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Caracterización endócrina y biología molecular del endometrio durante el diestro y la gestación temprana en la yegua

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“Both science and religion are needed to answer life's great questions”

Temple Grandin

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APENDICE

Esta tesis se ha basado en los siguientes artículos científicos originales, referidos como Artículos I, II y III que se adjuntan al final de la misma:

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II. I. Kalpokas, M. N. Martínez, D. Cavestany, F. Perdigón, R. Costa-Mattos and A. Meikle. Ipsilateral endometrial gene expression and endocrine profiles of early gestation-related components in the mare. Enviado a *Theriogenology.*

III. I. Kalpokas, M. N. Martínez, D. Cavestany, F. Perdigón, R. Costa-Mattos and A. Meikle. Cell-specific expression of early gestation-related components and inflammatory cellular infiltrations in equine endometrium during early pregnancy. Para enviar a su publicación.

RESUMEN

La hipótesis de esta tesis fue que la fisiología uterina (en términos de expresión génica y proteica de receptores esteroideos sexuales) depende de la localización del endometrio respecto del lado del cuerpo el lúteo (CL) (Artículo I). Además, postulamos que la presencia del embrión modula muy tempranamente la expresión endometrial de genes y proteínas que participan en procesos fisiológicos que contribuyen al desarrollo embrionario y a la mantención de la preñez (Artículos I, II y III). Finalmente, nos planteamos que este efecto es ejercido en tipos celulares endometriales de forma específica (Artículos I y III). Se realizaron biopsias transcervicales para recoger endometrio del cuerno uterino ipsilateral y contralateral (respecto al lado del CL) en el día 13 después de la ovulación en yeguas cíclicas (n = 6) y preñadas (n = 6) (Artículo I), y únicamente del cuerno ipsilateral en los días 7, 10 y 13 post-ovulación en yeguas cíclicas (n = 6 en cada día) y preñadas (n = 6 en cada día) (Artículos II y III). Las muestras de sangre se tomaron diariamente desde el día 0 hasta el día 13 para las determinaciones de 17ß-estradiol (E2) y progesterona (P4) (Artículo I) y para el factor de crecimiento insulínico 1 (IGF1), leptina y adiponectina (Artículo II). Se midió el inmunomarcado y niveles de transcriptos endometriales ipsi y contralaterales del receptor de estrógeno α (REα) y del receptor de progesterona (RP) al día 13 después de la ovulación (Artículo I). Los niveles de transcriptos endometriales ipsilaterales de *REα*, *RP*, progestin y adipoQ receptor family member V (*PAQR5*), receptor de oxitocina (*ROXT*), prostaglandina-endoperóxido sintasa 2 (*PTGS2*), factor de crecimiento fibroblastico tipo 9 (*FGF9*), IGF1 y su receptor (*RIGF1*), mucina 1 (*MUC1*), osteopontina (*OPN*), receptor de leptina (*RLEP*), receptores de adiponectina 1 y 2 (*R1* y *R2ADIPO*), proto-oncogen raf-1 (*RAF1*) y serine/threonine protein kinasa 6/p21-activated kinase 6 (*PAK6*) se evaluaron en los días 7 y 13 post ovulación (Artículo II). La cantidad de células del sistema inmune y la localización y abundancia de proteínas por inmunohistoquímica para RP, REα, ROXT, PTGS2, IGF1, IGF2, RIGF1 y MUC1 en los días 7, 10 y 13 después de la ovulación fueron analizadas en el Artículo III. Las concentraciones de E2, P4, IGF1, leptina o adiponectina no se vieron afectadas por el estado reproductivo (Artículos I y II) La expresión y localización REα, así como el mRNA del *RP* en yeguas preñadas, se redujeron en el cuerno ipsilateral en comparación con el contralateral (Artículo I). Las yeguas preñadas mostraron menor expresión génica de *REα* y *PR* que el grupo cíclico, mientras que para los demás genes hubo mayor expresión en las preñadas. La preñez afectó todos los genes al día 7, con la excepción del *RP* (Artículo II). La preñez y/o las interacciones con el status mostraron un efecto en la intensidad de tinción de todas las proteínas analizadas. Además, las yeguas preñadas mostraron mayor número de linfocitos, con una disminución hacia el día 13 de preñez. El efecto de la preñez sobre las células inflamatorias y los marcadores moleculares fue más evidente en los compartimentos superficiales, en los leucocitos al día 7 y en la localización al día 10 post ovulación (Artículo III). Los resultados confirman que el embrión equino ejerce una regulación de parácrina (sin cambios sistémicos en las hormonas determinadas) sobre genes y las proteínas que están vinculadas a procesos biológicos esenciales como la inmuno-modulación, la angiogénesis, el crecimiento y metabolismo endometrial y embrionario, sugiriendo que su fluctuación acompaña cambios y requerimientos específicos del desarrollo embrionario. Esta regulación comienza pronto después de entrar al útero, por lo que difiere del concepto más aceptado sobre una respuesta más tardía del endometrio a la presencia del embrión.

SUMMARY

Our hypothesis was that uterine physiology (in term of steroid receptors transcript expression and protein levels) depends endometrial localization regarding side of corpus luteum (CL) (Paper I). Besides, we postulated that the presence of the embryo could exert an early effect on several genes and proteins related to embryo development and pregnancy maintenance (Paper I, II and III). Finally, we hypothesized this effect could be exerted specifically in certain endometrial cell-types (Paper I and III). Transcervical biopsies were performed to collect endometrium ipsilateral and contralateral regarding the side of corpus luteum on day 13 post-ovulation in cyclic (n=6) and pregnant (n=6) mares (Paper I) and exclusively of the ipsilateral horn on days 7, 10 and 13 post ovulation in cyclic (n=6 on each day) and pregnant mares (n=6 on each day) (Papers II and III). Blood samples were collected daily from day 0 until the day 13 for 17ß-estradiol (E2) and progesterone (P4) determinations (Paper I) and for insulin growth factor 1 (IGF1), leptin and adiponectin determinations (Paper II). Immunohistochemical and transcriptional ipsi and contralateral endometrial levels of estrogen receptor α (ERα) and progesterone receptor (PR) where measured at day 13 post-ovulation in Paper I. Transcriptional ipsilateral endometrial expression of *ERα*, *PR*, progestin and adipoQ receptor family member V (*PAQR5*), oxytocin receptor (*OXTR*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), fibroblast growth factor family member 9 (*FGF9*), *IGF1* and its receptor (*IGF1R*), mucin 1 (*MUC1*), osteopontin (*OPN*), leptin receptor (*RLEP*), adiponectin receptors 1 and 2 (*RADIPO1* and *2*), raf-1 proto-oncogene (*RAF1*) y serine/threonine protein kinase 6/p21-activated kinase 6 (*PAK6*) was assessed at days 7 and 13 post ovulation in Paper II and ipsilateral endometrial immunological cell infiltration and immunohistochemical protein localization for PR, ERα, OXTR, PTGS2, IGF1, IGF2, IGF1R and MUC1 was done at days 7, 10 and 13 post-ovulation (Paper III).Serum E2,P4 IGF1, leptin and adiponectin concentrations were not affected by reproductive status (Papers I and II). ERα protein and transcript expression, as well as *PR* mRNA in pregnant mares was reduced in the ipsilateral horn as compared to the contralateral horn (Paper I). Pregnant mares showed lower *ERα* and *PR* mRNA transcript expression than the cyclic group, while the remaining genes showed an upregulation. Pregnancy affected all genes on day 7, with the exception of *PR* mRNA (Paper II).Pregnancy and/or the interactions found with status shows an effect in staining intensity of all proteins studied. Moreover, pregnant mares showed higher numbers of lymphocytes and a downregulation was observed towards day 13. The effect of pregnancy on immune cell population molecular markers was more evident on the superficial, at day 7 in leukocytes and at day 10 post ovulation in protein localization (Paper III). Results confirm that the equine embryo exert a paracrine regulation (without any systemic changes in the analysed hormones) on genes and proteins related to key biological process as immunomodulation, angiogenesis, endometrial and embryo growth and metabolism, suggesting their fluctuation escort specific embryo developmental changes and requirements. This regulations starts soon after the embryo enters the uterus, which challenged the more accepted knowledge regarding the endometrial later response to the presence of the embryo.

ABREVIACIONES

ADN: ácido desoxirribonucleico
ADNc: ácido desoxirribonucleico complementario
CL: Cuerpo lúteo
CV: coeficiente de variación
E2: 17ß-estradiol
EGP: epitelio glandular profundo
EGS: epitelio glandular superficial
EL: epitelio luminal
EP: estroma profundo
ES: estroma superficial
FGF9: fibroblast growth factor family member 9
IGF1: factor de crecimiento insulínico tipo 1
IGF2: factor de crecimiento insulínico tipo 2
IGFBP3: proteína de unión de IGFs tipo 3
HCG: gonadotrofina coriónica humana
RLEP: receptor de leptina
MFA: moléculas focales de adhesión
mRNA: ácido ribonucleico mensajero
MUC1: mucina 1
OPN: osteopontina
P4: progesterona
PAK6: serine/threonine protein kinasa 6/p21-activated kinasa 6
PAQR5: progestin and adipoQ receptor family member V
PGE2: prostaglandina E 2
PGF2α: prostaglandina F 2 alfa
PTGS2: prostaglandin-endoperoxide synthase 2
R1ADIPO: receptor 1 de adiponectina
R2ADIPO: receptor 2 de adiponectina
RAF1: raf-1 proto-oncogene, serine/threonine kinasa
REα: receptor de estrógeno α
RIGF1: receptor de tipo 1 del factor de crecimiento insulínico
RLEP: receptor de leptina
RMP: reconocimiento materno de la preñez
ROXT: receptor de oxcitocina
RP: receptor de progesterona

INTRODUCCIÓN

Desde la perspectiva de la mejora genética, los mejores reproductores equinos son aquellos que han demostrado su valor en la competencia o lo ha hecho su progenie; cuando han alcanzado estos objetivos, las yeguas suelen ser de edad avanzada y no son las mejores candidatas desde el punto de vista reproductivo (Rambags et al., 2003). De hecho, la tasa de pérdida embrionaria estimada entre los días 4 y 14 después de la ovulación para yeguas viejas subfértiles fue significativamente mayor (62%) que la de yeguas jóvenes sanas (9%) (Ball et al., 1989). Además, algunas yeguas jóvenes también tienen baja eficiencia reproductiva y comparten con las yeguas viejas los problemas de bajas tasas de recuperación embrionaria y calidad de embriones (Marinone et al., 2017). A nivel mundial, en los últimos 30 años ha habido avances significativos de la medicina y manejo reproductivo equino, resultando en importantes mejoras en las tasas de preñez por ciclo en yeguas. Sin embargo, la incidencia de la pérdida embrionaria temprana ha cambiado muy poco y sigue siendo una fuente importante de pérdida económica para la industria de la cría de caballos (Stout, 2016). Factores que contribuyen a lo anterior son probablemente el no conocer aún la señal del reconocimiento materno de la preñez (RMP) en esta especie (Klohonatz et al., 2019) y que muchas características de la preñez temprana equina son únicas y difieren marcadamente de los eventos observados en otras especies (Allen, 2001).

El endometrio

El endometrio equino es un tejido compuesto de varios tipos celulares inter-relacionados: epitelio luminal y glandular, estroma y vasos. El epitelio luminal se compone de una sola hilera de células. Las aperturas de las glándulas endometriales se pueden ver periódicamente en la superficie del epitelio luminal. Células epiteliales ciliadas anillan éstas aperturas. Una membrana basal separa el epitelio luminal del estroma subyacente. El estroma se puede dividir en dos estratos, el compacto o estroma superficial y el esponjoso o estroma profundo (Kenney, 1978). El estroma superficial presenta una alta densidad de células y contiene los ductos glandulares que se abren al epitelio luminal además de numerosos capilares debajo de la membrana basal. El estroma superficial es altamente vascularizado y es por lo tanto un importante sitio de exocitosis y acúmulo de células inflamatorias. El estrato profundo posee un tejido estromal laxo, con baja densidad de células que le da el aspecto esponjoso; da sostén a los cuerpos glandulares junto a vasos linfáticos, capilares sanguíneos, vénulas, arteriolas y ocasionalmente a pequeñas arterias musculares (Kenney, 1978). El epitelio glandular deriva del luminal y está conectado al mismo por ductos; su división arbitraria de superficial y profundo se distingue porque en esta última, los ductos son altamente ramificados. En combinación con el epitelio luminal, el epitelio glandular sirve para producir y liberar una serie de secreciones biológicas complejas (fluidos uterinos, leche uterina, histotrofo) (Kenney, 1978; Kenney and Doig, 1986; Van Camp, 1988; Schlafer, 2007) .

Cambios endometriales durante el ciclo estral

Los diferentes tipos celulares responden a los diferentes estímulos hormonales, fundamentalmente a los esteroides ováricos 17ß-estradiol (E2) y progesterona (P4) y por ende el endometrio varía en su apariencia histológica de acuerdo a la estación reproductiva y al momento del ciclo estral (Kenney, 1978; Kenney y Doig, 1986; Schlafer, 2007). Los cambios histológicos más notorios a través del ciclo estral se visualizan en la configuración de las glándulas, las características de las secreciones, la presencia de edema en la lámina propia y la altura del epitelio luminal (Leishman et al., 1982). Durante el diestro temprano (días 0 al 7 post ovulación) la altura del epitelio luminal puede variar, pero luego del día 7 la altura se incrementa progresivamente. Durante dicha etapa la densidad glandular aumenta por la reducción en el edema de la lámina propia y por el aumento de la tortuosidad de las glándulas (Kenney, 1978). Las principales acciones del E2 y la P4 están mediadas por receptores situados en los núcleos celulares (Couse et al., 2006). Las hormonas esteroideas ováricas son importantes moduladores de sus propios receptores. En general, los RE (específicamente el subtipo alfa, REα) y RP son relativamente altos en el endometrio durante el celo, cuando los niveles periféricos de E2 son altos, y relativamente bajos durante el diestro, cuando los niveles circulantes de P4 son altos, según lo demostrado en ovejas (Spencer y Bazer, 1995), cerdas (Sukjumlong et al., 2005) y yeguas (Tomanelli et al., 1991; Watson et al., 1992; McDowell et al., 1999). Ajustando la descripción por tipo celular, los antecedentes en la yegua describen que durante el estro los niveles de RE son altos en epitelio luminal y estroma mientras que son bajos en glandular; para el día 5-6 del diestro, coincidiendo con el pico en los niveles de P4, los patrones se invierten. En el caso de RP los máximos niveles en estroma y epitelio glandular se dan en el día 5-6 post ovulación para luego descender, mientras que los niveles de RP en epitelio luminal son menores al día 10 post ovulación y comienzan a ascender posteriormente (Aupperle et al., 2000; Kalpokas et al., 2010).

El mecanismo de regulación del E2 y la P4 sobre la proliferación y diferenciación endometrial es complejo y específico para cada tipo celular (Aupperle et al. 2000; Hartt et al., 2005). En forma general, se puede decir que el E2 estimula la proliferación epitelial mientras que la P4 causa la proliferación y diferenciación del estroma a la vez que presenta un efecto antiproliferativo epitelial llevando a estas células a un estado de diferenciación (Couse et al., 2006). Existen algunas diferencias en relación a este patrón cuando se observa la proliferación endometrial en la yegua: el E2 estimula la proliferación de todas las células (epiteliales y estromales) del estrato superficial durante el estro (hasta la ovulación) mientras la P4 estimula la proliferación de las células epiteliales glandulares profundas solo en los primeros días post ovulación (Gerstenberg et al., 1999). El E2 y la P4 también tienen efectos sobre la función inmune endometrial y se ha demostrado incluso que las células inflamatorias expresan receptores esteroideos (Acuña y Fumuso, 2013). En general el E2 ejerce un efecto de inmuno estimulación mientras que la P4 es inmunosupresora (Katila, 1996; Acuña and Fumoso, 2013). Si bien la infiltración inflamatoria es un hallazgo normal durante el estro (Kenney, 1978), la monta (Troedsson, 2006) y la preñez temprana (Keenan et al., 1987), las células de defensa encontradas son escasas durante el diestro (Kenney, 1978; Keenan et al., 1987; Acuña y Fumoso, 2013).

Las hormonas esteroideas pueden ejercer sus acciones a través de mecanismos alternativos, como la acción rápida de la P4 a través del receptor extranuclear progestin y adipoQ receptor family member V (PAQR5) (Gellersen et al., 2009). A su vez, existen otros mediadores de las acciones de las hormonas esteroideas. Ejemplos de lo anterior son el fibroblast growth factor family member 9 (FGF9) y los factores de crecimiento insulínico tipo 1 (IGF1), 2 (IGF2) y su receptor (IGF1R), mediadores de la acción de E2 con roles en la remodelación endometrial y angiogénesis en varias especies (Ghahary et al., 1990; Keller et al., 1998; Kaczmarek et al., 2008; Klein et al., 2010; Østrup et al., 2010; Šućurović et al., 2017). Las hormonas esteroideas también regulan a la mucina 1 (MUC1) y osteopontina (OPN) involucradas en funciones adhesivas y antiadhesivas del endometrio, así como constituyen componentes de secreciones glandulares (Gillies et al., 1999; Johnson et al., 2009).

Por otra parte, las hormonas esteroideas también están implicadas en la regulación de procesos que indirectamente influyen sobre sus niveles sanguíneos y por lo tanto sobre sus acciones en endometrio. Este es el caso de la regulación de los niveles del receptor de oxitocina (ROXT) y de la enzima prostaglandina-endoperóxido sintasa 2 (PTGS2), elementos claves de la cascada luteolítica en las especies domésticas (McCracken et al., 1999). El incremento de E2 y RE estimula la formación de ROXT en el endometrio de rumiantes (Spencer y Bazer, 1995), mientras que tanto el E2 como la P4 estimulan la secreción endometrial de PTGS2 *in vitro* en la yegua (Szostek et al., 2014). Por otro lado, diferentes estudios demostraron que el ROXT y la PTGS2 poseen funciones que se superponen a su vínculo con la cascada luteolítica, vinculadas a la angiogénesis y al crecimiento celular (Vanderwall et al., 1994; Bae y Watson, 2003; Cattaneo et al., 2009).

Paralelamente, existen otras hormonas que además de reflejar y regular el status metabólico (Collier et al., 1984; Radin, 2009), están implicadas en funciones reproductivas ejerciendo efectos sobre el endometrio. Tal es el caso de la IGF1, la leptina y la adiponectina, quienes a través de sus receptores (RIGF1, RLEP y RADIPO1 y 2) promueven el crecimiento celular, la angiogénesis, suprimen la apoptosis y median la señalización celular (Keller et al., 1998; Garofalo y Surmacz, 2006; Dos Santos et al., 2015; Pearson, 2015). Mientras algunos autores observaron mayores niveles de IGF1 (Derar et al., 2012) y leptina (Abdelnaby et al., 2016) circulante durante el estro en comparación con los niveles en diestro (medio y tardío), otros no encontraron tales diferencias (Deichsel et al., 2006; El-Maaty et al., 2013; Ferreira-Dias et al., 2005). Por otro lado, no encontramos referencias que compararan las concentraciones de adiponectina circulante durante el estro y diestro en la yegua.

Cambios endometriales durante la preñez temprana: particularidades del embrión equino y reconocimiento materno de la preñez

Tal como se describió, todos los anteriores elementos generan cambios durante el diestro; los mismos tienen como objetivo preparar un endometrio potencialmente capaz de recibir y sostener un embrión (Kenney, 1978; Couse et al., 2006). Sin embargo, la llegada del embrión genera modificaciones sobre este "modelo" endometrial y así lo confirman varios

experimentos que comparan y perfilan muestras endometriales del diestro y la preñez temprana en la yegua (Klein et al., 2010; Merkl et al., 2010; Klohonatz et al., 2015; de Ruijter-Villani et al., 2015; Scaravaggi et al., 2018). Sin embargo, los trabajos encontrados hasta el momento que describan diferencias histológicas y/o moleculares causadas por el embrión antes del período considerado como el RMP en la yegua (día 10 al día 14 post ovulación) son escasos (Klein y Troedsson, 2011). Esta carencia es especialmente llamativa si tenemos en cuenta por un lado que la señal del RMP no se ha identificado aún en la yegua (Klohonatz et al., 2019) y que el embrión equino muestra, desde momentos muy tempranos en su desarrollo, características morfológicas y fisiológicas únicas y que difieren marcadamente del resto de las especies domésticas estudiadas (Allen, 2004).

En este sentido, la cantidad y la variabilidad de las secreciones embrionarias constituyen una de las principales singularidades. Para empezar, el embrión sintetiza grandes cantidades de prostaglandina E2 (PGE2) al día 5 post concepción, para estimular la relajación del oviducto y la contracción y darse paso al útero (unas 24 horas después) (Weber et al., 1991). La secreción de PGE2 y PGF2α continúa y alcanza su máximo al día 10 (Stout y Allen, 2001). Por otra parte la esteroidogénesis en el embrión equino comienza al día 6 post ovulación, con un importante incremento entre los días 12 y 14 de preñez (Zavy et al., 1984; Paulo y Tischner, 1985). El embrión también produce 17-alpha-OH-progesterona entre los días 7 y 14, agonista de las acciones de la P4 a través de receptores de membrana (Goff et al., 1993; Smith et al., 2008). Los resultados de Consiglio et al. (2009) demostraron que la leptina se expresa en embriones equinos *in vitro* mientras que estudios recientes revelaron una importante cantidad de proteínas en el secretoma del embrión equino temprano (Swegen et al., 2017).

Para el momento en que el embrión entre en el útero, el desarrollo ha progresado hasta la mórula tardía o blastocisto en etapa temprana. En coincidencia con el momento de blastulación, se hace visible una cápsula glicoproteica acelular entre el trofoectodermo y la zona pelúcida (día 6,5) (Betteridge, 1989). La cápsula consiste en glicoproteínas similares a la mucina; de hecho no se descarta una contribución endometrial a la formación de la cápsula través del aporte de MUC1 (Gillies et al., 1999). Otro factor endometrial vinculado a la cápsula embrionaria es el IGF1; en el trabajo de Herrler et al. (2000) se describe la expresión de la proteína de unión 3 del IGF1 (IGBP3) en la cápsula embrionaria equina al día 10.

En el día 10 de gestación, el embrión comienza a moverse a lo largo del útero. Los días de movilidad máxima son entre el día 11-14, y el cese de movimientos en día 16 de gestación (Ginther, 1983). El papel propuesto para la extensa migración en todo el lumen uterino es para que el embrión distribuya el factor anti-luteolítico sobre la totalidad de la superficie del endometrio (McDowell et al., 1988) y es logrado gracias a la producción de PGE2 y PGF2α (Stout y Allen, 2001). Recientemente se postuló que el embrión en movimiento causa efectos moleculares activando moléculas focales de adhesión (MFA) (Klohonatz et al., 2015; 2016; 2017). En la yegua se describieron las MFA raf-1 proto-oncogene, serine/threonine kinase (RAF1) y serine/threonine protein kinase 6/p21-activated kinase 6 (PAK6) como los principales genes diferencialmente expresados en el endometrio durante la preñez temprana (Klohonatz et al., 2015; 2017).

Previo al día 10 de gestación, las referencias sobre cambios endometriales en la yegua muestran un efecto de preñez sobre el número de linfocitos y eosinófilos (aumento entre los días 6 y 9 de preñez vs. diestro) (Keenan et al., 1987). Contrariamente, no se encontraron cambios transcripcionales endometriales al día 8 de preñez (Merkl et al., 2010) o fue reportado en un solo gen al día 7 (de Ruijter-Villani et al., 2015). Más cerca del período del RMP, se describe una diminución de la expresión y localización de los REα alrededor del día 13 de gestación en la yegua (McDowell et al., 1999; Hartt et al., 2005; Klein et al., 2010; de Ruijter-Villani et al., 2015; Klohonatz et al., 2015;). Mientras que para de Ruijter-Villani et al. (2015) no existieron diferencias al día 14 ni en la inmunotinción ni en los niveles de mRNA de RP al comparar preñadas vs. cíclicas, otros autores encontraron una disminución de los transcriptos de RP al día 15 (McDowell et al., 1999). La regulación de los ROXT y PTGS2 durante el período del RMP está siendo aún debatida en la yegua (Boerboom et al., 2004; Atli et al., 2010; Merkl et al., 2010; de Ruijter-Villani et al., 2015; Rebordão et al., 2017). Se ha descrito que el receptor endometrial de oxitocina tiene una menor capacidad de unión a la misma en yeguas preñadas (Sharp et al., 1997). Por otro lado se ha descrito que tanto ROXT y PTGS aumentan luego de día 14 en yeguas preñadas, y se ha sugerido que el concepto enlentece el mecanismo luteolítico (más que una inhibición en si misma) (Starbuck et al., 1998; de Ruijter-Villani and Stout, 2015). El aumento en los niveles de transcriptos de *PAQR5* y *FGF9* en yeguas preñadas al día 12 (Merkl et al., 2010) se ha asociado a sus roles como mediadores de los efectos esteroideos.

Mientras algunos autores no encontraron efecto de la preñez sobre los genes de *IGF1* y *RIGF1* en ningún día evaluado (7, 14 y 21 post ovulación) (Gibson et al., 2015), otros demostraron que las yeguas preñadas expresan menores niveles de *IGF1 y RIGF1* al día 14 post ovulación en comparación con las yeguas cíclicas, pero mayores niveles para el mRNA de *IGF2* (Kurar et al., 2010). Asimismo, se evidenció un incremento de éste último gen entre los días 7,14 y 21 de preñez (Gibson et al., 2015). Lo anterior refleja que, al igual que en otras especies (Sosa et al., 2010), el gen del *IGF2* parece comportarse diferente al del IGF1 y al RIGF1. Si bien se ha descrito la expresión y localización de MUC1 tanto en el endometrio equino durante el estro, diestro (Maischberger et al., 2013) y preñez (días 20 a 68) (Wilsher et al., 2013), no hemos encontrado referencias sobre el efecto de la preñez temprana en esta glicoproteína. Se describe una menor expresión del mRNA de *OPN* en yeguas preñadas (días 14,18 y 21 post-ovulación) en comparación con cíclicas. Sin embargo los niveles de proteínas de OPN en yeguas preñadas no difirieron con los de las yeguas cíclicas al día 7 y 14 pero aumentaron al día 21 de preñez (Hermens, 2012). No hemos encontrado referencias del efecto del momento del ciclo estral o la preñez sobre el RLEP. El aumento de los receptores de adiponectina (RADIPO1 y 2) al día 16 de preñez equina (Pearson, 2016) sugiere un rol para la misma en el establecimiento de la preñez. Por otra parte, existe un aumento en la expresión de las MFA RAF1 y PAK6 en los días 9, 11 y 13 de la preñez y los autores postulan una vinculación de este fenómeno con el RMP en la yegua (Klohonatz et al., 2015; 2017).

En lo que respecta a las hormonas circulantes durante la preñez temprana, los estudios no muestran diferencias entre yeguas preñadas y cíclicas en los niveles de esteroides ováricos (Ousey, 2011). Mientras que varias revisiones describen el impacto de la obesidad y los niveles circulantes de la IGF1, leptina y adiponectina en el retorno a la ciclicidad post-anestro y la dinámica folicular en la yegua (Gastal, 2009; Radin et al., 2009; Sessions-Bresnahan et al.,

2018), los estudios comparativos de status reproductivo (preñada vs. cíclica) son escasos. Cuando se compararon los niveles sanguíneos de la IGF1, leptina y adiponectina en días específicos en yeguas preñadas y cíclicas, no se encontraron diferencias (El-Maaty et al., 2013; Daoud and Ezzo, 2014; Pearson, 2015). A su vez, no hemos encontrado referencias que determinen simultáneamente los niveles circulantes de estas hormonas y los receptores endometriales de las mismas. El estado de arte actual demanda un abordaje conjunto de estas variables de respuesta acorde al status reproductivo (gestación o ciclo estral) a lo largo de la gestación temprana.

