Efectos de la melatonina exógena sobre la viabilidad embrionaria en situaciones de subnutrición en la oveja

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EFECTOS DE LA MELATONINA EXÓGENA SOBRE LA VIABILIDAD EMBRIONARIA EN SITUACIONES DE SUBNUTRICIÓN EN LA OVEJA

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Fernando Forcada Miranda y José Alfonso Abecia Martinez, directores del trabajo de Tesis Doctoral de **María Isabel Vázquez** que lleva por título "***EFECTOS DE LA MELATONINA EXÓGENA SOBRE LA VIABILIDAD EMBRIONARIA EN SITUACIONES DE SUBNUTRICIÓN EN LA OVEJA***", consideran que dicho trabajo cumple los requisitos necesarios para poder ser presentado con el objetivo de optar al Grado de Doctor en Veterinaria. En este sentido, el presente trabajo se corresponde con el Proyecto de Tesis que fue aprobado el 16 de febrero de 2009.

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RESUMEN

A partir de los antecedentes que por un lado evidencian que la subnutrición afecta la eficiencia reproductiva en las ovejas, disminuyendo las tasas de gestación y/o causando un retraso en el desarrollo embrionario y que, por otra parte, se conoce que la melatonina es la hormona mediadora de los efectos entre la estacionalidad y el eje reproductivo, actuando con efectos benéficos tanto a nivel embrionario como del tracto reproductivo, nuestra hipótesis de partida planteó que el tratamiento con melatonina exógena podría ser una herramienta útil para paliar o revertir los efectos adversos que causa la subnutrición sobre la supervivencia embrionaria en las explotaciones ovinas. Por todo ello, la presente Tesis Doctoral investigó los efectos de la melatonina exógena tanto *in vivo* como *in vitro* sobre la competencia oocitaria y la viabilidad embrionaria en situaciones de subnutrición en la especie ovina, evaluando también dicho efecto sobre la población de receptores esteroideos en el endometrio, todo ello durante la estación reproductiva y el anoestro estacional y en hembras en diferentes estados fisiológicos (vacías y a los 45 días post-parto).

Por tal motivo se realizaron un total de cuatro experimentos utilizando ovejas adultas de raza Rasa Aragonesa. Cada uno de los experimentos se basó en un diseño factorial 2 x 2, considerando el tratamiento o no con melatonina exógena y el tratamiento nutricional (subnutrición o no) como factores fijos. De esta manera, los 4 grupos experimentales en cada uno de los experimentos realizados fueron: ovejas alimentadas con dieta control y que no recibieron implantes de melatonina (**C-MEL**), ovejas alimentadas con dieta control y que recibieron implantes de melatonina (**C+MEL**), ovejas subnutridas y que no recibieron implantes de melatonina (**B-MEL**) y

ovejas subnutridas y que recibieron implantes de melatonina (**B+MEL**). Las dietas control cubrían 1,5 veces las necesidades de mantenimiento, mientras que las dietas de subnutrición cubrían 0,5 veces dichas necesidades, en ambos casos durante un periodo de 21 días, desde el inicio de la sincronización de celos hasta el momento de la recuperación de los embriones u oocitos (Día 5 tras el celo).

Los experimentos 1 y 2 se realizaron con ovejas vacías durante la estación reproductiva y durante el anoestro estacional, respectivamente. Los experimentos 3 y 4 se realizaron con ovejas que se encontraban con más de 45 días post-parto, de nuevo durante la estación reproductiva y el anoestro estacional, respectivamente. Debido a las diferentes respuestas encontradas en los parámetros estudiados en cada estación, se introdujo al modelo original la época del año (estación reproductiva y/o anoestro estacional) como factor fijo, obteniéndose un diseño factorial final de 2x2x2. En cada uno de los experimentos se estudió el efecto de la melatonina exógena sobre la viabilidad de los embriones recuperados *in vivo,* de los embriones producidos *in vitro* a partir de los oocitos procedentes de los ovarios recuperados al momento del sacrificio (Día 5 tras el celo), así como también los efectos sobre la expresión de los receptores esteroideos de progesterona (RP) y de estrógenos (REα) en el endometrio. En todos los experimentos se observó que, en respuesta al tratamiento nutricional, todas las ovejas subnutridas (implantadas o no) presentaron pérdidas significativas de PV y CC en el momento del sacrifico respecto a las ovejas control ($P<0,01$).

En el **Experimento 1** se observó que la melatonina exógena perjudicó la viabilidad de los embriones recuperados *in vivo* de las ovejas subnutridas ($P<0,05$). No hubo efectos de los tratamientos nutricionales sobre la competencia oocitaria ni en el posterior desarrollo *in vitro* de los embriones, aunque hubo una interacción significativa melatonina x nutrición, de manera que la hormona pineal disminuyó el número de

oocitos no aptos para la maduración *in vitro* en las ovejas subnutridas (P<0,05), aumentándolo por otra parte en las ovejas control (P<0,05). También fue interesante observar que la melatonina exógena interaccionó con la nutrición disminuyendo la expresión de RP en los epitelios glandulares (superficiales y profundos) de las ovejas subnutridas (P<0,05) pero aumentando dicha expresión en el estroma superficial de las ovejas control (P<0,05). No hubo efectos significativos de los principales factores sobre la expresión de REα.

En el **Experimento 2**, la melatonina exógena tendió a mejorar la viabilidad de los embriones recuperados *in vivo*, independientemente del nivel nutricional que recibieron las ovejas (P<0,1). Respecto a la producción *in vitro* de embriones, se observó un efecto benéfico de la melatonina sobre la tasa de división y el posterior desarrollo embrionario hasta el estadio de blastocisto principalmente en los oocitos provenientes de los ovarios de las ovejas subnutridas (P<0,05). No se observaron efectos de los tratamientos ni interacción alguna sobre la expresión de RP y REα,

Los hallazgos procedentes de los efectos de la melatonina exógena sobre la viabilidad de los embriones obtenidos *in viv*o en los experimentos 1 y 2, durante ambas épocas del año estudiadas se describen en el ***Artículo I***. Se observó que durante el anoestro estacional la melatonina exógena tendió a mejorar la viabilidad embrionaria, principalmente sobre los embriones procedentes de las ovejas subnutridas (P=0,1) y que tuvo el efecto contrario durante la estación reproductiva (P<0,05). Se sugirió entonces que los mecanismos que determinan la interacción entre la melatonina exógena y el nivel nutricional sobre el desarrollo embrionario estarían regulados estacionalmente. En el ***Artículo II,*** hemos evaluado los efectos de la melatonina y la subnutrición sobre la competencia oocitaria, la producción y el desarrollo temprano *in vitro* durante la estación reproductiva y el anoestro estacional observados en las ovejas vacías de los

experimentos 1 y 2 de la presente tesis. Encontramos un importante efecto de la época del año sobre los parámetros evaluados: así, durante la estación reproductiva se obtuvo un mayor número de oocitos recuperados por oveja, mayor número y tasa de oocitos aptos para FIV respecto al anoestro estacional (P<0,01). Por el contrario, en el anoestro se observó que las tasas de división tendieron a ser mayores que durante la estación reproductiva, principalmente para las ovejas subnutridas implantadas con melatonina. Se concluyó que hubo un efecto perjudicial del anoestro estacional y de la subnutrición sobre la competencia de los oocitos, aunque la melatonina exógena mejoró dicha competencia oocitaria en las ovejas subnutridas durante el anoestro estacional.

En el **Experimento 3** encontramos un efecto benéfico de la melatonina exógena sobre las tasas de viabilidad embrionaria (P<0,05) y de gestación el día 5 en las ovejas subnutridas (P<0,05). Solo se observó efecto del tratamiento nutricional sobre los parámetros evaluados durante la producción *in vitro*, de manera que las ovejas subnutridas presentaron un menor número de embriones divididos y de blastocistos respecto de las ovejas control (P=0,09), aunque sólo en las ovejas subnutridas la melatonina exógena mejoró el número de embriones divididos (P<0,05). En general, no se detectaron efectos del tratamiento nutricional o de la melatonina sobre la expresión de RP y de REα, observándose solo un aumento en la expresión de RP en el epitelio glandular profundo de las ovejas subnutridas implantadas (P<0,05).

En el **Experimento 4**, hubo un efecto beneficioso muy evidente de la melatonina exógena sobre los parámetros evaluados en los embriones recuperados *in vivo*. Así, se observó que independientemente del nivel nutricional, la melatonina mejoró el número de embriones recuperados por cuerpo lúteo (CL) (P=0,08), el número de embriones viables por CL (P<0,01) y, particularmente en ovejas subnutridas, las tasas de viabilidad (P<0,05) y de gestación (P<0,05). Por el contrario, no hubo efecto de los tratamientos ni

interacción alguna sobre la competencia oocitaria y su posterior desarrollo *in vitro.* Solo se observaron efectos de la interacción de los tratamientos sobre la expresión de RP y de REα (P<0,01). De esta manera, en los epitelios glandulares y en los estromas (superficiales y profundos) de las ovejas subnutridas, el tratamiento con melatonina disminuyó la expresión de RP, al contrario que en las ovejas control, donde la hormona pineal aumentó la expresión de RP (P<0,01). Con respecto a REα, el tratamiento con melatonina disminuyó su expresión en el epitelio glandular profundo (EGp) de las ovejas subnutridas, mientras que la aumentó en el EGp de las controles (P<0,01).

En el ***Artículo III*** de esta Tesis se describieron los resultados del experimento 4, considerando los efectos de la melatonina exógena sobre la competencia oocitaria y el desarrollo *in vitro* de los embriones, así como también sobre la viabilidad de los embriones recuperados *in vivo* procedentes de ovejas en el período post-parto (más de 45 días) durante el anoestro estacional. Se concluyó que la melatonina exógena mejoró la viabilidad embrionaria durante dicha estación, particularmente en las ovejas post-parto subnutridas, y que estos efectos no estarían mediados por una acción de esta hormona sobre la competencia de los oocitos.

En conclusión, los resultados de la presente Tesis en general confirman nuestra hipótesis de trabajo, aunque los efectos de la melatonina exógena sobre los parámetros reproductivos estudiados en ovejas en condiciones de subnutrición fueron dependientes tanto del estado fisiológico como de la época del año en que se realizó el tratamiento. De esta manera, la melatonina exógena fue capaz de mejorar la viabilidad embrionaria *in vivo* así como también la competencia y el desarrollo de los oocitos *in vitro*, pero únicamente durante el anoestro estacional en las ovejas vacías y en condiciones de subnutrición. Este hecho estuvo acompañado de una ausencia de efectos de la

melatonina sobre la expresión de los receptores esteroideos (RP y RE) en el endometrio. En cambio, en las ovejas post-parto y subnutridas, los efectos benéficos de la melatonina se observaron únicamente sobre la viabilidad embrionaria *in vivo*, acompañados con cambios en la expresión de RP y REα tanto en la estación reproductiva como en el anoestro estacional.

SUMMARY

Previous studies have described the effect of undernutrition on reproductive efficiency in sheep, producing a reduction of pregnancy rates and/or delay of embryo development. On the other hand, it is well known that melatonin mediates the effects of season on the reproductive axis, with positive effects both at the embryo and the reproductive tract levels. Thus, our hypothesis was that treatment with exogenous melatonin could be a useful tool to prevent the adverse effects of undernutrition on the survival of sheep embryos. In this thesis, the effect of exogenous melatonin, both *in vivo* and *in vitro*, on oocyte competence and embryo viability has been studied, in a situation of undernutrition, evaluating such effect on the population of endometrial steroid receptor populations, both during the breeding and the anestrous seasons, in ewes in two different physiological stages (dry non pregnant ewes and ewes with a 45-day postpartum period).

Four experiments have been carried out using adult Rasa Aragonesa ewes. Each experiment was designed as a 2x2 factorial (treatment or not with melatonin and undernourished or control ewes as fixed factors). Thus, the four experimental groups were: ewes fed control diet and non implanted (**C-MEL**); ewes fed control diet and implanted with melatonin (**C+MEL**); ewes fed the low diet and non implanted (**B-MEL**), and ewes fed the low diet and implanted with melatonin (**B+MEL**). Control diet were designed as 1.5 times maintenance requirements (M) and low diets 0.5 M, and were offered for 21 days, from the onset of oestrous synchronizing treatments to the recovery of embryos or oocytes (Day 5 after oestrus).

Experiments 1 and 2 were carried out during the breeding season and seasonal anoestrus, respectively, using dry non pregnant ewes. Experiments 3 and 4 were done

using ewes with a postpartum period of more than 45 days, again during the breeding season and seasonal anoestrus, respectively. Due to the different responses obtained in each season, it was introduced in the original model (breeding or anoestrus season) as another fixed factor, obtaining a final design 2x2x2. In every experiment, the effect of exogenous melatonin on embryo viability *in vivo*, on *in vitro* produced embryos from recovered oocytes from the ovaries obtained at slaughter (Day 5), and on the expression of endometrial progesterone (PR) end oestrogen (ERα) receptors, was studied. After the nutritional treatment, all undernourished ewes (implanted or not) presented significant losses of live weight and body condition at slaughter, in comparison with control ewes ($P<0.01$).

In **Experiment 1**, melatonin was detrimental to *in vivo* embryo viability in undernourished ewes ($P<0.05$). No effects of nutrition on oocyte competence or embryo development *in vitro* was observed, although there was a significant interaction between melatonin and nutrition, so that this hormone reduced the number of non-healthy oocytes for *in vitro* maturation in undernourished ewes ($P<0.05$), and produced an increase in control ewes ($P<0.05$). Exogenous melatonin interacted with nutrition reducing the expression of RP in the glandular epithelia (superficial and deep) of undernourished ewes ($P<0.05$), increasing such expression in the superficial stroma of control ewes ($P<0.05$). No significant effects on ERα expression were observed.

In **Experiment 2**, melatonin showed a trend to improve embryo viability of recovered embryos *in vivo*, regardless of nutritional level ($P<0.1$). There was a positive effect of melatonin on division rate and embryo development of *in vitro* produced embryos up to the stage of blastocyst, specially from oocytes coming from undernourished ewes ($P<0.05$). No effects on PR and ERα expression were detected.

Results from the effect of melatonin on *in viv*o embryo viability obtained in

experiments 1 and 2, in both seasons, are described in ***Article I***. During the anoestrous season, melatonin tended to improve embryo viability (P=0,1), although it had the opposite effect during the breeding season, especially on embryos collected from undernourished ewes (P<0,05). It was suggested that the mechanisms determining the interaction between melatonin and nutrition are seasonality regulated. In ***Article II,*** we have evaluated the effects of melatonin and undernutrition on oocyte competence and *in vitro* production and early development during breeding and anestrous seasons from experiments 1 and 2. We found an important season effect: during the breeding season the number of recovered oocytes per ewe was higher, and so, the number and rate of healthy oocytes for IVF in comparison with anoestrus (P<0,01). On the other hand, during anoestrus division rate presented a trend towards being higher than during the breeding season. It was concluded that there was a negative effect of anoestrus and undernutrition on oocyte competence, although exogenous melatonin improved such competence in undernourished ewes during anoestrus.

In **Experiment 3**, some positive effect of melatonin on embryo viability (P<0.05) and pregnancy rate (P<0.05) on day 5 was observed in undernourished ewes. Nutrition only affected *in vitro* parameters, so undernourished ewes presented a lower number of cleaved embryos and blastoysts in comparison with control ewes (P=0.09), although only in undernourished ewes melatonin improved the number of cleaved embryos (P<0.05). In general, there were no effects of nutrition or melatonin on the expression of PR and ERα, with an increment of the expression of PR in the deep glandular epithelium in melatonin implanted undernourished ewes (P<0.05).

In **Experiment 4**, there was an evident positive effect of melatonin on the studied parameters in the recovered embryos *in vivo.* Thus, regardless of nutritional level, melatonin improved the number of recovered embryos per corpus luteum (CL)

(P=0.08), the number of viable embryos per CL (P<0.01) and, particularly in undernourished ewes, viability (P<0.05) and pregnancy (P<0.05) rates. On the opposite, there were no effects of treatments and interactions on oocyte competence and *in vitro* development. There was only an interaction between treatments on PR and REα expression (P<0.01). Thus, melatonin reduced the expression of PR in the glandular epithelium and in the stroma (deep and superficial) of undernourished ewes, in opposite than in control ewes, where melatonin increased the expression of RP (P<0.01). Regarding REα, melatonin reduced its expression in the deep glandular epithelium of undernourished ewes, increasing in control ewes (P<0.01).

Results obtained in experiment 4 were described in ***Article III***, considering the effects of melatonin on oocyte competence and *in vitro* embryo development, and on viability of recovered embryos *in vivo* collected from ewes in the postpartum period (more than 45 days) during seasonal anoestrus. It was concluded that melatonin improved embryo viability during anoestrus, particularly in postpartum ewes, althought the effect seems not to be mediated by an action of melatonin on oocyte competence.

In conclusion, results of this Thesis confirm our original hypothesis, although the effects of melatonin on the reproductive parameters under study in a situation of undernutrition were dependent both of physiological state and season. Thus, exogenous melatonin was able to improve embryo viability *in vivo* and oocyte competence and development *in vitro,* but only during seasonal anoestrus in dry non pregnant undernourished ewes. This fact was accompanied by a lack of effects of melatonin on endometrial steroid receptors population (PR and ER). On the other hand, in postpartum undernourished ewes, the beneficial effects of melatonin were observed only on *in vivo* embryo viability, together with changes in the expression of PR and ERα, both during the breeding and the anoestrous seasons.

TABLA DE CONTENIDOS

APÉNDICE

El presente trabajo se ha basado en los siguientes artículos científicos originales, los cuales adjuntamos al final:

Artículo I. Effects of melatonin and undernutrition on the viability of ovine embryos during anestrus and the breeding season. ***Animal Reproduction Science, 2008, 112: 83-94***. doi:10.1016/j.anireprosci.2008.04.004

Artículo II. Undernutrition and exogenous melatonin can affect the developmental competence of ovine oocytes. ***Reproduction in Domestic Animals, 2008.*** doi: 10.1111/j.1439-0531.2008.01329.

Artículo III. Effects of exogenous melatonin on in vivo embryo viability and oocyte competence of undernourished ewes after weaning during the seasonal anoestrus. ***Theriogenology (En revision desde 5/9/2009)***.

