UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
DEPARTAMENTO DE REPRODUÇÃO ANIMAL E RADIOLOGIA VETERINÁRIA

EFEITOS DA ADMINISTRAÇÃO DE ESTRADIOL E PROGESTERONA SOBRE A CONCENTRAÇÃO HORMONAL E EXPRESSÃO ENDOMETRIAL DOS RECEPTORES DE ESTRÓGENO E PROGESTERONA EM ÉGUAS RECEPTORAS ACÍCLICAS

ELISA SANT’ANNA MONTEIRO DA SILVA

BOTUCATU – SP
JUNHO/2015

UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
DEPARTAMENTO DE REPRODUÇÃO ANIMAL E RADIOLOGIA VETERINÁRIA

EFEITOS DA ADMINISTRAÇÃO DE ESTRADIOL E PROGESTERONA SOBRE A CONCENTRAÇÃO HORMONAL E EXPRESSÃO ENDOMETRIAL DOS RECEPTORES DE ESTRÓGENO E PROGESTERONA EM ÉGUAS RECEPTORAS ACÍCLICAS

ELISA SANT’ANNA MONTEIRO DA SILVA

Tese apresentada junto ao Programa de Pós-Graduação em Biotecnologia Animal para obtenção do título de Doutor.

Orientador: Prof. Dr. Cezinande de Meira

Co-orientador: Prof. Dr. José Paes de Oliveira Filho

BOTUCATU – SP
JUNHO/2015

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP

BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Silva, Elisa Sant'Anna Monteiro da.
Efeitos da administração de estradiol e progesterona sobre a concentração hormonal e expressão endometrial dos receptores de estrógeno e progesterona em éguas receptoras acíclicas / Elisa Sant'Anna Monteiro da Silva. - Botucatu, 2015

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina Veterinária e Zootecnia
Orientador: Cezinande de Meira
Coorientador: José Paes de Oliveira Filho
Capes: 50504002

1. Transferência de embriões. 2. Equino - Reprodução. 3. Estradiol. 4. Progesterona. 5. Reprodução animal. 6. Inseminação artificial. 7. Estrógenos - Receptores.

Palavras-chave: Equinos; Protocolos hormonais; Transferência de embriões.

Nome do autor: Elisa Sant'Anna Monteiro da Silva

Título: EFEITOS DA ADMINISTRAÇÃO DE ESTRADIOL E PROGESTERONA SOBRE A CONCENTRAÇÃO HORMONAL E EXPRESSÃO ENDOMETRIAL DOS RECEPTORES DE ESTRÓGENO E PROGESTERONA EM ÉGUAS RECEPTORAS ACÍCLICAS

COMISSÃO EXAMINADORA:

Prof. Dr. Cezinande de Meira
Presidente e Orientador
Departamento de Reprodução Animal e Radiologia Veterinária
Faculdade de Medicina Veterinária e Zootecnia – UNESP – Botucatu

Profa. Dra. Fabiana Ferreira de Souza
Membro
Departamento de Reprodução Animal e Radiologia Veterinária
Faculdade de Medicina Veterinária e Zootecnia – UNESP – Botucatu

Profa. Dra. Fernanda da Cruz Landim
Membro
Departamento de Reprodução Animal e Radiologia Veterinária
Faculdade de Medicina Veterinária e Zootecnia – UNESP – Botucatu

Profa. Dra. Paula de Carvalho Papa
Membro
Departamento de Cirugia
Faculdade de Medicina Veterinária e Zootecnia – USP – São Paulo

Prof. Dr. Guilherme Pugliesi

Membro

Departamento de Reprodução Animal

Faculdade de Medicina Veterinária e Zootecnia – USP – Pirassununga

Data da defesa: 22 de junho de 2015

DEDICATÓRIA

Aos meus pais, **Orlando Monteiro da Silva** e **Maria de Lourdes Sant'Anna Monteiro**, e irmão **Guilherme Sant'Anna Monteiro da Silva**, pelo exemplo, incentivo, apoio e amor. Por estarem sempre do meu lado nos momentos de dificuldade e de alegria. Sou eternamente grata a vocês.

AGRADECIMENTOS

Ao professor e orientador Prof. Cezinande de Meira, por todos os ensinamentos, confiança, oportunidades, amizade e agradáveis anos de convívio;

Ao professor e co-orientador José Paes de Oliveira Filho, pelos ensinamentos, paciência, confiança, oportunidades e por ceder o Laboratório de Biologia Molecular da Clínica Veterinária para que pudesse realizar a técnica de RT-qPCR;

Ao meu namorado Diego José Zanzarini Delfiol, por seu amor, amizade e companheirismo; pela paciência e compreensão durante os meses em que estive no exterior realizando o treinamento e posteriormente o estágio em pesquisa; por me tornar uma pessoa melhor e mais feliz;

Ao Samuel Catuci Fritsch, por toda a ajuda durante a coleta de dados do projeto, pelo companheirismo e parceria. Agradeço também a Anny Raissa Carolini Gomes, pela ajuda com a colheita de amostras durante o segundo experimento;

À Fernanda Saules Ignácio, pela amizade, pelas horas e horas de agradáveis conversas e principalmente pela ajuda na elaboração do projeto e coleta de dados do primeiro experimento;

À Yatta Linhares Boakari e ao Luiz Fernando Novaes Filho, pela amizade e ajuda na fase inicial da coleta de dados do primeiro experimento;

Ao Diogo Souza Zanoni, pela ajuda com a técnica de imunohistoquímica e com a avaliação das lâminas. Agradeço também ao Carlos Eduardo Fonseca Alves pela ajuda com a avaliação das lâminas e à Prof. Renée Laufer Amorim por ceder gentilmente o Laboratório de Imunohistoquímica para que pudéssemos realizar a técnica;

Ao professor José Carlos de Figueiredo Pantoja, pela realização das análises estatísticas e por sempre se dispor a me ajudar;

Ao Dr. Barry Ball, pesquisador do Gluck Equine Research Center – Lexington, KY – EUA, por me proporcionar a oportunidade de desenvolver um projeto em seu laboratório utilizando as técnicas de RT-qPCR e IHQ, o que me deixou mais preparada para a realização das técnicas durante o meu doutorado no Brasil.

Agradeço também à Kirsten Scoggin, técnica laboratorial do mesmo centro de pesquisa, pelos ensinamentos e acompanhamento durante a execução das técnicas.

À pesquisadora Dr. Janet Roser e técnica de laboratório Lillian Sibley, do Department of Animal Science - UC Davis, CA – EUA, por me receberem em seu laboratório para o desenvolvimento do projeto BEPE (Bolsa de Estágio e Pesquisa no Exterior) e pelos ensinamentos relativos a técnica de radioimunoensaio;

À amiga Denise Tsuzukibashi, pela amizade e companheirismo desde a época da estadia em Lexington até os dias atuais; pela paciência em me ouvir e por dividir os bons e maus momentos no dia-a-dia;

Às amigas Thatiane Kievitsbosch, Gabriela Dantas e Mariana Herman pela amizade, risadas, companheirismo e por tornar meus dias mais alegres;

À Faculdade de Medicina Veterinária e Zootecnia – UNESP – Botucatu, pelo apoio da instituição para o desenvolvimento desta tese;

À Fundação de Amparo a Pesquisa do Estado de São Paulo (Fapesp) pela concessão da bolsa de doutorado, o que tornou a execução do projeto possível; e pela concessão da bolsa BEPE, permitindo o aprimoramento do projeto.

LISTA DE TABELAS

LISTA DE FIGURAS

SUMÁRIO

SILVA, E.S.M. **Efeitos da administração de estradiol e progesterona sobre a concentração hormonal e expressão endometrial dos receptores de estrógeno e progesterona em éguas receptoras acíclicas**. Botucatu, 2015. 79p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho".

RESUMO

Com o objetivo de investigar os efeitos da administração de 2,5 mg de benzoato de estradiol (BE) seguido de 1500 mg de progesterona de longa ação (P4 LA) sobre a dinâmica molecular endometrial, características morfológicas do útero e concentrações plasmáticas de estrógeno e progesterona em éguas receptoras acíclicas, foram avaliados: a expressão proteica e as alterações na abundância relativa dos transcritos para os receptores de estrógeno (ER) e progesterona (PR) no endométrio por meio da RT-qPCR e imunohistoquímica; as características morfológicas do útero por ultrassonografia e palpação retal; e as concentrações plasmáticas do estrógeno e progesterona por meio de radioimunoensaio. A aplicação de 2,5 mg, 5 mg ou 10 mg de BE foram eficazes em causar edema uterino máximo 24 h após a administração. A dose de 2,5 mg de BE provocou picos de concentração de estradiol similares aos de éguas tratadas com 5 mg, 10 mg e aos de éguas cíclicas, sem elevar a concentração de estrógeno conjugado como observado com as outras doses de BE. Ainda, a concentração de P4 foi reduzida após a aplicação da P4 LA quando 10 mg de BE foram utilizados. A administração dos tratamentos hormonais alterou a abundância relativa dos transcritos para ERα, ERβ e PR de forma similar às alterações encontradas em éguas cíclicas quando o final do estro e início do diestro foram comparados. Em conclusão, o uso de 2,5 mg de BE seguido de 1500 mg de P4 LA provoca alterações similares na morfologia uterina, abundância relativa dos transcritos para ERα, ERβ e PR no endométrio, e de concentrações circulantes de estrógeno e progesterona às encontradas em éguas cíclicas.

Palavras-chave: Transferência de embriões; Protocolos hormonais; Equinos.

SILVA, E.S.M. **Effect of estradiol and progesterone administration on hormonal concentrations and endometrial expression of estrogen and progesterone receptors in non-cyclic recipient mares.** Botucatu, 2015. 79p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho".

ABSTRACT

To investigate the effects of the administration of 2.5 mg of estradiol benzoate (EB) followed by 1500 mg of long acting progesterone (LA P4) on endometrial molecular dynamics, uterine morphological characteristics, as well as estrogen and progesterone plasma concentrations in non-cyclic recipient mares, we evaluated: protein expression and changes in relative abundance of transcripts for estrogen (ER) and progesterone receptors (PR) in the endometrium by RT-qPCR and immunohistochemistry; uterine morphological characteristics by ultrasonography and rectal palpation; and estrogen and progesterone plasma concentrations by radioimmunoassay. The administration of 2.5 mg, 5 mg or 10 mg of EB were effective at promoting maximum uterine edema 24 h after their injection. The dose of 2.5 mg of EB caused similar estradiol concentration peaks to those observed in mares treated with 5 mg or10 mg of EB and to those observed in cyclic mares, which was not followed by increased concentrations of estrogen conjugate as observed for the other doses. Moreover, P4 concentration was reduced 24 h after LA P4 administration when the dose of 10 mg of EB was injected. The administration of the hormonal protocols altered the relative abundance of transcripts for ERα, ERβ and PR in non-cyclic mares as observed in cyclic ones when late estrus and early diestrus were compared. In conclusion, the use of 2.5 mg of EB followed by 1500 mg of LA P4 causes similar uterine morphology changes, relative abundance of transcripts for ERα, ERβ and PR, and estrogen and progesterone circulating concentrations to those found in cyclic mares.

Key-words: Embryo Transfer; Hormonal protocols; Equine.

Capítulo I

1 INTRODUÇÃO

A transferência de embriões (TE) em equinos é amplamente desenvolvida e disseminada no Brasil. A biotecnologia tem sido utilizada principalmente para a obtenção de múltiplos potros por ano de éguas doadoras de alto valor genético, além de possibilitar a produção de potros de éguas idosas, de animais que estejam em atividade esportiva ou que sejam subférteis por problemas adquiridos (ARRUDA et al., 2001).

A escolha da receptora é de fundamental importância para o sucesso da TE. Um importante fator limitante é o número de receptoras cíclicas disponíveis, principalmente no período de transição para a fase ovulatória, quando existe pressão econômica por algumas associações de criadores de equinos para que os potros nasçam o mais próximo possível do inicio do ano hípico. A influência do fotoperíodo sobre a incidência de ovulações ao longo do ano (GINTHER et al., 2004) e o manejo alimentar diferenciado oferecido à doadoras e receptoras (SILVA et al., 2014a) são importantes fatores que interferem na disponibilidade das receptoras cíclicas.

Com o objetivo de aumentar a oferta de receptoras nos programas de TE, diferentes protocolos hormonais utilizando estrógenos seguido de progesterona ou altrenogest são comumente aplicados para preparar o útero e assim estabelecer a gestação em éguas acíclicas (GRECO et al., 2012; ROCHA FILHO et al., 2004; SILVA et al., 2014a). A disponibilidade destes hormônios possibilita a indução de características uterinas similares às que ocorrem nas éguas cíclicas (PINTO, 2011), como a formação do edema endometrial causado pelos estrógenos e, posteriormente, aumento do tônus uterino provocado pela progesterona (VANDERWALL, 2011). Apesar de existirem protocolos que resultam em concentrações plasmáticas de progesterona conhecidas e adequadas para a manutenção da gestação (BRINGEL et al., 2003; ROCHA FILHO et al., 2004), não há estudos determinando a dose e/ou frequência de aplicação de estrógenos que, além de provocar edema endometrial, produzam perfil hormonal similar ao encontrado em éguas cíclicas durante o estro.

O estradiol e a progesterona exercem sua ação sobre o útero por meio da ligação a seus receptores, regulando a expressão gênica e a síntese de proteínas. Além disso, estes hormônios regulam a expressão dos seus próprios receptores, alterando o padrão de expressão durante o ciclo estral e início da gestação (HARTT et al., 2005). A modulação da expressão gênica e proteica é importante para o

estabelecimento e manutenção da prenhez (SPENCER;BAZER, 2002). Em éguas em anestro e transição, existem poucas descrições sobre o padrão de expressão dos receptores de estrógeno e progesterona no útero e, principalmente, sobre a expressão dos receptores em éguas acíclicas receptoras de embriões tratadas com protocolos hormonais.

Considerando que o útero é o órgão responsável pelo estabelecimento e manutenção da gestação, torna-se necessária a administração de protocolos utilizando estrógenos e progesterona/progestágenos que forneçam condições uterinas ideais para o pleno desenvolvimento do concepto, ou seja, condições similares ao observado em éguas cíclicas. Apesar disso, não há estudos que avaliem os efeitos das diferentes doses, hormônios ou protocolos comumente aplicados em éguas receptoras acíclicas sobre a dinâmica molecular uterina, características histomorfológicas e concentrações hormonais do estrógeno e progesterona.

Sendo assim, o objetivo deste estudo foi avaliar e comparar o efeito da administração do estradiol seguido de progesterona sobre a concentração hormonal e expressão gênica e proteica dos receptores endometriais de estrógeno e progesterona em éguas acíclicas. Além disso, também foram avaliadas as características ultrassonográficas e histológicas do útero após os tratamentos.

2 REVISÃO DE LITERATURA

2.1 Transferência de embriões em equinos e a importância das éguas receptoras

A TE é uma biotecnologia comumente empregada na indústria equina. Inicialmente, a técnica era realizada com o objetivo de se obter produtos de éguas com impossibilidade de se tornarem gestantes ou de levar uma gestação a termo. Nos últimos anos, a TE se tornou um meio para obtenção de múltiplos potros de doadoras geneticamente superiores ao longo do ano e de obter produtos de animais que estejam em competições esportivas. Desta forma, muitas associações de criadores de equinos podem obter e registrar mais de um produto por ano de uma mesma doadora com diferentes garanhões (SQUIRES, 2013).

A eficiência da TE é influenciada por diversos fatores que interferem no resultado final da biotécnica, como a experiência do técnico, qualidade do embrião, do sêmen, idade e histórico reprodutivo da doadora, e sincronia entre éguas doadora e receptora (CARNEVALE et al., 2000). Um dos fatores considerado ponto chave para o sucesso da técnica é a escolha e manejo das éguas receptoras, que serão encarregadas de levar a gestação do embrião transferido a termo (MCKINNON; SQUIRES, 2007).

A escolha da égua receptora deve se basear em alguns critérios de seleção relacionados à sanidade, desempenho reprodutivo e comportamental. As receptoras devem ser livres de enfermidades infecto-contagiosas, especialmente anemia infecciosa equina, babesiose, leptospirose e adenite equina (LOSINNO; ALVARENGA, 2006). Devem também apresentar boa conformação perineal (SQUIRES, 2003), cérvix íntegra, útero com ausência de líquido, cistos ou ar e ausência de anormalidades ovarianas (SQUIRES et al., 1999). Outro importante fator a se considerar é o comportamento da égua, pois um animal agitado e estressado representa risco para os profissionais e para o embrião (ALONSO, 2008).

Indica-se ainda a utilização de animais entre três e 10 anos de idade, uma vez que éguas mais velhas apresentam predisposição à degeneração do endométrio, o que pode comprometer a manutenção da gestação (RICKETS; ALONSO, 1991). Além disso, éguas jovens podem permanecer por mais tempo nos programas de TE

(LOSINNO; ALVARENGA, 2006). Preconiza-se também a utilização de receptoras com o peso semelhante ou superior ao da doadora, considerando que o tamanho do útero interfere no peso ao nascer do potro (ALLEN et al., 2002).

O manejo nutricional afeta diretamente a qualidade das receptoras. O fornecimento de alimentação inadequada ou a escassez de pastagens de qualidade interferem na ciclicidade das éguas, nas taxas de prenhez após a TE e, quando gestantes, afetam a qualidade do potro e comprometem a futura lactação (LOSINNO; ALVARENGA, 2006).

Diferentes categorias de receptoras podem ser utilizadas nos programas de TE, as ovariectomizadas (HINRICHS et al., 1985; MCKINNON et al., 1988), cíclicas (CARNEVALE et al., 2000; JACOB et al., 2012) ou acíclicas (anestro ou transição; LAGNEAUX;PALMER, 1993; SILVA et al., 2014a). Éguas ovariectomizadas foram muito utilizadas em pesquisas científicas nos primeiros anos do desenvolvimento da técnica, no entanto, em função da necessidade da realização de ovariectomia, não é mais utilizada. Há preferência por receptoras cíclicas, porém esta categoria é escassa, principalmente no início da estação reprodutiva, utilizando-se como alternativa as receptoras acíclicas tratadas com progesterona ou progestágenos.

2.2 Éguas receptoras acíclicas nos programas de TE

Éguas são caracterizadas como poliéstricas estacionais e a ocorrência de ciclos ovulatórios está associada aos períodos do ano de maior luminosidade diária. No entanto, aproximadamente 30% das éguas continuam a apresentar ciclos ovulatórios mesmo durante os períodos de menor luminosidade diária (AURICH, 2011). Além do fotoperíodo, a sazonalidade reprodutiva também depende de fatores como idade, raça, temperatura do ambiente e condição corporal (GINTHER, 1992; NAGY et al., 2000). Segundo Ginther (2004), a sazonalidade reprodutiva em éguas é dividida em quatro fases, baseada na dinâmica folicular: fase de anestro (inatividade ovariana), transição de primavera (aumento gradual do crescimento folicular), ovulatória (ciclos estrais regulares com folículos ovulatórios) e transição de outono (diminuição gradual da atividade ovariana).

Para algumas associações de criadores de equinos, o uso de receptoras na fase de transição de primavera é extremamente desejável (ROCHA FILHO et al.,

2004), uma vez que existe pressão econômica para que os potros nasçam o mais próximo possível do início do ano hípico (1° de janeiro no hemisfério norte e 1° de julho no hemisfério sul). No entanto, a oferta de alimentação diferenciada entre doadoras e receptoras nas propriedades rurais e centrais de TE é uma prática comum, fazendo com que éguas doadoras iniciem a atividade cíclica mais cedo no ano em relação as receptoras (SILVA et al., 2014a). Este fato tem estimulado pesquisadores a desenvolver métodos que antecipem a ciclicidade das éguas, por meio do uso da luz artificial para antecipar a primeira ovulação do ano (NAGY et al., 2000) ou que permitam a utilização das receptoras acíclicas (anestro ou transição) nos programas de TE por meio de tratamentos hormonais (BOTELHO et al., 2015; GRECO et al., 2012; ROCHA FILHO et al., 2004).

As taxas de gestação nas receptoras acíclicas são similares às das receptoras cíclicas na maioria das pesquisas realizadas (CARNEVALE et al., 2000; KAERCHER et al., 2013; ROCHA FILHO et al., 2004). Adicionalmente, existem algumas vantagens no uso de éguas acíclicas como receptoras, considerando que estas não precisam ser palpadas frequentemente para serem sincronizadas com as doadoras e não há necessidade de se realizar rufiações (HINRICHS; KENNEY, 1987). Em contrapartida, o uso de receptoras acíclicas implica em maiores custos com suplementação hormonal (PESSOA, 2012) e a administração de repetidas injeções intramusculares de P4 aumenta o risco de inflamações no local da aplicação (BERGFELT et al., 2007).

2.3 Protocolos hormonais para o preparo de receptoras acíclicas

Nos anos 80, utilizavam-se éguas ovariectomizadas como receptoras acíclicas. Hinrichs et al. (1985, 1986, 1987) realizaram as ovariectomias no mínimo três semanas antes de realizar as TEs, com o objetivo de reduzir o manejo com rufiações e palpações retais normalmente realizados em receptoras intactas.

A aplicação de apenas P4 ou altrenogest (progestágeno) foi realizada por Hinrichs et al. (1985,1986) para o preparo de receptoras ovariectomizadas. A administração de 300 mg diários de P4 por cinco dias antes da TE resultou em 75% (três gestações de quatro transferências) de taxa de gestação (Hinrichs et al., 1985). Ao comparar dois protocolos utilizando o altrenogest (22 mg diários por cinco dias ou 66 mg diários por seis dias antes da TE) com o protocolo utilizando 300 mg diários de

P4, Hinrichs et al. (1986) obtiveram 16% (1/6), 33% (2/6) e 40% (2/5) de prenhez. Os autores concluíram que o altrenogest foi capaz de estabelecer a gestação após a TE, apesar de que a dose de 22 mg pode ser insuficiente para a obtenção de taxas de prenhez adequadas. Ainda, Hinrichs e Kenney (1987) avaliaram a necessidade de realizar a sincronia entre a administração de P4 na receptora e ovulação da doadora. Foi observado que nas receptoras em que o tratamento hormonal foi iniciado dois dias após a ovulação da doadora as taxas de gestação foram superiores (6/8) em relação às taxas das éguas em que o início do tratamento hormonal ocorreu pelo menos quatro dias antes da ovulação da doadora (1/12), demonstrando assim a necessidade de sincronia.