Además de las interrogantes y contradicciones que existen en la información disponible sobre las interacciones embrio-maternas durante la preñez temprana en la yegua, existe un aspecto metodológico que cabe cuestionar. En las referencias mencionadas a lo largo de la introducción, la expresión génica y localización proteica endometrial en la yegua ha sido evaluada utilizando un pool de tejido de ambos cuernos o tejido de un cuerno sin hacer referencia sobre el lado de la ovulación. Existen estudios en la yegua que fundamentan la existencia de un efecto del lado de la ovulación sobre la funcionalidad endometrial. En primer lugar, se demostró el intercambio contra-corriente entre el drenaje venoso ovárico y la arteria utero-tubal (Barone, 2001). En segundo lugar, las concentraciones de P4 son mayores en el fluído oviductal ipsilateral comparado con el contralateral al día 16 (Nelis et al., 2016). Actualmente en los programas comerciales de transferencia embrionaria se utiliza la técnica no-quirúrgica en la cual el embrión es depositado en el cuerpo del útero con el fin de minimizar la manipulación y consecuente liberación de PGF2α uterina (Wilsher and Allen, 2004; Cuervo-Arango et al., 2018), por lo que no existen datos del efecto del lado de transferencia sobre las tasas de preñez. Sin embargo, McKinnon y Squires (1988) demostraron una mayor tasa de preñez cuando se transfirieron embriones quirúrgicamente al cuerno ipsilateral que cuando se transferían al contralateral. Por lo tanto, se podría postular que la funcionalidad endometrial está afectada por el lado de útero respecto de lado de ovulación / CL. Dado que las hormonas esteroideas sexuales, estradiol 17beta y progesterona son los principales moduladores de la función endometrial, un primer paso sería conocer si la sensibilidad endometrial a las mismas (receptores de estrógenos y progesterona) está afectada por el lado del cuerno uterino respecto a la presencia del cuerpo lúteo en yeguas cíclicas y preñadas.

HIPÓTESIS

La expresión de transcriptos e immunotinción de los receptores esteroideos gonadales en el endometrio equino está afectada por la localización del cuerno uterino respecto el cuerpo lúteo y por la presencia del embrión (Artículo I).

Durante la gestación temprana en la yegua, existen transformaciones en el transcriptoma endometrial y en el contenido y localización de proteínas involucradas en los procesos biológicos inherentes al desarrollo embrionario y el reconocimiento materno de la preñez (Artículos II y III).

OBJETIVOS

General

Evaluar los cambios endometriales que sostienen el diálogo embrio-maternal en el período que antecede al reconocimiento materno de la preñez en la yegua.

Específicos

1. Explorar si el lado de ovulación (presencia de CL) condiciona la expresión de transcriptos e immunotinción de los receptores de estrógenos y progesterona en el endometrio en yeguas vacías y gestadas (Artículo I).
2. Determinar el efecto de la presencia del embrión sobre los niveles de transcriptos y proteínas vinculadas a la preñez temprana en el cuerno ipsilateral al cuerpo lúteo previo al reconocimiento materno de la preñez (Artículos II y III).

METODOLOGÍA

Diseño experimental

El presente experimento se llevó a cabo durante la estación reproductiva (Diciembre- Marzo), en el campo experimental nº 1 de la Facultad de Veterinaria, Universidad de la República, Departamento de Canelones, Uruguay (latitud 34°22'23.1"S, longitud 55°36'10.3"O), previa aprobación del proyecto por el comité de Bioética de la Universidad de la República, Montevideo, Uruguay (CEUAFVET-PI-34/14 -Exp. 111130-001367-14).

Se utilizaron 30 yeguas, entre 5 y 10 años de edad, sin raza definida (fenotipo raza criolla), con un peso promedio aproximado de 400 kg, extraídas de una manada comercial. Las mismas fueron mantenidas en pasturas naturales, con agua ad libitum. Los animales presentaban condición corporal > 3 en una escala de 1 a 5 (escala de Henneke et al. -1983- modificada por Malschitzky et al. -2001-) y no poseían antecedentes de infertilidad. Se evaluó su estado sanitario mediante examen físico y ginecológico de rutina y se verificó su ciclicidad mediante examen ginecológico y ultrasonografía (presencia de CL y/o folículo mayor a 25mm) (SonoScape® A6v (China)) con un transductor lineal de 5 -7,5 MHz de frecuencia.
Ante la presencia de un CL en el examen ultrasonográfico, se procedió a administrar 250 µg cloprostenol vía intramuscular (Estrumate®, Schering-Plough, Essex Animal Health Friesoythe, Alemania), con el fin de provocar la luteólisis y el retorno al estro. Las yeguas fueron monitoreadas mediante ultrasonografía diariamente; cuando un folículo alcanzaba un diámetro ≥ 35 mm y existía edema uterino de grado 3 (Ginther y Pierson, 1984) se administró 2500 UI de gonadotrofina coriónica humana (hCG) vía intravenosa (Chorulon®, Intervet, Internacional, B.V., Holanda). Se continuó con control ecográfico diario, detectando el día de la ovulación (día 0 del ciclo 1). En el primer ciclo se extrajeron biopsias endometriales del cuerno ipsilateral en los días 7 (n=6), 10 (n=6) y de ambos cuernos al día 13 (n=6) post ovulación, constituyendo el grupo de yeguas cíclicas. Las muestras de biopsia endometrial se extrajeron de acuerdo al método descrito por Kenney (1978) utilizando pinza de Yeoman. Se tomó una muestra de cada cuerno uterino en el primer tercio dorsal de los mismos y entre cada extracción se procedió a limpiar y esterilizar la pinza de biopsia. Seguidamente se repitió el método de sincronización (250 ug de cloprostenol, seguimiento ultrasonográfico e inducción de ovulación con hCG).
En el segundo ciclo las yeguas fueron servidas por monta natural por un padrillo fértil 24 horas luego de la administración de hCG. Paralelamente se realizó control ecográfico diariamente para detectar la ovulación (día 0). A los días 7 (n=6), 10 (n=6) y 13 (n=6) post ovulación se realizó la recuperación embrionaria por lavado uterino. Solo fueron asignadas al grupo preñado las yeguas con visualización del concepto por ultrasonografía previo al lavado y/o recuperación embrionaria exitosa. Los conceptos se colectaron por lavado uterino transcervical con 1 a 3 litros (de acuerdo a la edad gestacional) de solución Ringer lactato a 37ºC. Los embriones de día 7 y 10 fueron recuperados por el método regular (Blanchard et al., 2003). Los embriones de día 13 fueron recuperados mediante una sonda uterina de fabricación casera con tubuladura de silicona de 1,5 cm de diámetro. Inmediatamente después de la recuperación embrionaria las biopsias endometriales fueron extraídas con la misma metodología descrita para las yeguas cíclicas.

Durante el experimento se extrajeron diariamente muestras de sangre a todas las yeguas desde el día de la ovulación hasta el día del muestreo endometrial en ambos ciclos. La extracción se realizó por venopunción yugular a través del sistema de Vaccutainer® en tubos secos. Dentro de los 15 minutos posteriores a la extracción, las muestras fueron centrifugadas a 3000g por 15 minutos (centrífuga Luguimac®, LC-15, Argentina). Se removió y almacenó el suero por duplicado en tubos ependorff identificados y se conservaron a -20°C hasta la determinación hormonal.

Determinaciones hormonales

Las determinaciones de las concentraciones de P4, E2, IGF1, leptina y adiponectina fueron realizadas mediante la técnica de radioinmunoanálisis.
El E2 fue determinado utilizando un Kit de RIA con doble anticuerpo (ImmuChem® Double Antibody 17β Estradiol 125 RIA Kit, ICN Biomedicals, Inc., Costa Mesa, CA). El límite de detección del análisis fue 2,6 pg/mL. El estudio presentó un coeficiente de variación (CV) intraensayo para el control (5,9 pg/mL) de 10,8% y un CV interensayo de 8,7%.
La P4 fue determinada utilizando el Kit PROG-RIA-CT KIP1458; DiaSource® (ImmunoAssays SA, Louvain la Neuve, Belgium). El límite de detección del análisis fue 0,074 ng/mL con un coeficiente de variación (CV) intraensayo para el control I (0,8 ng/mL) y II (2,6 ng/mL) de 14 y 7,5 % respectivamente, y un CV interensayo de 4,8 y 8% para el control I y II respectivamente.
Las concentraciones de IGF1 fueron determinadas mediante un kit comercial (Cisbio bioassays®, Codolet, France). El límite de detección del análisis fue 0,57 ng/mL, con un CV de 6,9 % y 4,1% para el control I (51 ng/mL) y II (367 ng/mL) respectivamente.
La leptina fue determinada un kit comercial (Linco®, Millipore). La sensibilidad del análisis fue 1,2 ng/mL, con un CV de 7,5 % y 11,4% para el control I (3,5 ng/mL) y II (9,6 ng/mL) respectivamente.
Las concentraciones de adiponectina fueron determinadas utilizando un kit comercial (HADP-61 HK, Linco®, Millipore). La sensibilidad del análisis fue 0,59 ng/mL, con un CV intraensayo de 8,8% y 18,6% para el control I (8,9 ng/mL) y II (75,9 ng/mL) respectivamente, mientras que el CV interensayo fue de 13,1% para el control I y 14,2% para el control II.

Determinaciones de transcriptos y proteínas en endometrio

Las muestras extraídas fueron divididas en 2: una porción fue congelada en nitrógeno líquido a -80 º C y la otra almacenada en tubos de ensayo de 5 mL conteniendo paraformaldehído bufferado al 4%.

Transcriptos
El RNA celular total fue extraído de las muestras endometriales utilizando reagente Trizol (Invitrogen, Carlsbad, CA) de acuerdo a las recomendaciones del fabricante, seguido por precipitación en cloruro de litio para remover los inhibidores de síntesis de DNAc y mediante el

tratamiento del DNA con DNA-FreeTM Kit (Ambion, Austin, TX, USA) para remover el DNA contaminante (Naderi et al., 2004). La concentración de RNA fue determinada mediante la medición de absorbancia a 260 nm, la pureza de todo el RNA aislado fue analizada desde un radio de absorbancia de 260/280. Para cada muestra, el DNAc fue sintetizado (en una misma corrida) mediante transcripción reversa utilizando un kit: SuperScript III First-Strand Synthesis System Kit (Invitrogen, Carlsbad, CA, USA) con primers al azar y un total de 1 µg de RNA total agregado como modelo.

Los primers para amplificar el DNAc de *REα, RP, PAQR5, ROXT, PGTS2, FGF9, IGF1, RIGF1, MUC1, OPN, RLEP, R1ADIPO, R2ADIPO, RAF1, PAK6* y de los controles endógenos se presentan en la tabla 1. Todos los primers fueron blasteados para confirmar su especificidad. Las reacciones de PCR-RT fueron llevadas a cabo utilizando 7.5 µL de SYBR® Green mastermix (KK4601; Kapa Biosystems, Wilmington, MA, USA), cantidades equivalentes de primers directos y reversos (0.45 µl; 10 µM, Macrogen, Rockville, MD, USA) y 2 µl de DNAc diluido (1:7.5 en agua libre de ARNasa/ADNasa) en un volumen final de 15 µL. Las muestras fueron analizadas por duplicado en un Rotor-GeneTM 6000 de 72 discos (Corbett Life Sciences, Sydney, Australia). Las condiciones de amplificación fueron 5 min a 95 ºC y 40 ciclos de 15 s a 95 ºC, 40 s a 60 ºC, y 20 s a 72 ºC. Luego de cada reacción de PCR, las correspondientes curvas de disociación fueron analizadas para asegurar que el amplicon fuese detectado y para descartar contaminación de ADN o de dímeros de primers. Las muestras de DNAc de todas las yeguas fueron pooleadas para obtener un control exógeno y fueron hechas diluciones de este pool (n=6, de 100 a 3.12 ng). Se aplicó la regresión lineal a las curvas de dilución de cada gen y muestra y la eficiencia (*E*) de los ensayos fue calculada de acuerdo a la fórmula % E = (10 – 1/pendiente – 1) x 100 (Rutledge y Côté, 2003) (Tabla 1). Para la cuantificación relativa, la expresión del gen objetivo fue normalizada con la expresión media de los genes controles endógenos (β-actina, HPRT y GAPDH) y teniendo en cuenta las respectivas eficiencias (Pfaffl, 2001). Los genes controles han sido utilizados como tales previamente (Tabla 1) y se mantuvieron incambiados en las muestras de este estudio.

Análisis histológico e inmunohistoquímica

Las muestras fueron acondicionadas hasta ser incluidas en parafina. Se cortaron secciones de 5 µm y fueron teñidas con hematoxicilina–eosina. Las láminas fueron fotografiadas con un microscopio electrónico (Nikon BM 2000®) utilizando un software (Micrometrics SE Premium®) y posteriormente evaluadas y clasificadas histológicamente de acuerdo al criterio de Kenney y Doig (1986).

Las muestras sometidas a paraformaldehido fueron mantenidas en etanol al 70% hasta ser incluidas en parafina. Para visualizar la inmunopositividad de los receptores se utilizó una técnica inmunohistoquímica (avidin-biotin-peroxidasa) previamente descrita (Kalpokas et al., 2010). Se cortaron las secciones de parafina (5 mm) que fueron colocadas en buffer citrato (pH 6.0) en el microondas a máximo poder (700W) por 10 min para exponer el antígeno. La actividad endógena de la peroxidasa se bloqueó tratando los cortes con peróxido de hidrogeno al 3 % en metanol por 10 min a temperatura ambiente. Para disminuir las uniones no

específicas, las secciones fueron expuestas a suero normal de cabra (Vectin S1000; Vector Laboratories, Burlingame, CA) en PBS por 30 minutos en una cámara húmeda a temperatura ambiente. Se utilizaron anticuerpos monoclonales de ratón y policlonales de cabra y conejo para visualizar los diferentes anticuerpos evaluados (REα, RP, ROXT, IGF1, IGF2 y MUC1: Santa Cruz, California, USA; PTGS2: Cayman Chemical Co., MI, USA y RIGF1: Abcam, Cambridge, UK), a diferentes diluciones (REα 1:25, RP 1:100, ROXT 1:50, PTGS2 1:100; IGF1 1:100; IGF2 1:50; RIGF1 1:50 y MUC1 1:25). Los controles negativos fueron obtenidos al reemplazar el anticuerpo primario por suero no inmune a concentraciones similares. Luego de la incubación las secciones se incubaron con anticuerpo biotinilado anti IgG de ratón (para Reα y RP), conejo (PTGS2 y RIGF1) y cabra (ROXT, IGF1, IGF2 y MUC1) (Vectastain, Vector Laboratories) diluido 1:200 en suero normal caprino. Las secciones se incubaron con el complejo avidin-biotin-peroxidasa (Vectastain Elite; Vector Laboratories). El sitio de unión a la enzima se visualizó por la aplicación del sustrato 3,3´-diaminobenzidina en H2O2 (DAB kit; Vector Laboratories). Los portaobjetos fueron teñidos con hematoxilina y luego deshidratados antes de ser montados con Pertex (Histolab, Gothenburg, Sweden). Todas las muestras de cada diseño experimental se corrieron en el mismo ensayo inmunohistoquímico.

Las láminas fueron fotografiadas de la misma manera que las muestras destinadas a análisis histológico. La expresión endometrial de las proteínas fue evaluada en compartimentos definidos por tipo celular y ubicación: epitelio luminal (EL), epitelio glandular superficial (EGS) y profundo (EGP) y estroma superficial (ES) y profundo (EP). Se analizaron 15 campos por compartimiento histológico por animal a un aumento de 1000X. El inmunomarcado fue clasificado según la intensidad de la tinción en una escala de: (-) ausente, (+) leve, (++) moderado, (+++) intenso; y expresado como promedio, según 1 x n1 + 2 x n2 + 3 x n3, donde n = proporción de células por campo que exhiben tinción leve (1), moderada (2) e intensa (3) (Boos et al., 1996). A partir de esta evaluación se estudiaron las variables proporción de células positivas (Artículo I) e intensidad de tinción (Artículo III).

Análisis estadístico

Fue realizado el análisis Univariate a todas las variables para identificar inconsistencias y valores extremos. El número de neutrófilos y linfocitos fue normalizado mediante una transformación logarítmica (logaritmo en base 2) y dado que el número de eosinófilos e intensidad de REα no eran normales aún transformados se analizaron por métodos no paramétricos. Las concentraciones de las hormonas en suero, los niveles de transcriptos y la abundancia y localización de las proteínas endometriales se analizaron por análisis de varianza utilizando un modelo mixto (Statistical Analysis System, SAS, Institute Inc., Cary, NC, USA). El modelo de análisis de las concentraciones hormonales (Artículos I y II) incluyó los efectos del status reproductivo, del día del ciclo/preñez y de su interacción. Estas variables se estudiaron por análisis de medidas repetidas. En el modelo de análisis de los niveles de transcriptos y la abundancia y localización de las proteínas endometriales se incluyeron los siguientes efectos fijos: status reproductivo, lado de ovulación/cuerpo lúteo (transcriptos y proteínas), tipo celular y ubicación (proteínas) e interacciones (Artículo I) y status reproductivo, día del

ciclo/preñez y su interacción y tipo celular cuando correspondiera (Artículos II y III). La comparación entre variables fue realizada mediante el test de Tukey Kramer. El estudio de la correlación entre las variables se realizó mediante la utilización de PROC CORR. El nivel de significación considerado fue siempre $P < 0.05$ y los valores de P comprendidos entre 0.05 y 0.1 se consideraron como tendencia.

RESULTADOS

Niveles de hormonas circulantes durante el diestro y preñez temprana (Artículos I y II).

Las concentraciones de E2, P4, IGF1 y adiponectina fueron afectadas por el día post-ovulación ($P < 0.05$, $P < 0.001$, $P < 0.05$ y $P < 0.05$, respectivamente), pero no por el status reproductivo o su interacción (Fig. 1). Las concentraciones de leptina no se vieron afectadas por ninguno de los efectos fijos estudiados (datos no mostrados, Artículo II). Las concentraciones de P4 fueron incrementándose lentamente, llegando a un pico entre los días 5 y 7. A partir del pico, la P4 se mantuvo, constante hasta el día 10, y luego las concentraciones disminuyeron hacia el día 13 en ambos grupos de yeguas.

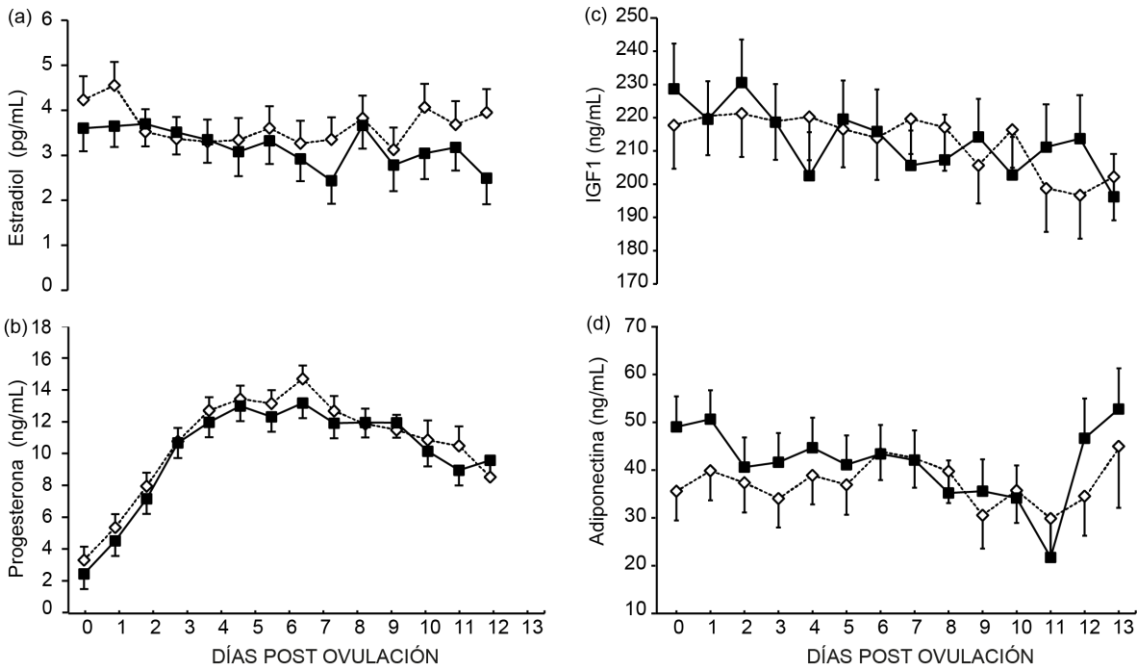

Figura 1. Concentraciones de 17ß estradiol (Estradiol) (a), progesterona (b), factor de crecimiento insulínico tipo 1 (IGF1) (c) y adiponectina (d) durante el diestro y la preñez temprana (día 0 = ovulación) en yeguas cíclicas (rombos blancos) y preñadas (cuadrados negros). La media (± SEM) de las concentraciones hormonales (eje de las y) se muestran para cada día (eje de las x).

Efecto del lado del cuerpo lúteo y la preñez sobre los expresión y localización de receptores esteroideos endometriales al día 13 post-ovulación (Artículo I)

El cuerno ipsilateral tendió a expresar menos mRNA para ambos receptores al día 13 que el contralateral (*REα*: 0.97 vs. 1.33 ± 0.17, P < 0.10; *RP:* 1.57 vs. 1.96 ± 0.52, P = 0.08). El cuerno ipsilateral de las yeguas preñadas presentó menores niveles para ambos transcriptos (P < 0.05) comparando con el contralateral, pero no existieron diferencias entre cuernos en el grupo cíclico (Fig. 2). Fue encontrada una correlación positiva entre la expresión de ambos transcriptos (r = 0.75, P < 0.0001).

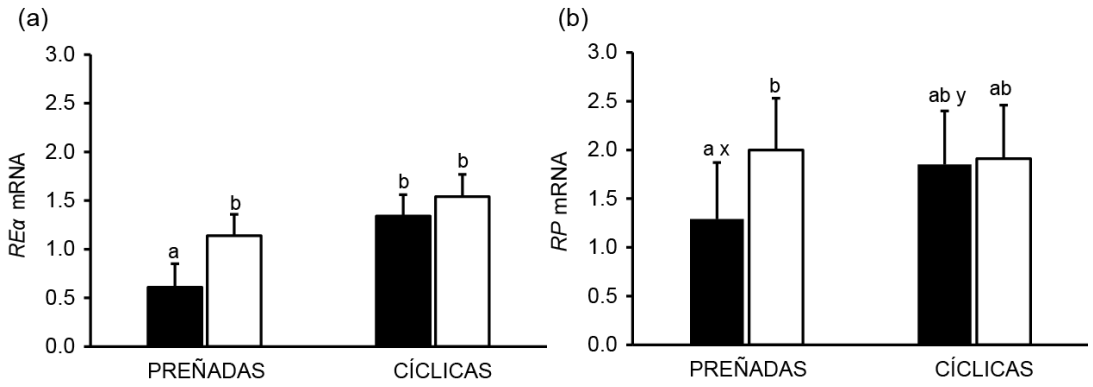

Figura 2. Expresión relativa de los transcriptos para *REα* (a) y *RP* (b) en cuernos ipsilaterales (barras negras) y contralaterales (barras blancas) de yeguas preñadas y cíclicas al día 13 post ovulación. Los datos se presentan como medias de mínimos cuadrados ± errores estándar. (a, b) los superíndices diferentes dentro del mismo gráfico difieren: P < 0.05. (x, y) los superíndices diferentes dentro del mismo gráfico tienden a diferir: 0.05 < P ≤ 0.10.

Se detectó escasa área positiva a REα en todos los tipos celulares y el cuerno ipsilateral presentó menor área de inmunomarcado que el contralateral (P < 0.05). En el grupo preñado, el REα presentó una tendencia a menor área de tinción en el ES y EP en el cuerno ipsilateral que en el contralateral (ES: 3.9 % vs 8.2 % ± 5.0; EP: 4.2 % vs 8.4 %± 5.0, P < 0.10). El lado del CL no afectó la expresión de RP y no hubo correlación de la abundancia entre ambos receptores.

El endometrio ipsilateral durante el diestro y preñez temprana (Artículos II y III)

El análisis histológico reveló que todas las yeguas pertenecían a la categoría I o IIA (yeguas sanas). Las infiltraciones celulares consistieron principalmente en linfocitos, eosinófilos y neutrófilos, que se distribuyeron en los compartimentos estromales (superficiales y profundos) (Fig. 3). Las yeguas preñadas mostraron un mayor número linfocitos en el ES (P < 0.001) y profundo (P= 0.0583) (Fig. 3). Los eosinófilos fueron observados casi exclusivamente al día 7 (P < 0.05) en el ES, y en este día las yeguas preñadas mostraron mayor número de éste tipo celular (4.51 ± 0.82 vs. 0.45 ± 0.31 eosifinófilos/5 campos; P < 0.05). Los neutrófilos también fueron más abundantes en yeguas preñadas en el ES (1.43 ± 0.41 vs. 0.71 ± 0.36/5 campos; P= 0.0554).

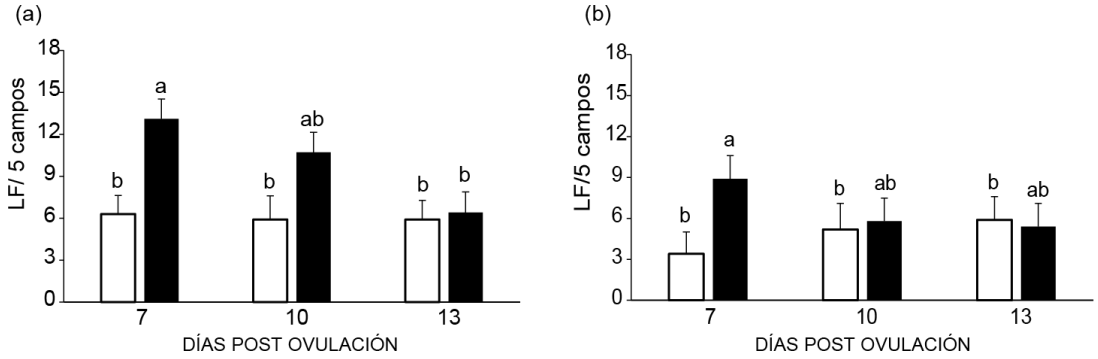

Figura 3. Número de linfocitos (LF) cada 5 campos en el estroma superficial (a) y profundo (b) en los días 7, 10 y 13 (día 0 = ovulación) en yeguas cíclicas (barras blancas) y preñadas (barras negras). Los datos se presentan como medias de los mínimos cuadrados ± S.E.M. (a, b, c) los superíndices diferentes dentro del mismo gráfico difieren: $P \leq 0.05$. (x, y) los superíndices diferentes dentro del mismo gráfico tienden a diferir: $0.05 < P \leq 0.10$.

Las concentraciones uterinas para los transcriptos *REα* y *RP* se muestran en la figura 4 (a,b). Las yeguas preñadas mostraron menor expresión de éstos transcriptos que las yeguas cíclicas ($P < 0.002$; $P = 0.053$, respectivamente). En ambos días, las concentraciones del mRNA de *REα* fueron menores en el grupo preñado, mientras que estas yeguas presentaron menor concentración del mRNA de *RP* únicamente al día 13. Se encontró menor nivel del transcripto *REα* al día 13 en comparación con el día 7 en el grupo preñado, mientras no hubo diferencias entre los días estudiados para el grupo cíclico. La expresión relativa del mRNA de *RP* aumentó hacia el día 13 del diestro, mientras no se evidenció diferencias entre los días muestreados en las yeguas preñadas.

El resto de los transcriptos se muestran en las figuras 4 (c,d,e), 5 y 6. Las yeguas preñadas mostraron mayor expresión que las cíclicas en todos estos genes (*PAQR5:* $P < 0.01$; *ROXT*: $P < 0.01$; *PTGS2*: $P < 0.001$; *FGF9:* $P < 0.001$; *IGF1:* $P < 0.01$; *RIGF1*: $P < 0.001$; *MUC1*: $P < 0.01$; *OPN:* $P < 0.001$; *RLEP:* $P < 0.05$; *R1ADIPO:* $P < 0.01$; *R2ADIPO*: $P < 0.001$; *RAF1:* $P < 0.001$ y *PAK6*: $P < 0.05$). En todos los casos se observó éste efecto al día 7 post ovulación. Al día 13 post ovulación las yeguas preñadas también presentaron mayores niveles de transcriptos que las cíclicas, excepto para los transcriptos de *IGF1*, *MUC1* y *RLEP*. En el grupo preñado, los niveles de mRNA de *PTGS2*, *IGF1*, *MUC1* y *RADIPO2* descendieron hacia el día 13 de preñez, mientras que los niveles relativos para el gen de *PAQR5* aumentaron hacia el día 13 de preñez. En las yeguas cíclicas, el *ROXT* y *PAQR5* fueron los únicos genes que mostraron fluctuaciones entre día 7 y 13 post ovulación, con un aumento hacia el final del diestro.

