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SUBNUTRICIÓN, MORTALIDAD EMBRIONARIA Y FUNCION UTERINA EN OVINOS

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NOTA: Las calificaciones de aprobación de la Defensa de Tesis pueden ser B.B.B.-6 o S.S.S.-12

“No puedes nadar hacia nuevos horizontes hasta que tengas el coraje de perder de vista la orilla”

W. Faulkner

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Finalmente se cierra una etapa, no es el punto final, pero es el fin de un proceso largo que comenzó hace ya algunos años con la maestría. Termina esta etapa, con la ilusión de que lo aprendido salga a flote para el inicio de nuevos desafíos.

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TABLA DE CONTENIDO

APENDICE

Los experimentos llevados a cabo fueron sintetizados en los artículos que se especifican debajo y que en la presente tesis se refieren como Artículos I, II y III:

I. V. de Brun, A. Meikle, A. Fernández-Foren, F. Forcada, I. Palacín, A. Mechaca, C. Sosa, J. A. Abecia. Failure to establish and maintain a pregnancy in undernourished recipient ewes is associated with a poor endocrine milieu in the early luteal phase: *Animal Reproduction Science, 2016: 173, 80-86.*

II. V. de Brun, JJ Loor, H. Naya, M. Vailati-Riboni, O. Bulgari, K. Shahzad, J. A. Abecia, C. Sosa, A. Meikle. The embryo affects day 14 uterine transcriptome depending on the nutritional status in sheep. 1. Immune system and uterine remodeling**.**

III. V. de Brun, JJ Loor, H. Naya, M. Vailati-Riboni, O. Bulgari, K. Shahzad, J. A. Abecia, C. Sosa, A. Meikle. The embryo affects day 14 uterine transcriptome depending on the nutritional status in sheep. 2. Metabolic adaptation to pregnancy in control and undernourished ewes.

Los siguientes artículos completos fueron los antecedentes directos de la tesis doctoral y/o fueron realizados durante el transcurso del Doctorado y se adjuntan como Anexos.

ANEXOS

I) V. de Brun, J.A. Abecia, A. Fernández-Foren, M. Carriquiry, F. Forcada, M.I. Vázquez, A. Meikle, C. Sosa. Undernutrition and Laterality of the Corpus Luteum Affects Gene Expression in Oviduct and Uterus of Pregnant Ewes. *Spanish Journal of Agricultural Research*, 2013: 114, 989-996.

II) V. de Brun, A. Meikle, A. Casal, M. Sequeira, I. Contreras-Solis, M. Carriquiry, F. Forcada, C. Sosa y J.A. Abecia. Periconceptional undernutrition modifies endocrine profiles and hepatic gene expression in sheep. *Journal of Animal Physiology and Animal Nutrition*, 2015*: 99(4), 710-718*

III) V. de Brun, A. Meikle, A. Casal, M. Carriquiry, C. Menezes, F. Forcada, C. Sosa, J.A. Abecia. Hepatic expression of insulin-like growth factor-1 in underfed pregnant ewes. *Journal of Agricultural Science and Technology A*, 2016: 6, 355-362.

RESUMEN

La hipótesis inicial de este trabajo fue que la mortalidad embrionaria asociada a la subnutrición en el ovino se debe principalmente a efectos sobre el ambiente materno, siendo de menor impacto la historia nutricional del embrión. Se transfirieron embriones de buena calidad provenientes de ovejas donantes sometidas a una dieta de mantenimiento (control) o alimentados a la mitad de los requerimientos nutrcionales diarios (con subnutrición) a ovejas receptoras controles o sometidas a subnutrición. Se determinaron los perfiles endocrino-metabólicos en ovejas receptoras con subnutrición que mantuvieron o no la preñez luego de ser transferidas con embriones de buena calidad. Se encontró que cuando se transfieren embriones de buena calidad, independiente del estado nutricional de la oveja donante, las receptoras con subnutrición presentaron una mayor mortalidad embrionaria que las receptoras controles (Experimento I). Las receptoras subnutridas que mantuvieron la preñez presentaron mayores concentraciones plasmáticas de insulina y progesterona respecto a las hembras subnutridas que perdieron el embrión entre los días 18 y 40 de preñez (Experimento I). En el siguiente experimento se evaluó si la presencia del embrión induce cambios en el transcriptoma uterino de manera dependiente del plano nutricional, utilizando animales preñados y cíclicos controles o sometidos a subnutrición (Experimento II). La presencia del embrión al momento de reconocimiento materno de la preñez (día 14) estimuló genes relacionados con la remodelación uterina y vías del sistema inmune en hembras controles, pero eso ocurrió en menor medida en madres subnutridas. No obstante existen genes diferencialmente expresados en útero acorde a la presencia del embrión sólo en animales controles y sólo en animales subnutridos preñados vs cíclicos. En cuanto a la adaptación metabólica del útero a la preñez, se encontró que la presencia del embrión aumentó el flujo de nutrientes hacia el útero en ambos planos de alimentación, siendo menor en las hembras con subnutrición. Asimismo, los datos encontrados en el transcriptoma uterino sugieren que el embrión induce diferentes estrategias en la utilización de nutrientes entre hembras controles y con subnutrición. Estos resultados reafirman el concepto de que la subnutrición modifica el ambiente endócrino materno (sistémico y local), lo que podría explicar las mayores tasas de mortalidad embrionaria encontrada en animales sometidos a un período corto de restricción alimenticia.

SUMMARY

The hypothesis of this thesis was that embryo mortality associated to undernourishment is mainly due to effects on the maternal environment, being the nutritional history of the embryo of smaller impact in ovine (Experiment I). Good quality embryos from control and undernourished donors ewes were transferred to control and undernourished recipient ewes. Metabolic profiles were determined in undernourished recipient that succeed or not to maintain pregnancy after embryo transfer. Data suggested that when good quality embryos are transferred, embryo survival appears to be dependent principally on the maternal environment in which the embryo is developing, regardless of the previousnutritional history of the embryo. Undernourished pregnant ewes presented higher concentrations of insulin and progesterone on day 7 than undernourished that fail to maintain pregnancy (Experiment I). In the Experiment II, we have studied if the presence of an embryo induces changes in the uterus transcriptome in a nutritional-dependent manner (Experiment II). We found that the presence of the embryo at the time of maternal recognition of pregnancy (day 14), both in control and, to a lesser extent, in undernourished ewes, influenced the expression of genes related to uterine remodelling and involved in pathways of the immune system. However, relevant genes for the adaptation of the uterus to the embryo were differentially expressed between control pregnant vs cyclic and undernourished pregnant vs cyclic ewes. Regarding the metabolic adaptation of the uterus to pregnancy, we found that the presence of the embryo increased the nutrients flux to the uterus independently of the nutritional treatment, being lower in the undernourished animals. Data found in the uterine transcriptome suggest that the embryo induces different strategies in the utilization of nutrients between control and undernourished animals. These results evidenced that undernutrition modifies the maternal endocrine environment, which could explain the higher rate of embryo mortality found in animals subjected to a short period of food restriction.

INTRODUCCIÓN

Nutrición y reproducción

La eficiencia productiva y por lo tanto económica de los sistemas de explotación ovina está determinada por varios factores, siendo la eficiencia reproductiva uno de los factores más importantes. En rumiantes, se ha observado que entre un 25 y un 55 % de los embriones se pierden durante la gestación temprana (Niswender y Nett, 1994). La mayor causa de mortalidad embrionaria en la etapa de pre-implantación se debe a problemas en la señalización entre el embrión y la madre, lo que conduce a un desarrollo asincrónico, con retraso en el crecimiento del embrión (Goff, 2002). Una sincronía estricta entre el ambiente materno y el embrión es esencial para asegurar la supervivencia embrionaria.

El estado nutricional del animal es uno de los factores más importantes que afectan la función reproductiva en la hembra, ya que su acción puede ser ejercida en todos los niveles del eje reproductivo (hipotálamo, hipófisis, ovarios, útero) y sobre el embrión. La información sobre el estado metabólico es traducida al sistema reproductivo mediante un complejo sistema de señales (p. ej., hormonas, metabolitos, opioides endogenos) que modulan la función reproductiva.

Adaptación metabólica a la subnutrición

La adaptación metabólica a la subnutrición implica ajustes en la partición de nutrientes, promoviendo el uso alternativo de los mismos por los tejidos. Entre las diversas fuentes de nutrientes, los ácidos grasos (movilizados a partir de grasa corporal o de la dieta), el propionato (a partir de la fermentación microbiana), lactato (producido a partir de glucosa en tejidos musculares, intestinales y ruminal) y los aminoácidos (a partir de la dieta y descomposición de proteínas) son combustibles importantes en rumiantes. Es así que, durante un período de subnutrición, el hígado desvía parte de la acetil-CoA procedente de la oxidación de los ácidos grasos no esterificados (NEFA), hacia la formación de cuerpos cetónicos, es decir, b-hidroxibutirato y acetoacetato, los cuales son transportados por la

sangre hacia diversos tejidos donde son oxidados para producir energía (Chilliard et al., 1998).

Durante la subnutrición en rumiantes, el eje somatotrófico que involucra a la hormona del crecimiento (GH) y al factor de crecimiento similar a la insulina (IGF) se desacoplan en el hígado, lo que resulta en una reducción del IGF-I circulante, a pesar de las altas concentraciones de GH (Kobayashi et al., 1999). Este desacople puede ser el resultado de un estado de resistencia hepática a GH debido a la disminución de la expresión del receptor de GH (GHR), especialmente su isoforma 1A, como ha sido reportado durante el balance energético negativo (BEN) en vacas de leche (Kobayashi et al., 1999). El aumento de la concentración de GH durante el BEN disminuye la sensibilidad tisular a la insulina (receptores de insulina) y promueve la movilización de NEFA del tejido adiposo y su uso oxidativo por parte del resto del organismo (Bauman, 2000; Block et al., 2001; de Brun et al., 2015: Anexo II). Por lo tanto, el anabolismo se inhibe directamente por la disminución de las concentraciones de insulina y el aumento de la resistencia a la insulina de los tejidos, e indirectamente por la falta de estimulación de insulina sobre GHR que limita la síntesis hepática de IGF-I (Butler et al., 2003; Rhoads et al., 2004). Sin embargo, se ha observado que el efecto de la subnutrición o BEN sobre el mecanismo molecular hepático que explica el desacoplamiento del eje GH-IGF difiere entre especies. En vacas de carne y ovejas, si bien las concentraciones de IGF-I en sangre disminuyeron durante BEN o subnutrición, no se observó una reducción en el ARNm de *GHR-1A* e *IGF-I* hepático (Sosa et al., 2006a; Soca et al., 2013; Astessiano et al., 2014; de Brun et al., 2015; de Brun et al., 2016: Anexo III). La actividad de IGF-I está modulada por complejas interacciones con proteínas de unión a IGF (IGFBP) que alteran la disponibilidad del factor de crecimiento activo y sus receptores celulares (Jones y Clemmons, 1995). La mayor parte del IGF-I se une en un complejo ternario a IGFBP3 y a la subunidad ácido-lábil, sin embargo, durante el BEN se produce una disminución de IGFBP3 y un aumento de IGFBP2 modificando esta unión ternaria hacia el complejo IGF-I / IGFBP2, reduciendo la vida media de IGF-I (Jones y Clemmons, 1995).

Asimismo, las concentraciones de leptina y adiponectina, hormonas secretadas principalmente por el tejido adiposo, se han propuesto como índices del estado metabólico, así como como señales metabólicas para el sistema reproductivo (Blache et al., 2000). Las

concentraciones plasmáticas de leptina disminuyen en ovejas y vacas sometidas a subnutrición (Delavaud et al., 2000; Ciccioli et al., 2003; Meikle et al., 2004; Sosa et al., 2006; de Brun et al., 2015), lo que provoca una disminución en la tasa metabólica y mejora el consumo voluntario de alimento (Ingvartsen y Boisclair, 2001). Los datos sobre adiponectina en hembras rumiantes con subnutrición son limitados. Se ha observado una asociación negativa entre las concentraciones de adiponectina y la condición corporal (CC) en vacas lecheras durante el período seco (De Koster et al., 2017) y durante el período posparto en vacas de carne (Astessiano et al., 2014).

Si bien las investigaciones sobre la adaptación metabólica a la restricción de consumo de energía se han centrado en tejidos que participan activamente en la regulación del metabolismo como son el hígado y el tejido adiposo, la partición de nutrientes también afecta a todos los tejidos periféricos, siendo de principal interés para esta tesis, el tracto reproductivo.

Efecto de la subnutrición sobre la mortalidad embrionaria, la concentración de progesterona y la funcionalidad del tracto reproductivo

En ovinos se ha demostrado que tras 25 días de subnutrición inducida por la administración de la mitad de los requerimientos nutricionales diarios, la mortalidad de los embriones aumenta al día 11 de la gestación (Rhind et al., 1989). En otros trabajos, se ha recuperado un porcentaje similar de embriones en ovejas con subnutrición y controles los días 4, 8 y 9 (aunque los embriones de ovejas sometidas a subnutrición presentaban un retraso en su desarrollo), pero fue menor en los días 14 y 15 de gestación (Abecia et al., 1997; 1999a; Lozano et al., 2003). Más recientemente, nuestro grupo ha demostrado que en animales alimentados con una dieta a la mitad de los requerimientos de mantenimiento (0.5M) durante 21 días, se produce una reducción en el número de embriones totales y embriones transferibles viables, en comparación con animales con una dieta de mantenimiento al día 7 de gestación (Abecia et al., 2015). A su vez, se ha demostrado previamente que la subnutrición afecta la funcionalidad de los folículos pre-ovulatorios, pudiendo comprometer la fertilidad (Sosa et al., 2010). Por lo tanto, las menores tasas de gestación encontradas en ovejas sometidas a subnutrición pueden deberse a un inadecuado desarrollo embrionario

ocasionado por problemas en el desarrollo del ovocito, lo que llevaría a tener menores tasas de fertilización, y/o a alteraciones en el tracto reproductivo que impiden un ambiente favorable para el desarrollo de la gestación. *Al momento de iniciar esta tesis no se encontraban estudios diseñados para aislar estos procesos y determinar el impacto de la subnutrición en los mismos, es decir, ¿el impacto de la subnutrición sobre las pérdidas embrionarias se debe principalmente a alteraciones a nivel del embrión o del ambiente materno del tracto uterino?*

La progesterona (P4) tiene un papel fundamental preparando al útero para una posible gestación, estimulando la producción de una variedad de secreciones endometriales necesarias para el desarrollo exitoso del embrión, entre los que se encuentran los factores de crecimiento. La literatura internacional es consistente que en ovinos, las concentraciones plasmáticas de P4 están inversamente relacionadas con el nivel nutricional (Williams y Cumming, 1982; Parr et al., 1987; Rhind et al., 1989; Lozano et al., 1998; O'Callaghan et al., 2001). Sin embargo, la producción *in vitro* de P4 por el cuerpo lúteo (CL) no se ha visto afectada por la subnutrición (Abecia et al., 1995; 1997; 1999), y se ha demostrado que ovejas con subnutrición tienen similares niveles de P4 en la vena ovárica y en la arteria uterina que ovejas controles (Abecia et al., 1997; Lozano et al., 1998). Por ello, se ha reportado que los mayores niveles plasmáticos de P4 en animales sometidos a subnutrición no se explicarían por una mayor síntesis de la hormona sino por una menor metabolización hepática de la misma, dado que las ovejas con subnutrición presentaban hígados más livianos y un menor flujo sanguíneo en la vena porta (Parr, 1992). A pesar de que se encontraron mayores niveles plasmáticos de P4 en ovejas sometidas a subnutrición, Lozano et al., (1998) observaron que estos animales tenían menores concentraciones de P4 en el tejido endometrial comparadas con las ovejas controles, lo que fue consistente con los menores contenidos de receptores de progesterona (RP) reportados en el útero al día 5 del ciclo estral (Sosa et al., 2004). Los autores sugirieron que los menores contenidos de P4 y de RP endometrial podrían estar relacionados con el retraso en el desarrollo de los embriones y las menores tasas de gestación que se han observado en ovejas sometidas a subnutrición. En el mismo sentido, la subnutrición en ovejas se asoció con una menor expresión de ARNm de *IGF-I* en el útero y de *IGF-II* en el oviducto al día 5 de la preñez (de Brun et al., 2013: Anexo I). Dado que estos

factores son promotores del crecimiento embrionario, estos hallazgos son consistentes con la función conocida de P4 sobre la supervivencia embrionaria y con las menores tasas de gestación reportadas en ovejas con subnutrición (Rhind et al., 1989ab).

Si bien existen escasos reportes, se ha demostrado que la subnutrición disminuye la capacidad de síntesis del interferón tau (IFNt - señal de reconocimiento materno en rumiantes) por parte del embrión (Abecia et al., 1999). Las acciones del IFNt se encuentran mediadas por el receptor de interferón (alfa y beta) (IFNAR), que consta de dos subunidades, IFNAR1 e IFNAR2, localizados principalmente en el epitelio luminal del endometrio uterino (Rosenfeld et al., 2002), aunque existe evidencia que puede alcanzar el estroma, el miometrio e incluso las células inmunes circulantes (Ott et al., 1998; Hicks et al., 2003; Shirasuna et al., 2012). Gran parte de la investigación sobre el IFNt se ha centrado en la manutención del CL (reconocimiento materno de la preñez) y la expresión génica uterina a través de la estimulación por IFNt; sin embargo, la función del IFNt en la aceptación inmunológica del embrión por la madre no ha sido bien caracterizada. Una de las funciones del IFNt es promover un delicado balance entre las respuestas pro- y anti-inflamatorias para mantener la integridad del sistema inmune materno y prevenir el rechazo del embrión (Hansen et al., 1997; Ott et al., 1998; Choi et al., 2003; Song et al., 2007; Mansouri-Attia et al., 2012). En este sentido, se ha reportado que el IFNt induce el aumento de expresión de *ISG15* (Ubiquitin-like modifier 15) (Austin et al., 2004; Johnson et al., 1999), *CXCL10* (quimoquina motivo C-X-C ligando 10), *MX1* (Myxovirus resistance 1), *MX2* (Myxovirus resistance 2) (Hicks et al., 2003) y *OAS1* (2',5'-oligoadenilato sintasa 1) (Short et al., 1991; Schmitt et al., 1993), los cuales presentan funciones antivirales y antiproliferativas.

Una alteración en el ambiente materno como consecuencia de una restricción alimenticia, puede comprometer una adecuada comunicación embrio-maternal, incidiendo sobre la mortalidad embrionaria. En este sentido, se ha estudiado en vacas en lactación la expresión génica uterina durante la preñez temprana, y se observó una expresión génica diferencial en el endometrio intercaruncular al día 17 (alrededor del reconocimiento materno en bovinos), respecto a la presencia del embrión y la lactancia (Cerri et al., 2012). Si bien la expresión endometrial de genes implicados en el sistema inmune se vio afectada de forma muy marcada debido a la presencia del embrión, la lactancia (subnutrición fisiológica) también modificó

genes relacionados con inmunoglobulinas, por lo que se sugirió que la misma podría causar un desequilibrio inmune con posibles efectos negativos sobre la supervivencia del concepto (Cerri et al., 2012). Además, también observaron que la lactancia afectó a la expresión de genes implicados en la homeostasis de la glucosa, lo que podría ser nocivo para el desarrollo del embrión.