Uma vez que o estrógeno estimula o aumento dos receptores uterinos de P4 e que o embrião equino secreta estradiol na fase inicial da gestação (ZAVY et al., 1979), McKinnon et al. (1988) utilizaram estradiol previamente e em conjunto à administração de progesterona ou altrenogest em três diferentes tratamentos. No primeiro protocolo foram administrados três a cinco dias de 17β-estradiol (E2) seguido da administração de 300 mg de P4 diários após a ovulação da doadora. O segundo protocolo foi similar ao primeiro, no entanto a aplicação do E2 foi continuada após a administração da P4. O terceiro tratamento também foi similar ao primeiro, com exceção da substituição da P4 por 0,044mg/kg de altrenogest diários. Os autores obtiveram 70% a 80% de taxa de gestação entre os protocolos testados (7/10, 8/10, 14/20), respectivamente, sugerindo que, independente da administração do E2, o requisito primário para o preparo e a manutenção da gestação é a concentração adequada de progesterona ou progestágenos.

A partir da década de 90 receptoras intactas acíclicas (anestro ou transição) foram introduzidas nos programas de TE. O uso desta categoria de receptoras dispensa a realização de cirurgia, tornando-se uma opção vantajosa. Tanto éguas ovariectomizadas quanto em anestro podem ser utilizadas com sucesso como receptoras de embriões (LAGNEAUX;PALMER, 1993).

Diferentes progestágenos disponíveis comercialmente foram avaliados por McKinnon et al. (2000) para avaliar a eficácia destes em manter a gestação em éguas após induzir a luteólise do corpo lúteo aos 18 dias de prenhez. Entre os diferentes hormônios testados, como a medroxiprogesterona, hidroxiprogesterona, altrenogest, norgestomet e megestrol, apenas o altrenogest foi eficiente em manter a gestação. Além disso, uma preparação de progesterona de longa ação (P4 LA) foi avaliada por

Bringel et al. (2003) em éguas sem o corpo lúteo primário, onde concentrações de P4 compatíveis com a fase luteal foram encontradas após a administração de 1500 mg a cada sete dias. A partir disso, Rocha Filho et al. (2004) administraram cipionato de estradiol (10 mg/dia por dois dias) seguido de P4 de curta (200mg/dia ou 400 mg a cada dois dias) ou longa ação (1500 mg a cada sete dias), com o objetivo de comparar as taxas de gestação em éguas acíclicas utilizando P4 de curta ou longa ação, observando taxas similares entre os diferentes tratamentos (em média 75,9%).

Apesar de existirem protocolos padronizados para a administração de progesterona ou altrenogest, que produzem concentrações plasmáticas de progesterona conhecidas e adequadas para a manutenção da gestação, não há estudos determinando a dose e/ou frequência de aplicação de estrógenos que, além de provocar edema endometrial, produzam perfil hormonal similar ao encontrado em éguas cíclicas durante o estro. Atualmente as receptoras recebem principalmente o benzoato de estradiol (BOTELHO et al., 2015; GRECO et al., 2008; KAERCHER et al., 2013; SILVA et al., 2014a), um éster do 17β-estradiol com meia vida de aproximadamente três dias (BÓ et al., 2006). Variadas doses e frequências de aplicação do benzoato de estradiol são descritas nas receptoras acíclicas, como 2,5 mg em dose única (SILVA et al., 2014a, 2015), 5 mg em doses decrescentes (GRECO et al., 2008) e 10 mg em doses decrescentes (KAERCHER et al., 2013), todos com o objetivo de causar edema uterino.

Após a observação do edema, as receptoras são tratadas com 1500 mg de P4 LA de três a oito dias antes das éguas receberem o embrião (BOTELHO et al., 2015; GRECO et al., 2012), ou altrenogest (0,044 mg/kg para Regumate® ou 22 a 44 ml para Progestal®) por quatro a seis dias antes da transferência (SILVA et al., 2014a, 2015). Uma vez confirmada a gestação, o tratamento com P4 ou altrenogest é comumente mantido até o período em que a unidade feto-placentária assume a manutenção da gestação, por meio da própria produção de progesterona/progestágenos aproximadamente aos 100 dias de gestação (HOLTAN et al., 1979), ou até a detecção dos corpos lúteos suplementares (SILVA et al., 2014a).

As taxas de gestação e morte embrionária em éguas acíclicas tratadas com protocolos hormonais variam de 53% a 76% e 11% a 46%, respectivamente (GRECO et al., 2012; KAERCHER et al., 2011, 2013; ROCHA FILHO et al., 2004; SILVA et al., 2015), enquanto as taxas descritas para éguas receptoras cíclicas variam em torno de 60% a 70% (JACOB et al., 2012), com taxa de mortalidade embrionária ao redor

de 16% (CARNEVALE et al., 2000). Vale salientar que as taxas de prenhez após a TE são influenciadas por diversos fatores, não só pelo tipo de protocolo hormonal utilizado, como a qualidade da receptora, experiência do técnico, qualidade do embrião, entre outros (CARNEVALE et al., 2000).

2.4 Mecanismos de ação dos estrógenos e progesterona

Os estrógenos (ex. estriol, estradiol, estrona), progesterona e progestágenos naturais, pertencentes ao grupo dos hormônios esteróides, são derivados do colesterol e sintetizados principalmente pelos ovários e placenta (BEATO; KLUG, 2000). Na espécie equina, o E2 e a P4 são os principais esteróides de origem ovariana (GINTHER, 1992).

A secreção de estrógenos envolve as células da teca e da granulosa. O colesterol atravessa a membrana plasmática das células da teca ligado a lipoproteínas, onde é armazenado em vacúolos citoplasmáticos e transportado para a membrana externa da mitocôndria. O LH liberado pela hipófise anterior se liga a seus receptores localizados na membrana das células da teca, mobilizando o colesterol para a via esteroidogênica. A proteína stAR (steroid acute regulatory protein) auxilia na transferência do colesterol para a membrana interna da mitocôndria, onde a enzima de clivagem de cadeia lateral do colesterol (P450scc) quebra o colesterol convertendo-o a pregnenolona (STOCKO;CLARK, 1996). A pregnenolona, por sua vez, é convertida a androstenediona, sendo então transportada através da membrana basal para as células da granulosa. Na granulosa, o hormônio FSH estimula a conversão da androstenediona a estrona e estradiol (CHRISTENSEN, 2011).

A síntese de progesterona é realizada principalmente pelas células do corpo lúteo. Assim como na síntese dos estrógenos, o colesterol é convertido a pregnenolona na mitocôndria. A pregnenolona é então transportada para o retículo endoplasmático liso, onde a enzima 3β-hidroxiesteróide desidrogenase a converte a P4.

Uma vez na circulação sanguínea, os hormônios esteróides são ligados a proteínas carreadoras e influenciam a atividade de células alvo que contêm receptores específicos para estrógeno e/ou P4. O modelo clássico que descreve o

mecanismo de ação do E2 e P4 envolve a ligação dos hormônios com receptores nucleares específicos (BEATO; KLUG, 2000). O complexo hormônio-receptor sofre ativação (processo que envolve alterações de conformação) e permite que o receptor se ligue a sítios específicos na cromatina. O complexo hormônio-receptor ativado interage com sequências específicas do DNA (chamadas “Steroid response elements” – SREs). A partir de então, o complexo atua como um fator de transcrição, modulando a síntese de RNAm e de proteínas, responsáveis pelos efeitos finais do E2 ou P4 (BAGCHI et al., 1992; DE FRANCO et al., 2002). Ao contrário do modelo clássico sobre o mecanismo de ação do E2 e P4, também conhecido como modelo genômico, existem evidências de que ambos os hormônios também atuam de forma rápida e não-genômica, por meio de receptores de membrana, não alterando a expressão gênica e síntese de proteínas. A ação genômica dos esteróides é geralmente detectada em horas ou dias, enquanto a não-genômica pode ser detectada em segundos (REVELLI et al., 1998). No entanto, estudos adicionais são necessários para elucidar o significado biológico das ações não-genômicas do estrógeno e P4 nos animais domésticos (BISHOP; STORMSHAK, 2008).

O E2 medeia várias de suas funções através da ligação e ativação dos receptores nuclares de estrógeno (ER), nas formas alfa (ERα) e beta (ERβ), expressos por diferentes genes (ENMARK et al., 1997). Estudos realizados em ratos relatam que o ERα é expresso na glândula hipofisária, ovários, útero, testículos, epidídimo e glândula adrenal (KUIPER et al., 1997), enquanto o ERβ é expresso principalmente nos ovários e próstata (ENMARK et al., 1997; KUIPER et al., 1996, 1997). No entanto, a dinâmica da expressão do ERβ também foi descrita no útero (MATSUZAKI et al., 1999; WEIHUA et al., 2000), incluindo no da espécie equina (HONNENS et al., 2011; SILVA et al., 2014). O importante papel do ERα foi confirmado em camundongos sem a presença do receptor, em que foi observada a ausência de resposta uterina à ação dos estrógenos (LUBAHN et al., 1993). Quanto ao ERβ, acredita-se que seja um modulador do ERα, apresentando efeito anti-proliferativo no útero, em oposição à ação do ERα. Adicionalmente, a ausência do ERβ em útero de camundongos experimentais causou redução na fertilidade (WEIHUA et al., 2000), o que demonstrou a sua importância no trato reprodutivo.

A P4 exerce suas funções genômicas principalmente por meio de dois tipos de receptores nucleares: isoforma A e isoforma B do receptor de progesterona (PR). Ambas isoformas são codificadas pelo mesmo gene, mas são reguladas por

promotores diferentes (MOTE et al., 2006). A isoforma A atua como inibidor e modulador da transcrição de outros receptores, enquanto os efeitos progestacionais típicos da P4 e derivados ocorrem por meio da ligação com o PR-B (GIANGRANDE; McDONNELL, 1999). No entanto, não existem informações sobre a expressão diferencial das isoformas do PR na espécie equina (PINTO, 2011).

Tanto o E2 quanto a progesterona P4 medeiam importantes alterações fisiológicas no útero. O E2, entre outras funções, tem participação no aumento da perfusão vascular durante o estro (BOLLWEIN et al. 2002; HONNENS et al., 2011), na formação do edema uterino em éguas (GINTHER, 1992) e na proliferação e diferenciação celular do endométrio e miométrio (AUPPERLE et al., 2000; MELLOR; THOMAS, 1995). Já a P4 está associada à quiescência do miométrio (THORBURN, 1993), ao aumento do tônus uterino e estímulo à secreção histotrófica (GINTHER, 1992; VANDERWALL, 2011), além da importante interação concepto-maternal (ALLEN, 2001; SPENCER; BAZER, 2002).

2.5 Dinâmica da expressão gênica e proteica dos receptores de estrógeno e progesterona

Os receptores de estrógeno e P4 são expressos de forma diferenciada durante o estro, diestro e início da gestação. A expressão gênica e proteica dos ER e PR são diferentes dentro dos tecidos que compõe o útero (endométrio, miométrio; SILVA et al., 2014b) e, ainda, entre os tipos celulares (epitélio luminal, epitélio glandular, estroma) que constituem os tecidos (AUPPERLE et al., 2000; HARTT et al., 2005; WATSON et al., 1992).

Estudos realizados em diversas espécies (GEISERT et al., 1993; MARTIN et al., 2008; OKUMU et al., 2010; OTT et al. 1993, SPENCER; BAZER, 1995, 2002), incluindo a espécie equina (HARTT et al., 2005; MCDOWELL et al., 1999; SILVA et al., 2014b; WATSON et al., 1992), mostram que durante o estro há aumento na expressão dos receptores para estrógeno e progesterona no endométrio, sendo encontrados no epitélio luminal, glandular e estroma. Durante o diestro, observa-se diminuição da expressão de ambos os receptores, principalmente no epitélio luminal e gladular. A dinâmica da expressão dos ER e PR aparenta estar positivamente e negativamente regulada pelo E2 e P4, respectivamente (HARTT et al., 2005). No

entanto, as alterações de expressão não podem ser atribuídas apenas às mudanças nas concentrações circulantes de estrógeno e P4. A produção local de fatores parácrinos também pode estar envolvida na regulação da expressão dos ER e PR (SPENCER; BAZER, 2002).

As alterações na expressão gênica e proteica dos ER e PR também são de extrema importância no ambiente uterino para o estabelecimento e manutenção da gestação. Ao contrário dos ruminantes, nos quais se observa a ausência do ER no epitélio luminal e glandular no início da gestação (BAZER et al., 2009; SPENCER; BAZER, 2002) os ER ainda são observados no endométrio de éguas, embora sua expressão esteja diminuída em relação ao período de estro (HARTT et al., 2005; SILVA et al., 2014b; WILSHER; ALLEN, 2001). Sabe-se que o útero de éguas gestantes é exposto a estrógenos, originados do embrião, aproximadamente desde o dia 10 pós-ovulação (CHOI et al., 1997). Em suínos, Ka et al. (2001) sugerem que o estrógeno de origem embrionária estimula o aumento na secreção histotrófica e que atua de forma parácrina no epitélio luminal e glandular para aumentar a produção de fatores de crescimento, os quais estimulam o desenvolvimento do trofoblasto. É possível que a presença dos ER no endométrio de éguas no período inicial da gestação também exerça importante papel na função secretória uterina, embora estes mecanismos ainda não tenham sido investigados em equinos (SILVA et al, 2014b).

Em relação aos PR, nos primeiros dias pós-ovulação (dias cinco a 14) observa-se redução em sua expressão, principalmente no epitélio luminal e glandular. McDowell et al. (1999) e Hartt et al. (2005) relataram que a expressão gênica dos PR no dia 14 do ciclo já é menor em éguas gestantes em relação às cíclicas. Os autores sugeriram que o referido momento é crítico para o estabelecimento da gestação e que, possivelmente, a menor concentração dos PR no endométrio seria uma resposta à sinalização do embrião presente no útero. Acredita-se também que a supressão do PR endometrial durante a fase inicial da gestação seja um pré requisito para a produção de proteínas secretórias e para o transporte seletivo de moléculas para o útero, de forma a suprir o concepto em desenvolvimento (BAZER et al., 2009). Em ruminantes, foi sugerido que a diminuição dos PR no epitélio glandular seja essencial para a hiperplasia e hipertrofia das glândulas endometriais durante o início da gestação (SPENCER;BAZER, 2002).

Apesar da redução observada nos epitélio luminal e glandular, a expressão dos PR ainda se mantém evidente no estroma durante o início da gestação tanto em

ruminantes (SPENCER; BAZER, 2002) quanto em equinos (SILVA et al., 2014b; WILSHER; ALLEN 2011). A ação da P4 sobre o endométrio durante a gestação parece ser mediada pelos seus receptores que se mantêm ativos no estroma e que de forma parácrina estimulam os demais tipos celulares do útero (SPENCER e BAZER, 2002).

É interessante mencionar que a administração de altrenogest ou P4 no início da gestação acelerou a supressão dos PR em éguas cíclicas (WILLMANN et al., 2011) e em vacas (OKUMU et al., 2010). Tal achado fortalece a hipótese de que altas concentrações de P4 reduzem a expressão do seus receptores e que a supressão destes no endométrio está associada com a receptividade uterina necessária para o desenvolvimento do concepto, reconhecimento materno da gestação e posterior implantação (BAZER et al., 2009; OKUMU et al., 2010; SPENCER; BAZER, 2002; WILMANN et al., 2011).

Para tentar esclarecer os mecanismos envolvidos na regulação dos estrógenos e/ou P4 sobre os receptores esteroidais no útero, estudos em animais acíclicos tratados com hormônios exógenos foram desenvolvidos. A expressão gênica e proteica dos ER e PR, após aplicação de estrógenos e P4, foram relatadas em éguas em anestro fisiológico (McDOWELL et al., 1999) e em vacas e ovelhas ovariectomizadas (KIMMINS; MACLAREN, 2001; WU et al., 1996). Apesar da utilização de diferentes protocolos hormonais (dose e duração do tratamento com estrógenos e/ou P4), foram encontrados resultados similares nas pesquisas realizadas nas fêmeas acíclicas. Foi observado aumento na abundância do RNAm que codifica o receptor de estrógeno somente após a aplicação do E2 (McDowell et al., 1999). Além disso, não se verificou efeito da administração de progesterona sobre a expressão dos ER ou dos próprios receptores de P4 no endométrio (KIMMINS; MACLAREN, 2001; WU et al., 1996).

Vale salientar que, nas fêmeas acíclicas que não receberam tratamento hormonal, foram observadas quantidades relativamente altas dos RNAm que codificam os ER e PR endometriais em éguas (McDOWELL et al., 1999) e dos respectivos receptores (proteínas) em vacas (KIMMINS; MACLAREN, 2001). Adicionalmente, as alterações na expressão gênica dos ER e PR das éguas em anestro tratadas com E2 e P4 não foram tão acentuadas quanto às observadas nas éguas cíclicas (McDOWELL et al., 1999).

3 REFERÊNCIAS

ALLEN, 2001. Luteal deficiency and embryo mortality in the mare. **Reproduction of Domestic Animals**, v. 36, p. 121-131, 2001.

ALLEN, W.R. et al. Influence of maternal size on placental, fetal and postnatal growth in the horse. I. Development in the uterus. **Reproduction**, v. 123; p. 445-453, 2002.

ALONSO, M.A. Seleção, manejo e fatores que influenciam as taxas de prenhez em éguas receptoras de embriões. **Acta Scientiae Veterinariae**, Supl. 2., v. 36, p. s207-s214, 2008.

ARRUDA, R. P. et al. Existem relações entre tamanho e morfoecogenicidade do corpo lúteo detectados pelo ultra-som e os teores de progesterona plasmática em receptoras de embrião equinos? **Brazilian Journal of Veterinary Research and Animal Science**, v. 38, n. 5, p. 233-239, 2001.

AUPPERLE, H. et al. Cyclical endometrial steroid hormone receptor expression and proliferation intensity in the mare. **Equine Veterinary Journal**, v. 32, n. 3, p. 228-232, 2000.

AURICH, C. Reproductive cycles of horses. **Animal Reproduction Science**, v.124, p. 220-228, 2011.

BAGCHI, M.K. et al. Analysis of the mechanism of steroid hormone receptor-dependent gene activation in cell-free systems. **Endocrine Reviews**, v. 13, n. 3, p. 525-535, 1992.

BAZER, F.W. et al. Comparative aspects of implantation. **Reproduction**, v. 138, p. 195-209, 2009.

BEATO, M.; KLUG, J. Steroid hormone receptors: an update. **Human Reproduction Update**, v.6, n.3, p. 225-236, 2000.

BERGFELT, D.R. et al. Ovulation synchronization following commercial application of ultrasound-guided follicle ablation during the estrous cycle in mares. **Animal Reproduction Science**, v. 68, p. 1183-1191, 2007.

BISHOP, C.V.; STORMSHAK, F. Non-genomic actions of progesterone and estrogens in regulating reproductive events in domestic animals. **Veterinary Jornal**, v. 176, p. 270–80, 2008

BOLLWEIN, H. at al. Uterine and ovarian blood flow during the estrous cycle in mares. **Theriogenology**, v. 57, p. 2129-2138, 2002.

BOTELHO, J.H.V. et al. Hormone supplementation protocol using estradiol benzoate and long-action progesterone is efficient in maintaining pregnancy of anovulatory recipient mares during autumn transitional phase. **Animal Reproduction Science,** v. 152, p. 39-43, 2015.

BRINGEL, B.A. et al. Biorelease progesterone LA 150 and its application to overcome effects of premature luteolysis on progesterone levels in mares. **Revista Brasileira de Reprodução Animal,** v. 27, p. 498-500, 2003.

CARNEVALE, E.M. et al. Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. **Theriogenology,** v.54, p. 965-79, 2000.

CHOI, S.J., ANDERSON, G.B., ROSER, J.F. Production of free estrogens and estrogen conjugates by the preimplantation equine embryo. **Theriogenology,** v. 47, p. 457-466, 1997.

CHRISTENSEN, B.W. Estrogens. In: MC KINNON, A.O.; SQUIRES, E.L.; VAALA, W.E.; VARNER, D.D. **Equine Reproduction**. 2 ed. Ames:Blackwell Publishing, 2011, vol.2, cap.169, pg. 1631-1636.

DE FRANCO, D.B. Navigating steroid hormone receptors through the nuclear compartment. **Molecular Endocrinology,** v. 16, p. 1449–1455, 2002.

ENMARK, E. et al. Human estrogen receptor b-gene structure, chromosomal localization, and expression pattern. **Journal of Clinical Endocrinology and Metabolism,** v. 82, p. 4258-4265, 1997.

GEISERT, R.D. et al. Immunocytochemical localization and changes in endometrial progestin receptor protein during the porcine oestrous cycle and early pregnancy. **Reproduction, Fertility and Development**, v. 6, p. 749-760, 1994.

GIANGRANDE, P.H.; McDONNELL, D.P. The A and B isoforms of the human progesterone receptor: two functionally different transcription factors encoded by a single gene. Recent Progress in Hormone Research, v. 54, p. 291–313, 1999.

GINTHER, O.J. **Reproductive Biology of the Mare: Basic and Applied Aspects**. 2. ed. Cross Plains:Equiservices, 1992. 642 p.

GINTHER, O.J. et al. Seasonal influence on equine follicle dynamics. **Animal Reproduction**, v. 1, n. 1, p. 31-44, 2004.

GRECO, G.M. et al. Effect of interrupting the progesterone treatment in pregnant acyclic recipient mares showing supplementary corpora lutea formation. **Acta Scientiae Veterinariae**, Supl. 2, v. 36, p. 590, 2008.

GRECO, G.M. et al. Use of long-acting progesterone to acyclic embryo recipient mares. **Revista Brasileira de Zootecnia,** v. 41, n. 3, p. 607-611, 2012.
HARTT, L.S. et al. Temporal and spatial associations of oestrogen receptor alpha and progesterone receptor in the endometrium of cyclic and early pregnant mares. **Reproduction,** v. 130, p. 241-250, 2005.