ANEXOS

Otros trabajos derivados del periodo de Tesis Doctoral

- Artículos científicos originales

Vázquez, M.I., Blanch, M.S., Alanis, G.A., Chaves, M.A., Gonzalez-Bulnes, A. Effects of treatment with a prostaglandin analogue on developmental dynamics and functionality of induced corpora lutea in goats. *Animal Reproduction Science, 2009*.
doi:10.1016/j.anireprosci.2009.05.016

Sosa, C., Abecia, J.A., Carriquiry, M., **Vázquez, M.I.**, Fernández-Foren, A., Talmon, M., Forcada, F., Meikle, A. Effect of undernutrition on the uterine environment during maternal recognition of pregnancy in sheep. R*eproduction, Fertility and Development 2009*, 21, 869-881.

Casao, A., Abecia, J.A., Cebrian-Perez, J.A., Muino-Blanco, T., **Vázquez, M.I.**, Forcada, F. The effects of melatonin on *in vitro* oocyte competence and embryo development in sheep. *Spanish Journal of Agricultural Research 2009*. Aceptado (ref. SJAR 170/09).

- Trabajos a Congresos

Vázquez, M.I., Sartore, I., Abecia, J.A., Forcada, F., Sosa, C., Palacín, I., Casao, A. y Meikle, A. **2008.** Melatonin treatment and undernutrition affect expression of uterine estrogen and progesterone receptors in ewes during the reproductive and the anestrous seasons. Reprod Dom Anim, vol 43 (3), 152.

Vázquez, M.I., Forcada, F., Casao, A., Sosa, C., Palacín, I., Abecia, J. A. **2008.** Undernutrition and exogenous melatonin affect ovine embryo viability on a seasonal basis. Reprod Dom Anim, vol 43 (3), 188.

Vázquez, M.I., Gonzalez-Bulnes, A., Alanis, G.A., Blanch, M.S., Chaves, M.A. **2008.** Morphological and functional features of corpora lutea induced by administration of cloprostenol in goats. Animal Reproduction (ISSN 1806-9614), vol 6 (1), 254.

Vázquez, M.I., Abecia, J.A., Forcada, F., Casao, A. **2008.** Influence of body condition previous to a short undernutrition period on embryo survival in ewes. Animal Reproduction (ISSN 1806-9614), vol 6 (1), 285.

Vázquez, M.I.; Forcada, F.; Casao, A.; Abecia, J.A. **2008.** Efecto de la condición corporal previa a un corto período de subnutrición sobre la supervivencia embrionaria en ovejas Rasa Aragonesa durante la estación reproductiva. *XXXIII Jornadas Científicas y XII Jornadas Internacionales de Ovinotecnia y Caprinotecnia (SEOC)*, 24-27 de setiembre, Almería, España.

Vázquez, M.I., Forcada, F., Casao, A., Abecia, J.A., Sosa, C. and Palacín, I. **2008.** Undernutrition and Exogenous Melatonin can affect the Developmental Competence of Ovine Oocytes. *6th International Congress on Farm Animal Endocrinology* (ICFAE), 14-16 de noviembre, Roanoke, Virginia, USA.

Vázquez, M.I., Abecia, J.A, Forcada, F., Casão, A. **2009.** El uso de melatonina exógena tras el parto en ovejas rasa aragonesa subnutridas mejora la viabilidad embrionaria durante la estación reproductiva. *XII Jornadas sobre Producción Animal, AIDA*, 12-13 de mayo, Zaragoza, España.

Vázquez, M.I., Forcada, F., Abecia, J.A, Casão, A. **2009.** Exogenous melatonin improves embryo viability of undernourished ewes during seasonal anoestrus. *60th EAAP Annual Meeting*, 24-27 de agosto, Barcelona, España. ***PREMIO Best Poster of Sheep and goat reproduction***

ABREVIATURAS Y TERMINOLOGÍA UTILIZADA

Oveja Vacía, se definió de esta manera a aquella oveja que en el momento de iniciar el experimento presentaba al menos 60 días de post-parto, no lactante y por supuesto no gestante, pudiendo estar cíclica durante la estación reproductiva (experimento 1) o no-cíclica durante el anoestro estacional (experimento 2).

Oveja Post-parto, aquella oveja que en el momento de recibir las dietas experimentales llevaba al menos 45 días de lactancia con un solo cordero, pudiendo estar cíclica durante la estación reproductiva (experimento 3) o no-cíclica durante el anoestro estacional (experimento 4).

Estructuras recuperadas tras el lavado uterino, se definió de esta manera al conjunto de estructuras recuperadas que incluían tanto a los oocitos como así también a los embriones.

AGNE, ácidos grasos no esterificados

BHB, beta-hidroxibutirato

CC, condición corporal

CL, cuerpo lúteo

E, estrógenos

eCG, gonadotrofina coriónica equica

E2, 17β-estradiol

EGp, epitelio glandular profundo

EGs, epitelio glandular superficial

EL, epitelio luminal

Ep, estroma profundo

Es, estroma superficial

FIV, fecundación *in vitro*

GnRH, hormona liberadora de gonadotrofinas

IFN-τ, interferon tau

MIV, maduración *in vitro*

P4, progesterona

PG, prostaglandina

PV, peso vivo

RE, receptor de estrógenos

RP, receptor de progesterona

INTRODUCCIÓN

Desde el punto de vista reproductivo, la oveja puede ser definida como una hembra poliéstrica estacional, cuya actividad reproductiva se caracteriza por presentar dos estaciones bien definidas en el año: una estación reproductiva durante el otoño-invierno y una estación de anoestro durante la primavera-verano. De este modo, y debido a la duración de la gestación en esta especie, los partos tienen lugar en la primavera, época más favorable para la supervivencia de las crías.

1. Breve descripción de la fisiología y la endocrinología del ciclo sexual ovino

La estación reproductiva se caracteriza por la sucesión de ciclos estrales regulares y su duración depende fundamentalmente de factores tales como el fotoperíodo, la nutrición y la genética (Karsch et al., 1984; Chemineau et al., 1988). Cada ciclo estral tiene una duración promedio de 16-17 días (14-19 días), y según la estructura predominante en el ovario se puede dividir en 2 fases: una fase folicular o estrogénica, que se caracteriza por la presencia de uno o más folículos en crecimiento continuo y con capacidad de secretar altas cantidades de estradiol hasta el momento de la ovulación y una fase luteal o progestativa, que se caracteriza por la presencia del cuerpo lúteo (CL) y su consecuente secreción de progesterona (P4).

La fase folicular es el período comprendido desde el momento de la regresión del CL (luteolisis) hasta la ovulación. En general, es una fase corta, ya que posee una duración aproximada del 20% del ciclo estral; la estructura predominante en el ovario durante esta fase es el folículo dominante que principalmente produce altos niveles de

estradiol-17β (E2) e inhibina (Senger, 1997). Durante esta fase se presenta un período de receptividad sexual llamado celo o estro, que en la oveja tiene una duración de 20-72 h (media de 36 h; Gordon, 1997). Por su parte, la fase luteal ocupa el período comprendido desde el momento de la ovulación hasta la luteolisis, por lo tanto, se caracteriza por la presencia de uno o más CL en el ovario, responsables de la secreción de P4 durante dicho período (Senger, 1997).

El eje hipotálamo-hipofisario-gonadal es el encargado del control neuroendocrino del ciclo estral en la hembra (Figura 1). De esta manera, el hipotálamo produce la hormona llamada hormona liberadora de gonadotropinas (GnRH), que es la encargada de estimular la secreción hipofisaria de las gonadotropinas folículo estimulante (FSH) y luteinizante (LH). Dichas hormonas, actuando en el ovario, son las responsables de la maduración final del folículo dominante durante la fase folicular del ciclo. Bajo la influencia de las gonadotropinas, el folículo produce y secreta varias hormonas y factores de crecimiento, siendo las más importantes el E2 y la inhibina, como se mencionó anteriormente. Durante la fase folicular, el estradiol ejerce una retroacción positiva a nivel de hipotálamo e hipófisis para lograr una mayor secreción de FSH y LH; en cambio, los niveles foliculares de inhibina causan una retroalimentación negativa en la secreción de FSH desde la hipófisis. De esta manera, se logra la maduración final del folículo, que ha producido los máximos niveles de estradiol que a su vez permiten que ocurra el pico preovulatorio de LH, dando como resultado la ovulación (Lucy et al., 1992; Senger, 1997).

Desde el momento en que se produce la ovulación, la LH es la hormona encargada de la formación y mantenimiento del CL (Niswender et al., 1981). Las

células de la teca interna y las células de la granulosa del folículo ovulatorio sufren una gradual y rápida transformación en tejido luteal (luteinización) dando lugar a la estructura denominada cuerpo lúteo, que será quien produzca y secrete P4. Dicha hormona prepara el útero para la implantación del embrión y el mantenimiento de la gestación (Hafez, 1989); para ello, la P4 ejerce un efecto inhibitorio a nivel de la secreción de GnRH en el hipotálamo y de gonadotrofinas (FSH y LH) en la hipófisis. Si la fecundación no se lleva a cabo, se producirá la regresión del CL, con el consiguiente cese en la producción de P4 para permitir el comienzo de un nuevo ciclo estral. La luteolisis tiene lugar al final de la fase luteal y está controlada principalmente por la prostaglandina (PG) F2α producida por el endometrio (Wathes y Lamming, 1995).

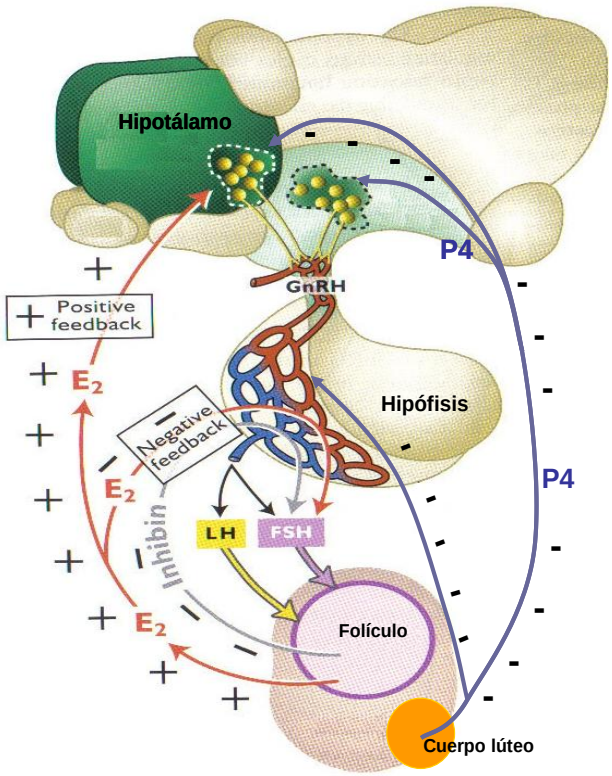


Figura 1. Relación entre el hipotálamo, la hipófisis y el ovario durante las fases folicular y luteal del ciclo estral en los rumiantes (adaptado de Senger, 1997).

Durante el ciclo estral, el tracto reproductivo de la hembra también sufre cambios morfológicos, bioquímicos y fisiológicos que tienden a establecer un microambiente adecuado que es esencial para la maduración final de los gametos, capacitación de los espermatozoides, transporte de los gametos y embriones, fecundación y divisiones tempranas en el desarrollo embrionario (Nancarrow y Hill, 1995; Buhi, 2002). Las hormonas esteroideas ováricas, la P4 y los estrógenos (E), tienen una función esencial regulando los cambios en la estructura y fisiología del tracto reproductivo (Geisert et al., 1992; Buhi et al., 1997). Durante el celo y los primeros días de gestación ocurren alteraciones estructurales (modificaciones en la altura, forma y secreción de las células) a lo largo de todo el oviducto (Murray, 1995). El endometrio también sufre una serie de transformaciones cíclicas en respuesta a la fluctuación de los niveles sanguíneos de las hormonas ováricas (Fawcett, 1988; Hafez, 1996). De esta manera, la progesterona y los estrógenos secretados por el ovario, se unen a sus receptores específicos (RP y RE, respectivamente) de localización nuclear, a fin de modular los cambios cíclicos que se producen a nivel de útero. Existen dos tipos de RE: REα y REβ, pero los primeros son los predominantes en el tracto resproductivo (Meikle et al, 2004). Los E producen un notable crecimiento uterino. Durante la fase folicular, las glándulas crecen, penetran profundamente en el estroma y comienzan a enrrollarse, pero su capacidad secretoria plena se da bajo la influencia de la P4 (Niswender y Nett, 1994). Las glándulas uterinas sufren una marcada hiperplasia e hipertrofia durante la gestación (Gray et al., 2001). El desarrollo y la función del útero dependen de interacciones entre el epitelio y el estroma (Gray et al., 2001). El estroma dirige el desarrollo epitelial, mientras que el epitelio influye en la organización del endometrio y en la diferenciación del miometrio (Cunha et al., 1989; Gray et al., 2001). La P4 actúa en el útero asegurando su quiescencia y estimulando y manteniendo las funciones

secretorias endometriales esenciales para el desarrollo temprano del embrión, implantación, placentación y un correcto desarrollo feto-placentario hasta el término de la gestación (Graham y Clarke, 1997).

La sensibilidad de un tejido a una hormona determinada depende de la expresión de los receptores específicos en dicho tejido (Clark et al., 1992). La modulación de la concentración de estas proteínas constituye el paso más probable en el control de las respuestas celulares (Clark y Mani, 1994). Las hormonas esteroideas ováricas son importantes moduladoras de sus propios receptores. La concentración de los receptores esteroideos en el tracto reproductivo de la hembra ha sido estudiada clásicamente en el útero y está demostrado que varía a lo largo del ciclo estral de la oveja (Miller et al., 1977). El E2, es el más importante de los E debido a su potencia biológica, estimula la síntesis de su propio receptor así como también la del RP, mientras que la P4 provoca la regulación a la baja (o *downregulation*) de ambos receptores (Clark y Mani, 1994). De esta manera, la concentración uterina de los RP y RE presenta valores máximos alrededor del celo como respuesta a la acción estimuladora de los E y mínimos durante la fase luteal tardía debido a la acción inhibitoria de la P4 (Miller et al., 1977).

En el caso en que la fecundación si se produzca, tendrá lugar un mecanismo de señalización entre el embrión, el endometrio y el ovario a fin de evitar la regresión del CL, asegurando de esta manera la producción y secreción de P4, lo que favorece el desarrollo temprano del embrión.

2. Desarrollo embrionario temprano y reconocimiento materno de la gestación

La gestación en las ovejas tiene una duración aproximada de 5 meses; sin embargo, al igual que en otros mamíferos, los eventos que transcurren durante los primeros días siguientes a la ovulación son determinantes para la supervivencia embrionaria. En condiciones normales, después del momento de la cubrición (natural o artificial), el oocito ovulado es fecundado durante su tránsito por el oviducto, produciendo un embrión que, aproximadamente el día 5 tras el celo, en el estadio embrionario de mórula compacta alcanza el cuerno uterino (Figura 2). Hacia el día 6 se forma el blastocisto que en su forma esférica esta rodeado de la membrana pelúcida pero alrededor del día 8 o 9 eclosiona. Luego comienza a adoptar una forma tubular y elongada para convertirse en un embrión filamentoso entre los días 12 y 16 tras el celo (Wintenberger-Torres y Flechon, 1974). Hasta el momento de la implantación (aproximadamente día 16 tras el celo), el embrión se desarrolla libremente tanto en el oviducto como en el útero, dependiendo de las secreciones de ambos órganos reproductivos para su supervivencia (Ashworth, 1995; Spencer et al., 2004).

Desde el momento en que se produce la fecundación, el ambiente reproductivo materno sufre transformaciones que deberán estar en total sincronía con el desarrollo del embrión para lograr llevar a término dicha gestación (Martal et al., 1997), de lo contrario aumentaría la incidencia de la mortalidad embrionaria (Roche et al., 1981). Por ello, es el propio embrión el encargado de iniciar los mecanismos que señalan su presencia en el útero y que evitan la regresión del CL. Dicho proceso se ha denominado reconocimiento materno de la gestación (Short, 1969) y se caracteriza por que el embrión se alarga, aumenta de tamaño y secreta hacia el lumen uterino una proteína de la familia de los interferones (IFN) llamada IFN-τ, que tiene como función evitar el desencadenamiento de la luteolisis. Por lo tanto, la elongación del embrión y su

producción de IFN-τ son eventos críticos para inhibir los mecanismos de la luteolisis, y por ende, favorecer la producción de P4 por el CL, asegurándose la continuidad del desarrollo embrionario (Spencer et al., 2007).

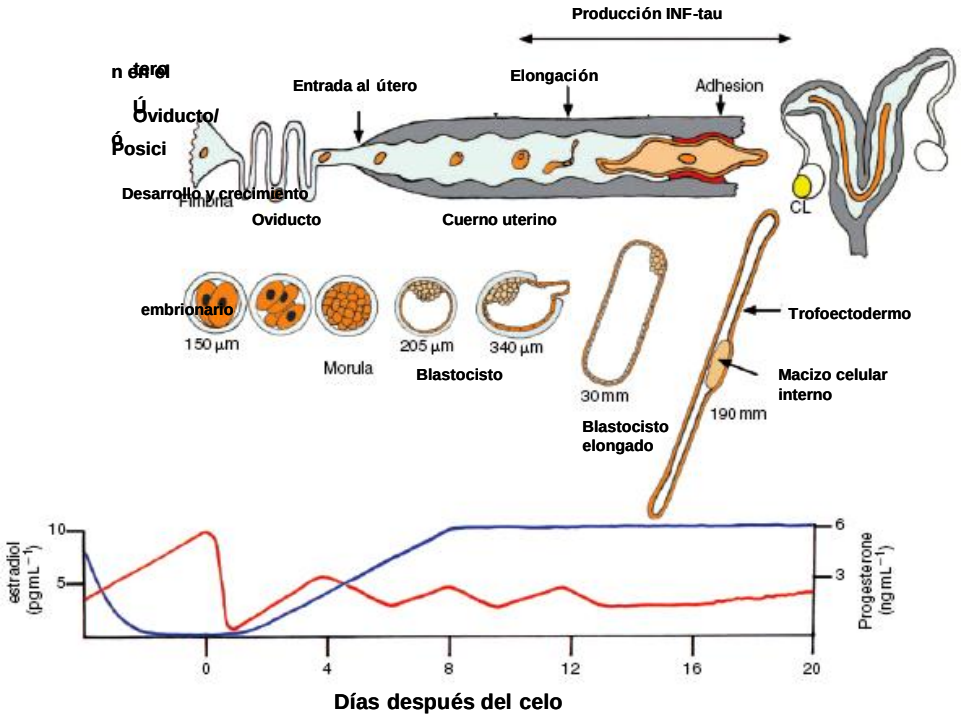


***Figura 2**. Esquema del desarrollo embrionario temprano ocurrido tras la fecundación en relación a la ubicación del blastocisto en el tracto reproductivo de la oveja y a los niveles circulantes de hormonas esteroideas ováricas (adaptado de Spencer et al., 2007).*

Es sabido que varios factores extrínsecos e intrínsecos influyen sobre la actividad reproductiva del ganado ovino. De los primeros, los dos más importantes parecen ser el fotoperiodo (duración de las horas de luz diarias y su evolución con el tiempo) y la nutrición. Ambos factores afectan directamente a la viabilidad embrionaria y en consecuencia influyen también sobre la eficiencia de la reproducción en los sistemas de explotación ovina.

