UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA ANIMAL:
EQUINOS

CARACTERÍSTICAS HISTOLÓGICAS DO ENDOMÉTRIO DURANTE O INÍCIO DO DESENVOLVIMENTO EMBRIONÁRIO EM ÉGUAS

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Tese apresentada ao Curso de Pós-Graduação da Faculdade de Veterinária da UFRGS como requisito para obtenção do grau de Doutor em Medicina Animal: Equinos na área de Reprodução Equina, sob a orientação da Dr[a]. Ricardo Macedo Gregory e co-orientação do Prof. Dr. Rodrigo Costa Mattos.

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GIOVANI CASANOVA CAMOZZATO

CARACTERÍSTICAS HISTOLÓGICAS DO ENDOMÉTRIO DURANTE O INÍCIO DO DESENVOLVIMENTO EMBRIONÁRIO EM ÉGUAS

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“*O sucesso não é a chave para a felicidade.*
A felicidade é a chave para o sucesso."

Albert Schweitzer

RESUMO

Características histológicas do endométrio durante o início do desenvolvimento embrionário em éguas

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Orientador: Ricardo Macedo Gregory

A gestação inicial da égua é um período fascinante que abrange numerosas e intensas mudanças em seu desenvolvimento, muitas das quais são únicas para a espécie equina. Esse desenvolvimento depende da manutenção da função lútea, do estabelecimento de um ambiente uterino e de uma interação precisa e orquestrada entre o concepto e o ambiente uterino. O objetivo deste estudo foi verificar as alterações histológicas do endométrio e a produção histotrófica em éguas cíclicas e prenhes nos dias 7, 10 e 13 pós-ovulação. No primeiro ciclo, biópsias endometriais de 30 éguas foram coletadas no dia 7 (n = 10), 10 (n = 10) e 13 (n = 10) constituindo o grupo éguas cíclicas. No segundo ciclo, as mesmas éguas foram cobertas por um garanhão fértil, acompanhadas diariamente até detectar a ovulação, considerada o dia 0. Foram coletadas biópsias endometriais nos dias7 (n 10), 10 (n 10) e 13 (n 10). Imediatamente após a coleta, o útero foi lavado e as éguas em que foi obtido embrião, foram inseridas no grupo de éguas prenhes. Um maior calibre dos vasos sanguíneos foi observado em prenhez comparados às éguas cíclicas do dia 7 aos 13. No sétimo dia pós-ovulação, uma grande perda de células ciliadas foi evidente no grupo de éguas prenhes, comparadas ao grupo de éguas cíclicas, as células do epitélio endometrial estavam mais protusas e uma pequena quantidade de secreção histotrófica entre as dobras endometriais foi observada. No décimo dia de prenhez, secreção histotrófica glandular e do epitélio luminal estavam mais presentes comparadas às éguas do grupo cíclico. No dia 13 de prenhes, foi observado um grande conteúdo de histotrofo nas aberturas glandulares que estavam cercadas por células ciliares. Ocorreram alterações no ambiente uterino logo após a entrada do embrião no útero. No estroma e no lúmen, essas modificações parecem visar fornecer a nutrição necessária para o desenvolvimento inicial do embrião e estas mudanças nas estruturas celulares irão interagir na sinalização embrionária, futura fixação, implantação e placentação.

Palavras-chave: embrião equino, ultraestrutura endometrial, histotrofo, período pré-implantação, células ciliadas, vasos sanguíneos.

ABSTRACT

Histological characteristics of the endometrium during early embryo development in mares

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The early pregnancy of mare is a fascinating period that encompasses numerous and intense changes in its development, many of which are unique to the equine species. This development depends on the maintenance of the luteal function, the establishment of a favorable uterine environment and a precise and orchestrated interaction between the concept and the uterine environment. The aim of this study was to evaluate histological changes in the endometrium in days 7, 10 and 13 post-ovulation in pregnant and cyclic mares. In the first cycle, endometrial biopsies from 30 cyclic mares (Cyclic group) were collected on days 7, 10 and 13 post-ovulation. In the second cycle, the same mares were bred by a fertile stallion. At days 7, 10 and 13 post-ovulation intrauterine biopsies were collected. Immediately after sample collection, the mare's uteri were flushed, and those mares with embryo recovery were assigned to the Pregnant group. A larger blood vessel caliber was observed in pregnant mares than in cyclic from day 7 to 13. On the 7th day a large loss of ciliated cells was evident in the group of pregnant mares in comparison with the Cyclic group and the superficial cells of the endometrium were more protruded, and a small amount of histotrophic material between the folds was observed. On the 10th day of pregnancy, the glandular histotrophic secretion and the secretion of luminal epithelium became more intense than the secretion of cyclic mares. On the 13th day of pregnancy, a very large amount of histotroph was observed within large glandular openings surrounded by ciliated cells. Changes occurred in the uterine environment thereupon the entry of the embryo into the uterus. In the stroma and in the lumen, these modifications seem aim to provide the necessary nutrition for the initial development of the embryo and to promote changes at cellular structures that will interact in the embryonic signaling and future fixation, implantation and placentation.

***Keywords**:* Equine embryo; Endometrium ultrastructure; Histotroph; Pre-implantation period; Ciliated cells; Blood vessel.

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1 INTRODUÇÃO

O agronegócio do cavalo ganha a cada ano maior espaço no mercado brasileiro, sendo uma importante atividade econômica e social. Em 2006 os estudos apontaram um PIB da equinocultura de 7,5 bilhões de reais que foram atualizados atingiram a marca histórica de 16 bilhões de reais (IBGE, 2015), ou seja, um crescimento bruto de 113% em 10 anos ou 11,3% ao ano. A atividade gera 610 mil empregos diretos e 2430 empregos indiretos, sendo responsável, assim, por 3 milhões de postos de trabalho (LIMA; CINTRA, 2016).

Os conhecimentos sobre a prenhez inicial nos equinos são rudimentares em alguns aspectos em comparação a outras espécies domésticas, mesmo assim alguns deles, únicos entre os animais domésticos, foram bem caracterizados (AURICH; BUDIK, 2015). É um fascinante período que envolve numerosos eventos e profundas mudanças em seu desenvolvimento (STOUT, 2009).

Uma interação recíproca (KLEIN, 2016a), ininterrupta e completa entre o útero e o concepto é essencial para o estabelecimento e manutenção da prenhez (MEIRA, 2012). O embrião jovem indica sua presença, interrompe o ciclo estral e mantém a gestação, num processo conhecido como “Reconhecimento Materno da Prenhez" (RMP) (SHORT, 1969). Até este momento não está claro como é esta mensagem celular que leva ao reconhecimento materno da gestação em éguas, entretanto, vários estudos demonstram que ambos, útero e embrião, garantem a vida útil e funções secretórias do corpo lúteo durante o início da prenhez (MAcDOWELL et al., 1988; SHARP et al., 1989; SILVA et al., 2005).

Em éguas, o embrião migra para o útero entre os dias 5 e 6 pós-ovulação começando sua mobilidade, através de todos os segmentos do útero (MEIRA et al., 2012), durante este período, o ambiente uterino está física e bioquimicamente dinâmico, realizando interações materno-embrionárias vitais ao embrião. Este migra continuamente através do lúmen uterino entre os dias 9 a 16 após a ovulação, deslocando-se para todas as regiões do útero 10 a 20 vezes por dia (GINTHER, 1986). Um movimento constante permite que o embrião entre em contato com a maior parte da superfície endometrial, provavelmente servindo para sinalizar sua presença uniformemente para todo o

endométrio e para captar secreções uterinas (SHARP, 2000). Antes da implantação, o concepto é mantido unicamente pelas secreções que se acumulam no lúmen uterino, um fenômeno conhecido como nutrição histotrófica (ASHWORTH, 1995; LEFRANC; ALLEN, 2007).

O objetivo deste estudo foi descrever alterações histológicas do endométrio de éguas cíclicas e éguas prenhes no início do desenvolvimento embrionário, dia 7, 10 e dia 13 pós-ovulação.

2 REVISÃO BIBLIOGRÁFICA

2.1 Anatomia uterina

O útero da égua apresenta, morfologicamente, três camadas. A parte mais interna é a mucosa ou endométrio que comporta o epitélio (superficial e glandular) e o estroma sendo que este pode ser subdividido em estroma superficial Stratum Compactum, estroma intermediário e profundo Stratum Spongiosum, de baixa densidade celular, mas com células fusiformes muito extensas, criando uma aparência esponjosa (KENNEY, 1978). O miométrio (camada muscular), no qual fibras musculares lisas se dispõem numa camada circular (interna) e longitudinal (externa), entre as quais se dispõe o estrato vascular comportando grandes artérias, veias, vasos linfáticos e nervos (ESTELLER-VICO; LIU; COUTO, 2012) e a serosa ou perimétrio, parte externa, revestido pelo mesotélio peritoneal, sendo constituído por tecido conjuntivo frouxo, vasos e nervos (LOVE, 2011).

2.2 Endométrio

O endométrio é formado por uma camada de epitélio luminal com sua lâmina basal e lâmina própria. Esta é convencionalmente dividida em duas partes. A porção junto ao epitélio luminal é designada *Stratum Compactum* (SC) e se caracteriza por uma maior densidade de células do estroma. Essa porção apresenta ductos glandulares que se abrem no epitélio luminal, mas não exibe glândulas. A outra porção é o *Stratum Spongiosum* (SS), no qual se encontram as glândulas em meio a um estroma com menor densidade de células. Nessas porções do endométrio, são observadas vênulas, capilares, vasos linfáticos, arteríolas, eventualmente pequenas artérias musculares e um discreto número de células mononucleares, particularmente no SC (BARROS; MASUDA, 2009).