Por otro lado, Lesage-Padilla et al. (2017) no encontraron cambios respecto al estado metabólico sobre la expresión endometrial de genes regulados por el concepto en bovinos al día 19, sugiriendo que la capacidad endometrial de responder a las señales embrionarias cuando ocurre la implantación no se ve afectada por el metabolismo materno (Lesage-Padilla et al., 2017). Estudios de expresión génica realizados en el endometrio ovino al día 14 de la preñez o ciclo estral, en animales subnutridos o no, mostraron que la subnutrición no produce grandes cambios de genes y proteínas relacionadas al reconocimiento materno en animales preñados (Sosa et al., 2009b). Es posible que los genes seleccionados para el estudio de expresión génica en el endometrio no sean los adecuados para evaluar cambios en la señalización embrio-maternal, por lo que se hace imprescindible evaluar los niveles de expresión génica a un nivel más detallado.

A pesar de que se conoce que la subnutrición aumenta la mortalidad embrionaria (Abecia et al., 2006), no están claras las bases de los mecanismos moleculares implicados, ni tampoco como las ovejas sometidas a una restricción alimenticia aguda son capaces de mantener la gestación. Asimismo, si bien se han reportado diferencias de expresión en el transcriptoma del endometrio entre animales preñados y cíclicos en bovinos y cerdos (Cerri et al., 2012; Ponsuksili et al., 2012; Kiewiszet al., 2014), *no hemos encontrado estudios sobre la interacción de la presencia de un embrión y el plano nutricional en el transcriptoma endometrial en ninguna especie. En la presente tesis se busca contribuir con el conocimiento respecto a los efectos de la subnutrición y la presencia del embrión sobre la expresión génica uterina.*

HIPÓTESIS

La mortalidad embrionaria asociada a la subnutrición en el ovino depende principalmente del ambiente materno en que se desarrolla el nuevo embrión, teniendo un menor impacto la historia nutricional previa del mismo.

La presencia del embrión alrededor del reconocimiento materno en ovinos induce cambios en el transcriptoma uterino y esto depende del plano nutricional.

La adaptación metabólica del útero a una restricción de nutrientes depende de la presencia del embrión.

OBJETIVOS

General

Estudiar los factores que afectan la sobrevivencia embrionaria haciendo énfasis en endocrinología metabólica y expresión génica uterina al momento de reconocimiento materno de la preñez.

Específicos

1. Indagar respecto del origen de las pérdidas embrionrias: ¿es debido a efectos producidos en el embrión, el ambiente materno o ambos? Determinar los perfiles endócrino metabólicos en ovejas receptoras que mantienen o no la preñez luego de ser transferidas con embriones de buena calidad bajo dos planos de alimentación (Experimento I, Artículo I).
2. Determinar el efecto de la presencia del embrión y la subnutrición sobre el transcriptoma en el cuerno uterino ipsilateral al cuerpo lúteo al momento del reconocimiento materno de la preñez (día 14) (Experimento II, Artículos II y III).

METODOLOGÍA

Diseños experimentales

Aspectos generales

Los experimentos I y II de esta tesis se realizaron en el Servicio de Experimentación Animal de la Facultad de Veterinaria de la Universidad de Zaragoza, España (Latitud 41º41'N), utilizando ovejas adultas de la raza Rasa Aragonesa durante la estación reproductiva descrita en cada uno de los artículos (Forcada et al.,1992). Los protocolos fueron aprobados por la Comisión Ética para la Experimentación Animal de la Universidad de Zaragoza y el Comité de Ética de la Facultad de Veterinaria, Uruguay.

Los animales se alimentaron una vez al día (por la mañana) en base a una dieta compuesta por concentrado y paja de cebada, con libre acceso al agua. El concentrado estuvo compuesto por cebada (85%) y soja (15%). En el Experimento I los animales se alimentaron con dietas para proveer 1,5 o 0,5 veces los requerimientos diarios de mantenimiento (M) (AFRC, 1993). De cuerdo a diversos antecedentes (Rhind et al., 1989; Abecia et al., 1995; 1997; Lozano et al.,1998; Sosa et al., 2004; 2006; 2009), la dieta 1,5 M ofrecida colectivamente asegura un mantenimiento del peso vivo (PV) y la condición corporal (CC) (grupo control), mientras que la dieta 0,5 M provoca una disminución en el mismo período de tiempo de aproximadamente un 10% tanto en el PV como en la CC (grupo bajo).

En el Experimento II los animales fueron alojados en corrales individuales, y se los alimentó para proveer una dieta de 1 M o 0.5M.

El PV y la CC de los animales se controlaron periódicamente durante los experimentos. La CC fue determinada por un único observador por palpación de las apófisis vertebrales en la región del lomo en una escala de 0 (emaciada) a 5 (obesa) (Russel et al., 1969).

Para sincronizar los celos se utilizó un tratamiento intravaginal de 14 días con esponjas impregnadas en progestágenos (Acetato de Fluorogestona, 40mg, Intervet S.A., Salamanca, España), al cabo del cual se administraron entre 285 y 300 UI de gonadotrofina coriónica equina (eCG, Intervet S.A., Salamanca, España) por vía intramuscular. Los celos (identificados como día 0 del ciclo estral o de la gestación) se detectaron cada 8 horas mediante machos vasectomizados.

Se tomaron muestras de sangre de la vena yugular en tubos con heparina. Las muestras se centrifugaron durante 10 min a 1000 g y el plasma se almacenó a –20ºC hasta su análisis.

En la Fig. 1 se muestra una representación esquemática de los dos experimentos.

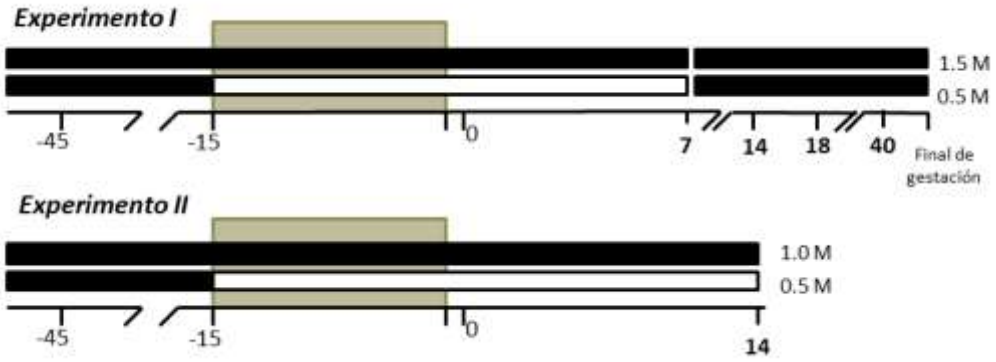

Fig. 1. Representación esquemática de los diseños experimentales. Grupo control (barras negras); grupo con subnutrición (barras blancas). El área gris indica el período del tratamiento con progestágenos para la sincronización de celos. El día 0 indica el inicio del celo, previo a la transferencia de embriones al día 7 (de hembras donantes controles y con subnutrición a hembras receptoras controles y sometidas a subnutrición), determinando tasas de gestación a los días 18 y 40 (Experimento I), o previo al sacrificio el día 14 (Experimento II).

Experimento I (Artículo I): "Failure to establish and maintain a pregnancy in undernourished recipient ewes is associated with a poor endocrine milieu in the early luteal phase".

Se seleccionaron 197 Ovejas Rasa Aragonesa con un PV aproximado de 57.1 ± 1.4 kg y una condición corporal de 2.7 ± 0.1. De estos animales 45 fueron utilizadas como donantes (20 Control y 25 con Subnutrición) y 52 (25 Control y 27 con Subnutrición) como receptoras de embriones. El resto de los animales no mostraron celo o no presentaron embrión al día 14. Las dietas diferenciales se iniciaron al momento de la colocación de las esponjas intravaginales y se mantuvieron hasta el día de la colecta de embriones (día 7). Las ovejas donantes fueron superovuladas previo a la remoción de las esponjas. Los detalles de los

protocolos y las dosis de hormonas para el tratamiento de superovulación y de procedimiento experimental se describen en el Artículo I.

Las ovejas receptoras recibieron al día 7 dos embriones de buena calidad (mórulas compactas y blastocitos tempranos y expandidos) mediante laparotomía ventral media. Los efectos del nivel de energía recibido en las ovejas donantes sobre los embriones y niveles hormonales y metabólicos se han descrito previamente (Abecia et al., 2015). Se colectaron muestras de sangre los días −14, −1, 7 y 18 para determinar las concentraciones plasmáticas de ácidos grasos no esterificados (NEFA), insulina, IGF-I, leptina y adiponectina. La progesterona se determinó los días 7 y 18. Preñez temprana se denominó cuando las concentraciones plasmáticas de progesterona al día 18 estaban por encima de 1 ng/mL. Al día 40, la tasa de preñez se definió por medio de ultrasonografía. Las pérdidas embrionarias tardías se definieron cuando una oveja estaba preñada el día 18, pero no el día 40.

Experimento II (Artículos II y III):

- *"The embryo affects day 14 uterine transcriptome depending on the nutritional status in sheep. 1. Immune system and uterine remodelling"*
- *"The embryo affects day 14 uterine transcriptome depending on the nutritional status in sheep. 2. Metabolic adaptation to pregnancy in control and undernourished ewes"*

Se utilizaron 46 ovejas Rasa Aragonesa (21 Controles y 25 con Subnutrición) con un PV de 61.5 ± 0.4 kg y una condición corporal de 3.4 ± 0.1. El día 0 fue definido como el día del estro detectado mediante el uso de machos vasectomizados, y hembras fueron o no sometidas a monta natural con machos fértiles. Al día 14 de gestación o del ciclo estral los animales fueron sacrificados y se extrajeron muestras del tercio craneal del útero. Los grupos experimentales fueron los siguientes: controles preñados (n=6), controles cíclicos (n=5), subnutrición preñados (n=7), subnutrición cíclicos (n=6). Los detalles del procedimiento experimental se describen en los artículos II y III.

Hormonas y metabolitos

Todas las hormonas fueron analizadas por radioinmunoanálisis (RIA) o usando un ensayo inmunorradiométrico (IRMA). Las concentraciones de IGF-I e insulina se determinaron por IRMA en fase sólida, utilizando kits comerciales según Sosa et al. (2009)y Fernández-Foren et al. (2011). Las concentraciones de adiponectina y leptina se determinaron según lo descrito por Raddatz (2008) y Fernández-Foren et al. (2011), respectivamente. Las concentraciones de progesterona se determinaron por RIA en fase sólida utilizando kits comerciales según Sosa et al. (2006) (Artículos I y II). La glucosa y los NEFA se analizaron mediante kits comerciales (Weiner Laboratory, Rosario, Argentina y Nefa-C, Wako Chemicals GmbH, Alemania, respectivamente) en un auto analizador Vitalab spectra 2 (Vital Scientific NV, Dieren, Países Bajos) (Artículo III). La información de la sensibilidad y precisión de los ensayos se encuentran en los artículos respectivos.

Extracción de ARN

El ARN total del útero se extrajo utilizando TRIzol (Invitrogen, Carlsbad, CA, EE. UU.), seguido de precipitación con cloruro de litio para eliminar los inhibidores de la síntesis de ADNc y ADNasa (Ambion, Austin, TX, EE. UU) para eliminar el ADN contaminante. La concentración del ARN se determinó midiendo la absorbancia a 260 nm, la pureza de todos los ARN extraídos se evaluó como la relación de absorbancia a 260/280 nm (A260 / 280) y la integridad del ARN se determinó mediante electroforesis (en un 1% gel de agarosa) y Agilent Bioanalyzer (tecnologías Agilent). Todas las muestras tenían un promedio de relación de A260 / 280 de 1.95 ± 0.21 y un RIN de 8 ± 1.4 (Artículo II y III)

Microarrays

Síntesis, etiquetado y purificación de ARNc

La plataforma de chip de microarray de expresión génica Agilent 15K Sheep (Agilent Technologies Inc.) se usó siguiendo los protocolos del fabricante. La muestra de referencia RNA consistió en un conjunto de todas las muestras de animales en el estudio. Brevemente,

se usaron un total de 200 ng de ARN por muestra (o grupo de referencia), para generar la primera hebra de cDNA, que se transcribió de forma inversa a ARNc usando el kit Low-Input Quick Amp Labeling kit (Agilent Technologies Inc). El ARNc resultante se marcó con Cy3 (muestras) o con colorante fluorescente Cy5 (referencia), se purificó usando columnas RNeasy Mini Spin (Qiagen), y posteriormente se eluyó en 30 μL de agua libre de DNasa-RNasa. El NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA) se utilizó para confirmar los criterios recomendados por el fabricante para el rendimiento y la actividad específica de al menos 0,825 μg y ≥6. El ARNc marcado se fragmentó y luego se hibridó con el portaobjetos de microarrays de acuerdo con el protocolo del fabricante. Los portaobjetos se lavaron de acuerdo con los procedimientos recomendados por el fabricante y se escanearon usando un escáner GenePix 4000B (Axon Instruments Inc., Sunnyvale, CA) y el software GenePix Pro v.6.1. Los puntos resultantes donde las características eran deficientes se marcaron y se excluyeron del análisis posterior. Se utilizaron 3 o 4 muestras por grupo, ya que dos slides tuvieron que ser descartados, correspondientes a los grupos control cíclico y control preñado.

Análisis del enriquecimiento de vías mediante Gene Ontology

Los genes regulados diferencialmente se anotaron con funciones biológicas y moleculares utilizando el sistema de clasificación PANTHER (Análisis de proteínas a través de relaciones evolutivas) (http://www.pantherdb.org/) (Mi et al., 2017). Para el mismo, se utilizaron las listas de genes diferencialmente expresados para cada comparación (efecto de la presencia del embrión y efecto del tratamiento nutricional), considerando un fold-change (FC) ± 1.5 veces, un valor de $p \leq 0.5$ y FDR ≤ 0.2. Se utilizó el homólogo bovino correspondiente al gen ovino que representa cada transcripto identificado como expresado diferencialmente.

PCR en tiempo real y verificación de microarray

Para verificar algunos de los hallazgos importantes del análisis de microarrays y bioinformática, se analizó, mediante PCR en tiempo real, el nivel de expresión de 5 genes diferencialmente expresados por grupo (n=6 muestras por grupo).

La expresión génica se determinó mediante PCR en tiempo real o cuantitativo (qPCR), utilizando el equipo Rotor-GeneTM 6000 (Corbett Life Sciences, Sydney, Australia). Los detalles de los procedimientos de las técnicas se describen en el artículo II. La eficiencia (E) de los ensayos fue calculada acorde a la fórmula E= (10-1/slope-1) según Rutledge y Côté (2003) (Artículo II). La expresión génica fue medida por cuantificación relativa al control exógeno (Pfaffl 2001) y normalizada a la media geométrica de los genes de control endógeno, tomando en consideración las respectivas eficiencias de amplificación.

Análisis estadístico

Artículo I

La tasa de preñez temprana y tardía y la mortalidad embrionaria tardía se analizaron utilizando el software SAS (versión 9.0, SAS Institute Inc., Cary, NC, Estados Unidos), mediante el procedimiento GENMOD. El modelo estadístico incluyó los efectos fijos del tratamiento nutricional (subnutrido vs. control) de las ovejas donantes, de las receptoras y la interacción entre ambas.

Para el análisis de medidas repetidas (hormonas), se incluyeron en el modelo estadístico el tratamiento, el día (observaciones) y el estatus (preñez o no), y las interacciones entre ellos. Se realizaron análisis de normalidad (procedimiento Univariate, SAS), en todas las variables para identificar valores atípicos y verificar la normalidad de los residuales.

Las diferencias se consideraron significativas cuando $P \leq 0.05$, tendencia cuando $0.05 \leq P \leq 0.10$ y $0.05 \leq P \leq 0.15$ para mortalidad embrionaria.

Artículos II y III

Para el procesamiento de datos de microarray se utilizó el paquete limma en R (http://www.r-project.org), donde se ingresaron los datos crudos para la corrección de fondo (Lowess) y normalización de los mismos utilizando el método de cuantiles. Luego, se utilizó el paquete limma para el análisis estadístico, aplicando el método empírico de Bayes propuesto por Smyth (Smyth, 2004). Este método calcula una estadística t moderada para la expresión

diferencial de cada gen al realizar un ajuste de modelo lineal en los datos. Se aplica un paso de Bayes empírico para moderar los errores estándar de los fold-change (FC) estimados y producir estimaciones más estables, especialmente cuando el número de réplicas es pequeño. Las diferencias en la expresión génica se consideraron significativas utilizando un valor de FC mayor o menor a 1.5, un $P \leq 0.05$ y un FDR < 0.2.

Las determinaciones de transcriptos se analizaron por análisis de varianza utilizando un modelo mixto del software SAS (versión 9.0, SAS Institute Inc., Cary, NC, Estados Unidos). La preñez, la nutrición y la interacción entre ambos se incluyó en el modelo estadístico.

RESULTADOS Y DISCUSIÓN

Experimento I (Artículo I): "*Failure to establish and maintain a pregnancy in undernourished recipient ewes is associated with a poor endocrine milieu in the early luteal phase*"

Para aislar los efectos de la subnutrición en el embrión o la madre se utilizó un modelo de transferencia embrionaria. En las ovejas donantes superovuladas, la subnutrición a la mitad de los requerimientos diarios resultó en una reducción del número de embriones recuperados y de embriones viables transferibles, de modo que las ovejas sometidas a subnutrición presentaron una tasa de preñez un 27% más baja y una tasa de transferibilidad un 30% más baja respecto a ovejas controles (Abecia et al., 2015). Al transferir embriones de buena calidad al día 7 de gestación (mórulas compactas y blastocitos expandidos) de madres donantes controles y sometidas a subnutrición a receptoras controles y sometidas a subnutrición, no se encontraron diferencias en el establecimiento de la preñez al día 18 (Tabla 1). Sin embargo, independientemente del origen del embrión, se encontró una tendencia a una mayor mortalidad embrionaria al día 40 en las ovejas receptoras sometidas a subnutrición respecto a las receptoras controles (35% vs. 14%, P= 0.11, Tabla 1). Por lo tanto, nuestros resultados sugieren que cuando se transfieren embriones de buena calidad, la supervivencia del embrión parece depender principalmente del ambiente materno en el que se desarrolla, independientemente de la historia nutricional previa del mismo.

Tabla 1. Tasa de preñez y mortalidad embrionaria tardía (diferencia entre las ovejas preñadas el día 18 y el día 40) en ovejas receptoras controles (Receptora C) y sometidas a subnutrición (Receptora S), luego de recibir embriones transferidos provenientes de donantes controles (Donante C) y subnutridas (Donante S).

	Donante C Receptora C	Donante S Receptora C	Donante C Receptora S	Donante S Receptora S	P valor
Tasa de preñez día 18	79% (11/14)	91% (10/11)	80% (12/15)	92% (11/12)	NS
Tasa de preñez día 40	71% (10/14)	73% (8/11)	53% (8/15)	58% (7/12)	0.17
Mortalidad embrionaria	9.1% (1/11)	20% (2/10)	33.3% (4/12)	36.4% (4/11)	0.11

Las ovejas receptoras sometidas a subnutrición que mantuvieron la preñez, presentaron mayor PV y mayores concentraciones plasmáticas de insulina, que los animales subnutridos que perdieron el embrión entre los días 18-40 (Artículo I, Tabla 2). La mayor concentración de insulina en estos animales al día 7, sugiere que estos animales fueron más eficientes en enfrentar la subnutrición (mejor estado energético). La menor muerte embrionaria en estos animales puede ser el resultado del mejor estado y/o de las acciones directas de la insulina actuando, a través de sus propios receptores o de IGF-I presentes en el endometrio de la oveja o del propio embrión (Fowden et al., 1989; Gluckman y Pinal, 2003; Dupont et al., 2014).