3. Efectos del fotoperiodo sobre el eje reproductivo

El fotoperiodo es el principal factor regulador de la estacionalidad reproductiva en las ovejas, siendo sus efectos más intensos a medida que nos alejamos del ecuador hacia latitudes más altas, ya sea norte o sur (Karsch et al., 1984). El traductor endógeno de la información fotoperiódica es la melatonina, hormona que es liberada por la glándula pineal como respuesta a la percepción de horas de oscuridad y que actúa a nivel de hipotálamo para modular la secreción pulsátil de GnRH (Karsch et al., 1984; Robinson et al., 1985). El conocimiento de los efectos de la melatonina sobre el hipotálamo y su función principal como mediadora endocrina de la regulación de la estacionalidad en las ovejas (Chemineau et al., 1996), ha permitido el desarrollo de nuevas técnicas para mejorar el control de la reproducción, como por ejemplo el uso de implantes subcutáneos de melatonina exógena a fin de lograr adelantar la estación reproductiva y mejorar la eficiencia reproductiva durante el anoestro tanto en ovejas de estacionalidad muy marcada (Haresign et al., 1990) como en ovejas de razas Mediterráneas (Chemineau et al., 1996; Zuñiga et al., 2002; Abecia et al., 2007). Los implantes subcutáneos de melatonina inducen altos niveles plasmáticos de la hormona durante las 24 horas del día pero sin suprimir la secreción endógena nocturna de la hormona pineal (O'Callaghan et al., 1991; Malpaux et al., 1997). La liberación de melatonina es eficaz más allá de los 70 días tras la colocación del implante (Forcada et al., 2002).

Como se ha comentado anteriormente, los primeros días de vida del embrión tras la fecundación son críticos para la supervivencia embrionaria, por lo que es muy importante conocer cuáles son los efectos de este tratamiento hormonal sobre el embrión y su ambiente.

3.1. Efectos de la melatonina sobre el establecimiento de la gestación

En general, los tratamientos con melatonina exógena aumentan la fertilidad y prolificidad en las ovejas (Forcada et al., 1995; Chemineau et al., 1996; Zuñiga et al., 2002; Abecia et al., 2007). Asimismo, se ha comprobado que es un método efectivo para inducir ciclicidad y para aumentar la tasa de ovulación (Forcada et al., 1995; Zúñiga et al., 2002; Luther et al., 2005).

3.1.1. A nivel ovárico

El efecto de la melatonina sobre el desarrollo folicular y en consecuencia sobre la tasa de ovulación, se basa en que dicha hormona aumenta el número de folículos ovulatorios debido a una disminución en el proceso de atresia de los folículos pequeños y medianos presentes en la onda folicular previa a la ovulación (Bister et al., 1999; Noël et al., 1999). Parece que la melatonina es capaz de modular el crecimiento folicular sin influir en la secreción de hormona FSH (Noël et al., 1999), tal vez debido a la capacidad de estimular la esteroidogénesis a través de una acción directa sobre el ovario (Fiske et al., 1984).

Por otra parte, distintas referencias destacan el efecto luteotrófico de la melatonina, que ha sido evidenciado tanto *in vivo* (Wallace et al., 1988; Durotoye et al., 1997) como *in vitro* (Abecia et al., 2002). De este modo, Abecia et al. (2006) observaron que el tratamiento con melatonina se asocia con una disminución significativa de la proporción de ovejas no cíclicas que responden al efecto macho con la presentación de un ciclo corto (en base a la formación de un cuerpo lúteo con la funcionalidad alterada), y por tanto con un aumento del número de hembras que presentan un ciclo de duración normal (cuerpo lúteo funcional) tras la introducción de los moruecos. Estos resultados refuerzan las propiedades luteotróficas de la melatonina

y podrían reflejar un efecto positivo de la melatonina en el establecimiento de la gestación, ya que los niveles de progesterona (P4) durante los primeros días siguientes a la fecundación son esenciales para la supervivencia e implantación del embrión (Watson et al., 1999) (Figura 3).

3.1.2. A nivel oviductal y uterino

No se conoce hasta el momento que la melatonina ejerza efectos tanto directos como indirectos a nivel oviductal. Sin embargo, a nivel uterino se ha observado que la hormona pineal es capaz de reducir la producción *in vitro* de PGF2α en ovejas subnutridas durante el anoestro (Abecia et al., 1999). A su vez, la demostración de la presencia de receptores uterinos específicos para melatonina en ratas (Zhao et al., 2000) y su regulación a través de las hormonas esteroideas (Clemmens et al., 2001), permite pensar en una posible acción directa de la hormona a nivel uterino, aunque tampoco existe en la literatura ninguna referencia que demuestre estos hechos en ovinos.

3.1.3. A nivel del embrión

Los resultados a este nivel son contradictorios. Si bien algunos autores, trabajando con ovulación normal o con superovulación, no encontraron diferencias en la viabilidad de los embriones recuperados en anoestro o en la estación reproductiva (López-Sebastián et al., 1990; Mitchell et al., 1999; González-Bulnes et al., 2003), Mitchell et al. (2002) observaron que la viabilidad y las características morfológicas de los embriones fueron mejores cuando éstos se recuperaban al inicio de la estación reproductiva que al final de la misma. Además, Abecia et al. (2002) demostraron que la melatonina añadida al medio de cultivo favoreció la viabilidad *in vitro* de embriones

descongelados. Es posible que los efectos beneficiosos de la melatonina sobre el desarrollo embrionario temprano pudieran deberse, al menos en parte, a las propiedades antioxidantes de la hormona sobre las células embrionarias (Chetsawang et al., 2006), protegiendo a las mismas del daño que causa el estrés oxidativo durante los procesos de fecundación y de desarrollo *in vitro*.

Sin embargo y hasta el presente, los mecanismos específicos por los cuales la melatonina podría actuar sobre la viabilidad embrionaria no han sido totalmente dilucidados.

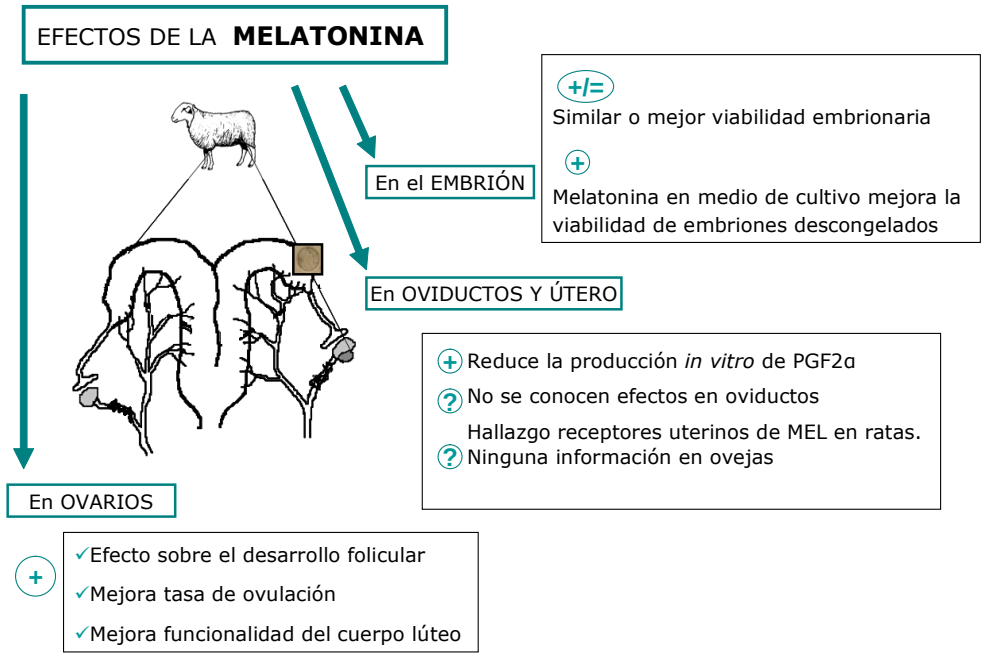


Figura 3. *Esquema de los principales efectos de la melatonina exógena sobre el establecimiento de la gestación en la especie ovina.*

4. Efectos de la nutrición sobre el eje reproductivo

Como ha sido mencionado previamente, la nutrición es uno de los principales factores que afectan la supervivencia embrionaria en los rumiantes. La compleja relación existente entre la nutrición y sus efectos sobre la reproducción ha sido objeto de numerosos estudios (Schillo, 1992; Rhind, 1992; Chilliard et al., 1998; O'Callaghan y Boland, 1999; Scaramuzzi et al., 2006). Aunque uno de los aspectos más estudiados de esa relación ha sido el efecto de la sobrealimentación o suplementación (flushing), como herramienta para incrementar la tasa de ovulación (Rhind, 1992), también existe documentación que avala el efecto de la subnutrición sobre el eje hipotálamo-hipofiso-gonadal en los rumiantes (Robinson, 1996; Boland et al., 2001; Forcada y Abecia, 2006). Al respecto, se ha observado que ovejas subnutridas, a largo y a corto plazo, presentaron una disminución de las concentraciones de FSH y LH, así como también una disminución en la frecuencia de pulsos de LH (Thomas et al., 1990; O'Callaghan et al., 2000). Dado que Foster et al. (1989) lograron revertir la baja frecuencia de descargas de FSH y LH con la administración de GnRH a corderas subnutridas, se sugiere que la deficiencia de gonadotropinas durante el período de subnutrición estaría causada por una deficiencia en la liberación endógena de GnRH, teniendo por tanto su origen a nivel hipotalámico. Algunos trabajos han encontrado una menor tasa de ovulación en ovejas subnutridas (McNeilly et al., 1987; Rhind et al., 1989a), mientras que otros autores no han encontrado diferencias (Lozano et al., 2003; Kakar et al., 2005).

La subnutrición es uno de los problemas que se presenta con mayor frecuencia en los rebaños ovinos, debido a que el esquema nutricional de los sistemas extensivos está

basado en el pastoreo, con lo cual la disponibilidad de alimento es muy fluctuante a lo largo del año (Lindsay et al., 1993).

4.1. Efecto de la subnutrición sobre el establecimiento de la gestación

Los mecanismos que deben llevarse a cabo para el establecimiento de la gestación son esenciales para la supervivencia del embrión (Robinson, 1996) (Figura 2), y la subnutrición afecta estos mecanismos aumentando la mortalidad embrionaria y disminuyendo las tasas de gestación (Rhind et al. 1989b, Abecia et al., 1995, 1999; Lozano et al., 2003), principalmente debido a una inadecuada calidad del ovocito o del desarrollo temprano del embrión; así como también afectando el ambiente uterino y el sistema de reconocimiento materno de la gestación (Abecia et al., 2006) (Figura 4).

4.1.1. A nivel ovárico

McNeilly et al. (1987) observaron en ovejas subnutridas un menor número de folículos grandes (≥ 3 mm) que en las controles. Además Viñoles et al. (2002) observaron que el patrón de la dinámica folicular era diferente según la condición corporal (CC) de los animales, donde ovejas con una CC más alta mostraron un número mayor de ondas durante el período inter-ovulatorio, y mayor cantidad de folículos antrales pequeños que en las ovejas con una CC baja. Cuando se estudió el efecto de la subnutrición sobre el número de folículos estrogénicos (aquellos que secretan cantidades > 500 pg de estrógeno/hora), no se encontraron diferencias entre animales subnutridos y controles (Abecia et al., 1995, 1999). En cuanto a la calidad de los oocitos, los resultados encontrados en la bibliografía son contradictorios. Algunos autores han encontrado una calidad similar, respecto a la morfología de los oocitos,

entre subnutridas y controles (O' Callaghan et al., 2000; Boland et al., 2001), mientras que otros han mostrado una alteración de la misma en animales subnutridos (Lozano et al., 2003). Además, se ha observado que, la tasa de fecundación de los oocitos en las ovejas subnutridas podía ser menor (O'Callaghan et al., 2000; Lozano et al., 2003) que las alimentadas según sus requerimientos de mantenimiento, probablemente como consecuencia de una alteración de la competencia oocitaria en el primer caso.

Otro importante aspecto a evaluar en el ovario es la función luteal. Al respecto, la literatura ha mostrado repetidamente que en los ovinos existe una relación inversa entre el nivel nutricional y las concentraciones plasmáticas de P4 (Parr et al., 1987; Rhind et al., 1992; Lozano et al., 1998; O'Callaghan et al., 2000). Sin embargo, la producción *in vitro* de P4 por el cuerpo lúteo (CL) no parece estar afectada por la subnutrición (Abecia et al., 1995, 1997, 1999), lo que indicaría que los mayores niveles de P4 no se deben a un aumento en su síntesis. Parr (1992) propuso que este fenómeno podría deberse a una menor metabolización hepática de la hormona en animales subnutridos. De esta manera, Rhind et al. (1989a) sugirieron que la medición de la hormona a nivel local (vena ovárica) daría un mejor reflejo de la situación que al tomar muestras de sangre de la vena yugular, y que además podría ayudar a explicar el hecho de que animales subnutridos, con mayores niveles plasmáticos de P4, presentaban una mayor tasa de mortalidad embrionaria. En este sentido, se ha podido comprobar que las ovejas subnutridas 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), aunque Lozano et al. (1998) observaron el día 5 del ciclo estral unas menores concentraciones de P4 en el tejido endometrial de ovejas subnutridas respecto a las controles, lo que podría explicar el desarrollo embrionario inadecuado que presentaron las primeras.

4.1.2. A nivel oviductal y uterino

Es conocido que un ambiente adecuado para el embrión, principalmente durante sus primeros estadios de formación, es esencial para alcanzar un desarrollo adecuado, y por ende, para asegurar su supervivencia (Watson et al., 1999). Los trabajos publicados recientemente por nuestro grupo de investigación muestran algunos de los efectos de la subnutrición sobre el ambiente en el cual podría desarrollarse un embrión ovino. Se observó que la subnutrición producía alteraciones en la expresión génica así como una disminución de la sensibilidad a las hormonas esteroideas ováricas (e.g. menor expresión de RP y REα) tanto en los oviductos como en el endometrio durante el día 5 después del estro (Sosa et al., 2004, 2006, 2008). En el útero en concreto observaron una menor expresión de los RP y una menor capacidad de unión de la hormona a los mismos durante el día 5 posterior al estro, aunque no encontraron diferencias los días 10 o 14 del ciclo estral para los parámetros mencionados.

4.1.3. A nivel del embrión

Los estudios que investigan los efectos directos de la subnutrición sobre el desarrollo embrionario son relativamente escasos. En ovinos, se ha demostrado que 25 días de subnutrición aumentan la mortalidad de los embriones con 11 días de gestación (Rhind et al., 1989c). Otros trabajos, con condiciones nutricionales comparables, han reportado un porcentaje de embriones recuperados similar en ovejas subnutridas y controles los días 4, 8 y 9, aunque los embriones de las ovejas subnutridas presentaban un retraso en su desarrollo (Abecia et al., 1997, 1999; Lozano et al., 2003). Además, se encontraron diferencias en las tasas de gestación debidas a la subnutrición durante los días 14 y 15, pero no en los días 8 y 9 de gestación (Abecia et al., 1995,1997, 1999).

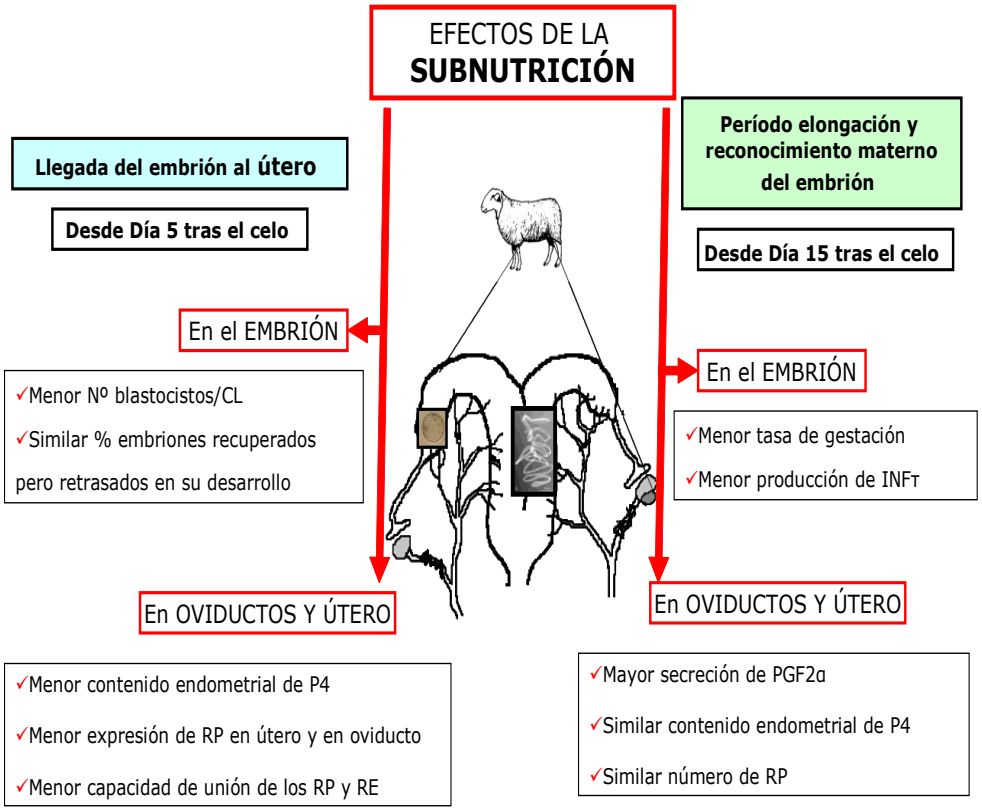


Figura 4. *Esquema de los principales efectos de la subnutrición sobre el establecimiento de la gestación en la especie ovina*

5. Efectos de la interacción melatonina-nutrición sobre la reproducción

No existe mucha información en la literatura respecto a los efectos combinados del tratamiento con melatonina y del plano de alimentación sobre los parámetros reproductivos. No obstante, en la especie ovina se han descrito algunos aspectos realmente interesantes. En concreto, distintos autores han mostrado que existe una notable y significativa interacción entre el tratamiento con melatonina y el nivel nutricional sobre la tasa de ovulación, de manera que la mejora que induce la hormona pineal sobre este parámetro es superior en aquellas ovejas sometidas a un bajo nivel nutricional (Robinson et al., 1991; Forcada et al., 1995) o con un reducido nivel de reservas (Zúñiga et al., 2001), siendo este efecto particularmente apreciable en los primeros ciclos que tienen lugar tras la colocación del implante (Rondón et al., 1996). Por otra parte y como ya se ha señalado previamente, la producción in vitro de PGF2α por el tejido endometrial de ovejas subnutridas el día 15 de gestación fue menor en presencia de melatonina que en su ausencia, indicando que la hormona pineal podría mejorar la supervivencia embrionaria (Abecia et al., 1999).