A estrutura endometrial modifica-se conforme o status reprodutivo das éguas (fase cíclica ou anestro) assim como dentro de sua fase cíclica (estro ou diestro) (KENNEY, 1978; LEISHMAN; MILLER; DOIG, 1982; SCHLAFER, 2007).

Dentre as técnicas que se pode avaliar o endométrio temos a microscopia óptica, a qual proporciona imagens do tecido, mostrando estruturas do *stratum compactum* e *stratum spongiopsum* incluindo epitélio luminal, glândulas endometriais, estroma e vasos sanguíneos (KENNEY, 1978), e a microscopia eletrônica de varredura, a qual proporciona uma representação tri-dimensional da ultraestrutura da superfície do tecido, exibindo o epitélio luminal. A MEV foi usada para estudar a anatomia da superfície do útero em seres humanos (JOHANNISSON; NELSON, 1972) coelho (KANAGAWA et al., 1972), éguas (SAMUEL et al, 1979) e ratos (SANFILIPPO et al., 1985). Em éguas, o padrão de atividades secretórias e ciliares do endométrio são similares a outras espécies (AURICH, 2011).

A superfície luminal é formada por células secretórias microvilosas poligonais, células ciliadas, aberturas de glândulas uterinas e alguns outros tipos de células secretórias (SAMUEL et al., 1979).

2.3 Aspectos histológicos do endométrio nas fases do ciclo estral

2.3.1 Estro

No estro, o epitélio luminal varia entre colunar alto ou pseudoestratificado, a altura do epitélio luminal varia de 20 a 30μm podendo alcançar até 50μm na fase inicial (BARROS; MASUDA, 2009) com vacuolização da porção basal das células epiteliais. É comum a presença de neutrófilos nos capilares subepiteliais e nas vênulas da lâmina própria (KENNEY, 1978). O edema do estroma é mais perceptível na lâmina própria, frequentemente está associado ao acúmulo de pequenas quantidades de líquido no lúmen uterino (TUNÓN et al., 1995). A ramificação das glândulas endometriais apresenta um diâmetro maior quando seccionados transversalmente, sendo pouco tortuosos em secção longitudinal (DOIG; WAELCHLI, 2011). As glândulas são menos tortuosas que no diestro, ocasionalmente as ramificações de glândulas individuais surgem em ninhos, que parecem ocorrer devido ao edema do estroma interglandular (KENNEY, 1978). No final do estro, em algumas éguas, o epitélio luminal retorna a colunar baixo, o que em certos animais apenas ocorre após cessar o comportamento de cio (DOIG; WAELCHLI, 2011). Nesta

fase as glândulas têm uma menor densidade (LEISHMAN; MILLER; DOIG, 1982), com ductos glandulares curtos e retilíneos, podendo conter material amorfo (DOIG; WAELCHLI, 2011), as células epiteliais são altas e pálidas, (RICKETTS, 1975).

Analisado por MEV, o epitélio luminal é formado na maior parte por células microvilosas poligonais. Células ciliadas e pró-ciliadas são encontradas ao longo da superfície microvilosa. Bolhas apicais de células secretórias são encontradas nas aberturas glandulares e nos cumes das dobraduras endometriais e aparentemente liberadas para o lúmen uterino (SAMUEL et al., 1979).

2.3.2 Diestro

No diestro o epitélio é colunar, medindo entre 15 a 20µm. Em alguns casos, pode ter somente 10µm (BARROS; MASUDA, 2009). O citoplasma das células apresenta uma coloração marcada, sendo as células hipercromáticas (RICKETTS, 1975). Há uma diminuição da vascularização e do edema endometrial. A densidade glandular aumenta durante o diestro e é notória a ramificação e enovelamento das glândulas, conferindo-lhes trajetos tortuosos e longos (KENNEY, 1978; LEISHMAN; MILLER; DOIG, 1982; BARROS; MASUDA, 2009).

Através da MEV, células secretórias diminuem rapidamente em número e as células ciliadas alcançam sua máxima densidade entre as células microvilosas na metade do diestro, diminuindo ao final, mas ainda assim encontradas em maior número que no estro (SAMUEL et al, 1979).

2.4 Interação do embrião com o útero no início da prenhez

O útero faz parte do sistema endócrino: um órgão alvo dos esteróides e produtor de hormônios. Estes controlam a atividade secretora uterina quantitativa e qualitativamente. A sincronia entre os hormônios maternos e o desenvolvimento embrionário é uma condição para que se estabeleça a prenhez (BLANCHARD et al., 2003).

Dentre as características fisiológicas incomuns da prenhez nos equídeos, os oócitos fertilizados e não fertilizados são diferentemente transportados pelo oviduto e só os primeiros atravessam a união istmo-ampola (FLOOD et al., 1979). O próprio embrião sintetiza grandes quantidades de prostaglandina E2 (PGE2) no dia 5 pós-concepção, para estimular o relaxamento e a contração do oviduto e passar para o útero (em torno de 24 horas depois) (WEBER et al., 1991; 1992; 1995).

Em éguas, o embrião migra para o útero entre os dias 5 e 6 pós-ovulação começando sua mobilidade, através de todos os segmentos do útero, que é essencial para o reconhecimento materno da prenhez (MEIRA et al., 2012). Gerstenberg et al. (1999) sugerem que o motivo do lento transporte do embrião no oviduto dos equinos está vinculado a este padrão de proliferação e propõem que o embrião chega ao útero no 6º dia para permitir à proliferação do epitélio glandular para posteriormente secretar histotrofo. Além disso, próximo a este dia ocorre a maior produção de progesterona pelo corpo lúteo. A progesterona é essencial em prover um apropriado ambiente ao desenvolvimento do concepto (SHARP, 2000) e induz a produção endometrial de histotrofo (AURICH; BUDIK, 2015).

Interações entre o concepto e várias células uterinas, especialmente do epitélio luminal, epitélio glandular, assim como células do estroma, coordenam mecanismos que estimulam o desenvolvimento do concepto. Dentre estes, o fluxo sanguíneo uterino, transporte de água e eletrólitos, reconhecimento materno da prenhez, transporte de nutrientes como glicose e aminoácidos, secreção ou transporte seletivo de componentes histotróficos pelo epitélio uterino para atender a demanda do crescimento e desenvolvimento do concepto (BAZER et al, 2012). Um concepto, individualmente, pode falhar em seu desenvolvimento ou não se desenvolver normalmente caso não responda a componentes do histotrofo que orquestram o desenvolvimento e eventos durante a peri-implantação embrionária (BAZER et al., 2011).

Na maioria das grandes espécies de animais domésticos, mas particularmente nos equídeos, a fixação do concepto ao epitélio uterino ocorre relativamente tarde após a sua entrada para a cavidade uterina. Durante este período, o ambiente uterino está física e bioquimicamente dinâmico, realizando interações materno-embrionárias vitais, evitando a luteólise e auxiliando no

processo de fixação e implantação do concepto (MEIRA et al., 2012). O embrião livre é dependente do ambiente uterino para a sua sobrevivência (GUILLOMOT et al., 1988) e este migra continuamente através do lúmen uterino entre os dias 9 a 16 após a ovulação, deslocando-se para todas as regiões do útero 10 a 20 vezes por dia (GINTHER, 1986).

A alta concentração de ácido siálico carregado negativamente com constituintes glicoprotéicos, podem regular o movimento intrauterino do embrião e auxiliar na captação de secreções exócrinas das glândulas endometriais, o chamado “leite uterino” ou histotrofo (ORIOL et al., 1993). A migração do concepto equino é extensa e isso permite que suas secreções afetem todo o endométrio e permitam o estabelecimento da gestação (GINTHER, 1983). Em ratas, é assumido que o meio uterino passa por mudanças simultaneamente com uma mudança endometrial em resposta a esteróides maternos e também a presença local de um concepto (NIEDER et al., 1987).

O desenvolvimento embrionário inicial, a implantação e a manutenção da prenhez dependem criticamente de uma orquestrada e precisa interação entre o embrião e o ambiente uterino (KLEIN; TROEDSSON, 2011a). O embrião jovem indica sua presença, interrompe o ciclo estral e mantém a gestação, num processo conhecido como “Reconhecimento Materno da Prenhez" (RMP) (SHORT, 1969). A diferença dos ruminantes e dos suínos em relação aos equinos é que estes são uma das poucas espécies domésticas na qual o sinal do RMP derivado do embrião ainda não foi identificado (KLOHONATZ et al., 2013).

Um endométrio funcional saudável é vital ao longo da gestação na égua, especialmente na implantação e durante o longo período que o precede, quando o concepto móvel é sustentado inteiramente por secreções exócrinas das glândulas endometriais (LEFRANC; ALLEN, 2007). Estudos em ovinos, usando um modelo de bloqueio de glândulas uterinas, demonstraram o papel fundamental do histotrofo como o concepto não consegue alongar-se após a eclosão da zona pelúcida em úteros sem glândulas, devido à falta de certas moléculas de adesão epiteliais ou incapacidade do endométrio em responder os sinais do concepto (GRAY et al., 2002). Portanto, a composição e a qualidade do meio ambiente uterino são críticos para o contínuo suporte e

desenvolvimento do concepto a partir do 6º dia pós-ovulação (KENNEY, 1978; GINTHER, 1992; BALL, 1993; CROSSET; ALLEN; STEWART, 1996).