Además, se observó que las ovejas receptoras subnutridas que mantuvieron la preñez presentaron mayores concentraciones plasmáticas de P4 al día 7 que las sometidas a subnutrición que perdieron el embrión entre los días 18 y 40 (Tabla 2). Los altos niveles plasmáticos de P4 encontrados al día 7 en estas ovejas receptoras subnutridas preñadas, se asocian con la mayor tasa ovulatoria encontrada en estos animales respecto a los subnutridos no preñados (Artículo I). Esta mayor tasa ovulatoria en animales subnutridos que mantuvieron la preñez es consistente con resultados recientes de nuestro grupo (Fernández-Foren et al., en prensa), donde reportamos que una mayor proporción de ovejas con mejores reservas corporales presentaron más de un CL, mayores concentraciones plasmáticas de P4 y una tendencia a presentar un mayor número de embriones transferibles viables. Asimismo, las mayores concentraciones de progesterona en ovejas con subnutrición que mantuvieron la preñez también se pueden explicar por las mayores concentraciones de insulina en este grupo, ya que se ha demostrado que la síntesis de P4 en las células luteales es estimulada por la

insulina (Wathes et al., 1995). La progesterona tiene la habilidad de modificar la relación embrio-maternal estimulando los cambios en el estado fisiológico del útero para influir sobre la sobrevivencia embrionaria (Lawson y Cahill, 1983). De hecho, altos niveles plasmáticos de P4 post concepción, se han asociado con la promoción de la elongación del embrión, aumentos en la producción de IFNt y mayores tasas de preñez en ovejas (Ashworth y Bazer, 1989; Satterfield et al., 2006). Sin embargo, los niveles plasmáticos de P4 no siempre son un reflejo de las concentraciones de P4 en el tracto reproductivo. En efecto, si bien la progesterona circulante se encuentra inversamente relacionada con el nivel de nutrición, se ha reportado que animales subnutridos presentan menores concentraciones de P4 en el útero, respecto a animales controles asociado a una menor concentración de su receptor (RP) (Lozano et al., 1998; Sosa et al., 2006a; Fernández-Foren et al., en prensa).

Tabla 2. Peso vivo (PV, kg) y concentraciones plasmáticas de ácidos grasos no esterificados (AGNE, mM), insulina (uUI/mL) al día 7 y progesterona (ng/mL) al día 7 y 18, en ovejas preñadas controles y con subnutrición y ovejas con subnutrición que presentaron mortalidad embrionaria entre los días 18-40.

	Control Preñadas (n=18)	Subnutridas Preñadas (n=15)	Mortalidad embrionaria (n=8)	Error estándar
PV	57.70^{a}	55.70^{b}	49.3^{c}	±1.7
AGNE	0.12^{a}	0.18^{b}	0.20^{b}	±0.2
Insulina	35.32^{a}	33.46^{a}	29.27^{b}	±1.5
P4 día 7	5.73^{abx}	9.62^{ya}	5.12^{b}	±1.9
P4 día 18	9.43	11.53	8.76	±2.0

Diferentes superíndices dentro de la misma fila diferencian a vs. b en $P < 0.05$ y x vs. y en $P \leq 0.1$.

Por otro lado, la subnutrición no afectó las concentraciones plasmáticas de IGF-I en los animales de este estudio, lo cual contradice previos resultados de nuestro grupo donde encontramos una menor concentración plasmática de esta hormona en animales subnutridos respecto a controles (de Brun et al., 2015: Anexo II), asociado a un menor peso hepático (de Brun et al., 2016: Anexo III). Por otro lado, se debe tener en cuenta que la concentración

local de IGF-I en el útero que modifica la funcionalidad uterina, y que estimula el desarrollo embrionario, no solo depende de la síntesis hepática de IGF-I, sino también de la síntesis local del endometrio. En este sentido, en estudios previos (de Brun et al., 2013) hemos demostrado que la subnutrición a la mitad de los requerimientos diarios disminuye la expresión génica de *IGF-I* en útero y de *IGF2* en oviducto.

Es interesante notar que no se encontraron diferencias en las concentraciones de insulina (datos no mostrados) ni de progesterona al día 18 (Tabla 2) entre las ovejas subnutridas que mantuvieron la preñez y ovejas subnutridas con mortalidad embrionaria tardía. Esto sugiere que el ambiente endocrino temprano (día 7) podría haber condicionado el desarrollo del embrión en ovejas con subnutrición, lo que llevaría a la pérdida del embrión después del día 18 de preñez, lo cual es consistente con reportes previos donde se observan cambios en el ambiente uterino debido a la subnutrición durante la fase luteal temprana (día 5), pero no se observan alteraciones en el ambiente materno frente a una restricción alimenticia al día 10 o 14 post estro (Sosa et al., 2004; 2006a; 2009).

En resumen, los resultados preliminares de este trabajo indican que la supervivencia embrionaria cuando embriones de buena calidad son transferidos, es dependiente principalmente del ambiente materno en el que se desarrolla el embrión, siendo independiente del origen del mismo (donantes controles o sometridas a subnutrición). Por lo tanto, el estudio de la funcionalidad uterina cuando está presente el embrión, el tratamiento nutricional, y su interacción, pueden facilitar la comprensión de los mecanismos que influyen en el éxito de la preñez.

Experimento II (Artículos II y III): "*The embryo affects day 14 uterine transcriptome depending on the nutritional status in sheep. 1. Immune system and uterine remodeling*" y "*2. Metabolic adaptation to pregnancy in control and undernourished ewes*"

Peso vivo, condición corporal y concentración plasmática de hormonas y metabolitos

Los animales subnutridos mostraron una disminución progresiva de peso vivo y condición corporal durante el experimento, mientras que el grupo control mantuvo estos parámetros estables (Artículo III).

Las concentraciones de NEFA fueron mayores en animales subnutridos respecto a los controles. La subnutrición redujo o tendió a reducir las concentraciones plasmáticas de insulina y leptina al día 13, consistente con reportes previos (Chilliard et al., 1998; Abecia et al., 2006; Fernández-Foren et al., 2011). La disminución de estas hormonas anabólicas es el resultado de la restricción de energía y permite el ahorro energético por disminución de la síntesis biomolecular. Las concentraciones plasmáticas de IGF-I tendieron a ser menores en los animales preñados respecto a los cíclicos al día 13 (Artículo II) lo que puede deberse a una mayor captación por parte del útero, ya que el IGF-I durante la preñez temprana actúa directamente sobre el embrión o indirectamente modulando las secreciones uterinas y estimulando la proliferación celular (Wathes et al., 1998). Por otro lado, las concentraciones de P4 no fueron afectadas por la presencia del embrión o el tratamiento nutricional al día 13 del ciclo estral o preñez.

La presencia del embrión al día 14 promovió la expresión diferencial de 85 genes (DEG) en animales controles y 38 en animales subnutridos (Fig. 2A). El tratamiento nutricional promovió la expresión diferencial de 1 gen en ovejas cíclicas y 18 DEGs en ovejas preñadas (Fig. 2B). Dado el gran número de genes y vías metabólicas afectadas, para explicar los hallazgos se proponen modelos de funcionalidad asociados a la presencia del embrión, y como esto se afecta con la subnutrición. Los datos específicos se encuentran en los artículos correspondientes y los archivos suplementarios.

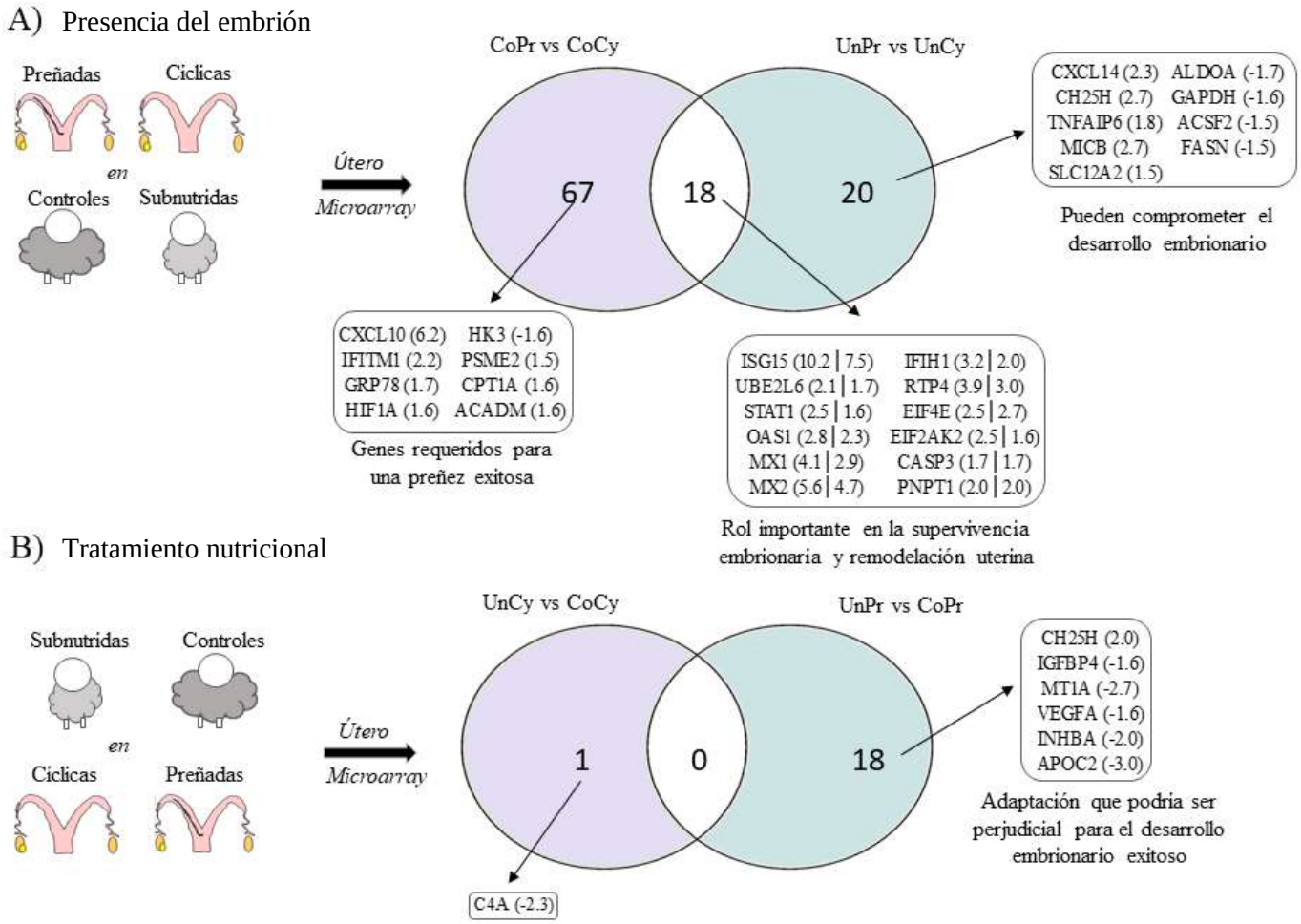

Fig. 2. Diagrama de Venn con genes diferencialmente expresados (FDR <0.2) para el efecto de la presencia de un embrión (A) y tratamiento nutricional (B). CoPr (Control Preñada), CoCy (Control Cíclica), UnPr (Subnutrida Preñada), UnCy (Subnutrida Cíclica). Ver explicación en el texto.

Genes vinculados al sistema inmune y remodelación uterina estimulados por la presencia del embrión independientemente del estado nutricional

La presencia del embrión durante la fase de reconocimiento materno, induce cambios en la morfología y fisiología del endometrio, de manera de generar un ambiente adecuado para su desarrollo. Tanto en animales controles como en subnutridos, la presencia del embrión promovió la expresión de los mismos genes relacionados con el sistema inmune en útero, y

si bien el FC fue distinto entre grupos, resalta la importancia de la modulación de estos genes para el mantenimiento de la preñez (Fig. 3).

La presencia del embrión en animales controles y subnutridos estimuló la expresión de *ISG15* (Fig. 3, Artículo II), como ha sido previamente reportado durante la preñez temprana (Haq et al., 2016; Meyerholz et al., 2016; Han et al., 2018). Se conoce que la principal función celular de esta proteína es la ISGilación, un proceso reversible en el que ISG15 es capaz de unirse covalentemente a residuos de lisina de sus proteínas diana con el fin de alterar su funcionalidad, estabilidad o localización dentro de la célula (Loeb y Haas, 1992; Lenschow, 2010; Zhang y Zhang, 2011). Este proceso se realiza, al igual que el de ubiquitinación, a través de la acción secuencial de tres enzimas, E1 activadora, E2 conjugadora (UBE2L6) y E3 ligasa (Falvey et al., 2017). En concordancia con estos resultados, *UBE2L6*, fue regulado positivamente tanto en ovejas preñadas controles como en subnutridas (Fig. 3, Artículo II).

La ISGilación es una respuesta materna frente a la presencia del embrión, y actúa modulando las acciones de STAT1, el cual activa la transcripción de genes con funciones antiproliferativas, proapoptóticas y proinflamatorias, que influencian la remodelación uterina para el embrión (van Boxel-Dezaire et al., 2006; Joyce et al., 2007). Entre estos genes se encuentran OAS1 (2'-5'-Oligoadenilato sintetasa 1) y MX1 (Mixovirus1) (Malakhova et al., 2003). En este trabajo la expresión de *STAT1* aumentó en animales preñados controles y subnutridos, indicando la relevancia de este factor para la inmunomodulación del ambiente materno (Binelli et al., 2001; Marconato et al., 2012; Hansen y Pru, 2014) (Fig. 3, Artículo II). OAS1 presenta un rol importante en la diferenciación celular y regulación de la expresión génica, así como modulador de la respuesta inmune innata (Kumar et al., 2000; Li et al., 2000; Mandal et al., 2011; Pulit-Penaloza et al., 2012). La expresión de este gen fue mayor en animales controles respecto a subnutridos preñados vs cíclicos (2.8 vs 2.3) (Fig. 2, Artículo II),y si bien por qPCR encontramos que la expression de *OAS1* fue dos veces mayor en animales controles preñados vs cíclicos, no encontramos diferencias asociadas a la presencia del embrión en los animales subutridos, por lo que la nutrición parece ser un regulador importante de este gen. Asimismo, en concordancia con otros estudios, la expresión de *MX1* y *MX2*, quienes regulan la secreción de células epiteliales uterinas (Racicot

et al., 2012), también se encontró aumentada debido a la presencia del embrión, en animales controles y subnutridos (Fig. 3, Artículo II).

Asimismo, la presencia del embrión en animales controles y subnutridos estimuló la expresión de *IFIH1* (Interferon Induced With Helicase C Domain 1), el cual es estimulado directamente por IFNt, y está implicado en el establecimiento de un estado antiviral mediante la modulación de células inmune del endometrio (Song et al., 2007).

Por otro lado, se ha reportado que *RTP4* (proteína transportadora de receptores 4) aumenta durante la preñez temprana, consistente con la mayor expresión de este gen en animales controles y subnutridos preñados vs cíclicos (Fig. 3, Artículo II). Esta proteína se ha asociado con el transporte de receptores acoplados a proteína G en humanos y roedores, que son el tipo de receptores a los que se unen la mayoría de las quimoquinas (Murdoch y Finn 2000; Saito et al. 2004; Behrens et al., 2006). En este sentido, *CXCL10* (C-X-C motif chemokine ligand 10) una quimoquina estimulada por IFNt, con efectos sobre el crecimiento del trofectodermo y adhesión en rumiantes (Gray et al., 2006; Brooks et al., 2016), se encontró regulada positivamente en animales controles (+6). En ovejas subnutridas preñadas CXCL10 aumentó 2 veces respecto a las ovejas subnutridas cíclicas (*p-raw* value <0.005), aunque no fue diferente por FDR (0.36), consistentemente con los datos obtenidos de este transcripto mediante PCR en tiempo real (Artículo II), lo cual puede ser detrimental para el embrión.

Es importante destacar que el impacto de la presencia de un embrión sobre el sistema inmunológico fue menor en ovejas subnutridas preñadas, lo cual se evidencia por el menor grado de cambio en la mayoría de los genes relacionados con el sistema inmunitario (Fig. 3).

Uno de los principales procesos de remodelación del ambiente materno es el recambio proteico en el fluído uterino, promoviendo la síntesis de proteínas necesarias para potenciar el desarrollo del embrión. En este sentido, genes involucrados en la síntesis proteica (*EIF4E*: Eukaryotic Translation Initiation Factor 4E, y *EIF2AK2*: Eukaryotic Translation Initiation Factor 2 Alpha Kinase 2), fueron estimulados en animales preñados tanto controles como subnutridos. Asimismo, *CASP3* (Caspasa 3), quien presenta un rol en apoptosis, proceso

importante para la remodelación del endometrio, también se encontró estimulada en animales preñados controles y subnutridos (Artículo II).

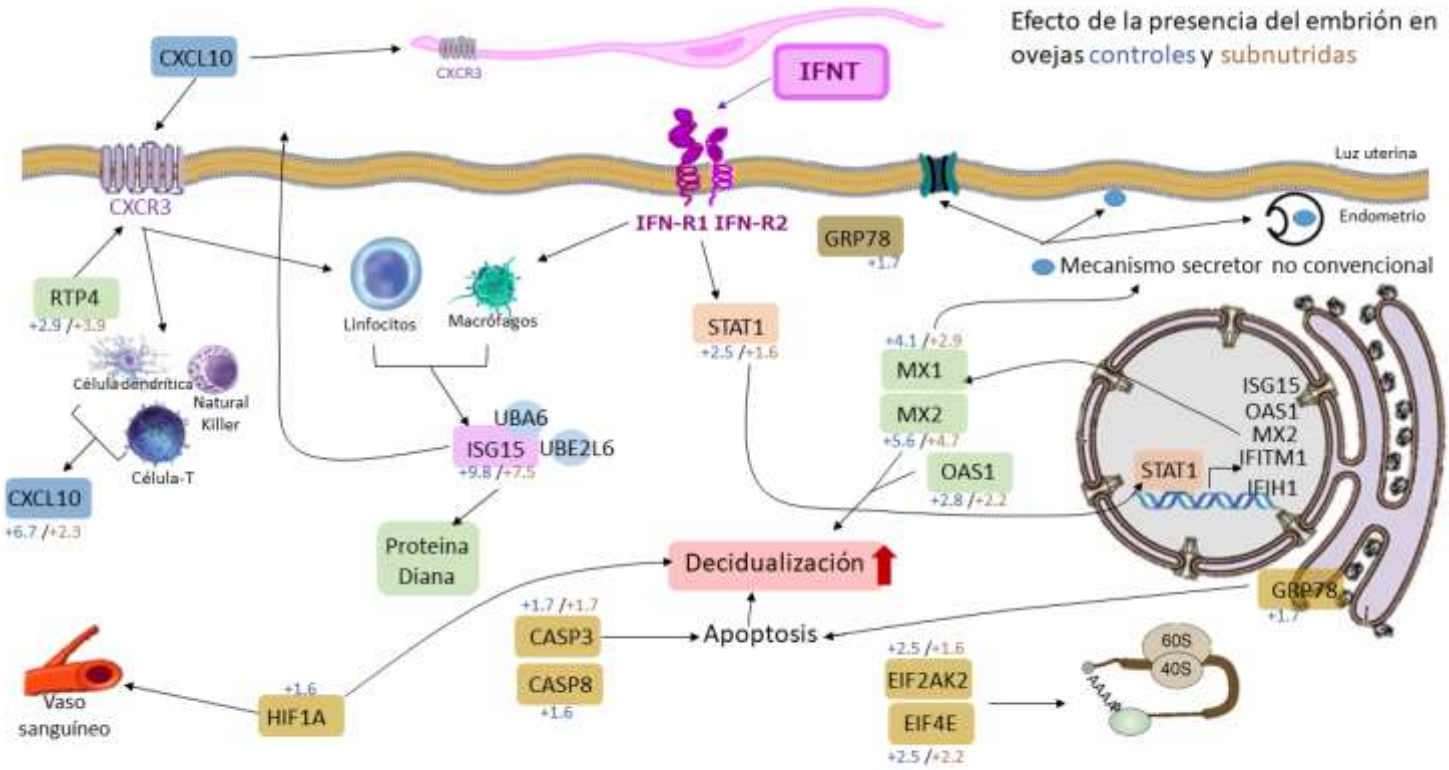

Fig. 3. Efecto de la presencia del embrión en útero de animales controles y subnutridos sobre la expresión génica de vías relacionadas con el sistema inmune y remodelación uterina. Se muestra el cambio de aumento de expresión para cada gen; en azul para controles preñados vs cíclicos, en marrón para animales subnutridos preñados vs cíclicos. Ver explicación en el texto.