6. Efecto del anoestro post-parto sobre la reproducción

6.1. Breve descripción de la fisiología y de la endocrinología del anoestro post-parto

El periodo de anoestro postparto se caracteriza por la ausencia de ciclicidad ovárica, con la finalidad de preparar al útero para iniciar una nueva gestación. Los factores de mayor relevancia que influyen sobre la duración de este periodo son diversos, desde la estacionalidad, el nivel nutricional y la condición corporal (Randel, 1990; Short et al., 1990), la raza (Tervit et al., 1977), pasando por la edad y la dificultad al parto (Dziuk y Bellows, 1983) y por supuesto el propio estímulo del amamantamiento

(Williams, 1990). El amamantamiento es un estímulo exteroceptivo que tiene un papel fundamental en la regulación de la reproducción de los mamíferos (McNeilly, 1988), pero su efecto parece ser irrelevante en la oveja (Lamming, 1978).

El anoestro posparto se caracteriza por una baja secreción de LH, tanto en la concentración media como en la pulsatilidad (Short et al., 1990; Wallace et al., 1989). Fray et al. (1995) demostraron que la funcionalidad ovárica tras el parto permanece intacta, y que luego de una infusión de gonadotropinas la oveja era capaz de ovular, respondiendo a las hormonas hipotalámicas. A fin de dilucidar el papel de las gonadotrofinas hipofisarias en el anoestro post-parto, se estudiaron las concentraciones séricas de hormona luteinizante (LH) determinándose que las mismas son bajas durante el último tercio de la gestación y el período temprano del post-parto. La elevación en las concentraciones séricas basales y el inicio de un patrón episódico en la liberación de LH preceden al inicio de la ciclicidad estral (Lamming et al., 1981). Las concentraciones plasmáticas de hormona folículo estimulante (FSH) son bajas en el período previo al parto, pero se regularizan al día 5 post-parto (Lamming et al., 1981). Por esta razón se considera que la FSH no sería limitante para el reinicio de la ciclicidad ovárica.

Existen diferentes componentes del eje hipotálamo-hipofisiario que podrían provocar una disminución en la secreción de LH durante el periodo post-parto. No parece que la causa de la insuficiente secreción de LH sea una disminución en el contenido hipotalámico de GnRH, pues se ha visto que el contenido de esta hormona no varía en el área preóptica, en el hipotálamo mediobasal o en la eminencia media desde el parto hasta que se reanuda la actividad cíclica (Moss et al., 1980). Otra posible causa podría deberse a una menor sensibilidad de la hipófisis a la GnRH, sobretodo debido a un menor número de receptores de dicha hormona. Pero se ha visto que la cantidad de LH liberada se incrementa progresivamente con la dosis de GnRH, y que aunque dicho

incremento es mayor conforme avanza el periodo post-parto, la dosis de GnRH que provocó la máxima secreción de LH fue similar en cada momento (Moss et al., 1980), hecho que indica que el número de receptores hipofisiarios para la GnRH es similar en cada momento tras el parto. También la menor disponibilidad de LH podría ser una causa de la escasa secreción de esta hormona durante el anoestro, ya que se ha visto que durante la gestación la LH disminuye en un 20% sus cantidades en los almacenes hipofisiarios con respecto a los niveles en ovejas vacías (Jenkin et al, 1977). La respuesta a la infusión de GnRH se incrementa con el tiempo postparto, siendo este incremento mayor en las ovejas secas que en las lactantes (Pelletier y Thimonier, 1975). Gordon (1997) observó que las ovejas lactantes tienen una menor producción de esteroides ováricos, especialmente estrógenos, los cuales sensibilizan la hipófisis a la GnRH.

Parece ser que, la depleción hipofisiaria de LH es consecuencia de las elevadas concentraciones de E2 y de P4 presentes en la oveja durante el último tercio de la gestación. La P4 por sí sola no modifica el contenido hipofisiario de LH, pero el estradiol o ambos sí disminuyen dicho contenido (Moss et al., 1981). Por otra parte, para que se produzca la síntesis de LH es necesaria la secreción pulsátil de GnRH (Fraser et al., 1975). La P4 reduce la frecuencia de pulsos de LH mientras que el estradiol reduce la amplitud en ovejas ovariectomizadas (Goodman y Karsch, 1980). Por lo tanto, puede ser que el estradiol placentario inhiba directamente la síntesis de LH a nivel de hipófisis o inhiba más directamente la secreción hipotalámica de GnRH, lo que provocaría una insuficiente estimulación trópica para la síntesis de LH (Nett, 1977). Estos factores en conjunto serían los que provocarían la depleción de los almacenes de LH durante la última fase de la gestación, lo que impide la secreción de LH al comienzo del anoestro post-parto. Una vez que se recuperan los almacenes de LH, es el escaso

número de receptores hipotálamo-hipofisiarios de estradiol lo que impide la reanudación de la actividad cíclica. Estos receptores se van recuperando paulatinamente y en torno al mes tras el parto alcanzan la concentración habitual, permitiendo que se produzca un pico de LH en respuesta a los niveles crecientes de estradiol (Wise et al., 1986).

Tampoco el útero parece estar preparado para acoger a una nueva gestación pues presenta un escaso número de receptores de P4 y de E, los cuales son necesarios para la supervivencia del embrión. Un experimento realizado con ovejas sacrificadas a diferentes días tras el parto encontró que en los días 1 a 5 post-parto las concentraciones endometriales de RP y RE fueron mas bajas que en los días 17 y 30 tras el parto (Tasende et al., 1996). Dicho hallazgo evidencia que existe una recuperación paulatina de estos receptores, los cuales presentarían una concentración adecuada para permitir la gestación a partir de la tercera semana post-parto.

A nivel ovárico, el cuerpo lúteo presenta una función anormal durante el inicio del periodo post-parto, a pesar de que el ovario es capaz de ovular tras el parto en respuesta a la administración de hormonas gonadotropas exógenas. Dicha función luteal alterada es debida a los altos niveles de PGF2α secretados por el útero durante el proceso de involución (Cooper et al., 1991). Al respecto, se ha observado en ovejas que la primera ovulación tras el parto generalmente es una ovulación silenciosa, no acompañada de conducta de celo, en la que generalmente se produce un cuerpo lúteo de vida media corta debido a la luteolisis temprana ejercida por los mencionados niveles elevados de PGF2α (Goodman, 1994).

6.2. Influencia de la nutrición sobre el anoestro post-parto

Debido a los efectos que ejercen el nivel nutricional y la CC sobre el reinicio de la ciclicidad tras el parto, su control se considera una importante herramienta a fin de lograr minimizar la duración de dicho periodo de anoestro, algo especialmente importante en los sistemas intensificados de reproducción que podemos encontrar en el ámbito mediterráneo.

El comienzo de la lactación significa para la oveja un repentino aumento de sus necesidades energéticas, las cuales intenta compensar con un aumento de la ingestión de alimentos. No obstante, para lograr un aumento en el consumo de alimentos, previamente se requieren ciertos cambios anatómicos y fisiológicos del tracto digestivo y/o reproductivo que son lentos y están desfasados en el tiempo respecto al aumento de las necesidades energéticas de la hembra lactante. Así, se produce una movilización de las reservas energéticas de la oveja, lo que lleva a un balance energético negativo durante el primer tercio de la lactación, ya que la producción de leche es prioritaria. Sin embargo, durante el segundo tercio de la lactación, la capacidad de ingestión es máxima y la producción de leche comienza a declinar, siendo posible entonces una cierta recuperación de las reservas energéticas.

Al respecto, en la raza Rasa Aragonesa se ha observado que la tasa de ovulación en el primer ciclo tras el parto en ovejas destetadas en primavera está correlacionada negativamente con las pérdidas de peso durante el periodo de lactancia; de esta manera, a menores pérdidas de peso mayor tasa de ovulación. Por lo tanto, la ganancia de peso desde el destete hasta la aparición del primer celo no tuvo efecto sobre la misma (Abecia et al., 1993).

Es importante separar los efectos de la nutrición sobre el anoestro de lactación de los efectos derivados sobre la época del año en que se producen los partos (estación

reproductiva o anoestro), pues la época natural de partos de la oveja es la estación de anoestro. Así, la duración del periodo post-parto tras los partos de primavera es superior a la de los partos de otoño, de manera que en el primer caso las ovejas no son capaces de reactivar su actividad sexual inmediatamente tras el destete (Whiteman et al., 1972). Además, en el Valle Medio del Ebro (lugar donde se ha desarrollado la presente Tesis) son frecuentes los sistemas de intensificación de la reproducción, donde la inducción de celos y gestaciones subsiguientes durante el periodo de anoestro estacional permite obtener partos en la estación sexual y una nueva cubrición en un corto período tras el destete.

HIPÓTESIS DE TRABAJO

Bajo la premisa de aumentar la eficiencia reproductiva en la especia ovina y teniendo en cuenta que tanto la nutrición como la estación del año son factores muy importantes que influyen en la reproducción de esta especie, nuestro equipo de investigación viene desarrollando, desde 1988, una línea de investigación con el objeto de profundizar en el conocimiento de los mecanismos que intervienen en las relaciones entre nutrición y reproducción en la oveja. Además, y a partir de 1991, se incorporó una línea paralela con el objeto de determinar los mecanismos de acción de la melatonina sobre el eje reproductivo en la especie ovina. Uno de los aspectos de mayor interés en los que han confluido ambas líneas de trabajo ha sido el conocimiento de los mecanismos que determinan la viabilidad embrionaria y el reconocimiento materno de la gestación en la especie ovina, poniendo al descubierto el papel que la subnutrición y la estacionalidad juegan en los mismos.

Hemos comentado anteriormente que la oveja es una especie con estacionalidad reproductiva y que la melatonina es la hormona que interviene como mediadora entre el medio ambiente externo y el eje reproductivo, actuando tanto a nivel del embrión como del tracto reproductivo, con efectos benéficos para la eficiencia reproductiva. **Nuestra hipótesis de trabajo** es que el tratamiento con melatonina exógena podría ser una herramienta útil para paliar o revertir los efectos adversos que causa la subnutrición sobre la supervivencia embrionaria en las explotaciones ovinas. Además, debido a que un inadecuado ambiente uterino podría determinar un retraso en el desarrollo e incluso la muerte del embrión, consideramos un punto clave y esencial para el entendimiento de los mecanismos involucrados en la supervivencia embrionaria, evaluar los efectos sobre

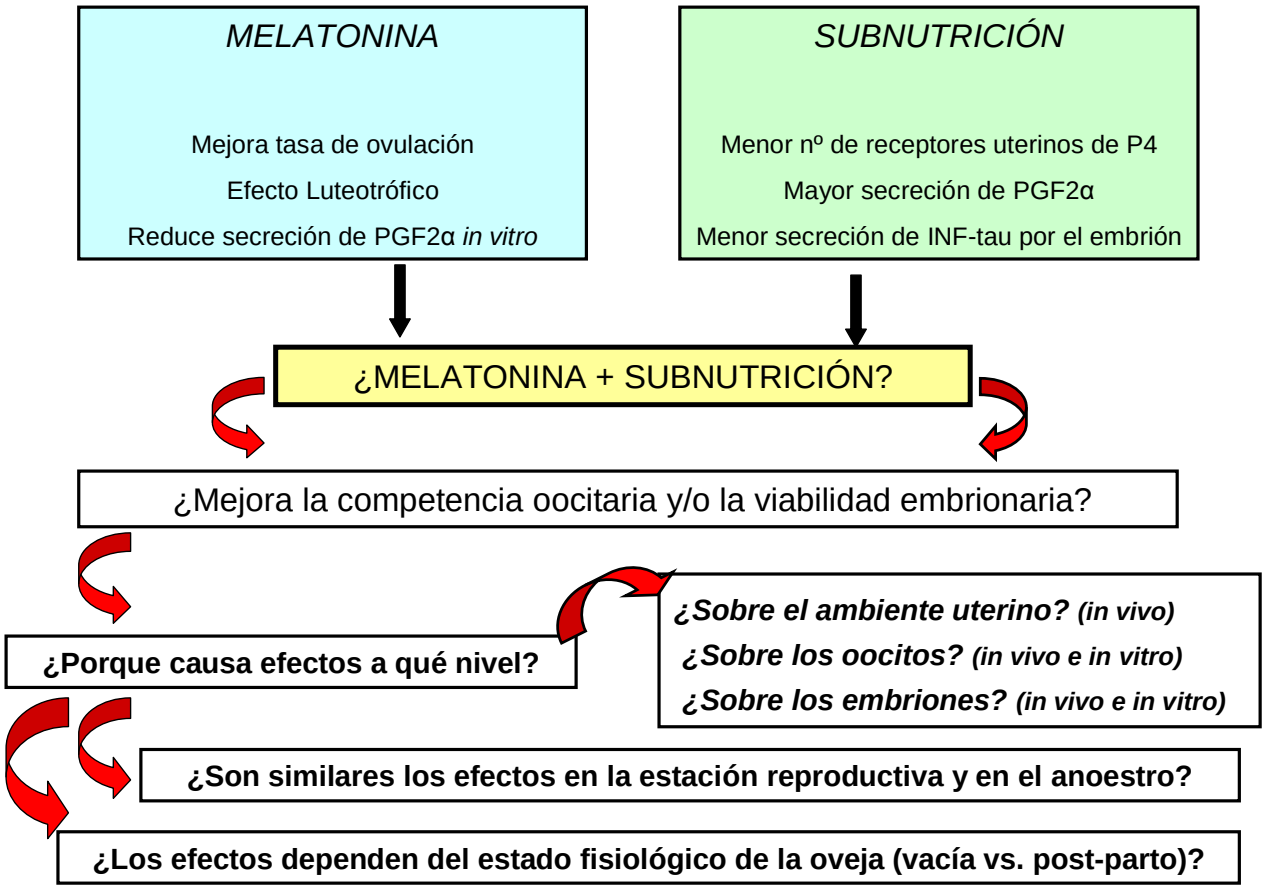
la competencia oocitaria y el embrión tanto *in vivo* como *in vitro*, así como también los efectos sobre el endometrio.

En las condiciones de los sistemas de producción ovinos en el área mediterránea, el manejo nutricional del rebaño es un punto fundamental a considerar al determinar la rentabilidad de las explotaciones. Debido a las variaciones en la disponibilidad de alimentos, estacionales o coyunturales, es muy frecuente observar la presentación de periodos más o menos prolongados de subnutrición. Esta situación puede agravarse en los momentos en que las hembras presentan requerimientos máximos para cubrir sus necesidades fisiológicas, como por ejemplo durante la lactación. De esta manera, nos pareció importante comprobar nuestra hipótesis de trabajo en hembras que estuvieran en diferentes estados fisiológicos (vacías secas o recién paridas), lo que podría influir en la respuesta al efecto de la melatonina exógena sobre la viabilidad embrionaria y en la posible mejora de la eficiencia de producción en las explotaciones ovinas.

En la actualidad existen pocos trabajos en los que se evalúen de manera conjunta los efectos de la estación del año y de la subnutrición sobre el establecimiento de la gestación en una especie con una notable estacionalidad reproductiva como la ovina. Por lo que profundizar sobre el tema nos permitiría comprender de manera global los mecanismos implicados en la viabilidad embrionaria y por tanto en la mejora de la productividad en dicha especie.

En resumen, el presente trabajo pretende aportar información precisa sobre los efectos del tratamiento exógeno con melatonina a ovejas mediterráneas subnutridas sobre la viabilidad de oocitos y de embriones, así como también del ambiente uterino, tanto durante la estación reproductiva como durante el anoestro estacional.

ESQUEMA DE LA HIPOTESIS DE TRABAJO PLANTEADA



OBJETIVOS ESPECÍFICOS

I- Evaluar los efectos de la melatonina exógena sobre la viabilidad embrionaria en ovejas subnutridas de Rasa Aragonesa, en dos diferentes estados fisiológicos (vacías y con al menos 45 días tras el parto) y durante dos épocas diferentes del año (estación reproductiva y anoestro estacional).

II- Evaluar los efectos de la melatonina exógena sobre la competencia de los oocitos recuperados de ovejas subnutridas, vacías y con 45 días tras el parto, durante la estación reproductiva y el anoestro estacional.

III- Evaluar los efectos de la melatonina exógena sobre la localización y abundancia de los receptores esteroideos de progesterona (RP) y estrógenos (REα) en el endometrio de ovejas subnutridas, vacías y con 45 días tras el parto, durante la estación reproductiva y el anoestro estacional.

MATERIALES Y MÉTODOS

1. Aspectos generales

1.1. Instalaciones

Todos los experimentos se realizaron en la granja experimental del Servicio de Experimentación Animal de la Facultad de Veterinaria de la Universidad de Zaragoza, España (latitud 41º 41'N), cumpliendo con los requerimientos del Comité Ético para la Experimentación Animal de la Universidad de Zaragoza. Se utilizaron ovejas adultas de la raza Rasa Aragonesa, durante la estación reproductiva y durante el anoestro estacional (definido para dicha raza por Forcada et al., 1992).