2.5 Histotrofo

Histotrofo é um material extracelular derivado do endométrio e das glândulas uterinas que se acumula no espaço entre os tecidos materno e fetal. É fagocitado inicialmente pelo trofoectoderma do blastocisto e, posteriormente, pelo trofoblasto do endoderma do saco vitelínico (BURTON et al, 2002).

O útero de todos os mamíferos contém glândulas endometriais que produzem ou transportam seletivamente uma matriz complexa de proteínas. Estas substâncias, chamadas de histotrofo, são uma mistura complexa de enzimas, fatores de crescimento, citocinas, linfocinas, hormônios, proteínas de transporte, açúcares, aminoácidos, água e outros nutrientes que afetam o desenvolvimento e função do trofoectoderma (BAZER et al, 2012). Em um estudo com ovinos, Bazer et al. (2011) verificou que um tratamento inicial com progesterona exógena, aumenta seus níveis no sangue que por sua vez aumenta os níveis de nutrientes, particularmente arginina e glucose no lúmen uterino, acelerando o desenvolvimento e crescimento do concepto e adiantando a sinalização do reconhecimento materno da gestação.

Antes da implantação, o concepto é mantido unicamente pelas secreções que se acumulam no lúmen uterino (ASHWORTH, 1995). Considerando o longo período pré-implantação, o histotrofo é particularmente importante na espécie equina (REILAS, 2001).

As proteínas constituem uma parte substancial dos produtos gênicos supra-regulados em éguas gestantes e contribuem para a formação do histotrofo (KLEIN et al, 2010). As principais proteínas encontradas no fluido uterino são a uteroglobina (UGL), a uteroferrina (UF) e uma das maiores proteínas, secretada unicamente pelo endométrio da égua, a uterocalina (UCA ou proteína P19) (BEIER- HELLWIG et al., 1995; HOFFMAN et al., 2009). Progesterona dependente, grandes quantidades de UCA são secretadas no lúmen uterino durante o ciclo estral e a prenhez inicial, auxiliando a formação da cápsula embrionária em equinos. A detecção mais precoce da mesma se evidencia em biópsias uterinas coletadas dois dias pós-ovulação, e a coloração

revelou que se encontrava nas células epiteliais glandulares (CROSSET et al., 1998).

As mucinas, outro exemplo, são uma família de glicoproteínas de alto peso molecular que se encontram geralmente cobrindo as superfícies luminais dos órgãos epiteliais, incluindo o útero na maioria das espécies. No trabalho de Al-Ramadan et al. (2002) foi determinada por imunofluorescência a expressão da proteína de MUC1. Os resultados mostraram que a mesma se expressou uniformemente nas superfícies apicais do epitélio luminal e glandular, entretanto, a intensidade de coloração de MUC1 foi superior no corno gravídico que no não gravídico nos dias de obtenção das amostras (dias 14, 17, 21, 27, 37 da prenhez/ciclo estral).

Estudos em ovelhas, ratas, porcas e vacas indicam que a progesterona aumenta a abundância de certos nutrientes no histotrofo, o qual avança o desenvolvimento do concepto e aumenta a sobrevivência de embriões via ativação de nutrientes de sinalização celular (BAZER et al., 2011). Há fortes evidências que sustentam um crucial papel da progesterona em eventos iniciais regulares nos equinos, incluindo a fase de pré-implantação, desenvolvimento embrionário e reconhecimento materno da gestação. A progesterona é essencial para prover um apropriado ambiente uterino para o desenvolvimento do concepto (SHARP, 2000) e produção de histotrofo endometrial, nutrição do embrião até a placentação (AURICH; BUDIK, 2015). A diminuição ou privação dos níveis de progesterona no início da prenhez em éguas, gerou menos embriões, com tamanho menor e qualidade inferior aos embriões de éguas controle (LEISINGER et al., 2018), demonstrando o papel crucial da progesterona no desenvolvimento embrionário em éguas.

ARTIGO SUBMETIDO Á THERIOGENOLOGY

ULTRASTRUCTURAL AND HISTOLOGICAL CHARACTERISTICS OF THE ENDOMETRIUM DURING EARLY EMBRYO DEVELOPMENT IN MARES

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Abstract

The aim of this study was to evaluate ultrastructural and histological changes in the endometrium on days 7, 10 and 13 post-ovulation in pregnant and cyclic mares. Mares were routinely examined by transrectal palpation and ultrasonographic examination of the reproductive tract until estrus was detected. In the first cycle, endometrial biopsies from 30 cyclic mares (Cyclic group) were collected on days 7, 10 and 13 post-ovulation. In the second cycle, the same mares were bred by a fertile stallion. At days 7, 10 and 13 post-ovulation intrauterine biopsies were collected. Immediately after sample collection, the mare's uteri were flushed, and those mares with embryo recovery were assigned to the Pregnant group. From ovulation detection until day of uterine biopsy, blood samples to measure Progesterone concentrations were collected daily in cyclic and pregnant mares. A larger blood vessel caliber was observed in pregnant mares than in cyclic from day 7 to 13. On the 7th day of pregnancy a large loss of ciliated cells was evident in the group of pregnant mares in comparison with the Cyclic group and the superficial cells of the endometrium were more protruded, and a small amount of histotrophic material between the folds was observed. On the 10th day of pregnancy, the glandular histotrophic

secretion and the secretion of luminal epithelium became more intense than the secretion of cyclic mares. On the 13th day of pregnancy, a very large amount of histotroph was observed within large glandular openings surrounded by ciliated cells. The concentrations of P4 were affected by day ($P < 0.001$), but were not affected by group. Changes occurred in the uterine environment thereupon the entry of the embryo into the uterus. In the stroma and in the lumen, these modifications seem aim to provide the necessary nutrition for the initial development of the embryo and to promote changes at cellular structures that will interact in the embryonic signaling and future fixation, implantation and placentation.

Keywords: Equine embryo; Endometrium ultrastructure; Histotroph; Pre-implantation period; Ciliated cells; Blood vessel.

1. Introduction

Early pregnancy in the mare is a fascinating period that encompasses numerous profound developmental changes and events, many of which are unique to the horse [1]. Several features of pregnancy in the mare and other equids are unusual and differ markedly from equivalent events in other, well-studied large domestic animal species [2]. In mares, the embryo migrates to the uterus between days 5 and 6 post-ovulation, beginning its mobility through all uterine segments, which is essential for the maternal recognition of pregnancy [3].

The term ‘maternal recognition of pregnancy’ is used to refer to the physiological process by which the lifespan of the corpus luteum is prolonged in the pregnant mare [4]. The horse is one of the few domestic species in which the conceptus-derived pregnancy recognition signal has not been identified, and equids appear to be distinct from ruminants and pigs in the signals used by the conceptus to communicate its presence [5]. Early embryonic development in the mare is dependent upon both the maintenance of luteal function and the establishment of a propitious uterine environment [6,7]. A precisely orchestrated interaction between the conceptus and the uterine environment, if successful,

prompts continued progestin support, leading to a receptive uterine environment [8].

The uterus is part of the endocrine system, a target organ for steroids [9]. The morphological and physiological changes that occur in the cells during the estrous cycle are thought to be controlled by the relative concentrations of the ovarian steroid hormones estradiol and progesterone, and by the local actions of various growth factors [10]. Secretory products from maternal uterine epithelia (histotroph), hematotrophic transfer of essential gases and nutrients, and coordinated signaling between conceptus trophectoderm and uterine epithelia are critical to conceptus growth and development, pregnancy recognition signaling, implantation, and placentation [11]. In temporal association with the occurrence of these phenomena, several profound functional and morphologic changes occur in the uterus [12].

Several studies were conducted to describe the surface anatomy and fine structure of the endometrium of the cyclic mare [13-15], placenta and uterine glands [16-19], endometrial surface and ultrastructural changes during early to mid-pregnancy [20]. However, to our knowledge, there are no publications on such assessments of the mentioned structures during the period of maternal recognition of pregnancy, which is believed to occur between days 12 to 14 post-ovulation [21]. The aim of this study was to verify the ultrastructural and histological changes in the endometrium in days 7, 10 and 13 post-ovulation in pregnant and cyclic mares.

2. Material and methods

2.1. *Animals*

The present experiment was conducted during the southern hemisphere breeding season (December to March) in the south of Uruguay (34°22 S, 55°36' W). This study was carried out with an Animal Ethical Use Committee approved protocol at the Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (protocol number 27316 from July 17, 2014) and by the Bioethics Committee of the Universidad de la República, Montevideo, Uruguay (CEUAFVET-PI-34/14 – protocol number 111130-001367-14).

Thirty mixed breed Criollo type mares (mean age, 7 and range between 5 and 10 years old) from a commercial herd, weighting approximately 400 kg, were used. Mares were kept in natural pastures with free access to mineral supplement and water. All mares remained healthy and with an average body condition score of 3.5 (scale 1 to 5) [22]. Mares were examined for reproductive soundness by evaluation of perineal conformation, transrectal palpation and ultrasound of the genital tract (SonoScape A6v, China), vaginal examination with speculum, endometrial biopsy and bacteriological culture and endometrial cytology using a guarded swab. Only cyclic and clinically normal mares, with endometrium classified as category I or IIA [23], without evidence of endometritis - absence of polimorphonuclear neutrophils in the cytology smear at magnification 400x and negative culture [24] were selected.