Genes diferencialmente expresados por la presencia del embrión de manera dependiente del estatus nutricional

Los animales controles preñados presentaron genes estimulados por IFNt que no se encontraron en los animales subnutridos, como *IFITM1* (proteína transmembrana 1 inducida por interferón), *GRP78* (Glucose-Regulated Protein, 78kDa) y *HIF1A* (Hypoxia Inducible Factor 1 Subunit Alpha). Estos genes presentan roles importantes para el desarrollo embrionario como es la participación en la preparación del útero para promover un ambiente y un flujo de nutrientes adecuado para el desarrollo del embrión (Caniggia et al., 2000;

Goessling et al., 2009; Clarke et al., 2012; Klymiuk et al., 2012; Lesage-Padilla et al., 2017) (Fig. 3, Artículo II).

El hecho de que la presencia de un embrión regule diferencialmente estos genes en ovejas bien alimentadas, pero no en animales sometidos a subnutrición, indica una respuesta diferente acorde al plano nutricional, lo cual puede facilitar la comprensión sobre los mecanismos de remodelación uterina necesarios para mantener una preñez exitosa. Por lo tanto, la ausencia de expresión de estos genes en animales subnutridos preñados, puede ser un indicador de fallas reproductivas, consistente con las mayores pérdidas embrionarias que ocurren luego del reconocimiento materno de la preñez en animales subnutridos (Artículo I).

Por otro lado, la presencia del embrión en animales sometidos a subnutrición estimuló la expresión de *CH25H* (Cholesterol 25-Hydroxylase) respecto a cíclicos. CH25H es estimulado por interferón, y actúa reduciendo la acumulación de colesterol, produciendo 25-hidroxicolesterol, y alterando la composición de membrana (Chirala et al., 2001; Watkins y Ellis, 2012). El colesterol juega un papel importante el en la proliferación, diferenciación y comunicación celular, por lo que una reducción en su contenido puede ser perjudicial para el desarrollo embrionario (Wolf, 1999; Stec, 2015). Se ha demostrado que CH25H reprime la producción de interleuquina 1 (IL-1), una citoquina pro-inflamatoria, que juega un papel importante en la inmuno-tolerancia en la interface embrio-maternal (Geisert et al., 2012), por lo que la mayor expresión en animales preñados subnutridos respecto a cíclicas, puede ser indicativo de un ambiente nocivo para el embrión. (Artículos II y III). Por otro lado, *TNFAIP6* (TNF Alpha Induced Protein 6) quien presentan un rol en la estabilidad de la matriz extracelular y en el mantenimiento de la motilidad celular (Petit et al., 2003; Ashworth et al., 2010), se encontró regulado positivamente en animales preñados con subnutrición respecto a cíclicos. De hecho, se ha demostrado que la concentración proteica de TNFAIP6 aumenta luego del día 10 de preñez en el endometrio de cerdas (Ashworth et al., 2010). Asimismo, *MICB* (MHC Class I Polypeptide-Related Sequence B) se encontró estimulada en animales sometidos a subnutrición preñados respecto a cíclicos. Se ha demostrado que esta proteína, inducida por estrés, inhibe las acciones de receptores involucrados en actividad citotóxica, favoreciendo la supervivencia embrionaria (Mincheva-Nilsson et al., 2006). Es posible que

los animales subnutridos preñados induzcan vías alternativas de remodelación uterina de manera de mantener la preñez.

Efecto del tratamiento nutricional en animales preñados y cíclicos

En el mismo sentido que para la presencia del embrión en animales subnutridos, la subnutrición estimuló la expresión de *CH25H* en animales preñados respecto a controles (Fig. 4), pero no en ovejas cíclicas, y estos datos fueron confirmados por PCR en tiempo real (Artículo II). Los datos sugieren una adaptación específica a la subnutrición en el útero que es promovida por la presencia del embrión. Mas aún, se ha demostrado que CH25H es estimulado por TNFa (Tumor Necrosis Factor alpha) (Eibinger et al., 2013; Martel et al., 2018), el cual aumenta en balance energético negativo como es el caso de las ovejas subnutridas.

Asimismo, la subnutrición tendió a estimular *GNLY* (Granulisina) en animales preñados pero no en los cíclicos (Artículo II). Granulisina es una proteína citolítica presente en las células T citotóxicas y en las células Natural Killer (NK), es quimioatrayente de células inflamatorias, activando la expresión de muchas citoquinas proinflamatorias (Deng et al., 2005; Pulit-Penaloza et al., 2012). Se ha demostrado que la actividad citotóxica de las NK durante la preñez temprana es perjudicial para un desarrollo embrionario exitoso (Lee et al., 1987), por lo que la mayor expresión de este gen en animales preñados subnutridos respecto a controles es indicativo de un ambiente poco favorable para el embrión.

La subnutrición en animales preñados también disminuyó la expresión de *IGFBP4* en el útero respecto a las ovejas controles. Previamente hemos reportado que animales subnutridos que perdieron el embrión presentaron una menor expresión de *IGFBP4* en hígado, respecto a los que mantuvieron la preñez (de Brun et al., 2014: Anexo II). A su vez, se ha observado en embriones de ratones que IGFBP4 optimiza las acciones de IGF-II, quien presenta funciones esenciales durante las etapas tempranas de desarrollo embrionario (Ning et al., 2008), por lo que la menor expresión de este transcripto en animales subnutridos preñados puede ser perjudicial para el desarrollo exitoso. En el mismo sentido, *VEGFA* (Vascular endotelial growth factor A; -1.6) e *INHBA* (Inhibin subunit beta a; -1.9), quienes ejercen un rol en la angiogénesis, y aumentan las propiedades adhesivas de las células epiteliales del endometrio

y el crecimiento del embrión (Roberts et al., 2004; Binder et al., 2014), se encontraron negativamente reguladas en animales preñados subnutridos vs. controles (Fig. 4, Artículo II). Por lo tanto, proponemos que la menor expresión de estos genes en animales preñados subnutridos vs controles, podría comprometer el desarrollo embrionario exitoso, a través de la disminución de la vascularización del endometrio, indispensable para un adecuado suministro de nutrientes hacia el útero y el embrión, lo que puede dar lugar a pérdidas embrionarias tardías como ha sido previamente reportado (Artículo I).

Los radicales libres participan normalmente en procesos reproductivos como el desarrollo embrionario temprano (Rizzo et al., 2012), y se conoce que funcionan como segundos mensajeros en la señalización intracelular de varios procesos biológicos como ser la angiogénesis y la mortalidad celular (Staiculescu et al., 2014), mecanismos involucrados en la remodelación uterina durante la preñez temprana. Sin embargo, un desbalance entre radicales libres y antioxidantes puede causar estrés oxidativo, el cual es perjudicial para la supervivencia del embrión (Rizzo et al., 2012). Se ha observado que durante un período de balance energético negativo, el estrés oxidativo aumenta en bovinos (Pedernera et al., 2010). En este sentido, *GSR* (Glutathione-Disulfide Reductase) una de las principales enzimas involucradas en la defensa antioxidante celular (Masella et al., 2005), se encontró disminuida en animales preñados subnutridos vs controles. Asimismo, *MOXD1* (Monooxygenase DBH Like 1) y *MT1A* (metalotioneína 1A) quienes participan como sensores del estrés oxidativo y presentan un rol antioxidante protegiendo contra radicales libres (Andrews, 2000), también se encontraron disminuídos en animales preñados subnutridos vs controles. Por lo tanto, la disminución de estos genes involucrados en el control del estrés oxidativo en animales preñados subnutridos vs controles puede comprometer el desarrollo embrionario luego del reconocimiento materno de la preñez, como hemos observado previamente (Artículo I).

Por otro lado, también se ha demostrado una relación directa con la presencia de metales en el útero y la hiperplasia o proliferación celular (Guyot et al., 2015), procesos necesarios para la adaptación del útero a la preñez. En este sentido, la menor expresión de *MT1A*, la cual también presenta un rol en la regeneración de la capa funcional del endometrio, puede

comprometer una adecuada adaptación uterina al embrión y por lo tanto afectar la comunicación embrio-materna (Artículo II).

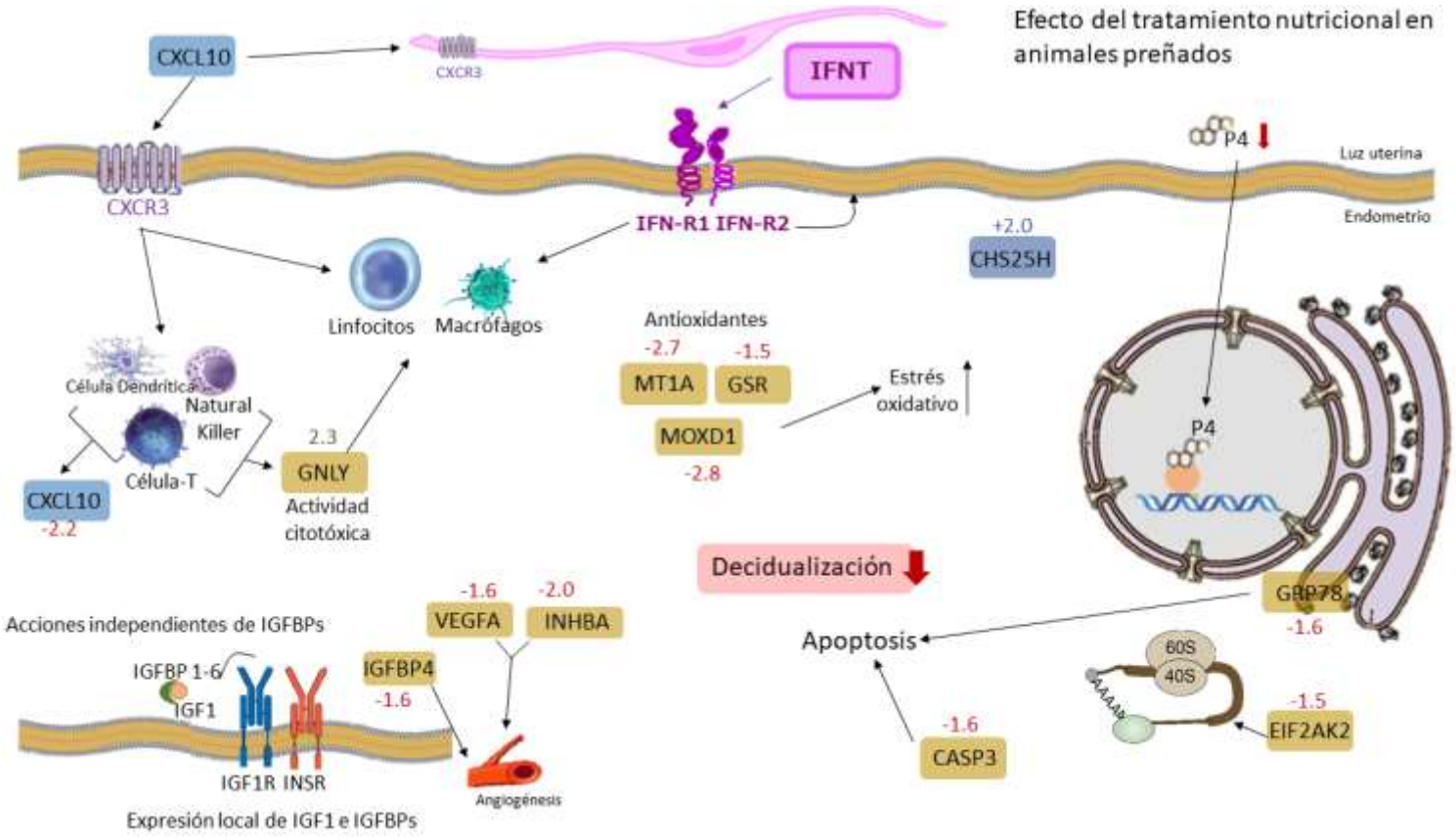

Fig. 4. Efecto del tratamiento nutricional en animales preñados sobre la expresión génica de vías relacionadas con el sistema inmune y remodelación uterina. Se identifica el cambio de aumento de expresión para cada gen (UnPr vs CoPr). Ver explicación en el texto.

En animales cíclicos, subnutridos vs controles, el único gen diferencialmente expresado por FDR fue *C4A* (Complement 4A) (Artículo II). En concordancia con nuestros resultados se ha visto una correlación positiva entre los niveles plasmáticos de C4A y el índice de masa corporal (Yang et al., 2004). El sistema del complemento es parte del sistema inmunitario innato y desempeña un papel en la regulación de la inflamación y la respuesta a los patógenos, por lo que la regulación negativa de este gen en animales subnutridos cíclicos puede ser perjudicial para la salud uterina del animal (Artículo II).

Estos datos describen por primera vez cuales son los mecanismos de adaptación uterina frente a la presencia del embrión, que son dependientes de la restricción de energía. Los datos sugieren que la presencia del embrión estimula procesos celulares como apoptosis y angiogénesis y vías del sistema inmunitario en animales controles y, en menor medida, en animales subnutridos. Por otro lado, el tratamiento nutricional en animales cíclicos afectó la expresión de un solo gen, el cual se encontró relacionado con el sistema inmune, sin embargo, en animales preñados, el tratamiento nutricional afectó negativamente la expresión de varios genes involucrados en las vías de remodelación uterina.

Adaptación metabólica del útero a la preñez y nutrición

La preñez es un estado dinámico que implica múltiples adaptaciones. Además de los cambios en el sistema inmune uterino para el reconocimiento materno y los cambios en la morfología y fisiología del útero, es necesario garantizar un suministro continuo de metabolitos esenciales para apoyar el crecimiento y el desarrollo del embrión.

La presencia del embrión en animales controles indujo la expresión uterina de transportadores de glucosa y aminoácidos respecto a ovejas cíclicas (*SLC15A1* y *SLC16A1*). Asimismo, las ovejas preñadas sometidas a subnutrición presentaron menor expresión de estos transportadores respecto a animales controles (UnPr vs CoPr), lo que sugiere una menor captación de glucosa y aminoácidos en el útero de animales subnutridos, consistentemente con la partición de nutrientes durante el balance energético negativo. Por otro lado, los requerimientos nutricionales del útero aumentan durante la preñez, lo que es consistente con el aumento de la expresión de transportadores de glucosa (*SLC12A2*) en los animales subnutridos preñados respecto a cíclicos. Sin embargo el grado de expresión del mismo fue menor que lo observado para animales controles (+1.5 vs. +3.8, respectivamente, Fig. 5, Artículo III). Debido a que la presencia del embrión tanto en animales controles como subnutridos promovió una disminución en la expresión de enzimas involucradas en la glucólisis (*HK3* en controles y *AldoA* y *GAPDH* en subnutridos), se sugiere que la preñez

induce un mecanismo compensatorio al flujo positivo (transportadores) de glucosa hacia el endometrio, disminuyendo el metabolismo de glucosa, posiblemente para dejar disponible este nutriente para el embrión (Fig. 5, Artículo III).

En animales controles, la presencia del embrión estimuló la expresión de *CPT1A* (Carnitine Palmitoyltransferase 1A) y *ACADM* (Acyl-CoA Dehydrogenase Medium Chain) (Fig. 5, Artículo III), lo que sugiere un aumento en la degradación de ácidos grasos, posiblemente como requerimiento energético para la preñez. De hecho, se ha observado previamente en ovinos que la concentración uterina de lípidos es menor en animales preñados respecto a cíclicos al día 15 de preñez o ciclo estral, asociados a los cambios uterinos durante la implantación, o a las necesidades metabólicas para el rápido crecimiento del embrión (Meier et al., 1997).

En animales subnutridos, si bien no se encontraron genes diferencialmente expresados involucrados en la β-oxidación, la presencia del embrión inhibió la expresión de genes involucrados en la síntesis de ácidos grasos, evidenciado por la regulación negativa de *FASN* (Fatty acid synthetasa -1.5) y *ACSF2* (Acyl-CoA Synthetasa -1.5) (Fig. 5), probablemente como mecanismo de ahorro energético.

El tratamiento nutricional en animales preñados reguló negativamente la expresión de *APOC2* en animales preñados subnutridos vs controles (Artículo III, Fig. 5). Si bien no se encontraron reportes de APOC2 en útero, se ha demostrado que diferentes apolipoproteínas se expresan en la superficie de células epiteliales del útero, y presentan un rol en el intercambio lipídico a través de endocitosis del complejo apolipoproteína-lípido (Argraves y Morales, 2004), mecanismo por el cual se podría proveer ácidos grasos libres a las células. Se ha demostrado que durante la preñez, los triglicéridos se desvían de la absorción por el hígado a la absorción por el útero (Ghio et al., 2011), para proporcionar la energía necesaria para el embrión en desarrollo y para el útero (Bazer et al., 2012). Por lo tanto, la disminución en la expresión de estos genes en animales preñados subnutridos vs. controles, sugiere que la absorción celular de ácidos grasos se encuentra disminuida, posiblemente impactando en la generación de energía, lo que podría explicar en parte las pérdidas embrionarias que ocurren luego del reconocimiento materno de la preñez en animales subnutridos (Artículo I).

Consistentemente con el aumento en el flujo de nutrientes hacia el útero en animales preñados, se estimularon genes involucrados en la cadena transportadora de electrones, en animales preñados vs. cíclicos (*COX7A1:* (Cytochrome C Oxidase Subunit 7A1, +2.0 fold-change) y en animales preñados controles y subnutridos vs cíclicos (*PNPT1* Polyribonucleotide Nucleotidyltransferase 1), probablemente en repuesta al aumento de las demandas energéticas asociadas a la preñez (Piwowarski et al., 2003; Alodaib et al., 2016; Signes y Fernandez-Vizarra, 2018) (Artículo III).