1.2. Alimentación

Los animales se alimentaron una vez al día, por la mañana, en base a una dieta compuesta por paja de cebada y concentrado, con libre acceso al agua. El concentrado de la dieta consistió en una mezcla de cebada (73%), soja (22%) y suplemento mineral (5%).

Desde la colocación de los implantes de melatonina hasta el inicio de las dietas experimentales, todos los animales recibieron una dieta que cubría 1,0 veces los requerimientos diarios de mantenimiento (M) (Agricultural and Food Research Council, 1993) (experimentos 1 y 2) o los requerimientos de lactancia para un solo cordero (experimentos 3 y 4).

En todos los experimentos y desde el momento de la colocación de las esponjas intravaginales hasta el sacrificio (día 5 tras el celo), las ovejas fueron asignadas para ser

alimentadas en grupo con una de dos dietas con diferentes niveles energéticos: dieta control y dieta baja, que aportaron 1,5 o 0,5 veces los requerimientos diarios de M, respectivamente. La dieta control (C) estuvo compuesta por 0,60 kg de concentrado y 1,0 kg de paja de cebada por oveja y por día, aportando 12,4 MJ de energía metabolizable (EM) y 9,3% de proteína bruta (PB). La dieta baja (B) consistió en 0,20 kg de concentrado y 0,35 kg de paja de cebada por oveja y por día (4,1 MJ de EM y 9,1% PB). Durante el periodo de oferta de los tratamientos nutricionales (20 días), la dieta 1,5 M aseguró el mantenimiento del PV y la CC, mientras que la dieta 0,5 M provocó una disminución de aproximadamente un 12% tanto en el PV como en la CC (Abecia et al., 1995, 1997; Lozano et al., 1998; Sosa et al., 2004, 2006).

El PV y la CC fueron determinados al momento de colocación y de retirada de las esponjas intravaginales, y al sacrificio de los animales. Adicionalmente en los experimentos 3 y 4, también se determinaron al momento del parto y del destete.

1.3. Tratamientos hormonales

En cada uno de los experimentos, la mitad de las ovejas fueron seleccionadas para recibir un implante subcutáneo de melatonina (18 mg melatonina, Melovine®, CEVA Salud Animal, Barcelona, España) en la base de la oreja, 42 d antes de realizar la sincronización de los celos.

La sincronización de los celos se realizó utilizando un tratamiento intravaginal con esponjas impregnadas con progestágenos (30 mg de acetato de fluorogestona, Sincropart®, CEVA Salud Animal, Barcelona, España) durante 14 días, al cabo de los cuales se administraron 400 UI (Experimentos 1 y 3, durante la estación reproductiva) y 480 UI (Experimentos 2 y 4, durante el anoestro) de gonadotropina coriónica equina (eCG) (Sincropart® PMSG, CEVA Salud Animal, Barcelona, España) por vía

intramuscular. Desde las 24 h posteriores a la retirada de las esponjas intravaginales, y cada 8 h, se realizó la detección del celo (día 0) con machos enteros dotados de arneses con pintura marcadora.

2. Diseño experimental

Cada uno de los experimentos se basó en un diseño factorial 2 x 2; considerando el tratamiento con melatonina exógena (con o sin implantes de melatonina) y el tratamiento nutricional (dietas control y baja) como factores fijos.

De esta manera, los 4 grupos experimentales en cada uno de los experimentos realizados fueron: ovejas alimentadas con dieta control y que no recibieron implantes de melatonina (**C-MEL**), ovejas alimentadas con dieta control y que recibieron implantes de melatonina (**C+MEL**), ovejas alimentadas con dieta baja y que no recibieron implantes de melatonina (**B-MEL**) y ovejas alimentadas con dieta baja y que recibieron implantes de melatonina (**B+MEL**).

La Figura 5 muestra un esquema del diseño experimental en los 4 experimentos realizados.

3. Animales

3.1. Experimento 1 (Artículos I y II-Ovejas vacías, Estación reproductiva)

Se utilizaron 42 ovejas adultas, cíclicas y vacías de la raza Rasa Aragonesa, que al comienzo del experimento tuvieron un peso vivo (PV) medio (± ES) de 64,1±1,7 kg y una condición corporal (CC) media (± S.E.) de 3,1±0,1. A principios de diciembre, las ovejas fueron distribuidas en 2 grupos: el primer grupo (n=21) incluyó ovejas que recibieron un implante subcutáneo de melatonina el segundo grupo (n=21) quedó sin

implantar. A mediados de enero, 42 días después de la colocación de los implantes de melatonina, los celos de las hembras fueron sincronizados, y cada hembra fue distribuída en uno de los 4 grupos experimentales, iniciándose el mismo día los tratamientos nutricionales correspondientes.

3.2. Experimento 2 (Artículos I y II-Ovejas vacías, Anoestro)

Se utilizaron 31 hembras no-cíclicas, vacías de raza Rasa Aragonesa que tuvieron un PV (± S.E.) medio de 57,2±1,2 kg y una CC media (± S.E.) de 2,9±0,04. El 26 de marzo, 16 de esas ovejas recibieron un implante subcutáneo de melatonina y el resto de las ovejas (n=15) no fueron implantadas. Todas las hembras fueron sincronizadas en mayo, 42 días después de la colocación de los implantes de melatonina, iniciándose el mismo día con el suministro de las dietas experimentales.

3.3. Experimento 3 (Ovejas post-parto, Estación reproductiva)

Se utilizaron 24 ovejas adultas, de la raza Rasa Aragonesa, con un peso vivo (PV) medio (± S.E.) de 65,9±1,8 kg y una condición corporal (CC) media (± S.E.) de 2,80±0,06, durante los meses de noviembre a febrero. En el momento del parto (noviembre), las ovejas fueron distribuidas en 2 grupos: el primer grupo recibió un implante subcutáneo de melatonina, el segundo grupo incluyó las ovejas sin implantar. Tras un periodo de amamantamiento de 45 días, con un solo cordero, se realizaron los destetes y los celos de las ovejas fueron sincronizados Desde el momento de la colocación de las esponjas hasta el sacrificio (día 5 post estro), las ovejas fueron alimentadas con una de las dos dietas experimentales.

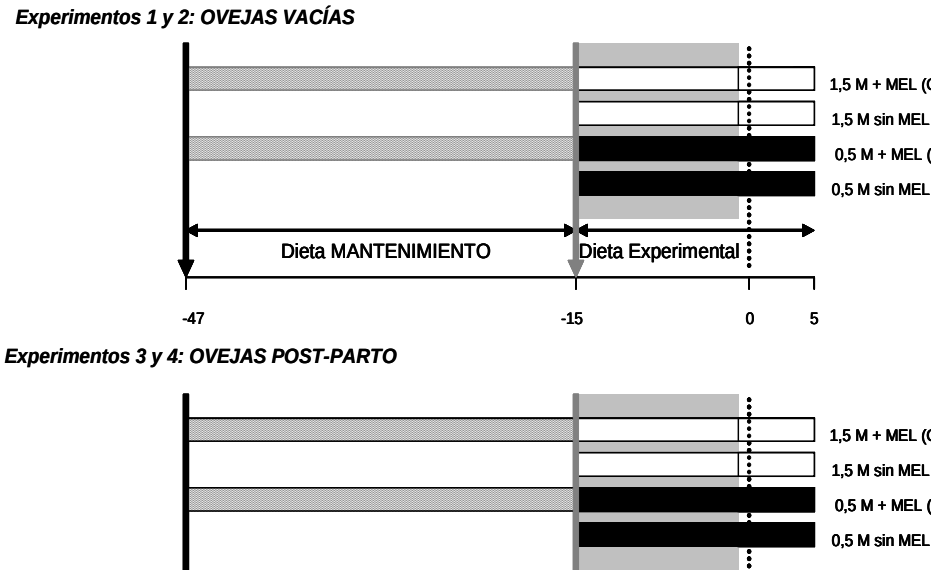


Figura 5. E*squema de los diseños experimentales utilizados. Grupos Control (barras blancas); grupos Bajos (barras negras). Flechas negras indican el día de la colocación de los implantes de melatonina (barras oblicuas). Flechas grises indican el día de inicio de los tratamientos nutricionales (dietas control y bajo). El área sombreada de gris indica la duración del tratamiento de sincronización de los celos utilizando progestágenos intravaginales. La línea punteada indica el día del celo (día 0 del ciclo estral) previo al sacrificio el día 5, en todos los experimentos.*

3.4. Experimento 4 (Artículo III-Ovejas post-parto, Anoestro)

Se utilizaron 31 ovejas adultas, de la raza Rasa Aragonesa, con un peso vivo (PV) medio (± S.E.) de 61,0 ± 1,4 kg y una condición corporal (CC) media (± S.E.) de 2,9 ± 0,07, durante los meses de noviembre a febrero. En el momento del parto (mediados de febrero), las ovejas fueron distribuidas en 2 grupos: el primer grupo recibió un implante subcutáneo de melatonina y el segundo grupo incluyó las ovejas sin implantar. Tras un periodo de amamantamiento de 45 días, con un solo cordero, se realizaron los destetes tras los cuales, los celos de las ovejas fueron sincronizados, momento en el que se inició el suministro de las dietas experimentales hasta el día del sacrificio (Día 5 tras el celo).

4. Obtención de embriones producidos in vivo *(Artículos I y III)*

El día 5 tras el celo, los embriones fueron recuperados por laparotomía ventral y lavado uterino. Las ovejas fueron anestesiadas utilizando una dosis i.m. de xylazina al 2% (0.4 ml, Xilagestic 2%®, Calier, Barcelona, España) y 5 minutos después una inyección i.v. de tiopental sódico (10 ml, Thiobarbital Braun Medical®, Jaén, España). La respuesta ovárica fue cuantificada con el número de cuerpos lúteos (CL) observados con un aspecto congruente con una fase lútea activa. Los cuernos uterinos fueron expuestos y lavados utilizando una sonda catéter tipo Foley y una solución tampón salina fosfatada (PBS) suplementada con 1% de albúmina sérica bovina (BSA; Sigma, St. Louis, MO, USA) y antibióticos (penicilina y estreptomicina), atemperada a 36ºC. Las ovejas fueron posteriormente sacrificadas utilizando una dosis de tiopental sódico (T-61®; Intervet, Salamanca, España).

Las estructuras recuperadas (oocitos y embriones) fueron examinadas bajo una lupa microscópica (aumentos de 20-40X) y clasificadas según su morfología y estado de desarrollo (Winterberger-Torres y Sevellec, 1987). Se consideró como embriones viables a los estadios embrionarios de mórula y mórula compacta, de acuerdo al día de gestación en el que los mismos fueron recuperados. La viabilidad *in vitro* de los embriones fue evaluada basada en la proporción de embriones que fueron capaces de desarrollarse hasta estadios de blastocisto expandido o eclosionado después de 48 h de cultivo a 39º C con 5% de CO_2. El medio de cultivo estuvo compuesto por fluido oviductal sintético (SOF) sin glucosa, amino ácidos esenciales BME, amino ácidos no-esenciales MEM (Walker et al., 1996), 10% (v/v) de suero fetal bovino, 1 mM de L-glutamina, 100 UI/ml de penicilina-G y 100 μg/ml de sulfato de estreptomicina.

Se obtuvieron las siguientes variables para cada oveja: número de cuerpos lúteos (CL), número de estructuras recuperadas (oocitos+embriones), tasa de recuperación (número de estructuras recuperadas/número de CL), número de embriones fertilizados, número de embriones viables (como mórula compacta), y número de embriones viables después de 48 h de cultivo *in vitro* (como blastocisto expandido o eclosionado). Se consideró como tasa de fecundación al número de embriones dividido por el número de estructuras recuperadas, y como tasa de viabilidad al número de embriones viables dividido por el número de estructuras recuperadas. Las ovejas de las cuales no se recuperó ninguna estructura fueron excluidas del cálculo de tasa de fecundación y tasa de viabilidad.

5. Obtención de ovarios y maduración in vitro (MIV) de oocitos *(Artículos II y III)*

Inmediatamente después del sacrificio de las ovejas se procedió a recuperar ambos ovarios, que fueron mantenidos en PBS suplementado con 100 UI/ml de penicilina-G y 100µg/ml de sulfato de estreptomicina y atemperado a 39ºC hasta su posterior procesamiento, que se realizó dentro de la media hora desde su recuperación. A excepción de que sea indicado, todos los reactivos utilizados en el proceso de producción *in vitro* de embriones procedían de la firma Sigma-Aldrich Co., St. Louis, MO, USA. Para recuperar los oocitos se utilizó una combinación de las técnicas de punción y *slicing* (Wani et al., 1999) de los ovarios colocados en una placa de Petri parcialmente cubierta con medio de manejo (Solución tampón Hepes TCM-199 suplementada con 0,1% de alcohol polivinílico (PVA), 0,004% de bicarbonato de sodio, 25 UI/ml de heparina, 100 UI/ml de penicilina-G y 100µg/ml de sulfato de estreptomicina). Los oocitos obtenidos se clasificaron de acuerdo al criterio de Wani et al. (2000) observando la morfología del citoplasma y el número de capas celulares que conforman el *cumulus*; se definió como oocitos aptos para maduración *in vitro (MIV)* a aquéllos oocitos que presentaban varias capas celulares conformando el *cumulus* y un citoplasma uniforme y homogéneo.

El medio de maduración estuvo constituído por una solución tampón de bicarbonato TCM-199 suplementada con 10% (v/v) de suero de oveja en celo, 0,1 µg/ml de FSH y de LH, 100 µM de cisteamina, 0,3 mM de piruvato de sodio, 100 UI/ml de penicilina G y 100 µg/ml de sulfato de estreptomcina. Los oocitos colocados en el medio de maduración fueron cubiertos con aceite mineral e incubados a 39º C durante 24 h, en una atmósfera saturada de humedad y con 5% de CO_2.

6. Fecundación in vitro (FIV) y cultivo embrionario *(Artículos II y III)*

Al finalizar la maduración *in vitro*, los oocitos fueron liberados de las células del cumulus y transferidos a un medio de fecundación. Dicho medio estaba compuesto por fluido oviductal sintético (SOF) sin glucosa (Tervit y Whittingham, 1972) y suplementado con 2% (v/v) de suero de oveja en celo (Hopper et al., 1993; Huneau et al., 1994; Li et al., 2006), 10 μg/ml de heparina (Cox et al., 1992) y 1 μg/ml de hipotaurina.

El día de la fecundación, el semen utilizado provenía de un pool de 4 machos de Rasa Aragonesa diluido 1:10 en un medio salino de 0,25 mol/l de sucrosa, 10 mmol/l de hepes, 2 mmol/l de hidróxido de potasio, 5 mmol/l de glucosa, 0,5 mol/l de fosfato monobásico de sodio y 100 mmol/l de ácido tetra acético glicol etileno (EGTA); y mantenido a 15º C hasta el momento de su utilización. Los espermatozoides fueron seleccionados utilizando la técnica de Swim-up (Wani et al., 2000; Luther et al., 2005). La dosis de fecundación fue de 1 x 10^{-6} espermatozoides/ml. El semen fue agregado al medio que contenía los oocitos, cubierto con aceite mineral e incubado a 39º C durante 24 h en una atmósfera con 5% de CO_2, 5% de O_2 y 90% de N_2.

A las 24 h y 36 h después de la fecundación, los presuntos zigotos fueron colocados en un medio de cultivo que contenía SOF suplementado con amino ácidos esenciales y no esenciales en una concentración oviductal (Walker et al., 1996), 0,4% de BSA, 1 NM de L-glutamina, 100 UI/ml de penicilina G y 100 μg/ml de sulfato de estreptomicina. El medio fue cubierto con aceite mineral y mantenido a 39º C en una atmósfera saturada de humedad y con 5% CO_2, 5% de O_2 y 90% de N_2, durante 8 días hasta el estadio de blastocisto.

Los embriones divididos fueron colocados en una placa, y los no divididos fueron examinados bajo una lupa estereomicroscópica para evaluar su estado de maduración.

Oocitos que exhibieron el primer corpúsculo polar se consideraron maduros, y aquéllos que presentaron los dos corpúsculos polares se consideraron fecundados pero no divididos.

En cada grupo experimental, se obtuvieron los siguientes datos de cada oveja: número de oocitos recuperados, número de oocitos aptos para MIV, tasa de oocitos aptos (número de oocitos aptos/número de oocitos recuperados), número de oocitos no aptos para madurar, número de embriones divididos, tasa de división (número de embriones divididos/número de oocitos aptos para madurar), número de blastocistos, tasa de blastocistos (número de blastocistos/número de embriones divididos). Todos los valores han sido expresados por oveja, y todas las tasas se han expresado como porcentajes.

7. Determinación de receptores de hormonas esteroideas en el endometrio

7.1. Abundancia y localización tisular de PR y REα

Inmediatamente tras el sacrificio, se extrajo el útero y se fijó una sección transversal del cuerno ipsilateral al CL en formaldehído al 4%, manteniéndose en etanol hasta el momento de ser incluido en bloques de parafina por técnicas histológicas de rutina. Para localizar las diferentes proteínas sobre las secciones de útero y estimar su abundancia, se utilizó la técnica de inmunohistoquímica (sistema avidina-biotina-peroxidasa) previamente descrita por Meikle et al. (2000). Los cortes de tejido (3 μm) se desparafinaron y rehidrataron en concentraciones decrecientes de etanol. Las secciones se sumergieron en citrato de sodio 0,01 M (pH 6) pre-calentado y se llevaron al microondas (a 900 watt de potencia) durante 5 minutos, para mejorar la exposición

antigénica. Tras un lavado en solución tampón (PBS 0,01 M, pH 7,5), se bloqueó la actividad inespecífica de las peroxidasas endógenas con peróxido de hidrógeno al 3% en metanol durante 10 minutos a temperatura ambiente. Después de un lavado de 10 minutos en PBS, los preparados se incubaron con suero de caballo (Vectastain; Vector Laboratories, Burlingame, CA, USA) durante 60 minutos en una cámara húmeda a temperatura ambiente. Luego, se los incubó durante 1 hora con el anticuerpo primario correspondiente para la detección de cada receptor, diluido en PBS: anti-RP (Zymed, South San Francisco, CA, USA) y anti-REα (Santa Cruz, California, USA). Los controles negativos se obtuvieron reemplazando el anticuerpo primario por un suero inespecífico a una concentración equivalente. Tras la unión al anticuerpo primario, los tejidos se incubaron durante 60 minutos con un anticuerpo secundario biotinilado (Vectastain, Vector) diluido en suero de caballo, y luego 60 minutos más con el complejo avidinabiotina-peroxidasa (Vectastain Elite; Vector). El sitio de unión del complejo enzimático al tejido se visualizó por medio de 3,3'-diaminobencidina (DAB, Vector), un cromógeno que produce un precipitado marrón insoluble cuando se incuba junto con la enzima. Las secciones de tejido se colorearon con hematoxilina y se deshidrataron en etanol antes de ser montadas con cemento sintético.