2.2. *Experimental design*

Mares were routinely examined by transrectal palpation and ultrasonographic examination of the reproductive tract until estrus was detected. Once estrus was confirmed (ovarian follicle > 35 mm in diameter and marked uterine edema), mares were examined daily to detect ovulation, considered day 0. In the first cycle, endometrial biopsies of 30 cyclic mares (Cyclic group) were collected on days 7 (n = 10), 10 (n = 10) and 13 (n = 10).

In the second cycle, the same mares were bred by a fertile stallion. After breeding, the 30 mares were examined daily to confirm ovulation, considered day 0. At days 7 (n=10), 10 (n=10) and 13 (n=10) intrauterine biopsies were collected. Immediately after sample collection, mares' uteri were flushed, and those with embryo recovery were assigned to the Pregnant group. Of the 30 mares flushed, embryos were recovered in 6 mares on the 7^{th} day, 6 on the 10^{th} day and 6 on the 13^{th} day. Samples from the mares without embryo were excluded from both experimental groups.

Embryo recovery was performed by three sequential uterine flushes with 1 L of Ringer Lactate solution at 37º C in each flush [25]. An embryo flushing catheter with 10.7 mm of diameter (32 Fr, ref 19009, Minitube, Brazil) was used in the mares on day 7 and 10. Mares on day 13 were flushed employing a silicone catheter with an internal diameter of 15 mm.

2.3. *Endometrial biopsy*

Endometrial biopsies samples were collected at the dorsal region [23,26,] from both uterine horns (ipsi and contralateral to ovulation).

The samples of each horn, measuring 0.5 to 1 cm, were stored separately in 5 mL tubes containing 4% buffered formalin for light microscopy and morphometry, and 2,5% glutaraldehyde containing 0.01mol/L phosphate buffer (pH 7.3) for scanning electron microscopy.

2.4. *Scanning Electron Microscopy (SEM)*

Scanning Electron Microscopy gives a three-dimensional representation of tissue surface ultrastructure, exhibiting the epithelium of the uterus which is folded into long ridges and troughs. The samples for SEM were dehydrated through a graded series of ethanol and subsequently acetone. The tissue was dried in a critical-point drier (CPD030 – Balzers) using carbon dioxide. The dried pieces were attached to stubs with double-sided adhesive tape and were sputter-coated with gold/palladium (Sputter Coater SCD050 – Balzers). Samples were scanned and photographed with a JEOL (JSM 6060) digital scanning electron microscope. For the analysis, five pre-defined squares were superimposed to SEM images. For each day and group, five images were evaluated, totalizing 25 areas/day/group. Flattened, protruded and ciliated cells were counted and the percentage of area occupied by the different cells and by the secretion was calculated.

2.5. *Light microscopy and morphometry*

Light microscopy provides transverse images of the tissue, showing structures in the compact and spongiosum stractum of endometrium including luminal epithelium, endometrial glands, stroma and blood vessels. Samples conserved in formalin were processed to be included in paraffin. Paraffin blocks were cut with an automated microtome (Leica, RM165) at 5 µm thickness, adhered in histology slides and kept on a 60ºC incubator. After deparaffinization, cuts were stained using routine techniques for tissue samples

[27], using H-E and periodic acid Schiff (PAS). All sample slides were analyzed by an experienced pathologist blinded from any information, under light microscopy at 400x and 1000x magnification. The morphological features were photo-documented and structures were measured using an image analysis system (Infinity analyze®, Toronto, Canada) connected to an optical microscope (Olympus®, BX50, Japan).

The following measures were performed in both uterine horns:

a) Blood vessel diameter: measured in five fields at x1000 magnification. Total blood vessel area was calculated multiplying the vessel diameter by the number observed in the studied field.

b) Intraglandular secretion: quantified using the mean of the two largest diameters of the secretion of 10 randomly selected spherical glands at x400 magnification.

c) Height of the luminal epithelium: measured from the basement membrane to the apical membrane of the cells, recorded at 400x magnification in five randomly selected fields.

d) Height of the glandular epithelium: measured from the basement membrane of the cells to the apical membrane of the cells recorded at 400x magnification in 10 randomly selected spherical stratum spongiosum glands.

e) Glandular diameter: obtained using the mean of two perpendicular diameters of each gland (from a basement membrane to the opposite one). Measured in 10 randomly selected spherical stratum spongiosum glands at 400x magnification.

f) Glandular lumen: measured in the same way as the previous variable, measuring the space between the apical membranes of the epithelial cells, recorded in 10 randomly selected spherical stratum spongiosum glands at 400x.

An average of the records of each variable for each uterine horn was calculated.

2.6. *Progesterone assay*

From day 0 (ovulation detection) until day of uterine biopsy, blood samples were collected daily by jugular venipuncture in cyclic and pregnant mares. Blood samples were centrifuged at 500 x *g* for 20 min and serum was

stored at -20ºC in cryogenic tubes. Progesterone (P4) concentrations were measured by radioimmunoassay kit (PROG-RIA-CT KIP1458; DiaSource InmunoAssays SA, Louvain la Neuve, Belgium). The sensitivity of the assay was 0.074 ng/mL with intra-assay coefficients of variation (CVs) of 14%, and 7.5%, and inter-assay CVs of 4.8% and 8% for controls 1 (0.84 ng/ml) and 2 (2.65 ng/ml) respectively.

7. *Statistical analysis*

Variables were evaluated using the Statistical Analysis System (SAS, Cary, NC, USA). P4 concentration, percentage of polygonal microvilli secretory cells (flattened and protruded), ciliated cells, level of secretion over the epithelium, diameter of endometrial blood vessels, total vessel area, intraglandular secretion, height of luminal and glandular epithelium, glandular diameter and glandular lumen were considered as dependent variables. Groups 'Cyclic' and "Pregnant" (group) and the subgroups, 7d, 10d and 13d (day) and uterine horn (ipsi or contralateral) were considered as independent variables. Variables were evaluated for normality using the PROC UNIVARIATE procedure, those not meeting normal distribution were transformed using natural logarithm and those still not meeting normality by this method were evaluated by non-parametric statistics.

Variables with normal distribution were analyzed using the PROC GLM procedure, which evaluates non balanced variables, testing for interactions between treatment (cyclic or pregnant), and if these had a statistical effect on the variables. Means were evaluated using Tukey's test, using the LSMEANS procedure. Variables not meeting normal distribution were analyzed by the PROC NPAR1WAY procedure, to evaluate the means by Wilcoxon and Kruskal-Wallis tests. Data are presented as means ± standard error. Differences $P \leq 0.05$ were considered significant. Differences > 0.05 and ≤ 0.1 were considered tendency.

3. Results

Biopsies from the 18 pregnant mares, were classified [23] as Category I (n = 4, 22.2%) and as Category IIA (n = 14, 77,8%) similar percentage (P > 0.709) observed in mares that were bred, but with no detectable pregnancy (Category I, n = 2, 16.7%; Category IIA, n = 10, 83.3%).

The mean diameter of the endometrial stromal blood vessels was influenced by day (P = 0.046) and group (P < 0.001), but no interaction day by group was observed (P = 0.167).). In pregnant mare, blood vessels diameter was greater at days 7 and 10 (18.4 ± 0.8 and 18.6 ± 1.0 respectively) than in cyclic ones at the same days (12.0 ± 0.7 and 15.1 ± 1.1). In pregnant mares the diameter did not vary (P = 0.576) during the three days of the experiment, however, in cyclic mares an increase (P = 0.024) in blood vessel caliber was observed between day 7 (12.0 ± 0.7) and day 13 (16.5 ± 1.3) (Fig. 1). Vessel area was not influenced by day (P = 0.170) nor by group (P = 0.713). No interaction day by group was observed (0.079).

The percentage of area occupied by flattened secretory cells was influenced by group (P < 0.001). Flattened cells were observed by SEM in greater proportion (P < 0.001) in the cyclic mares at days 7, 10 and 13 (69.1 ± 70.9 ± 2.7 % and 69.0 ± 3.0 % respectively) when compared to pregnant mares at day 7 (59.6 ± 3.2 %), day 10 (61.1 ± 3.1 %) and day 13 (53.8 ± 4.2 %) (Fig. 2a, 2d and 2e).

Day (P = 0.068) and group (P = 0.060) tended to influence the proportion of endometrial surface occupied by protruded secretory cells. An interaction day by group was observed (P = 0.044). Protruded cells observed by SEM were more abundant (P = 0.017) at day 7 in pregnant (9.0 ± 2.3 %) than in cyclic mares (2.2 ± 1.2 %), and no differences were detected in the other days studied (Fig. 2b, 2d and 2e).

The presence of ciliated cells observed by SEM was influenced by group (P < 0.001). Cyclic mares presented a large percentage of ciliated cells homogeneously disseminated by the endometrial epithelium at days 7 (27.2 ± 2.0 %), 10 (24.8 ± 2.8%) and 13 (21.5 ± 3.8%). The percentage of ciliated cells in the endometrial epithelium was lower (P < 0.001) in pregnant mares at 7^{th} day (15.1 ± 1.8 %) when compared to cyclic mares at the same day. On the 10^{th} (18.9 ± 1.5%) and 13^{th} (14.1 ± 1.7%) days, pregnant mares presented a trend

(P < 0.090) to lower presence of ciliated cells in the endometrial epithelium than cyclic mares (Fig. 2c, 2d, 2e, 2f and 2g).