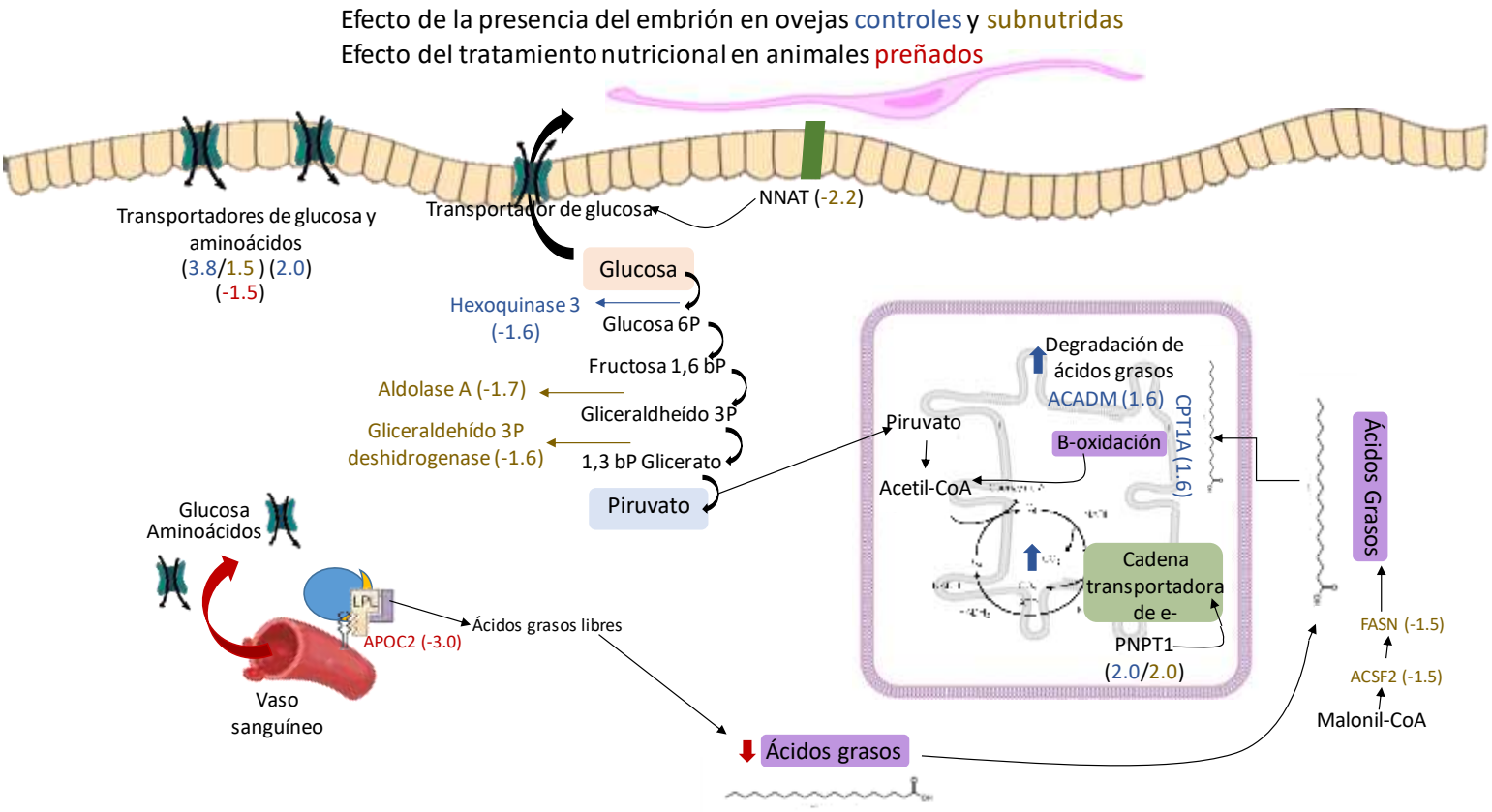

Fig. 5. Efecto de la presencia del embrión sobre genes vinculados a vías metabólicas en animales controles y subnutridos. En azul se muestran los genes (fold-change entre paréntesis) afectados en animales controles. En mostaza se muestran los genes afectados en animales subnutridos. En rojo se muestra la comparación del efecto del tratamiento nutricional en animales preñados (UnPr vs CoPr). Ver explicación en el texto.

La ausencia de expresión de genes involucrados en vías metabólicas relacionadas con la generación de energía en animales subnutridos preñados vs cíclicos, y animales preñados subnutridos vs controles es sorprendente, sin embargo, como es sabido, la expresión del transcripto no siempre refleja el contenido proteico real en la célula. Se ha postulado que en

respuesta a diversos estímulos, como la subnutrición, la vida media de los transcriptos se incrementa, mostrando niveles similares de transcriptos pero modificando el contenido proteico (Sheu et al., 1994; Ross, 1996). Consistentemente, genes relacionados con la síntesis proteica como *EIF4E* y *EIF2AK2* se encontraron diferencialmente expresados en animales preñados controles y subnutridos (Artículo II). De manera similar, previamente hemos demostrado que durante un período corto de subnutrición, los niveles circulantes de IGF-I se vieron reducidos en relación a animales controles, sin presentar cambios en el transcripto en el hígado (de Brun et al. 2016: Anexo III). Posiblemente, la ausencia de cambios en los transcriptos relacionados con la generación de precursores energéticos en animales subnutridos, se deba a que la subnutrición pudo haber causado un aumento en la vida media de estos transcriptos (Artículo III).

Por lo tanto, los cambios en el transcriptoma uterino acorde a la presencia del embrión fueron dependientes del plano nutricional, incidiendo sobre diferentes vías metabólicas para mantener la preñez. La identificación de genes que afectan el metabolismo uterino inducido por embrión da luz sobre importantes procesos biológicos y redes funcionales que influyen el éxito de la preñez en el ovino.

CONCLUSIONES

- En embriones de buena calidad al momento de la transferencia, la mortalidad embrionaria hasta el día 40 de gestación asociada a la subnutrición se debe principalmente a efectos sobre el ambiente materno, y no depende de la historia nutricional previa del embrión (Artículo I).
- Los animales con subnutrición que lograron mantener la preñez presentaron un mejor estado energético y tendieron a presentar mayores concentraciones de progesterona e insulina al día 7 de la preñez, en relación a animales subnutridos que presentaron mortalidad embrionaria en los primeros 40 días de gestación (Artículo I).
- La presencia del embrión estimula genes similares relacionados con las vías del sistema inmune en animales controles y, en menor medida, en subnutridos. No obstante, genes relevantes para la adaptación del útero al embrión se expresaron de forma distinta en animales controles y sometidos a subnutrición, lo que refleja una respuesta adaptativa a la restricción nutricional (Artículo II).
- La presencia del embrión aumentó el flujo de nutrientes hacia el útero, independientemente del tratamiento nutricional, aunque en menor medida en animales subnutridos (Artículo III). Los cambios provocados por la presencia del embrión en el transcriptoma uterino sugieren diferentes estrategias en la utilización de nutrientes entre animales controles y subnutridos (Artículo III).

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Artículo I

Artículo II

The embryo affects day 14 uterine transcriptome depending on the nutritional status in sheep. a. Immune system and uterine remodeling

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Abstract

Transcriptomics and bioinformatics were used to investigate the potential interactions of undernutrition and the presence of the conceptus at the time of maternal recognition of pregnancy on uterine immune system and remodeling. Adult Rasa Aragonesa ewes were allocated to one of two planes of nutrition for 28 days: maintenance energy intake (control; 5 cyclic, 6 pregnant ewes) providing 7.8 MJ of metabolisable energy and 0.5 maintenance intake (undernourished; 6 cyclic, 7 pregnant ewes) providing 3.9 MJ of metabolisable energy per ewe. RNA from intercaruncular uterine tissue was harvested at slaughter on day 14 of estrus or pregnancy, and hybridized to the Agilent 15K Sheep Microarray chip. Validation of gene expression was performed by qPCR. Functional bioinformatics analyses were performed using PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System. Eighteen genes were similar between control and undernourished pregnant ewes, underscoring the relevance for embryo-maternal interactions. Immune system evidenced by classical interferon stimulated genes were activated in control and -in a lesser extent- in undernourished pregnant vs cyclic ewes. Genes involved in uterine remodeling such as protein metabolism were also upregulated with the presence of an embryo in control and undernourished ewes. However, relevant genes for the adaptation of the uterus to the embryo were differentially expressed between control pregnant vs cyclic and undernourished pregnant vs cyclic ewes. Undernutrition alone led to an overall weak activation of immune system pathways both in cyclic and pregnant ewes. Data revealed that cellular and immune adaptations of the uterus to nutrient restriction are dependent on the presence of the conceptus.

Key words: ovine, pregnancy, transcriptome, undernutrition, uterus

Introduction

A precisely-timed uterine environment is crucial for the establishment of pregnancy and the development of the conceptus; alterations in the communication between conceptus and endometrium are the main cause of early embryo losses (Spencer et al. 2008). Nutritional status is one of the most important factors affecting reproductive losses, e.g. feeding sheep at levels below estimated nutritional requirements for maintenance decreases pregnancy rates and affects embryo quality and development (Abecia et al. 1997, 2015; Rhind et al. 1989). Although we have recovered the same number of embryos from underfed ewes and control ewes on days 8–9 after mating, reduced pregnancy rates were observed as pregnancy progressed, specifically on days 14–15 (around the time of maternal recognition of pregnancy in sheep) (Abecia et al. 1997; Abecia et al., 1999).

At day 14, transcription profiling using custom ovine endometrial cDNA arrays in pregnant and cyclic ewes identified candidate genes regulating uterine receptivity and conceptus growth (Gray et al. 2006). As far as we know there are no reports of the effect of the presence of an embryo on the sheep uterine transcriptome using high-throughput technology and bioinformatics. In cattle, it has been reported that the intercaruncular endometrial transcriptome profile does not change due to the presence of an embryo until approximately day 16 (day of maternal recognition of pregnancy) (Forde and Lonergan 2012). Recent work from Sponchiado et al. (2017) challenged this concept, as local changes in the endometrial transcription were measured as early as day 7 post-estrus in pregnant cows.

In spite of the impact of nutrient restriction in embryo mortality (Abecia et al. 2006), reports on the effects of negative energy balance (NEB) on uterine functionality

are scarce (Lesage-Padilla et al. 2017). In cyclic dairy cows, Astessiano et al. (2017) reported greater intercaruncular endometrial expression of genes of the somatotrophic axis on day 7 when cows were fed a high herbage allowance than to medium or low herbage allowance cows. In pregnant cows, lactation upregulated genes related to immunoglobulins, and the authors suggested that lactation could cause an immune imbalance with potential negative effects on conceptus survival (Cerri et al. 2012).

We have studied the effects of pregnancy and undernutrition on endometrial candidate gene expression in ewes fed all or half their maintenance requirements, nevertheless pregnant ewes did not presented differences in uterine expression at day 14 in response to different planes of nutrition (Sosa et al. 2009). Clearly, large-scale analysis of the endometrium transcriptome could provide a broad view of molecular alterations, complementing existing knowledge of temporal changes and facilitating a better understanding of conceptus-maternal cross talk at the time of maternal recognition of pregnancy. Thus, in the present study, we hypothesized that intercaruncular endometrial gene expression responses to the presence of an embryo depends on maternal nutrition. Consequently, we investigated the effects of the plane of nutrition (maintenance vs half maintenance requirements) and the presence of the conceptus on the uterine transcriptome at the time of maternal recognition of pregnancy in sheep.

Materials and Methods

Experimental Design and Animal Management

The study was performed at the experimental farm of the University of Zaragoza (Zaragoza, Spain; latitude 41º41_N) using a protocol approved by the Ethics Committee of the University of Zaragoza following the requirements of the European Union for

Scientific Procedure Establishments. Details of the experimental design have been published previously (Sosa et al., 2009b). Briefly, 46 adult multiparous Rasa Aragonesa ewes were housed in individual pens and offered a diet (once daily) that provided 1 x live weight maintenance requirements for 1 month prior to the beginning of the experimental procedures (Agricultural and Food Research Council 1993). Diet comprised 0.42 kg pellets and 0.70 kg barley straw per day, providing 7.8 MJ of metabolizable energy per ewe. The pelleted diet consisted of barley (85%) and soybean (15%). The animals had unrestricted access to water and a mineral supplement. Estrus cycles were synchronized using intravaginal progestagen pessaries (Fluorogestone acetate 40 mg, Intervet S.A., Salamanca, Spain), which were inserted for 14 days. At the time of pessaries insertion, ewes were allocated to one of two planes of nutrition: a control (n= 21; 7.8 MJ of metabolisable energy per ewe), which continued to receive the same diet for 28 days, and a low plane of nutrition (n = 25; 3.9 MJ of metabolisable energy per ewe), which was offered at 50% of estimated daily requirements until the end of the experiment. At the time of sponge withdrawal, ewes were injected with 300 IU, i.v., equine chorionic gonadotropin (Intervet, Salamanca, Spain), and the occurrence of estrus (Day 0) was monitored every 8 h. Thirteen control ewes and 18 ewes in the low plane of nutrition were mated to vasectomized or intact rams to establish a cyclic and pregnant group, respectively, within each plane of nutrition. Two ewes from each nutritional group (all of which were to be mated) did not show oestrus and one cyclic control ewe presented health problems and so were excluded from the experiment. Two cyclic control ewes (CoCy), and one cyclic ewe from the low group (UnCy) that did not exhibit a normal luteal phase (as determined by plasma progesterone profiles) or ewes that were mated but did not conceive (no embryos observed when ewes were killed; five in the control group and nine in the low group) were also excluded from the study. On day 14 of the estrus cycle or

pregnancy, uterine horns were flushed with 10 mL saline solution (0.9% w/v NaCl) and pregnancy was defined as the presence of an apparently normal conceptus. After embryo recovery, animals were subjected to general anesthesia induced by sodium thiopental (Tiobarbital, Braun Medical, Jaen, Spain) and sacrificed with an euthanasia agent (5–10 mL 50 kg−1 T-61®, Intervet S.A., Salamanca, Spain), and the medial region of the uterine tissue (including the endometrium and myometrium) ipsilateral to the corpus luteum was dissected. The intercaruncular tissue was selected due to the relevance of the endometrial glandular secretions and their role in the process of maternal recognition of pregnancy (Bazer et al. 2012). Tissues collected were snap frozen in liquid nitrogen and stored at −80ºC until RNA extraction and microarray analysis. The final treatment groups consisted of 5 cyclic and 6 pregnant control ewes, and 6 cyclic and 7 pregnant ewes in the low group.

RNA extraction

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by precipitation with lithium chloride to remove inhibitors of cDNA synthesis and DNase treatment with a DNA-*Free* kit (Ambion, Austin, TX, USA) to remove contaminating DNA (Naderi et al. 2004). The concentration of the RNA was determined by measuring absorbance at 260 nm, the purity of all RNA isolates was assessed as the ratio of absorbance at 260/280 nm (A260/280) and the integrity of the RNA was determined by electrophoresis (on a 1% agarose gel) and the Agilent Bioanalyzer (Agilent technologies). All samples had an average of A260/280 ratios of 1.95 ± 0.21 and an RNA Integrity Number of 8 ± 1.4.

Microarrays

cRNA Synthesis, Labeling, and Purification. The Agilent 15K Sheep gene expression microarray chip platform (Agilent Technologies Inc.) was used following the manufacturer's protocols. The reference sample RNA consisted of a pool of all the samples from animals in the study. Briefly, a total of 200 ng of RNA per sample (or reference pool) were used to generate first-strand cDNA (4 samples per group), which was reverse-transcribed to cRNA using the Low-Input Quick Amp Labeling kit (Agilent Technologies Inc). The resulting cRNA was labeled with either Cy3 (samples) or Cy5 (reference) fluorescent dye, purified using RNeasy Mini Spin columns (Qiagen), and subsequently eluted in 30 μL of DNase-RNase-free water. The NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA) was used to confirm the manufacturer's recommended criteria for yield and specific activity of at least 0.825 μg and ≥6.

Hybridization and Scanning. The labeled cRNA was fragmented and then hybridized to the microarray slide according to manufacturer's protocol. Briefly, 825 ng of Cy3 (sample) and Cy5 (reference) labeled cRNA were combined, mixed with 11 μL of 10× Blocking Agent (Agilent Technologies Inc.), 2.2 μL of 25× Fragmentation Buffer (Agilent Technologies Inc.), and nuclease-free water (to a final volume of 55 μL); and then fragmented at 60°C for 30 s. The reaction was then stopped by adding 55 μL of 2× GEx Hybridization Buffer (Agilent Technologies Inc.), and the samples were loaded onto the slide. These were hybridized in a rotating hybridization oven (Agilent Technologies Inc.) at 65°C for 17 h. The slides were washed according to the manufacturer's recommended procedures and scanned using a GenePix 4000B scanner (Axon Instruments Inc., Sunnyvale, CA) and GenePix Pro v.6.1 software. Resulting spots where features were substandard were flagged as bad and excluded from subsequent analysis.

Quantitative Real Time PCR

Sequences and the expected product lengths of primers to amplify cDNA of the target genes *ISG15*, *OAS1*, *SLC15A1*, *CXCL10*, *GNLY*, *SLC16A1*, *CH25H* and *IGFBP1* and of the endogenous controls hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and ribosomal protein L19 (*RPL19*) are presented in Additional file 1. Real-time PCRs were performed using 7.5 ul Quantinova SYBR_Green PCR Kit (Qiagen), equimolar amounts of forward and reverse primers (200 nM; Operon Biotechnologies GmbH, Cologne, Germany) and 2 ul diluted cDNA (1:10 in RNase/DNase free water) in a final volume of 15 ul. Samples (n= 5 or 6 per group) were analysed in duplicate in a 72-disk Rotor-GeneTM 6000 (Corbett Life Sciences, Sydney, NSW, Australia). Standard amplification conditions were 2 min at 95 °C and 40 cycles of 15 s at 95 °C, 40 s at 60 °C, and 10 s at 72 °C. At the end of each run, dissociation curves were analysed to ensure that the desired amplicon was being detected and to discard contaminating DNA or primer dimers. Samples of cDNA were pooled to provide an exogenous control, and five dilutions (from 100 to 6.25 ng/tube) of this pool were used to perform linear regression for each gene. The efficiency (E) of the assays was calculated according to the formula E = (10 _1/slope –1) (Additional File 1) (Rutledge and Cote, 2003). Gene expression was measured by relative quantification (Pflaffl, 2009) to the exogenous control and normalized to the geometric mean expression of the endogenous control genes (HPRT and RPL19), taking into account the respective efficiencies (Pflaffl, 2009). Expression of endogenous control genes remained unchanged among samples in this study.

Statistical Analysis

Computational and statistical analyses were carried out using Bioconductor (http://www.bioconductor.org/) packages of R software (version 3.0.3).

Raw data were background corrected with normexp method and normalized for dye and array effects with Lowess and A-quantile method respectively, before statistical analysis. A linear regression model was fitted using R software. The model consisted of plane of nutrition, pregnancy and the interaction of treatment x pregnancy as fixed effects.

The false discovery rate for differentially expressed transcripts was controlled according to the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) with an adjusted $P \leq 0.2$.

Transcripts determination were subjected to analysis of variance using a mixed model (Statistical Analysis System, SAS, Institute Inc., Cary, NC, USA), that included in the model the pregnancy, the nutritional treatment and interaction between both. Tukey–Kramer tests were conducted to analyse differences between groups. Means were considered different when $p < 0.05$, and tendency to differ when $0.05 < p \leq 0.10$.

Gene function and pathway identification

Differentially regulated genes were annotated with biological and molecular functions using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System (http://www.pantherdb.org/) (Mi et al. 2017). Gene lists containing differentially expressed genes for each comparison (Effect of the presence of an embryo and nutritional treatment, 1.5-fold differential expression, P-value ≤ 0.5, and FDR ≤ 0.2) were used for analyses. The bovine homologue corresponding to the ovine gene representing each transcript identified as being differentially expressed was used.

Verification of microarray results

In order to evaluate correlation between microarray and qPCR results for some differentially expressed genes, a fold-change was calculated from the normalized relative

mRNA abundance obtained from each gene, and compared with the fold-change from the microarray analysis. The relative expressions were based on two technical replicates.