HINRICHS, K. et al. Pregnancy in ovariectomized mares achieved by embryo transfer. **Equine Veterinary Journal,** Suppl. 3, p. 74-75, 1985.

HINRICHS, K.; SERTICH, P.L.; KENNEY, R.M. Use of altrenogest to prepare ovariectomized mares as embryo transfer recipients. **Theriogenology**, v.26, p. 455-60, 1986.

HINRICHS, K.; KENNEY, R.M. Effect of timing of progesterone administration on pregnancy rate and embryo transfer in ovariectomized mares. **Journal of Reproduction and Fertility Supplement**, v. 35, p. 439-443, 1987.

HOLTAN, D.W.; NETT, T.M.; ESTERGREEN, V.L. Plasma progesterone in pregnant mares. **Journal of Reproduction and Fertility**, Supl. 23, p. 419-424, 1975.

HONNENS, A. et al. Relationships between uterine blood flow, peripheral sex steroids, expression of endometrial estrogen receptors and nitric oxide synthases during the estrous cycle in mares. **Journal of Reproduction and Development**, v. 57, n.1, p. 43-48, 2011

JACOB, J.C.F. et al. Effect of embryo age and recipient asynchrony on pregnancy rates in a commercial equine embryo transfer program. **Theriogenology**, v. 77, p. 1159-1166, 2012.

KAERCHER, F. et al. Embryo Transfer in no Cycling Crioula and Quarter Horse Breeds Treated with Estradiol Cipionate and Long-acting Progesterone. **Biology and Technology,** v. 54, n. 6, p. 1147-1150, 2011.

KA, H. et al. Keratinocyte growth factor is up-regulated by estrogen in the porcine uterine endometrium and functions in trophectoderm cell proliferation and differentiation. **Endocrinology**, v. 142, p. 2303-2310, 2001.

KAERCHER, F. et al. Embryo transfer in anovulatory recipient mares treated with estradiol benzoate and long-acting progesterone. **Journal of Equine Veterinary Science,** v. 33, n. 3, p. 205-209, 2013.

KIMMINS, S.; MACLAREN M.A. Oestrous cycle and pregnancy effects on the distribution of oestrogen and progesterone receptors in bovine endometrium. **Placenta**, v. 22, p. 742-748, 2001.

KUIPER, G.G. et al. Cloning of a novel receptor expressed in rat prostate and ovary. **Proceedings of the National Academy of Sciences,** v. 93, p. 5925–5930, 1996.

KUIPER, G.G. et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. **Endocrinology,** v. 138, p. 863-870, 1997.

LAGNEAUX, D.; PALMER, E. Embryo transfer in anoestrous recipient mares: attempts to reduce altrenogest administration period by treatment with pituitary extract**. Equine Veterinary Journal**, Supl. 15, p. 107-110, 1993.

LOSINNO, L; ALVARENGA, M.A. Fatores críticos em programas de transferência de embriões em equinos no Brasil e Argentina. In: Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, 2006, Araxá. **Acta Scientiae Veterinariae**, v. 34, p. 39-49, 2006.

LUBAHN, D.B. et al. Alteration of reproduction function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. **Proceedings of the National Academy of Sciences**, v. 90, p. 11162-6, 1993.

MARTIN, I. et al. Immunohistochemical detection of receptors for oestrogen and progesterone in endometrial glands and stroma during the oestrous cycle in nelore (*Bos Taurus indicus*) cows. **Reproduction of Domestic Animals**, v. 43, p. 415-421, 2008.

MATSUZAKI, S. et al. Oestrogen receptor alpha and beta mRNA expression in human endometrium throughout the menstrual cycle. **Molecular Human Reproduction,** v. 5 n. 6, p. 559-64, 1999.

McDOWELL, K.J. et al. Changes in equine endometrial oestrogen receptor α and progesterone receptor mRNAs during the oestrous cycle, early pregnancy and after treatment with exogenous steroids. **Journal of Reproduction and Fertility,** v. 117, p. 135-142, 1999.

McKINNON, A.O. et al. Ovariectomized steroid-treated mares as embryo transfer recipient and as a model to study the role of progestins in pregnancy maintenance. **Theriogenology,** v. 29, p. 1055-63, 1988.

McKINNON, A.O. et al. The inability of some synthetic progestogens to maintain pregnancy in the mare. **Equine Veterinay Journal**, v. 32, p. 83-85, 2000.

McKINNON, A.O.; SQUIRES, E.L. Embryo transfer and related technologies. In: SAMPER, J.C.; PYCOCK, J.F.; MCKINNON, A.O. **Current therapy in equine reproduction**. Philadelphia: W.B. Sounders, 2007, p. 319-334.

MELLOR, S.J.; THOMAS, E.J. Interactions between oestradiol and epidermal growth factor in endometrial stromal proliferation and differentiation. **Journal of Reproduction and Fertility**, v.1 04, p. 157-164, 1995.

MOTE, P.A. et al. Overlapping and distinct expression of progesterone receptors A and B in mouse uterus and mammary gland during the estrous cycle. **Endocrinology**, v. 147, p. 5503-5512, 2006.

NAGY, P.; GUILLLAUME, D.; DAELS, P. Seasonality in mares. **Animal Reproduction Science**, 60-61, p.245-62, 2000.

OKUMU, L.A. et al. Expression and localization of progesterone and oestrogen receptors in the bovine uterus. **Reproduction**, v. 140, p. 143-153, 2010.

OTT, T.L. et al. Changes in progesterone and oestrogen receptor mRNA and protein during maternal recognition of pregnancy and luteolysis in ewes. **Journal of Molecular Endocrinology,** v. 10, p. 171-183, 1993.

PESSOA, M.A. Custos envolvidos em central de reprodução equina (central de TE). In: Conferência Anual da Abraveq, 2012, Campinas. **Revista Brasileira de Medicina Veterinária Equina**, v. 41, p. 99-102, 2012.

PINTO, C.R.F. Progestagens and Progesterone. In: MC KINNON, A.O.; SQUIRES, E.L.; VAALA, W.E.; VARNER, D.D. **Equine Reproduction**. 2 Ed. Ames:Blackwell Publishing, 2011, vol.2, cap.189, pg. 1811-1817.

REVELLI, A.; MASSOBRIO, M., TESARIK, J. Nongenomic actions of steroid hormones in reproduction tissues. **Endocrine Reviews**, v. 19, p. 3-17, 1998.

RICKETS, S.W.; ALONSO, S. The effect of age and parity on the development of equine chronic endometrial disease. **Equine Veterinary Journal**, v. 23, n. 3, p. 189-192, 1991.

ROCHA FILHO, A.N. et al. Transfer of equine embryos into anovulatory recipients supplemented with short or long acting progesterone. **Animal Reproduction**, v. 1, n. 1, p. 91-95, 2004.

SILVA, E.S.M. et al. Supplementary corpora lutea monitoring allows progestin treatment interruption on day 70 of pregnancy in non-cyclic recipient mares. **Animal Reproduction Science**, v. 144, p. 122-128, 2014a.

SILVA, E.S.M. et al. Expression of receptors for ovarian steroids and prostaglandin E2 in the endometrium and myometrium of mares during estrus, diestrus and early pregnancy. **Animal Reproduction Science**, v. 151, p. 169-181, 2014b.

SILVA, E.S.M. et al. Ultrasonography of the conceptus development from days 15 to 60 of pregnancy in non-cyclic recipient mares. **Ciência Rural**, v. 45, n. 3, p. 512-518, 2015.

SPENCER, T.E.; BAZER, F.W. Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. **Biology of Reproduction**, v. 53, p.1527-1543, 1995.

SPENCER, T.E.; BAZER, F.W. Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. **Frontiers in Bioscience**, v. 7, p. 1879-1898, 2002.

SQUIRES, E.L.; MCCUE, P.M.; VANDERWALL, D. The current status of equine embryo transfer. **Theriogenology**, v. 51, n. 1, p. 91-104, 1999.

SQUIRES, E.L. Management of the embryo donor and recipient mare. In: ROBINSON, N.E. **Current therapy in equine medicine**. Philadelphia: Saunders, 2003, p. 277-279.

SQUIRES, E.L. Embryo transfer challenges and perspectives. Brazilian Journal of Animal Reproduction, v. 37, n. 2, p. 105-107, 2013.

STOCCO, D.M.; CLARK, B.J. Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. **Biochemical Pharmacology**, v. 51, p. 197–205, 1996.

THORBURN, G.D. A speculative review of parturition in the mare. **Equine Veterinary Journal Supplement**, v. 14, p. 41-49, 1993.

VANDERWALL, D.K. Progesterone. In: MC KINNON, A.O.; SQUIRES, E.L.; VAALA, W.E.; VARNER, D.D. **Equine Reproduction**. 2 Ed. Ames:Blackwell Publishing, 2011, vol.2, cap.170, pg. 1637-1641.

WATSON, E.D.; SKOLNIK, S.B.; ZANECOSKY, H.Z. Progesterone and estrogen receptor distribution in the endometrium of the mare. **Theriogenology,** v. 38, p. 575-580, 1992.

WEIHUA, Z. et al. Estrogen receptor (ER) α, a modulator of ERβ in the uterus. **Proceedings of the National Academy of Sciences**, v. 97, n. 11, p. 5936-5941, 2000.

WILLMANN, C. et al. Influences of treatment of early pregnant mares with the progestin altrenogest on embryonic development and gene expression in the endometrium and conceptus. **Theriogenology**, v. 76, p. 61-73, 2011.

WILSHER, S., GOWER, S., ALLEN, W.R. Immunohistochemical local-ization of progesterone and oestrogen receptors at the placentalinterface in mares during early pregnancy. **Animal Reproduction Science**, v. 129, p. 200-208, 2011

WU, W.X. et al. Regulation of the estrogen receptor and its messenger ribonucleic acid in the ovariectomized sheep myometrium and endometrium: the role of estradiol and progesterone. **Biology of Reproduction**, v. 55, p. 762-768, 1996.

ZAVY, M.T et al. An investigation of the uterine luminal environment of non-pregnant and pregnant pony mares. **Journal of Reproduction and Fertility Supplement,** v. 27, p. 403-11, 1979.

Capítulo II

O artigo a seguir foi redigido de acordo com as normas do periódico científico 'Domestic Animal Endocrinology': http://www.elsevier.com/journals/domestic-animal endocrinology/0739-7240/guide-for-authors.

Comparison of different regimens of estradiol benzoate treatments followed by long-acting progesterone to prepare non-cycling mares as embryo recipients

E.S.M. Silva[a], J.F. Roser[b], A.R.C. Gomes[a], S.C. Fritsch[a], J.C.F. Pantoja[c], J.P. Oliveira-Filho[d], C. Meira[a]

[a] *School of Veterinary Medicine and Animal Science, Univ. Estadual Paulista (UNESP), Department of Animal Reproduction and Veterinary Radiology, Botucatu, São Paulo, Brazil*
[b] *Department of Animal Science, University of California, Davis, California, USA*
[c] *School of Veterinary Medicine and Animal Science, Univ. Estadual Paulista (UNESP), Department of Veterinary Hygiene and Public Health, São Paulo State University, Botucatu, São Paulo, Brazil*
[d] *School of Veterinary Medicine and Animal Science, Univ. Estadual Paulista (UNESP), Department of Veterinary Clinical Science, São Paulo State University, Botucatu, São Paulo, Brazil*

Abstract

The present study evaluated the influence of different regimens of estradiol benzoate (EB) treatments followed by a single dose of long acting progesterone (LA P4) on plasma estrogen and progesterone (P4) concentrations in non-cyclic mares prepared as embryo recipients. Twenty one anestrous mares were distributed into three groups (n=7 mares/group), according to the EB dose received (single dose of 2.5 mg, total of 5 mg in decreasing doses for two consecutive days, and total of 10 mg in decreasing doses in three consecutive days), which was followed by a single administration of 1500 mg of LA P4 in all groups. Mares were reevaluated during the ovulatory phase and seven of them became part of the cyclic non-treated control group. Mares were evaluated by ultrasonography to monitor endometrial edema and blood samples were collected to measure estradiol (E2), estrogen conjugate (EC) and P4 by radioimmunoassay. After EB injection, maximum uterine edema was achieved 24 h after administration in all treated groups. Maximum E2 concentrations were observed 24 h after the

first EB injection in treated groups and there were no differences ($P > 0.05$) among treatments. Maximum EC concentration was observed 24 h after the single EB injection in the 2.5 mg group, whereas in 5 mg and 10 mg groups EC peaks were observed 48 h after the first EB administration. Maximum P4 concentrations were detected 24 h after LA P4 injection, although higher P4 concentrations were observed in the group treated with 2.5 mg of EB than in group 10 mg of EB ($P < 0.05$). Because P4 concentrations were reduced after administration of high doses of EB, we also measured 17α-hydroxyprogesterone (17-OH-P) to test the hypothesis that high concentrations of EB would accelerate the conversion of P4 to 17-OH-P. However, 17-OH-P concentrations paralleled P4 profile in all groups, irrespective of EB doses. In summary, the three EB treatment regimens induced similar E2 peaks, although the observation of EC peaks 24 h after E2 peaks in groups 5 and 10 mg indicate that an excess of E2 was given, which was converted into EC to be inactivated. Administration of 10 mg of EB reduced P4 concentrations 24 h after LA P4 was given. We demonstrated that the mechanism by which this reduction occurred was not by an increase in progesterone metabolism to 17α-OH-P. In conclusion, the use of 2.5 mg of EB followed by 1500 mg of LA P4 appears to be the appropriate regimen to prepare non-cyclic mares as embryo recipients.

Key-words: Equine Embryo Transfer, Recipient Mares, Hormonal Protocols.

1. Introduction

Equine embryo transfer (ET) is a worldwide technique in which the highest number of transferred embryos/year is found in Brazil, Argentina and the United States of America [1]. However, a major limiting factor in the ET programs is the reduced number and quality of recipient mares during the breeding season, especially during the spring transitional period. For some horse breed associations, the use of recipient mares during the spring transitional period

at the beginning of the breeding season is very desirable [2], since there is economic pressure for foals to be born early in the foaling season to enhance their athletic performance.

In order to increase the offer of recipient mares in ET programs, exogenous estrogen and progesterone (P4) treatments are usually administered to non-cyclic mares [2-7]. These hormones (natural or synthetic) induce similar uterine changes to those which occur in cyclic mares that become pregnant [8], such as endometrial edema caused by estradiol followed by increased uterine tone and stimulus to histotrophic secretion as a result of exogenous progestin treatment [9].

Currently, non-cyclic recipient mares often receive estradiol benzoate (EB) [7, 10, 11], an ester of the natural estrogen 17β-estradiol (E2) with an approximate half-life of three days [12]. After the observation of endometrial edema, long acting progesterone (LA P4; half-life of seven days) is administered three to eight days prior to embryo transfer [2, 13], or altrenogest is given during four to six days before transfer [7, 14], in order to achieve an appropriate progesterone concentration (≥ 2.5 ng/mL) [15] at the time of ET.

Although protocols for exogenous P4 are already described, which produce known and suitable P4 concentrations for pregnancy maintenance [16], EB administration is empirical, with different reports of dose and administration frequencies for preparing non-cyclic recipient mares; a single dose of 2.5 mg [7, 14] and decreasing doses of 5 mg [17] or 10 mg [10, 11].

To our knowledge, there are no studies comparing EB regimens to select the treatment that causes endometrial edema and produce similar estrogen concentrations to those found in cyclic mares during estrus. The knowledge of an exogenous estrogen protocol that is more compatible with endogenous estrogen concentrations found in cyclic mares would allow an optimization of the hormone use in non-cyclic embryo recipient mares. Therefore, the objective of this study was to evaluate the influence of different regimens of EB followed by a single

dose of LA P4 on plasma estrogen and P4 concentrations in non-cyclic mares prepared for ET, as well as to evaluate the degree of endometrial edema after treatments.

2. Material and methods

2.1 Animals and experimental groups

Twenty one cross-bred mares from five to 15 years and weighing between 350 and 450 kg were used in the study. Mares were maintained on coast-cross hay (*Cynodon dactylon*) with water and trace-mineralized salt *ad-libitum*. The experiment was conducted from June to December at the Equine Reproduction and Biotechnology Center – Lageado Farm, School of Veterinary Medicine and Animal Science, Botucatu – São Paulo, Brazil. Animal procedures were approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, Univ. Estadual Paulista (CEUA-200/2014).

The same mares were used during anestrous and cyclic phases. Anestrous mares were those showing ovarian follicles < 20 mm in diameter, absence of a corpus luteum and progesterone concentrations < 1ng/mL on three evaluations at seven day intervals. Cyclic mares were those showing regular estrous cycles and ovulatory follicles. The twenty one anestrous mares selected to the experiment were distributed into three groups (n=7 mares/group), according to estradiol benzoate regimen followed by the same dose of LA P4: 2.5 mg EB + LA P4, 5 mg EB + LA P4, and 10 mg EB + LA P4. The anestrous mares were reevaluated during the cyclic phase and seven of them became part of the control group. A cyclic control group was used to compare artificially induced reproductive phases to physiological reproductive phases.

2.2 Hormonal treatments

Mares from group 10 mg EB + LA P4 received 10 mg of EB (Estrogin®, Farmavet, SP, Brazil) intramuscularly in decreasing doses (5, 3 and 2 mg on consecutive days) and 24 h after the last EB administration 1500 mg of intramuscularly LA P4 (Sincrogest Injetável®, Ourofino, SP, Brazil) was given (Fig. 1A). Group 5 mg EB + LA P4 received 5 mg of EB in decreasing doses (3 and 2 mg on consecutive days) and 24 h after the last EB administration, 1500 mg of intramuscularly LA P4 was given (Fig. 1B). Group 2.5 mg EB + LA P4 received a single 2.5 mg dose of EB and 48 h after its administration 1500 mg of intramuscularly LA P4 was injected (Fig. 1C). Control group consisted of cyclic mares which did not receive EB and LA P4 treatments. During the cyclic phase of the control group mares, ovulation induction was performed using 1500 IU of hCG (Vetecor®, Hertape Calier, MG, Brazil) after detection of a ≥ 35 mm follicle and uterine edema, to synchronize evaluation days between natural and artificial cycles (Fig. 1D).

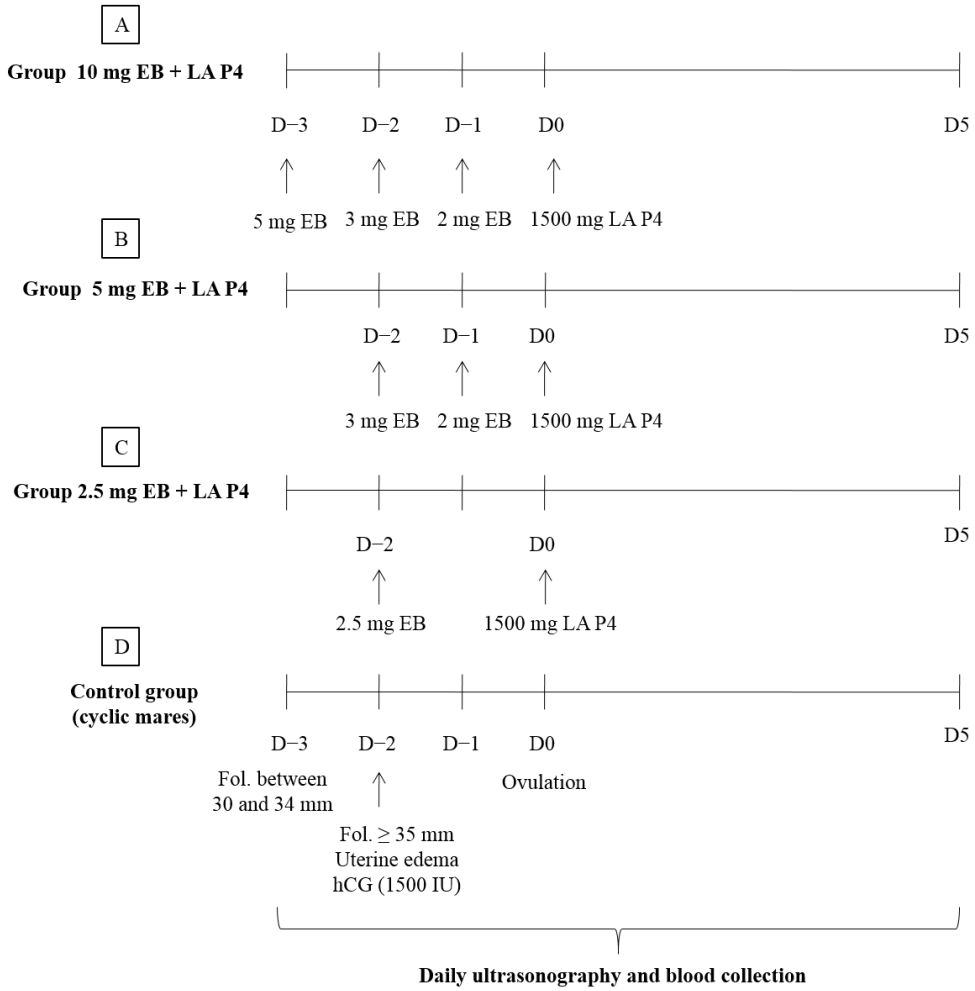

Figure 1: Representative scheme of the estradiol benzoate (EB) followed by long acting progesterone (LA P4) treatments administered to groups: (A) 10 mg EB + LA P4, (B) 5 mg EB + LA P4 and (C) 2.5 mg EB + LA P4. During the cyclic phase of the control group mares, ovulation induction was performed after detection of a ≤ 35 mm follicle and uterine edema (D), to synchronize evaluation days between natural and artificial cycles. Daily ultrasound examinations and blood collections were performed in all groups.

2.3 Ultrasound examinations

Mares were examined with B-mode ultrasonography (Mindray®DP-3300Vet, Shenzhen, China) to monitor their reproductive status (anestrous and subsequently cyclic phase). Moreover, when data collection was initiated, ultrasonography was performed daily, immediately before the first EB administration until the D5 after LA P4 administration in treated groups and from the detection of a 30 mm follicle until the D5 after ovulation in control group. During this period, endometrial edema was quantified and scored as 0 (no edema), 1 (slight edema), 2 (moderate edema) or 3 (high edema) [18].

