-steroidal regulation on aromatase and its mRNA in the male rat brain: immunohistochemical and in situ hybridization analyses. **J Comp Neurol**, v. 500, n. 3, p. 557-573, 2007.

CONSIDERAÇÕES

3 CONSIDERAÇÕES

O objetivo do presente trabalho foi de avaliar as ações exercidas pelo 17 β -estradiol sobre seus receptores (ER α e ER β) em células de corpo lúteo da cadela não prenhe. Este objetivo nos permitiu as seguintes conclusões:

Sobre os experimentos in vivo:

- O CL canino possui perfis de expressão diferentes para cada gene do receptor de estradiol (*ESR1* e *ESR2*), sendo o *ESR1* predominante no início do diestro e o *ESR2* a partir do meio até o final do diestro.
- Os genes e proteínas pró-apoptóticas aumentam a partir do dia 40 após a ovulação, o que ocorre junto com a diminuição do gene marcador de proliferação celular (*MKI67*).

Sobre os experimentos in vitro:

- O CL canino tem respostas diferentes para o estímulo com E2, de acordo com o receptor que estava bloqueado.
- O tratamento com E2 juntamente com o bloqueio do ER α estimula os genes pró-apoptóticos das vias extrínseca e intrínseca da apoptose. Este tratamento também diminui o *SLC2A4* e o *HSD3B1*, podendo assim diminuir a captação de glicose e a produção de P4.
- O tratamento com E2 com o bloqueio do ER β estimula os genes responsáveis pela esteroidogênese (*CYP11A1*, *CYP19A1* e *HSD3B1*) e pela captação de glicose (*SLC2A4*).

A partir destas conclusões podemos inferir que o E2 tem um papel importante no CL canino, com papéis fisiológicos diferentes e importantes em cada fase do diestro. O estradiol pode estimular o crescimento celular e o metabolismo no início do diestro, como também iniciar o processo de regressão do CL.

REFERÊNCIAS

REFERÊNCIAS

CASAI, M.; VALLCANERAS, S. S.; CAMPO VERDE ARBOCCO, F.; DELGADO, S. M.; HAPON, M. B.; SOSA, Z.; TELLERIA, C. M.; RASTRILLA, A. M. Estradiol promotes luteal regression through a direct effect on the ovary and an indirect effect from the celiac ganglion via the superior ovarian nerve. **Reprod Sci**, v. 19, n. 4, p. 416-422, 2012.

CHIMENTO, A.; SIRIANNI, R.; DELALANDE, C.; SILANDRE, D.; BOIS, C.; ANDÒ, S.; MAGGIOLINI, M.; CARREAU, S.; PEZZI, V. 17 beta-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ER alpha. **Mol Cell Endocrinol**, v. 320, n. 1-2, p. 136-144, 2010.

CONCANNON, P. W. Endocrinologic control of normal canine ovarian function. **Reprod Domest Anim**, v. 44, p. 3-15, 2009. Supplement, 2.

DAVIS, J. S.; RUEDA, B. R. The corpus luteum: an ovarian structure with maternal instincts and suicidal tendencies. **Front Biosci**, v. 7, n., p. d1949-1978, 2002.

DUFFY, D. M.; CHAFFIN, C. L.; STOUFFER, R. L. Expression of estrogen receptor alpha and beta in the rhesus monkey corpus luteum during the menstrual cycle: regulation by luteinizing hormone and progesterone. **Endocrinology**, v. 141, n. 5, p. 1711-1717, 2000.

FILIGHEDDU, N.; SAMPIETRO, S.; CHIANALE, F.; PORPORATO, P. E.; GAGGIANESI, M.; GREGNANIN, I.; RAINERO, E.; FERRARA, M.; PEREGO, B.; RIBONI, F.; BALDANZI, G.; GRAZIANI, A.; SURICO, N. Diacylglycerol kinase α mediates 17- β -estradiol-induced proliferation, motility, and anchorage-independent growth of Hec-1A endometrial cancer cell line through the G protein-coupled estrogen receptor GPR30. **Cell Signal**, v. 23, n. 12, p. 1988-1996, 2011.

KOWALEWSKI, M. P.; MUTEMBEI, H. M.; HOFFMANN, B. Canine prostaglandin E2 synthase (PGES) and its receptors (EP2 and EP4): expression in the corpus luteum during dioestrus. **Anim Reprod Sci**, v. 109, n. 1-4, p. 319-329, 2008.

KUMAR, A.; KLINGE, C. M.; GOLDSTEIN, R. E. Estradiol-induced proliferation of papillary and follicular thyroid cancer cells is mediated by estrogen receptors alpha and beta. **Int J Oncol**, v. 36, n. 5, p. 1067-1080, 2010.

LEWIS-WAMBI, J. S.; JORDAN, V. C. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? **Breast Cancer Res**, v. 11, n. 3, p. 206, 2009.

NISWENDER, G. D.; JUENGEL, J. L.; SILVA, P. J.; ROLLYSON, M. K.; MCINTUSH, E. W. Mechanisms controlling the function and life span of the corpus luteum. **Physiol Rev**, v. 80, n. 1, p. 1-29, 2000.

PELUFFO, M. C.; STOUFFER, R. L.; TESONE, M. Activity and expression of different members of the caspase family in the rat corpus luteum during pregnancy and postpartum. **Am J Physiol Endocrinol Metab**, v. 293, n. 5, p. E1215-1223, 2007.

SCAFFIDI, C.; FULDA, S.; SRINIVASAN, A.; FRIESEN, C.; LI, F.; TOMASELLI, K. J.; DEBATIN, K. M.; KRAMMER, P. H.; PETER, M. E. Two CD95 (APO-1/Fas) signaling pathways. **EMBO J**, v. 17, n. 6, p. 1675-1687, 1998.

SCHLEIPEN, B.; HERTRAMPF, T.; FRITZEMEIER, K. H.; KLUXEN, F. M.; LORENZ, A.; MOLZBERGER, A.; VELDERS, M.; DIEL, P. ER β -specific agonists and genistein inhibit proliferation and induce apoptosis in the large and small intestine. **Carcinogenesis**, v. 32, n. 11, p. 1675-1683, 2011.

SKARZYNSKI, D.; PIOTROWSKA-TOMALA, K.; LUKASIK, K.; GALVÃO, A.; FARBEROV, S.; ZALMAN, Y.; MEIDAN, R. Growth and regression in bovine corpora lutea: regulation by local survival and death pathways. **Reprod Domest Anim**, v. 48, p. 25-37, 2013. Supplement, 1.

STOCCO, C.; TELLERIA, C.; GIBORI, G. The molecular control of corpus luteum formation, function, and regression. **Endocr Rev**, v. 28, n. 1, p. 117-149, 2007.

VASKIVUO, T. E.; TAPANAINEN, J. S. Apoptosis in the human ovary. **Reprod Biomed Online**, v. 6, n. 1, p. 24-35, 2003.

ZALDIVAR, V.; MAGRI, M. L.; ZÁRATE, S.; JAITA, G.; EIJO, G.; RADL, D.; FERRARIS, J.; PISERA, D.; SEILICOVICH, A. Estradiol increases the Bax/Bcl-2 ratio and induces apoptosis in the anterior pituitary gland. **Neuroendocrinology**, v. 90, n. 3, p. 292-300, 2009.