Results and Discussion

Overall uterine transcriptome expression patterns

The DEG with a FDR ≤ 2 and a p-value ≤ 0.05 are reported in Additional files 2-7 (for the effect of the presence of an embryo and nutritional treatment) and Venn Diagrams are shown in Fig. 1A and B. The lowest number of DEG (taking into account the FDR and the p-value < 0.05) among comparisons was detected in undernourished compared with control cyclic ewes.

When an embryo was present, there was an overall upregulation of genes enriched in metabolic processes (mainly protein metabolism: GO: 0019538), cellular processes (like cell communication GO: 0007154 and signal transduction), genetic information processes like nucleobase-containing compound metabolic process (GO:0006139) and RNA metabolic process (GO:0016070), immune system (GO: 0002376) and response to stimulus (GO: 0050896) (Table 1). Eighteen genes were similar between controls and undernourished pregnant ewes when compared to their cyclic counterparts, underscoring the relevant pathways that need to be activated in uterus for a successful embryo development (Fig. 1A), nevertheless, the fold-change among pregnant and cyclic in undernourished ewes was lower.

We identified an agreement between the microarray and the qPCR results for the selected genes, showing the relative expression of array data compared to the relative expression of qPCR data (Fig. 2).

Common genes upregulated by pregnancy in control and undernourished ewes

The presence of an embryo promoted the upregulation of immune-related genes both in control and undernourished ewes, underscoring their importance for the maintenance of pregnancy (Fig. 1A). IFNt acts in a paracrine manner on the endometrium to induce the expression of a large number of IFN-stimulated genes (ISGs), in order to modify the uterine environment for maintenance of pregnancy. Among these ISGs, Interferon stimulated gene 15 (ISG15), 2´,5´-oligoadenylate synthetase 1(OAS1), myxovirus resistance 1 (MX1), myxovirus resistance 2 (MX2), receptor transporter protein 4 (RTP4) and signal transducer and activator of transcription (STAT) are particularly important (Johnson et al. 1999; Johnson et al. 2001; Hicks et al. 2003; Oliveira et al. 2012; Buragohain et al. 2016; Haq et al. 2016; Meyerholz et al. 2016). Among those genes, it is particularly noteworthy that the presence of an embryo upregulated the expression of *ISG15* in control (+10.2) and undernourished ewes (+7.5 fold-change) (Fig. 1A), which is consistent with the qPCR results (Fig. 3). Indeed, it was previously demonstrated in sheep that intrauterine administration of recombinant IFNt significantly increased the expression of ISG15 in endometrium, corpus luteum and liver (Bott et al. 2010). Although the precise biological role for ISG15 during pregnancy remains elusive, it is clear that it may impart protection of the conceptus against inflammatory insults. It is known that ISG15 acts as an extracellular cytokine (D'Cunha et al. 1996) secreted by lymphocytes and monocytes present in endometrium. It can also act as an intracellular ubiquitin homolog, covalently adding cytoplasmic and nuclear proteins, which is termed ISGylation (Zhang and Zhang 2011).

ISGylation is a maternal response to the developing conceptus, implantation and placentation, and is conserved across mammalian species (Hansen and Pru 2014). Consistent with this, *UBE2L6* (Ubiquitin/ISG15-Conjugating Enzyme E2 L6) was

also upregulated in control and undernourished ewes (+2.1 vs. +1.7, respectively, additional files 2 and 3; Fig. 1A). This is a protein coding gene that conjugates to ISG15 and is involved in ISGylation (Falvey et al. 2017). ISGylation enhances the cellular response to interferon through STAT1 (signal transducer and activator of transcription 1), which then translocates to the nucleus where it acts as a transcription activator (Malakhova et al. 2003) to render the uterus receptive to the embryo. We observed that the presence of an embryo upregulated *STAT1* in control (+2.5 fold-change) and undernourished ewes (+1.6 fold-change) (Additional files 2 and 3; Fig. 1A), suggesting the relevance of this factor during the immunomodulation of pregnancy as proposed previously (Binelli et al. 2001; Marconato et al. 2012; Hansen and Pru 2014). STAT1 is required for the activation and induction of expression of certain ISGs such as *OAS1* and *MX1*, which is consistent with their upregulation in control and undernourished pregnant ewes in this study. Indeed, *OAS1* was more than two-fold upregulated in control and undernourished pregnant ewes (Additional files 2 and 3). The FC observed by microarray according to pregnancy in undernourished animals was lower than in control ewes (2.3 vs 2.8), and while *OAS1* mRNA expression by qPCR in control pregnant ewes was more than two fold respect cyclic ewes (Fig. 3), no differences were observed in undernourished ewes, thus, nutritional status seems to be an important regulator of this gene. This idea is supported by the lower expression of *STAT1* in undernourished pregnant ewes. As OAS1 is involved in cell growth, differentiation and apoptosis, processes relevant for uterine adaptation to pregnancy and nutrition, the lower expression in undernourished pregnant animals could be indicative of a less suitable uterine environment (Kumar et al. 2000; Li et al. 2000)(Cheon and Stark 2009; Pulit-Penaloza et al., 2012).

As previously reported (Buragohain et al., 2016), *MX1* and *MX2* were upregulated in the endometrium with the presence of an embryo, both in control (4.1 and 5.6 fold-change respectively) and undernourished (2.9 and 4.7 fold-change respectively) animals (Additional files 2 and 3; Fig. 1A). It is known that MX1 is secreted into the uterine lumen during early pregnancy in sheep, and may play a role in regulating protein secretion via one of the unconventional secretory mechanisms (Racicot et al., 2012). Proteins secreted via unconventional pathways function in a wide range of cellular processes including angiogenesis, immune modulation, apoptosis and cell signaling (Perillo et al. 1995; Andrei et al. 1999; Denzer et al. 2000; Racicot et al. 2012).

The presence of an embryo both in control and undernourished ewes upregulated the expression of *IFIH1* (Interferon Induced with Helicase C Domain 1) (Fig. 1), which has been associated with the promotion of an antiviral responses including the induction of proinflammatory and enhancing immune cells function (Song et al., 2007).

Receptor Transporter Protein 4 (*RTP4*) was 4- and 3- fold upregulated in control and undernourished pregnant ewes, respectively (Additional files 2 and 3, Fig. 1A). It has been demonstrated that *RTP4* is upregulated during early pregnancy, and is thought to transport chemosensory G-protein coupled receptors (major chemokine receptors) in humans and rodents (Murdoch and Finn 2000; Saito et al. 2004; Behrens et al. 2006). The uterine environment during early pregnancy is characterized, at least in part, by the differential expression and secretion of chemokines that induce selective trafficking of leukocyte subsets to the maternal–fetal interface and regulate multiple events for uterus adaptation to pregnancy. In this sense, upregulation of *CXCL10* (C-X-C motif chemokine ligand 10), a chemokine stimulated by IFNt with biological effects on trophectoderm growth and adhesion in ruminants, was upregulated by 6-fold in control pregnant ewes by microarray (Fig. 1A), consistent with the almost 4-fold by q PCR in the same

comparison (Fig. 3), which agrees with previous data (Additional File 2) (Gray et al. 2006; Brooks et al. 2014). In undernourished pregnant ewes, CXCL10 was 2.3- fold upregulated by microarray (*p-raw* value <0.005, FDR: 0.36) when compared to undernourished cyclic ewes, and was not different by qPCR in the same comparison (Fig. 3). The CXCL10 data obtained by microarray and qPCR suggest an altered uterine functionality due to undernutrition.

In order to stimulate decidualization and render the uterus suitable for pregnancy maintenance, the activation of these factors in response to IFNt acts in the uterus during early pregnancy through ISGylation. In this sense, the increased expression of *EIF4E* (Eukaryotic Translation Initiation Factor 4E), *EIF2AK2* (Eukaryotic Translation Initiation Factor 2 Alpha Kinase 2) and *CASP3* (Caspase 3) (all involved in protein synthesis and apoptosis needed for decidualization) by the presence of an embryo in control and undernourished ewes underscores their importance (Additional files 2 and 3; Fig. 1A) (Sonenberg and Gingras 1998; Forde and Lonergan, 2012; Suzuki et al. 2018). It is noteworthy that the impact of the presence of an embryo on the immune system was lower in undernourished pregnant animals, evidenced by the lower fold change in immune related genes (Fig. 1 and Fig. 3), which could be associated with embryo losses that occur after maternal recognition of pregnancy (de Brun et al. 2016) (Fig. 1A).

The presence of the embryo promotes a differential gene expression depending on nutritional status

In our study, *IFITM1* (interferon-induced transmembrane protein 1, +2.2 fold-change) was upregulated by pregnancy only in control ewes suggesting its involvement in embryo development through different pathways required for proper formation of important

tissues during embryo development (Goesling et al., 2009; Hansen, 2011; Klymiuk et al., 2012). Furthermore, *GRP78* (Glucose-Regulated Protein, 78kDa) was upregulated in control pregnant compared with cyclic ewes (Additional file 2 and Fig. 1A), and in well-fed pregnant ewes compared with undernourished pregnant animals (-1.6 fold-change in UnPr vs CoPr, Fig. 1B). It has been demonstrated that GRP78 is highly expressed and localized in the glandular epithelium during early pregnancy and participates in monitoring protein transport through the cell (Clarke et al. 2012; Lin et al. 2014). It has been suggested that it plays a role in preparing a receptive endometrium inducing endometrial cell apoptosis, providing a suitable environment for the embryo. In this sense, *HIF1A (*Hypoxia Inducible Factor 1 Subunit Alpha) was also upregulated by the presence of an embryo in control ewes (Additional file 2 and Fig. 1A). This gene encodes a protein that is relevant for an adequate nutrient supply to the uterus and the embryo (Caniggia et al. 2000).

Overall, the fact that the presence of an embryo upregulated these genes in control but not in undernourished ewes sheds light on maternal factors that are necessary for a successful pregnancy at day 14 of embryo development. The altered uterine physiology of undernourished pregnant animals could explain embryo mortality after maternal recognition of pregnancy (de Brun et al. 2016).

In undernourished ewes, pregnancy upregulated *CXCL14*, which is consistent with the reported role in angiogenesis and recruitment of NK cells into the uterus during early pregnancy (Cao et al. 2013) (Benarafa and Wolf 2015)(Additional files 2 and 3; Fig. 1A). Undernutrition upregulated *CH25H* (Cholesterol 25-Hydroxylase), by 2.0 fold in microarray and by ~4-fold in the qPCR analysis (Fig. 1B and Fig. 3) in pregnant vs cyclic

ewes. CH25H promotes cholesterol hydrolysis and has been associated with an innate immune response (Griffiths and Wang 2018). It has been demonstrated that this protein represses the production of the pro-inflammatory cytokine IL-1, which was suggested to play a role in immunotolerance at the embryo-maternal interface (Geisert et al. 2012). In addition, as cholesterol is important in the composition of cell membranes and steroid hormone synthesis, upregulation of this gene in undernourished ewes may promote an inadequate uterine environment for proper embryo development.

TNFAIP6 (TNF Alpha Induced Protein 6) was also upregulated in undernourished pregnant vs cyclic ewes, and is involved in cell-cell and cell-matrix interactions during inflammation (Petit et al., 2003; Ashworth et al. 2010). Consistent with our results it has been demonstrated that TNFAIP6 increases in the endometrium during early pregnancy in pigs (Ashworth et al. 2010). This gene is induced by TNFa (Tumor Necrosis Factor Alpha)- which increases during negative energy balance-, and consistent with our results, it has been showed that stimulates the expression of CH25H (Eibinger et al. 2013; Martel et al. 2018). In the same sense, *MICB* (MHC Class I Polypeptide-Related Sequence B) was upregulated in undernourished pregnant vs cyclic ewes, and it has been demonstrated that under stress conditions, this protein inhibit the actions of receptors involved in cytotoxic activities, which are harmful for embryo growth (Mincheva-Nilsson et al. 2006). Thus, data suggest the presence of an embryo in undernourished ewes induces different pathways of the immune system and uterus remodeling in order to maintain pregnancy.

Effect of undernutrition in pregnant and cyclic ewes

Consistent with the upregulation of *CH25H* in undernourished pregnant vs cyclic ewes, this gene was also affected by the nutritional treatment in pregnant ewes (greater

expression in undernourished vs control pregnant ewes). As mentioned above, CH25H participates regulating inflammatory processes, and the marked increased expression consistently found by microarray and qPCR in undernourished pregnant ewes when compared to both control pregnant ewes and undernourished cyclic ewes (Fig. 1B and Fig. 3), supports the specific uterine adaptation to undernutrition exerted by the presence of the embryo.

Undernutrition upregulated the expression of *GNLY* (Granulisin) in pregnant ewes, and this was confirmed by qPCR results (Fig. 3). Granulysin is a protein present in cytotoxic granules of cytotoxic T cells and natural killer cells, and activates the expression of a number of cytokines (Deng et al. 2005; Pulit-Penaloza et al. 2012). As the cytotoxic activity of natural killer cells during early pregnancy is deleterious for a proper embryo development (Lee et al. 1987), the greater expression of this gene in undernourished vs control pregnant ewes is indicative of an inadequate uterine environment in food restricted animals.

Undernutrition downregulated mRNA expression of *IGFBP4*. Growth factors such as IGFs and their binding proteins are potential mediators of tissue turnover and remodelling in the uterus (Robinson et al. 2000). It has been postulated in rats, that IGFBP4 is detected in the blood vessels of decidual endometrium probably promoting angiogenesis (Cerro and Pintar 1997), thus downregulation of this gene could impair embryo-maternal communication. Indeed, *VEGFA* (Vascular Endothelial Growth Factor A) and *INHBA* (Inhibin Subunit Beta A), which are involved in angiogenesis, were downregulated in undernourished compared with control pregnant ewes (Additional file 5 and Fig. 1B). It has been demonstrated that the absence or reduction in levels of VEGF during the preimplantation period affects key events during embryo development and implantation (Binder et al. 2014; Roberts et al. 2004). Clearly, such adaptation could be detrimental

for successful uterine development, a scenario that agrees with the embryo mortality occurring after the period of maternal recognition of pregnancy (de Brun et al. 2016) (Fig. 1B).

Different studies have confirmed the presence of reactive oxygen species (ROS) and the transcripts of the various antioxidant enzymes in the female reproductive tract (Rizzo et al. 2012). ROS are known to serve as a second messenger in intracellular signal transduction cascades for various physiological cellular processes, such as angiogenesis and cell death (Staiculescu et al. 2014), processes involved in uterine remodelling associated with pregnancy. When ROS production overwhelms antioxidant defences, such as during a negative energy balance, oxidative stress occurs, which may deeply threaten the functional integrity of the reproductive tract (Pedernera et al. 2010). In this sense, *GSR* (Glutathione-Disulfide Reductase), a central enzyme of cellular antioxidant defence, was downregulated in undernourished vs control pregnant ewes (Fig. 1B). Indeed, MOXD1 (Monooxygenase DBH like 1) and *MT1A* (Metallothionein 1A), which act as an anti-oxidant, protect against hydroxyl free radicals, and are important in homeostatic control of metal in the cell (Andrews 2000; Duprez et al. 2012), were also downregulated in undernourished vs control pregnant ewes (Fig. 1B). Thus, diminished expression of genes involved in response to oxidative stress in undernourished pregnant ewes, could compromise embryo development after maternal recognition of pregnancy, leading to embryo mortality as has been previously demonstrated (de Brun et al. 2016).

In cyclic ewes, undernutrition led to only 58 DEG ($p<0.05$) and only 1 was differentially expressed if the FDR threshold is taken into account. This gene (C4A; complement C4A) is related to the immune system (Fig. 1B) and its concentrations in

plasma are positively correlated with body mass index in humans (Yang et al. 2003). Such association would be consistent with the lower expression of C4A in undernourished cyclic ewes. The complement system is part of the innate immune system and plays a role in regulating inflammation and response to pathogens (Copenhaver et al. 2018). Furthermore, *IGL@* (Immunoglobulin Lambda Locus, -2.0 fold-change, *p raw-value*: 0.0001, FDR: 0.53), also downregulated in undernourished compared with control cyclic ewes (Additional file 4), encodes a protein that recognizes foreign antigens and initiates immune responses such as phagocytosis and the complement system (Combriato and Klobeck 1991). Thus, downregulation of these genes in undernourished cyclic ewes could be in detriment for uterine health.

Conclusions

The present work sheds light on the biological processes of uterine adaptation to energy restriction, which is dependent on the presence of the conceptus. The presence of the embryo upregulated cellular processes and pathways of the immune system in control and to a lesser extent in undernourished ewes. The plane of nutrition affected uterine remodeling pathways, which were dependent on the presence of an embryo.

Ethics approval

The Ethical Committee of the University of Zaragoza (Zarazoga, Spain) approved all animal manipulations in accordance with Protection RD1201/05, which meets the European Union Directive 2010/63 related to the protection of animals used for experimental and other scientific purposes

Availability of data and materials

The array data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (accession number: GSE108176; BioProject ID: PRJNA422883).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

VDB extracted and quality-assessed the RNA for the uterus samples, wrote the main draft of the manuscript with inputs of AM MVR and JJL. OB performed the microarray experiment and HN and KS handled the statistical analysis of the array results. AG performed the qPCR validation. AM, JAA and CS designed the study and participated in its coordination. All authors read and approved the final manuscript.

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Table 1. Most descriptive categories of functional classification, with the number of genes involved and the fold enrichment for each processes for the effect of the presence of an embryo.

Most descriptive categories of PANTHER functional classification	CoPr vs CoCy		UnPr vs UnCy	
	# Genes	Fold enrichment	# Genes	Fold enrichment
Response to stimulus	29	1.56	18	1.63
Immune system process	13	2.80	7	2.53
Cytokine-mediated signaling pathway	3	7.21	0	<0.01
Signal transduction	20	1.24	11	1.5
Cell communication	22	1.18	13	1.17
Apoptotic process	6	2.57	3	2.16
RNA metabolic process	13	1.19	8	1.23
Mitochondrion organization	2	3.13	2	5.26

Table 2. Most descriptive categories of functional classification, with the number of genes involved and the fold enrichment for each processes for the effect of nutritional treatment.

Most descriptive categories of PANTHER functional classification	UnCy vs CoCy		UnPr vs CoPr	
	# Genes	Fold enrichment	# Genes	Fold enrichment
Response to stimulus	7	.92	23	2.23
Immune system process	7	3.67	8	3.11
Cytokine-mediated signaling pathway	0	<0.01	2	8.66
Signal transduction	8	1.21	10	1.12
Cell communication	9	1.18	15	1.45
Apoptotic process	1	3.13	2	1.55
RNA metabolic process	5	1.12	2	.50
Chromatin organization	3	3.85	0	<0.01
Angiogenesis	0	<0.01	2	14.61

Fig 1. Venn Diagramm with DEGs (FDR < 0.2) for the effect of the presence of an embryo (A) and nutritional treatment (B). CoPr (Control Pregnant), CoCy (Control Cyclic), UnPr (Undernourished Pregnant), UnCy (Undernourished Cyclic). When the effect of the presence of an embryo in control and undernourished ewes were compared (18 genes) straight line divide the fold-changes for the control and undernourished groups: righ fold-change is for CoPr vs CoCy and left fold-change is for UnPr vs UnCy group.

Fig. 2. Correlation between qPCR and microarray expression fold change results.

Fig. 3. Quantitative real-time PCR analysis of selected genes for microarray validation for the effect of the presence of an embryo and nutritional treatment, the expression pattern of the selected genes obtained by qPCR was consistent with the results from the microarray analysis. For each transcript, a vs b $P < 0.05$, and x vs y $0.05 < P < 0.10$. CoPr (Control Pregnant), CoCy (Control Cyclic), UnPr (Undernourished Pregnant), UnCy (Undernourished Cyclic). P=Pregnancy, T= Nutritional Treatment, I= Interaction Pregnancy*Nutritional Treatment.