Secretions observed in the uterine lumen by SEM were influenced by day (P = 0.002) and by group (P < 0.001). An interaction day by group was observed (P = 0.046). Secretion occupied a lower endometrial area in cyclic mares at days 7 (1.1 ± 0.5 %), 10 (1.3 ± 0.7 %) and 13 (2.7 ± 0.5%), whereas, in pregnant mares secretion was higher (P < 0.001). In the latter group of mares an increase (P = 0.026) in the percentage of endometrial area filled with histotroph was observed from day 7 (8.6 ± 1.3 %) to day 13 (17.7 ± 2.6 %) (Fig. 3a).

Openings of glandular ducts were difficult to identify in cyclic mares, but in pregnant mares they were larger, usually surrounded by ciliated cells and by histotrophic secretion (Fig. 3c and 3e). On day 13 of pregnancy it was possible to observe an increase in the size of many secretory cells, releasing all their cellular content (Fig. 3g).

Intraglandular secretion observed by light microscopy increased (P < 0.049) in pregnant mares from day 7 (3.1 ± 0.4 µm) to day 10 (4.5 ± 0.5 µm) and day 13 (4.7 ± 0.5 µm). Pregnant mares presented more intraglandular secretion at day 10 and 13 than cyclic mares (2.6 ± 0.3 µm and 3.1 ± 0.4 µm respectively). Intraglandular secretion was influenced by group (P = 0.011) and an interaction day by group was observed (P = 0.041) (Fig. 3b, 3d, 3f and 3h).

Histological examination revealed that the height of the luminal epithelium was not influenced by day (P = 0.393) nor by group (P = 0.856) (Fig. 4a). No interaction day by group was observed (P = 0.372).

The glandular epithelium measured by light microscopy was influenced by day (P < 0.001) and by group (P = 0.002) and no interaction day by group was observed (P = 0.359). Glandular epithelium was higher (P < 0.05) in pregnant mares at days 7 and 10 (15.7 ± 0.2 µm and 15.0 ± 0.3 µm respectively) than in cyclic mares at the same days (14.7 ± 0.2 µm and 14.0 ± 0.3 µm). In pregnant and cyclic mares a decrease (P <0.001) in glandular epithelium was observed at day 13 (Fig. 4b).

Group and day influenced the glandular diameter (P < 0.001) observed by light microscopy. No interaction day by group was observed (P = 0.621) (Fig. 4c). A higher (P < 0.047) glandular diameter was observed in pregnant mares at

7, 10 and 13 days (42.7 ± 1.2 µm, 39.4 ± 1.3 µm and 37.5 ± 0.7 µm respectively) (Fig. 4f and 4h) than in the cyclic group at the same days (37.8 ± 0.4 µm, 36.2 ± 0.9 µm and 33.0 ± 0.6 µm) (Fig. 4e and 4g). At day 13 in both groups the glandular diameter was lesser ($P < 0.012$) than the observed at day 7.

The diameter of the glandular lumen was influenced by day ($P = 0.009$) and group ($P < 0.001$). An interaction day by group was observed ($P = 0.030$). Glandular lumen was larger ($P < 0.001$) at day 13 in pregnant mares (11.3 ± 0.7) than in cyclic ones (7.5 ± 0.3) (Fig 4d, 4g and 4h). In pregnant mares glandular lumen was greater ($P = 0.062$) at day 13 than at days 7 (8.2 ± 0.8) and 10 (8.2 ± 0.8) (Fig. 4f and 4h).

The concentrations of P4 were affected by day ($P < 0.001$), but were not affected by group neither by the day by group interaction. P4 concentrations increased slowly reaching a peak between days 5 and 7, with a plateau until day 10, when the concentration started to decrease until day 13 in both pregnant and cyclic groups (Fig. 5).

Percentage of polygonal microvilli secretory cells (flattened and protruded) ciliated cells, level of secretion over the epithelium, height of glandular and luminal epithelium, glandular diameter, glandular lumen and intraglandular secretion were not affected in pregnant and cyclic mares ($P > 0.05$) by uterine horn (ipsi and contralateral uterine horn to ovulation).

4. Discussion

Successful establishment of pregnancy is characterized by temporally and spatially regulated events in the endometrium and conceptus [8,14,15]. In this study changes in the endometrial environment at Day 7 in pregnant mares were observed, immediately after the entry of the embryo into the uterus. One of these changes was the increase of blood vessel diameter in endometrial stroma in relation to mares of the Cyclic group at Day 7. The endometrium is supplied with nutrients via its vascular system, which is especially critical for the developing embryo [28,29]. The mechanism for this conceptus-induced increase in vascular perfusion is unknown, but it appears likely that the conceptus produces vasoactive substances. Many interesting candidate genes

and biological processes were identified as potentially important for endometrial remodeling in response to the early embryo [30]. The equine conceptus produces a number of different secretory products during early pregnancy, including steroids, prostaglandins, different proteins, and peptides [31], such as interferon delta, a member of the type I interferon family [32]. In a two-dimensional electrophoresis study from the uterine fluid of the same mares two proteins, Haptoglobin and Chloride intracellular channel protein, with angiogenic function, were more abundant in the Pregnant group than in the Cyclic group [33]. Estrogen production by the conceptus is temporally associated with stimulation of uterine vascularity in horses [34,35]. Movement of the conceptus during the mobile phase is associated with a temporary increase in vascular perfusion following the pattern of conceptus movement [36]. The blastocysts of many species also secrete a variety of prostaglandins, and it has been proposed that conceptus prostaglandins, principally PGE2, stimulate increased uterine blood flow [35] which may explain the increase in vessel sizes on day 7, since PGE2 is released by the embryo while still in the oviduct, in day 5 after ovulation [37].

Conceptus secretory products also act to allow survival of the conceptus allograft, stimulate vasodilatation and angiogenesis to increase uterine blood flow and substrate delivery to the pregnant uterus, stimulate active transport of nutrients into the uterine lumen from maternal tissues and fluids, regulate intrauterine migration of embryos and placentation as well as other events associated with establishment and maintenance of pregnancy [38]. Using transrectal color Doppler ultrasonography, early and transient changes on uterine hemodynamics of pregnant mares are observed on day 4 [39] and a gradual increase in the uterine vascular perfusion is observed on day 12 of pregnancy [36]. In the present study, an increase in blood vessel caliber in the histological samples were observed in the Pregnant group comparing to vessels in the Cyclic mares and this increase remained constant from day 7 to 13. Endometrial vascular remodeling in mares is modulated by the conceptus, creating a uterine environment that will support further conceptus development, and assuring optimal conditions for the dynamic events of mobility, fixation, maternal recognition of pregnancy, uterine immune modulation, implantation, and eventually placentation [40].

Other changes in the stromal morphology and architecture of the uterine environment were also observed from day 7 of pregnancy using SEM and light microscopy analysis. On the 7th day of gestation a large loss of ciliated cells was evident in the group of pregnant mares. Previous studies [13,15] showed that the ratio of secretory (nonciliated) and ciliated cells in the equine endometrium varies throughout the cycle, since ciliated cells reached maximum density during mid-diestrus and few secretory cells could be identified. In contrast, in this study, samples from pregnant mares exhibited more secretory cells than ciliated as soon as embryo reaches the uterus. According to Winter et al., [41], the most striking feature of endometrial transformation or adaptation to pregnancy during embryo stage (21 to 42 d) was the almost complete disappearance of ciliated cell on the epithelial surface in endometrium, providing an environment with more accumulated histotrophic material. In addition, a surface with more cells with microvilli and less cilia will provide a greater contact between the embryo and the endometrium to absorb more nutrients, signal its presence and establish its future fixation and implantation. The constant movement allows the embryo to get in contact with most of the endometrial surface, likely serving to signal its presence uniformly to the entire endometrium and to garner uterine secretions [42]. Probably this loss of ciliated cells in the pregnant mare is due to a rapid replacement of ciliated cells by secretory cells, which will produce material for nutrition of the blastocyst, including oxytocin (OT) which stimulates conceptus growth by action on OT receptors (OTR) in the trophectoderm [21]. According to Bae and Watson [43], the mechanism of secretion of uterine oxytocin in the equine endometrium is merocrine. In merocrine secretion, the contents of small secretory granules are released as the secretory product by exocytosis on the apical surface [43], which would explain erosions in secretory cells seen through scanning electron microscopy.

Even in the 7th day of pregnancy, the superficial cells of the endometrium are more protruded and there is a small amount of histotrophic material between the folds, probably resulting from the merocrine secretions of the epithelial cells. The height of the glandular epithelium and glandular diameter are increased, this hypertrophy probably is due to the production and accumulation of glandular secretion, which will be released in the following days

of gestation. The spherical equine conceptus migrates continuously throughout the uterine lumen between days 9 and 16 after ovulation, traveling to all portions of the uterus 10 to 20 times per day [44]. Intrauterine migration of the horse conceptus is extensive and this may enable its secretions to affect the entire endometrium and allow for establishment of pregnancy [45]. The equine conceptus is dependent upon nutrient uptake from uterine secretions during the preimplantation period. Secreted proteins make up a substantial portion of the gene products up-regulated in pregnant mares, and contribute to formation of the so-called histotroph [46], which has its production increased from the 10th day in this study.