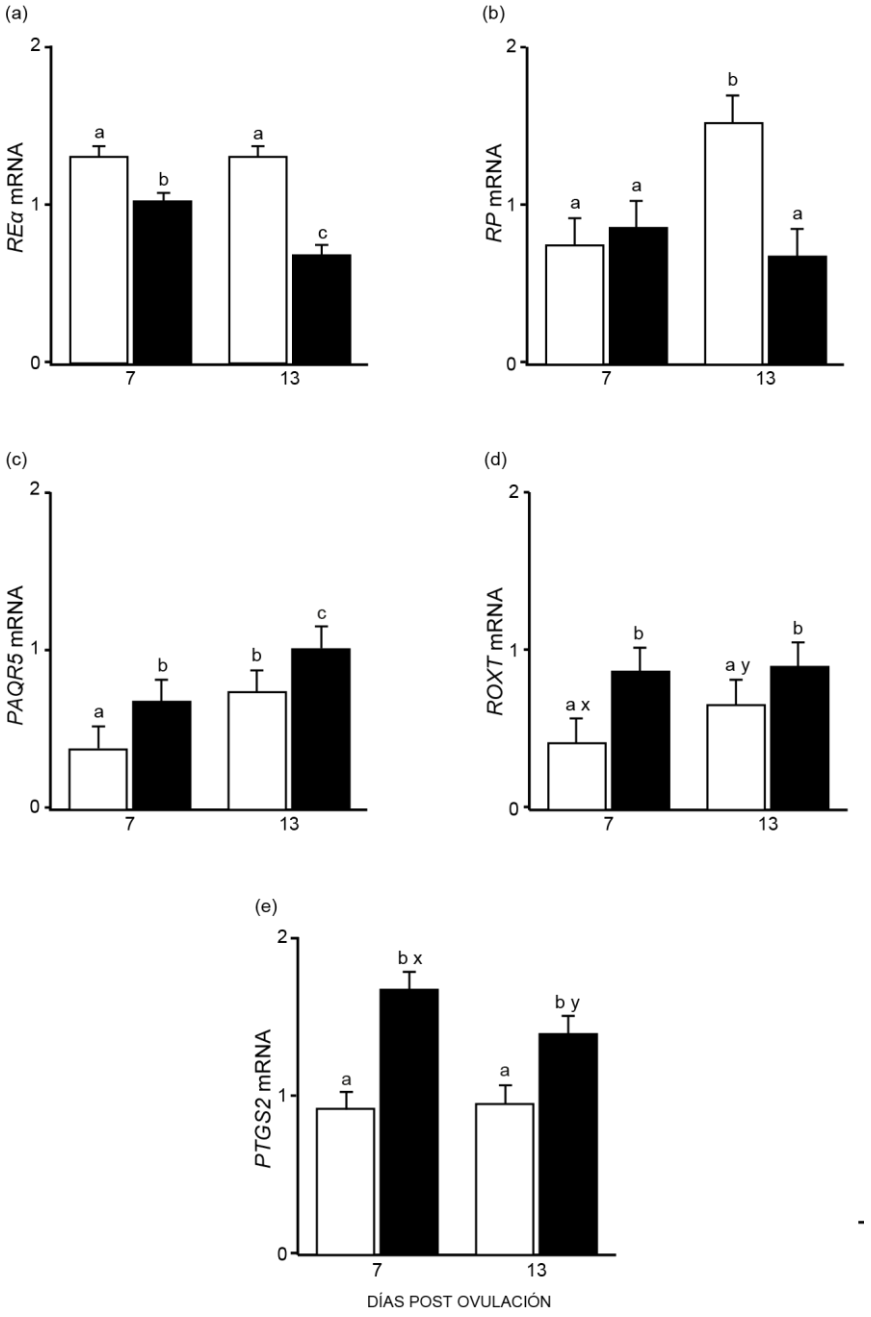

Figura 4. Expresión endometrial ipsilateral relativa de los transcriptos para el receptor de estrógeno alfa (*REα*) (a), receptor de progesterona (*RP*) (b), progestin and adipoQ receptor family member V (*PAQR5*) (c), receptor de oxitocina (*ROXT*) (d) y prostaglandin-endoperoxide synthase 2 (*PTGS2*) (e) en yeguas cíclicas (barras blancas) y preñadas (barras negras) en los días 7 y 13 post ovulación. Los datos se presentan como medias de los mínimos cuadrados ± S.E.M. (a, b, c) los superíndices diferentes dentro del mismo gráfico difieren: $P \leq 0.05$. (x, y) los superíndices diferentes dentro del mismo gráfico tienden a diferir: $0.05 < P \leq 0.10$.

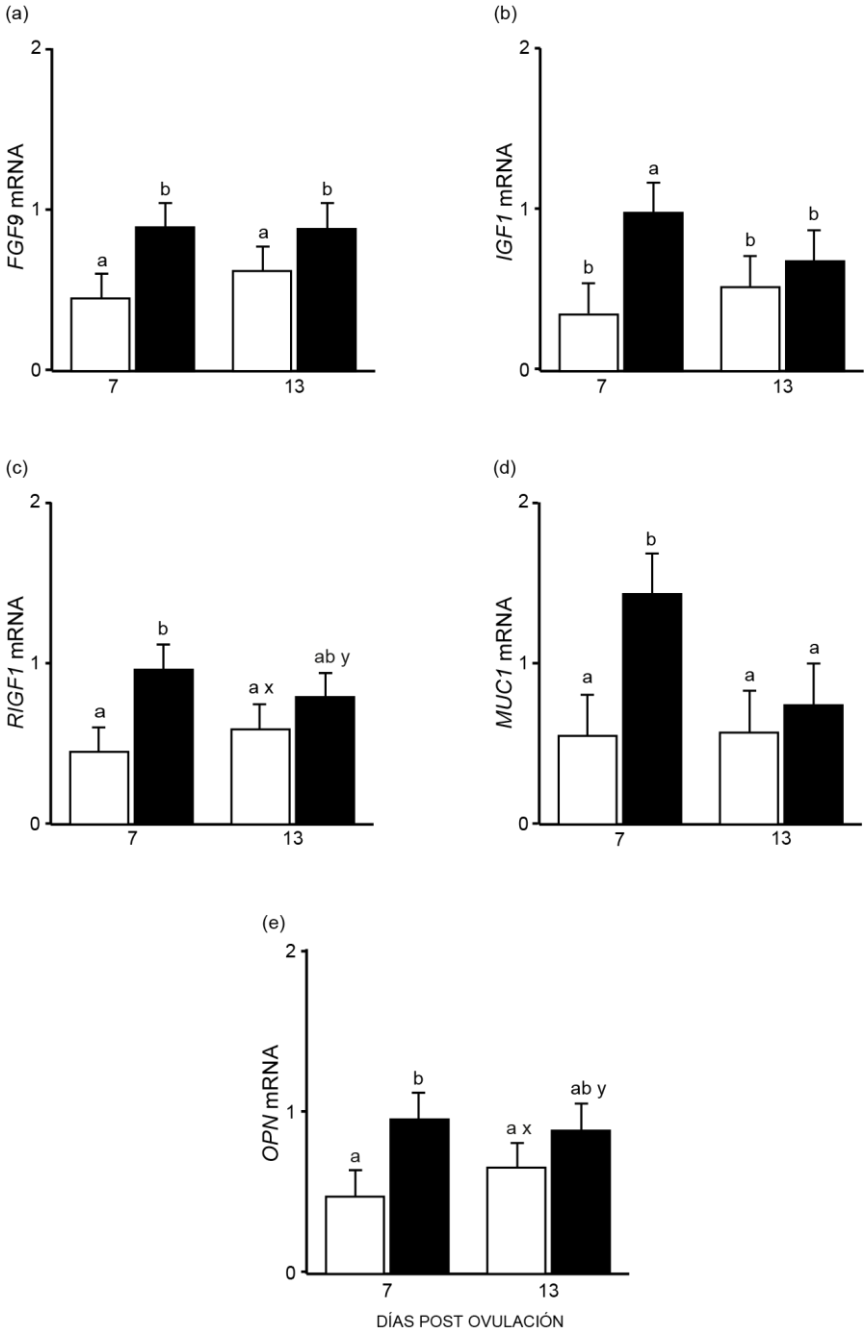

Figura 5. Expresión endometrial ipsilateral relativa de los transcriptos para el fibroblast growth factor family member 9 (*FGF9*) (a), factor de crecimiento insulínico tipo 1 (*IGF1*) (b), receptor de IGF1 (*RIGF1*) (c), mucina 1 (*MUC1*) (d) y osteopontina (*OPN*) (e) en yeguas cíclicas (barras blancas) y preñadas (barras negras) en los días 7 y 13 post ovulación. Los datos se presentan como medias de los mínimos cuadrados ± S.E.M. (a, b, c) los superíndices diferentes dentro del mismo gráfico difieren: $P \leq 0.05$. (x, y) los superíndices diferentes dentro del mismo gráfico tienden a diferir: $0.05 < P \leq 0.10$.

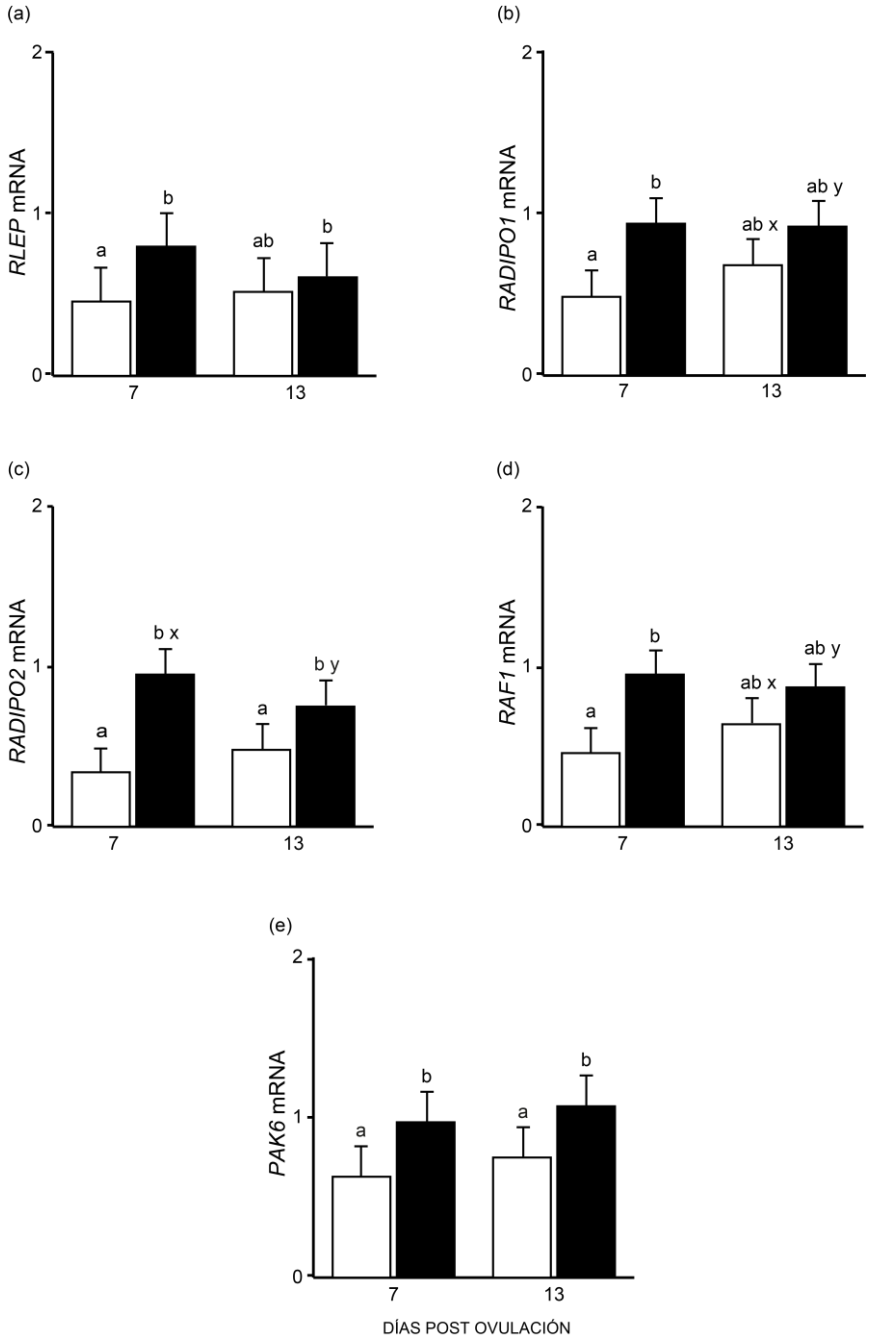

Figura 6. Expresión endometrial ipsilateral relativa de los transcriptos para el receptor de leptina (*RLEP*) (a), receptor de adiponectina 1 (*R1ADIPO*) (b), *R2ADIPO* (c), raf-1 proto-oncogene, serine/threonine kinase (*RAF1*) (d) y serine/threonine protein kinase 6/p21-activated kinase 6 (*PAK6*) (e) en yeguas cíclicas (barras blancas) y preñadas (barras negras) en los días 7 y 13 post ovulación. Los datos se presentan como medias de los mínimos cuadrados ± S.E.M. (a, b, c) los superíndices diferentes dentro del mismo gráfico difieren: $P \leq 0.05$. (x, y) los superíndices diferentes dentro del mismo gráfico tienden a diferir: $0.05 < P \leq 0.10$.

Hubo un efecto del estado en la intensidad de tinción del REα ($P < 0.001$) ya que las yeguas preñadas estaban casi desprovistas de tinción para este receptor. También hubo un efecto del día de extracción ($P < 0.001$): en las yeguas cíclicas, hubo una disminución de la intensidad de tinción desde el día 7 hacia el día 13 en el epitelio glandular y el ES, mientras que en el EL y ES

este descenso comenzó en el día 10. La mayor intensidad de tinción del grupo cíclico en comparación con las yeguas preñadas se evidenció en los días 7 y 10, pero no en el día 13 post-ovulación. Las yeguas cíclicas mostraron una mayor intensidad de tinción que las yeguas preñadas en todos los tipos celulares. El grupo preñado mostró una baja intensidad de tinción restringida a las células estromales (Fig. 7).

La intensidad de tinción del RP no se vió afectada por el estado, pero hubo un efecto del día ($P < 0.001$) y del tipo celular ($P < 0.001$). Incluyendo ambos grupos de yeguas, hubo una mayor intensidad de tinción para esta proteína en el día 7 con una disminución hacia el día 13 post-ovulación. En este día, ambos grupos estaban casi desprovistos de tinción en el EL (datos no mostrados). El patrón de regulación a la baja del día 7 visto para el RP se observó en el EL, EGS y EGP en ambos grupos (Fig. 7).

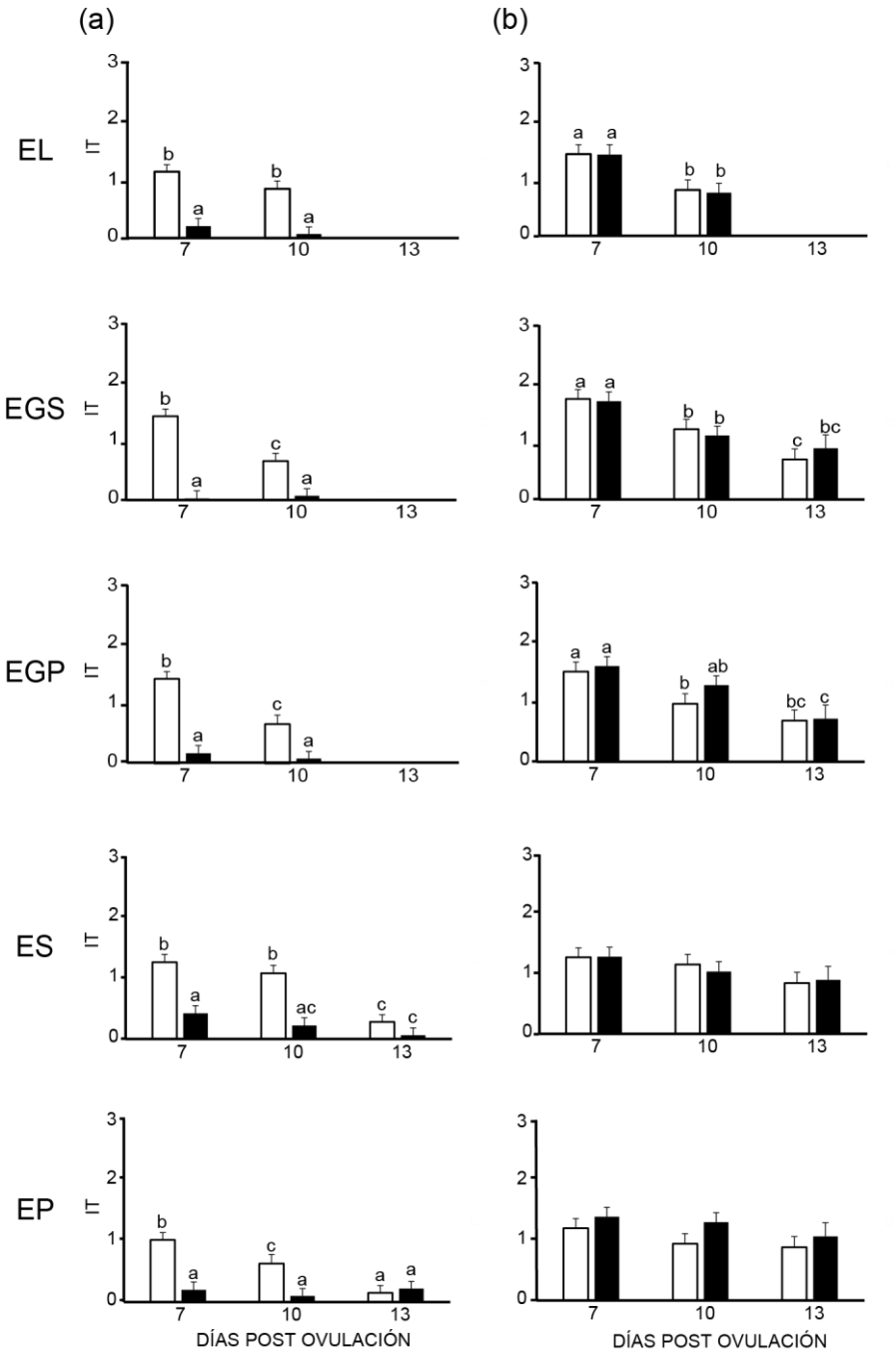

Figura 7. Intensidad de tinción (IT) para el receptor de estrógeno α (a) y el receptor de progesterona (b) en los días 7, 10 y 13 post ovulación en el endometrio de yeguas cíclicas (barras blancas) o preñadas (barras negras). EL, epitelio luminal; EGS, epitelio glandular superficial; EGP, epitelio glandular profundo; ES, estroma superficial; EP, estroma profundo. Los datos son la media de mínimos cuadrados ± SEM. (a, b) los superíndices diferentes dentro del mismo gráfico difieren: $P < 0.05$.

El grupo preñado tuvo menor intensidad de tinción para el ROXT que las cíclicas al día 10 post ovulación en los compartimentos superficiales EL, EGS y ES. Por otro lado, en este mismo día el grupo preñado mostró una mayor intensidad de tinción a la enzima PTGS2 en ubicaciones profundas (EGP y EP) en comparación con las yeguas cíclicas, no encontrándose diferencias en otros días.

En comparación con el grupo cíclico, las yeguas preñadas mostraron una menor intensidad de tinción para IGF1 en el EGS al 10 post- ovulación y en el EL al día 7 para el RIGF1. Para ambas proteínas, la intensidad de tinción en el EGP disminuyó del día 7 al 13 en yeguas tanto cíclicas como preñadas, mientras que el mismo patrón se observó en las yeguas cíclicas para el RIGF1 en el EGS. Si bien las yeguas preñadas también mostraron menor intensidad de tinción para IGF2 al día 10 post-ovulación en EL, se observó un aumento de la inmunotinción de éste factor a lo largo de los días de estudio en EL y EGS en ambos grupos de yeguas y para las yeguas preñadas en ES.

También se observó para la proteína MUC1 una menor intensidad de tinción del grupo preñado en comparación con el cíclico al día 10 en el EGS y ES. En el grupo preñado además aumentó la intensidad de tinción desde el día 10 hasta el día 13 después de la ovulación en todos los tipos celulares.

DISCUSIÓN

Niveles de hormonas circulantes durante el diestro y preñez temprana (Artículos I y II)

Las concentraciones séricas de E2 y P4 no se vieron afectadas por la preñez, lo que concuerda con trabajos previos (Ousey, 2011) y sugiere que las acciones diferenciales de éstas sobre el endometrio en yeguas preñadas estaría asociada a una sensibilidad endometrial a las mismas (es decir, receptores) modificada debido a la presencia del embrión. El aumento y posterior disminución de P4 desde el día 10 hasta el día 13 observado en nuestro estudio, ya ha sido previamente reportado (Daels et al., 1991; Allen, 2001; Ousey, 2011; Kelleman, 2013). Dicha disminución podría estar relacionada con una señal luteotrófica débil en las yeguas preñadas (Allen, 2001; Ousey, 2011) o como sucede en otras especies, que el embrión pueda rescatar al cuerpo lúteo de una luteólisis ya activada (Aba et al., 1995).

En nuestro conocimiento, este es el primer trabajo en yeguas que reporta simultáneamente las concentraciones de IGF1, leptina y adiponectina durante la preñez temprana y el diestro. La ausencia del efecto del estado en las concentraciones de estas hormonas coincide con trabajos previos (Daoud y Ezzo, 2014; El-Maaty et al., 2013; Pearson, 2015). Ninguna de estas tres

hormonas fue afectada por la preñez temprana en oveja (de Brun et al., 2015), por lo que coincidimos con lo propuesto por otros autores en que las demandas del embrión temprano alteran muy poco el balance energético de la madre a nivel sistémico (Sosa et al., 2009).

Efecto del lado del cuerpo lúteo sobre la expresión y localización de receptores esteroideos endometriales al día 13 de gestación o ciclo estral

Por lo que sabemos, este es el primer estudio que muestra un efecto del lado del cuerpo lúteo sobre la expresión y localización del REα y/o RP en el endometrio de yeguas sanas dentro del período del reconocimiento materno de la preñez/luteólisis en la yegua. El lado del cuerpo lúteo afectó a ambos receptores, disminuyendo los niveles de transcriptos y proteína de REα y el mRNA de *RP*. Esto podría estar relacionado con la distribución local de secreciones ováricas al cuerno ipsilateral mediante el intercambio entre el drenaje venoso ovárico y la arteria tubárica uterina (Barone, 2001; Nelis et al., 2015), lo que llevaría a un aumento en la concentración de P4 en el cuerno ipsilateral. Takahashi et al. (2016) sugieren que la dinámica de las concentraciones de P4 en el tejido endometrial puede diferir de las concentraciones séricas. De hecho, la expresión de mRNA de *REα* y *RP* en el oviducto ipsilateral ovino al día 5 (de Brun et al., 2013) o cuerno uterino en ovejas y vacas al día 5, 7 y 14 post-ovulación (Araújo et al., 2014; Sosa et al., 2006) fue menor que en el contralateral, lo que se asoció con concentraciones de P4 más altas (Einer-Jensen and Mc Cracken, 1981; Weems et al., 2011). Por otro lado, no se encontraron diferencias de lado sobre la inmunotinción de RP. Previamente se demostró que el contenido de mRNA del *RP* puede presentar el perfil opuesto al de la proteína (Meikle et al., 2000), y que la transcripción de genes es un proceso que probablemente no proporcione efectos medibles dentro de la célula hasta horas o incluso días después de la estimulación esteroidea (Couse et al., 2006).

Los cambios en la sensibilidad del endometrio a las hormonas esteroides son críticos para la regulación de la función uterina. Junto con los resultados que muestran mejores tasas de preñez de embriones equinos transferidos al cuerno uterino ipsilateral (McKinnon y Squires, 1988), nuestros resultados plantean la posibilidad de una posible diferencia en la funcionalidad de los cuernos uterinos. Entendemos además que lo anterior fundamenta la recomendación de especificar el lado de la ovulación/CL en la metodología del análisis endometrial en yeguas y de forma consecuente todas las variables de esta tesis fueron determinadas en el endometrio ipsilateral.

Efecto de la preñez sobre células inflamatorias en el endometrio

Así como fue postulado en rumiantes (Ott et al., 2014), ni la madre está inmunodeprimida durante la preñez temprana, ni el concepto semialogénico escapa de ser detectado por el sistema inmune materno, que detecta la presencia de antígenos en el concepto y además es activado por citokinas secretadas por el embrión (Hansen, 2011). El mayor número de linfocitos encontrado en las yeguas preñadas al día 7 respondería entonces a esta detección y

activación, ya que en otras especies se describe la presencia de poblaciones de linfocitos (linfocitos T y natural killers) en el endometrio durante la preñez temprana (Ott et al., 2014). Paralelamente, varios transcriptos que tuvieron mayores niveles en yeguas preñadas al día 7 han sido vinculados en otras especies y/o tejidos con un rol pro-inflamatorio relacionado a los linfocitos (*RLEP*-Dos Santos et al., 2015-; *OPN* -Johnson et al., 2014-; *IGF1 y RIGF1* -Smith, 2010). Por otra parte, esto también explicaría la mayor presencia de los eosinófilos al día 7, si tenemos en cuenta su función como presentadores de antígeno a los linfocitos T (Blanchard y Rothenberg, 2009). De la misma manera, existió un mayor número de linfocitos y eosinófilos en yeguas preñadas al día 6-9 post ovulación en comparación con yeguas servidas no preñadas y con yeguas no servidas (Keenan et al., 1987). Además, existe evidencia de que los productos del sistema inmunológico pueden ser beneficiosos para el desarrollo embrionario y la remodelación tisular y vascular endometrial en varias especies (Jeziorska et al., 2005; Hansen, 2011). Sin embargo, no encontramos diferencias en la cantidad de células inmunes entre los grupos al día 10 ni 13 post-ovulación, en línea con estudios previos en yeguas al día 14 (Keenan et al., 1987; Watson y Dixon, 1993). Esta regulación negativa coincide con la formación al día 7 de la cápsula del embrión equino, que podría "ocultar" al embrión de la detección del sistema inmune materno (Betteridge, 2007). Paralelamente podrían estar actuando los efectos inmunosupresores de la PGE2 (Low and Hansen, 1988) y el IFNγ (Cochet et al., 2009) secretados por el concepto. El perfil proteómico del histótrofo durante los días 7, 10 y 13 también describe una inmunomodulación en yeguas preñadas (Bastos et al., 2018) ya que las mismas mostraron menores niveles de fibrinógeno, un marcador de inflamación (Salini et al., 2011).

Expresión y localización endometrial de factores asociados a cambios histológicos y funcionales del útero

La expresión endometrial de todos los genes estudiados se vió afectada por la preñez, revelando el impacto del embrión equino temprano en la regulación local de la funcionalidad endometrial. En varias especies incluyendo la equina, se ha demostrado que tanto a través de los productos secretados (principalmente estrógenos y prostaglandinas) o por un efecto físico, el concepto equino regula los genes evaluados en el presente estudio (Zavy et al., 1984; Simmen et al., 1995; Spencer et al., 1995; Surveyor et al., 1995; Johnson et al., 2004; Consiglio et al., 2009; Smolinska et al., 2014; Szóstek et al., 2014; Tsai et al., 2014; Klohonatz et al., 2017). Excepto por el mRNA del RP, todos los genes se expresaron en el endometrio diferencialmente de acuerdo a la preñez ya en el día 7, lo cual es consistente con los cambios histológicos encontrados en las mismas yeguas en este día (Camozzato et al., 2018), pero en desacuerdo con otros trabajos (Merkl et al., 2010; de Ruijter-Villani et al., 2015) que no encuentran cambios en la expresión génica al día 7 y 8. Sin embargo, los autores mencionados anteriormente tomaron muestras sin hacer referencia al lado de la ovulación / cuerpo lúteo. Las muestras de cuerno ipsilateral del presente estudio representan un escenario que garantiza no solo la presencia del embrión al día 7 (Griffin et al., 1993), sino también probablemente un entorno hormonal distinto, como se sugirió en el Artículo I.