Additional file 1. Oligonucleotide primer sequences used for quantification of target and endogenous control genes cDNA.

Additional file 2. Differentially expressed genes with fold change (FC) ≤ -1.5 or $\geq +1.5$ at day 14 in uterine tissue of control pregnant compared with control cyclic ewes (CoPr vs. CoCy).

Additional file 3. Differentially expressed genes with fold change (FC) ≤ -1.5 or $\geq +1.5$ at day 14 in uterine tissue of undernourished pregnant compared with undernourished cyclic ewes (UnPr vs. UnCy).

Additional file 4. Differentially expressed genes with fold change (FC) ≤ −1.5 or ≥ +1.5 at day 14 in uterine tissue of undernourished cyclic compared with control cyclic ewes (UnCy vs. CoCy).

Additional file 5. Differentially expressed genes with fold change (FC) ≤ −1.5 or ≥ +1.5 at day 14 in uterine tissue of undernourished pregnant compared with control pregnant ewes (UnPr vs. CoPr).

Fig. 1

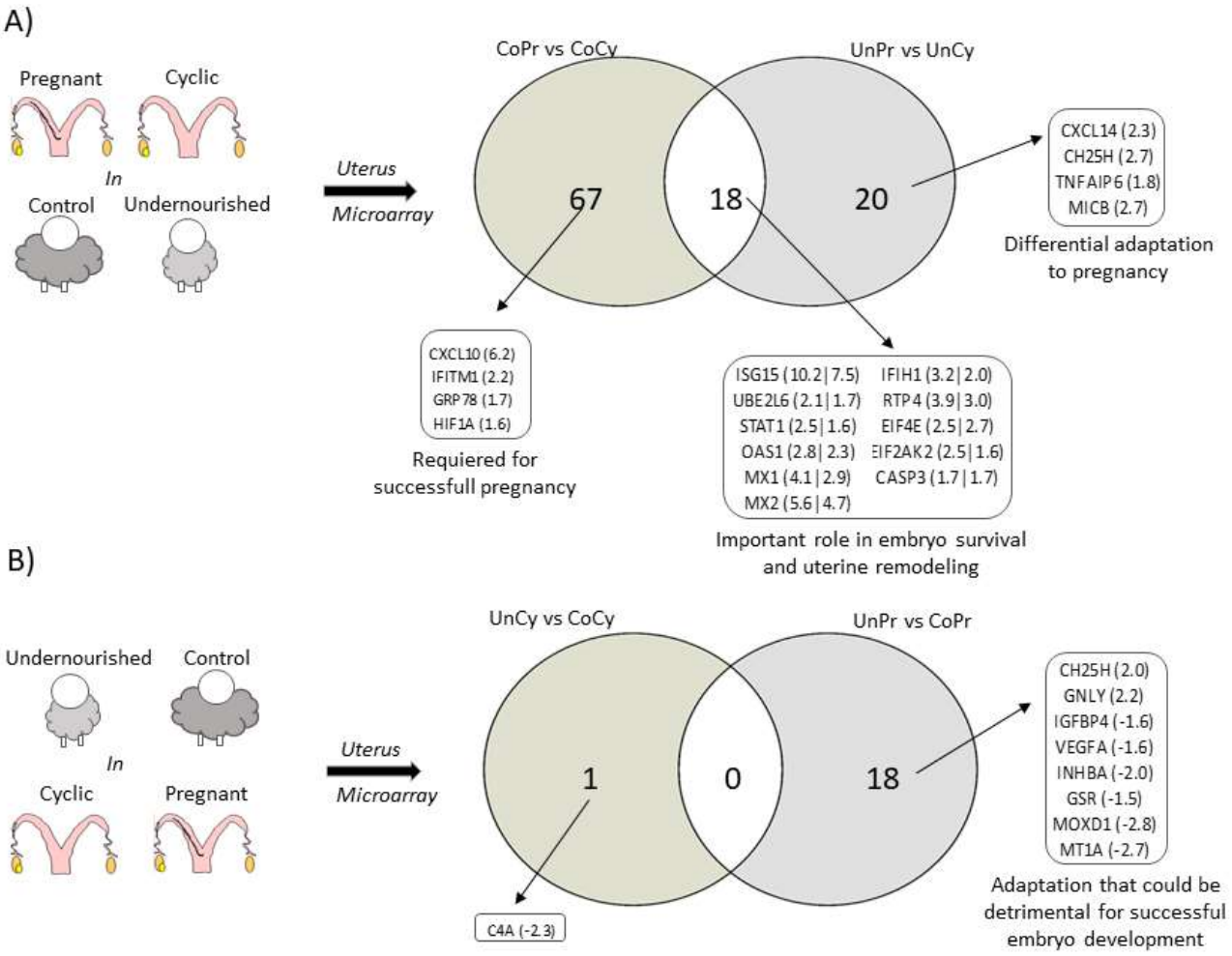

A)
Pregnant
Cyclic
In
Control
Undernourished
Uterus
Microarray
CoPr vs CoCy
UnPr vs UnCy
67
18
20
CXCL14 (2.3)
CH25H (2.7)
TNFAIP6 (1.8)
MICB (2.7)
Differential adaptation to pregnancy
CXCL10 (6.2)
IFITM1 (2.2)
GRP78 (1.7)
HIF1A (1.6)
Requiered for successfull pregnancy
ISG15 (10.2|7.5)
IFIH1 (3.2|2.0)
UBE2L6 (2.1|1.7)
RTP4 (3.9|3.0)
STAT1 (2.5|1.6)
EIF4E (2.5|2.7)
OAS1 (2.8|2.3)
EIF2AK2 (2.5|1.6)
MX1 (4.1|2.9)
CASP3 (1.7|1.7)
MX2 (5.6|4.7)
Important role in embryo survival and uterine remodeling
B)
Undernourished
Control
In
Cyclic
Pregnant
Uterus
Microarray
UnCy vs CoCy
UnPr vs CoPr
1
0
18
CH25H (2.0)
GNLY (2.2)
IGFBP4 (-1.6)
VEGFA (-1.6)
INHBA (-2.0)
GSR (-1.5)
MOXD1 (-2.8)
MT1A (-2.7)
Adaptation that could be detrimental for successful embryo development
C4A (-2.3)

Fig. 2

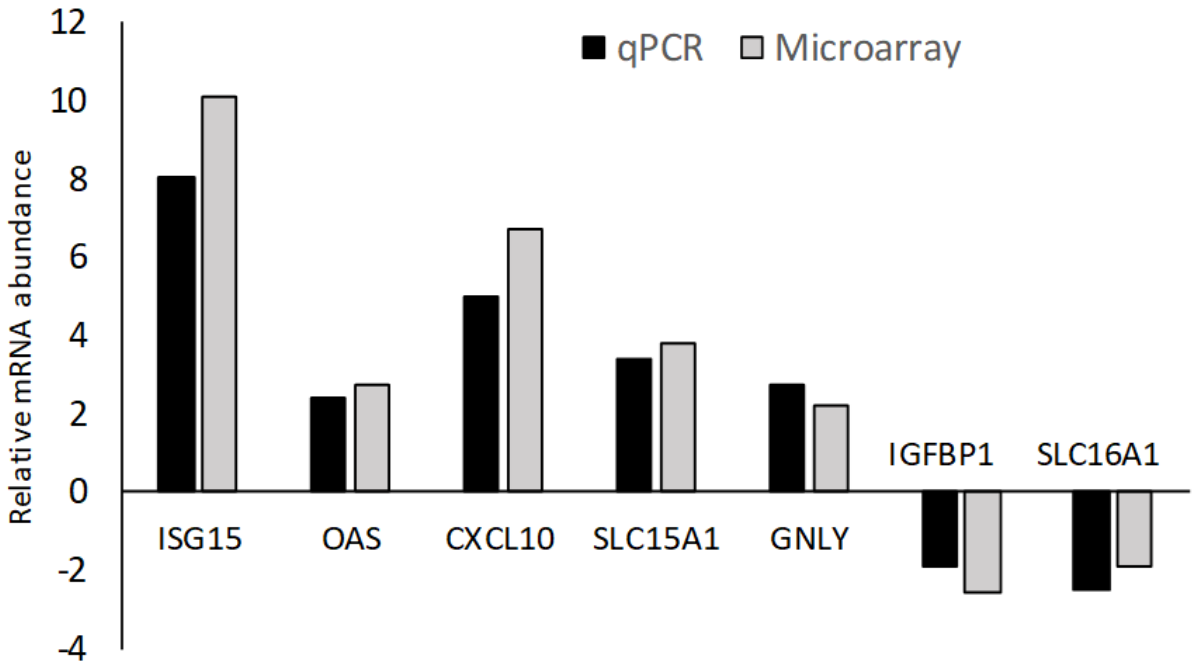
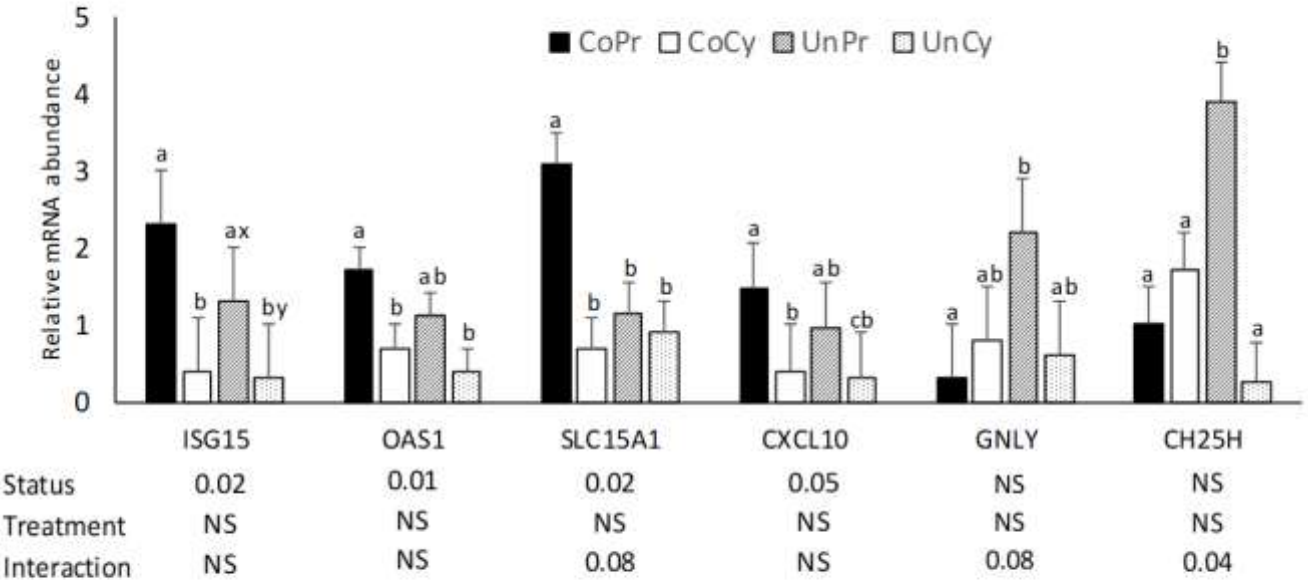

qPCR
Microarray
Relative mRNA abundance
12
10
8
6
4
2
0
-2
-4
ISG15
OAS
CXCL10
SLC15A1
GNLY
IGFBP1
SLC16A1

Fig. 3


CoPr
CoCy
UnPr
UnCy
Relative mRNA abundance
5
4
3
2
1
0
ISG15
OAS1
SLC15A1
CXCL10
GNLY
CH25H
Status 0.02 0.01 0.02 0.05 NS NS
Treatment NS NS NS NS NS NS
Interaction NS NS 0.08 NS 0.08 0.04

Artículo III

The embryo affects day 14 uterine transcriptome depending on nutritional status in sheep. b. Metabolic adaptation to pregnancy in nurished and undernourished ewes

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Abstract

This study investigated the effects of undernutrition and the presence of the conceptus at the time of maternal recognition of pregnancy on uterine indicators of metabolism. Adult Rasa Aragonesa ewes were allocated to one of two planes of nutrition for 28 days: maintenance energy intake (control; 5 cyclic, 6 pregnant ewes) providing 7.8 MJ of metabolisable energy and 0.5 maintenance intake (undernourished; 6 cyclic, 7 pregnant ewes) providing 3.9 MJ of metabolisable energy per ewe. RNA from intercaruncular uterine tissue was harvested at slaughter on day 14 of estrus or pregnancy, and hybridized to the Agilent 15K Sheep Microarray chip. The presence of the embryo upregulated expression of genes encoding glucose transporters regardless of nutritional treatment, although the degree of change was lower in undernourished ewes. Control and undernourished ewes had downregulation of genes encoding enzymes involved in glycolysis, probably as a compensatory mechanism for the increased glucose transport to the uterus. Compared with control cyclic ewes, control pregnant ewes had greater expression of genes involved in oxidation of fatty acids, suggesting increased uterine energy demands. This was not observed in undernourished pregnant animals when compared to undernourished cyclic ewes; nevertheless, those animals had lower uterine expression of enzymes involved in fatty acid biosynthesis. The presence of the embryo upregulated genes involved in electron transport probably as a result of increased energy demands for pregnancy. Overall, the data indicate that depending on the nutritional status of ewe, pregnancy alters gene expression of metabolic pathways related to energy generation in the uterus. An impairment in nutrient transport and metabolism in the uterus of pregnant undernourished ewes help explain the greater embryo mortality associated with undernutrition.

Key words: ovine, pregnancy, transcriptome, undernutrition, uterus

Introduction

It is well known that undernutrition affects reproductive performance and the reproductive axis at various anatomical sites (Meikle et al., 2018). Nutrient partitioning is regulated by endocrine signals, which in turn, modulate reproductive function accordingly. Although the preimplantation embryo is to a certain extent metabolically autonomous, the metabolic status of the mother at the onset of pregnancy is crucial to provide a suitable environment for early development of the embryo and may tip the balance towards pregnancy success or failure (Abecia et al., 2006; D'Occhio et al., 2019).

The effect of negative energy balance at the time of maternal recognition of pregnancy in sheep (around day 14) and cows (around day 17) has been addressed previously. In dairy cows, Cerri et al. (2012) and Thompson et al. (2012) reported that at day 17 the intercaruncular endometrium transcriptome is affected by pregnancy and lactation. Lactation affected the expression of genes involved in glucose homeostasis, an effect that authors suggested could be deleterious for the embryo. In pregnant ewes at day 14, Sosa et al. (2009) reported no effects of undernutrition on the intercaruncular endometrial expression of candidate genes involved in mechanisms of maternal recognition of pregnancy. It was suggested that conceptus present in the uterus of undernourished mothers could elicit effects similar to those in well-fed pregnant ewes.

In order to gain in-depth knowledge of the underlying mechanisms, we studied the uterine transcriptome in the same animals used in Sosa et al. (2009). We hypothesized that intercaruncular endometrial gene expression responses to the presence of an embryo depends on maternal plane of nutrition.

Materials and Methods

Experimental Design and Animal Management

The study was performed at the experimental farm of the University of Zaragoza (Zaragoza, Spain; latitude 41º41_N) using a protocol approved by the Ethics Committee of the University of Zaragoza following the requirements of the European Union for Scientific Procedure Establishments. Details of the experimental design have been published previously (Sosa et al., 2009b; de Brun et al., 2019a). Briefly, 46 adult multiparous Rasa Aragonesa ewes (Ovis aries), with a mean (±s.e.m.) body weight (BW) of 61.2±2.2 kg and a mean body condition score (BCS) of 3.4±0.1 (scale 0–5; Russel et al. 1969), were housed in individual pens and offered a diet (once daily) that provided 1 × live weight maintenance requirements for 1 month prior to the beginning of the experimental procedures (Agricultural and Food Research Council 1993). Estrus cycles were synchronized using intravaginal progestagen pessaries (Fluorogestone acetate 40 mg, Intervet S.A., Salamanca, Spain), which were inserted for 14 days. At the time of pessaries insertion, ewes were allocated to one of two planes of nutrition: a control (n= 21; 7.8 MJ of metabolisable energy per ewe), which continued to receive the same diet for 28 days, and a low plane of nutrition (n = 25; 3.9 MJ of metabolisable energy per ewe), which was offered at 50% of estimated daily requirements until the end of the experiment. At the time of sponge withdrawal, ewes were injected with 300 IU, i.v., equine chorionic gonadotropin (Intervet, Salamanca, Spain), and the occurrence of estrus (Day 0) was monitored every 8 h. Thirteen control ewes and 18 ewes in the low plane of nutrition were mated to vasectomized or intact rams to establish a cyclic and pregnant group, respectively, within each plane of nutrition. On day 14 of the estrus cycle or pregnancy, uterine horns were flushed with 10 mL saline solution (0.9% w/v NaCl) and pregnancy was defined as the presence of an apparently normal conceptus.

After embryo recovery, animals were subjected to general anesthesia induced by sodium thiopental (Tiobarbital, Braun Medical, Jaen, Spain) and sacrificed with an euthanasia agent (5–10 mL 50 kg−1 T-61®, Intervet S.A., Salamanca, Spain), and the medial region of the uterine tissue (including the endometrium and myometrium) ipsilateral to the corpus luteum was dissected. The intercaruncular tissue was selected due to the relevance of the endometrial glandular secretions and their role in the process of maternal recognition of pregnancy (Bazer et al. 2012). Tissues collected were snap-frozen in liquid nitrogen and stored at −80ºC until RNA extraction and microarray analysis. The final treatment groups consisted of 5 cyclic and 6 pregnant control ewes, and 6 cyclic and 7 pregnant ewes in the low group.

Blood sampling, hormone and metabolite assays

Jugular blood was sampled 1 h before feeding (08:00 h) every 2 days, from 1 week before the start of nutrition treatments until the end of the experiment. Samples were collected in heparinized vials and centrifuged within 15 min of collection, and plasma was separated and stored at −20 °C until analysis. Glucose and non-esterified fatty acids (NEFA) were assayed with commercial kits (Weiner Laboratory, Rosario, Argentina and Nefa-C, Wako Chemicals GmbH, Germany, respectively) on a Vitalab spectra 2 auto analyzer (Vital Scientific NV, Dieren, The Netherlands). The coefficient of variation (CV) values were below 3% and 8% for glucose and NEFA, respectively. Plasma insulin and IGF-1 was assayed by a double-antibody radioimmunoassay previously validated for sheep plasma (Gluckman et al. 1983; Miller et al. 1995), their sensitivity was 5.6 pmol/L and 0.05 ng/mL, respectively, and the intra-assay CVs were below 8.2% and 7.4%, respectively. Leptin concentrations were analyzed by RIA using an antiserum raised against recombinant bovine leptin in an emu and validated for its use in sheep (Blache et

al. 2000), and the intra-assay CVs quality controls were below 3.8%. Plasma progesterone concentration was determined using a direct solid-phase commercial RIA (Coat-A-Count, DPC, Los Angeles, CA, USA) previously used for sheep (Meikle et al. 1997), and the intra-assay coefficient of variation was below 6.5%.

RNA extraction

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by precipitation with lithium chloride to remove inhibitors of cDNA synthesis and DNase treatment with a DNA-*Free* kit (Ambion, Austin, TX, USA) to remove contaminating DNA (Naderi et al. 2004). The concentration of RNA was determined by measuring absorbance at 260 nm, the purity of all RNA isolates was assessed as the ratio of absorbance at 260/280 nm (A260/280) and the integrity of the RNA was determined by electrophoresis (on a 1% agarose gel) and the Agilent Bioanalyzer (Agilent technologies). All samples had an average of A260/280 ratios of 1.95 ± 0.21 and an RNA Integrity Number of 8 ± 1.4.