7.2. Análisis de imagen

Para estimar la expresión de los receptores de interés en el endometrio, se realizó un análisis de imagen subjetivo llevado a cabo por dos observadores independientes que desconocían la conformación de los grupos. Sólo se evaluaron las regiones intercarunculares del endometrio. La expresión de RP y REα se estudió en 5 compartimientos histológicos. El primero de ellos fue el epitelio luminal. Tanto el epitelio glandular como el estroma, se dividieron según su ubicación en: superficial y

profundo. Se analizaron 10 campos por compartimiento histológico por animal a un aumento de 1000X. La intensidad de tinción se clasificó en negativa, leve, moderada o intensa y cada categoría se expresó como porcentaje de la cantidad total de células (Thatcher et al., 2003). A partir de esta evaluación se estudiaron dos variables: la proporción de células positivas y la intensidad de tinción. La intensidad de tinción se calculó mediante la siguiente fórmula: 1 x n1 +2 x n2 + 3 x n3, donde n es igual a la proporción de células con intensidad leve (1), moderada (2) o intensa (3) (Boos et al., 1996).

8. Determinación de niveles plasmáticos de hormonas y metabolitos

En todos los experimentos se obtuvieron muestras de sangre de la vena yugular en tubos heparinizados (BD Vacutainer®, Franklin Lakes, NJ, USA). Todas las muestras fueron centrifugadas dentro de los 15 minutos desde su obtención (1000 g, 10 minutos) y el plasma obtenido fue almacenado a -20ºC hasta su posterior procesamiento.

8.1. Progesterona

Previo al inicio de los experimentos y para confirmar la ciclicidad ovárica (experimentos 1 y 3) o el anoestro (experimentos 2 y 4), se procedió a la determinación de los niveles de progesterona (P4) plasmática los días 7 y 14 antes de la colocación de los implantes de melatonina. Todas las ovejas que presentaron niveles de P4 mayores a 1 ng/ml en al menos una de las muestras, se consideraron cíclicas. Estos niveles han sido demostrados como propios de una fase luteal del ciclo estral en esta especie y con la analítica empleada. En todos los experimentos se obtuvieron muestras de sangre en tubos heparinizados los días 0, 3 y 5 (sacrificio) para determinar los niveles plasmáticos

de P4.

Las concentraciones plasmáticas de P4 fueron determinadas utilizando un kit comercial de RIA en fase sólida y directo (Count-A-Count TKPG;DPC) (Meikle et al., 1997). La sensibilidad de la prueba fue de 0,02 ng/ml. El coeficiente de variación (CV) intra-ensayo para los controles de concentraciones bajas (3ng/ml), medias (15 ng/ml) y altas (30 ng/ml) fue 14%, 8,5% y 7,5%, respectivamente. El CV inter-ensayo fue menor al 15% para todos los controles de las concentraciones utilizados.

8.2. Melatonina

A fin de comprobar la efectividad de los implantes de melatonina, se obtuvo una muestra diurna de sangre. Las concentraciones plasmáticas de melatonina fueron analizadas en un único ensayo de radioinmunoanálisis (RIA) directo y en fase sólida, utilizando un kit comercial (Bühlmann RK-MDI; Bühlmann Lab; Switzerland). La sensibilidad del ensayo fue de 1,3 pg/ml. Los CV intra-ensayo fueron de 10,9 % y 7,5 % para los controles de concentraciones bajas (3,3 pg/ml) y altas (20,6 pg/ml), respectivamente.

8.3. Metabolitos

Desde el inicio de las dietas experimentales hasta el día del sacrificio (día 5 post celo) de los animales, se obtuvieron muestras de sangre cada 2 días. Posteriormente se realizó la determinación de los niveles plasmáticos de glucosa, ácidos grasos no esterificados (AGNE) y de β-hidroxibutirato (BHB) en un autoanalizador (Gernonstar, Transasia, Bombay, India). La glucosa se analizó utilizando un kit enzimático (Gernon®, RAL for Laboratories Techniques, Barcelona, Spain). También se utilizaron

kits comerciales para analizar AGNE (NEFA®, Randox Laboratories Ltd., Crumlin, United Kingdom) y para BHB (RANBUT®, Randox Laboratories Ltd., Crumlin, United Kingdom). Los CV intra e inter-análisis para glucosa y BHB fueron 2,2 % y 3,1%, respectivamente (siendo los CV similares para ambos metabolitos). En cambio los CV intra e inter-análisis para AGNE fueron 11,7% y 13%, respectivamente.

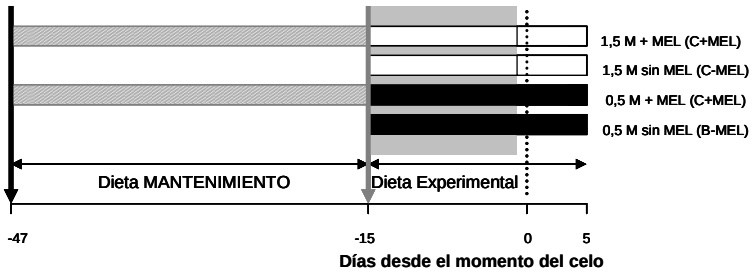
9. Análisis estadísticos

Cada experimento se basó en un diseño factorial 2x2, con el nivel nutricional y el tratamiento con melatonina como efectos fijos. En un análisis posterior y complementario, los experimentos se agruparon con un diseño factorial 2x2x2, con el nivel nutricional, el tratamiento con melatonina y la época del año (estación reproductiva o anoestro estacional) como efectos fijos. En los artículos I y II, los efectos de los tratamientos en el desarrollo y calidad de los oocitos y de los embriones (obtenidos *in vivo* e *in vitro)* fueron evaluados usando el PROC GEN MOD (Statistical Analysis System, SAS, Institute Inc., Cary, NC, USA) con una distribución Poisson especificada en un modelo que incluyó la estación del año (reproductiva o anoestro), el nivel nutricional (dieta control o dieta baja), el tratamiento con melatonina (tratadas o no con implantes de melatonina) y sus interacciones. Las concentraciones de metabolitos y hormonas plasmáticas, la expresión de receptores en el endometrio, el PV y la CC, se analizaron por análisis de varianza utilizando un modelo mixto (Statistical Analysis System, SAS, Institute Inc., Cary, NC, USA), que incluyó los efectos del tratamiento nutricional, del tratamiento con melatonina, del día del ciclo y de sus interacciones. Para el caso concreto de la expresión de receptores, se incluyó además, el efecto del compartimiento histológico estudiado. Estas variables se estudiaron por análisis de medidas repetidas, y las observaciones previas al inicio del tratamiento se

incluyeron en el análisis como covariables. Los valores expresados como porcentajes fueron comparados con la prueba de Chi cuadrado. El nivel de significación se estableció en $P<0,05$ y los valores de P comprendidos entre 0,05 y 0,1 se consideraron como tendencia. Todos los resultados se expresaron como medias ± S.E.

RESULTADOS

EXPERIMENTO 1. Ovejas vacías, Estación reproductiva *(Artículos I y II).*



1.1. Efecto de los tratamientos sobre el peso vivo y condición corporal

Los PV en las ovejas de los grupos C-MEL y C+MEL no experimentaron cambios durante el periodo experimental de las dietas (20 días), pero las ovejas B-MEL y B+MEL mostraron una pérdida media de peso significativa ($P<0,001$), de 7,5 y 9.3 kg, respectivamente (Figura 6a). Así mismo, las ovejas B-MEL y B+MEL disminuyeron significativamente la CC media (0,3 y 0,5 puntos, respectivamente); mientras que en los grupos C-MEL y C+MEL la CC de las ovejas permaneció sin variaciones (Figura 6b).

1.2. Efecto de los tratamientos sobre los perfiles hormonales

1.2.1. Melatonina

El día 45 posterior a la colocación de los implantes, todos los implantes de melatonina indujeron altos niveles plasmáticos de melatonina durante las horas diurnas (enero: 53,6±4,2 pg/ml), lo cual indicó que la hormona fue liberada por los implantes de manera apropiada.

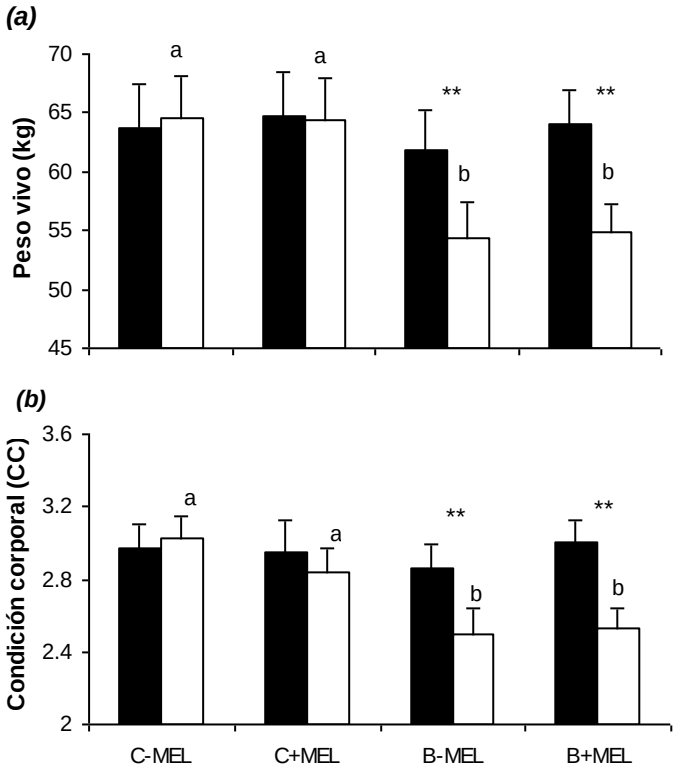


Figura 6. *Medias (± S.E.) de peso vivo* ***(a)*** *y de CC* ***(b)*** *en ovejas raza Rasa Aragonesa alimentadas 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con implantes de melatonina, desde el inicio (barras negras) hasta el final (barras blancas) de los 20 d de periodo experimental de las dietas, durante la estación reproductiva.* Barras con diferentes letras (a, b) entre grupos indican P<0,01. Asteriscos dentro de cada grupo indican P<0,001 (**).

1.2.2. Progesterona

En todas las ovejas, los niveles de P4 incrementaron gradualmente desde el momento del celo hasta el día del sacrificio. Las concentraciones plasmáticas de P4 el día del sacrificio (día 5), aunque no presentaron diferencias significativas entre grupos, indicaron que todas las ovejas exhibieron un CL funcional. De esta manera, los niveles medios de P4 en plasma el día 5 tras el celo fueron: 3,7±0,5 ng/ml (C-MEL); 3,8±0,5 ng/ml (C+MEL), 4,7±0,7 ng/ml (B-MEL) y 4,0±0,8 ng/ml (B+MEL) (Figura 7).

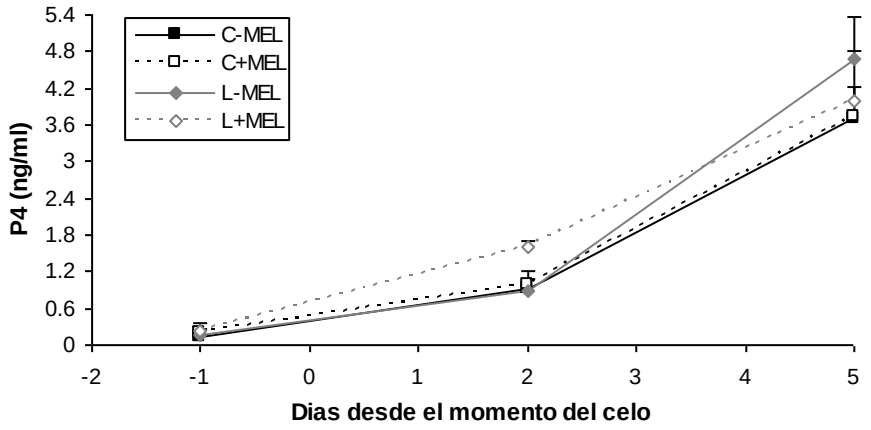


Figura 7. *Concentraciones plasmáticas medias (± S.E.) de progesterona (P4) desde el día del retiro de la esponja con progestágenos hasta el día del sacrificio (día 5 tras el celo) en ovejas raza Rasa Aragonesa alimentadas 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con implantes de melatonina durante la estación reproductiva*. (Día 0=celo).

1.3. Efecto de los tratamientos sobre los perfiles plasmáticos de glucosa, ácidos grasos no esterificados y β-hidroxibutirato

La melatonina exógena no tuvo efectos sobre la concentración media de glucosa, aunque se observó que el tipo de dieta influyó sobre el perfil de secreción de este metabolito. Así, de manera global y a lo largo de la experiencia, los animales que consumieron la dieta C presentaron una mayor concentración media de glucosa que las ovejas con la dieta B (C: 3,6±0,03 vs. B: 3,4±0,03 mmol/L; P<0,01). Es de destacar que, a excepción del grupo C-MEL, los demás grupos presentaron la aparición de un pico pronunciado en la secreción de glucosa el día previo a la aparición del estro (Figura 8a).

Con respecto a la secreción de AGNE y coincidiendo con las pérdidas de peso vivo observadas, las ovejas de los grupos B-MEL y B+MEL presentaron concentraciones entre 5 a 6 veces mayores que las observadas en los grupos C-MEL y C+MEL. Un hallazgo interesante en este experimento fue que hubo un efecto significativo de la melatonina sobre las concentraciones de AGNE en las ovejas subnutridas, siendo el grupo B+MEL el que presentó menores niveles de este metabolito durante los 21 d de tratamiento nutricional (B+MEL: 0,30±0,01 vs. B-MEL: 0,38±0,01 mmol/L, P<0,01) (Figura 8b).

Al evaluar el perfil de secreción de BHB, se observó que las ovejas que consumieron la dieta C presentaron una menor concentración de este metabolito respecto a las ovejas subnutridas (C: 0,24±0,006 vs. B: 0,29±0,006 mmol/L; P<0,001). Se puede observar en la Figura 8c que fue el grupo B-MEL el que presentó los mayores niveles de BHB a lo largo de todo el experimento.

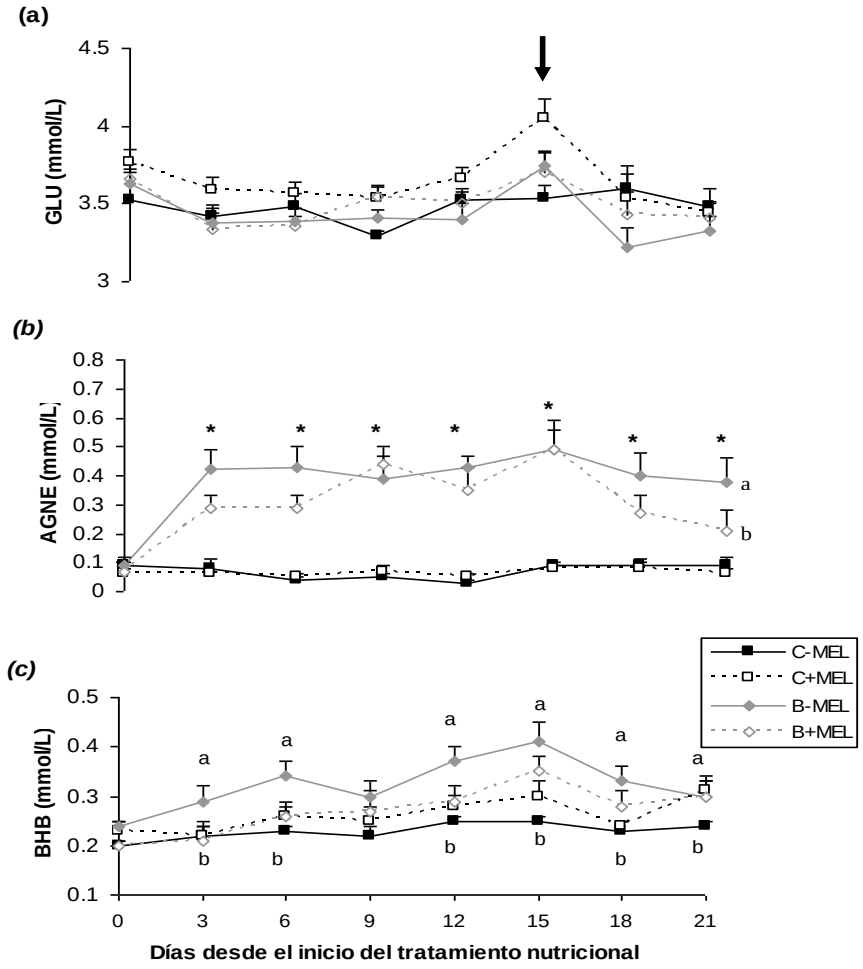


Figura 8. *Concentraciones plasmáticas medias* (± *S.E.) de glucosa* ***(a)****, ácidos grasos no esterificados* ***(b)*** *y β-hidroxibutirato* ***(c)*** *en ovejas raza Rasa Aragonesa alimentadas 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con implantes de melatonina, durante los 20 d de periodo experimental de las dietas, en la estación reproductiva.* La flecha señala el día previo al celo. Letras diferentes (a, b) para BHB indican P<0,01 entre grupos C-MEL y B-MEL. Asterisco indica P<0,01 entre grupos C y B.

1.4. Efectos de los tratamientos sobre la recuperación de embriones obtenidos in vivo

La tasa de ovulación no difirió entre grupos (Tabla 1). Se observó un efecto del nivel de nutrición sobre el número de embriones viables obtenidos (C: 1,1±0,2 y B: 0,6±0,2; P<0,05); además se observó que en el grupo de ovejas subnutridas el tratamiento con melatonina redujo aún más el número de embriones viables (P<0,05) (Tabla 1). El tratamiento con melatonina redujo significativamente la tasa de fecundación en el grupo B+MEL (P<0,05) y, en consecuencia, tanto el número de embriones totales como el número de embriones viables fueron mas bajos en el grupo de ovejas subnutridas y tratadas con melatonina respecto a los demás grupos. Con respecto a la viabilidad *in vitro,* se observó que fue el grupo B+MEL el que presentó la tasa más baja (40,0%), que fue diferente de las observadas en las ovejas de los grupos C-MEL y C+MEL (77,8% y 80,0%, respectivamente; P<0,05), y tendió a ser diferente de la del grupo B-MEL (66,7%; P=0,07).