Con excepción de los receptores esteroideos, el efecto de la preñez en la localización de elementos evaluados en el Artículo III no reflejó los resultados de los transcriptos (Artículo II). Lo anterior podría explicarse en parte por las diferentes técnicas utilizadas en cada caso: el PCR-RT a tiempo real es una técnica cuantitativa que arroja resultados de todo el tejido, mientras que la inmunohistoquímica es una técnica semicuantitativa que informa sobre el contenido en cada tipo celular. A modo de ejemplo, el aporte del EL en términos de masa de tejido es muy bajo y por ende los cambios en este tipo celular pueden no detectarse con algunas técnicas. De hecho Scaravaggi et al. (2018) reportaron que al día 12 post-ovulación es en el EL donde se encuentran los cambios más importantes en términos de genes diferencialmente expresados al comparar yeguas preñadas vs. no preñadas. Por otro lado, esto depende del momento de la preñez, ya que en rumiantes fue el epitelio glandular el que mostró esta característica durante la pre-implantación (Brooks et al., 2016). Por otra parte, existen efectos post transcripcionales que pueden afectar los niveles de proteínas (Couse et al., 2006). Debe además tenerse en cuenta que se vieron resultados contradictorios en las proteínas localizadas a nivel citoplasmático, cuya evaluación pudo verse influida por las diferencias y fluctuaciones de la altura del epitelio observadas en el grupo preñado (Camozzatto et al., 2019). Por lo tanto, entendemos que sería de mucha utilidad realizar mediciones histomorfológicas simultáneamente y aplicarlas como corrección a la hora de analizar proteínas mediante inmunohistoquímica.

Cambios vinculados a la proliferación y diferenciación endometrial durante el diestro y preñez temprana

Las yeguas preñadas mostraron menores niveles endometriales de transcriptos y proteínas del REα en todos los días estudiados, coincidiendo con los antecedentes en la especie (de Ruijter-Villani et al., 2015). La esteroidogénesis en el embrión equino comienza al día 6 después de la ovulación, con un aumento dramático entre los días 12 y 14 de preñez (Paulo y Tischner, 1985; Zavy et al., 1984). Esto podría explicar no solo la disminución del transcripto al día 7 y la disminución hacia el día 13 en yeguas preñadas sino también el efecto de “knock out” observado en la proteína. El patrón observado para el REα en el grupo cíclico coincide con estudios previos (Watson et al., 1992; McDowell et al., 1999; Aupperle et al., 2000; de Ruijter et al., 2015) y se estima que es el efecto de regulación en menos ejercido por la P4 y su receptor correspondiente (Clark et al., 1992).

Ni el mRNA ni la proteína del RP se vieron afectadas por la preñez al día 7, coincidiendo con estudios previos en la yegua (de Ruijter-Villani et al., 2015). El aumento hacia el día 13 del mRNA de *RP* en el grupo cíclico, coincide con lo reportado para la especie (McDowell et al., 1999; Ruijter-Villani et al., 2015) y posiblemente esté vinculado con la relación E2 / P4 más alta en el día 13 encontrada en el grupo cíclico (Artículo I) y con la correlación positiva entre el mRNA del *RP* y el E2 encontrado en este estudio (Artículo II). Sin embargo, existió una disminución de la intensidad de tinción del RP hacia el día 13 para ambos grupos en las células epiteliales, que se ha observado anteriormente (Watson et al. 1992; Aupperle et al., 2000;

Kalpokas et al., 2010; de Ruijter Villani et al., 2015) y nuevamente expone el desfasaje entre el proceso de traducción y la estimulación esteroidea (Couse et al., 2006). La ausencia de tinción en el epitelio luminal al día 13 para ambos grupos está en línea con estudios previos (de Ruijter-Villani et al., 2015) y con la paradójica regulación negativa del RP, necesaria para la secreción histotrófica uterina (Bazer et al., 2009).

El patrón observado para ambos receptores esteroideos explica los cambios histológicos observados en los dos grupos de yeguas durante el experimento (Camozzato et al., 2019). Las tasas de proliferación celular endometrial disminuyen después del pico de P4 (día 7 post ovulación) (Gestenberg et al., 1999) y esto es un patrón común al diestro y la preñez temprana (Camozzatto, et al., 2019). Además de la disminución del efecto proliferativo del REα, el efecto antiproliferativo de P4 en las células epiteliales se obtiene a través de RP estromal (Hantak et al., 2014), cuya tinción se mantuvo en ambos grupos durante los días evaluados en el presente estudio. Sin embargo, tal como se plantea en rumiantes (Binelli et al., 2014), en yeguas preñadas se observan cambios histológicos vinculados a una anticipada diferenciación celular (pérdida de cilias y aumento de células protrusas en EL y aumento de secreción glandular) (Camozzatto et al., 2019) que se sobreponen al patrón encontrado en diestro. Estos podrían explicarse por efectos progestacionales mediados por progestomedinas y receptores de P4 de membrana descritos en otras especies animales (Fernandes et al., 2005; Gellersen et al., 2009; Keator et al., 2012). En rumiantes, la estimulación estromal del RP es responsable de la secreción de leche uterina a través de la expresión de factores de crecimiento (Spencer et al., 1999; 2002). Nosotros observamos un aumento del mRNA del *FGF9* en el grupo preñado, coincidiendo con antecedentes en yeguas (Klein et al. 2010; Merkl et al. 2010). Encontramos además mayores niveles del transcripto del gen *PAQR5*, que codifica para el receptor de membrana mPRγ (Tang et al., 2005) y que fue el único gen que aumentó los niveles hacia el día 13 de preñez. Cabe mencionar que la 17-alfa-OH-progesterona producida por el concepto entre los días 7 y 14, y agonista de las acciones de la P4 (Goff et al., 1993) activa el receptor de membrana pero no el receptor nuclear (Smith et al., 2008). Paralelamente, los resultados de intensidad de tinción para IGF1 y el RIGF1 (ver debajo) también podrían aportar a los cambios histológicos observados en ambos grupos, ya que estos elementos son ejecutores de la proliferación epitelial inducida por el E2 (Hantak et al., 2014). Asimismo, los mayores niveles de transcriptos para *RAF1* y *PAK6* encontrados en las yeguas preñadas también podrían estar vinculados a la disminución en la proliferación celular de las yeguas preñadas si tenemos en cuenta los resultados de Klohonatz et al. (2019) en donde en muestras endometriales la presencia del embrión se asoció a mayores niveles de estas MFA y menores niveles de proteínas mecano-mediadoras que estimulan la progresión del ciclo celular.

La angiogénesis y la remodelación vascular son procesos muy importantes durante la RMP (Merkl et al., 2010) y existe un aumento de proteínas relacionadas con estas funciones así como del calibre de los vasos sanguíneos en muestras histológicas de yeguas preñadas (Bastos et al., 2019; Camozzato et al., 2019). Lo anterior va en línea con la alta correlación encontrada entre varios genes que presentan propiedades angiogénicas (Artículo II). En nuestro trabajo encontramos en yeguas preñadas una mayor expresión de genes que, en varias especies incluída la equina, han demostrado estar vinculados a la estimulación de la angiogénesis: *FGF9* (Šućurović et al., 2017), *ROXT* (Cattaneo et al., 2009), *PGTS2* (Spencer et al., 2013; Tsujii et al.,

2004), *IGF1* (Kaczmarek et al., 2008; Keller et al., 1998), *OPN* (Du et al., 2009), *RLEP* (Dos Santos et al., 2015). Si bien es controversial el efecto de la adiponectina sobre la angiogénesis en el endometrio (Dos Santos et al., 2015), estudios previos en yeguas mostraron una regulación positiva simultánea de factores anti y pro-angiogénicos en el endometrio gestante al día 12 (Merkl et al., 2010).

Las yeguas preñadas también presentaron mayores niveles de transcriptos vinculados a la estimulación del crecimiento embrionario (*FGF9* -Østrup et al., 2010-, *PTGS2* -Spencer et al., 2013; Tsujii et al., 1998-, *IGF1* -Kaczmarek et al., 2008; Keller et al., 1998- y *RLEP* -Consiglio et al., 2009-). Sin embargo, esta función parece estar ajustada a diferentes etapas del desarrollo del concepto: la disminución observada del mRNA del *IGF1* hacia el día 13 de preñez puede estar asociada a que entre los días 11 y 16, el crecimiento embrionario depende más de la entrada de agua que de la multiplicación celular (Betteridge, 2007). La disminución en los niveles proteicos de IGF1 e IGF2 al día 10 de preñez podría deberse a un secuestro de este factor por parte del embrión, el que expresa proteína transportadora de IGFs tipo 3 (IGFBP3) al día 10 de desarrollo (Herrler et al., 2000). La contradicción entre los niveles de transcripto y proteínas de IGF1 (Artículos II y III) puede explicarse porque los niveles de transcriptos revelan la producción *in-situ* de este péptido, mientras que el análisis de la localización proteica captura diferentes momentos en la dinámica del aporte de diferentes fuentes de IGF1 al endometrio: la materna (sistémica y la producida *in-situ)* y la embrionaria. La fluctuación divergente de la intensidad de tinción de IGF1 / IGF2 (Artículo III), respalda el postulado de que estos factores tienen diferentes funciones (Simmen et al., 1995), siendo el IGF2 más necesario en estadíos más avanzados de la preñez (Pantaleon et al., 2003; Sosa et al., 2010). De hecho, existió un aumento secuencial del mRNA del *IGF2* (pero no del *IGF1*) en el endometrio de yeguas preñadas (del día 7 al 21 de preñez) (Gibson et al., 2015).

Otro elemento vinculado a los requerimientos del desarrollo embrionario equino es la MUC1; el notable aumento en los niveles de mRNA de *MUC1* en el día 7 del preñez evitaría la adherencia temprana del embrión ya que coincide con momento de formación de la cápsula embrionaria equina, que es muy "pegajosa" hacia otras superficies (Betteridge, 2007). Además, es probable que la cápsula esté formada por un aporte de mucina endometrial (Gillies et al., 1999). Si bien los resultados de inmunomarcado de MUC1 del presente trabajo no reflejan un mayor nivel en yeguas preñadas, lo anterior podría ser explicado porque el anticuerpo utilizado en el presente trabajo no se une a la isoforma MUC1/SEC (Wilsher et al., 2013), que está presente en grandes cantidades en las glándulas y fluido uterino durante la fase secretoria del ciclo menstrual (Hey et al., 2003). La disminución del mRNA de *MUC1* hacia el día 13 de preñez y la falta de efecto de status sobre el gen en este día, nos hace especular que una molécula antiadhesiva sería menos necesaria a medida que nos acercamos a la fijación y al final del período de movilidad (día 16) (Betteridge, 2007). Coincidiendo con lo anterior, los mayores niveles de mRNA de *OPN*, *R1ADIPO* y *R2ADIPO* al día 13 pueden deberse a la proximidad de la fijación del embrión: la OPN ancla integrinas del trofoblasto y el endometrio favoreciendo la adhesión en otras especies (Johnson et al., 2014) mientras que tanto R1ADIPO como R2ADIPO se consideran marcadores de éxito en la implantación humana (Dos Santos et al., 2015; Pearson, 2015). Por otro lado, en ovejas, los requerimientos nutricionales del útero aumentan durante la preñez y la presencia del embrión aumenta el flujo de nutrientes hacia el

útero (de Brun, 2019). En nuestro trabajo, las yeguas preñadas presentaron mayores niveles de transcriptos vinculados a la mayor captación de glucosa endometrial (*RIGF1* -da Silva Xavier et al., 2015- y *R1ADIPO* y *R2ADIPO* -Yamauchi et al., 2003-), lo que concuerda con un estudio reciente que muestra mayores niveles de proteínas relacionadas con la producción de energía en el fluído uterino de yeguas preñadas al día 13 (Bastos et al., 2019). De forma consistente se encontraron mayores niveles de *R1ADIPO* y *R2ADIPO* en endometrio bovino de vacas lecheras en mejor estatus energético (Astessiano et al., 2017).

Cambios vinculados al metabolismo de la prostaglandina F2 alfa

Contrariamente a lo hallado para los niveles de transcriptos del *ROXT*, la inmunoexpresión del ROXT en el grupo preñado se vio disminuida, aunque ya ha sido reportado en la yegua resultados de contradicciones similares entre el transcripto y la proteína (de Ruijter-Villani et al., 2015). Si bien la inhibición de la formación de ROXT por inhibición de la expresión de REα está bien descrita en ovinos (Spencer y Bazer, 1995), la menor intensidad de tinción para ROXT se produjo casi exclusivamente en el día 10 de preñez y en los compartimentos superficiales. El día 10 coincide por un lado con el comienzo de la capacidad de respuesta endometrial a la oxcitocina (Goff et al., 1987) y por otro con el inicio de la fase de alta movilidad embrionaria impulsada por las PGE2 y PGF2α secretadas por el concepto (Stout y Allen, 2001). Quizás la diminución del ROXT en este día y en estos tipos celulares sea un mecanismo de prevención de producción excesiva de prostaglandina. La liberación *in vitro* de ambas prostaglandinas por mg de tejido de concepto equino es muy alta en el día 10 después de la ovulación y posteriormente disminuye (Stout y Allen, 2001). La recuperación de la inmunotinción hacia el día 13 en las yeguas preñadas podría estar relacionada con este perfil de secreción de prostaglandinas embrionarias así como con la postulación de que el RMP en la yegua implica un retraso, más que una completa abolición del aumento del ROXT (Stout y Allen 2001; de Ruijter Villani et al., 2015). Además, la luteólisis en la yegua debe involucrar mecanismos alternativos porque no encontramos una regulación positiva del ROXT en el grupo cíclico hacia el día 13. De hecho, Klonohatz et al. (2019) postulan que el embrión en movimiento activa las MFA y una vez activadas las mismas impactan múltiples procesos (Vogel y Sheetz, 2006) incluyendo la prevención de la liberación de PGF2α. En el presente estudio encontramos niveles más altos para el mRNA de *RAF1* y *PAK6* en ambos días estudiados en yeguas preñadas. Estas moléculas además han demostrado ser marcadores importantes de un endometrio receptivo en otras especies (Burghardt et al., 2009; Ponsuksili et al., 2012; Yotova et al., 2012). Por lo que sabemos, este es el primer estudio que describe la regulación positiva de MFA tan pronto como al día 7 de preñez en la yegua, lo que puede indicar que el primer contacto físico con el embrión proporciona una regulación mecánica de la función endometrial, como se demostró en bovinos (Sponchiado et al., 2017).

Así como para el transcripto, también existió mayor inmunotinción de la enzima PTGS2 en las yeguas preñadas. Las diferencias observadas en la intensidad de tinción al día 10 en los compartimentos profundos podrían relacionarse con la contribución endometrial de PGF2α requerida para la contracción localizada miometrial y, por tanto, el inicio de la fase de alta movilidad del embrión (Stout y Allen, 2001). De esta manera, coincidimos con la postulación de

Atli et al. (2010) en que durante el RMP existe en el útero una interacción balanceada entre las enzimas y receptores involucrados en la síntesis de prostaglandinas, ya que simultáneamente encontramos al embrión retrasando el inicio de la cascada luteolítica en los compartimentos superficiales, mientras estimula las capas más profundas para estimular su movimiento. En lo que refiere a la disminución de tinción encontrada entre los días 10 y 13 post-ovulación observada en el EGS y ES en el grupo cíclico, la misma podría explicarse parcialmente por una regulación hormonal esteroidea según lo propuesto por Charpigny et al., (1997) en ovejas. No obstante, es necesario realizar más estudios para comprender completamente la fluctuación de esta enzima, siendo que no encontramos estudios que describieran la localización de PTGS2 endometrial en etapas tempranas del diestro en yeguas.

CONCLUSIONES

- Existe un efecto de la localización del cuerno uterino respecto del ovario que contiene el cuerpo lúteo sobre la expresión endometrial de transcriptos e immunotinción de los receptores esteroideos al día 13 post-ovulación. Estos hallazgos marcan la relevancia en la toma de muestra para estudiar la fisiología endometrial y los efectos provocados por el embrión y convoca a revisar los hallazgos previos que no tomaron en consideración este aspecto.
- La preñez no afectó los niveles circulantes de estradiol, progesterona, IGF-I, leptina o adiponectina, mientras que afectó la expresión endometrial de los receptores de los mismos, sugiriendo que el embrión modifica la sensibilidad endometrial a las hormonas mencionadas. Además, la presencia del embrión afectó genes vinculados a la angiogénesis endometrial, crecimiento y metabolismo embrionario.
- Los cambios histológicos endometriales de la preñez que se superponen a los observados en el diestro están regulados de una manera específica al tipo celular, en algunos casos con efectos opuestos simultáneos que se asocian con procesos relevantes del desarrollo del embrión equino.

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ANEXO I

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Effect of side of the corpus luteum and pregnancy on estrogen and progesterone receptor expression and localization in the endometrium of mares

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ABSTRACT

The effect of side of corpus luteum on uterine gene expression and protein localization of estrogen receptor α (ERα) and progesterone receptor (PR) in healthy cyclic and pregnant mares 13 days after ovulation (day 0) was investigated. Transcervical biopsies were performed to collect endometrium ipsilateral and contralateral regarding the side of corpus luteum on day 13 post-ovulation in cyclic (n = 6) and pregnant (n = 6) mares. Blood samples were collected daily from day 0 until the day of biopsy for 17β-estradiol (E2) and progesterone (P4) determinations. Receptor expression was determined by immunohistochemistry and transcript expression by real time RT-PCR. Serum E2 and P4 concentrations were not affected by reproductive status. The contralateral horn presented higher percentage of positive cells for ERα than the ipsilateral horn ($P < .05$), but side did not affect PR. ERα showed low staining and no main effect of pregnancy was found, but pregnant mares had lower protein expression of PR (19.8 vs. 40.4 ± 5.3%, $P < .01$). The contralateral horn tended to present higher expression of *ERα* mRNA (1.33 vs. 0.97 ± 0.17, $P < .10$) and *PR* mRNA (1.96 vs. 1.57 ± 0.52, $P < .09$). *ERα* mRNA relative expression was lower in the pregnant group (0.88 vs. 1.44 ± 0.19, $P < .05$). The interaction of reproductive status and side of corpus luteum tended to affect *PR* mRNA expression as pregnant mares had a lower *PR* mRNA content in the ipsilateral horn than cyclic mares. To our knowledge, this is the first study that describes the behavior of steroid receptors in the endometrium of mares regarding side of corpus luteum.

1. Introduction

Early pregnancy failure remains a substantial source of economic loss to the equine breeding industry [1]. Pregnancy rates are significantly lower in subfertile than in normal mares at day 14 after ovulation [2], coincidently with the time frame of maternal recognition of pregnancy, when the embryo signals its presence to prevent luteolysis, through a mechanism yet unidentified for this species [1,3–5]. Therefore, a better understanding of the endocrine and molecular mechanisms regulating this critical period is needed. In this regard, it is known that the endometrium responds to ovarian hormones through specific receptors: the estrogen receptor alpha (ERα) and the progesterone receptor (PR) [6]. In mares, the steroid receptor's expression varies in relation to hormone and cell-type [7–12].

During maternal recognition of pregnancy in sheep, luteolysis is prevented through ERα down-regulation, exerted through interferon-t, wich is the embryo signal [13]. In mares down-regulation of ERα protein and mRNA occurs around day 13 of pregnancy [9,14–17]. On day 14 no differences were found in PR staining neither in mRNA concentration when comparing cyclic vs. pregnant mares [9], while less *PR* mRNA levels were found for pregnant mares on day 15 [14]. Besides this discrepancy, it is not yet known how this regulation of nuclear ERα and PR during pregnancy relates to the prevention of luteolysis in the mare [9,18]. Clearly, estrogen -signaling plays an important role during the

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establishment of pregnancy in mares since there is a large number of estrogen-regulated genes or genes involved in estrogen signaling that are either up or down-regulated during early pregnancy [16].

Uterine protein and gene expression has been studied using tissue from a single uterine horn, a pool of tissues from both horns, or making no reference to side of ovulation/corpus luteum is made [7–12,14–20]. However, the following recent findings suggest that the side of ovulation/corpus luteum would have an effect on the functionality of uterine horns. Firstly, although the mare has a systemic pathway where the ovarian artery does not contact the utero-ovarian vein [21], a counter-current exchange was suggested to occur between ovarian venous drainage and the uterine tubal arteria [22]. Secondly, P4 concentrations were greater in the ipsilateral than in the contralateral oviductal fluid and tissue on day 16 [23]. Thirdly, better pregnancy rates have been found when equine embryos were transferred surgically to the ipsilateral uterine horn as compared to those transferred to the contralateral horn, suggesting a local beneficial effect of P4 on the embryo immediately post transfer [24]. Besides, data available on protein and gene expression for ruminants show contradictory results: greater *ERα* and *PR* mRNA concentrations on days 5 and 14 in sheep [25] and greater *PR* mRNA expression on day 7 in cows [26] was recorded in the contralateral horn, while no differences were found also on day 5 in sheep [27] and on days 5 and 6 in cows [28] when comparing both horns. Thus, the aim of the present study was to investigate the effect of side of corpus luteum on uterine gene and protein expression of ERα and PR in healthy cyclic and pregnant mares on day 13 post ovulation.

2. Materials and methods

2.1. Animals and treatments

This experiment was performed during the breeding season (December–March), at the experimental farm number 1 of the Veterinary Faculty, University of Uruguay (34°22'"S, 55°36′W). This study was carried out following a protocol approved by the Animal Ethical Use Committee at the Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (protocol number 23716 from July 17, 2014) and by the Bioethics Committee of the Universidad de la República, Montevideo, Uruguay (CEUAFVET-PI-34/14 -Exp. 111130-001367-14). Criollo Horse crossbreed mares aged from 5 to 10 years, weighting an average of 400 ± 50 kg, from a commercial herd, with no history of fertility problems and proven fertility were used in this study. All mares (n = 10) were cyclic and clinically normal, as indicated by physical and ultrasonographic examination (SonoScape® A6v, 5–7,5 MHz, SonoScape, China), and histopathological classification of endometrial samples (see histological analysis). Mares were kept in natural pasture, with unrestricted access to water.

The estrus cycles were synchronized using two injections of cloprostenol (250 μg i.m.) (Estrumate®, Schering-Plough Animal Health Friesoythe Essex, Germany) with a 14 day interval. After the second injection, mares were teased with a stallion and follicular development was monitored daily by the same operator by transrectal ultrasonography. Once the dominant follicle reached 35 mm in diameter, mares were given 2500 IU of hCG i.m. (Chorulon®, Intervet International BV, Netherlands) and 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 daily examined by ultrasound to detect the ovulation (considered day 0) and side of ovulation/corpus luteum was registered. In the first cycle, intrauterine samples were collected on day 13 and constituted the cyclic group (n = 6). Mares were treated with another dose of cloprostenol (250 μg i.m.) and follicular monitoring and ovulation induction was repeated as for the first cycle.

In the second cycle, the same mares were bred to a fertile stallion 24 h after the second ovulation induction and they were monitored daily to confirm ovulation and to register the side of ovulation/corpus luteum. They were bred one time and if they did not ovulate by 48 h, they were re bred one last time. At day 13, intrauterine samples were collected. Immediately after sample collection mares' uterus were flushed, and those with embryo recovery were assigned to the pregnant group. Of the 10 mares flushed embryos were recovered in 6 mares. Samples from the mares without an embryo were excluded from the experiment. Embryo recovery was performed by flushing the uterus three times sequentially with 1 L of ringer lactate solution at 37 °C in each flush. A homemade embryo flushing catheter with an internal diameter of 15 mm was used.

Endometrial biopsy samples were collected on day 13 post ovulation in cyclic (n = 6) and pregnant (n = 6) mares. In both groups, endometrial samples were taken as transverse sections from the mid-portion of the left and right uterine horns, and they were registered as ipsilateral and contralateral regarding the side of corpus luteum [29]. Samples were divided in halves: one was fixed in 4% paraformaldehyde solution while the other was snap-frozen in liquid nitrogen and stored at −80 °C.

Blood samples from the jugular vein were collected daily from day 0 until the day of biopsy sampling in each cycle. Samples were centrifuged (Rolco®, model 197, Argentina) 10 min at 400 × g and serum was stored at −20 °C.

2.2. Histological analysis

Endometrial samples fixed in 4% paraformaldehyde were embedded in paraffin. Paraffin blocks were serially sectioned at 5 μm and stained with haematoxylin-eosin. Stage of the estrous cycle and pathological status were evaluated following Kenney and Doig [29]. All mares belonged to Class I or IIA (healthy mares).

2.3. Determination of hormone concentrations

E2 and P4 concentrations were measured using radioimmunoassay (RIA) kits (ImmuChem® Double Antibody 17β estradiol 125 RIA Kit, ICN Biomedicals, Inc., CA and PROG-RIA-CT KIP1458; DiaSource InmunoAssays SA, Belgium, respectively). The sensitivity of the E2 assay was 2.6 pg/ml. The intra-assay coefficient of variation (CV) was 10.8% whereas the inter-assay CV was 8.7% (control sample: 5.9 pg/ml). For P4, the sensitivity of the assay was 0.074 ng/mL. The intra-assay CVs were 14%, and 7.5%, whereas the inter-assay CVs were 4.8% and 8% for controls 1 (0.84 ng/ml) and 2 (2.65 ng/ml), respectively.

2.4. ERα and PR protein localization and transcript expression

Avidin-biotin-peroxidase immunohistochemical technique was used to visualize immunostaining [8]. Slides were photographed with an electronic microscope (Nikon BM 2000®) using a software (Micrometrics SE Premium®) and evaluated by an image processing software (FIJI®). Staining of estrogen and progesterone receptors was evaluated in five uterine compartments defined by cell type and location: luminal epithelium (LE), superficial and deep glandular epithelium (SGE and DGE), and superficial and deep stroma (SS and DS). Fifteen fields were analyzed for each cell type at a magnification of 1000 ×. The extent of staining was scored according to Boos et al. [30] and Thatcher et al. [31].

RNA isolation and reverse transcription were performed

following the method described by de Brun et al. [27]. Primers to amplify cDNA of the target genes *ERα*, *PR* and of the endogenous controls *β-actin*, *HPRT* and *GAPDH* were used (Table 1). Real time PCR reactions were performed following the method described by de Brun et al. [27]. For relative quantification, target gene expression was normalized to the mean expression of the endogenous control genes [37] (Table 1) which remained unchanged among samples in this study.

2.5. Statistical analysis

All variables were subjected to analysis of variance using a mixed model (Statistical Analysis System, NC, USA). Residuals derived from all linear regression models were normally distributed. For hormone concentration the model included the reproductive status (pregnant or cyclic), the day after ovulation and their interaction as fixed effects. The variable studied in the analysis of receptor localization by immunohistochemistry was the proportion of total positive cells of the 15 fields. The statistical model included the effects of reproductive status, side of corpus luteum (ipsi or contralateral), cell type and location (LE, SGE, DGE, SS and DS) and their interactions. For relative quantification of target gene expression statistical model included the fixed effects of reproductive status and side of corpus luteum, and their interaction, and PCR plate as a random effect. Data are presented as least square means ± pooled standard errors. Pearson correlation tests were performed to describe the relationships among variables. Means were considered different when $P < .05$, and with tendency to differ when $0.05 < P \leq .10$.

3. Results

Results of the analysis of variance for steroid receptor levels and transcript expression are shown in Table 2.

3.1. Hormone concentrations

Serum E2 and P4 levels are shown in Fig. 1. The concentrations of E2 and P4 were affected by day after ovulation ($P < .02$ and $P < .001$, respectively), but were not affected by reproductive status (E2: $P = .41$; P4: $P = .54$) neither by the interaction (E2: $P = .61$; P4: $P = .98$). The highest E2 concentrations were detected on days 0 and 1 (D0-1 vs. D5: $P < .05$; D0-1 vs. D9: $P < .01$; D0-1 vs. D13: $P < .1$), while P4 concentrations increased slowly reaching a peak between days 5 and 7 (D0 vs. D5-7: $P < .001$), with a plateau until day 10, when the concentration started to decrease until day 13 in both pregnant and cyclic groups (D10 vs. D13: $P < .01$).

3.2. ERα and PR protein localization and transcript expression

Immunoreactive ERα and PR were detected exclusively in the nuclei of all endometrial cell types. When specific monoclonal antibodies were substituted with a non-immune mouse IgG, the absence of staining confirmed the high specificity of immunostaining for both receptors (Fig. 2). There was no correlation between ERα and PR expression.

Very low staining of ERα was found in most cell types. While the main effect of side respect ovulation was significant as the contralateral horn presented more staining for ERα than the ipsilateral horn ($P < .05$), the reproductive status (cyclic vs. pregnant mares) did not affect the immune-reactivity of this receptor (Table 2). In LE, SGE and DGE only few positive cells were found, as most samples presented no positive staining. The stroma was the cell type more stained (Fig. 3). Positive ERα staining tended to be lower in the superficial and deep stroma of the ipsilateral horn of pregnant mares than in the contralateral ($P < .1$) (Fig. 2). A similar finding was observed in cyclic mares in deep stroma, as the ipsilateral horn presented lower ERα contents ($P < .05$). Moreover, half of the samples of both uterine horns in cyclic and pregnant mares showed scarce infiltrated immune cells (mononuclear and polymorphonuclear cells) stained positive for ERα (data not shown).