On the 10th day of pregnancy, the glandular histotrophic secretion becomes more intense than the secretion of luminal epithelium. There was a decrease in the number of protruded cells, which appear to have already expelled their contents, collaborating with the initial nutrition of the blastocyst. Glands became smaller with a decrease in the size of the glandular cells, but an increase in glandular lumen diameter resulting in the bigger amount of secretion (histotroph) observed by scanning electron microscopy and through histology. Histotroph is an extracellular material derived from the endometrium and the uterine glands that accumulates in the space between the maternal and fetal tissues. It is phagocytosed initially by the trophectoderm of the blastocyst, and later by the trophoblast of the endoderm of the yolk sac [47]. Histotroph is produced in all mammalian uteri and consists of a complex mixture of proteins and molecules dependent on progesterone action [11]. Estrogens have also been proposed to be responsible for endometrial glandular secretion [30,48]. The importance of progesterone for histotroph production and maintenance of pregnancy in the horse has been long emphasized [42]. A pronounced rise in progestins during the early post-ovulatory phase in pregnant mares contributes to improved development of the conceptus [49,50] while deprivation of progesterone due to luteolysis leads to immediate changes in endometrial protein secretion [51] leading to the formation of a small and low quality embryo [7]. Progesterone levels in cyclic and pregnant mares had no statistical difference [15] as in this study.

On the 13th day of pregnancy, the endometrial architecture still undergoes modifications comparing to cyclic mares. The modifications aim to increase the

supply of nutrients and growth factors to the embryo. Before implantation, nutrition of the mammalian conceptus is essentially histotrophic [47]. At this moment, a very large amount of histotroph diffused abundantly throughout the epithelium is observed through scanning electron microscopy. The glandular openings are large and are surrounded by ciliated cells that help to eliminate histotrophic secretion. The endometrial glands are smaller in diameter, compared to days 7 and 10, the cells of the glandular epithelium are low columnar, increasing glandular lumen. All of these alterations are due to abundant production and secretion of histotrophic material and probably to the action of prostaglandins and oxytocin that cause myometrial contraction, also facilitating the expulsion of the glandular content to the uterine lumen. Maximum uterine contractility in the early pregnant mare occurs on days 11–14 during maximum mobility of the embryonic vesicle [52], when Watson et al. [53] reported high levels of immunostaining for oxytocin in the endometrium. At this time, the images showed endometrial cells of apocrine secretion, which lose their shape, lengthen and release all their contents to the uterine lumen. These secretions also probably contain nutritional and growth substances such as proteins, which can provide nutrients for the growth of the embryo until its fixation.

Maternal recognition of pregnancy, and embryo-maternal communication in a wider sense is a reciprocal process involving both the embryo and the uterine environment [54]. The events between the 7th and 13th day of gestation are a concatenated process like a full harmony in a song where more than one note is played at a time, for instance by playing a chord.

In the present study, modifications occurred in the uterine environment thereupon the entry of the embryo into the uterus. In the stroma and in the lumen, modifications occurred to provide nutrition necessary for the initial development of the embryo and to promote changes at cellular structures that will interact in the embryonic signaling and future fixation, implantation and placentation.

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Conflict of interest

None of the authors has any conflict of interest to declare.

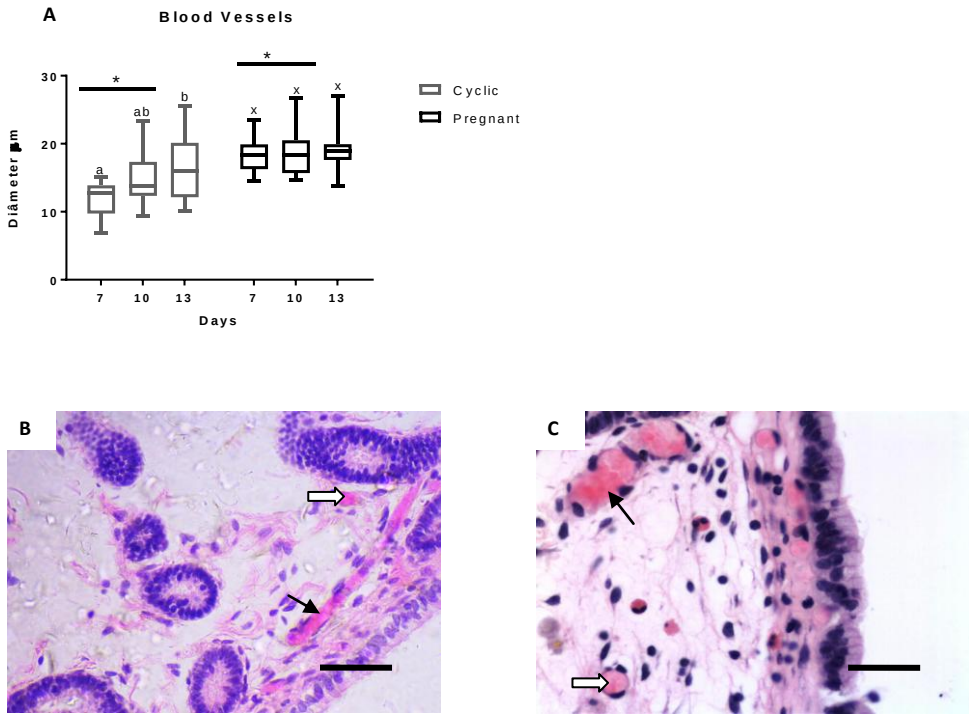

Fig. 1. **A** Box-plots (whiskers represent min to max) of the endometrial stroma blood vessels diameter in the cyclic and pregnant mares on days 7, 10 and 13 after ovulation. Different letters (a, b) or (x, y) represent significant differences (P<0.05) within the same group (cyclic or pregnant). Asterisks (*) represent a significant difference between groups (P <0.05) on the respective days. **B** (7-day Cyclic mare) histological section of endometrial stroma demonstrating blood capillaries in longitudinal cut (black arrow) and transversal cut (white arrow) (H-E 400x, bar = 50µm). **C** (7-day Pregnant mare) histological section of endometrial stroma demonstrating blood capillaries in longitudinal cut (black arrow) and transversal cut (white arrow) (H-E 400x, bar = 50µm).