2.4 Blood collection

Blood samples were collected via jugular venipuncture into heparinized tubes every 24 h, immediately before the first EB administration until the D5 after LA P4 administration in treated groups and from the detection of a 30 mm follicle until D5 after ovulation in control group. Plasma samples were centrifuged and stored at -20°C until assayed for E2, EC and P4 by radioimmunoassay (RIA).

2.5 Radioimmunoassay

2.5.1 Estradiol radioimmunoassay

The concentrations of plasma E2 were measured by a validated RIA [19]. The primary antibody was a sheep anti-estradiol-17β-6-BSA (#244, Niswender; Colorado State University, Fort Collins, CO, USA) used at a 1/60,000 dilution, and the trace was ^{3}H-estradiol (1, 2, 6, 7-^{3}H-E2, NET-317, specific activity 70-115 Ci/mmol; Perkin Elmer Life Science, Boston, MA, USA). Standards (E950; Steraloids, Wilton, NH, USA) ranged from 6.25 to 500 pg/mL. Two hundred microliters of plasma or control samples were extracted with 2 mL of fresh reagent grade anhydrous ethyl ether (EMD-Millipore, Gibbstown, NJ, USA) and reconstituted with 300 µL of PBS with 0.1% porcine skin gelatin (PBS-G, Sigma, St Louis, MO, USA). The sensitivity of the assay was 8 pg/mL and the intra- and inter-assay coefficients of variation were 3.9% (n=6) and 8.9% (n=8), respectively. The extraction efficiency was 88%.

2.5.2 Estrogen conjugate radioimmunoassay

The concentrations of plasma EC were measured by a validated RIA [20]. The primary antibody was a rabbit anti-estrone-3-glucuronide (R-583, C.J. Munro, Clinical Endocrinology Lab, University of California, Davis, CA) used at a 1/12,000 dilution, and the trace was estrone sulfate [6,7 ^{3}H(N), NET-203, Perkin Elmer Life Science, Boston, MA, USA]. Estrone sulfate

was also used as standards (E0251; Sigma-Aldrich, St Louis, MO, USA), ranging from 0.05 to 12.5 ng/mL. Tris/gel was added to 50 µL of plasma or control samples to bring volume to 300 µL.

When using estrone-3-sulfate as the standard (100%), the antibody cross-reacted with estrone (200%), estradiol-17β (100%), equilin (50%), estrone-3-glucuronide (38%), estradiol-3-sulfate (21%), estradiol-3-glucuronide (6.8%) and cross reaction was less than 0.5% with all the non-estrogenic steroids tested. The sensitivity of the assay was 0.1 ng/mL and the intra- and inter-assay coefficients of variation were 3.0% (n=6) and 7.3% (n=4), respectively.

2.5.3 Progesterone and 17α-hydroxyprogesterone radioimmunoassays

Plasma P4 concentrations were measured using a commercial kit (Immunotech Beckman Coulter – Brea, CA, USA) according to manufacturer's recommendations. Measurements were performed in three assays and the antiserum was specific for P4, with minimal sensitivity of 0.05 ng/mL. The antiserum presented low cross-reactivity to other steroid hormones, as follows: 5α-pregnanedione (15.02%), 5β-pregnanedione (8.12%), 6β-hydroxiprogesterone (5.1%) corticosterone (4.07%), 11-desoxycorticosterone (2.56%), 16α-hydroxiprogesterone (1.82%) and 17α-hydroxiprogesterone (1.15%). The intra- and inter-assay coefficients of variation were 12% (n=6) and12.6% (n=3), respectively.

Because P4 concentrations were reduced after the administration of higher doses of EB, we hypothesized that high estrogen concentrations could be accelerating P4 metabolism. In this regard, we quantified 17α-hydroxyprogesterone (17-OH-P) plasma concentrations starting from D0 (immediately before LA P4 administration or on ovulation day in treated and control groups, respectively). Concentrations of 17-OH-P were also measured using a commercial kit (ImmuChem 17a-hydroxyprogesterone CT RIA kit, MP Biomedicals, Orangeburg, NY) according to manufacturer's recommendations. Measurements were performed in two assays

and the antiserum was specific for 17-OH-P. Minimal sensitivity was 0.1 ng/mL. The intra- and inter-assay coefficients of variation were 4.3% (n=6) and 12.5% (n=2), respectively.

2.6 Statistical Analysis

Descriptive statistics (measures of central tendency and dispersion) were produced to characterize the study sample. All response variables were not normally distributed and were log-transformed for analysis, therefore achieving normality. Data were presented as geometric means with confidence intervals to show values in the original scale. A repeated measures model [21] was constructed with PROC MIXED [22] to compare the mean E2, EC, P4 and 17-OH-P concentrations among collection days and treatments. The auto regressive covariance structure provided the best fit to the data and was used to model the correlation between repeated observations within the same mare. Statistical significance was defined as $P < 0.05$.

3. Results

3.1 Ultrasound examinations

The median scores of uterine edema found during treatments with EB and LA P4, as well as the edema scores found in cyclic mares (control group) are shown in Table 1. A lack of uterine edema was observed before treatments. After EB injection, high uterine edema was achieved 24 h after administration in all treated groups, remaining high until LA P4 injection. After LA P4 administration, reduction in the edema scores were observed, wherein 2.5 mg EB group presented slight edema from D1 onwards, group 5 mg EB showed slight to moderate level from D3 onwards, and group 10 mg EB exhibited moderate level from D2. In the control group, high edema was observed three and two days before ovulation, after which a gradual decrease to a slight level on D1 after ovulation was detected. On D5, a slight increase in the edema (slight to moderate level) was observed in the control group.

Table 1: Median scores of uterine edema found in non-cyclic mares (n=7/group) after EB treatment using: a single dose of 2.5 mg on D-2; a total of 5 mg in decreasing doses (3 and 2 mg on consecutive days, D−2 and D−1, respectively); and a total of 10 mg in decreasing doses (5, 3 and 2 mg on consecutive days, D−3, D−2 and D−1, respectively), followed by the injection of 1500 mg of LA P4 on D0. The median scores for cyclic mares (control group) at the corresponding days to treated mares are also shown.

Uterine Edema									
	Days relative to progesterone injection or ovulation (D0)								
Groups	**D−3**	**D−2**	**D−1**	**D0**	**D1**	**D2**	**D3**	**D4**	**D5**
2.5 mg EB + LA P4	-	0[a]	3	3	1	1	1	1	1
5 mg EB + LA P4	-	0[a]	3	3	2.5	2	1.5	1.5	1.5
10 mg EB + LA P4	0[a]	3	3	3	2.5	2	2	2	2
Control	3	3	2	1.5	1	1	1	1	1.5

[a] Uterine edema immediately before EB administration.

3.2. Plasma concentrations of estradiol and estrogen conjugate

Maximum concentrations of E2 were observed 24 h after the single or first EB injection in treated groups and there were no differences ($P > 0.05$) among treatments (Fig. 2A,B,C), despite the different doses that were initially given. In the cyclic group, higher concentrations of E2 were observed on D−2 (Fig. 2D) and there were no differences ($P > 0.05$) when compared to E2 maximum concentrations of the treated groups. When E2 concentrations were compared after LA P4 injection (from D1 onwards), significant difference was only observed between 10 mg EB and cyclic groups on D1 ($P < 0.05$; Fig. 2C,D).

Maximum concentrations of EC were observed 24 h after the single EB injection in the 2.5 mg group (Fig. 2A), whereas in 5 mg and 10 mg groups higher concentrations were observed 48 h after the first EB administration (or 24 h after the second injection; Fig. 2B, 2C). After the peak, concentrations declined to minimal sensitivity levels on D1 in group 2.5 mg

(Fig. 2A) and on D4 in groups 5 mg and 10 mg of EB (Fig. 2B, 2C). As for E2 maximum concentration, EC peak was observed on D−2 in the cyclic group and thereafter declined continuously until D1, remaining relatively constant until D5 (Fig. 2D). Differences were not observed ($P > 0.05$) when EC maximum concentrations were compared among treated groups and between treated and cyclic groups. After LA P4 injection, there were no differences on EC concentrations between groups ($P > 0.05$).

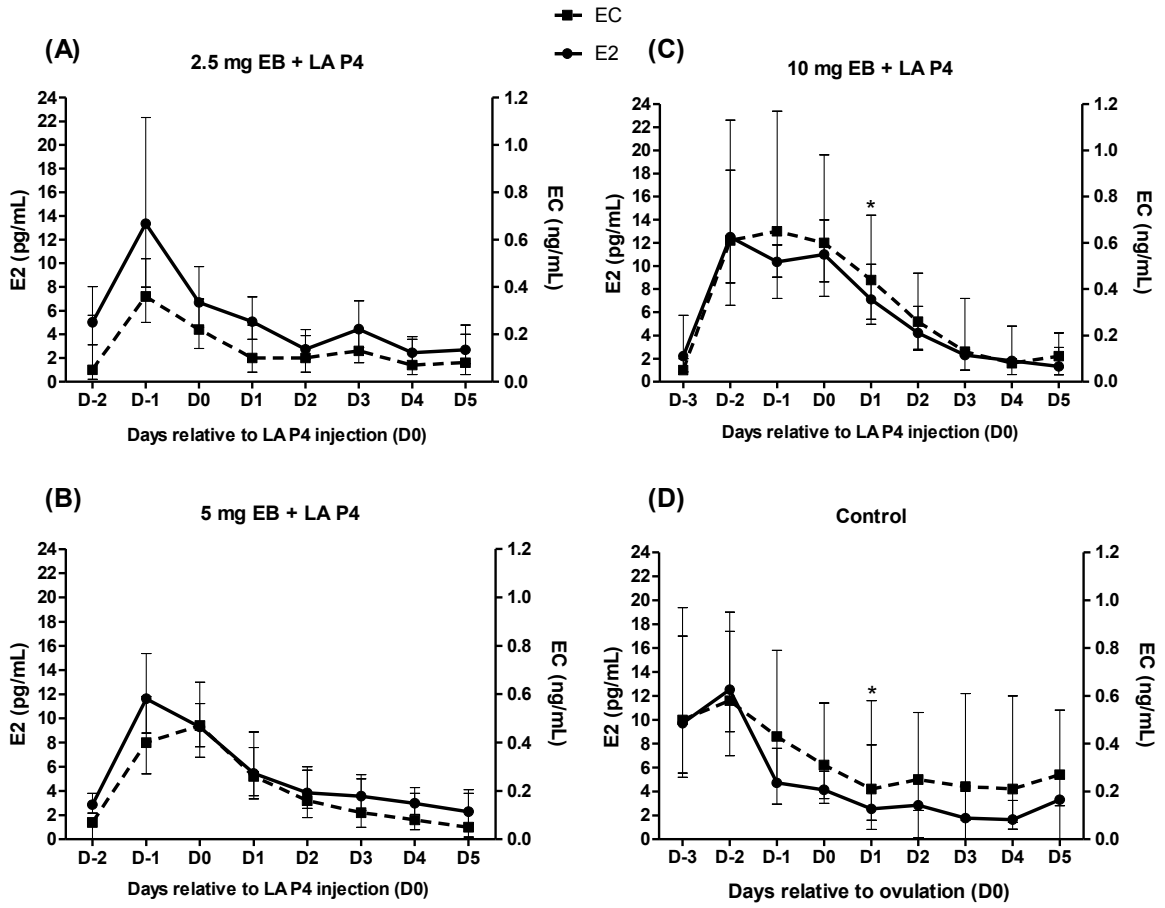

Figure 2: Estradiol (E2) and estrogen conjugate (EC) concentrations (geometric means with confidence intervals) in (A) non-cyclic mares treated with a single dose of 2.5 of EB on D−2; (B) total of 5 mg of EB in decreasing doses for two consecutive days starting from D−2; (C) total of 10 mg of EB in decreasing doses for three consecutive days starting from D−3. The injection of 1500 mg of LA P4 on D0 was performed in all treated groups. (D) Concentrations of E2 and EC in non-treated cyclic mares (control group), where D0 was considered as the day of ovulation. Asterisks within days between groups indicate $P < 0.05$.

3.3 Plasma concentrations of progesterone and 17α-hydroxyprogesterone

Maximum concentrations of P4 were observed 24 h after injection of 1500 mg of LA P4 in non-cyclic treated groups (Fig. 3A). Despite the same dose of LA P4 given, different P4 concentrations were detected between groups treated with different EB concentrations. Concentration of P4 was higher on D1 (24 h after P4 injection) in the group treated with 2.5 mg of EB than in groups treated with 5 mg or 10 mg of EB, although statistical difference was only detected ($P < 0.05$) when groups 2.5 and 10 mg of EB were compared. There was no difference ($P > 0.05$) on D1 between groups 5 mg and 10 mg of EB (Fig. 3A).

After peak, P4 concentrations showed a sharp decrease on D2 after LA P4 injection, after which it showed a less pronounced decrease until D5 in the non-cyclic treated groups. There were no statistical differences between treated groups from D2 onwards (Fig. 3A). In the control group, concentration of P4 was low before ovulation and started to increase from D1, increasing gradually until D5. Concentrations of P4 were significantly lower ($P < 0.05$) in the cyclic group on D1 and significantly higher on D5 when compared to the treated groups ($P < 0.05$; Fig. 3A).

The 17-OH-P concentrations paralleled P4 profile in both treated and control groups. There appeared to be an increase 24 h after LA P4 injection and gradual decrease on the subsequent days. In the control group, the P4 metabolite concentration increased gradually until D5. When the hormone concentrations were compared between groups, higher values were observed in the control group as compared to group 10 mg EB ($P < 0.05$) starting from D2 onwards and when compared to group 5 mg EB on D5 (Fig. 3B).

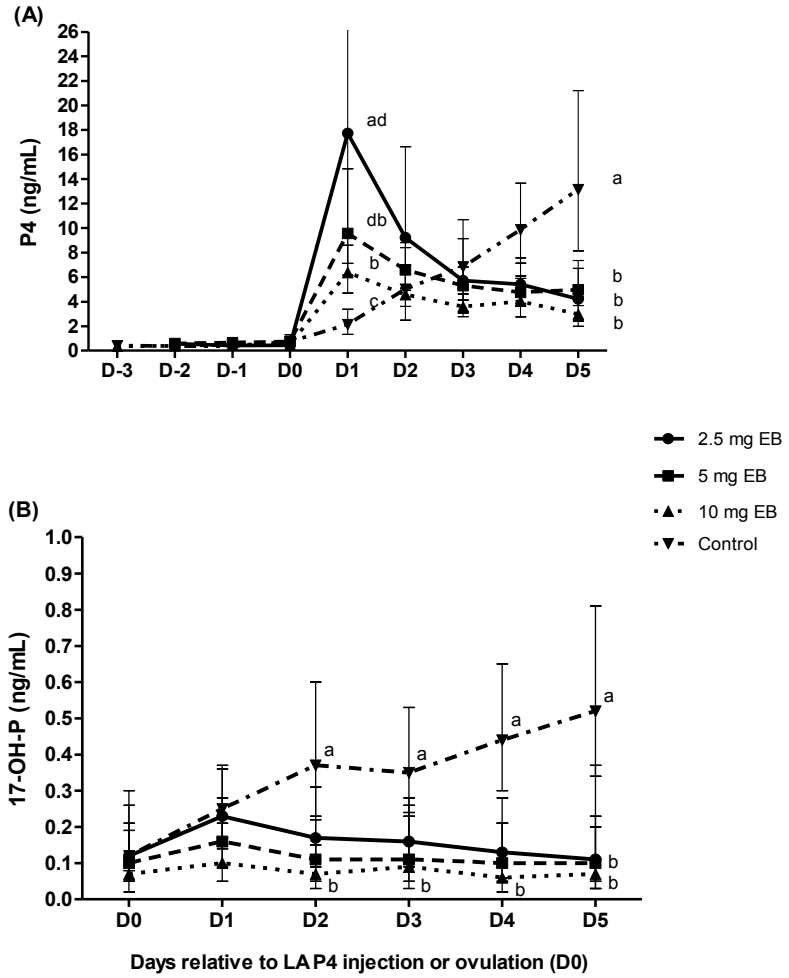

Figure 3: Progesterone (P4) and 17α-hydroxyprogesterone (17-OH-P) concentrations (geometric means with confidence intervals) in the control and non-cyclic treated groups. (A) P4 profile in the non-cyclic groups treated with a single dose of 2.5 of EB on D−2; total of 5 mg of EB in decreasing doses starting from D−2 or total of 10 mg of EB in decreasing doses starting from D−3, followed by the injection of 1500 mg of LA P4 on D0; as well as P4 concentrations of the control group (cyclic mares) three days before ovulation until D5. (B) Concentrations of 17-OH-P in the non-cyclic treated and control groups from D0 to D5. Different letters within the same day between groups indicate $P < 0.05$.

4. Discussion

In view of the fact that recipient mares are key factors for the success of embryo transfer programs and that cyclic recipients are not always available, the use of non-cyclic mares as embryo recipients has become of growing interest. However, the administration of hormonal protocols to non-cyclic recipients has not been deeply investigated, especially the use of estrogen. In the present study, we evaluated and compared plasma E2 and P4 concentrations

after injection of different regimens of EB treatments followed by a single dose of LA P4 in non-cyclic mares, aiming to evaluate which protocol would produce similar uterine and endogenous hormonal conditions to those observed in cyclic mares during the end of estrus until the beginning of diestrus.

Administration of initial doses of 2.5 mg, 3mg and 5mg of EB from groups 2.5 mg EB, 5 mg EB or 10 mg EB, respectively, were effective at promoting high endometrial edema 24 h after injection. Moreover, edema remained high in all treated groups, regardless of additional EB injections, until LA P4 administration. High edema was also observed in the control group, three and two days before ovulation, in agreement with previous reports on cyclic mares [23]. After P4 administration, uterine edema declined in all treated groups, whereas in the control group edema began to reduce one day before ovulation. Uterine edema accompanied estrogen profile in the control group, while in treated groups, mainly in the ones treated with higher EB concentrations, decrease in estrogen concentrations did not necessarily decrease uterine edema in the same extent. It is likely that the higher EB concentrations given to groups 5 and 10 mg EB continued to stimulate the uterus even after P4 injection and decline on plasma estrogen concentrations.

A slight edema and estrogen rise was observed on D5 after ovulation in the control group. Ultrasonic uterine echotexture studies have revealed the presence of a slight increase in edema in early diestrus during the first half of the ovulatory season but not during the second half [24], which may reflect follicular growth that occurs in some mares in early diestrus or estrogen production by the developing corpus luteum [20]. In the present study, diestrus data were collected on the third estrous cycle of the breeding season. However, we do not have accurate information on the follicular activity after ovulation. Herein, the reason for the slight rise in uterine edema on D5 after ovulation is inconclusive.

It is generally considered that measuring the active form of estrogen, the E2, provides the best information in terms of hormone signal delivered to the target cell. However, because circulating E2 concentrations are low in some animals, the accuracy of this method is sometimes limited [20]. In the present study, only concentrations after EB injections were above the E2 assay sensitivity. On the other hand, EC concentrations are 100-fold higher than plasma E2 concentrations [20]. In addition, it has been reported that the increase of conjugated estrogens parallels plasma E2 and thus the same physiological information can be obtained by EC (estrone sulphate) analysis in cyclic mares [20]. For this reason, we also provided the EC profile in treated and control mares.

As observed in the control group, EC profile paralleled E2 profile in group 2.5 mg EB, where maximum EC and E2 concentrations were detected 24 h after EB administration, after which both hormone concentrations declined. However, EC peaks in groups 5 and 10 mg EB were observed 24 h after E2 peak. In women, it has been described that estrogens are mainly present in their inactive sulfated form in serum and tissue [25], being estrone-3-sulfate, an EC, an important precursor for the active biological form, E2. As EC has longer half-life than E2, it has been considered as storage form for estrogens, to form estrone and E2 [26]. Considering that sulfated estrogens are unable to bind to the estrogen receptors, sulfonation of estrogens results in their inactivation [25]. Therefore, conjugation with sulfate protects cells and tissues from an excess of active estrogens. In addition, sulfate conjugation and removal of the sulfate by specific enzymes are important for a balance in the organism [27]. In this context, as EC maximum concentrations were observed after E2 maximum concentrations in the higher EB concentrations groups in this study, we suggest that there would be an excess of E2 in these groups, which were sulfated into EC to be inactivated. Moreover, the similar E2 maximum concentrations found between treated groups, irrespective of the initial dose given, indicates

that the mare's organism was able to control and protect itself from an excess of active hormone.

When estrogen concentrations of the treated groups were compared to the control group, peak concentrations of EC and E2 were similar, regardless of the EB dose administered. Estrogen concentrations started to decline prior to LA P4 administration in group 2.5 mg EB, while in groups 5 and 10mg EB estrogen concentrations began to decline on the day of LA P4 injection or on the day after, respectively. However, E2 concentrations were as low as observed in cyclic mares from day two after LA P4 administration onwards, in all treated groups, which are the usual days in which embryo transfer are performed. In this regard, the different doses of EB administered produced similar estrogen concentrations to those found in cyclic mares when comparing maximum estrogen concentrations and after day two of LA P4 injection.

Surprisingly, there was an effect of EB on P4 concentrations, despite the same LA P4 dose given to all treated groups. Higher EB doses led to lower P4 peaks 24 h after LA P4 administration. In the steroids biosynthetic pathways, cholesterol is converted to pregnenolone by the cholesterol side-chain cleavage enzyme, which in turn can be converted to P4 via 3β-hydroxysteroid dehydrogenase and steroid delta-isomerase enzymes. Furthermore, the enzyme 17α-hydroxylase can convert P4 to 17-OH-P4. Subsequently, androstenedione is formed from 17-OH-P4, followed by conversion into testosterone and to estrogens (including E2) [28]. However, a direct effect of estrogen on P4 following administration of exogenous hormones is still not known.