Side of corpus luteum did not affect the expression of PR ($P = .34$), but there was an effect of pregnancy ($P < .01$), cell type ($P < .01$) and location ($P < .05$) (Table 2). Pregnant mares had lower expression of PR (19.8 ± 5.3 vs. $40.4 \pm 5.3\%$, $P<.01$) (Fig. 2). Including both uterine horns, pregnant mares had lower PR contents in DGE (23.5 ± 8.9 vs. $50.4 \pm 8.9\%$, $P<.05$) and LE (0.2 ± 7.9 vs. $20.5 \pm 7.9\%$, $P < .05$), but no differences were detected in SS (33.1 ± 7.5 vs. 44.2 ± 7.5; $P = .29$) and SGE (15.9 ± 9.1 vs. 30.4 ± 9.1; $P = .23$). Least square differences of the means showed that pregnant mares had or tended to have lower PR content in LE (data not shown) and DS of the ipsilateral horn ($P < .10$, Fig. 4). Likewise, pregnant mares had lower PR content in DGE of the contralateral horn ($P < .05$).

Side of corpus luteum tended to affect *ERα* mRNA expression as the contralateral horn tended to have greater expression than the ipsilateral horn (1.33 vs. 0.97 ± 0.17, $P < .10$) (Table 2). The mRNA relative expression of *ERα* was affected by status, with cyclic mares presenting more transcript abundance than pregnant mares (1.44 vs. 0.88 ± 0.19, $P < .05$). The ipsilateral horn of pregnant mares presented a lower *ERα* mRNA expression ($P < .05$), but no differences among horns were observed in cyclic mares ($P = .47$) (Fig. 5). In the ipsilateral horn, *ERα* mRNA concentrations were lower in pregnant mares than in cyclic mares ($P < .05$), and no differences were observed in the contralateral one ($P = .22$) (Fig. 5).

Side of corpus luteum tended to affect *PR* mRNA expression, as it tended to be greater in the contralateral horn (1.96 vs. 1.57 ± 0.52, $P = .08$). While no main effect of pregnancy was observed ($P = .39$) a trend for an interaction among side and status was found ($P < .1$)

Table 1
Primer sequences and references used for real-time reverse transcription-polymerase chain reaction assays. (*ERα*, estrogen receptor; *PR*, progesterone receptor; *β-ACTIN*, Beta-actin; *HPRT*, Hypoxanthine-guanine phosphoribosyltransferase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase). Efficiency: reported values of reaction efficiency.

Gene	Primer sequences	Efficiency	Reference
ERα	Forward: GGGGAGGGCAGGAATGAAGT Reverse: GGGACAAGGCTGGGCTGTT	0.99	Colitz et al. (2009) [32]
PR	Forward: TGTGCTGGAAGAAACGATTGC Reverse: GACAGGACTTTCTAAGGCGACAT	1.09	Sosa et al. (2009) [33]
β-ACTIN	Forward: GCGTGGCTACAGCTTCACC Reverse: TTGATGTCACGGACGATTTC	1.00	Bionaz and Loor (2007) [34]
HPRT	Forward: TGGAGAAGGTGTTTATTCCTCATG Reverse: CACAGAGGGCCACAATGTGA	0.97	Carriquiry et al. (2009) [35]
GAPDH	Forward: GCATCGTGGAGGGACTTATGA Reverse: GGGCCATCCACAGTCTTCTG	0.89	Stabel et al. (2009) [36]

Table 2
Level of significance of fixed effects and interactions studied in the statistical model. Fixed effects shown for serum17β-estradiol (E2) and progesterone (P4) are day of the extraction, status and their interaction. For estrogen and progesterone receptor (ERα and PR) fixed effects are status, side of corpus luteum (SCL), their interactions, cell type (luminal epithelium, glandular epithelium, stroma) and location (superficial or deep). For estrogen and progesterone transcripts (*ERα* mRNA; *PR* mRNA) fixed effects are status, SCL and their interaction.

	Day	Status	Day x Status	Side of corpus luteum (SCL)	Status x SCL	Cell type	Location
E2	*	NS	NS				
P4	***	NS	NS				
ERα		NS		*	NS	NS	*
PR		**		NS	NS	**	*
ERα mRNA		*		T	NS		
PR mRNA		NS		T	T		

NS: not significant, T: .05 < p ≤ .10, *p < .05, **p < .01, ***p < .001.

(Table 2). Lower *PR* mRNA concentrations were found in the ipsilateral than in the contralateral horn of pregnant mares (P < .05), but no differences were observed in cyclic mares (P = .84). In the ipsilateral horn, a tendency for a greater *PR* mRNA expression was found in cyclic mares when compared to pregnant mares (P < .1) (Fig. 5).

There was a significant positive correlation between *ERα* and *PR* relative expression (r = 0.75, P < .0001).

4. Discussion

As far as we know, this is the first study that showed an effect of side of corpus luteum and pregnancy on protein and gene expression of ERα and PR in the endometrium of healthy mares around the time of maternal recognition of pregnancy (day 13 post ovulation). Our study was conducted on a representative sample allowing for statistical analysis and using the minimum animals necessary.

Serum E2 and P4 concentrations were not affected by reproductive status, and peak values for both hormones as well as the decrease of P4 concentration from day 10 until day 13 in both pregnant and cyclic mares, in agreement with previous results [38–41]. Such decrease could be related to a weak luteotrophic signal in early pregnant mares [39,40] or to the embryo rescuing the corpus lutheum from an already triggered luteolysis [42]. Regarding E2, despite the large quantities of estrogens produced by the early embryo, our results seem to reinforce the concept that they become systemic after the vitelline blood circulation gets established [43].

Side of the corpus luteum affected both receptors; there was a reduction of ERα content and its mRNA, as well as a reduction of *PR* mRNA expression in the ipsilateral horn. This could be related to the local distribution of ovarian products to the ipsilateral horn by exchange between the ovarian venous drainage and the uterine tubal arteria [22,23] leading to an increased P4 concentration on day 13 in the ipsilateral horn. As proposed by Takahashi et al. [28], the dynamics of the endometrial tissue concentrations for P4 on days 5–6 may be different from those of the serum concentrations. Indeed, P4 down-regulates *ERα* and *PR* mRNA expression in sheep ipsilateral oviduct on day 5 [27]. Similar results analyzing uterine horns showed that both *ERα* and *PR* mRNA expression were lower in the ipsilateral uterine horn of sheep (day 5 and 14) and cows (day 7) [25,26]. Changes in the sensitivity of the endometrium to steroid hormones are critical for the regulation of uterine function. In ruminants, following a determined period of exposure to P4, endometrial PR downregulation is key to allow luteal regression mechanism through oxytocin receptors (OTR) [42]. It is also clear that both P4 and E2, acting via both genomic and non-genomic pathways [44], are central to the regulation of the endometrial responsiveness to OTR [45]. Regarding the mare, although the luteolytic mechanism is scarcely identified, regulation of OTR and prostaglandin-endoperoxide synthase 2 (PTGS2) between days 7 and 14 of the diestrus appear to contribute to it [9]. Prolonged exposure of the endometrium to P4 may decrease oxytocin activity through desensitization of OTR [18], although PTGS2 expression at day 11 of pregnancy is not affected by a progestin treatment [11]. Changes in expression of the reproductive steroid hormone receptors between days 7 and 14 of the diestrus/pregnancy are not sufficient per se to determine how they influence OTR and PTGS2 and the development of the luteolytic cascade during diestrus [9]. Hence, further studies should include OTR and PTGS2, along with receptors studied herein.As highlighted before, no studies regarding side of ovulation or corpus luteum on endometrial

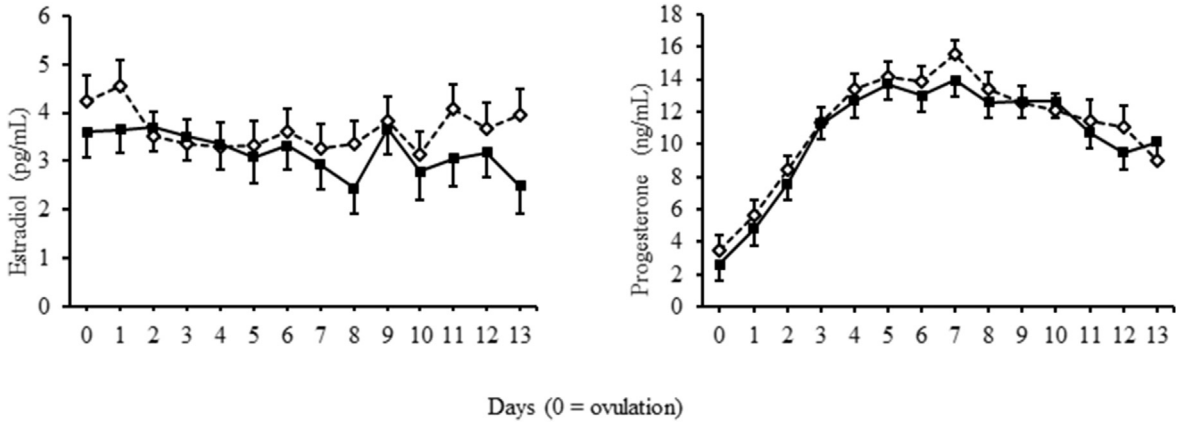

Fig. 1. Serum estradiol (right plot) and progesterone (left plot) concentrations of cyclic (white rhombus) and pregnant (black squares) mares along 13 days after ovulation. Mean (±standard error of the mean) hormone concentration (y axis) is shown for each day (x axis).

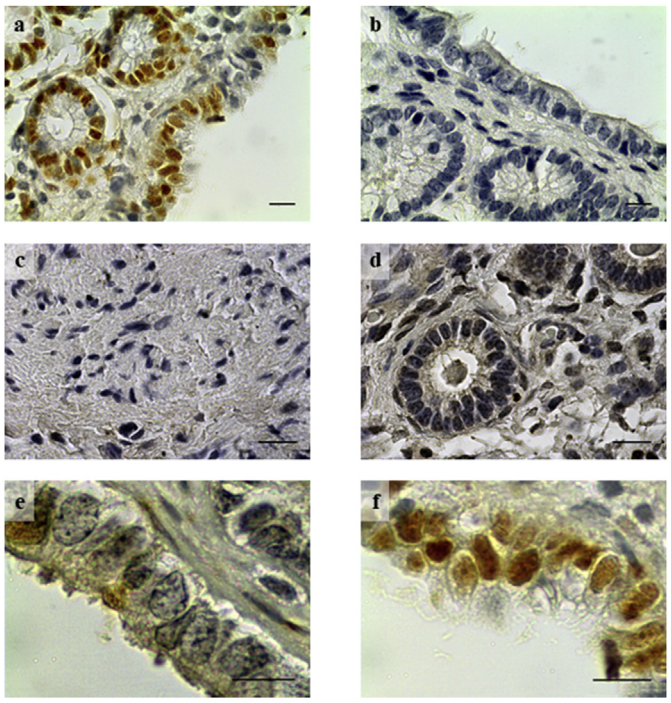

Fig. 2. Photographs of immunohistochemical localization of steroid receptors (a), negative control (b) and representative endometrial cross sections of de ipsilateral and contralateral horn of the pregnant group for estrogen receptor (c; d) and the ipsilateral horn of the cyclic and pregnant group for progesterone receptor (e; f). Original magnification x 1000. Scale bars= 10 μm.

receptors expression in the mare were found. A differential sensitivity of the reproductive tract to steroids may result in a distinct environment for the embryo through different growth factors concentrations for instance, as these are mainly regulated by steroids [46]. Along with results showing better pregnancy rates of equine embryos transferred to the ipsilateral uterine horn [24], our results raise the question of a possible difference in functionality of uterine horns.

Moreover, in the present study, side of corpus luteum did not affect the expression of PR protein, so PR immunoexpression in the endometrium is more likely to reflect the concentrations of P4 and E2 in peripheral serum (Fig. 1), rather than an effect of ipsilateral hormonal milieu. In fact, *PR* mRNA content presents the opposite profile to PR protein, as determined by binding assays and immunohistochemistry for the first 24 h [47], reinforcing the idea that gene transcription is a process not likely to provide measurable effects within the cell until hours or even days after steroid stimulation [6].

We found an effect of pregnancy on *ERα* mRNA expression, such as the one reported in previous studies [14–17]. A decrease of ERα on the days of maternal recognition of pregnancy has already been suggested to be part of the regulation of such complex mechanism in the mare [16] as described in ruminants [13]. Moreover, decreased *ERα* mRNA on day 15 of pregnancy and subsequent decreased expression of OTR was suggested for the mare [14], as opposed to results obtained on day 14 of pregnancy [9]. In face of the large quantities of estrogens secreted by the equine conceptus as early as day 10 after ovulation [48] and the observation that estrogens can negatively regulate the expression of its own receptor in uterine tissue [49], the conceptus-derived estrogens are the most likely cause of the observed down-regulation of ERα in pregnant mares at day 13,5 [16]. We consider further investigation is necessary to fully understand this mechanism, including sampling before day 10 of the cycle/pregnancy.

Furthermore, there was an effect of the reproductive status on PR: less PR staining and a trend for lower *PR* mRNA expression in the pregnant group. The development of the endometrium and its capacity to secrete essential nutrients, growth factors and enzymes for correct embryo development are largely controlled by P4 but paradoxically, the down-regulation of PR is a prerequisite to achieve this capacity [50]. In the present study, immunoexpression of PR in pregnant mares remained low or absent in the LE, in accordance with previous reports in mares and ruminants and in line with a possible local effect exerted by the embryo considering LE is the cell type most intimately related to the conceptus [13,20,33]. In fact, this could also be attributed to conceptus-derived estrogens; the down regulation of PR by E2 has been scarcely reported, but differences in E2 regulation of PR may depend on the species, tissues and/or the cell type [47].

Nuclear expression of ERα was scarce, as described in previous studies [8,9,15,16,19]. This receptor was greatly confined to stromal cells, in line with previous findings [7]. Nuclear expression of PR was high in the stroma and deep locations, wich does not agree

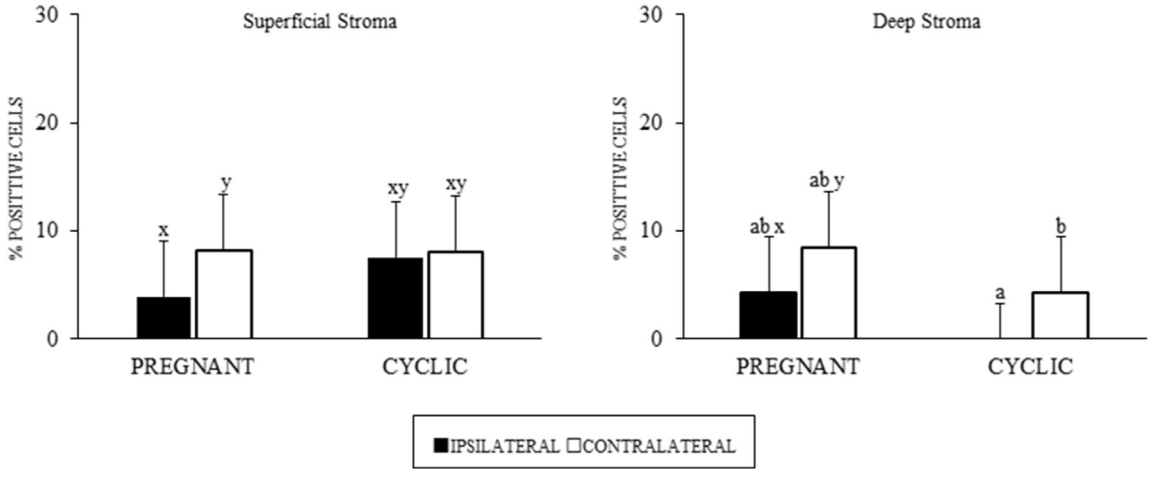

Fig. 3. Percentage of immunostained cells for estrogen receptor α (ERα) in pregnant and cyclic mares on the ipsilateral (black bars) and contralateral (white bars) horns on day 13 post ovulation in superficial (left) and deep (right) stroma. Data are the least square mean ± pooled standard error of the mean. (a,b) different superscripts within the same graph differ: $p < .05$. (x,y) different superscripts within the same graph tend to differ: $0.05 < p \leq .10$.

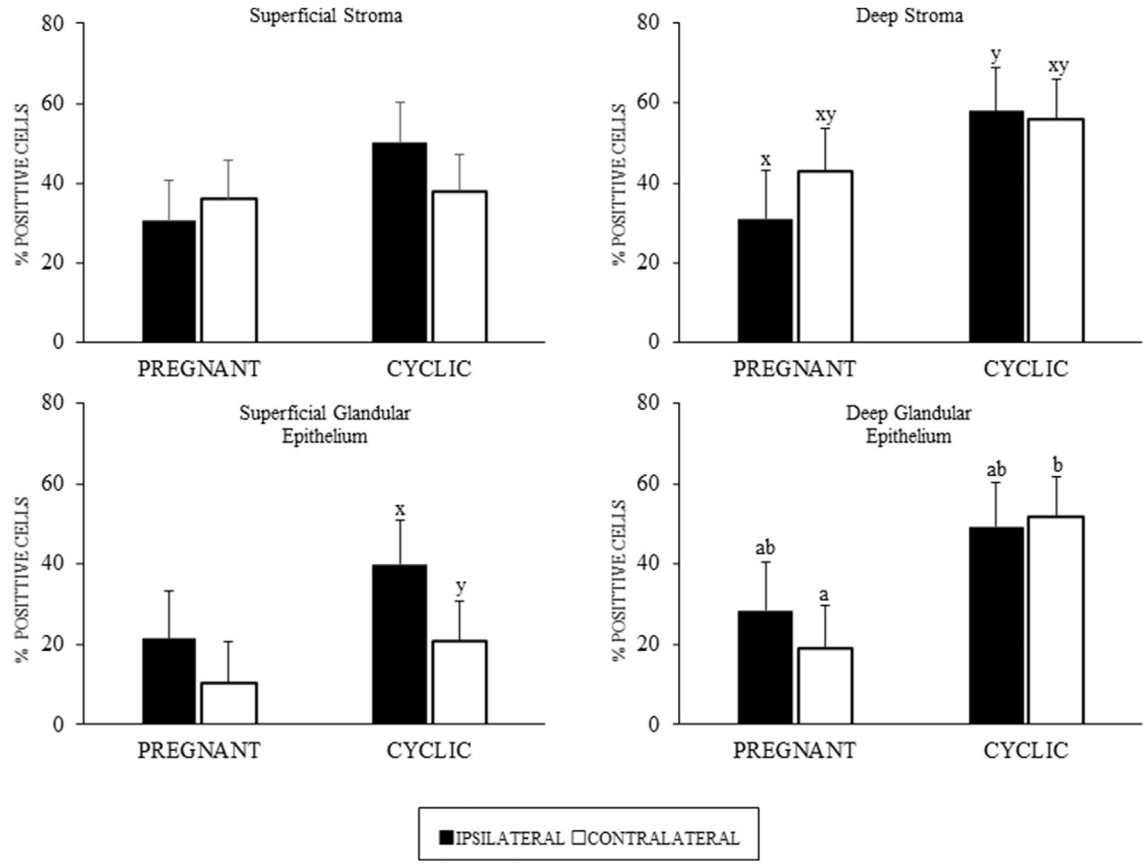

Fig. 4. Percentage of immunostained cells for progesterone receptor (PR) in pregnant and cyclic mares on the ipsilateral (black bars) and contralateral (white bars) horns on day 13 post ovulation in superficial stroma (upper left), deep stroma (upper right), superficial glandular epithelium (lower left) and deep glandular epithelium (lower right). Data are the least square mean ± pooledstandard error of the mean. (a,b) different superscripts within the same graph differ: $p < .05$. (x,y) different superscripts within the same graph tend to differ: $0.05 < p \leq .10$.

with PR being largely confined to the glandular epithelium in mares on day 14 of the cycle/pregnancy [9]. Meanwhile, the lowest staining for PR was observed in LE, wich goes in line with previous findings [9]. The effect seen in cell type and location for both steroid receptors reinforces the concept that their expression throughout the estrus cycle and pregnancy is cell-type dependent and that the endometrial sensitivity for steroid hormones seems to be different between stromal and epithelial cells [7–9,18–20].

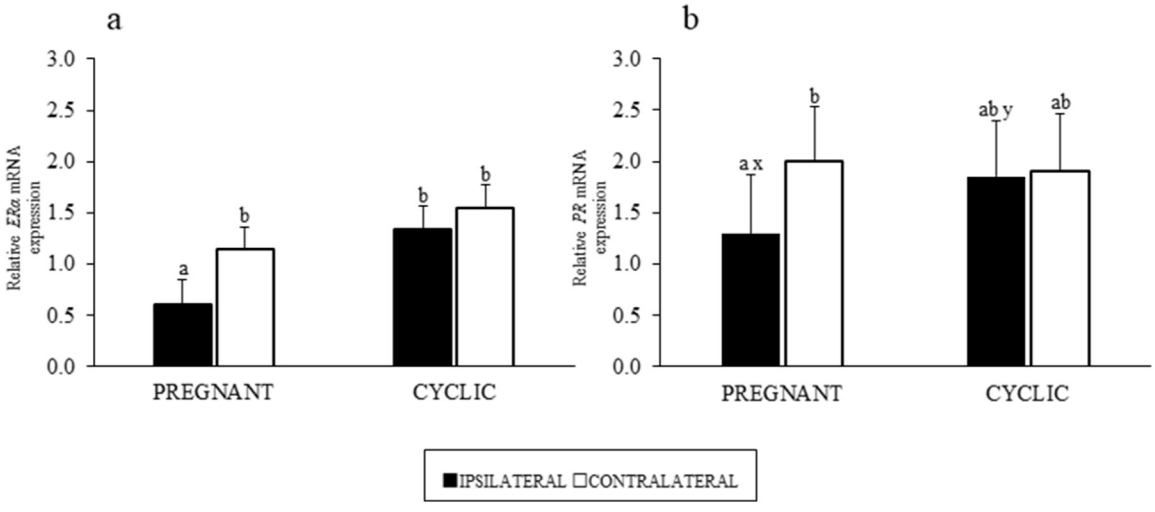

Fig. 5. Relative expression of *ERα* (a) and *PR* (b) transcripts in ipsilateral (black bars) and contralateral (white bars) horns of pregnant and cyclic mares. Data are presented as least square means ± standard errors. (a,b) different superscripts within the same graph differ: $p < .05$. (x,y) different superscripts within the same graph tend to differ: $0.05 < p \leq .10$.

In conclusion, ERα protein and transcript expression, as well as *PR* mRNA in pregnant mares was reduced in the ipsilateral horn as compared to the contralateral horn. Additionally, an effect of reproductive status on PR protein localization in different cell types on day 13 of pregnancy was found, suggesting that this could be the consequence of a differential hormonal endometrial milieu depending on the side of corpus luteum or an embryo-derived product.

Conflicts of interest

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

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ANEXO II

REVISED

Ipsilateral endometrial gene expression and endocrine profiles of early gestation-related components in the mare

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Abstract

The effect of embryo presence on transcript expression in the endometrium was investigated during early pregnancy in the mare. We analysed the endometrial expression of estrogen receptor α (*ERα*), progesterone receptor (*PR*), progestin and adipoQ receptor family member V, oxytocin receptor, prostaglandin-endoperoxide synthase 2, fibroblast growth factor family member 9, insulin-like growth factor-1 (*IGF1*), type 1 IGF receptor, mucin 1, osteopontin, leptin receptor, adiponectin receptors 1 and 2, raf-1 proto-oncogene, serine/threonine kinase and serine/threonine protein kinase 6/p21-activated kinase 6. Transcervical biopsies of the ipsilateral horn (regarding side of corpus luteum) were performed on days 7 and 13 post-ovulation in cyclic (*n* = 6 on each day) and pregnant (*n* = 6 on each day) mares. Blood samples were collected daily from day 0 until day 13 for IGF1, leptin and adiponectin determinations. Endometrial gene expression was determined by real time RT-PCR. Pregnancy did not affect any circulating hormone concentration. Pregnant mares showed lower *ERα* and *PR* mRNA transcript expression than the cyclic group, while the remaining genes showed an upregulation. Pregnancy affected all genes on day 7, with the exception of *PR* mRNA. Fluctuations in gene expression of the pregnant group could be explained by stage-specific requirements and/or characteristics of the equine embryo development, along with overlapping roles of elements studied herein. Our results suggest that endometrial transcript changes are related to conceptus physical contact and/or derived factors acting locally, even before maternal recognition of pregnancy in mares.

Keywords
Mare; Pregnancy; Endocrine; Endometrium; Ipsilateral; Genes

1. Introduction

Successful gestation depends on preventing lysis of the corpus luteum (CL) by the conceptus, an event known as the maternal recognition of pregnancy (MRP) [1]. A broader definition includes the sum of events resulting in a precisely orchestrated interaction between the conceptus and the uterine environment, leading to pregnancy preparation and maintenance; it is a time-specific interaction, solving embryonic development requirements at each stage [2,3]. In this regard, the early equine conceptus shows unique morphological and physiological features, such as the formation of an acellular capsule and an intense mobility phase until fixation (day 16) [4,5]. The above mentioned singularities not only assure the maintenance of pregnancy in mares, but should be considered when evaluating uterine molecular markers at the different stages of gestation.

Estradiol (E2) and progesterone (P4) are central to the regulation of many key events related to MRP at the endometrium level, and they act through their specific receptors, mainly the estrogen receptor alpha (ERα) and the progesterone receptor (PR), which vary during estrous cycle and pregnancy [6]. Around the days of prevention of luteolysis in pregnant mares, there is a down-regulation of ERα [7–10]. However, as to PR, there is no such compliance [7,10]. Besides, P4 can act via the progestin and adipoQ receptor family member V (PAQR5), which is upregulated during pregnancy in the mare [11]. As for ruminants, a differential regulation of oxytocin receptors (OXTR) and prostaglandin-endoperoxide synthase 2 (PTGS2) has been associated with equine luteolysis [7,12,13]. Nevertheless, the regulation of these elements is still debated [7,11,14,15], and studies in the mare suggest overlapping functions of both OXTR and PTGS2, which are beneficial for pregnancy [16–18].

Steroid hormones also regulate other elements; fibroblast growth factor family member 9 (FGF9), insulin growth factor 1 (IGF1) and type 1 IGF receptor (IGF1R) are mediators of estrogen-signalling with potential roles in endometrial remodeling and angiogenesis as in embryo growth in several species [9,11,19–23]. However, regarding the mare, there is scarce information available to conclude a pattern for the regulation of these factors in the endometrium during pregnancy [24,25] and for IGF1 plasma levels [26]. Ovarian steroids also regulate mucin 1 (MUC1) and osteopontin (OPN), which are potential mediators of accurate conceptus attachment [27,28]. There is also an endometrial contribution of MUC1 to the embryonic capsule formation [27], and OPN is an important histotroph component with pro-angiogenic and embryo development stimulatory effects in other species [29–31]. Despite this, only few reports in the mare show divergent and/or inconclusive results regarding OPN and MUC1 gene and protein expression and localization when comparing pregnant vs. cyclic endometrium [27,28,32,33].

Hormones released from adipose tissue, which were firstly associated with regulation of energy metabolism, are also implicated in reproductive processes in several species [34]. Leptin stimulates angiogenesis and acts as a mitogen in the human endometrium [35]. Leptin receptor (LEPR) transcript and protein are present in equine preimplantation embryos and increase with embryo development [36], but we have not found reports regarding endometrial LEPR expression according to pregnancy. Adiponectin also plays an important role in preimplantation embryo development and uterine receptivity by autocrine and paracrine pathways in other species [37,38], while the gene expression of adiponectin receptors 1 (ADIPOR1) and 2 (ADIPOR2) increases after 16 days of pregnancy, compared to cyclic mares [39]. The interaction of sex hormones and these adipokines is being explored in horses (for review, see [34]), and scarce information regarding early-pregnancy endocrine profiles in the mare shows they are neither affected by early pregnancy nor by day [39–41].