1.5. Efecto de los tratamientos sobre la población de oocitos como fuente de embriones in vitro

No hubo efecto del nivel nutricional ni del tratamiento con melatonina sobre el número de oocitos recuperados, aptos para MIV o divididos; tampoco se observaron diferencias sobre la tasa de oocitos aptos para MIV o la tasa de división (Tabla 2); sin embargo, la interacción dieta x melatonina exógena tuvo un efecto significativo sobre el número de oocitos no aptos para MIV (P<0,05), mostrando que los implantes de melatonina tuvieron un efecto negativo sobre éste parámetro en las ovejas del grupo C+MEL pero un efecto beneficioso en las ovejas del grupo B+MEL.

Tabla 1. *Respuesta ovárica y producción de embriones en la estación reproductiva de ovejas raza Rasa Aragonesa alimentadas con 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena 62 d antes de la recuperación de los embriones (medias* ± S.E.).

	GRUPOS			
	C-MEL	**C+MEL**	**B-MEL**	**B+MEL**
No. de ovejas	10	11	11	10
No. ovejas en estro	8/10	9/11	9/11	9/10
Tasa de ovulación	1,9±0,4	2,2±0,3	2,2±0,4	1,8±0,4
No. de estructuras recuperadas	1,1±0,3	1,7±0,3	1,1±0,2	0,9±0,4
No. de embriones	1,1±0,3 ^{c}	1,3±0,3 ^{c}	1,1±0,3 ^{c}	0,5±0,3 ^{d}
Tasa de fecundación (%)	100 ^{a}	87,5 ^{b}	100 ^{a}	70 ^{b}
No. de embriones viables	1,0±0,3 ^{a}	1,2±0,3 ^{a}	0,9±0,3 ^{a}	0,2±0,3 ^{b}
Tasa de viabilidad (%)	83,3 ^{c}	81,3 ^{c}	75,0 ^{c}	40,0 ^{d}
Tasa de gestación (%)*	75,0 (6/8)	77,7 (7/9)	88,8 (8/9)	44,4 (4/9)

Diferentes superíndices (a, b) o (c, d) en la misma fila indican $P<0,05$ o $P<0,1$; respectivamente. Estructuras recuperadas = oocitos + embriones. *Porcentaje de ovejas con embriones viables el día 5.

Tabla 2. *Resultados de la fecundación in vitro (FIV) de los oocitos recogidos de ovejas raza Rasa Aragonesa alimentadas con una dieta 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena durante la estación reproductiva (medias* ± S.E.)

	C-MEL	**C+MEL**	**B-MEL**	**B+MEL**
No. de ovejas con CL funcional	8	9	9	9
Tasa de ovulación	1,9 ± 0,4	2,2 ± 0,3	2,2 ± 0,4	1,8 ± 0,4
No. de oocitos recuperados	17,1 ± 2,3 ^{c}	22,2 ± 2,2 ^{d}	21,3 ± 2,2 ^{d}	17,8 ± 2,2
No. de oocitos aptos para MIV	13,3 ± 1,4	15,3 ± 1,3	14,1 ± 1,0	13,0 ± 1,6
Tasa de oocitos aptos (%)	77,7 ^{c}	68,9 ^{d}	66,2	73,0
No. de oocitos no aptos para MIV	3,9 ± 1,2 ^{a}	6,9 ± 1,1 ^{b}	7,2 ± 1,1 ^{b}	4,8 ± 1,1 ^{a}
No. de embriones divididos	6,1 ± 0,9	9,3 ± 1,2	8,1 ± 1,0	6,8 ± 1,3
Tasa de división (%)	45,8 ^{c}	60,8 ^{d}	57,5	52,3
No. de blastocistos	0,8 ± 0,5	1,2 ± 0,3	2,6 ± 0,6	0,8 ± 0,5
Tasa de blastocistos (%)	13,1 ^{d}	12,9	32,0 ^{c}	11,8 ^{d}

Todos los valores han sido expresados por oveja. Diferentes superíndices (a, b) o (c, d) en la misma fila indican diferencias significativas ($P<0,05$) o ($P<0,1$), respectivamente.

1.6. Efecto de los tratamientos sobre la expresión de los receptores esteroideos (RP y REα) en el endometrio

No se observaron efectos de la nutrición, la melatonina o la interacción entre ambas sobre el porcentaje de células positivas para REα ni para RP. No hubo efectos de la nutrición ni de la melatonina sobre la intensidad de tinción de ambos receptores, pero si hubo efectos de la interacción entre ambos efectos ($P<0,01$). Las ovejas de los grupos C experimentaron una mayor intensidad de tinción de REα que las ovejas subnutridas en el epitelio luminal ($P<0,05$). Sin embargo al evaluar la intensidad de tinción de RP, se observó que esta fue mayor en las ovejas del grupo C+MEL que en las ovejas B+MEL en el estroma superficial ($P<0,05$). El tratamiento con melatonina en las ovejas subnutridas disminuyó la expresión de RP en los epitelios glandular superficial y profundo, así como también en el estroma superficial ($P<0,05$) (Figura 9).

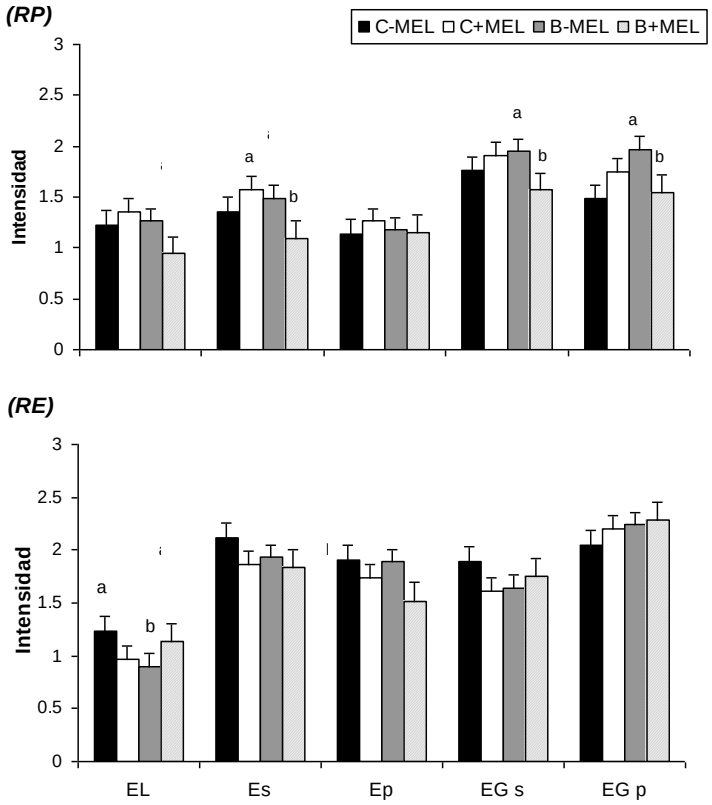
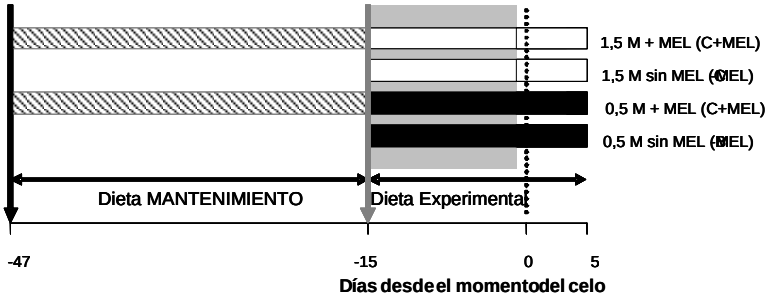


Figura 9. *Intensidad de tinción de RP (arriba) y REα (abajo) en el endometrio de ovejas raza Rasa Aragonesa alimentadas con una dieta 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena durante la estación reproductiva.* EL=epitelio luminal, Es=estroma superficial, Ep=estroma profundo, EGs=epitelio glandular superficial, EGp=epitelio glandular profundo. Letras diferentes indican diferencias dentro del mismo gráfico (a, b; P< 0,05) (*Medias* ± S.E.).

EXPERIMENTO 2. Ovejas vacías, Anoestro estacional *(Artículos I y II).*



2.1. Efecto de los tratamientos sobre el peso vivo y condición corporal

Las ovejas de los grupos C-MEL y C+MEL mantuvieron el PV y la CC, pero las ovejas de B-MEL y B+MEL disminuyeron significativamente el PV (pérdidas medias de 2,5 y 3,8 kg, respectivamente) y la CC (pérdidas medias de 0,2 y 0,3 puntos, respectivamente) durante los 20 d de periodo experimental de las dietas ($P<0,05$) (Figura 10).

2.2. Efecto de los tratamientos sobre los perfiles hormonales

2.2.1. Melatonina

El día 45 posterior a la colocación de los implantes, todos los implantes de melatonina indujeron altos niveles plasmáticos de melatonina durante las horas del día (mayo: 62,4±4,9 pg/ml), lo cual indicó que la hormona fue liberada por los implantes de manera apropiada.

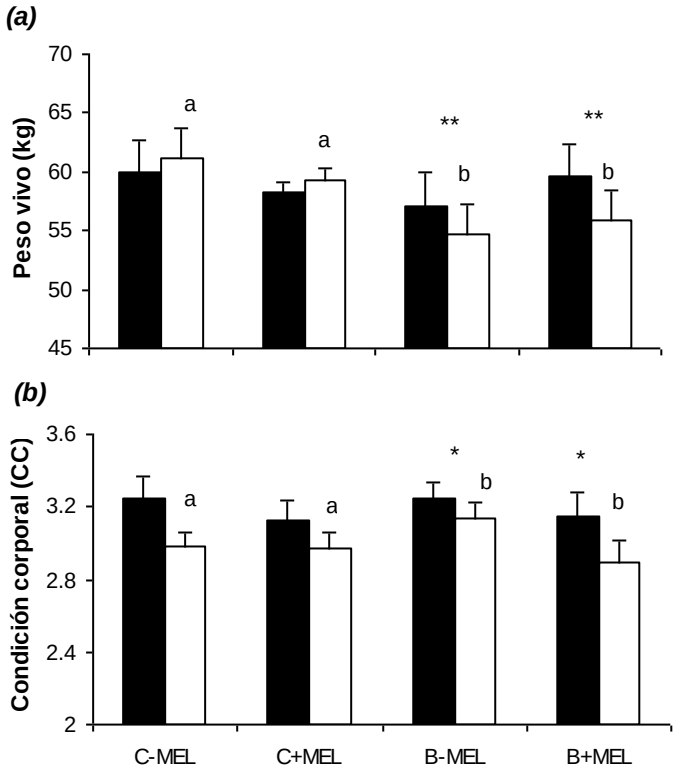


Figura 10. *Medias (± S.E.) de peso vivo* ***(a)*** *y de CC* ***(b)*** *en ovejas raza Rasa Aragonesa alimentadas 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con implantes de melatonina, desde el inicio (barras negras) hasta el final (barras blancas) de los 20 d de periodo experimental de las dietas, durante el anoestro.* Barras con diferentes letras (a, b) entre grupos indican P<0,01. Asteriscos dentro de cada grupo indican P<0,001 (**) o P<0,05 (*).

2.2.2. Progesterona

En todas las ovejas, los niveles de P4 incrementaron gradualmente desde el momento del estro hasta el día del sacrificio (día 5), indicando que todas las ovejas exhibían un CL funcional al momento del sacrificio. Las concentraciones plasmáticas de P4 el día 5 post-estro en los diferentes grupos fueron: 3,6±0,6 ng/ml (C-MEL); 3,8±0,6 ng/ml (C+MEL), 2,6±0,7 ng/ml (B-MEL) y 3,4±0,7 ng/ml (B+MEL), sin diferencias significativas entre grupos (Figura 11).

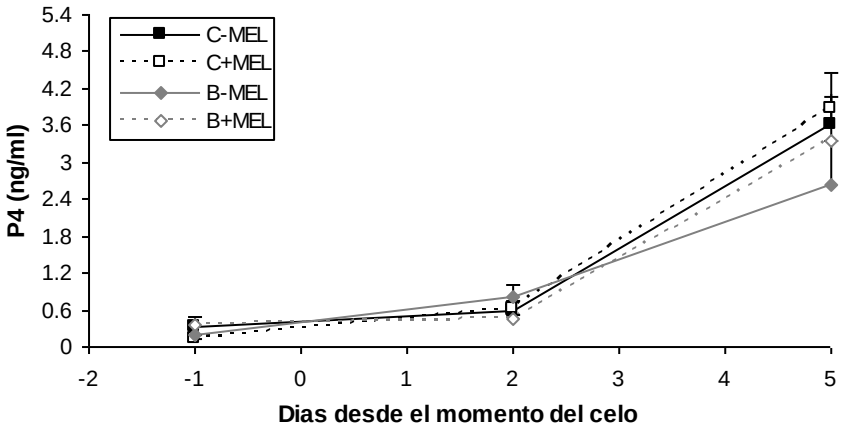


Figura 11. *Concentraciones plasmáticas medias (± S.E.) de progesterona (P4) desde el día del retiro de la esponja con progestágenos hasta el día del sacrificio (día 5 tras el celo) en ovejas raza Rasa Aragonesa alimentadas 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con implantes de melatonina durante el anoestro estacional.* (Día 0=celo).

2.3. Efecto de los tratamientos sobre los perfiles plasmáticos de glucosa, ácidos grasos no esterificados y β-hidroxibutirato

Las ovejas alimentadas con la dieta C presentaron mayores niveles de glucosa que las ovejas de los grupos B, expresado como media general del periodo experimental (C: 3,8±0,03 vs. B: 3,5±0,03 mmol/L; P<0,001) (Figura 12a). Se observó un efecto de la melatonina sobre las concentraciones de este metabolito, siendo las ovejas implantadas las que presentaron mayores niveles de glucosa durante los 21 días de periodo experimental (+MEL: 3,74±0,04 vs. –MEL: 3,4±0,03 mmol/L; P<0,05). En la Figura 12a también se puede observar que todos los grupos, excepto las ovejas B+MEL presentaron un pico de secreción alrededor de la aparición del estro.

Un hallazgo inesperado se observó en la secreción de AGNE, ya que a pesar de las pérdidas significativas de peso vivo observadas en ambos grupos de ovejas subnutridas, solo en el grupo L+MEL se observó un incremento de los niveles plasmáticos de este metabolito, de manera que las ovejas del grupo L-MEL tuvieron un perfil de secreción semejante a las ovejas de los grupos C. Además, en la Figura 12b se puede observar que todas las ovejas presentaron una elevada concentración de este metabolito el día del inicio del tratamiento nutricional, el cual fue seguido por un descenso del nivel, leve en el caso de los grupos C-MEL y L-MEL y pronunciado en el grupo C+MEL.

No hubo efecto de la nutrición ni de la melatonina exógena sobre la concentración plasmática de BHB durante todo el periodo experimental (Figura 12c).

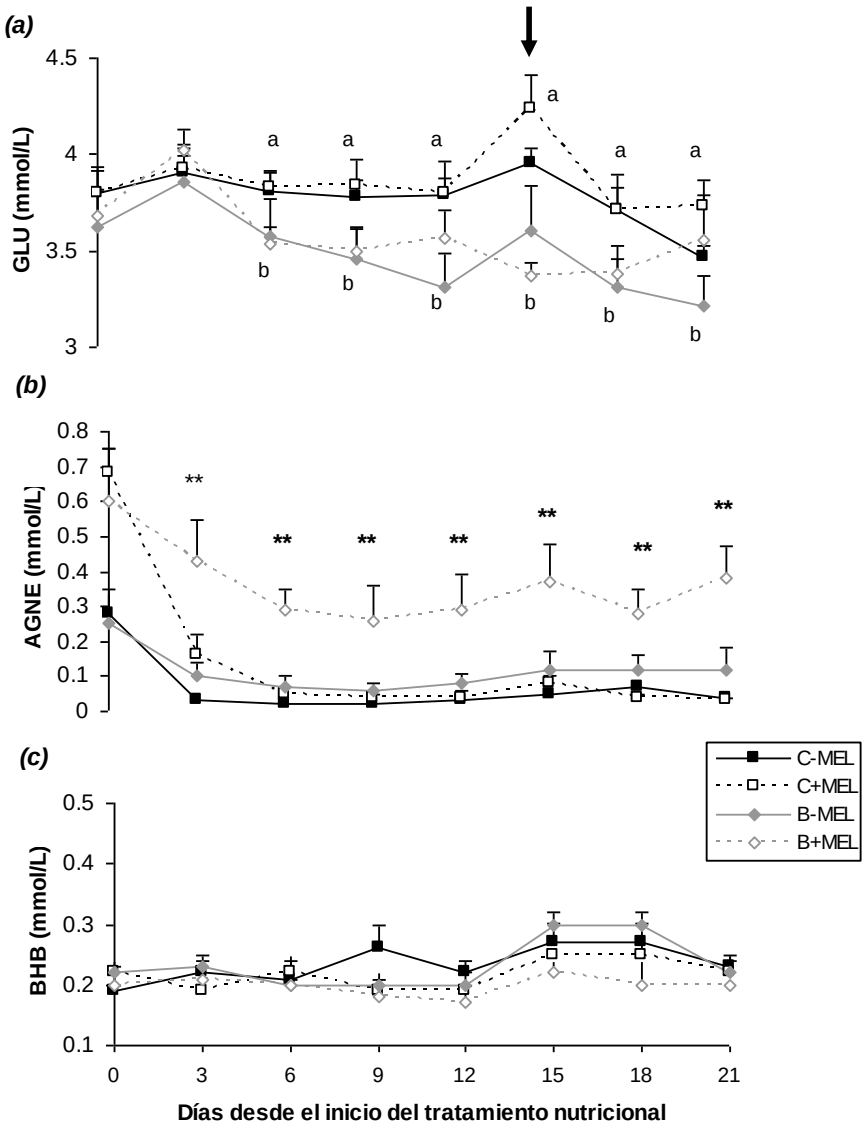


Figura 12. *Concentraciones plasmáticas medias (± S.E.) de glucosa* ***(a)****, ácidos grasos no esterificados* ***(b)*** *y β-hidroxibutirato* ***(c)*** *en ovejas raza Rasa Aragonesa alimentadas 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con implantes de melatonina, durante los 20 d de periodo experimental de las dietas en el anoestro estacional.* Letras diferentes en glucosa (a, b) indican P<0,01entre grupos C y B. La flecha señala el día previo al celo. Los asteriscos indican P<0,001 entre L+MEL y los demás grupos.