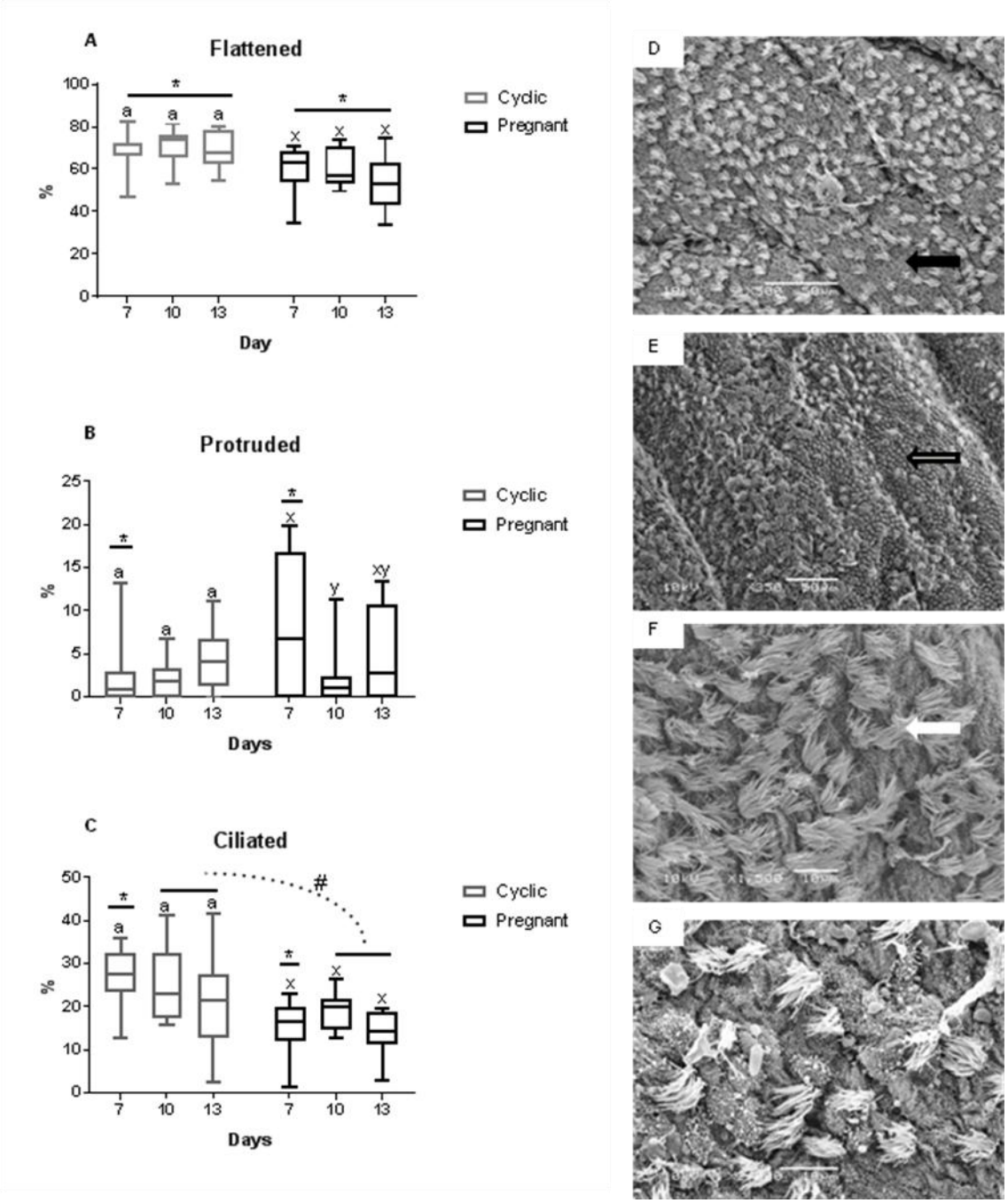

Fig. 2. Box-plots (whiskers represent min to max) of the endometrial surface showing **(A)** flattened secretory cells **(B)** protruded secretory cells and **(C)** ciliated cells of cyclic and pregnant mares on days 7, 10 and 13 after ovulation. Different letters (a, b) or (x, y) represent significant differences ($P < 0.05$) within the same group (cyclic or pregnant). Asterisks (*) represents significant difference between the groups ($P < 0.05$) on the respective days. Hashtag (#) represents a trend ($P > 0.05 < 0.1$) between the groups on the respective days. **D** (7-day Cyclic mare) scanning electron micrograph (500x) of the endometrium showing ciliated cells and equivalent number of flattened secretory cell (black arrow). **E** (7-day Pregnant mare): scanning electron micrograph (350x) of the endometrium showing area with ciliated cells, protruded secretory cells (gray arrow) and histotrophic secretion. **F** (10-day Cyclic mare) scanning electron micrograph (1500x) of the endometrium showing the surface of endometrium with abundant ciliated cells (white arrow). **G** (10-day Pregnant mare) scanning electron micrograph (2000x) of the endometrium showing few ciliated cells and droplets of histotroph on the epithelium.

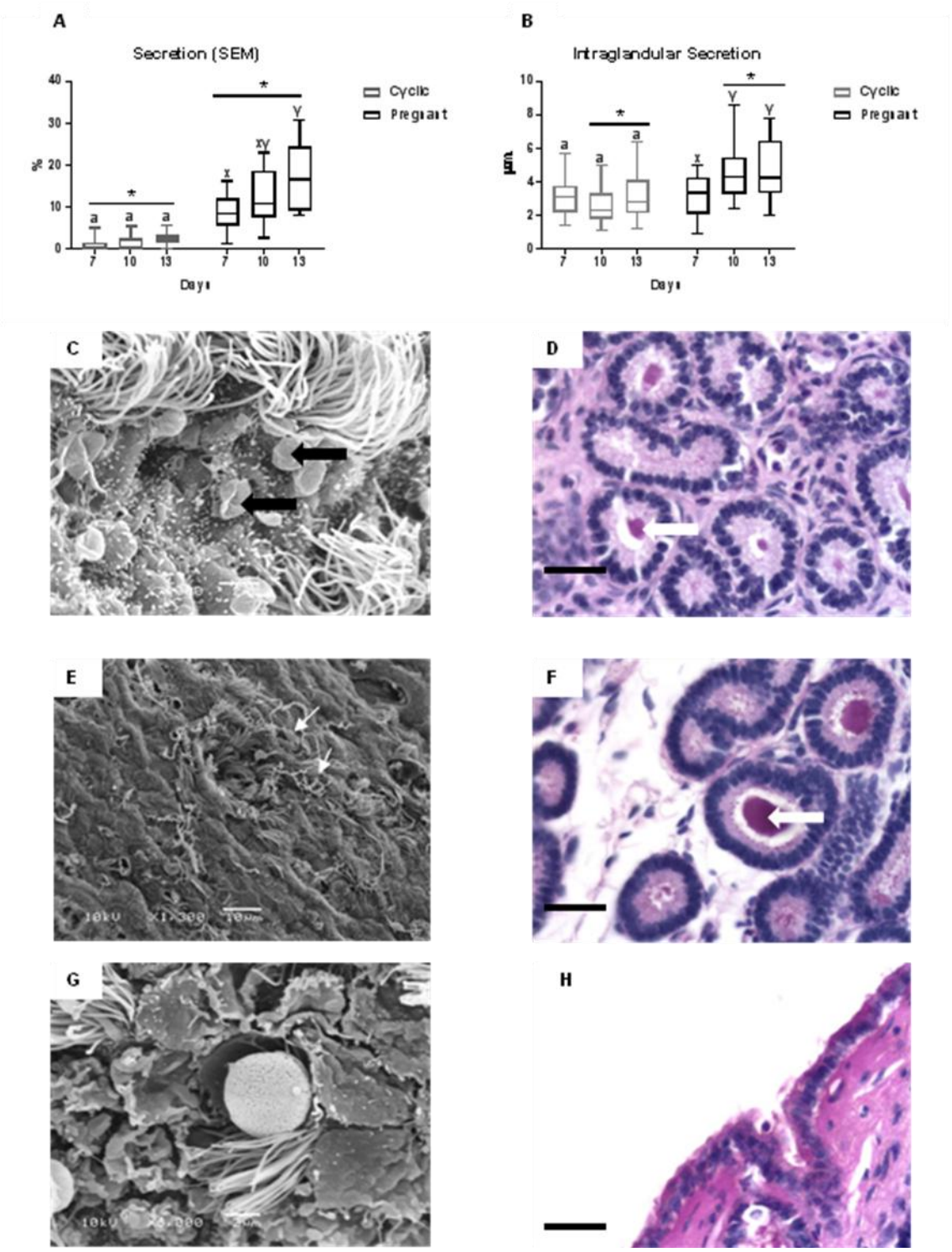

Fig. 3. Box-plots (whiskers represent min to max) demonstrating **(A)** areas with luminal secretion (SEM) and **(B)** intraglandular secretion (light microscopy) in the cyclic and pregnant mares on days 7, 10 and 13 after ovulation. Different letters (a, b) or (x, y) represent significant differences ($P < 0.05$) within the same group (cyclic or pregnant). Asterisks (*) represents a significant difference between groups ($P < 0.05$) on the respective days. **C** (10-day Pregnant mare): scanning electron micrograph (7000x) of the endometrium showing the surface of endometrium with an glandular opening and drops of histotrophic secretion (black arrow), **D** (10-day Pregnant mare): histological section of endometrial glands composed of medium columnar cells, presenting secretory content (white arrow) (PAS, 400x, bar = 50µm). **E** (13-day Pregnant mare): scanning electron micrograph (1300x) of the endometrium showing the glandular opening surrounded of ciliated cells and histotrophic secretion (white arrows). **F** (13-day

Pregnant mare): histological section of endometrial glands composed of medium and cubical columnar cells, presenting lots of secretory content (white arrow) (PAS, 400x, bar = 50µm). **G** (13-day Pregnant mare): scanning electron micrograph (6000x) of the endometrium showing the endometrial surface with high cells releasing histotroph drops. **H** (13-day Pregnant mare): histological section of endometrial epithelium composed of medium columnar cells, presenting the opening of an endometrial gland with secretory content (PAS, 400x, bar = 50µm).