Recently, it has been suggested that the moving embryo causes molecular effects through the focal adhesion pathway, leading to MRP [42–44]. Focal adhesion molecules (FAMs) are

macromolecular complexes which compose the interface between cells and the extracellular matrix (ECM) [45]; ECM interacts with membrane-associated complexes that control many functions related to cell growth, development and proliferation [46]. In the mare, from all FAMs analysed, Raf-1 proto-oncogene, serine/threonine kinase (*RAF1*) and serine/threonine protein kinase 6/p21-activated kinase 6 (*PAK6*) genes are differentially expressed in the endometrium on days 9, 11 and 13 of pregnancy [42,47].

Understanding the embryo-maternal crosstalk will not only bring us closer to identifying the MRP signal, but has the potential to expand our clinical capacity in early pregnancy management [48]. Moreover, a methodological aspect should be addressed because endometrial gene expression has been studied using a pool of tissues from both horns, or making no reference to side of ovulation/corpus luteum [7–10,12,13,42,49–52]. However, recent findings in the mare revealed an effect of side of CL on endometrial transcript levels, probably related to a differential hormonal endometrial milieu and/or an embryo-derived product [53]. Therefore, the aim of the present study was to analyse the effect of pregnancy on ipsilateral endometrial expression of selected candidate genes related to gestation and luteolysis at days 7 and 13 post-ovulation, as well as on IGF1, leptin and adiponectin circulating concentrations in mares.

2. Materials and methods

2.1. Animals and treatments

This experiment was performed during the breeding season (December-March), at the experimental farm number 1 of the Veterinary Faculty, University of Uruguay (34°22'"S, 55º36'W). This study was carried out following a protocol approved by the Animal Ethical Use Committee at the Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (protocol number 23716 from July 17, 2014) and by the Bioethics Committee of the Veterinary Faculty, Montevideo, Uruguay (CEUAFVET-PI-34/14 -Exp. 111130-001367-14). Criollo Horse crossbreed mares from a commercial herd were used in this study. They were from 5 to 10 years old, weighed an average of 400 ± 50 kg, and had proven fertility with no history of fertility problems. All mares (*n* = 20) were cyclic and clinically normal, as indicated by physical and ultrasonographic examination (SonoScape® A6v, 5-7.5 MHz, SonoScape, China), and histopathological classification of endometrial samples (see histological analysis). Mares were kept in natural pasture, with unrestricted access to water.

The estrous cycles of 20 mares were synchronized and groups were managed following Kalpokas et al. [53]. In the first cycle (cyclic group), ten mares were sampled on day 7 post ovulation and the remaining ten mares were sampled on day 13 post ovulation. In the second cycle, the same 20 mares were bred to a fertile stallion and constituted the putative pregnant group. Again, ten mares were sampled on day 7 post ovulation and the remaining ten mares were sampled on day 13 post ovulation. Immediately after sample collection, mares' uterus were flushed, and those with embryo recovery were assigned to the pregnant group. Out of the 20 mares, flushed embryos were recovered in 12 mares, 6 at each day of collection. Samples from the mares without an embryo were excluded from the experiment (*n* = 8). Thus, we adjusted the sample size in the cyclic group and the final sample sizes were: cyclic group day 7 post-ovulation *n* = 6, day 13 post ovulation *n* = 6 and pregnant group day 7 post-ovulation *n* = 6, day 13 post ovulation *n* = 6. Embryo recovery was performed flushing the uterus three times sequentially with 1 L of ringer lactate solution at 37º C in each flush. Embryos recovered on day 7 consisted in expanded blastocysts with a visible capsule. A

homemade embryo flushing catheter with an internal diameter of 15 mm was used for collecting embryos on day 13.

Endometrial biopsy samples were taken as transverse sections from the mid-portion of both uterine horns, but only the ipsilateral sample was used in the present study [54]. Samples were divided in halves: one was fixed in 4% paraformaldehyde solution while the other was snap-frozen in liquid nitrogen and stored at -80 °C. Samples were taken on specific days before the maternal recognition of pregnancy day limit (day 14 of pregnancy) (for review, see [55]): at day 7 the embryo has recently arrived to the uterus and the capsule is formed at this stage, while at day 13 the embryo has already started its mobile phase but has not fixated yet [56].

Blood samples from the jugular vein were collected daily from day 0 until the day of biopsy sampling in each cycle in all mares (n= 20). Hormone determinations were performed only in mares that remained in the experimental design (cyclic and pregnant as described before, *n* = 12). Samples were centrifuged (Rolco®, model 197, Argentina) 10 minutes at 400 x g and serum was stored at -20 º C.

For histological analysis, endometrial samples fixed in 4% paraformaldehyde were embedded in paraffin, paraffin blocks were serially sectioned at 5 µm and stained with haematoxylin-eosin. Stage of the estrous cycle and pathological status were evaluated following Kenney and Doig [54]. All mares belonged to category I or IIA (healthy mares).

2.2. Determination of hormone concentrations

Estradiol and progesterone determinations are described in Kalpokas et al. [53]. Determinations of IGF1 concentrations were performed using the RIA kit Cisbio bioassays®, Codolet, France, with a limit of detection of 0.57 ng/mL. The intra-assay CVs were 6.9 % and 4.1% for controls 1 (51 ng/mL) and 2 (367 ng/mL). For leptin, Linco®, Millipore kit was used, with a detection limit of 1.2 ng/mL and the intra-assay CVs were 7.5 % and 11.4% for controls 1 (3.5 ng/mL) and 2 (9.6 ng/mL). For adiponectin, HADP-61 HK, Linco®, Millipore kit was used, with a detection limit of 0.59 ng/mL.The intra-assay CVs were 8.8% and 18.6%, whereas the inter-assay CVs were 13.1% and 14.2% for controls 1 (8.9 ng/mL) and 2 (75.9 ng/mL) respectively.

2.3. RNA isolation and reverse transcription

RNA isolation and reverse transcription were performed following Kalpokas et al. [53]. Primers used to amplify cDNA of the target genes *ERα*, *PR*, *PAQR5*, *OXTR*, *PTGS2*, *FGF9*, *IGF1*, *IGF1R*, *MUC1*, *OPN*, *LEPR*, *ADIPOR1*, *ADIPOR2*, *RAF1* and *PAK6,* and of the endogenous controls *β-actin*, *HPRT* and *GAPDH* are shown in Table 1. Real-time PCR reactions were performed following de Brun et al. [57]. For relative quantification, target gene expression was normalized to the mean expression of the endogenous control genes [58] (Table 1), which remained unchanged among groups and days in this study.

Table 1. Primer sequences and references used for real-time reverse-transcription polymerase chain reaction assays. *ERα*, estrogen receptor α; *PR*, progesterone receptor; *PAQR5*, progestin and adipoQ receptor family member V; *OXTR*, oxytocin receptor; *PTGS2*, prostaglandin-endoperoxide synthase 2; *FGF9*, fibroblast growth factor family member 9; *IGF1*; insulin-like growth factor 1; *IGF1R*, type 1 IGF receptor; *MUC1*, mucin 1; *OPN*, osteopontin; *LEPR*, leptin receptor; *ADIPOR1*, *ADIPOR2*, adiponectin receptors 1 and 2; *RAF1*, raf-1 proto-oncogene, serine/threonine kinase; *PAK6*, serine/threonine protein kinase 6/p21-activated kinase 6; *β-ACTIN*, Beta-actin; *HPRT*, Hypoxanthine-guanine phosphoribosyltransferase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase. Efficiency: reported values of reaction efficiency.

Gene	Primer sequence - forward	Primer sequence - reverse	Efficiency	Reference
ERα	GGGGAGGGCAGGAATGAAGT	AGGGACAAGGCTGGGCTGTT	0.99	[59]
PR	TGTGCTGGAAGAAACGATTGC	GACAGGACTTTCTAAGGCGACAT	1.09	[60]
PAQR5	CGCACGTGCAGATGGAAGCCATA	CCGAGGCTGAAGACAAGGCACA	1.09	[11]
OXTR	TGCAGATGTGGAGCGTCTGGGA	TGGAAGAGGTGGCCCGTGAACA	1.04	[11]
PTGS2	GAGGTGTATCCGCCCACAGT	AGCAAACCGCAGGTGCTC	0.88	[12]
FGF9	ACGTCAGCTCCACTGTTGCCAAA	AAGCAAGTGGGCACAGGCAGT	1.04	[11]
IGF1	TGCTTCCGGAGCTGTGATCT	CCGACTTGGCAGGCTTGA	0.84	[12]
IGF1R	TCCAGACAGGAGTACAGG AA	AGAAGAACACGGGATCCGTC CA	0.96	[61]
MUC1	TGCTGGTCTGTGTTCTGGTC	TCCAGCTGCCCATAGTTCTT	0.83	[33]
OPN	CCA GTT AAT CAG GCC GAC TCT	TGG GCA CAG CTG GTG TAA AA	0.94	[62]
LEPR	TGCTTTTGACTCCAGATCTT	CAGGCCTTCTGAGAATGGAA	1.01	[63]
ADIPOR1	GGCTCTACTACTCCTTCTAC	ACACCCCTGCTCTTGTCTG	1.04	[64]
ADIPOR2	GGCAACATCTGGACACATC	CTGGAGACCCCTTCTGAG	0.88	[64]
RAF1	GTCCCTCCTCTGAAGGTTCC	GACAAGGCTGAAGGTGAAGC	1	This paper
PAK6	TGCAGTCTGAGAGCCAGAGA	CAGATGGTTTGCACAAATGG	1.12	This paper
BACT	GCGTGGCTACAGCTTCACC	TTGATGTCACGGACGATTTC	1	[65]
HPRT	TGGAGAAGGTGTTTATTCCTCATG	CACAGAGGGCCACAATGTGA	0.97	[66]
GAPDH	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG	0.89	[67]

2.4. Statistical analysis

All variables were subjected to analysis of variance using a mixed model (Statistical Analysis System, NC, and USA). Residuals derived from all linear regression models were normally distributed. For hormone concentration, the model included the reproductive status (pregnant or cyclic), the day after ovulation and their interaction as fixed effects. For relative quantification of target gene expression, the statistical model included the same fixed effects and PCR plate as a random effect. Data are presented as least square means and their pooled standard errors. Pearson correlation tests were performed to describe the relationships among variables. Means were considered significantly different when $P \leq 0.05$, and with a tendency to differ when $0.05 < P \leq 0.10$.

3. Results

The effects of reproductive status, day and their interaction on each variable are shown in Table 2.

Table 2. Level of significance of fixed effects and interactions on variables in the analysis of variance using a mixed model. Variables are hormone concentrations (hormone) and mRNA expression (mRNA), fixed effects are status (cyclic, pregnant), day post ovulation (day 7 and day 13) and the interactions between them. IGF1, insulin-like growth factor-1; *ERα*, estrogen receptor alpha; *PR*, progesterone receptor; *PAQR5*, progestin and adipoQ receptor family member V; *OXTR*, oxytocin receptor; *PTGS2*, prostaglandin-endoperoxide synthase 2; *FGF9*, fibroblast growth factor family member 9; *IGF1*; insulin-like growth factor 1; *IGF1R*, type 1 IGF receptor; *MUC1*, mucin 1; *OPN*, osteopontin; *LEPR*, leptin receptor; *ADIPOR1*, *ADIPOR2*, adiponectin receptors 1 and 2; *RAF1*, Raf-1 proto-oncogene, serine/threonine kinase; *PAK6*, serine/threonine protein kinase 6/p21-activated kinase 6.

Variable	Status	Day	Status x Day
Hormone			
IGF1	NS	*	NS
Leptin	NS	NS	NS
Adiponectin	NS	*	NS
mRNA			
ERα	*	0.10	*
PR	*	NS	**
PAQR5	**	***	NS
OXTR	***	0.10	NS
PTGS2	***	NS	NS
FGF9	***	NS	NS
IGF1	**	NS	**
IGF1R	***	NS	0.07
MUC1	**	*	*
OPN	***	NS	0.12
LEPR	*	NS	NS
ADIPOR1	**	NS	NS
ADIPOR2	***	NS	0.06
RAF1	***	NS	0.12
PAK6	**	NS	NS

NS: not significant, *P < 0.05, ** P < 0.01, ***P < 0.001

3.1. Hormone concentrations

No differences in E2 and P4 concentrations were found according to status [53]. Levels of IGF1, leptin and adiponectin concentration from day 0 to day 13 are depicted in Fig. 1. The concentrations of IGF1 and adiponectin were affected by day of collection (P = 0.04 and P = 0.03, respectively), but were not affected by the reproductive status neither by the interaction. Meanwhile, leptin concentration was not affected by any of the fixed effects studied.

Levels of IGF1 concentrations decreased (P < 0.01) from the day of ovulation towards day 13 in both groups (Fig 1 a). Adiponectin concentrations decreased (P < 0.05) from day 0 until day 11, when it started to increase reaching maximum values by day 13, both in pregnant and cyclic groups (Fig. 1 c).

(Figure 1)

3.2. Endometrial gene expression

Uterine concentrations of mRNA for *ERα* and *PR* are shown in Fig. 2 (a, b). Pregnant mares presented lower transcript expression than cyclic mares (P < 0.002; P = 0.053, respectively); in

the pregnant group, *ERα* mRNA concentrations were lower in both days, while *PR* mRNA concentrations were lower only at day 13. There was less *ERα* mRNA on day 13 than on day 7 in the pregnant group, but no differences between days 7 and 13 were observed in cyclic mares. *PR* mRNA expression increased by day 13 of diestrus, but no difference between days 7 and 13 of pregnancy was observed.

The main effects of pregnancy and day were significant in *PAQR5* mRNA relative expression (Fig. 2 c). *PAQR5* mRNA relative endometrial expression was upregulated towards day 13 in both diestrus ($P < 0.01$) and pregnancy ($P < 0.005$). Pregnant mares had higher levels for this transcript at both days, as compared to cyclic mares (day 7: $P < 0.01$, day 13: $P < 0.05$).

There was a higher level of *OXTR* mRNA ($P < 0.001$) and *PTGS2* mRNA ($P < 0.0001$) in pregnant mares (Fig. 2 d, e) at both days. *OXTR* mRNA levels tended to increase towards day 13 of diestrus ($P < 0.10$), while no differences were observed in the pregnant group. In this group, *PTGS2* mRNA relative expression tended to be higher at day 7 than at day 13 ($P < 0.10$), while no difference among days was observed in cyclic mares.

(Figure 2)

There was an effect of pregnancy on *FGF9* transcript levels ($P < 0.0001$) (Fig. 3 a). Pregnant mares had higher levels for this transcript at both days of extraction compared to cyclic mares (day 7: $P < 0.001$; day 13: $P < 0.05$). Likewise, pregnant mares showed higher expression of *IGF1* and *IGF1R* mRNA than cyclic mares ($P < 0.01$; $P < 0.0001$, respectively) (Fig. 3 b, c) on day 7, while only a tendency for greater *IGF1R* mRNA was found on day 13. No differences among days in cyclic mares were observed for neither of the genes, while *IGF1* mRNA was downregulated at day 13 in the pregnant group ($P < 0.05$).

The relative expression of *MUC1* and *OPN* mRNA in pregnant mares was higher (Fig. 3, d, e) than in cyclic ones. There was also a significant effect of day on *MUC1* mRNA levels ($P < 0.01$). At day 7, pregnant mares showed higher *MUC1* mRNA transcript levels than at day 13 (Fig. 3, d). Both transcripts were upregulated in pregnant mares vs. cyclic mares at day 7, and a trend for a higher *OPN* mRNA relative expression was observed at day 13 in pregnant mares (Fig. 3, e) as compared to cyclic mares.

(Figure 3)

Pregnant mares showed higher relative expression for *LEPR*, *ADIPOR1* and *ADIPOR2* than cyclic ones ($P < 0.05$; $P < 0.01$; $P < 0.001$; respectively) (Fig. 4 a, b, c). A higher relative expression of *LEPR*, *ADIPOR1* and *ADIPOR2* transcripts was detected at day 7 of pregnancy than at the same day of diestrus ($P < 0.05$; $P < 0.005$; $P < 0.001$, respectively). Similarly, *ADIPOR2* mRNA was higher ($P < 0.05$) and *ADIPOR1* tended to be higher ($P < 0.10$) in pregnant mares at day 13 than in cyclic ones (Fig. 4 b, c). In pregnant mares, *ADIPOR2* mRNA tended to be higher at day 7 than at day 13, but no differences among days 7 and 13 of pregnancy was observed neither in *ADIPOR1* nor in *LEPR*.

There was also an effect of pregnancy on *RAF1* and *PAK6* mRNA relative expression ($P < 0.0001$ and $P < 0.005$, respectively) (Fig. 4 d, e). The pregnant group had greater *RAF1* mRNA levels at day 7 and a trend ($P = 0.072$) was found at day 13 compared to the cyclic group (Fig. 4 d).

Likewise, pregnant mares had higher mRNA levels of *PAK6* at both days ($P < 0.05$) (Fig. 4 e). Both transcripts remained unchanged during the days studied in diestrus as in pregnancy.

(Figure 4)

Correlations between genes are shown in Table 3, except for *ERα* and *PR* which were only correlated between them ($r = 0.74$; $P < 0.005$), and *PTGS2* which did not show any correlation. Table 3 also shows significant and high correlations between genes and circulating hormones.

Table 3. Summary of Pearson correlations between relative expression of targeted genes and between gene expression and circulating hormones: *ERα*, estrogen receptor alpha; *PR*, progesterone receptor; *PAQR5*, progestin and adipoQ receptor family member V; *OXTR*, oxytocin receptor; *PTGS2*, prostaglandin-endoperoxide synthase 2; *FGF9*, fibroblast growth factor family member 9; *IGF1*; insulin-like growth factor 1; *IGF1R*, IGF1 receptor; *MUC1*, mucine 1; *OPN*, osteopontin; *LEPR*, leptin receptor; *ADIPOR1*, *ADIPOR2*, adiponectin receptors 1 and 2; *RAF1*, raf-1 proto-oncogene, serine/threonine kinase; *PAK6*, serine/threonine protein kinase 6/p21-activated kinase 6. The P value is given in parentheses. NS, not significant.

Genes/genes	*OXTR*	FGF9	*IGF1*	*IGF1R*	*MUC1*	*OPN*	*RLEP*	*ADIPOR1*	*ADIPOR2*	*RAF1*	*PAK6*
PAQR5	0.869 (<.0001)	0.831 (<.0001)	NS	0.696 (0.0039)	NS	0.783 (<.0001)	NS	0.832 (<.0001)	0.650 (0.0086)	0.781 (<.0001)	0.862 (<.0001)
OXTR		0.993 (<.0001)	0.881 (<.0001)	0.950 (<.0001)	0.768 (<.0001)	0.983 (<.0001)	0.667 (0.0091)	0.991 (<.0001)	0.920 (<.0001)	0.983 (<.0001)	0.942 (<.0001)
FGF9			0.901 (<.0001)	0.966 (<.0001)	0.804 (<.0001)	0.989 (<.0001)	0.667 (0.0091)	0.994 (<.0001)	0.945 (<.0001)	0.990 (<.0001)	0.954 (<.0001)
IGF1				0.975 (<.0001)	0.956 (<.0001)	0.945 (<.0001)	NS	0.917 (<.0001)	0.974 (<.0001)	0.946 (<.0001)	0.763 (0.0009)
IGF1R						0.988 (<.0001)	NS	0.972 (<.0001)	0.982 (<.0001)	0.989 (<.0001)	0.868 (<.0001)
MUC1							0.728 (0.0031)	0.820 (<.0001)	0.917 (<.0001)	0.868 (<.0001)	0.661 (0.0073)
OPN							NS	0.994 (<.0001)	0.961 (<.0001)	0.999 (<.0001)	0.916 (<.0001)
RLEP								0.717 (0.0039)	0.706 (0.0048)	0.732 (0.0029)	0.551 (0.041)
ADIPOR1									0.939 (<.0001)	0.993 (<.0001)	0.937 (<.0001)
ADIPOR2										0.965 (<.0001)	0.837 (<.0001)
RAF1											0.914 (<.0001)

Genes/hormones	Estradiol	Progesterone	IGF1	Adiponectin
ERα	0.676 (0.0015)	NS	NS	- 0.535 (<0.05)
PR	0.635 (0.0062)	- 0.4 (P < 0.10)	NS	NS
PAQR5	NS	- 0.684 (0.0035)	- 0.831 (<0.001)	0.620 (0.010)
OXTR	- 0.442 (0.087)	NS	- 0.641 (0.0075)	NS
PTGS2	- 0.628 (0.0122)	NS	NS	NS

4. Discussion

This is the first report characterizing IGF1, leptin and adiponectin concentrations simultaneously during early pregnancy and diestrus in the mare, and their levels did not differ according to reproductive status. In contrast, the endometrial expression of all genes studied herein were affected by pregnancy, probably revealing the impact of the early equine embryo in locally regulating endometrial functionality.

As for serum E2 and P4 concentrations [53], IGF1, leptin and adiponectin levels were not affected by pregnancy, in accordance with previous reports in mares [39–41]. IGF1 concentrations decreased from the day of ovulation towards day 13 in both groups, as shown before [26]. However, we did not observe a positive correlation between plasma E2 levels [53] and IGF1 reported by the abovementioned authors, possibly because our samples were taken only during diestrus. Adiponectin increased from day 11, as opposed to P4 concentrations [53], as in cattle [68]. The interaction of sex hormones and adiponectin is being explored in horses (for review, see [34]), so further studies are necessary to fully understand this interplay. Altogether, these results suggest that processes at this stage of the equine pregnancy in the

endometrium are gained mainly with autocrine/paracrine actions. Moreover, and as discussed below, the circulating hormone concentrations may not reflect the local endometrial hormone concentrations, neither the tissue sensitivity to them (e.g. receptors).

Most genes were differentially expressed in the endometrium according to pregnancy as early as day 7, which is consistent with the histological changes found in the same mares on this day [69]. These findings contrast the non-detectable differences on day 8 according to status reported by Merkl et al. [11], and the weak effect of pregnancy on a single gene at day 7 described by de Ruijter-Villani et al. [7]. However, the abovementioned authors took samples making no reference to side of ovulation/corpus luteum. The ipsilateral horn samples of the present study represents a guaranteed scenario not only in terms of the embryo presence at day 7 [70], but also probably due to a distinct hormonal milieu as suggested previously [53]. In this regard, since side of CL affected steroid receptor expression and localization [53], it would be interesting to assess contralateral endometrial samples for the rest of the genes studied herein, as well as samples collected on day 7 post-ovulation.

Pregnant mares showed less *ERα* transcript endometrial levels on both days studied which is consistent with previous findings [7–10,42,53]. Steroidogenesis in the equine embryo begins at day 6 after ovulation, with a dramatic increase between days 12 and 14 of pregnancy [71,72], which could explain the downregulation of this transcript between days 7 and 13 in the pregnant group. Regarding *PR* mRNA levels, fluctuations between days were seen only in the cyclic group, with an upregulation towards day 13. It agrees with earlier data [10] and could be related to the higher E2/P4 ratio at day 13 found in the cyclic group [53], and is also supported by the positive correlation between E2 and *PR* mRNA found in this study. Likewise, de Ruijter-Villani et al. [7] found this same pattern, but between day 14 and 21 after ovulation. We also agree with the abovementioned authors in that *PR* mRNA at day 7 was not affected by pregnancy. The behavior of *PR* mRNA expression in the pregnant group is in line with the paradoxical downregulation of PR, necessary for uterine milk secretion [73]. Besides, P4 can also elicit its effects through *PAQR5*, which was upregulated in pregnant mares in the present study, in line with previous findings [11]. *PAQR5* was the only gene that showed higher levels at day 13 than at day 7 in pregnant mares. Interestingly, the 17-alpha-OH-progesterone produced by the conceptus between days 7 and 14, agonist of P4 actions [74], activates the membrane but not the nuclear receptor [75]. Different endometrial levels of *PAQR5* at days 7 and 13 could also explain the negative correlation of this transcript and circulating P4 / IGF1 found in this study. Regarding the positive correlation with circulating adiponectin, potentially target receptors of adiponectin in the PAQR family have been suggested in other species [38].

With the development of more precise transcript level measurement techniques, studies showed no differences in *OXTR* mRNA expression between pregnant and non-pregnant mares on day 13.5 [9] and 14 [7] after ovulation, whereas Merkl et al. [11] described a modest upregulation in *OXTR* mRNA expression at day 12 of pregnancy. Coincidently, we found higher *OXTR* mRNA expression in pregnant mares at day 13, but also at day 7, which could be related to the pro-angiogenic role proposed for uterine oxytocin [18]. As in other species, conceptus-derived estrogens could be responsible for such upregulation [76] and for *PTGS2* mRNA upregulation as well [77]; however, no positive correlation between *PTGS2* mRNA and E2 was observed. Moreover, PTGS2 is necessary for PGE2 secretion. The enzyme PGE2 has embryo growth, luteoprotective and angiogenic stimulatory effects in other species [78,79] and several genes related to PGE2 signaling are upregulated during equine pregnancy [11,80]. Overall, the

results of genes involved in the luteolytic pathway suggest other overlapping functions for these elements.

The upregulation of *FGF9* mRNA expression seen in the pregnant group is in agreement with previous reports in mares [9,11] and with its potential role as an embryonic growth, endometrial remodeling and angiogenic promoter in other species [22,23]. Conceptus-derived E2 and PGE2 upregulate this factor in human endometrium [81,82]. No differences were found between day 7 and 13 in the cyclic group, which disagree with previous reports [9,11]. We have no explanation for this discrepancy but, given the scarce information available on this factor regulation, further investigation is needed.

Angiogenesis and vascular remodeling has proven to be important processes during MRP [11]. Recent findings show an increase in the abundance of certain proteins related to those processes, as well as an increase in blood vessel caliber in the histological samples of pregnant vs. cyclic mares during early gestation [69,83]. Therefore, it was not surprising to find a high correlation among several genes with angiogenic properties.

The higher mRNA levels of *IGF1* and *IGF1R* in the pregnant group agree with previous studies in heifers [84] and could also be explained by an embryo-derived estrogen modulation (for review, see [85]). *IGF1* is an active mediator of endometrial angiogenesis, cell proliferation and conceptus development in other species [20,21], and the early horse conceptus secretes significant quantities of IGF 1-binding protein 3 towards the conceptus capsule [86]. Moreover, *IGF1R* can increase glucose transport/uptake in humans [87], which is in line with a recent study showing higher levels of proteins related to energy production in pregnant mares at day 13 [83]. The decrease of *IGF1* mRNA towards day 13 of pregnancy may be associated to the rapid embryo growth between days 11 and 16, dependent on fluid intake rather than on cellular multiplication [56]. In our study, several genes which regulate embryo and uterine growth/differentiation and glucose uptake were highly correlated.

The pronounced upregulation of *MUC1* mRNA levels at day 7 of pregnancy agrees with previous results in cattle [3], and could be related to the timing of the equine capsule formation: due to its negative electrostatic charge, the outer surface of the capsule is very “sticky” towards other surfaces [4]. Thus, we hypothesize that a high *MUC1* mRNA expression would prevent early embryo attachment. Moreover, the capsule is likely formed by an endometrial mucin contribution [27]. This glycoprotein is upregulated by E2 in the murine model [88], although some other factor must also be implicated in its control, since we found a downregulation of *MUC1* mRNA towards day 13 of pregnancy and no effect of pregnancy at this day. It is tempting to speculate that an anti-adhesive molecule would be less necessary as we approach the fixation and the end of the mobility period (day 16) [89].

Our results show higher endometrial *OPN* expression in pregnant mares at both days studied. Osteopontin regulates endometrial angiogenesis in human endometrium [31] and in the porcine uterus; studies suggest it binds to the surface receptors/integrins on the embryo to induce cell proliferation [30]. Endometrial OPN protein is induced by conceptus-secreted estrogens in pigs [90], and *OPN* mRNA is expressed by uterine epithelial cells during porcine pregnancy with a complex fluctuating temporal and spatial pattern [91]. The increased OPN expression during early pregnancy suggests a role for this protein in the pre-implantation period in the mare.

As far as we know, this is the first report comparing the expression of *LEPR* mRNA between pregnant and cyclic equine endometrium. Although the expression of endometrial *LEPR* has been compared according to the degree of obesity in mares [92], the effect of the embryo on the endometrial expression of this gene has not been described before. The presence of LEPR in reproductive tissues has linked it with reproductive processes in several species [93] including endometrial angiogenesis [35]. The presence of leptin and its receptor in 3-day-old equine embryos suggests an autocrine/paracrine process in horse embryo early development [36]. Moreover, human blastocysts secrete leptin [94]. Altogether, these findings could explain the observed upregulation of *LEPR* mRNA at day 7 of pregnancy. However, no differences among groups were seen at day 13. Indeed, our results regarding *LEPR* mRNA levels in the cyclic group differ from the variation found during diestrus in cattle [95]. Thus, further investigation is needed to better understand this gene expression, function and modulation in equine cyclic and pregnant endometrium.