In cultures of swine granulosa cells, estrogen inhibited total P4 in a time- and dose-dependent fashion [29]. The predominant locus of estrogen action was the blockade of pregnenolone's conversion to P4, rather than accelerated metabolism of P4 to its metabolites. According to the authors, increasing concentrations of E2 attained in antral follicular fluid during the later stages of follicle maturation could effectively limit the premature secretion of

large quantities of P4 before ovulation. We believe that a regulation between estrogen and progesterone could also take place in the mare. Because we provided exogenous P4 to non-cyclic mares, we hypothesized that the most likely mechanism by which estrogen would decrease P4 concentrations would be by accelerating P4 metabolism. In this way, we sought to evaluate whether the higher EB doses given to the anestrous mares would generate higher P4 metabolite, namely the 17-OH-P. However, the opposite was observed, the metabolite concentrations accompanied P4 profile and although concentrations were not statistically different between treated groups, the higher the EB dose administered, the lower the P4 metabolite peak after LA P4 administration. It has been reported that large doses of E2 can decrease 17α-hydroxylase activity in the ovaries of immature hypophysectomized rats [30], thereafter reducing 17-OH-P concentrations. Alternatively, P4 metabolite concentrations may be only the reflection of the higher or lower P4 concentrations in the treated groups.

Considering that accelerated P4 metabolism is not the mechanism by which high doses of estrogen reduces P4 concentrations herein, the steroidogenesis conversion pathway left is through conversion of P4 to deoxycorticosterone via the enzyme 21-hydroxylase [28]. In pregnant women, estrogen treatment increased conversion of plasma P4 to deoxycorticosterone through regulation of extra-adrenal 21-hydroxylase activity [31]. Therefore, it is very likely that in the groups in which mares received high doses of EB, mainly group 10 mg EB, P4 was converted to deoxicorticosterone. This is yet to be determined in future studies.

In conclusion, administration of total doses of 2.5 mg, 5 mg and 10 mg of EB were effective at promoting maximum uterine edema until LA P4 injection. However, uterine edema in group 10 mg EB was not decreased to minimum levels after P4 injection as observed in the control group after ovulation. The three doses induced similar E2 peaks after administration, however, the observation of EC peaks 24 h after E2 peaks in groups 5 and 10 mg indicate that an excess of the active form E2 was given, which was converted into EC to be inactivated. In

addition, administration of 10 mg of EB significantly reduced P4 concentrations 24 h after LA P4 was given and we could demonstrate that the mechanism by which high doses of estrogen reduce P4 concentrations is not by increased P4 metabolism to 17-OH-P. Therefore, we conclude that 2.5 mg of EB followed by 1500 mg of LA P4 is an appropriate regimen to prepare non-cycling mares as embryo recipients.

Ackowledgments

The FAPESP foundation (Fundação de Apoio a Pesquisa e Ensino do Estado de São Paulo) is acknowledged for supporting the first author during the execution of this study through the Research Internship Abroad Fellowship (Processo Fapesp n. 2014/08207-6) in the Department of Animal Science, University of California, Davis, USA. The authors also thank Lillian Sibley (University of California, Davis) for assistance with the hormonal assays.

References

[1] Stroud B. The year 2011 worldwide statistics of embryo transfer in domestic farm animals. Statistics and Data Retrieval Committee Report. IETS Embryo Transfer Newsl 2012, 30:16-26

[2] Rocha Filho AN, Pessôa MA, Gioso MM, Alvarenga MA. Transfer of equine embryos into anovulatory recipients supplemented with short or long acting progesterone. Anim Reprod 2004; 1(1):91-95.

[3] Hinrichs K, Sertich PL, Cummings MR, Kenney MR. Pregnancy in ovariectomized mares achieved by embryo transfer. Eq Vet J. 1985**;** Suppl 3:74-75.

[4] Hinrichs K, Sertich PL, Kenney RM. Use of altrenogest to prepare ovariectomized mares as embryo transfer recipients. Theriogenology. 1986; 26:455-60.

[5] McKinnon AO, Squires EL, Carnevale EM, Hermenet MJ. Ovariectomized steroid-treated mares as embryo transfer recipient and as a model to study the role of progestins in pregnancy maintenance. Theriogenology. 1988; 29:1055-63.

[6] Carnevale, EM, Ramirez RJ, Squires EL, Alvarenga MA, Vanderwal DK, McCue PM. Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. Theriogenology. 2000; 54:965-979.

[7] Silva ESM, Ignácio FS, Pantoja JCF, Puoli Filho JNP, Meira C. Supplementary corpora lutea monitoring allows progestin treatment interruption on day 70 of pregnancy in non-cyclic recipient mares. Anim Reprod Sci. 2014; 144:122-128.

[8] Pinto CRF. Progestagens and Progesterone. In: McKinnon AO, Squires EL, Vaala WE, Varner DD. Equine Reproduction. 2nd ed. Ames, IA:Blackwell Publishing, 2011:1811-1817.

[9] Vanderwall DK. Progesterone. In: McKinnon AO, Squires EL, Vaala WE, Varner DD. Equine Reproduction. 2nd ed. Ames, IA:Blackwell Publishing; 2011:1637-1641.

[10] Kaercher F, Kozicki LE, Camargo CE, Weiss RR, Santos IW, Muradas PR, Bertol MAF, Abreu RA. Embryo transfer in anovulatory recipient mares treated with estradiol benzoate and long-acting progesterone. J Eq Vet Sci. 2013; 33:205-209.

[11] Botelho JHV, Pessoa GO, Rocha LGP, Yeste M. Hormone supplementation protocol using estradiol benzoate and long-action progesterone is efficient in maintaining pregnancy of anovulatory recipient mares during autumn transitional phase. Anim Reprod Sci. 2015; 152:39-43.

[12] Bó GA, Colazo MG, Martínez MF, Kastelic JP, Mapletoft RJ. Sincronizacion de la emergencia de la onda folicular y la ovulacion en animales tratados con progestagenos y diferentes esteres de estradiol. In: 2° Simposio Internacional de Reprodução Animal Aplicada -Biotechnology in Bovine Reproduction, 2006:71-84.

[13] Greco GM, Burlamaqui FLG, Pinna AE, Queiroz FJR, Cunha MPS, Brandão FZ. Use of long acting progesterone to acyclic embryo recipient mares. Braz J Anim Sci. 2012; 41:607-611.

[14] Silva ESM, Pantoja JCF, Puoli Filho JNP, Meira C. Ultrasonography of the conceptus development from days 15 to 60 of pregnancy in non-cyclic recipient mares. Ciência Rural. 2015; 45:512-518.

[15] McKinnon AO, Squires EL, Carnevale EM, Hermenet MJ. Ovariectomized steroid-treated mares as embryo transfer recipient and as a model to study the role of progestins in pregnancy maintenance. Theriogenology. 1988; 29:1055-1063.

[16] Burns PJ, Morrow C, Abraham J. Evaluation of biorelease P4 LA 300 in the mare. In: Proceedings of the 7th International Symposium on Equine Embryo Transfer, Cambridge, UK 2008:82-83.

[17] Greco GM, Avanzi BR, Fioratti EG, Queiroz FJR, Meira C, Alvarenga MA. Effect of interrupting the progesterone treatment in pregnant acyclic recipient mares showing supplementary corpora lutea formation. Acta Sci Vet. 2008; Supl 2:590.

[18] McCue PM, Scoggin CF, Lindholm AFG. Estrus. In: McKinnon AO, Squires EL, Vaala WE, Varner DD. Equine Reproduction. 2nd ed. Ames, IA:Blackwell Publishing, 2011:1716-1727.

[19] Parlevliet JM, Pearl CA, Hess MF, Famula TR, Roser JF. Immunolocalization of estrogen and androgen receptors and steroid concentrations in the stallion epididymis. Theriogenology. 2006; 66:755-65.

[20] Daels PF, Ammon DC, Stabenfeldt GH, Liu IKM, Hughes JP, Lasley BL. Urinary and plasma estrogen conjugates, estradiol and estrone concentrations in non-pregnant and early pregnant mares. Theriogenology. 1991; 35:1001-1017.

[21] Kleinbaum DG, Kupper LL, Nizam A, Muller KE. Applied Regression Analysis and Multivariable Methods. Duxbury Press, Belmont, California, 2007.

[22] SAS Institute. 2011. SAS/STAT User's Guide. Version 9.2, SAS Institute Inc., Cary, NC.

[23] Ginther OJ. Reproductive Biology of the Mare: Basic and Applied Aspects. 2nd ed. Cross Plains WI:Equiservices; 1992.

[24] Hayes KEN, Pierson RA, Scraba ST, Ginther OJ. Effects of estrous cycle and season on ultrasonic uterine anatomy in mares. Theriogenology. 1985; 24:465-477.

[25] Secky L, Svoboda M, Klameth L, Bajna E, Hamilton G, Zeillinger R, Jager W, Thalhammer T. The sulfatase pathway for estrogen formation: targets for the treatment and diagnosis of hormone-associated tumors. J Drug Deliv. 2013; 2013:1-13.

[26] Sasano H, Miki Y, Nagasaki S, Suzuki T. *In situ* estrogen production and its regulation in human breast carcinoma: from endocrinology to intracrinology. Pathol Int. 2009; 59:777-789.

[27] Purohit A, Woo LWL, Potter BVL. Steroid sulfatase: a pivotal player in estrogen synthesis and metabolism. Mol Cell Endocrinol. 2011; 340(2):154-160.

[28] Stanczyk, F. Glob. libr. women's med., (ISSN: 1756-2228) 2009; DOI 10.3843/GLOWM.10278. Last acess: 05/18/2015.

[29] Veldhuis JD, Azimi P, Garmey J, Juchter D. Bipotential actions of estrogen and progesterone biosynthesis by ovarian cells. II. Relation of estradiol's stimulatory actions to cholesterol and progestin metabolism in cultured swine granulosa cells. Endocrinology. 1985; 117:1076-1083.

[30] Johnson DC, Martin H, Tsai-Morris CH. The in vitro and in vivo effect of estradiol upon the 17 apha-hydroxylase and C17,20-lyase activity in the ovaries of immature hypophysectomized rats. Mol Cel Endocrinol. 1984; 35:199-204.

[31] MacDonald P, Cutrer S, MacDonald S, Casey ML, Parker CR. Regulation of extra adrenal steroid 21-hydroxylase activity. J Clin Invest. 1981; 69:469-478.

Capítulo III

O artigo a seguir foi redigido de acordo com as normas do periódico científico 'Reproduction, Fertility and Development': http://www.publish.csiro.au/nid/47/aid/358.htm.

Administration of 2.5 mg of estradiol followed by 1500 mg of progesterone to anovulatory mares promote similar uterine morphology, hormone concentrations and molecular dynamics to those observed in cyclic mares

E.S.M. Silva[A], F.S. Ignácio[A], S.C. Fritsch[A], D.S. Zanoni[B], J.C.F. Pantoja[C], J.P. Oliveira-Filho[D], C. Meira[A]

[A] Department of Animal Reproduction and Veterinary Radiology, São Paulo State University, Botucatu, São Paulo, Brazil
[B] Department of Veterinary Clinical Science, São Paulo State University, Botucatu, São Paulo, Brazil
[C] Department of Veterinary Hygiene and Public Health, São Paulo State University, Botucatu, São Paulo, Brazil
[D] Department of Veterinary Clinical Science, São Paulo State University, Botucatu, São Paulo, Brazil

Abstract

To test the hypothesis that the administration of 2.5 mg of estradiol benzoate (EB) followed by 1500 mg of long acting progesterone (LA P4) causes similar uterine changes and molecular dynamics in anovulatory mares to those observed in cyclic ones, we evaluated the changes in transcripts abundance and protein immunostaining of estrogen (ERα and ERβ) and progesterone receptors (PR) in anestrous, transitional and cyclic mares by RT-qPCR and immunohistochemistry. In addition, we evaluated uterine edema, tonus and estrogens and progesterone plasma profile. Endometrial biopsies were taken from anestrous and transitional mares immediately before EB injection, 48 h after EB administration and five days after LA P4 was given. In cyclic mares, biopsies were collected at estrus and at five days after ovulation. Similar estrogen peaks were achieved after the injection of the single EB dose between treated and cyclic groups, as well as maximum uterine edema. Uterine tone was increased to diestrus levels after administration of 1500 mg of LA P4. Changes in relative abundance of transcripts

for PR, ERα and ERβ when progesterone stimulated endometrium was compared to estrogen stimulated endometrium were similar between cyclic and non-cyclic treated mares. However, apparent decreased PR in the endometrial glandular epithelium was not observed in non-cyclic mares five days after LA P4 administration as observed at five days after ovulation in cyclic mares. The protocol produced similar endometrial edema, uterine tonus and changes in relative abundance of PR, ERα and ERβ transcripts to those observed in cyclic mares during late estrus and early diestrus, as well as similar estradiol and estrogen conjugate plasma concentrations.

Key-words: Embryo transfer, Hormonal protocols, Equine, Steroid Receptors.

Introduction

Embryo transfer (ET) is a worldwide technology in the equine industry. The technique is a valuable tool to increase the number of foals from genetically valuable donor mares, for obtaining foals from subfertile mares and to obtain embryos from mares that are competing in equestrian modalities (Squires 2013). The selection of the recipient mare has a major effect on the embryo transfer success. However, the availability of recipient mares has become a limiting factor, especially during the beginning of the breeding season, as part of the recipients are in anestrous due to seasonal reproductive activity. In addition, differentiated feed management provided to donor and recipient mares can account on a delayed onset of cyclicity in the recipient ones (Silva *et al*. 2014).

In order to increase the offer of recipient mares in ET programs, estrogen and/or progesterone (P4) treatments can be administered to non-cyclic recipient mares. Several studies reporting the pregnancy rates after estrogen and/or progestins injections have been described (Hinrichs *et al*. 1985, 1986; McKinnon *et al*. 1988; Carnevale *et al*. 2000; Rocha Filho *et al*. 2004; Greco *et al*. 2012). Because it has been demonstrated that estradiol (E2) increases the

expression of P4 uterine receptors and that the equine embryo secretes estrogens during the early gestational phase (Zavy *et al*. 1979), McKinnon *et al*. (1988) administered E2 prior to and together with P4 to ovariectomized mares, obtaining 70 to 80% pregnancy rates within the different evaluated hormonal protocols. It was suggested that, regardless of E2 injection, the primary requirement for pregnancy establishment and maintenance in non-cyclic recipient mares is an adequate concentration of exogenous progestins.

In the past few years, E2 has been given to intact non-cyclic recipient mares and after observation of uterine edema, long acting progesterone (LA P4; Rocha Filho *et al*. 2004; Greco *et al*. 2012) or altrenogest (Silva *et al*. 2014, 2015) has been administered three to eight days prior to embryo transfer. Exogenous E2 has been empirically given, since the dose of E2 that produces an estrogen concentration similar to that found in cyclic mares is still not known. In this regard, E2 injection of doses ranging from 2.5 mg to 20 mg prior to P4 administration have been reported (Greco *et al*. 2012; Silva *et al*. 2015; Botelho *et al.* 2015). On the other hand, it is known that plasma P4 concentration after injection of 1500 mg of LA P4 at seven day intervals is compatible with P4 concentrations found in cyclic mares during the luteal phase (>4 ng/mL; Bringel *et al*. 2003).

Estrogen and P4 mediate many of their effects through nuclear receptors, which are transcription factors that regulate expression of target genes (Simoncine and Genazzani 2003). Estrogen can bind and activate two types of nuclear receptors (i.e. ERα and ERβ) encoded by different genes, *ESR1* and *ESR2* (Enmark *et al*. 1997). Estrogen receptor α (ERα) is the predominant estrogen receptor (ER) in the uterus (Weihua *et al*. 2000). It has been suggested that ERβ modulates the uterotrophic effects of ERα (Weihua *et al*. 2000). Estrogen acts via its receptors to mediate cellular proliferation and secretory protein production during estrus. In addition, it is widely accepted that estrogen increases the expression of its own receptor and P4 receptor (PR) in the endometrium during the estrous cycle in mares (Aupperle *et al*. 2000; Hartt

et al. 2005; Gebhardt *et al*. 2012). On the other hand, P4 is required for maternal support of conceptus survival and development during pregnancy (Spencer and Bazer 2002). The actions of P4 are mediated by PR. In ruminants, loss of PR in the endometrial luminal and glandular epithelium prior to the stages of uterine receptivity and implantation seems to be required for onset of differentiated functions in terms of secretory proteins during early pregnancy (Spencer and Bazer 2002).

Considering that recipient mares are responsible for carrying a pregnancy to term, it is important to provide an appropriate uterine environment for the conceptus development in non-cyclic ones. The protein immunostaining and changes in abundance of transcripts for ER and PR have not yet been described in non-cyclic mares treated with hormonal protocols to become embryo recipients. In previous studies (Silva *et al*. 2014, 2015), we could demonstrate that the administration of 2.5 mg of estradiol benzoate (EB) followed by progestin administration was able to establish and maintain pregnancy in either anestrous or transitional mares. Therefore, to test the hypothesis that the administration of a single dose of 2.5 mg of EB followed by 1500 mg of LA P4 in anestrous and transitional mares promote similar uterine edema, tone and molecular dynamics in anovulatory mares to those observed in non-treated cyclic mares, we described and compared protein immunolocalization and changes in relative abundance of transcripts for ERα, ERβ and PR, as well as the uterine characteristics and estrogen and P4 profile during the hormonal treatment.

Material and Methods

Animals and experimental design

Twelve mares of different breeds, ranging from five to 15 years and weighing 350 to 450 kg were used in the study. Mares were selected based on histological evaluations of endometrial inflammation and fibrosis by biopsy (Kenney and Doig 1986), which only mares

classified as categories I (normal endometrium) and IIA (mild inflammation and/or fibrosis) were included. Mares were maintained on coast-cross hay (*Cynodon dactylon*) with water and trace-mineralized salt *ad-libitum*. The experiment was conducted from June to December at the Equine Reproduction and Biotechnology Center – Lageado Farm, School of Veterinary Medicine and Animal Science, Botucatu – São Paulo, Brazil. Animal procedures were approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, Univ. Estadual Paulista (CEUA-95/2012).

Mares were assigned to anestrous, transition or cyclic (control) groups. Anestrous mares were those presenting ovarian follicles <20 mm in diameter, absence of a corpus luteum (CL) and progesterone concentration <1 ng/mL on three evaluations at seven day intervals. Transitional mares were those showing multiple follicles of 20 to 30 mm in diameter, absence of a CL and progesterone concentration <1 ng/mL, considering that treatment was started when at least one of the follicles reached 30 mm. Cyclic group consisted of non-treated cyclic mares showing regular estrous cycles and ovulatory follicles. The same twelve mares were used throughout the experiment, i.e., anestrous mares were reevaluated during the transitional period and afterwards during the cyclic phase.

Hormonal treatment

Anestrous and transition groups were treated with a single dose of 2.5 mg of intramuscular estradiol benzoate (EB; Estrogin®, Farmavet, SP, Brazil) and 48 h after its administration 1500 mg of long acting progesterone (LA P4; Sincrogest Injetável®, Ourofino, SP, Brazil) was given intramuscularly. Cyclic group did not receive EB and LA P4 treatments, although ovulation was induced with 1500 IU of hCG (Vetecor®, Hertape Calier, MG, Brazil) after detection of at least a 35 mm follicle and uterine edema, to synchronize evaluation days between natural and artificial cycles (Fig. 1).

Blood collection and steroid hormones assay

Blood samples were obtained via jugular venipuncture into heparinized tubes every 24 h, starting from three days before ovulation (D−3) until five days after (D5) in the cyclic group. In anestrous and transition groups, samples were obtained immediately before EB injection (D-2) until five days after LA P4 administration (D5). Samples were collected into heparinized tubes, centrifuged (900g/10 min) and plasma was harvested and stored at − 20º until assayed (Fig. 1).

The concentrations of plasma 17β-estradiol (E2), estrogen conjugate (EC) and progesterone (P4) were measured by validated radioimmunoassay (RIA) for E2 (Parlevliet *et al*. 2006), EC (Daels *et al*. 1991), and using a commercial RIA kit according to manufacturer's recommendations (Immunotech Beckman Coulter – Brea, CA, USA) for P4. When using estrone-3-sulfate as the standard (100%) for EC assay, the antibody cross-reacted with estrone (200%), 17β-estradiol (100%), equilin (50%), estrone-3-glucuronide (38%), estradiol-3-sulfate (21%), estradiol-3-glucuronide (6.8%) and cross reaction was less than 0.5% with all the non-estrogenic steroids tested. The antiserum for P4 assay presented low cross-reactivity to other steroid hormones, as follows: 5α-pregnanedione (15.02%), 5β-pregnanedione (8.12%), 6β-hydroxiprogesterone (5.1%) corticosterone (4.07%), 11-desoxycorticosterone (2.56%), 16α-hydroxiprogesterone (1.82%) and 17α-hydroxiprogesterone (1.15%).

The extraction efficiency for the E2 assay was 88%. The sensitivity of the assays were 8 pg/mL for E2, 0.1 ng/mL for EC and 0.05 ng/mL for P4. The intra- and inter-assay coefficients of variation were 3.9% (n=6) and 8.9% (n=8) for E2, 3.0% (n=6) and 7.3% (n=4) for EC, and 12% (n=6) and 12.6% (n=3) for P4.

Rectal palpation and ultrasound examinations

Rectal palpation and B-mode ultrasonography (5 MHz linear transducer; Mindray® DP-3300Vet, Shenzhen, China) were performed daily prior to and during hormonal treatment in anestrous group (Fig. 1) After the end of treatment in anestrous group, ultrasound examinations were performed at two day intervals until the transitional phase was characterized. Once in transition, mares were transrectally palpated and scanned by ultrasound as described for anestrous group. After treatment in transition group was completed, ultrasound evaluations were also performed at two day intervals until the detection of the first ovulation of the season. In the cyclic group, ultrasonography was performed in a daily basis, starting from the second ovulation of the season (D=0) until five days after the following ovulation (third ovulation of the season).

Rectal palpation was performed immediately before and during hormonal treatments to evaluate uterine tone, scored as 0 to 3 (0=lack of uterine tone; 1=minimum uterine tone; 2=intermediate uterine tone; 3=maximum uterine tone; Hayes and Ginther, 1986), and ultrasound examinations were performed to evaluate uterine edema, which also received a scoring system from 0 to 3 (0=lack of uterine edema; 1=minimum uterine edema; 2=intermediate uterine edema; 3=maximum uterine edema; McCue *et al*., 2011). Uterine tone and edema were also evaluated in the cyclic group, starting from the detection of a 35 mm follicle until five days after ovulation.