Microarrays

cRNA Synthesis, Labeling, and Purification. The Agilent 15K Sheep gene expression microarray chip platform (Agilent Technologies Inc.) was used following the manufacturer's protocols. The reference sample RNA consisted of a pool of all the samples from animals in the study. Briefly, a total of 200 ng of RNA per sample (or reference pool) were used to generate first-strand cDNA (4 samples per group), which was reverse-transcribed to cRNA using the Low-Input Quick Amp Labeling kit (Agilent Technologies Inc). The resulting cRNA was labeled with either Cy3 (samples) or Cy5 (reference) fluorescent dye, purified using RNeasy Mini Spin columns (Qiagen), and subsequently eluted in 30 μL of DNase-RNase-free water. The NanoDrop ND-1000

(Thermo Fisher Scientific Inc., Waltham, MA) was used to confirm the manufacturer's recommended criteria for yield and specific activity of at least 0.825 μg and ≥6.

Hybridization and Scanning. The labeled cRNA was fragmented and then hybridized to the microarray slide according to manufacturer's protocol. Briefly, 825 ng of Cy3 (sample) and Cy5 (reference) labeled cRNA were combined, mixed with 11 μL of 10× Blocking Agent (Agilent Technologies Inc.), 2.2 μL of 25× Fragmentation Buffer (Agilent Technologies Inc.), and nuclease-free water (to a final volume of 55 μL); and then fragmented at 60°C for 30 s. The reaction was then stopped by adding 55 μL of 2× GEx Hybridization Buffer (Agilent Technologies Inc.), and the samples were loaded onto the slide. These were hybridized in a rotating hybridization oven (Agilent Technologies Inc.) at 65°C for 17 h. The slides were washed according to the manufacturer's recommended procedures and scanned using a GenePix 4000B scanner (Axon Instruments Inc., Sunnyvale, CA) and GenePix Pro v.6.1 software. Resulting spots where features were substandard were flagged as bad and excluded from subsequent analysis.

Statistical Analysis

Computational and statistical analyses were carried out using Bioconductor (http://www.bioconductor.org/) packages of R software (version 3.0.3).

Raw data were background corrected with normexp method and normalized for dye and array effects with Lowess and A-quantile method respectively, before statistical analysis. A linear regression model was fitted using R software. The model consisted of plane of nutrition, pregnancy and the interaction of treatment x pregnancy as fixed effects.

The false discovery rate for differentially expressed transcripts was controlled according to the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) with an adjusted $P \leq 0.2$.

Gene function and pathway identification

Differentially regulated genes were annotated with biological and molecular functions using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System (http://www.pantherdb.org/) (Mi et al. 2017). Gene lists containing differentially expressed genes for each comparison (Effect of the presence of an embryo and nutritional treatment, 1.5-fold differential expression, P-value ≤ 0.05, and FDR ≤ 0.2) were used for analyses. The bovine homologue corresponding to the ovine gene representing each transcript identified as being differentially expressed was used.

Results and Discussion

Body weight, body condition and plasma concentrations of metabolites and metabolic hormones

Undernourished animals had a progressive decrease in BW during the experiment (initial BW: 61.5 ± 0.4 kg, final BW: 56.3 ± 0.4 kg, P < 0.0001), while the control group maintained a stable BW (initial BW: 61.5 ± 0.4 kg, final BW: 60.7 ± 0.4 kg). A decrease in BCS was observed in undernourished ewes (initial BCS: 3.35 ± 0.06, final BCS: 2.86 ± 0.06, P < 0.0001), but it remained constant in control (initial BCS: 3.32 ± 0.07, final BCS: 3.17 ± 0.07).

There was no effect of the presence of the embryo, nutritional treatment or the interaction on glucose concentrations at day 13 of the estrus cycle or pregnancy. Concentrations of NEFA were affected only by treatment, with greater values in undernourished ewes (0.79 vs. 0.47 ± 0.06 mM, p<0.001), reflecting a rapid increase in lipolytic activity, as reported previously (Sosa et al., 2009a). No effect on plasma progesterone concentration was

detected with the presence of an embryo, plane of nutrition, or their interaction at day 13 of estrus cycle or pregnancy (25.7 ± 3 nmol/L). Plasma concentration of IGF-1 tended to be lower in pregnant ewes at day 13 (32.2 ± 6.5 vs. 49.7 ± 7.1 ng/mL, p=0.08), which could be due to a higher endocrine uptake, since during the preimplantation period IGF-1 may act directly on the embryo or indirectly via modulation of the uterine secretions, stimulating embryo cell proliferation (Wathes et al., 1998). On the other hand, undernutrition tended to reduce or reduced plasma insulin and leptin concentrations at day 13 (43.1 ± 6.7 vs. 61.8 ± 6.8 pmol/L, p=0.06 or 1.2 vs. 2.2 ± 0.2 ng/mL, p<0.001; respectively), as was reported previously (Abecia et al. 2006; Chilliard et al., 1998), consistent with the negative energy balance in this group.

Uterine metabolic adaptation to pregnancy and nutrition

The DEG with an FDR ≤ 2 and a p-value ≤ 0.05 for the effect of the presence of an embryo and nutritional treatment are reported in Additional files 1-6. The presence of an embryo in control and undernourished ewes caused changes for genes enriched in Carbohydrate Metabolism (GO: 0005975) including Glycolysis (GO: 0061621), Protein Metabolism, like Proteolysis (GO: 0006508) and Cellular protein modifications (GO: 0006464), and Fatty acid metabolic processes (GO: 0006631) (Table 1). Despite that, only few genes involved in Glycolysis were affected by the presence of an embryo both in control and undernourished ewes (Table 1). Nutritional treatment modified Protein metabolism including Proteolysis (GO: 0006508) and Cellular amino acid metabolic processes (GO: 0006520), and Fatty acid metabolic processes (GO: 0006631) (Table 1).

Transporters expressed in the endometrium are important for tailoring the composition of nutrients in uterine histotroph that are required to support blastocyst growth and rapid

development into an elongated and filamentous conceptus (Satterfield et al. 2010). Compared with cyclic ewes, in control ewes the presence of the embryo upregulated transcripts of glucose and amino acid transporters such as *SLC15A1* and *SLC16A1* by 3.8- and 2.0-fold, respectively, (Fig. 1), as reported previously (Gao et al. 2009). Furthermore, undernourished pregnant ewes had a lower expression of *SLC15A1* (-3.1) and *SLC16A1* (-1.9) when compared with control pregnant ewes (UnPr vs. CoPr, Fig. 1), suggesting a lower uptake of glucose and amino-acids in undernourished ewes which is consistent with the nutrient partitioning that takes places during negative energy balance. On the other hand, uterine nutrient requirements during early pregnancy are increased, and this is consistent with the upregulation of a glucose transporter (*SLC12A2*, 1.5 fold-change, Fig. 1) in undernourished pregnant compared with undernourished cyclic ewes. Nevertheless, the fold change due to the presence of the embryo was lower than observed in control pregnant ewes. In contrast, expression of *NNAT* (Neuronatin, -2.2 fold change, Fig. 1), which has been reported to stimulate glucose transport in pig placenta (Gu et al. 2012), was downregulated in undernourished pregnant ewes. This seemingly conflicting data in undernourished pregnant compared with cyclic ewes (upregulation of *SLC12A* and downregulation of *NNAT*) suggest that glucose flux in undernourished pregnant ewes is tightly regulated. Thus, the lower expression of these transporters in undernourished pregnant ewes may be implicated in nutrient restriction for the uterine environment and may partly explain embryo losses (de Brun et al. 2016). Overall, data suggest that the presence of an embryo induces an increase in nutrient flux to the uterus both in control and undernourished animals, but to a lesser degree in undernourished animals. The proposed greater supply of nutrients in control compared with undernourished pregnant ewes, based on the greater expression of nutrient transporters, is consistent with the

upregulation of genes involved in angiogenesis (vascularization) in pregnant control compared with undernourished ewes (de Brun et al., 2019a, companion paper).

The presence of an embryo in control ewes downregulated the expression of *HK3* (Hexoquinase 3; -1.6) (Fig. 1), the first enzyme involved in the control of glycolysis. In contrast, in undernourished ewes the presence of an embryo downregulated the expression of *AldoA* (Aldolase A; -1.7, *p-raw value*: 0.025, FDR: 0.56) and *GAPDH* (Glyceraldehyde 3 phosphate dehydrogenase; -1.6, *p-raw value*: 0.0036, FDR: 0.33) (Fig. 1), enzymes involved in reversible steps of glycolysis. Our data suggest that the presence of an embryo both in control and undernourished ewes induced a compensatory mechanism in response to increased nutrient flux to the uterus, diminishing the use of glucose (the main fuel for the embryo) by the uterus, in order to spare this nutrient for the embryo.

The presence of an embryo in control ewes upregulated the expression of *CPT1A* (Carnitine Palmitoyltransferase 1A) and *ACADM* (Acyl-CoA Dehydrogenase Medium Chain), enzymes involved in mitochondrial uptake of medium-chain fatty acids and subsequent beta-oxidation (Lim et al. 2018; Jogl et al. 2004). It has been observed that uterine concentration of total lipids are lower in pregnant compared with cyclic ewes at day 15, suggesting that these changes during early pregnancy could be associated with uterine implantation or metabolic needs (e.g., ATP) to support the fast growth of the embryo (Meier et al. 1997). When compared with undernourished cyclic ewes, no differentially expressed genes involved in the degradation of fatty acids were observed in undernourished pregnant ewes. In spite of this, genes involved in fatty acid biosynthesis *FASN* (Fatty Acid Synthase) and intracellular transport (*ACSF2*, Acyl-CoA Synthetase

Family Member 2) were downregulated in undernourished pregnant compared with cyclic ewes, probably as a mechanism of energy saving.

When the effect of nutritional treatment is compared in pregnant ewes, we found that *APOC2* (Apolipoprotein C2) was negatively regulated according to undernutrition. It has been reported that apolipoproteins are expressed in the apical regions of uterine epithelial cells, presenting a role in lipid exchange mediated by endocytosis (Argraves and Morales 2004). Thus, data suggest that lipid consumption by cells could be negatively regulated in undernourished ewes. It has been shown that during pregnancy in well fed ewes, triglycerides are diverted from uptake by the liver to uptake by the uterus (Ghio et al., 2011), in order to provide the necessary energy and building blocks not only for the developing embryo, but for the uterus as well (Bazer et al. 2012; Meier et al. 1997). Thus, downregulation of this gene in undernourished vs control pregnant ewes suggest that cellular uptake of fatty acids are diminished, impacting possibly in energy generation, and thus, in the embryo development, possibly explaining future embryo losses (de Brun et al. 2016).

It should also be taken into account that mRNA expression does not always predict protein content. Indeed, it has been demonstrated that in response to adverse growth conditions, mRNA stability globally increased in cultured and in bacterial cells (Sheu et al. 1994; Ross 1995, 1996; Redon et al., 2005). Thus, it is possible that absence of marked changes in genes related to generation of energy in undernourished pregnant compared with cyclic or undernourished compared with control pregnant ewes, could be due to a prolonged half-life of mRNAs, such response probably spares energy. In this sense, pregnancy alone upregulated the expression of *COX7A1* (Cytochrome C Oxidase Subunit 7A1, +2.0 fold-change, Additional file 5), which is the terminal component of the

mitochondrial respiratory chain (Signes and Fernandez-Vizarra 2018). Also, *PNPT1* (Polyribonucleotide Nucleotidyltransferase 1) which regulates the expression of components of the electron transport chain at the mRNA and protein levels (Alodaib et al. 2016; Piwowarski et al. 2003) was also upregulated by the presence of an embryo in control and undernourished ewes (Additional file 1 and 2, Fig. 1), probably in response to an increase in energy during pregnancy.

Although few genes were differentially expressed according to pregnancy in undernourished ewes and nutritional status, cholesterol metabolism is of particular interest. The upregulation of Cholesterol-25-hydroxylase (*CH25H*, +2.7) found in pregnant undernourished compared with pregnant control ewes, as well as in undernourished pregnant compared with undernourished cyclic ewes is noteworthy (Fig. 1, de Brun et al., 2019a, companion paper). This gene is an Interferon Stimulated Gene (ISG) that encodes an enzyme which converts cholesterol to the soluble oxysterol 25-hydroxycholesterol (25-HC) and, thus, reduces cholesterol accumulation which in turn results in alteration of the cellular membrane composition (Chirala et al. 2001; Watkins and Ellis 2012). The fact that 25-HC can affect glycosylation agrees with upregulation of *UBE2L6* in undernourished pregnant compared with cyclic ewes (de Brun et al., 2019a, companion paper). In addition, the upregulation of *IL-6ST* (Interleukin 6 Signal Transducer) in undernourished pregnant compared with cyclic ewes (IL-6ST, p raw value: 0.01, and FDR: 0.43, Additional file 4) also supports the increase in 25-HC, because a previous study demonstrated that this compound can increase production of IL-6 (Raniga and Liang 2018).

In summary, the presence of the embryo upregulated gene expression of glucose transporters regardless the nutritional treatment, although the fold change was lower in undernourished ewes. Control and undernourished pregnant ewes presented a diminished gene expression of enzymes involved in glycolysis, probably as a compensatory mechanism for the increased glucose transport to the uterus in order to leave glucose for the embryo. In lipid metabolism, differential gene expression according to pregnancy and treatment was found, and data suggest that pregnancy in well fed ewes supplies metabolic needs by increasing the degradation of fatty acids while in undernourished ewes an inhibition of fatty acid biosynthesis is involved. Data suggest that undernutrition diminished nutrient uptake by the uterus, explaining embryo mortality due to undernutrition (de Brun et al., 2016).

CONCLUSION

The effect of the presence of the embryo on uterine transcriptomics was dependent on the nutritional status, suggesting that the plane of nutrition induces differential metabolic and cellular pathways in order to maintain pregnancy. The identification of genes that affect metabolic changes induced by the embryo, may uncover important biological processes and functional networks that influence the success of pregnancy.

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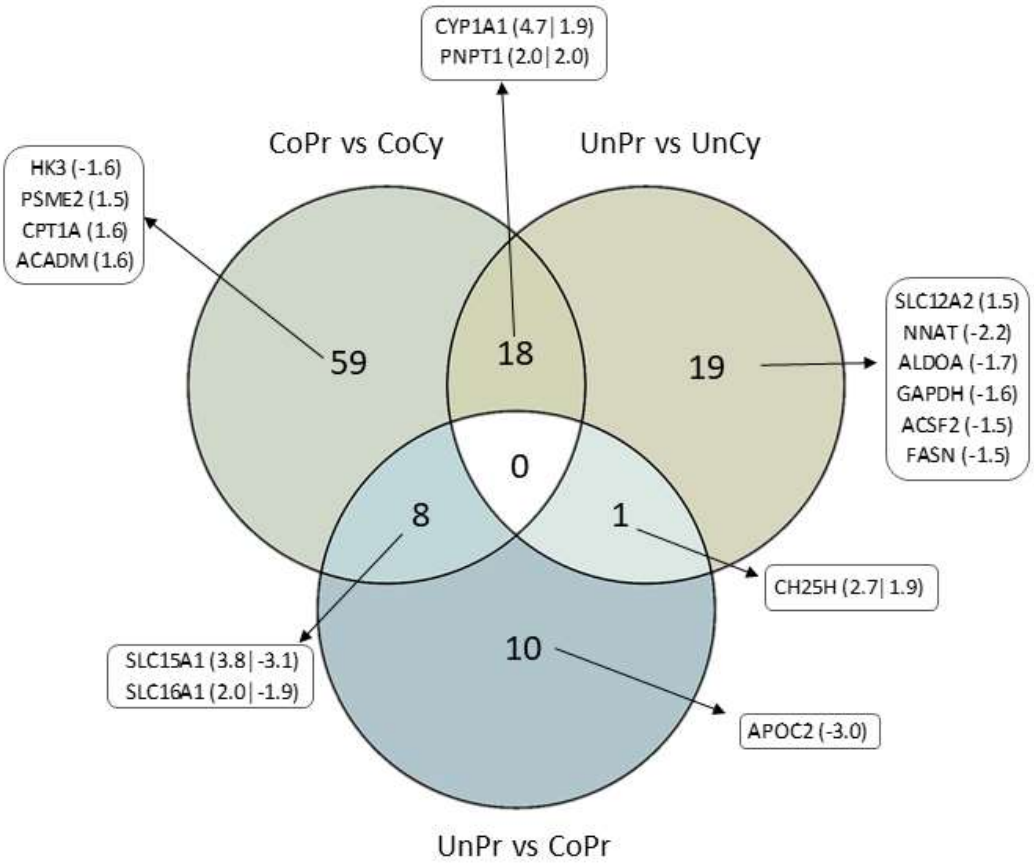
Table 1. Most descriptive categories of functional classification, with the number of genes involved and the fold enrichment for each processes for the effect of the presence of an embryo and nutritional treatment in pregnant ewes.

Most descriptive categories of PANTHER functional classification	CoPr vs CoCy		UnPr vs UnCy		UnPr vs CoPr	
	# Genes	Fold enrichment	# Genes	Fold enrichment	# Genes	Fold enrichment
Glycolysis	1	5.15	3	17.28	0	0
Protein metabolic process	22	2.00	6	0.92	9	1.48
↳ Proteolysis	7	2.25	2	1.08	2	1.74
↳ Protein glycosylation	2	2.75	1	2.30	0	0
↳ Cellular amino acid metabolic process	4	2.51	2	2.10	1	1.14
Purine nucleobase metabolic process	4	10.11	0	<0.01	0	0
Lipid metabolic process	2	0.37	3	1.84	3	1.98
↳ Fatty acid metabolic process	1	0.88	2	2.95	0	<0.01
Generation of precursor metabolites and energy	1	0.84	2	2.83	0	0

Legends to Figures

Fig. 1. Venn diagram with DEGs (FDR < 0.2) for the effect of the presence of an embryo and nutritional treatment in pregnant ewes. CoPr vs CoCy (Control Pregnant vs Control Cyclic), UnPr vs UnCy (Undernourished pregnant vs Undernourished Cyclic), UnPr vs CoPr (Undernourished Pregnant vs Control Pregnant). Straight lines among comparisons dive the fold change between groups. For *SLC15A*1 and *SLC16A1* right fold-change is for CoPr vs CoCy and left fold-change for UnPr vs CoPr. For *CYP1A1* and *PNPT1* right fold-change is for CoPr vs CoCy and left fold-change for UnPr vs UnCy. For *CH25H* right fold-change is for UnPr vs UnCy and left fold-change is for UnPr vs CoPr.

Fig. 1


CYP1A1 (4.7| 1.9)
PNPT1 (2.0| 2.0)
CoPr vs CoCy
UnPr vs UnCy
HK3 (-1.6)
PSME2 (1.5)
CPT1A (1.6)
ACADM (1.6)
59
18
19
SLC12A2 (1.5)
NNAT (-2.2)
ALDOA (-1.7)
GAPDH (-1.6)
ACSF2 (-1.5)
FASN (-1.5)
0
8
1
CH25H (2.7| 1.9)
10
SLC15A1 (3.8| -3.1)
SLC16A1 (2.0| -1.9)
APOC2 (-3.0)
UnPr vs CoPr

UNIVERSIDAD DE LA REPUBLICA URUGUAY

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