In our study, *ADIPOR1* and *ADIPOR2* mRNA were upregulated in pregnant mares, in agreement with previous results [39]. The upregulation observed at day 7 could be related to the reported mediation for increasing glucose uptake in other species [96], while higher levels at day 13 may be due to the proximity of embryo fixation [39], as both receptors are considered human implantation success markers [35]. Studies in sow suggest adiponectin system is regulated by local steroids and prostaglandins; indeed, adiponectin endometrial concentration was higher on days of MRP suggesting there could be an autocrine/paracrine action of this hormone stimulated by the embryo [97]. Furthermore, there is controversial data regarding angiogenesis effects of adiponectin in the endometrium [35]. Nevertheless, previous studies in mares have shown simultaneous upregulation of anti and pro-angiogenic factors in pregnant endometrium at day 12 [11]. In this study, the trend for lower levels of *ADIPOR2* mRNA towards day 13 of pregnancy, when compared to day 7, could be related to the abovementioned complex regulation mechanisms and functions.

We found higher levels for *RAF1* and *PAK6* mRNA on both days studied in pregnant mares, in agreement with previous studies [42,47]. These molecules have proven to be important markers of an embryo-receptive endometrium in other species [98–100]; through the transmission of force at cell adhesion sites, FAMs affect pathways regulating uterine growth, development and proliferation [101]. As far as we know, this is the first study to report the upregulation of FAMs as early as day 7 of pregnancy in the mare, which may indicate that the first physical contact with the embryo provides mechanical regulation of endometrial function, as it was demonstrated in day-7 bovine embryos [3]. Noteworthy, given that FAMs communicate the interface between cells and ECM [45], and that OPN is an ECM molecule, a high correlation between these transcripts was not surprising.

5. Conclusions

Our results show the effect of embryo presence on several pregnancy-related genes in the endometrium. High correlations between genes related to endometrial angiogenesis, embryo growth and metabolism were found, suggesting their role to escort specific embryo developmental changes and requirements. We conclude the conceptus and its location exert stage-dependant paracrine and mechanical regulation on endometrial responsiveness, which probably begins soon after the embryo enters the uterus.

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7. Conflict of interest

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

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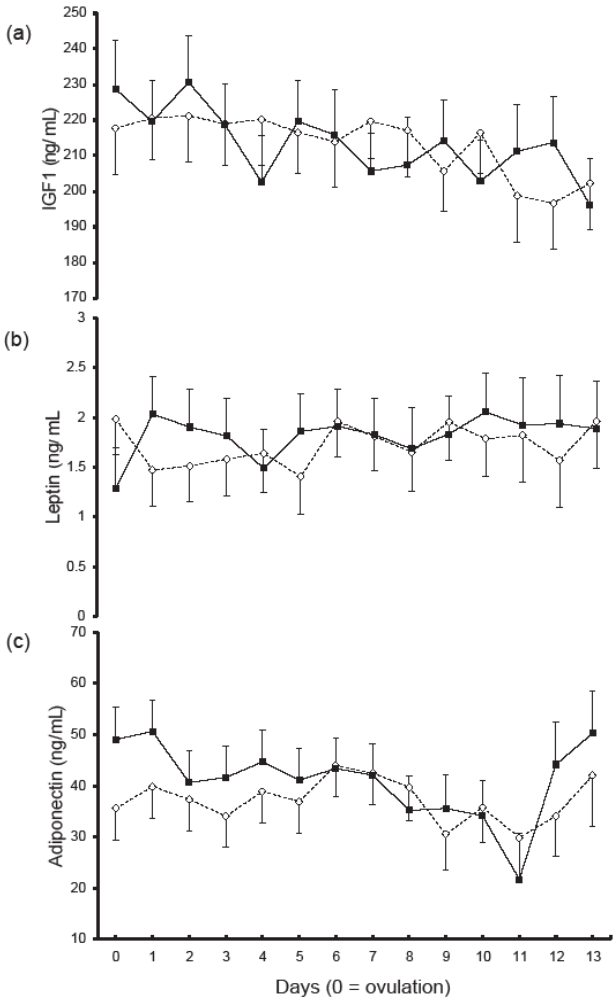
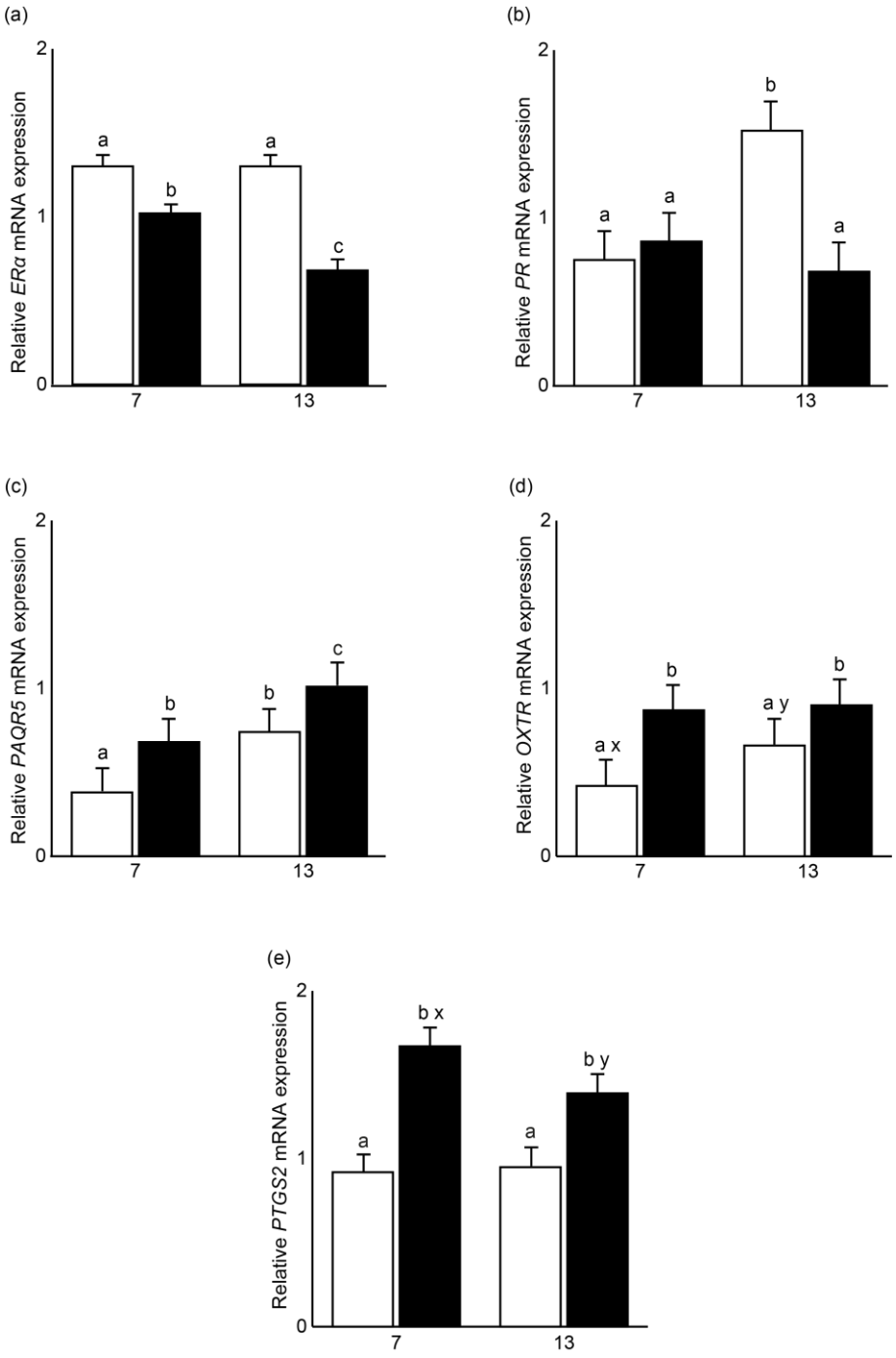
790 **7. Figures.**



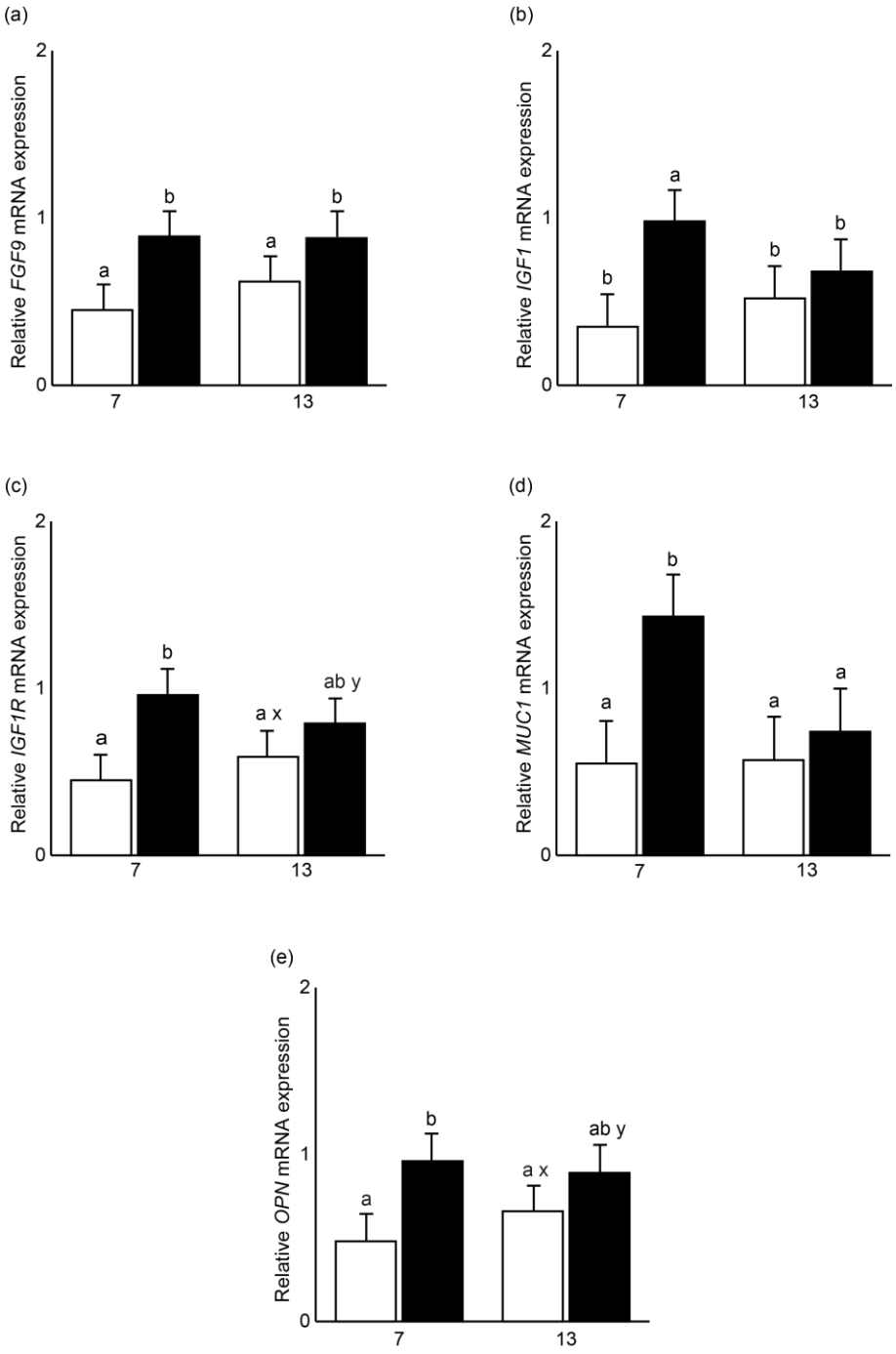
791

792 Fig. 1. Concentrations of insulin-like growth factor 1 (IGF1) (a), leptin (b) and adiponectin (c) during
793 diestrus or early pregnancy (day 0 = ovulation). Mean (± S.E.M.) hormone concentration (y axis) is shown
794 for each day (x axis) for cyclic (white rhombus) and pregnant (black squares) mares.



795

796 Fig. 2. Ipsilateral endometrial relative expression of estrogen receptor alpha (*ERα*) (a), progesterone
797 receptor (*PR*) (b), progestin and adipoQ receptor family member V (*PAQR5*) (c), oxytocin receptor
798 (*OXTR*) (d) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) (e) transcripts in cyclic (white bars) and
799 pregnant (black bars) mares. Data are presented as least square means ± standard error of the mean
800 (S.E.M.). Superscripts a, b, c show significant differences $P \leq 0.05$. Superscripts x, y show tendency to
801 differ $0.05 < P \leq 0.10$.



802

803 Fig. 3. Ipsilateral endometrial relative expression of fibroblast growth factor family member 9 (*FGF9*) (a),
804 insulin-like growth factor 1 (*IGF1*) (b), type 1 IGF receptor (*IGF1R*) (c), mucin 1 (*MUC1*) (d) and
805 osteopontin (*OPN*) (e) transcripts in cyclic (white bars) and pregnant (black bars) mares. Data are
806 presented as least square means ± standard error of the mean (S.E.M.) Superscripts a, b show significant
807 differences $P \leq 0.05$. Superscripts x, y show tendency to differ $0.05 < P \leq 0.10$.

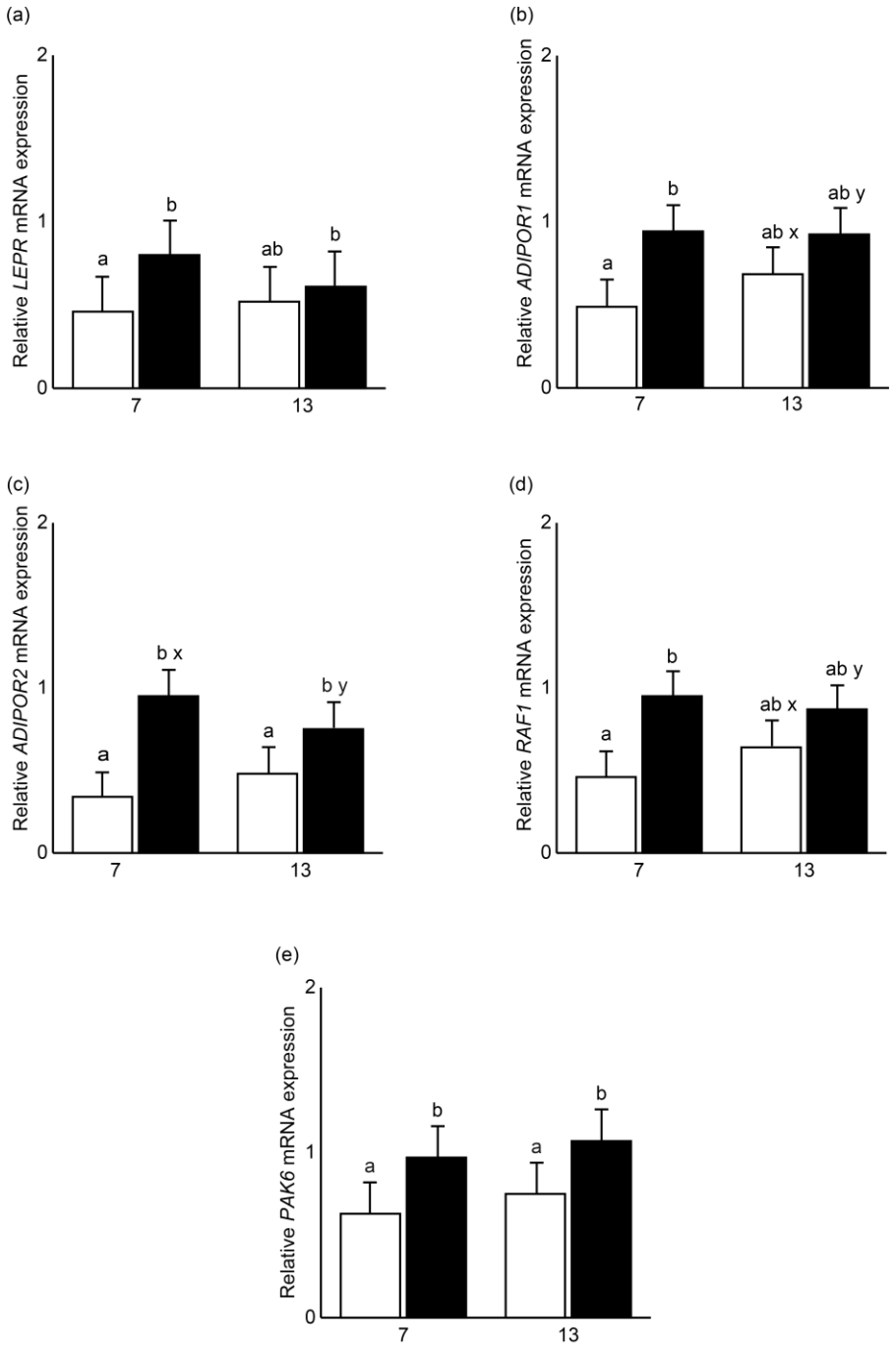


Fig. 4. Ipsilateral endometrial relative expression of leptin receptor (*LEPR*) (a), adiponectin receptors 1 and 2 (*ADIPOR1*) (b); (*ADIPOR2*) (c), raf-1 proto-oncogene, serine/threonine kinase (*RAF1*) (d) and serine/threonine protein kinase 6/p21-activated kinase 6 (*PAK6*) (e) transcripts in cyclic (white bars) and pregnant (black bars) mares. Data are presented as least square means ± standard error of the mean (S.E.M.) Superscripts a, b show significant differences $P \leq 0.05$. Superscripts x, y show tendency to differ $0.05 < P \leq 0.10$.

ANEXO III

Cell-specific expression of early gestation-related components and immune cell response in equine endometrium during diestrus and early pregnancy

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Abstract

The aim of the present study was to analyse the immune cell response and protein localization by immunohistochemistry during diestrus and early pregnancy in the equine endometrium. Transcervical biopsies of the ipsilateral horn (regarding side of corpus luteum) were performed on days 7, 10 and 13 post-ovulation (day 0) in cyclic (n = 6 on each day) and pregnant (n = 6 on each day) mares. Pregnant mares showed higher numbers of lymphocytes and a downregulation of this cells was observed towards day 13. Likewise, in superficial stroma there was a trend for higher number of neutrophils in pregnant mares while at day 7 more eosinophils were found for this group. Pregnancy strongly downregulated estrogen receptor α (ERα) in all cell types but did not affect progesterone receptor (PR). Pregnant mares showed less staining intensity than cyclic mares for oxytocin receptor (OXTR), mucin 1 (MUC1), insulin growth factor 1 (IGF1) and 2 (IGF2) at day 10 in superficial compartments, while type 1 insulin growth factor receptor (IGF1R) show this effect on day 7. Staining intensity for prostaglandin-endoperoxide synthase 2 (PTGS2) was upregulated in pregnant mares at day 10 in deep locations and on day 13 on superficial glandular epithelium. A downregulation towards day 13 was seen for ERα protein localization in the cyclic group and for PR in both groups, in all cell types. Likewise, a downregulation towards day 13 was seen in IGF1 and IGF1R (for both groups in deep glandular epithelium) and for PTGS2 (cyclic group in superficial glandular epithelium and stroma). Conversely, an up-regulation towards day 13 was seen for IGF2 (for both groups in the luminal and superficial glandular epithelium) and for mucin 1 (MUC1) (in the pregnant group for all cell types, except deep glandular epithelium), while no differences were found between days 7 and 13 in none of the groups regarding OXTR immunostaining. Immunohistochemical temporal and spatial fluctuations seen during diestrus and early pregnancy are probably related to a complex regulation-mechanism of endometrial cell proliferation and differentiation, which could not be explained by the classical steroid nuclear receptors levels in the pregnant mares. The overlapping changes seen in the endometrium of the pregnant group are possible consequence of differences found in the immune cells counts and ERα-negative epithelium.

Key words: mare; uterus; leukocytes; proteins; localization.

1. Introduction

It has been shown that the embryo regulates in a paracrine and/or mechanical manner the endometrial gene expression prior to the maternal recognition of pregnancy onset. In pregnant animals, endometrial fluctuations in the expression of genes related to several biological process, seem to escort specific embryo developmental changes and requirements (Sponchiado et al., 2017; Kalpokas et al., submitted). In this regard, there are unique morphological and physiological features of the horse conceptus that should be considered: 1) the formation of an acellular capsule at day 7; 2) the synthesis and secretion of several hormones and enzymes and 3) a high mobility phase from day 10 to day 14 (for review, see Betteridge, 2007). As for global tissue gene expression, there are studies showing cell-specific alterations in the localization of some uterine early gestation-related components during the estrous cycle and early pregnancy in the mare (de Ruijter-Villani et al., 2015; Scaravaggi et al., 2018). This cell type specific alterations occur simultaneously to endometrial histological remodelling, including changes in tissular proliferation and differentiation (Kenney RM., 1978; Aupperle et al., 2000; Hoffmann et al., 2009; Camozzato et al., 2018) as to immunomodulation, a crucial common feature of pregnancy in mammals (Hansen, 2011). In this regard, studies show an increase in the infiltration of lymphocytes and eosinophils in the endometrium of pregnant mares when compared to their cyclic counterparts (Keenan et al., 1987; Martínez, 2019)

In mares, several studies showed that estrogen receptor alpha (ERα) and progesterone receptor (PR) expression throughout the estrous cycle and pregnancy is cell-type dependent being different between stromal and epithelial cells (Aupperle et al., 2000; Kalpokas et al., 2010, 2018; de Ruijter-Villani et al., 2015). However, little and inconclusive information was found regarding endometrial location of steroid receptors during the first 10 days of pregnancy (de Ruijter-Villani et al., 2015). Notwithstanding, in ruminants is well stablished how the differential cell-type sensitivity allow steroid receptors to regulate several process during early pregnancy, including the inhibition of luteolysis: the ruminant embryo prevents increases in oxytocin receptor (OXTR) gene expression by repression of ERα in the endometrial luminal epithelium during days 11 to 15 of pregnancy (Spencer et al., 1995). Conversely, a higher *OXTR* mRNA expression is found in pregnant mares during this same period (Merkl et al., 2010: Kalpokas et al., submitted), though de Ruijter-Villani et al. (2015) found low levels of OXTR protein expression at day 14 of pregnancy compared to diestrus samples. In ruminants, the downregulation of OXTRs in the endometrium inhibits the conversion of arachidonic acid to prostaglandin F2 alpha (PGF2alpha) by the enzyme PTGS2 (Fortier et al., 2008) preventing lysis of the corpus luteum (CL). However, studies in sheep reported that pregnancy caused an upregulation, a downregulation or even no changes in the endometrial levels of PTGS2 (Charpigny et al., 1997; Chen et al., 2006; Sosa et al., 2009). Likewise in the mare, while immunohistochemical expression of PTGS2 is downregulated at day 15 of pregnancy in luminal epithelium (LE) (Boerboom et al., 2004), recent findings showed no changes in *PTGS2* mRNA on LE at day 12 of pregnancy (Scaravaggi et al., 2018).

IGF1, 2 IGF2 and IGF1R are mediators of estrogen-signalling with potential roles in endometrial remodelling and angiogenesis as for embryo growth in several species (Byrne et al., 2002; Keller et al., 2005; Kaczmarek et al., 2008; Ghahary et al., 2009; Klein et al., 2010; Østrup et al., 2010;

Šućurović et al., 2017). Recent findings showed that the equine embryo upregulates endometrial expression of *IGF1* and *IGF1R* mRNA during early gestation (Kalpokas et al., submitted). In other species, pregnancy affected IGF 1 and 2 endometrial protein levels at the luminal and glandular epithelium compartments (Persson et al., 2002; McCarthy et al., 2012). However, we have not found information regarding endometrial protein localization during pregnancy in the mare.

A gene found to be upregulated in the endometrium by the bovine and equine embryo is MUC1 (Sponchiado et al., 2017; Scaravaggi et al., 2018) which is a constitutive element of the embryonic capsule preventing premature implantation (Gillies et al., 1999). Recent findings show *MUC1* mRNA was mainly detected in LE and increased from day 12 pregnant mares (Scaravaggi et al., 2018), but we have not found reports regarding endometrial MUC1 protein localization according to equine pregnancy.

As pointed out before, there is lack of information concerning endometrial spatial characterization of several elements related to important biological process during equine early pregnancy. Therefore, the aim of the present study was to analyse the effect of pregnancy on endometrial immune cell response and protein localization of ERα, PR, OXTR, PTGS2, IGF1, IGF2, IGF1R and MUC1 at days 7, 10 and 13 post-ovulation in mares.

2. Materials and methods

2.1. Animals and treatments

This experiment was performed during the breeding season (December-March), at the experimental farm number 1 of the Veterinary Faculty, University of Uruguay (34°22'"S, 55º36'W). This study was carried out following a protocol approved by the Animal Ethical Use Committee at the Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (protocol number 23716 from July 17, 2014) and by the Bioethics Committee of the Republic University, Montevideo, Uruguay (CEUAFVET-PI-34/14 -Exp. 111130-001367-14). Criollo Horse crossbreed mares aged from 5 to 10 years, weighting an average of 400 ± 50 kg, from a commercial herd, with no history of fertility problems and proven fertility were used in this study. All mares (n = 30) were cyclic and clinically normal, as indicated by physical and ultrasonographic examination (SonoScape® A6v, 5-7.5 MHz, SonoScape, China), and histopathological classification of endometrial samples (see histological analysis). Mares were kept in natural pasture, with unrestricted access to water.

The estrous cycles were synchronized and groups were managed following Kalpokas et al., (2018). In the first cycle, intrauterine samples of 30 cyclic mares were collected and they constituted the cyclic group. In the second cycle, the same mares were bred to a fertile stallion and constituted the pregnant group. On both groups at each cycle, endometrial biopsies were collected on day 7 (n=6 on each cycle), day 10 (n=6 on each cycle) and 13 post ovulation (n=6 on each cycle). Samples from mares without an embryo were excluded from the experiment (n=12).

2.2. Biopsy samples and histological analyses

Endometrial biopsy samples were taken as transverse sections from the mid-portion of the ipsilateral uterine horn regarding the side of corpus luteum (Kenney and Doig, 1986). Samples were fixed in 4% paraformaldehyde solution. Samples were taken on specific days before the maternal recognition of pregnancy day limit (day 14 of pregnancy) (for review, see Klein and Troedsson, 2011) because at day 7 the embryo has recently arrived to the uterus and also the capsule is formed at this stage while at day 13 the embryo has already started its highly mobile phase but is still not fixated (Betteridge, 2007). Day 10 is the midpoint between 7 and 13 days.

For histological analysis, endometrial samples fixed in 4% paraformaldehyde were embedded in paraffin. Paraffin blocks were serially sectioned at 5 µm, stained with haematoxylin-eosin. Samples were evaluated following Kenney and Doig (1986). This included an evaluation of the stage of the estrous cycle and the pathological findings. Infiltrations of immune cells were evaluated based on the presence of neutrophils, lymphocytes, eosinophils, macrophages and plasma cells, their distribution (into superficial stroma – SS or deep stroma - DS) and their frequency (average in linear fields of 5.5 mm, five fields per endometrial cellular section described above) (Schoon et al., 1992).

For localization of proteins, an avidin-biotin-peroxidase immunohistochemical technique was used to visualize immunostaining (Kalpokas et al., 2010). Type of antibodies, dilutions, suppliers and time of incubation are described in Table 1. Slides were photographed with an electronic microscope (Nikon BM 2000®) using a software (Micrometrics SE Premium®). Receptor staining intensity was evaluated in five endometrial compartments; the luminal epithelium (LE), glandular epithelium (arbitrarily divided in two portions; the superficial glandular (SG) epithelium next to the uterine lumen, and the deep glandular (DG) epithelium next to the myometrium) and stroma (also divided into superficial (SS) and deep (DS) regions using the same criteria as that used to divide the glandular epithelium). The amount of immunoreactive protein in the different compartments was estimated subjectively. Fifteen fields were analyzed for each compartment at a magnification of 1000×. The extent of staining was scored according to Boos et al. (1996) and Thatcher et al. (2003).