Tissue collection

Endometrial tissue samples were collected immediately before EB treatment, 48 h after EB administration and five days after LA P4 injection in anestrous and transition groups. In cyclic group, samples were collected during estrus, when a follicle of ≥35 mm and uterine edema (score 2 to 3) were observed, and during diestrus, on day five after ovulation (Fig. 1).

Endometrial samples were recovered transcervically using an alligator jaw biopsy forceps (Botupharma, Botucatu, SP, Brazil) from the base of one of the uterine horns. Biopsies were divided in two parts and one part was frozen in liquid nitrogen and stored at − 80º C for RT-qPCR, while the other part was fixed for 24 h in 10% formalin and stored at 70% alcohol for histology and immunohistochemistry (IHC).

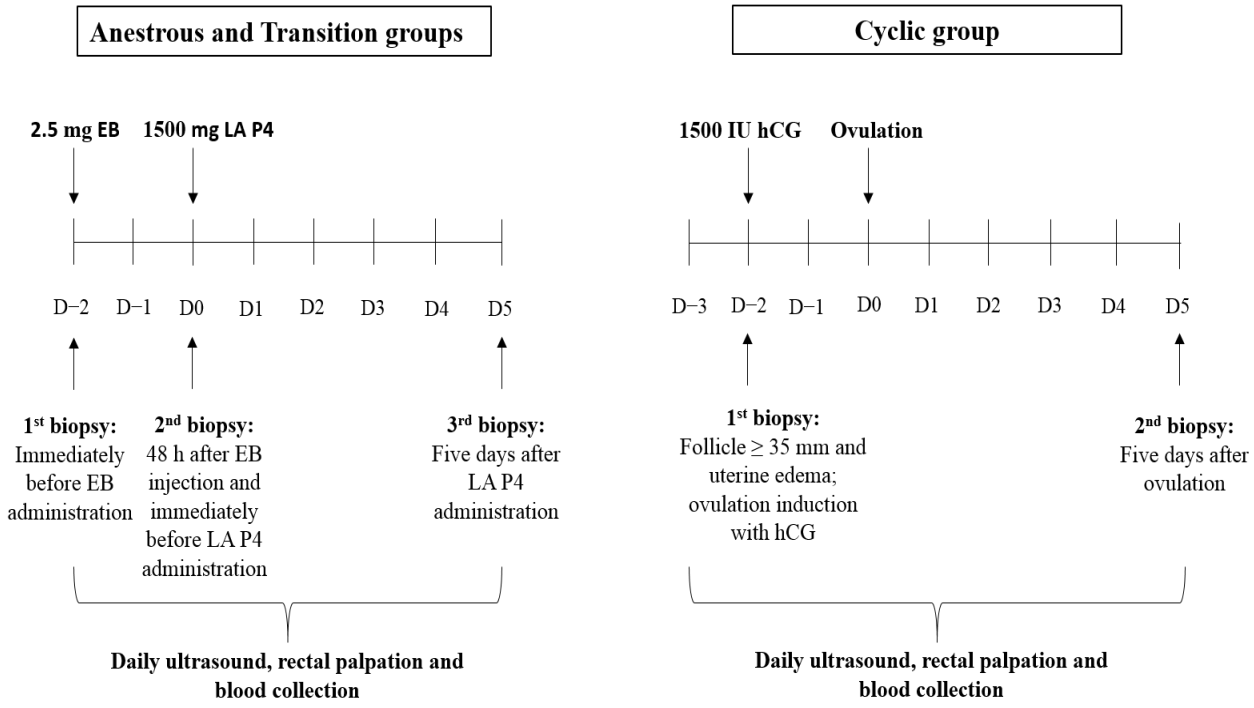

Figure 1: Representative scheme of the days in which endometrial tissues were collected by biopsy and hormonal treatments were administered using 2.5 mg of estradiol benzoate (EB) followed by 1500 mg of long acting progesterone (LA P4) in anestrous and transition groups. In the cyclic group, ovulation was induced after detection of a ≤ 35 mm follicle and uterine edema, to synchronize evaluation days between natural and artificial cycles. Daily ultrasound, rectal palpation and blood collections were performed in all groups.

Reverse transcription qPCR (RT-qPCR)

Total cellular RNA was extracted from endometrium samples using trizol (Trizol® Reagent, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendation. The extracted RNA was diluted in 40 µL of RNase-*free* water. The RNA (1 µL) was quantified via spectrophotometry (NanoDrop ND1000®) and samples with a 260/280 ratio of 1.90 or greater were used for analysis. The RNA samples (2 µg/reaction) were treated with RNase-free DNase I (Ambion®, Life Technologies, Carlsbad, CA, USA) for 30 min at 37º C, followed by treatment with DNase Inactivation Reagent (room temperature for 2 min). Quantification of RNA was performed once more by spectrophotometry and treated samples were normalized to the same final concentration of 2400 ng using endonuclease free water. Synthesis of cDNA was performed with 2400 ng of total RNA per 40 µL of reaction using random hexamers and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Primers specific for estrogen receptor α (*ESR1*), estrogen receptor β (*ESR2*), progesterone (*PGR*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta actin (*ACTB*) and beta-2-microglobulin (*B2M*) were selected based on primers previously validated for the mare's endometrium (Klein *et al*., 2011; Silva *et al*., 2014; Table 1). Primers efficiencies ranged from 93.8% to 99.3%.

Table 1: Primer sequences and gene accession numbers used for estrogen receptor α (*ESR1*), estrogen receptor β (*ESR2*), progesterone receptor (*PGR*), glyceraldehyde-3- phosphate dehydrogenase (*GAPDH*), beta actin (*ACTB*) and beta-2-microglobulin (*B2M*) transcripts.

Gene	Accession number	Primer sequence (5`-3`)	Product size (bp)
ESR1[1]	NM 001081772	Forward:TCCATGGAGCACCCAGGAAAGC Reverse: CGGAGCCGAGATGACGTAGCC	125
ESR2[1]	XM 001915519	Forward: TCCTGAATGCTGTGACCGAC Reverse: GTGCCTGACGTGAGAAAGGA	116
PGR[1]	XM 001498494	Forward: CTTCCCCGACTGCGCGTACC Reverse: TTGTGTGGCTGGAAGTCGCCG	81
GAPDH[1]	NM 001163856	Forward: AGAAGGAGAAAGGCCCTCAG Reverse: GGAAACTGTGGAGGTCAGGA	87
ACTB[2]	NM_001081838	Forward: CGACATCCGTAAGGACCTGT Reverse: CAGGGCTGTGATCTCCTTCT	99
B2M[2]	NM_001082502	Forward: GTGTTCCGAAGGTTCAGGTT Reverse: ATTTCAATCTCAGGCGGATG	102

[1] Primers validated for the mare's endometrium according to Silva *et al*. (2014).
[2] Primers validated for the mare's endometrium according to Klein *et al*. (2011).

Each PCR reaction was performed in duplicate, using final volume of 20 µL: 2 µL of cDNA, 10 µL of GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), 1 µL of each primer (forward and reverse) and endonuclease free water q.s.p. A 'no template' control was included in duplicate on each plate to show that amplicon contamination was absent. Quantitative PCR was completed using 7500 fast Real Time PCR systems (Applied Byosystems®), with the following cycling conditions: 10 min at 95°C (initial denaturation) followed by 40 cycles for 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension). Amplification of specific transcripts was confirmed by melting curve profiles generated at the end of each run. A standard curve was made using serial 10-fold dilution of a "pool" of equine endometrium cDNAs used as template in the RT-qPCR reactions.

Changes in gene expression were calculated by mean quantification cycle (Cq) and then normalized for the endogenous reference gene *B2M* to generate delta Cq values. *GAPDH*

and *ACTB* were also tested as endogenous reference genes, since *GAPDH*, *ACTB* and *B2M* have been shown to be stably expressed across equine endometrium (Klein *et al*. 2011), however B2M was the most stably expressed gene in our experimental conditions. Changes in relative abundance of transcripts were calculated by the relative expression ratio (*R*) method (Pfaffl 2001). Data were analysed in SDS 7500 v.2.05 software (Applied Byosystems®).

Immunohistochemistry

Formalin fixed samples were dehydrated, embedded in paraffin and cut at 4 μm. Paraffin sections were deparaffinized and rehydrated in serial ethanol dilutions. Antigens retrieval were performed by heating tissue sections for 30 min in citrate buffer solution at pH 6. After heat treatment all slides were left to cool down for 20min. Endogenous peroxidases were inhibited with 8% H_20_2 in methanol for 20 min. Thereafter, incubation with 12% skimmed milk powder for 1h at 27º C was performed to minimize non-specific antibody binding. Sections were incubated with the primary antibodies ERα (1:100, mouse monoclonal SC-311, Santa Cruz Biotechnology), PR (1:100, mouse monoclonal PR-2C5, Invitrogen) and ERβ (1:100, mouse monoclonal PPG5/10, Abcam) for 18 hours (overnight). The next day, section were washed with dilution buffer at ph 7.4 and treated with secondary antibody (N-Histofine Simple Stain® - Nichirei Biosciences Inc.) for 30 min at 27º C. Sections were washed and developed with diaminobenzidine substrate (Liquid DAB Cromogen® – Dako) for 3 min in room temperature. Subsequently, sections were washed and counterstained with Mayer's Hematoxylin for 90 sec, washed with tap water for 5 min, dehydrated and mounted for analysis. Negative controls were prepared using dilution buffer instead of primary antibody.

Slides were observed at 10×, 20× and 40× magnification under a light microscope. Staining intensity and distribution of ERα, ERβ and PR in luminal epithelium, glandular

epithelium and stroma were described by three observers blinded to mare reproductive status and treatment.

Statistical Analysis

The mean relative expression ratio for ESR1, ESR2 and PGR were compared between treatments within anestrous and transition groups, as well as within treatments or cycle stages between groups, with the mixed model for repeated measures. The Tukey´s test was used to adjust the P-values that resulted from multiple comparisons. The analysis was performed with PROC MIXED (SAS Institute, 2011).

Descriptive analysis were produced (measures of central tendency and dispersion) for the hormone concentration results to characterize the study sample. All response variables were not normally distributed and therefore were log-transformed for analysis. Data were presented as geometric means with confidence intervals to show values in the original scale. A repeated measures model (Kleinbaum *et al*. 2007) was constructed with PROC MIXED (SAS Institute, 2011) to compare the mean estradiol, estrogen conjugate, and progesterone concentrations among collection days and treatments. The auto regressive covariance structure provided the best fit to the data and was used to model the correlation between repeated observations within the same mare. A *P*-value of 0.05 or less was considered statistically significant

Results

Intervals between treatments

The mean interval between the last hormonal treatment (LA P4) in anestrous group to the onset of treatment in transition group was 64.6 ± 9.2 days (mean ± SEM). The mean interval between the last hormonal treatment (LA P4) in transition group to the first ovulation of the season in the cyclic group was 27.4 ± 2.9 days (mean ± SEM).

Steroid concentrations

Estradiol and estrogen conjugate peaks were observed 24 h after EB injection in treated groups and there were no differences ($P > 0.05$) on estrogens average concentrations between treatments (Fig. 2*a*,*b*). In the cyclic group, estradiol and estrogen conjugate peaks were observed on D2 before ovulation and there were no differences ($P > 0.05$) when compared to estrogens peaks of the treated groups. There were no differences on estrogens concentrations after LA P4 injection (from D1 onwards) between groups ($P > 0.05$; Fig. 2*a*,*b*).

Progesterone peaks were observed 24 h after injection of 1500 mg of LA P4 in non-cyclic treated groups. After peak, progesterone concentrations showed a sharp decrease on D2, followed by a less pronounced decrease until D5 in treated groups. There were no statistical differences between treated groups after P4 administration (Fig. 2*c; P* > 0.05). Progesterone concentration in the cyclic group was low before ovulation and started to increase from D1, rising gradually until D5. Concentration of P4 was significantly lower ($P < 0.05$) in the cyclic group on D1 and significantly higher on D5 when compared to the treated groups ($P < 0.05$; Fig. 2*c*).

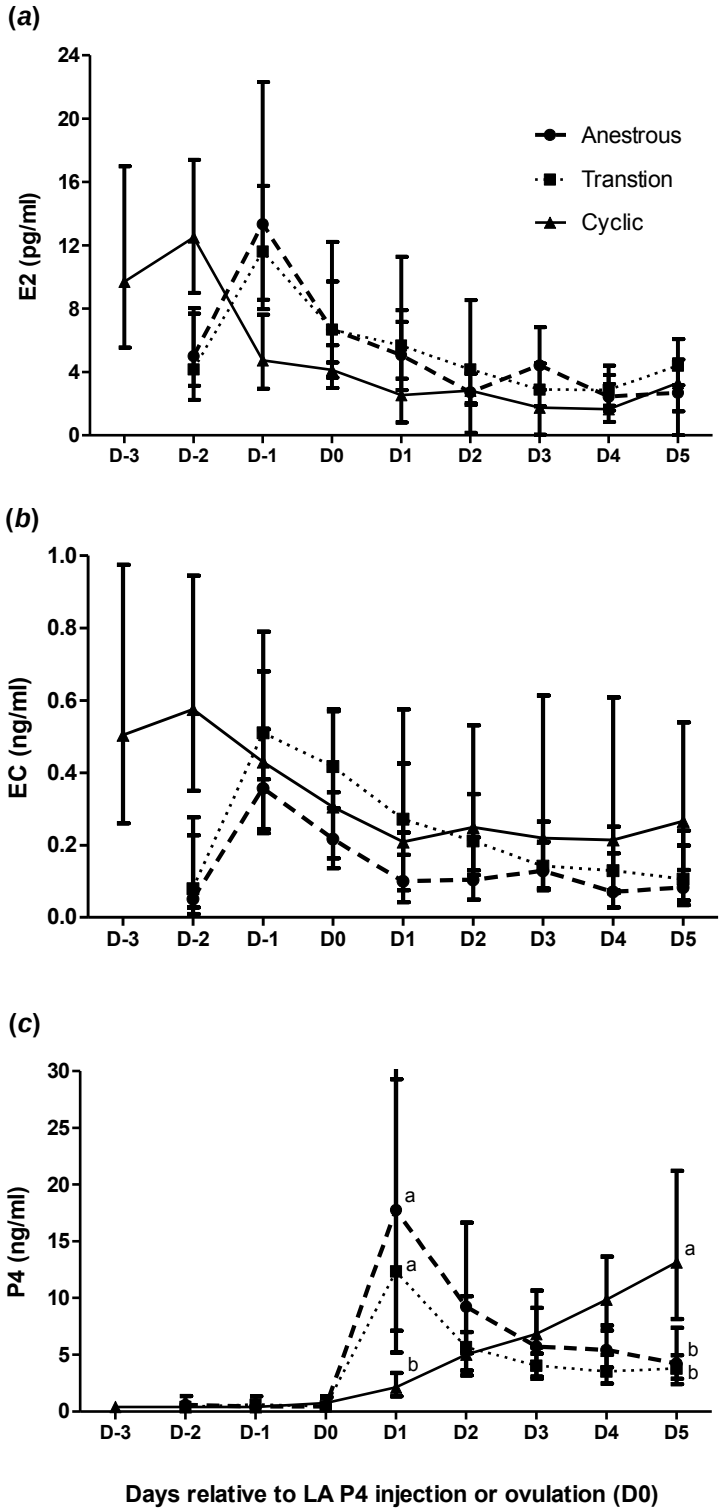

Figure 2: Hormone concentrations of (*a*) estradiol (E2), (*b*) estrogen conjugate (EC) and (*c*) progesterone (P4) after injection of 2.5 mg of estradiol benzoate (EB) on day −2 (D−2) followed by 1500 mg of long acting progesterone (LA P4) on day 0 (D0) in anestrous and transition groups. Hormone concentrations for E2, EC and P4 from the Control group are also shown, starting three days before ovulation (D−3). Different letters between groups within correspondent days indicate statistical difference ($P < 0.05$).

Uterine edema and tone

The scores given to uterine edema and tone in cyclic and non-cyclic treated mares are shown in Table 2**.** In the anestrous group, a lack of uterine edema was observed immediately before initiation of treatment (on D−2). After EB was given, maximum edema was found 24 and 48 h after injection. Following LA P4 injection, a sharp reduction was observed on D1, remaining low until D5. With regards to uterine tone, an increase was detected starting from D2 after LA P4 injection, reaching the intermediate level on D4.

In the transition group, a minimum to intermediate edema was observed before treatment initiation, reaching a maximum level 24 to 48 h after EB injection. After LA P4 administration, there was a gradual edema decrease, which was found in a minimum level on D5. An increase in uterine tone after LA P4 administration was observed sooner in the transition group as compared to anestrous group, reaching the intermediate level on D2.

Uterine edema in the cyclic group was found at a maximum level two days before ovulation, decreasing gradually to an intermediate to moderate level on ovulation day (D0). After ovulation, edema was observed at a minimum level, showing a slight increase on D5, where an intermediate to moderate edema was detected. An increase in uterine tone was observed starting from day one after ovulation, reaching an intermediate level on D2.

Table 2: Median scores of uterine edema and tone in anestrous or transitional non-cyclic mares after estradiol benzoate treatment (EB) using a single dose of 2.5 mg on D-2, followed by the injection of 1500 mg of long acting progesterone 48 h after EB administration (D0). The median scores for cyclic mares at the corresponding days to treated mares are also shown.

	Uterine edema								Uterine tone					
	Days relative to progesterone injection or ovulation (D0)													
Groups	D-2	D-1	D0*	D1	D2	D3	D4	D5	D0*	D1	D2	D3	D4	D5
Anestrous	0	3	3	1	1	1	1	1	1	1	1.5	1.75	2	2
Transition	1.5	3	3	2	1.5	1.5	1.5	1	1	1.5	2	2	2	2
Cyclic	3	2	1.5	1	1	1	1	1.5	1	1.5	2	2	2	2

* Uterine edema and tone observed immediately before progesterone injection.

Changes in relative abundance of transcripts for PGR, ESR1 and ESR2

In anestrous group, there was an increase in relative abundance of *PGR* and *ESR1* transcripts (1.74-fold and 2.07-fold, respectively) after EB administration (Fig. 3*a*,*b*), which was not different ($P > 0.05$) from the slight decrease in relative abundance of *PGR* and *ESR1* transcripts (−0.66 fold and −0.43 fold, respectively) after EB injection in transition group (Fig 3*a*,*b*). In both anestrous and transition groups, there was a decrease in relative abundance of *ESR2* transcripts after EB administration (−0.89 and −1.89, respectively; Fig. 3*c*), which was not significantly different between groups ($P > 0.05$). After LA P4 administration in anestrous group, a slight increase in relative abundance of *PGR* (0.34 fold), *ESR1* (2.67 fold) and a lack of change of *ESR2* (−0.004 fold) transcripts were observed, which were not different ($P > 0.05$) from the decrease in relative abundance of *PGR* (−0.42 fold), *ESR1* (−0.27 fold) and *ESR2* (−2.78 fold) transcripts after LA P4 injection in transition group (Fig. 3). In addition, within groups, there were no differences ($P > 0.05$) when changes in relative transcripts abundance were compared after EB and after LA P4 administration (Fig. 3).

When changes in transcripts abundance between five days after LA P4 injection and 48 h after EB treatment were compared to changes between five days after ovulation and estrus, the decrease observed on *PGR* mRNA expression was not different ($P > 0.05$) between cyclic (−1.25 fold), anestrous (−1.28 fold) and transitional mares (−0.62 fold; Fig. 4*a*). There were no differences ($P > 0.05$) between anestrous and transition groups when changes in relative abundance of transcripts for *ESR1* (Fig. 4*b*) and *ESR2* (Fig. 4*c*) were compared between the moments after LA P4 injection and after EB administration. In addition, when changes in relative abundance five days after ovulation were compared to estrus, there were no differences between treated and cyclic group for *ESR1* and *ESR2* transcripts ($P > 0.05$; Fig. 4*a*, 4*b*).