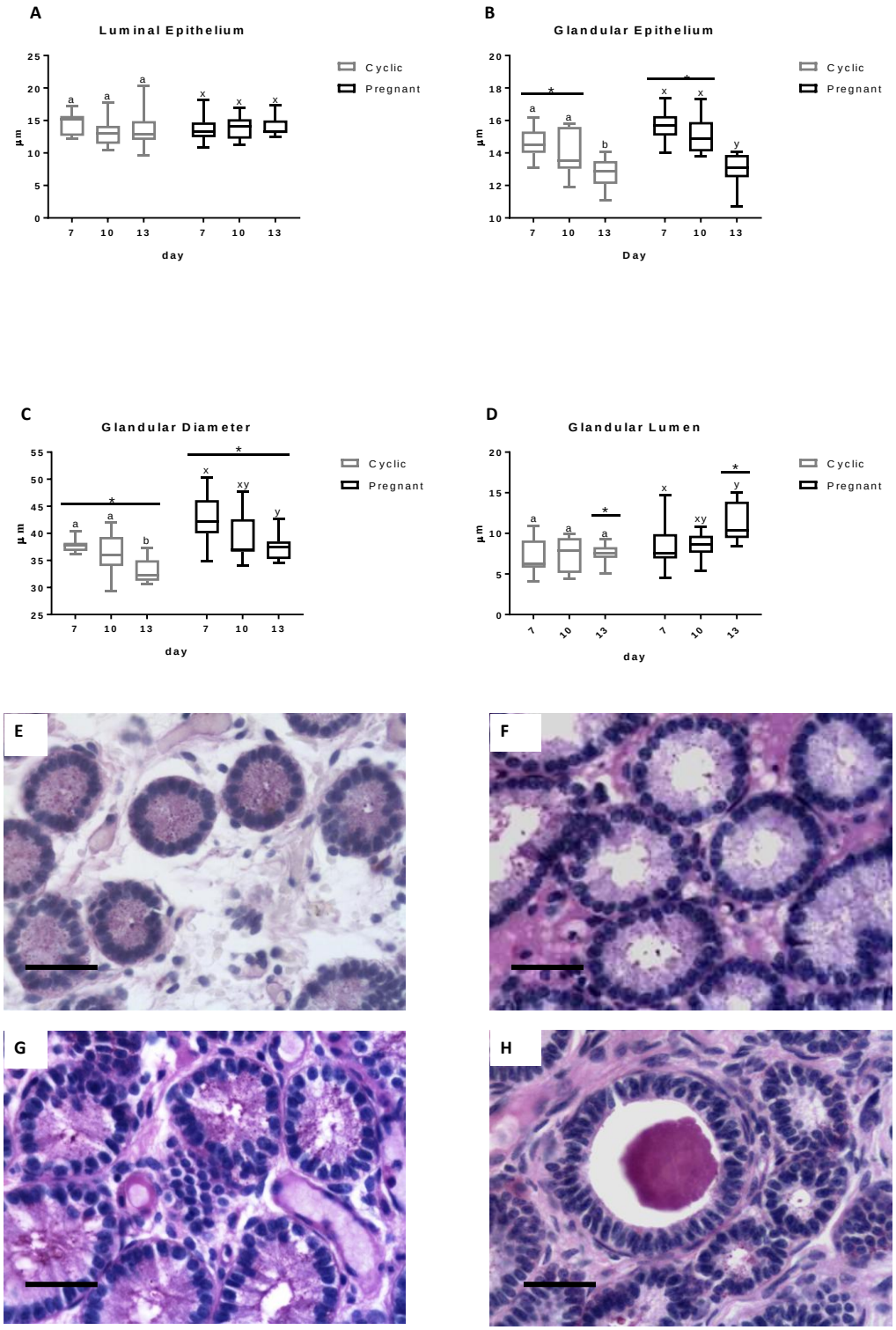

Fig. 4. Box-plots (whiskers represent min to max) of **(A)** Luminal epithelium, **(B)** glandular epithelium **(C)** glandular diameter and **(D)** glandular lumen of cyclic and pregnant mares on days 7, 10 and 13 after ovulation. Different letters (a, b) or (x, y) represent significant differences (P <0.05) within the same group (cyclic or pregnant). Asterisks (*) represents significant difference between the groups (P <0.05) on the respective days. **E** (7-day Cyclic mare): histological section of endometrial glands composed of medium and high columnar cells (PAS, 400x, bar = 50µm). **F** (7-day Pregnant mare): histological section of endometrial glands presenting a moderate lumen composed of high columnar cells (PAS, 400x, bar = 50µm). **G** (13-day Cyclic mare): histological section of endometrial glands composed of high columnar cells (PAS, 400x, bar = 50µm). **H** (13-day Pregnant mare): histological section of endometrial glands composed of cubical columnar cells presenting a high luminal diameter and a lot histotrophic secretion (PAS, 400x, bar = 50µm).

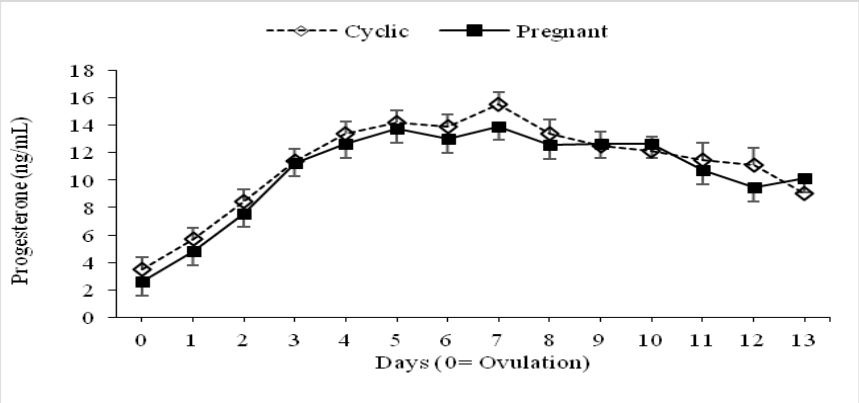

Fig. 5. Concentration of serum P4 (Mean ± standard error of the mean (ng / mL)) from ovulation (day 0) to the end of sampling (day 13).

4 CONCLUSÕES

Foi verificado maior calibre dos vasos sanguíneos no endométrio de éguas prenhes do que de éguas cíclicas a partir do dia 7 após ovulação.

O diâmetro glandular foi maior em éguas prenhes, comparadas com as éguas cíclicas nos dias 7, 10 e 13 pós-ovulação.

Foi observada uma grande perda de células ciliadas no grupo de éguas prenhes a partir do dia 7 comparadas ao grupo de éguas cíclicas.

No dia 13 de prenhez foi observada uma grande quantidade de histotrofo no lúmen uterino.

Alterações ultraestruturais ocorreram no endométrio assim que o embrião entra no útero.

5 CONSIDERAÇÕES FINAIS

O propósito deste trabalho foi observar as alterações ultraestruturais do endométrio no início do desenvolvimento embrionário, para tanto, foi utilizado o mesmo grupo de éguas em um período de tempo restrito. Os resultados obtidos das éguas prenhes foram comparados a um grupo de éguas em diestro (éguas cíclicas) nos mesmos dias. As amostras foram coletadas no mesmo período para não haver nenhuma interferência de sazonalidade, ou qualquer outro tipo de interferência externa.

Foram encontradas alterações logo no dia 7 de prenhez, colocando em dúvida os autores do trabalho se estas são mediadas sistemicamente desde o período em que o embrião se encontra no oviduto, ou o endométrio responde muito rapidamente aos estímulos físicos e químicos a partir da chegada do embrião ao útero.

Qualquer tipo de transformação tecidual depende de um suporte nutricional celular, o qual é sustentado por irrigação sanguínea (transporte de gases e nutrientes) que no presente estudo já está aumentado também no dia 7 de prenhez, dando mais validade aos dados observados.

A presença de histotrofo no dia 7 de prenhez sinaliza a importância da nutrição inicial do concepto, desde sua chegada ao útero. Isso fez com que os autores do trabalho questionassem a sobrevivência ou desenvolvimento embrionário em programas de transferência de embrião, nos quais o concepto é inserido a um ambiente praticamente nulo de nutrientes.

Todas as alterações observadas no endométrio visam prover nutrientes e um ambiente favorável ao desenvolvimento embrionário. Os maiores suportes destas alterações são os níveis de progesterona, que no estudo foi similar nos dois grupos, e a produção de grande quantidade de histotrofo pelas glândulas endometriais e pelo epitélio luminal, estimuladas física e/ou quimicamente pelo embrião.

Este trabalho, por fim, esclarece alguns aspectos relacionados ao ambiente uterino e ao provimento de nutrientes à vida inicial embrionária. Outros estudos poderão ser realizados para esclarecer dúvidas que se formaram no decorrer deste trabalho.

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ANEXO B

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PRODUÇÃO ENDOMETRIAL DE HISTOTROFO DURANTE O INÍCIO DO DESENVOLVIMENTO EMBRIONÁRIO EM ÉGUAS

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A gestação inicial da égua é um período fascinante que abrange numerosas e intensas mudanças em seu desenvolvimento, muitas das quais são únicas para a espécie equina (Stout TAE. Equine breeding management and artificial insemination 2nded;223-240,2009). Esse desenvolvimento depende da manutenção da função lútea, do estabelecimento de um ambiente uterino propício (Leisinger CA, Theriogenology;105;178-183,2017) e de uma interação precisa e orquestrada entre o concepto e o ambiente uterino (Klein C. Biol Reprod;84;872–885,2011). O objetivo deste estudo foi verificar as alterações ultraestruturais do endométrio e a produção histotrófica em éguas cíclicas e prenhes nos dias 7, 10 e 13 pós-ovulação. No primeiro ciclo, biópsias endometriais de 30 éguas foram coletadas no dia 7 (n = 10), 10 (n = 10) e 13 (n = 10) constituindo o grupo éguas cíclicas. No segundo ciclo, as mesmas éguas foram cobertas por um garanhão fértil, acompanhadas diariamente até detectar a ovulação, considerada o dia 0. Foram coletadas biopsias endometriais nos dias7 (n=10), 10 (n=10) and 13 (n=10). Imediatamente após a coleta, o útero foi lavado e as éguas em que foi obtido embrião, foram inseridas no grupo de éguas prenhes. As amostras foram desidratadas com dióxido de carbono e analisadas em um microscópio eletrônico de varredura. Células ingurgitadas foram mais abundantes no grupo de éguas prenhes no dia 7. A secreção endometrial foi maior nas éguas prenhes, aumentando sua quantidade do dia 7 ao 13. No sétimo dia pós-ovulação, uma grande perda de células ciliadas foi evidente no grupo de éguas prenhes. O grupo de éguas prenhes tem menos células ciliadas comparado como o grupo cíclico, provavelmente isso ocorre devido a rápida substituição de células ciliadas por células secretórias, que produzirá substâncias para a nutrição do blastocisto. A superfície com mais células com microvilosidades e menos cílios pode providenciar um maior contato entre o endométrio e o embrião, permitindo que o embrião absorva os nutrientes que compõem a secreção uterina, sinalizando sua presença e estabelecendo sua future fixação e implantação. Ainda no sétimo dia de prenhez, as células superficiais do endométrio são mais ingurgitadas e apresentam uma quantidade significativa de material histotrófico, provavelmente resultado de secreções merócrinas das células epiteliais. Antes da implantação, o concepto equino é dependente dos nutrientes provenientes das secreções uterinas que são dependentes da ação da progesterona. Provavelmente a produção de histotrofo nas éguas prenhes pode estar relacionada a estímulos químicos ou físicos do concepto.
O reconhecimento materno de gestação e a comunicação materno embrionária é um processo recíproco. No presente estudo, as modificações que ocorreram no ambiente uterino tiveram o propósito de prover nutrientes para receber e manter o embrião.

Palavras-chave: embrião, microscopia eletrônica de varredura, endométrio