2.3 Statistical analysis

All variables were subjected to analysis of variance using a mixed model (Statistical Analysis System, NC, and USA). Residuals derived from all linear regression models were normally distributed or normalized through a logarithm transformation (log base 2), except for ERα and eosinophils (which data was subjected to PROC GENMOD). Immune cells data from the SS an SD were separately subjected to the analysis and the statistical model included the effects of reproductive status (cyclic or pregnant), day of extraction (7^{th}, 10^{th} or 13^{th}) and their interaction. The variable studied in the analysis of protein localization by immunohistochemistry was the average staining intensity of the 15 fields. The statistical model included the effects of reproductive status (cyclic or pregnant), day of extraction (7^{th}, 10^{th} or 13^{th}) and endometrial compartment (LE, SGE, DGE, SS and DS) and their interactions. Data are presented as least square means ± pooled standard errors. Pearson correlation tests were performed to describe

the relationships among variables. Means were considered significant when $P \leq 0.05$ and a tendency when $0.05 < P \leq 0.10$.

3. Results

Histological analysis revealed all mares belonged to category I or IIA (healthy mares). Cellular infiltrations consisted mainly in lymphocytes, eosinophils and neutrophils which were distributed in the stroma (superficial and deep) (Fig 1, A). There was an effect of reproductive status on lymphocytes, as pregnant group showed higher number of these cells at the 7th day in SS ($P < 0.001$) and DS ($P= 0.058$) (Fig. 2). We found very few eosinophils at days 10 and 13 post ovulation in both endometrial compartments and experimental groups, as this cell type was mainly observed at day 7 ($P < 0.05$) in SS. At this day, higher count for eosinophils was found for the pregnant group in SS compared to the cyclic group (4.51 ± 0.82 vs. 0.45 ± 0.31; $P < 0.05$). There was an effect of status in neutrophils´ count at SS, as the pregnant group showed higher number of this cell type (1.43 ± 0.41 vs. 0.71 ± 0.36; $P= 0.055$) than the cyclic group. There was no effect of status in macrophagues neither in plasma cells. We found scarce immune cells stained positive for all proteins studied herein in all days studied and both groups.

When specific monoclonal antibodies were substituted with a non-immune mouse IgG, the absence of staining confirmed the high specificity of immunostaining for all proteins (Fig. 1, B). Immunoreactive ERα and PR were detected exclusively in the nuclei of all endometrial cell types (Fig 1, C, D), whereas OXTR, PTGS2, IGF1 and 2, IGF1R and MUC1 were observed in the cytoplasm of the cells (Fig 3).The effects of status, day and their interaction on each variable are listed in Table 2.

There was an effect on reproductive status in ERα ($P < 0.001$) as pregnant mares were almost devoid of staining for this receptor (Table 2). There was also an effect of day ($P < 0.001$): in cyclic group, there was a staining intensity downregulation from day 7 towards day 13 at SGE, DGE and DS, while at LE and SS this downregulation started at day 10. The higher staining intensity found for the cyclic group compared to pregnant group was present at days 7 and 10, but not at day 13 post-ovulation. Cyclic group showed higher staining intensity than pregnant group in all compartments. The pregnant group showed a low intensity of staining restricted to the stromal cells (Fig 4).

The PR staining intensity was not affected by reproductive status, but there was an effect of day ($P < 0.001$) and cell type ($P < 0.001$) (Table 2). Including both experimental groups, there was a higher staining intensity for this protein at day 7 with a downregulation towards day 13 post-ovulation. At this day, both groups were almost devoid of staining in the LE (data not shown). The downregulation pattern from day 7 seen for PR was observed at LE, SGE and DGE on both groups (Fig 4).

Pregnancy decreased ($P < 0.05$) OXTR protein but there was no effect of day in this receptor (Table 2). The glandular cells showed less staining intensity for OXTR ($P < 0.001$). Pregnant mares showed less staining intensity in LE, SGE and SS at day 10 post-ovulation when compared to the cyclic group, while this was observed at day 13 in DS. Staining intensity increased again in superficial compartments by day 13 post-ovulation (Fig 4).

There was an effect of reproductive status ($P < 0.01$) and compartment ($P < 0.001$) for PTGS2 staining intensity: higher levels were detected in pregnant mares and staining was observed mainly in the superficial endometrial compartments (LE, SGE and SS) (Table 2). The pregnant group showed a higher staining intensity when compared to their cyclic counterpart at day 13 in SGE and at day 10 in deep locations (DGE and DS). In the cyclic mares, there was a downregulation from day 10 compared to day 13 post-ovulation at the superficial locations (SGE and SS) (Fig 4).

The staining intensity for IGF1 was affected by status as pregnant mares had less staining ($P < 0.01$). There was also an effect of compartment ($P < 0.001$) with less staining in the stromal cells (Table 2). Pregnant mares showed less staining intensity in SGE at day 10 post-ovulation when compared to the cyclic group. Staining intensity in deep glandular epithelium decreased from day 7 to 13 in both cyclic and pregnant mares (Fig 5).

Day and cell type affected IGF2 staining intensity ($P < 0.001$) and the interactions found with status shows a different profile according to pregnancy (Table 2). The pregnant group showed less staining intensity for LE at day 10 ($P < 0.001$). This group had an upregulation from day 10 towards day 13 in superficial endometrial compartments (LE, SGE and SS), while an upregulation was observed in the cyclic group in LE from day 7 to 10, and in SGE from day 7 to 13 (Fig 5).

Pregnant mares had less staining intensity for IGF1R ($P < 0.05$) (Table 2). There was also a significant effect for the rest of fixed effects studied (day: $P < 0.01$ and compartment $P < 0.001$), because less staining intensity was observed at day 13 and at the stroma cells, respectively. Pregnancy downregulated the staining intensity for this receptor on day 7 at the LE. In both groups of mares, there was a downregulation towards day 13 at the DGE compartment, while the cyclic group had the same pattern at the SGE as well (Fig 5).

Pregnancy affected staining intensity of MUC1 (Table 2), while there was an effect of the rest of the fixed effects studied: there was higher staining intensity in the cyclic group and at day 13 post-ovulation, while the higher intensity was observed in LE cells and in superficial locations (Table 2). There was a downregulation of staining intensity at day 10 on the SGE in the pregnant group and an upregulation on the SS in the cyclic group. In pregnant group, there was an upregulation from day 10 towards day 13 post-ovulation in all compartments, while the only fluctuation seen in the cyclic group was an up regulation between days 7 and 10 in SS (Fig 5).

4. Discussion

Recent findings in the cattle and mare stated that early embryo mechanical and paracrine actions affect endometrial global tissue gene transcription as soon as day 7 post-ovulation (Sponchiado et al., 2018; Kalpokas et al. submitted), which challenged the more accepted knowledge regarding the endometrial later response to the presence of the embryo (Klein and Troedsson, 2011). Results from the present study show these embryo endometrial changes are also regulated in a cell-specific manner, because the effect of pregnancy on immune cell population and most molecular markers was predominantly in the superficial compartment.

The pattern of cellular infiltration found in both groups agrees with previous reports in mares (Keenan et al., 1987). The higher immune reaction found at day 7 in pregnant mares was

expected, since the equine embryo affected the oviductal expression of immune response-related genes at day 4 post ovulation (Smits et al., 2016). Likewise, there was an increase in neutrophils, lymphocytes and eosinophils in the equine endometrium 48 hours after conceptus fragments were transferred at day 5 of the estrous cycle (Camacho et al., 2018). Moreover, pregnant mares showed higher infiltration of lymphocytes and eosinophils at days 6-9 compared to mated mares with no embryo recovery and with non-mated mares (Keenan et al., 1987). Although products of the immune system can enhance embryonic development and endometrial tissue remodelling in several species (Hansen, 2011), harnessing of these beneficial effects would be transient since we found no difference between groups at day 10 or 13 post-ovulation in the population of immune cells, in line with previous studies in mares at day 14 (Keenan et al., 1987; Watson and Dixon, 1993). This could be explained by the day-7 formed equine capsule, that could sheld the embryo from maternal immune detection (for review, see Betteridge, 2007). Likewise, the decreased immune cell counts found after day 7 could be the result of the immunosuppression exerted by conceptus-derived PGE2 or interferon (IFN) delta (Low and Hansen, 1988; Cochet et al., 2009). Recent studies revealed the superficial compartments of equine endometrium and uterine histotroph show an up-regulation for immunosuppressive genes and proteins at day 12-13 of pregnancy (Bastos et al., 2018; Scaravaggi et al., 2018).

Effect of pregnancy and both groups-day regulation on ERα and PR localization agrees with previous reports in mares (Watson et al., 1992; Aupperle et al., 2000; Kalpokas et al., 2010; De Ruijter-Villani et al., 2015), while status effect was the same as for transcript levels (Kalpokas et al., submitted).The behaviour of steroid receptors explain endometrial histological changes shared by cyclic and pregnant mares (Camozzatto et al., 2018), regarding downregulation of epithelial E2- induced proliferation and the antiproliferative effect of P4 on epithelial cells mediated through PR-positive stromal cells (Gerstenberg et al., 1999; Bazer et al., 2009; Hantak et al., 2014). The absence of ERα seen in epithelium of pregnant mares towards day 13 of pregnancy is associated with a more differentiated endometrium with abundance of protruded cells in the luminal epithelium at day 7, diminishment of ciliated cells and increasing amounts of histotroph (Camozzato et al., 2018). The latter overlapping effects are probably consequences of progestational effects mediated via progestomedins and membrane-bound progesterone receptors (Fernandes et al., 2005; Gellersen et al., 2009; Keator et al., 2012). In ruminants stimulation of stromal PR is responsible for uterine milk secretion through the expression of growth factors (Spencer et al., 1999; 2002), while we previously reported an upregulation of a growth factor transcript levels (fibrobalst growth factor 9-*FGF9*-) in pregnant mares (Kalpokas et al., submitted). Our previous work also showed an upregulation of progestin binding receptor 5 (*PAQR5)* mRNA in the pregnant group, which was the only upregulated gen towards day 13 of pregnancy (Kalpokas et al., submitted).

In the present study the presence of the embryo down-regulated OXTR staining intensity at day 10, coincidently with the beginning of endometrial responsiveness to oxytocin (Goff, et al., 1987) and the embryonic high mobility phase (Ginther, 1983), propelled by conceptus-derived prostaglandins (Stout and Allen, 2001). This downregulation in the superficial compartments could be a way of preventing excessive prostaglandin production, which would not be that critical as the conceptus prostaglandin secretion decreases (Stout and Allen, 2001), in line with

the recovering of OXTR seen towards day 13 in pregnant mares in the present study. Moreover, the above theory escort the postulation that MRP in the mare involves a delay, rather than a complete avoidance, of OXTR upregulation (de Ruijter Villani et al., 2015). Indeed, Klonohatz et al. (2019) proposed that the embryo activates focal adhesion molecules preventing PGF2alfa release, while we previously demonstrated the upregulation of *RAF1* and *PAK6* transcripts levels during pregnancy (Kalpokas et al., submitted).

Differences seen at day 10 for PTGS2 protein localization in deep compartments could be related to the endometrial contribution of PGF2alfa required for myometrial localized contraction and embryo mobility (Stout and Allen, 2001). Therefore, we agree with Atli et al. (2010) in that a balanced interplay among the enzymes and receptors involved in PG synthesis exists inside the uterus during the early pregnancy in the mare, since we found the embryo delaying the luteolytic process in superficial compartments while stimulating the deeper endometrium PG secretion for its movement. There was a downregulation between days 10 and 13 post-ovulation seen at the SGE and SS in the cyclic group, that could be partially explained by a hormonal steroid regulation as proposed by Charpigny et al. (1997) in sheep. Notwithstanding, further investigation is needed to fully understand this cell-specific fluctuation during diestrus in the mare.

In the present study, the effect of pregnancy differs from our previous gene expression findings (Kalpokas et al., submitted) because pregnant mares showed less IGF1 protein levels at day 10 in SGE. Notwithstanding, the transcript levels reveals exclusively the in-situ production of this peptide, while the protein analysis capture different moments in the dynamic of diverse sources of IGF1 passing through the endometrium. Therefore, the conceptus could be up-taking uterine IGF1 through a binding protein: IGFBP3, secreted by the equine conceptuses at day 10 of pregnancy (Herrler et al., 2005). The downregulation after day 7 seen in glandular epithelium in both groups may be related to the pattern seen for ERα, as is suggested for IGF1 mRNA in cattle (Meikle et al., 2001). Moreover, there may be a smaller systemic contribution to the uterine content of this protein (Kalpokas et al., submitted). This downregulation fits the diminishment in cellular proliferation seen in mares (Camozzato et al., 2018; Gerstenberg et al., 1999), since this factor is an executer of E2-induced epithelial proliferation (Hantak et al., 2014). Indeed, *IGF1* mRNA was downregulated in the pregnant group towards day 13 in our previous study (Kalpokas et al., submitted) and studies in ruminants show a preferential role for IGF1 in the early stages of embryonic development (Byrne et al., 2002; Sosa et al., 2010). The sequestering effect of IGFB3 suggested before can also explain the downregulation seen for IGF2 regarding pregnancy on day 10. We found an upregulation of IGF2 towards day 13 in superficial compartments in both groups, in line with previous results in cattle (McCarthy et al., 2012). The divergent IGF1/IGF2 day pattern is also seen in cattle during estrous cycle (Sosa et al., 2010) and support the concept that IGF1 and IGF2 have distinct functions (Simmen et al., 1995), where IGF2 may be more involved in later stages of pregnancy (Pantaleon et al., 2003; Sosa et al., 2010). Pregnant mares showed less staining intensity for IGF1R at day 7, opposite to what was found for this receptor transcriptional levels (Kalpokas et al., submitted), thought in agreement with previous studies in cattle showing both a downregulation regarding pregnancy and divergent transcript/protein results (McCarthy et al., 2012). However, the pattern seen for this receptor in pregnant mares contrast its potential role of enhancing secretory activity in endometrial glands (for review, see Wathes et al., 1998). On another hand, the downregulation towards day 13 seen

for both groups in glandular epithelium aligns with this receptor-mediation of E2 actions (Hantak et al., 2014).

As far as we know, this is the first report of equine endometrial MUC1 protein localization according to pregnancy. The pronounced levels of *MUC1* mRNA present at day 7 of pregnancy (Kalpokas et al., submitted) were not reflected in the present protein assessment. Is worth noting that the antibody used in the present study had shown not to bind to MUC1/SEC (Wilsher et al., 2013), an isoform of MUC1 which was found to be very abundant in the glands and uterine fluid during the secretory phase of human menstrual cycle (Hey et al., 2003) and that probably contributed to the high *MUC1* transcript levels in pregnant mares. Moreover, in the present study pregnant mares showed an upregulation towards day 13 post ovulation, in line with results showing no loss of MUC1 in equine endometrium during the initial conceptus–endometrium attachment (Wilsher et al., 2013). Indeed, studies in women reveal concomitant MUC1 pro-adhesive capacities (Carson, et al., 2006). There was an upregulation of MUC1 in the cyclic group between days 7 and 10 in SS; given the scarce information available for this glycoprotein localization in equine endometrium and that its regulation seems to differ from other species (de Ruijter-Villani and Stout, 2015), interpretation of this finding requires further investigation.

5. Conclusions

The present study show endometrial temporal and spatial immune cell response and protein localization of gestation-related components in cyclic and pregnant mares, being the more marked differences the immune cells counts and ERα-negative epithelium found in the pregnant group. The conceptus stage-dependant regulation on endometrial responsiveness is also cell-type specific and it is accomplished through different simultaneous and balanced mechanisms, encompassing the requirements of equine early pregnancy.

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7. Conflict of interest

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

8. Legends of the figures:

Table 1. Antibodies, type, suppliers and dilutions used for immunohistochemical analysis of estrogen receptor alfa (ERα), progesterone receptor (PR), oxytocin receptor (OXTR), prostaglandin-endoperoxide synthase 2 (PTGS2), insulin growth factor 1 (IGF1), insulin growth factor 2 (IGF2), type 1 IGF1 receptor (IGF1R) and mucin 1 (MUC1)

Primary antibody	Type	Catalog n°/supplier	Dilution	Secondary antibody type	Dilution
ERα	Mouse monoclonal	(C-311) sc-787/ Santa Cruz, USA	1:25	Anti mouse	1:200
PR	Mouse monoclonal	No.18-0172(PR-2C5)/ Zymed, USA	1:100	Anti mouse	1:200
OXTR	Goat policlonal	(C-20) sc-8102/ Santa Cruz, USA	1:50	Anti goat	1:200
PTGS2	Rabbit policlonal	Cat.160106/ Cayman, USA.	1:100	Anti rabbit	1:200
IGF1	Goat policlonal	(G-17)sc-1422/ Santa Cruz, USA	1:100	Anti goat	1:200
IGF2	Goat policlonal	(N-20)sc-1415/ Santa Cruz, USA	1:50	Anti goat	1:200
IGF1R	Rabbit policlonal	cat# ab 5497/ Abcam, UK.	1:50	Anti rabbit	1:200
MUC1	Goat policlonal	(C-20)sc-6827/ Santa Cruz, USA	1:25	Anti goat	1:200

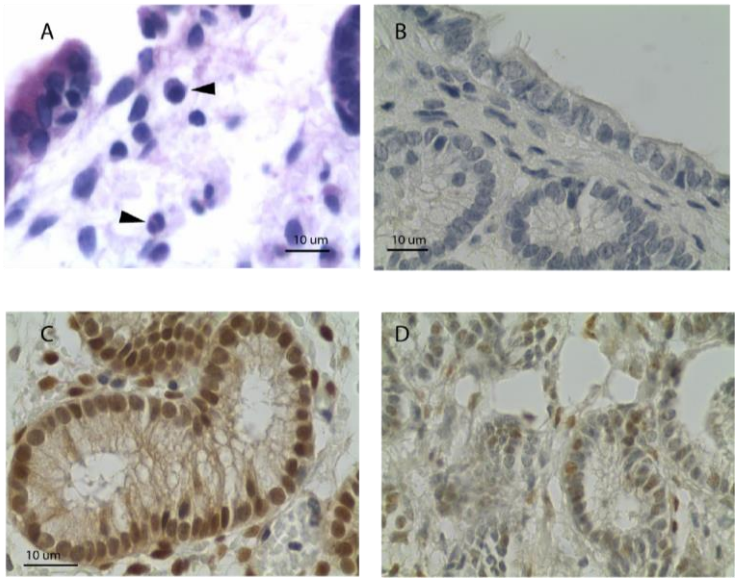
Table 2. Level of significance of fixed effects and interactions studied in the statistical model. Fixed effects shown for estrogen receptor α (ERα), progesterone receptor (PR), oxytocin receptor (OXTR), prostaglandin-endoperoxide synthase 2 (PTGS2), insulin growth factor 1 (IGF1), I insulin growth factor 2 (IGF2), type 1 IGF1 receptor (IGF1R) and mucin 1 (MUC1) are reproductive status, day, compartment (cmpt) and their interactions.

	Status	Day	Cmpt	*status*day*	*day*cmpt*	*status*day*cmpt*
ERα	***	***	NS	**	NS	NS
PR	NS	***	**	NS	**	0.057
OXTR	*	0.101	***	**	NS	*
PTGS2	**	NS	***	NS	NS	NS
IGF1	**	**	***	NS	NS	NS
IGF2	NS	**	***	*	*	*
IGF1R	*	**	***	NS	0.090	NS
MUC1	**	*	***	NS	NS	NS

NS: not significant, T 0.05 < P <0.10 *P < 0.05, ** P < 0.01, ***P < 0.001

Figure 1

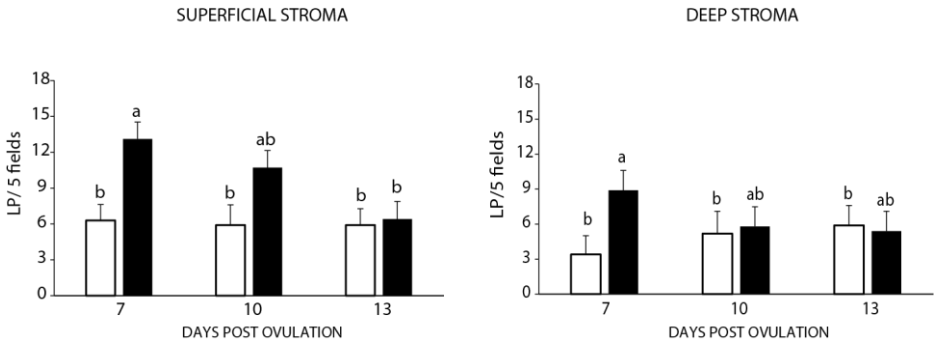
Microphotographs of H&E staining (with arrows indicating inflammatory cells) (A) and of immunohistochemical localisation. (B), negative control, (C), representative endometrial cross sections of de ipsilateral horn, deep glandular epithelium for progesterone receptor of the pregnant group at day 7 and (D) day 13 post ovulation. Original magnification x 1000.



393

394 Figure 2

395 Number of lymphocytes (LP) every 5 fields on day 7, 10 and 13 (day 0 = ovulation) in the
396 superficial and deep stroma of cyclic (white bars) or pregnant (black bars) mares. Data are the
397 least square mean ± pooled s.e.m. (a,b) different superscripts within the same graph differ: P <
398 0.05.



399

400 Figure 3

401 Microphotographs of immunohistochemical localisation of (A), representative endometrial
402 cross sections of de ipsilateral horn for oxytocin receptor at day 10 post ovulation of the cyclic
403 and (B) the pregnant group. (C), representative endometrial cross sections of de ipsilateral horn
404 for insulin growth factor 2 at day 10 post ovulation of the cyclic and (D) the pregnant group. (E),
405 representative endometrial cross sections of de ipsilateral horn for mucin 1 at day 10 post
406 ovulation of the cyclic and (F) pregnant group. Original magnification x 1000.

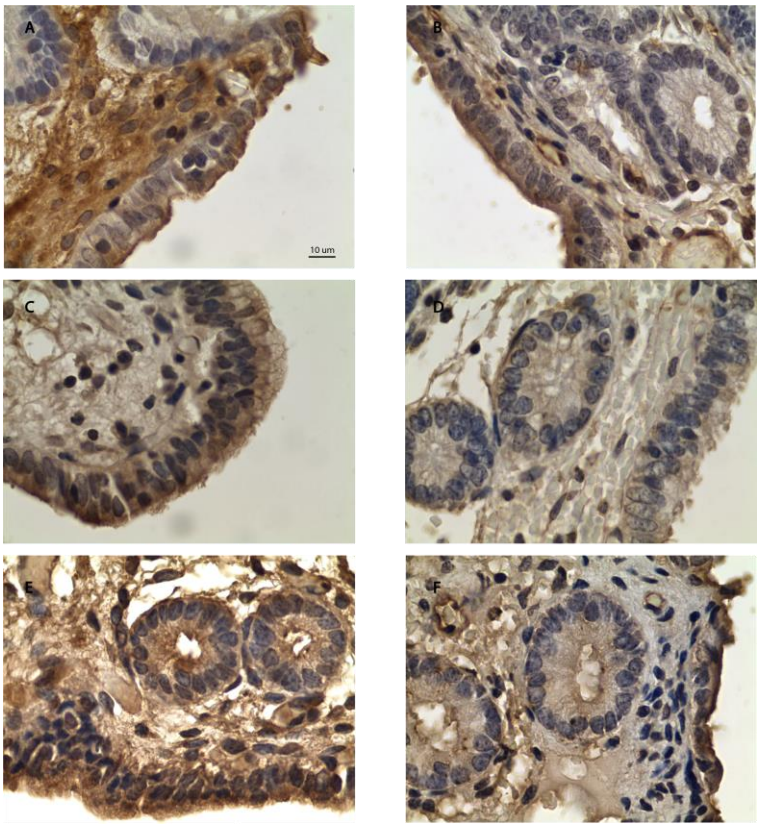


Figure 4

Staining intensity for oestrogen receptor α (ERα), progesterone receptor (PR), oxytocin receptor (OXTR) and prostaglandin-endoperoxide synthase 2 (PTGS2) on day 7, 10 and 13 (day 0=ovulation) in the endometrium of cyclic (white bars) or pregnant (black bars) mares. LE, luminal epithelium; SGE, superficial glandular epithelium; DGE, deep glandular epithelium; SS, superficial stroma; DS, deep stroma. Data are the least square mean±pooled s.e.m. (a,b) different superscripts within the same graph differ: $P < 0.05$.

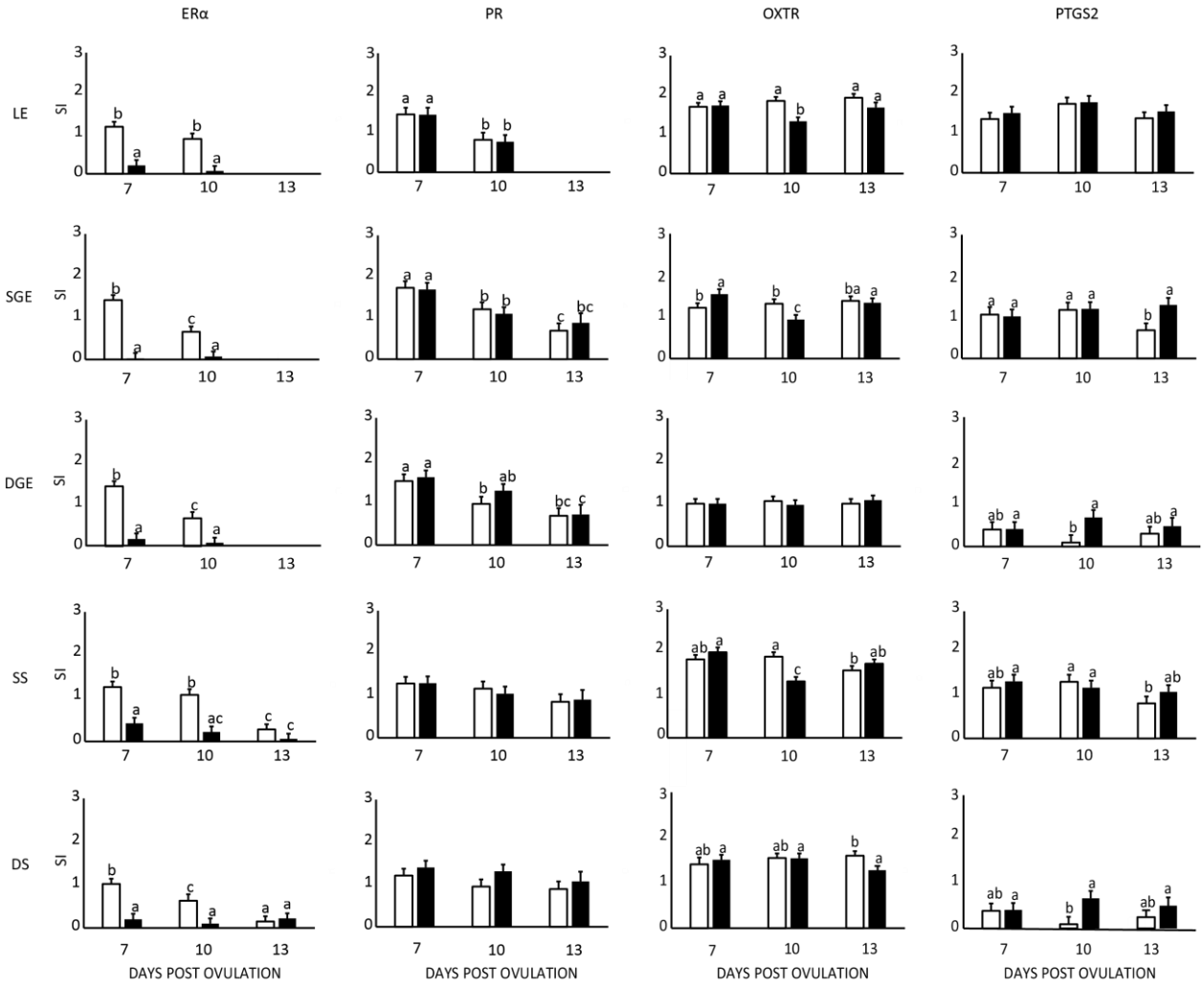


Figure 5

Staining intensity for insulin growth factor 1 (IGF1), insulin growth factor 2 (IGF2), type 1 IGF1 receptor (IGF1R) and mucin 1 (MUC1) on day 7, 10 and 13 (day 0=ovulation) in the endometrium of cyclic (white bars) or pregnant (black bars) mares. LE, luminal epithelium; SGE, superficial glandular epithelium; DGE, deep glandular epithelium; SS, superficial stroma; DS, deep stroma. Data are the least square mean ± pooled s.e.m. (a,b) different superscripts within the same graph differ: $P < 0.05$.

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