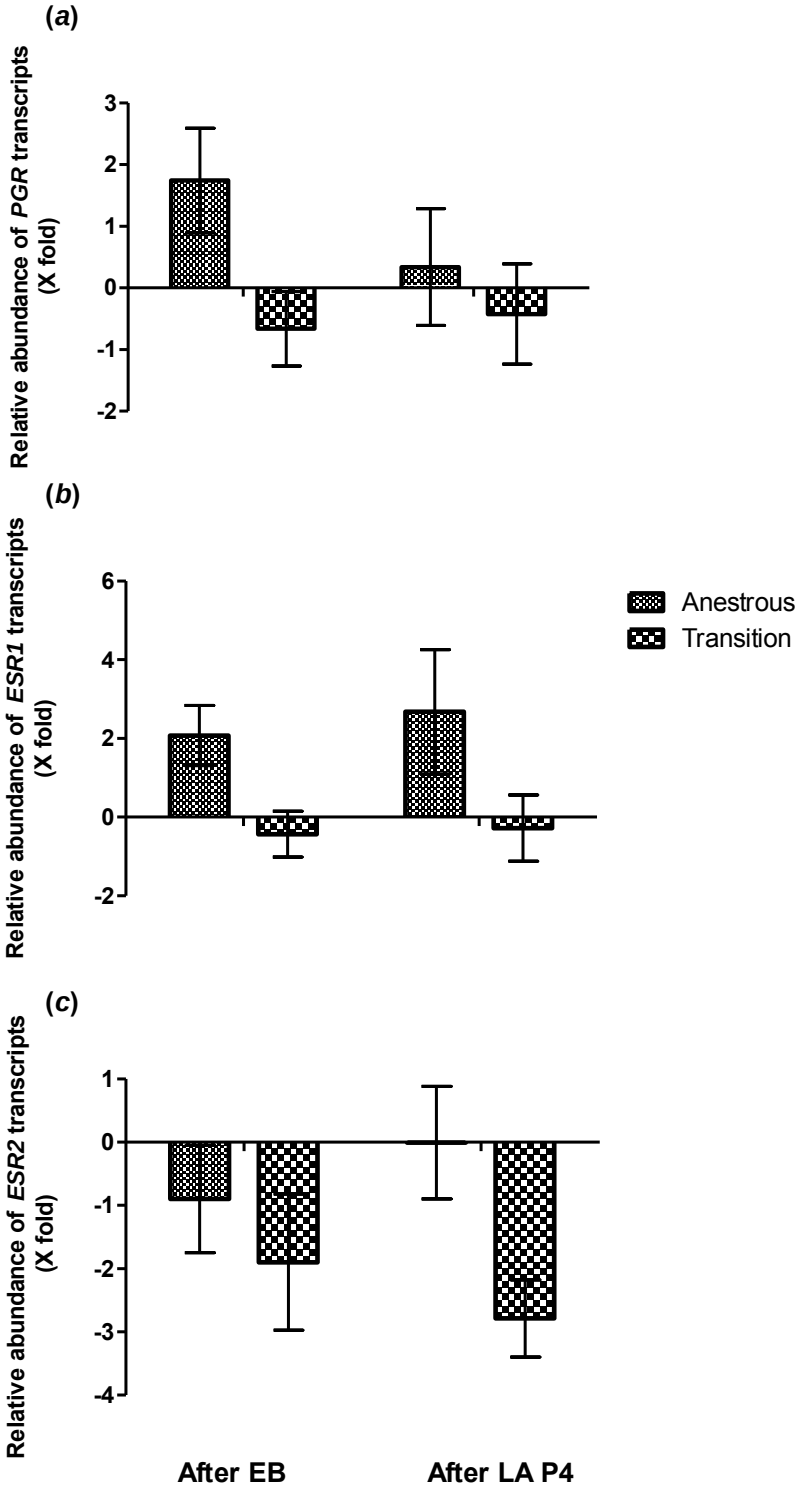

Figure 3: Changes in relative abundance of transcripts for (*a*) progesterone receptor – *PGR*, (*b*) estrogen receptor α – *ESR1*, (*c*) and estrogen receptor β - *ESR2* at 48 h after estradiol benzoate treatment (After EB) and after long-acting progesterone treatment (after LA P4), in anestrous and transition groups. Data are expressed as mean ± SEM of the relative expression ratio of each transcript.

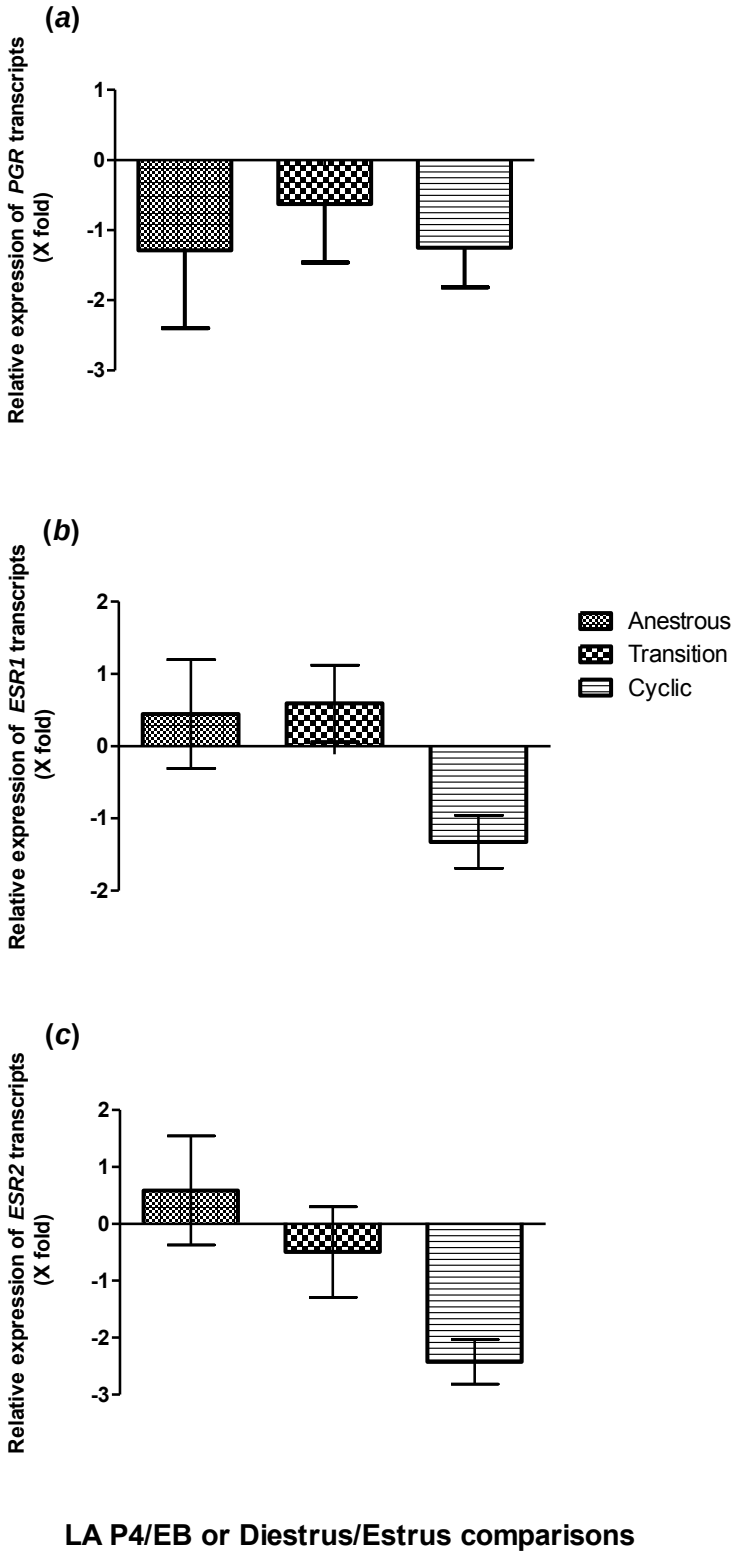

Figure 4: Changes in relative abundance of transcripts for (*a*) progesterone receptor – *PGR*, (*b*) estrogen receptor α – *ESR1*, (*c*) and estrogen receptor β - *ESR2* when transcripts abundance after LA P4 treatment were compared to abundance after EB treatment (LA P4/EB) or abundance five days after ovulation was compared to estrus (Diestrus/Estrus), in anestrous, transition and cyclic groups. Data are expressed as mean ± SEM of the relative expression ratio of each transcript.

Endometrial immunostaining and distribution of PR, ERa and ERβ

In anestrous and transition groups, ERα and ERβ immunostaining were mostly localized to the nucleus of luminal epithelium, glandular epithelium and stroma before (Fig. 4*a,d* and 5*a,d*) and after treatment with EB (Fig. 5*b*,*e* and 6*b*,*e*). Following LA P4 injection, ERα and ERβ immunoreaction were also found in the glandular epithelium cytoplasm in both treated groups (Fig. 5*c*,*f* and 6*c*,*f*). Immunolabeling for PR in anestrous and transition groups were detected in the nuclei of the stroma, luminal epithelium and mostly in the glandular epithelium nuclei, prior to (Fig. 7*a*,*d*) and after EB treatment (Fig. 7*b*,*e*). After LA P4 treatment, PR immunostaining was localized to the nuclei of stroma and luminal epithelium, and in the nuclei and cytoplasm of glandular epithelium in both treated groups (Fig. 7*c*,*f*). In the cyclic group, immunolabeling for ERα, ERβ and PR were detected in the nuclei of stroma and luminal epithelium, as well as in the cytoplasm and nuclei of glandular epithelium at estrus and five days after ovulation (Fig. 5*h*,*i*; 6*h*,*i* and 7*h*,*i*).

In anestrous group, ERα and ERβ immunostaining five days after LA P4 administration (Fig. 5*c*, 6*c*) appeared to be more intense than prior to treatment initiation (Fig. 5*a*, 6*a*). In addition, apparently greater ERα, ERβ and PR immunolabeling, particularly in the glandular epithelium cytoplasm, were detected five days after LA P4 administration (Fig. 5*c*, 6*c*, 7*c*) as compared to 48 h after EB injection (Fig. 5*b*, 6*b*, 7*b*). In transition group, ERα cytoplasmic immunolabeling appeared to be stronger five days after LA P4 administration (Fig. 5*f*) than prior to treatments (Fig. 5*d*) and when compared to 48 h after EB injection (Fig. 5*e*). Subjectively, immunostaining for ERβ was more intense five days after P4 injection (Fig. 6*f*) as compared to 48 h after EB was given (Fig. 6*e*). For the cyclic group, cytoplasmic PR immunostaining appeared to be greater at estrus ($P < 0.05$; Fig. 7*h*) than five days after ovulation (Fig. 7*i*). There were no apparent differences observed for ERα and ERβ immunolabeling between cycle stages (Fig. 5*h,i* and 6*h,i*).

When the effects of treatment on receptors immunostaining were compared between treated groups (anestrous and transition) and between treated and control groups, ERα and PR cytoplasmic immunolabeling at 48 h after EB injection appeared to be greater in transition group when compared to anestrous group (Fig. 5*e*,*b* and 7*e*,*b*). Subjectively, immunostaining for ERα and PR were more intense at estrus in cyclic group when compared to 48 h after EB administration in anestrous group (Fig. 5*h*,*b* and 7*h*,*b*). In addition, staining for ERα at estrus appeared to be greater than at 48 h after EB injection in transition group (Fig. 5*h*,*e*). There were no apparent differences on ERβ immunostaining between groups (Fig. 5). Immunolabeling before initiation of treatments were also compared between treated groups and subjectively a more intense staining for ERα was found before treatments in transition group as compared to anestrous group (Fig. 5*d*,*a*).

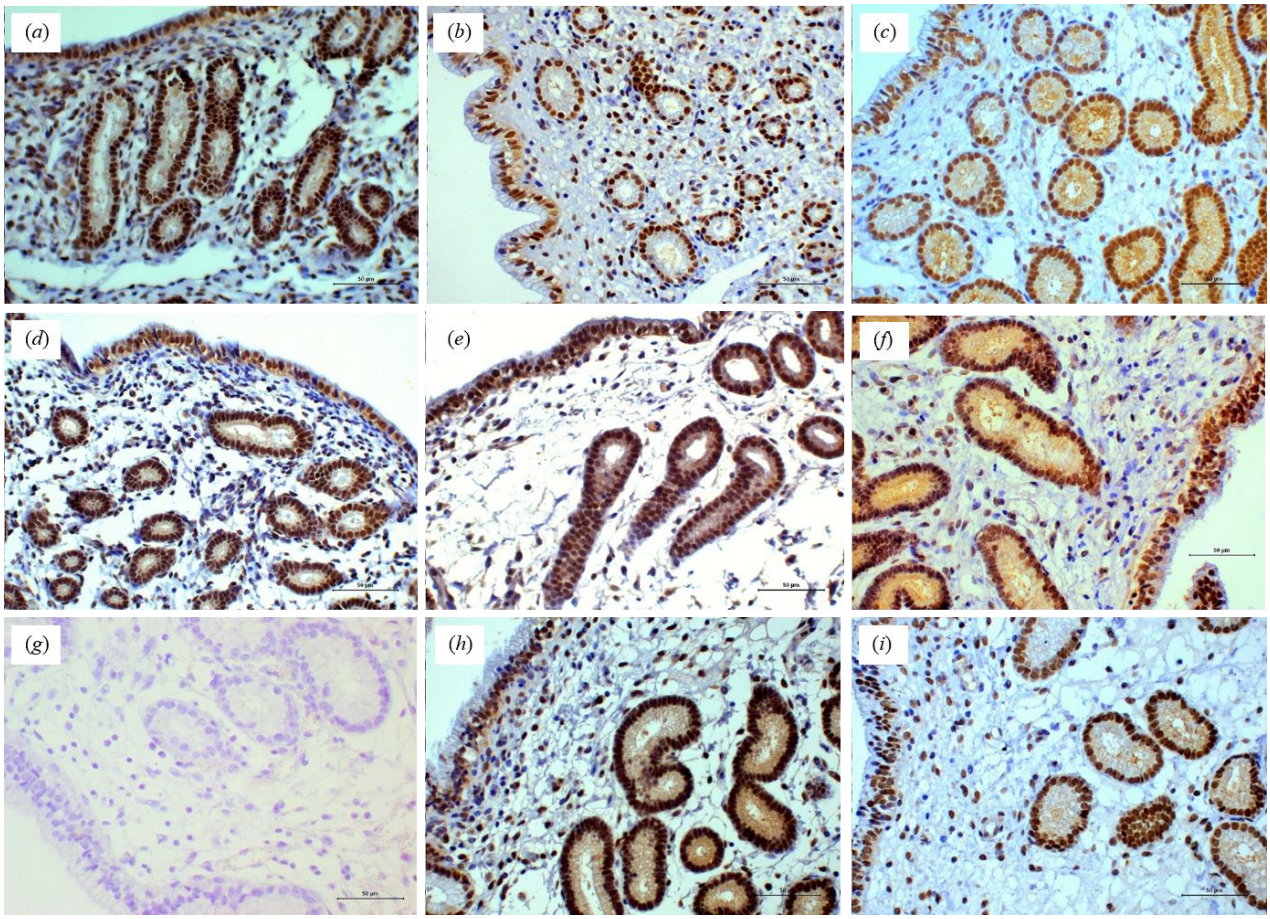

Figure 5: Photomicrographs of the equine endometrium immunostained for estrogen receptor α (ERα). The figure panels show immunoexpression before treatments in anestrous (*a*) and transition (*d*) groups, at 48 h after estradiol benzoate treatment in anestrous (*b*) and transition (*e*) groups, and at five days after long acting progesterone administration in anestrous (*c*) and transition (*f*) groups. Figures (*h*) and (*i*) show immunohistochemical expression at estrus and five days after ovulation, respectively, in the cyclic group. Figure (*g*): negative control. Bars = 50 µm.

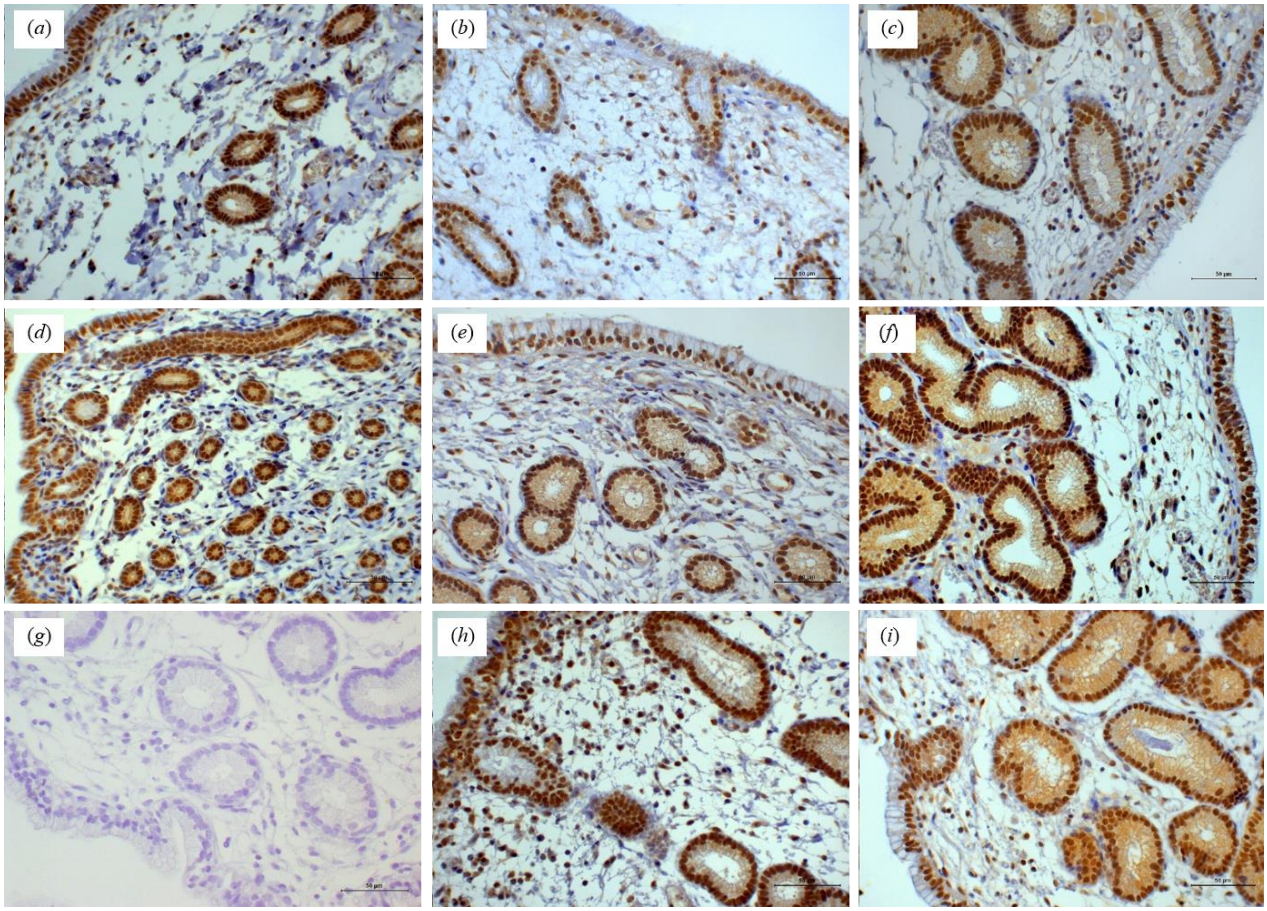

Figure 6: Photomicrographs of the equine endometrium immunostained for estrogen receptor β (ERβ). The figure panels show immunoexpression before treatments in anestrous (*a*) and transition (*d*) groups, at 48 h after estradiol benzoate treatment in anestrous (*b*) and transition (*e*) groups, and at five days after long acting progesterone administration in anestrous (*c*) and transition (*f*) groups. Figures (*h*) and (*i*) show immunohistochemical expression at estrus and five days after ovulation, respectively, in the cyclic group. Figure (*g*): negative control. Bars = 50 µm.

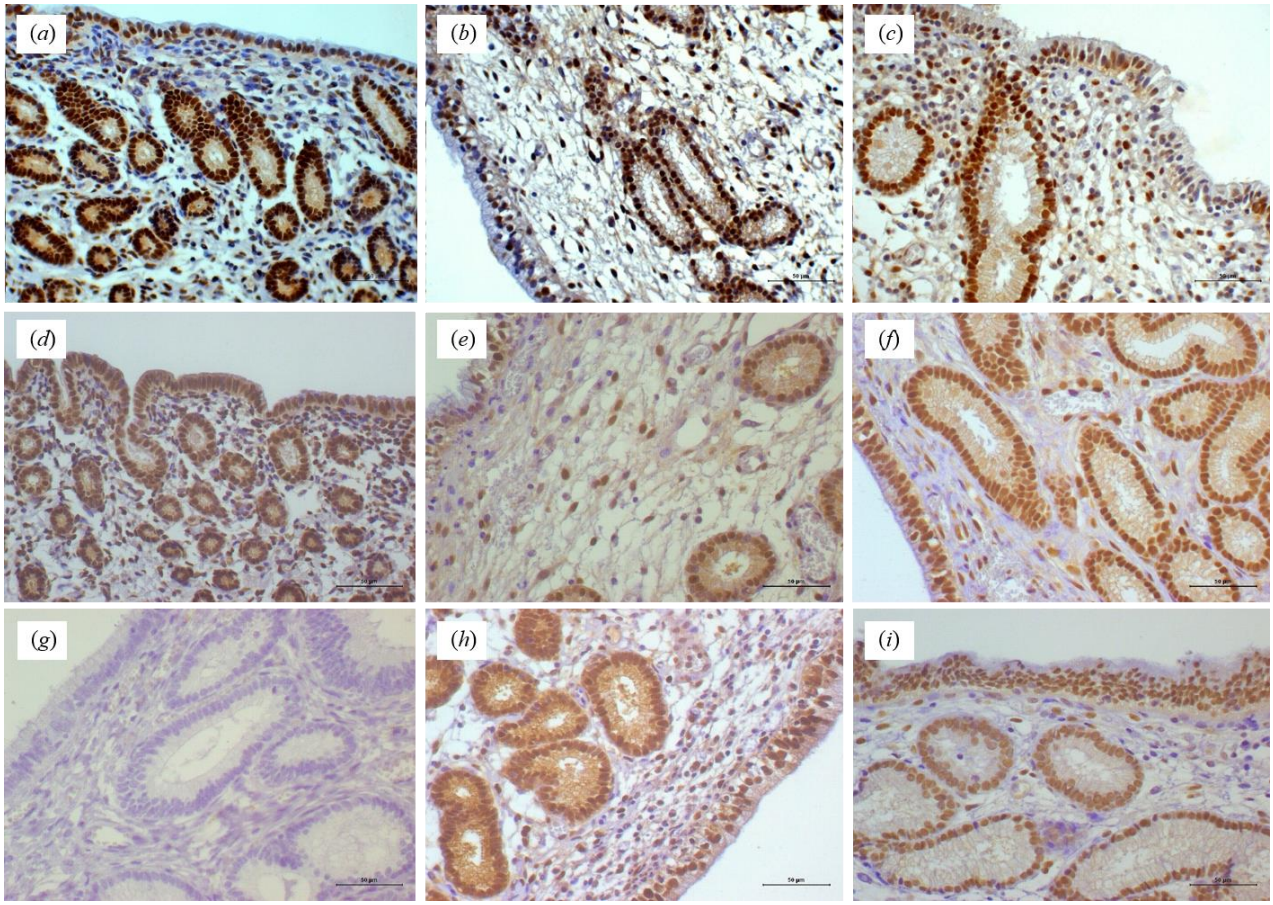

Figure 7: Photomicrographs of the equine endometrium immunostained for progesterone receptor (PR). The figure panels show immunoexpression before treatments in anestrous (*a*) and transition (*d*) groups, at 48 h after estradiol benzoate treatment in anestrous (*b*) and transition (*e*) groups, and at five days after long acting progesterone administration in anestrous (*c*) and transition (*f*) groups. Figures (*h*) and (*i*) show immunohistochemical expression at estrus and five days after ovulation, respectively, in the cyclic group. Figure (*g*): negative control. Bars = 50 µm.

Discussion

Here we report, for the first time, protein immunostaining and changes in relative abundance of transcripts for ERα, ERβ and PR in non-cyclic mare's endometrium after the administration of 2.5 mg of EB followed by 1500 mg of LA P4 for preparing anovulatory mares to become embryo recipients. Moreover, we could demonstrate that the aforementioned protocol was effective at causing similar uterine edema, tone and plasma estradiol concentrations to those observed during the late estrus and early diestrus in cyclic mares.

Similar estradiol and estrogen conjugate peaks were achieved after the injection of the single dose between treated and cyclic groups. It is generally considered that measuring the active form of estrogen, the 17β-estradiol, provides the best information in terms of hormone signal delivered to the target cell. However, because circulating E2 concentrations are low in some animals, the accuracy of this method is sometimes limited. In contrast, EC concentrations are 100-fold higher than plasma E2 concentrations and are therefore easier to measure (Daels *et al*. 1991). In the present study, only the peak values were above the E2 assay sensitivity. Because conjugated estrogens parallels plasma E2 and the same physiological information is obtained by estrogen conjugate (estrone sulphate) analysis (Daels *et al*. 1991), we also provided the EC profile in non-cyclic treated and cyclic mares.

The dose of 2.5 mg of EB was effective at causing high endometrial edema 24 h after injection in anestrous and transitional mares. High edema was also observed in the cyclic group, two days before ovulation, in agreement with previous reports on cyclic mares (Ginther, 1992). Moreover, a greater edema median score was detected prior to treatment initiation in transition group as compared to anestrous, probably due to the different follicular population size detected in anestrous (follicles <20 mm) and transitional mares (20 to 30 mm follicles). Considering that the larger the follicle the greater the ability to synthesize estrogen (Gastal *et al*. 1999), the presence of a discrete to intermediate edema in transition group as compared to

a lack of edema in the anestrous group could be explained. However, no difference on peripheral plasma E2 concentration was detected at this time between treated groups. A study was performed comparing estrogen and P4 concentrations between uterine fluid and peripheral blood of non-pregnant and pregnant pony mares, in which the authors indicated that changes occurring within the uterine environment are not discernible in the systemic circulation, especially during early pregnancy (Zavy *et al*. 1984). It is likely that local estrogens in the endometrium were not detected either by peripheral plasma concentrations during transitional phase in the present study.

After P4 administration, uterine edema and estrogen concentrations from both treated groups decreased to low levels, similarly to cyclic mares' concentrations after ovulation. However, a slight edema and estrogen rise was observed on day five after ovulation in the cyclic group. Ultrasonic uterine echotexture studies have revealed the presence of a slight increase in edema in early diestrus during the first half of the ovulatory season but not during the second half (Hayes *et al*. 1985), which may reflect follicular growth that occurs in some mares in early diestrus or estrogen production by the developing corpus luteum (Daels *et al*. 1991). In the present study, diestrus data were collected on the third estrous cycle of the breeding season, although we do not have accurate information on the follicular activity after ovulation.

Administration of 1500 mg of LA P4 in treated mares increased uterine tone to the intermediate level characteristic of diestrus in cyclic mares (Ginther, 1992). In addition, the dose given was effective in terms of reaching the minimum P4 concentrations required for pregnancy establishment and maintenance (>2.5 ng/mL; McKinnon *et al*. 1988) until day five after its injection, although P4 profiles were different between cyclic and non-cyclic treated mares. Despite the gradual P4 decrease after peak in the treated groups, for embryo transfer purposes, performed on average on day four to six after P4 injection, another LA P4 dose is

commonly given after transfer if an embryo is recovered, what ensures appropriate levels until pregnancy diagnosis.

It is well established that mRNA abundance of estrogen and P4 receptors in the mare's endometrium are stimulated by E2 and down-regulated by P4 (Watson *et al*. 1992; Aupperle *et al*. 2000; Kimmins and MacLaren 2001; Spencer and Bazer 2002; Hartt *et al*. 2005). Accordingly, *ESR1*, *ESR2* and *PGR* transcripts were decreased at early diestrus when compared to estrus in this study. When relative transcripts abundance five days after LA P4 injection were compared to transcripts abundance 48 h after EB administration within treated groups, the decrease found in *PGR* transcripts was similar to the decrease observed in the cyclic group when diestrus was compared to estrus. On the other hand, *ESR1* transcripts were increased in anestrous and transition groups, although such increase was not statistically different from the decrease found in the cyclic group.

As for *ESR1* transcripts, when *ESR2* transcripts at five days after LA P4 injection were compared to 48 hs after EB administration in treated groups, there were no differences from the changes found in the cyclic group, when diestrus and estrus were compared. Moreover, changes in relative abundance of transcripts for *PGR*, *ESR1* and *ESR2* were similar after EB administration or LA P4 injection between anestrous and transition groups, although a variation in the changes pattern (increase or decrease) was observed between groups after both hormone treatments. The similarity in transcripts changes observed in treated and cyclic groups suggest that the hormonal protocol was able to provide similar dynamics to that observed in cyclic mares.

As observed for mRNA abundance and as previously described (Aupperle *et al*. 2000; Hartt *et al*. 2005), immunostaining for PR appeared to be more intense at estrus when compared to diestrus in cyclic mares. However, no apparent differences for ERα and ERβ immunostaining were observed. Increased levels of ERα during the pre-ovulatory phase in

relation to late diestrus (8 to 13 days after ovulation) have been reported in mares (Aupperle *et al*. 2000; Silva *et al*. 2014) although no evidence for differential regulation of ERβ among cycle stages has been described (Silva *et al*. 2014; Ruijter-Villani *et al*. 2014). It is possible that the lack of difference on ERα protein immunostaining between estrus and diestrus herein could be due to the early stage of diestrus (five days after ovulation) in which tissues were collected.

In addition, there were no apparent differences on receptor staining intensity after EB injection for ERα and PR in anestrous and transition groups. Interestingly, ERα and ERβ immunostaining in the glandular epithelium cytoplasm appeared to be more intense after LA P4 injection than prior to or after EB treatment, in both treated groups. Staining for ERα was observed in both the cell nuclei and cytoplasm of the glandular epithelium during estrus and diestrus in cyclic mares, with a pronounced staining observed in the nucleus, in agreement with recent reports (Silva *et al*. 2014). Although ER and PR have been identified as nuclear receptors, it has been reported that they are continuously shuttled between the cytoplasm and cell nucleus (Guiochon-Mantel *et al*. 1996; Levin 2005). In addition, predominant cytoplasmic ER has been found in early pregnant mares (Wilsher *et al*. 2011). Furthermore, it is now widely accepted that both nuclear/cytoplasmic ERα and ERβ exist and, regardless to their location in the cell, the receptors have similar affinity for the steroid ligand (Levin 2001). Alternatively, cytoplasmic ER may function as membrane associated ER to mediate non-genomic actions of estrogen (Levin 2001, 2005). To identify the effects of E2 and P4 on ERα and PR expression, ovariectomized mice were treated with exogenous hormones (Tibbets *et al*. 1998). Among other findings, the authors observed that ERα is dependent on P4 stimulation as it was found to be upregulated in the glandular epithelium after administration of estrogen and progesterone. It appears that glandular epithelium ERα and ERβ can also be upregulated by P4 in non-cyclic treated mares.

The surge in hormone levels and the differential expression pattern of the steroid hormone receptors play a critical and important role during estrous cycle and in pregnancy establishment and maintenance. Several studies in mares have determined that both PR and ER are evident during estrus when P4 concentrations are low and estrogen concentrations are increased (Aupperle *et al*. 2000; Hartt *et al*. 2005; Silva *et al*. 2014). Thereafter, PR and ER both decline in late diestrus, when circulating P4 levels are high. Spatially, the PR and ER located in the nuclei of the lumenal and glandular epithelia and stromal fibroblasts during estrus, whereas in late diestrus and early pregnancy these are reduced in the luminal and glandular epithelium but persist in the stroma (Aupperle *et al*. 2000; Spencer and Bazer 2002).

In sheep the loss of PR in the glandular epithelia is a pre-requisite for the expression of genes for secretory proteins. Loss of PR in the luminal and glandular epithelium, not in the stroma, were found to be reduced or absent as early as day 20 of pregnancy also in the mare (Wilsher *et al*. 2011). In the present experiment, PR expression in the cyclic group was aapparently decreased in early to mid diestrus (day five after ovulation), particularly in the cytoplasm, but still showed pronounced labeling in the luminal and glandular epithelium nuclei. However, such decrease was not evidenced on day five after LA P4 administration in treated groups. Perhaps the different hormonal profile between non-cyclic progesterone treated groups and cyclic mares could have interfered on the PR expression. Moreover, successful pregnancies after embryo transfer are achieved using these LA P4 protocols. However, additional injections of LA P4 are given, especially after embryo transfer and pregnancy diagnosis, which may cause a further reduction on PR expression in luminal and glandular epithelium. Additional studies are needed to clarify these events in non-cyclic treated embryo recipient mares.

In conclusion, the dose of 2.5 mg of EB caused consistent endometrial edema and estrogens concentrations to those observed at late estrus in cyclic mares. Additionally, the

administration of 1500 mg of LA P4 increased uterine tone to characteristic levels of diestrus found in cyclic mares and acceptable P4 concentrations to that observed during the early luteal phase. Changes in relative abundance of *PGR*, *ESR1* and *ESR2* transcripts were similar between cyclic and non-cyclic treated mares when P4 stimulated endometrium was compared to estrogen stimulated endometrium. However, decreased PR immunostaining in the glandular epithelium was not subjectively observed in the non-cyclic mares five days after LA P4 administration, as observed at five days after ovulation in the cyclic mares. Furthermore, the apparent increase in ERα and ERβ immunostaining in the glandular epithelium cytoplasm after LA P4 injection suggest that P4 can upregulate ER protein expression after the injection of 2.5 mg of EB in anestrous and transitional mares, as opposed to what is observed in cyclic mares.

Acknowledgments

The authors thank the FAPESP foundation (Fundação de Apoio a Pesquisa e Ensino do Estado de São Paulo, Processo Fapesp n. 2012/00298-7) for the financial support and Lillian Sibley for assistance with the hormonal assays.

References

Aupperle, H., OzgenSshoon, H.A., Scoon, D., Hoppen, H.O., Sieme, H., and Tannapfel, A. (2000). Cyclical endometrial steroid hormone receptor expression and proliferation intensity in the mare. *Eq. Vet. J.* **32**, 228-232.

Botelho, J.H.V., Pessoa, G.O., Rocha, L.G.P., and Yeste, M. (2015). Hormone supplementation protocol using estradiol benzoate and long-action progesterone is efficient in maintaining pregnancy of anovulatory recipient mares during autumn transitional phase. *Anim. Reprod. Sci*. **152**, 39-43.

Bringel, B.A., Jacob, J.C.F., Zimmerman, M., Alvarenga, M.A., and Douglas, R.H. (2003). Biorelease progesterone LA 150 and its application to overcome effects of premature luteolysis on progesterone levels in mares. *Rev. Bras. Reprod. Anim*. **27**, 498-500.

Carnevale, E.M., Ramirez, R.J., Squires, E.L., Alvarenga, M.A., Vanderwal, D.K., and McCue, P.M. (2000). Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. *Theriogenology*. **54**, 965-979.

Daels, P.F., Ammon, D.C., Stabenfeldt, G.H., Liu, I.K.M., Hughes, J.P., and Lasley, B.L. (1991) Urinary and plasma estrogen conjugates, estradiol and estrone concentrations in nonpregnant and early pregnant mares. *Theriogenology*, **35**(5), 1001-1017.

De Sousa Abreu, R., Penalva, L.O., Marcotte, E., and Vogel, C. (2009). Global signatures of protein and mRNA expression levels. *Mol. Biosyst*. **5**, 1512-1526.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J.A. (1997). Human estrogen receptor b-gene structure, chromosomal localization, and expression pattern. *J. Clin. Endocr. Metab*. **82**, 4258-4265.

Gastal, E.L., Gastal, M.O, Wiltbank, M.C., and Ginther, O.J. (1999). Comparison of circulating estradiol-17β and folliculogenesis during the breeding season, autumn transition and anestrus in the mare. *Biol. Reprod*., **61**(1), 31-39.

Gebhardt, S., Merkl, M., Herbach, N., Wanke, R., Handler, J., and Bauersachs, S. (2012). Exploration of global gene expression changes during the estrous cycle in equine endometrium. *Biol. Reprod*. **87**(6):136, 1-13.

Ginther, O.J., Gastal, E.L, Gastal, M.O., and Beg, M.A. (2004). Seasonal influence on equine follicles dynamics. *Anim. Reprod*., **1**(1), 31-44.

Ginther, O.J. (1992). 'Reproductive Biology of the Mare: Basic and Applied Aspects'. 2. ed. (Cross Plains WI:Equiservices).

Guiochon-Mantel, A., Delabre, K., Lescop, P., and Milgrom, E. (1996). Intracellular traffic of steroid hormone receptors. *J. Steroid Biochem. Mol. Biol*. **56,** 3-9.

Greco, G.M., Burlamaqui, F.L.G., Pinna, A.E., Queiroz, F.J.R., Cunha, M.P.S., and Brandão, F.Z. (2012). Use of long acting progesterone to acyclic embryo recipient mares. *Braz. J. Anim. Sci*. **41**(3), 607-61.

Hartt, L.S., Carling, S.J., Joyce, M.M., Johnson, G.A., Vanderwall, D.K., and Ott, T.L. (2005). Temporal and spatial associations of oestrogen receptor alpha and progesterone receptor in the endometrium of cyclic and early pregnant mares. *Reproduction*. **130**, 241-250.

Hayes, K.E.N., Pierson, R.A., Scraba, S.T., and Ginther, O.J. (1985). Effects of estrous cycle and season on ultrasonic uterine anatomy in mares. *Theriogenology*. **24**(4), 465-477.

Hayes, K.E.N. and Ginther, O.J. (1986). Role of progesterone and estrogen in development of uterine tone in mares. *Theriogenology*. **25**, 581-590.

Hinrichs, K., Sertich, P.L., Cummings, M.R., and Kenney, M.R. (1985). Pregnancy in ovariectomized mares achieved by embryo transfer. *Equine Vet. J*. **3**, 74-75.

Hinrichs, K., Sertich, P.L., and Kenney, R.M. (1986). Use of altrenogest to prepare ovariectomized mares as embryo transfer recipients. *Theriogenology*. **26**, 455-460.

Kaercher, F., Siqueira, M., Mercadante, A., Figueiredo, T., Weiss, R.R., Siqueira, F., and Kozicki, L.E. (2011). Embryo transfer in no cycling crioula and quarter horse breeds treated with estradiol cipionate and long acting progesterone. *Braz. Arch. Biol. Techn*. **54**(6), 1147-1150.

Kaercher, F., Kozicki, L.E., Camargo, C.E., Weiss, R.R., Santos, I.W., Muradas, P.R., Bertol, M.A.F., and Abreu, R.A. (2013). Embryo transfer in anovulatory recipient mares treated with estradiol benzoate and long-acting progesterone. *J. Eq. Vet. Sci*. **33**(3), 205-209.

Kenney, R.M., and Doig, P.A. (1986). Equine endometrial biopsy. 'Current therapy in theriogenology 2'. (Philadelphia: WB Saunders). 723-9.

Kimmins, S., and MacLaren, L.A. (2011). Oestrous cycle and pregnancy effects on distribution of oestrogen and progesterone receptors in bovine endometrium. *Placenta*. **22**, 742-748.

Klein, C., Rutllant, J., and Troedsson, M.H.T. (2011). Expression stability of putative reference genes in equine endometrial, testicular and conceptus tissues. *BMC Res. Notes.* **4**, 120-129.

Kleinbaum, D. G., Kupper, L. L., Nizam, A. and Muller, K. E. (2007). 'Applied Regression Analysis and Multivariable Methods'. Duxbury Press, Belmont, California.

Levin, E.R. (2001). Cell localisation, physiology and non-genomic actions of estrogen receptors. *J. Appl. Physiol*. **91**, 1860-1867.

Levin, E.R. (2005). Integration of the extranuclear and and nuclear actions of estrogen. *Mol Endocrinol.* **19**(8), 1951-1959.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real Time Quantitative PCR and the 2^{-ddCT} Method. *Methods.* **25**, 402-408.

McCue PM, Scoggin CF, Lindholm AFG. Estrus. In: McKinnon AO, Squires EL, Vaala WE, Varner DD. Equine Reproduction. 2nd ed. Ames, IA:Blackwell Publishing, 2011:1716-1727.

McKinnon, A.O., Squires, E.L., Carnevale, E.M., and Hermenet, M.J. (1988). Ovariectomized steroid-treated mares as embryo transfer recipient and as a model to study the role of progestins in pregnancy maintenance. *Theriogenology*. **29**, 1055-1063.

Parlevliet, J.M., Pearl, C.A., Hess, M.F., Famula, T.R., and Roser, J.F. (2006). Immunolocalization of estrogen and androgen receptors and steroid concentrations in the stallion epididymis. *Theriogenology.* **66**, 755-65.

Rocha Filho, A.N., Pessôa, M.A., Gioso, M.M., and Alvarenga, M.A. (2004). Transfer of equine embryos into anovulatory recipients supplemented with short or long acting progesterone. *Anim. Reprod*. **1** (1), 91-95.

Ruijter-Villani, M., van Tol, H.T.A., and Stout, T.A.E. (2014). Effect of pregnancy on endometrial expression of luteolytic pathway components in the mare. *Reprod. Fertil. Dev*. http://dx.doi.org/10.1071/RD13381.

SAS Institute. (2011). SAS/STAT User's Guide. Version 9.2, SAS Institute Inc., Cary, NC.

Silva, E.S.M., Ignácio, F.S., Pantoja, J.C.F., Puoli Filho, J.N.P., and Meira, C. (2014). Supplementary corpora lutea monitoring allows progestin treatment interruption on day 70 of pregnancy in non-cyclic recipient mares. *Anim. Reprod. Sci*. **144**, 122-128.

Silva, E.S.M., Pantoja, J.C.F., Puoli Filho, J.N.P., and Meira, C. (2015). Ultrasonography of the conceptus development from days 15 to 60 of pregnancy in non-cyclic recipient mares. *Cien. Rural*. **45**(3), 512-518.

Simoncini, T., and Genazzani, A.R. (2003). Non-genomic actions of sex steroid hormones. *Eur. J. Endocrinol*. **148**, 281-292.

Spencer, T.E., and Bazer, F.W. (2002). Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. *Front. Biosci*. **7**, 1879-1898.

Squires, E.L. (2013). Embryo transfer challenges and perspectives. *Braz. J. Anim. Reprod*. **37**(2), 105-107.

Tibbets, T.O., Mendoza-Meneses, M., O'Malley, B.W., and Conneely, O.M. (1998). Mutual and intercompartmental regulation of estrogen receptor and progesterone receptor expression in the mouse uterus. *Biol. Reprod*. **59**, 1143-1152.

Watson, E.D., Skolnik, S.B., and Zanecosky, H.Z. (1992). Progesterone and estrogen receptor distribution in the endometrium of the mare. *Theriogenology.* **38**, 575-580.

Weihua, Z., Saji, S., Makine, S., Cheng, G., Jensen, E.V., Warner, M., and Gustafsson, J.A. (2000). Estrogen receptor (ER) β, a modulator of ERα in the uterus. *PNAS*. **97**(11), 5936-5941.

Wilsher, S., Gower, S., and Allen, W.R. (2011). Immunohistochemical localization of progesterone and oestrogen receptors at the placental interface in mares during early pregnancy. *Anim. Reprod. Sci*. **129**, 200-208.

Zavy, M.T., Mayer, R., Vernon, M.W., Bazer, F.W., and Sharp, D.C. (1979). An investigation of the uterine-luminal environment of nonpregnant and pregnant Pony mares. *J. Reprod. Fertil. Suppl*. **27**, 403-411.

Zavy, M.T., Vernon, M.W., Sharp, D.C., and Bazer, F.W. (1984). Endocrine aspects of early pregnancy in pony mares: a comparison of uterine luminal and peripheral plasma levels of steroids during the estrous cycle and early pregnancy. *Endocrinology*. **115**(1), 214-219.

Capítulo IV

CONSIDERAÇÕES FINAIS

Fornecer um ambiente uterino compatível com a sobrevivência e desenvolvimento de um concepto é essencial quando se prepara éguas acíclicas como receptoras de embriões. A partir dos resutados encontrados no presente estudo, concluimos que a aplicação de 2,5 mg de benzoato de estradiol (BE) provocou edema uterino e tônus similares aos que ocorrem em éguas cíclicas no final do estro e início do diestro. A mesma dose provocou picos de concentrações de estradiol similares aos de éguas tratadas com doses de 5 ou 10 mg de BE e aos de éguas cíclicas, sem elevar a concentração de estrógeno conjugado como observado com as outras doses. Após a administração da progesterona de longa ação (P4 LA), a concentração máxima da P4 plasmática não foi reduzida pela administração prévia de 2,5 mg de BE quando comparada ao pico da concentração de P4 após aplicação de 10 mg de BE.

Além disso, a administração de 2,5 mg de BE seguida de 1500 mg de P4 LA foi capaz de provocar alterações na abundância relativa dos transcritos para ERα, ERβ e PR similares às encontradas em éguas cíclicas, quando o dia cinco pós administracão da P4 LA foi comparado ao dia cinco pós ovulação. No entanto, o mesmo não foi observado para a imunomarcação, uma vez que a redução aparente na imunomarcação do receptor de P4 encontrada durante o diestro não foi detectada cinco dias após a administração de uma única dose de 1500 mg de P4 LA nas éguas acíclicas.

A partir do exposto, indicamos o uso de 2,5 mg de BE seguido de 1500 mg de P4 LA para o preparo de éguas receptoras acíclicas, por ser eficiente em provocar alterações nas éguas acíclicas similares às encontradas nas cíclicas, principalmente no que diz respeito às alterações morfológicas uterinas, na abundância dos transcritos para ERα, ERβ e PR no endométrio, e de concentrações circulantes de estrógeno e P4.