2.4. Efectos de los tratamientos sobre la recuperación de embriones obtenidos in vivo

No hubo diferencias entre grupos en la tasa de ovulación (Tabla 3). Sólo una oveja, en el grupo B+MEL, no presentó comportamiento de celo después de retirada la esponja. Además, dos ovejas del grupo B-MEL (n=5) y dos ovejas del grupo B+MEL (n=6) fueron excluidas del experimento debido a que no presentaron CL de apariencia normal en el momento del sacrificio. No se observaron diferencias significativas en el número de embriones fertilizados ni viables el día 5 tras el celo, lo que indica que durante el anoestro ni la subnutrición ni el tratamiento con melatonina exógena tuvieron efecto sobre estos parámetros (Tabla 3). No hubo efecto significativo de la subnutrición ni de la melatonina exógena en la viabilidad *in vitro* de los embriones recuperados; de esta manera, los porcentajes de embriones que fueron capaces de alcanzar el estadio de blastocisto expandido o eclosionado fueron los siguientes: 87,5% (C-MEL), 83,3% (C+MEL), 83,3% (B-MEL) y 88,9% (B+MEL).

2.5. Efecto de los tratamientos sobre la población de oocitos como fuente de embriones in vitro

Se observó una tendencia a que la subnutrición redujera el número de oocitos recuperados por oveja (C: 15,6±1,4 vs. B: 13,3±1,8; $P<0,1$) (Tabla 4). En los grupos que no recibieron implantes de melatonina, la subnutrición provocó una significativa disminución en el número de embriones divididos observados por oveja (C: 7,2±0,8 vs. B: 4,1±0,9; $P<0,05$). Por contra, ninguno de los parámetros evaluados estuvieron afectados por el tratamiento de melatonina exógena ni por la interacción entre el nivel nutricional y el tratamiento con melatonina; sin embargo, en los grupos de ovejas

subnutridas, la melatonina exógena pareció incrementar el número de oocitos aptos para IVM (B+MEL: 9,4±1,0; B-MEL: 7,6±1,4; P<0,1), aumentando el número de oocitos divididos (B+MEL: 7,0±0,7; B-MEL: 4,1±0,9; P<0,05) así como también la tasa de blastocistos (B+MEL: 37,2%, B-MEL: 21,9%; P<0,05), Además, el tratamiento con melatonina mejoró la tasa de blastocistos en las ovejas de los grupos C (C+MEL 26,4%; C-MEL: 11,3%; P<0,05).

Tabla 3. *Respuesta ovárica y producción de embriones en el anoestro de ovejas raza Rasa Aragonesa alimentadas con 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (MEL) o no (-MEL) con melatonina exógena 62 d antes de la recuperación de los embriones (medias* ± S.E.)

	GRUPO			
	C-MEL	**C+MEL**	**B-MEL**	**B+MEL**
No. de ovejas	8	7	5	6
No. de ovejas en estro	8/8	7/7	5/5	5/6
Tasa de ovulación	2,4±0,4	2,1±0,3	2,0±0,6	1,8±0,2
No. estructuras recuperadas	1,0±0,3	1,6±0,3	1,2±0,4	1,4±0,4
No. de embriones recuperados	0,6±0,3 [c]	1,3±0,3 [d]	0,8±0,4 [c]	1,2±0,4 [d]
Tasa de fecundación (%)	66,7±18	83,3±18	75,0±22	87,5±22
No. de embriones viables	0,6±0,3 [c]	1,1±0,3 [d]	0,8±0,4 [c]	1,2±0,4 [d]
Tasa de viabilidad (%)	66,7±18,1	75,0±18,1	75,0±22,2	87,5±22,2
Tasa de gestación (%)*	50 (4/8)	71,4 (5/7)	60 (3/5)	80 (4/5)

Diferentes superíndices (c, d) en la misma fila indican P<0,1.
Estructuras recuperadas = oocitos + embriones. *Porcentaje de ovejas con embriones viables el día 5.

Tabla 4. *Resultados de la fecundación in vitro (FIV) de los oocitos recogidos de ovejas raza Rasa Aragonesa alimentadas con una dieta 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena durante el anoestro estacional (medias* ± S.E.)

	C-MEL	**C+MEL**	**B-MEL**	**B+MEL**
No. de ovejas con CL funcional	11	9	8	8
Tasa de ovulación	2,3 ± 0,4	2,4 ± 0,4	2,5 ± 0,5	1,9 ± 0,5
No. de oocitos recuperados	16,1 ± 1,8	15,2 ± 1,9	12,0 ± 2,1	14,6 ± 2,1
No. de oocitos aptos para MIV	9,6 ± 0,4 [d]	9,4 ± 0,8	7,6 ± 1,4 [e]	9,4 ± 1,0 [d]
Tasa de oocitos aptos (%)	59,6	61,8	63,3	64,4
No. de oocitos no-aptos para MIV	6,5 ± 0,9	5,8 ± 1,0	4,4 ± 1,1	5,2 ± 1,1
No. de embriones divididos	7,2 ± 0,8 [a]	5,3 ± 0,9	4,1 ± 0,9 [b]	7,0 ± 0,7 [a]
Tasa de división (%)	75,0	56,4	53,9 [d]	74,5 [e]
No. de blastocistos	0,8 ± 0,4	1,4 ± 0,5	0,9 ± 0,6	2,6 ± 0,8
Tasa de blastocistos (%)	11,3 [a]	26,4 [b, d]	21,9 [b]	37,2 [c, e]

Todos los valores han sido expresados por oveja. Diferentes superíndices (a, b) o (c, d) en la misma fila indican diferencias significativas (P<0,05) o (P<0,1), respectivamente.

2.6. Efecto de los tratamientos sobre la expresión de los receptores esteroideos (RP y REα) en el endometrio

El porcentaje de células positivas para RP y REα no estuvo afectado por el tratamiento nutricional ni por la melatonina exógena ni tampoco por la interacción entre ambos. Tampoco hubo efectos de los tratamientos ni de su interacción sobre la intensidad de tinción de RP y REα en ninguno de los tipos celulares evaluados (Figura 13). Hubo un efecto muy claro de la ubicación (superficial vs. profundo) en EG para REα ($P<0,01$).

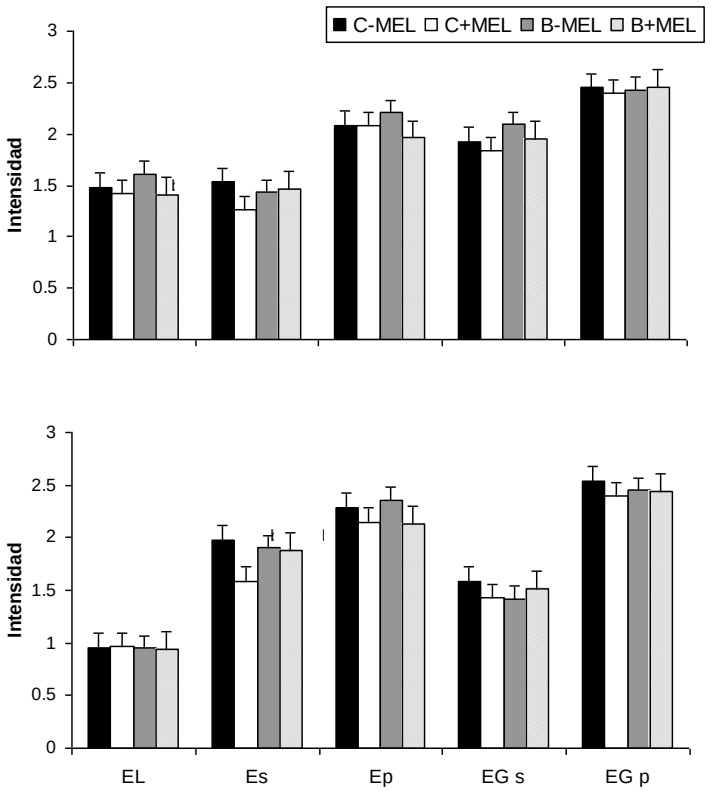


Figura 13. *Intensidad de tinción de RP (arriba) y REα (abajo) en el endometrio de ovejas raza Rasa Aragonesa alimentadas con una dieta 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena durante el anoestro estacional (medias* ± S.E.)

Efecto de la época del año. Experimentos 1 y 2

Efecto de la época del año sobre los parámetros estudiados

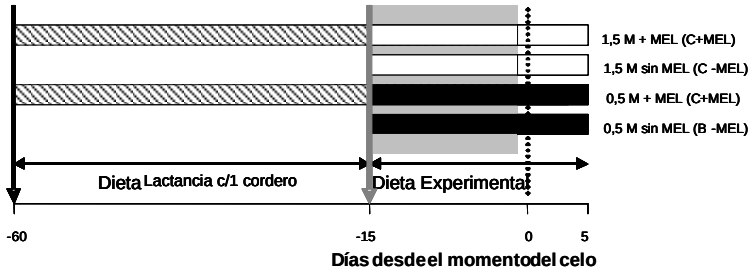
Tal como se comentó en la sección de Metodología, una vez estudiados los parámetros en cada estación y dadas las diferentes respuestas encontradas en función de la estación del año en algunos de ellos, se introdujo en el modelo estadístico original la estación del año (estación reproductiva y/o anoestro estacional) como factor fijo. De esta manera, el diseño factorial final fue 2x2x2, con la estación del año, el nivel nutricional y el tratamiento con melatonina como factores fijos. Los principales efectos observados se resumen en la Tabla 5.

Tabla 5. *Efectos de la época del año sobre las principales variables estudiadas en los experimentos 1 y 2 con ovejas vacías raza Rasa Aragonesa alimentadas con una dieta 1,5x (C) o 0,5x (B) veces los requerimientos de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena.*

	ESTACIÓN	NUTRICIÓN	MELATONINA	INTERACCIÓN (A)
PESO VIVO	**	**	ns	ns
CC	**	**	ns	ns
Metabolitos				
GLUCOSA	**	**	**	P<0.1
AGNE	**	**	**	**
BHB	ns	**	**	**
PROGESTERONA	ns	ns	ns	ns
Parámetros de la recuperación de embriones in vivo				
TASA DE OVULACIÓN	ns	ns	ns	ns
No. DE EMBRIONES TOTALES	*	ns	ns	*
TASA DE FECUNDACIÓN	*	ns	ns	* (estac x mel)
No. DE EMBRIONES VIABLES	ns	ns	ns	ns
TASA DE VIABILIDAD	ns	ns	ns	P<0.1
TASA DE PREÑEZ (a día 5 tras el celo)	ns	ns	ns	P<0,1
Parámetros de la producción in vitro de embriones				
No. OOCITOS RECUPERADOS	ns	ns	ns	**
No. OOCITOS APTOS PARA MIV	**	ns	ns	**
No. EMBRIONES DIVIDIDOS	ns	ns	ns	ns
TASA DE DIVISIÓN	P<0,1	ns	ns	P<0,1
No. DE BLASTOCISTOS	ns	ns	ns	*(estac x mel)
TASA DE BLASTOCISTOS	ns	ns	ns	* (estac x mel)
Receptores de hormonas esteroideas				
Receptor de Progesterona (RP)	**	ns	*	*
Receptor de Estrógenos (REα)	P<0,1	ns	**	*

(**) indica P< 0,01 y (*) indica P<0,05. (A)=Interacción se consideró como la intervención global de los tres efectos fijos del modelo estadístico (estación, nutrición y melatonina), excepto en las aclaraciones que figuran en la tabla.

EXPERIMENTO 3. Ovejas post-parto, Estación reproductiva



3.1. Efecto de los tratamientos sobre el peso vivo y condición corporal

En la figura 14a se puede observar que durante el intervalo parto-inicio de las dietas experimentales (50 días), a pesar de que los animales tuvieron acceso a una dieta calculada para lactancia con un solo cordero, las ovejas C+MEL y B-MEL experimentaron una ganancia media de PV de 4,5 kg ($P<0,01$); mientras que las ovejas C-MEL y B+MEL tuvieron una pérdida de peso de 3,5 y 4 kg, respectivamente ($P<0,01$). Durante el periodo experimental de las dietas (20 días), los PV en las ovejas de los grupos C-MEL y C+MEL no tuvieron cambios significativos, pero las ovejas B-MEL y B+MEL experimentaron una pérdida media de peso significativa ($P<0,001$), de 9,6 y 10,5 kg, respectivamente. La CC durante el período experimental presentó un patrón de evolución semejante al observado con los pesos vivos (Figura 14b).

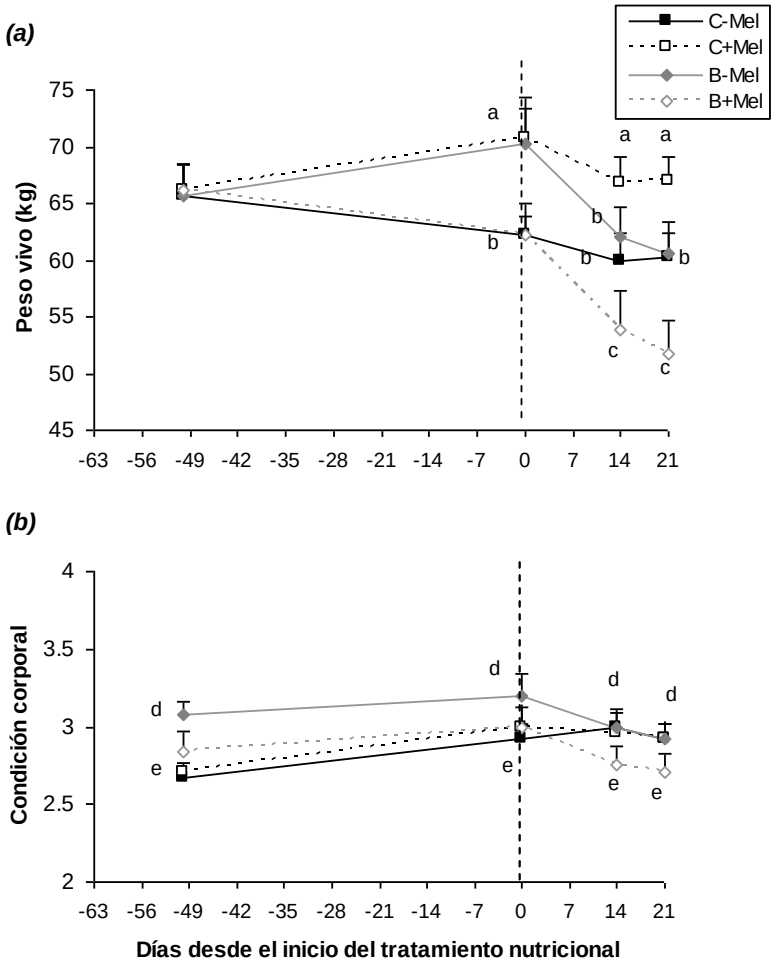


Figura 14. *Medias (± S.E.) de peso vivo* ***(a)*** *y de CC* ***(b)*** *en ovejas raza Rasa Aragonesa alimentadas durante 45 días de periodo post-parto con una dieta lactancia para un solo cordero, y tras el destete alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto, durante el anoestro.* Letras diferentes entre grupos para un mismo día de tratamiento indican P<0,001 (a, b, c) y P<0,05 (d, e).

3.2. Efecto de los tratamientos sobre los perfiles hormonales

3.2.1. Melatonina

El día 45 posterior a la colocación de los implantes, todas las ovejas implantadas con melatonina presentaron altos niveles plasmáticos de dicha hormona durante las horas diurnas (enero: 125,1±24,2 pg/ml), indicando que los implantes liberaron la hormona de manera apropiada.

3.2.2. Progesterona

Los niveles de P4 aumentaron gradualmente desde el momento del celo hasta el día del sacrificio (día 5 tras el celo) en todas las ovejas, indicando la presencia de un CL funcional en el momento del sacrificio. En la Figura 15 se puede observar que las concentraciones plasmáticas de P4 el día 5 tras el celo fueron: 4,3±0,9 ng/ml (C-MEL); 4,8±1,2 ng/ml (C+MEL), 3,2±0,7 ng/ml (B-MEL) y 7,1±1,8 ng/ml (B+MEL). No hubo efecto del tipo de dieta sobre los niveles de P4. Sin embargo, hubo una tendencia en las ovejas implantadas con melatonina a presentar mayores niveles de P4 respecto a las ovejas sin implantar (+MEL: 5,9±0,9 vs. –MEL: 3,7±0,8 ng/ml; P=0,09), este efecto fue más evidente entre las ovejas subnutridas, siendo las del grupo B+MEL las que presentaron mayores niveles de P4 el día 5 tras el celo (B+MEL vs. B-MEL, P=0,03).

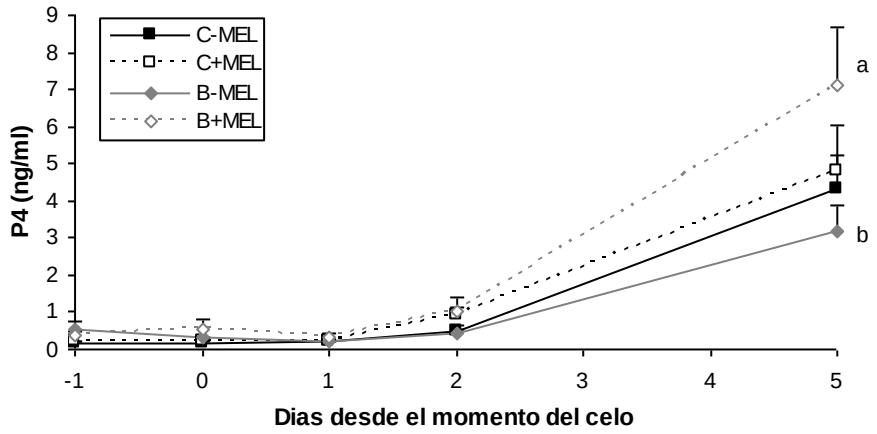


Figura 15. *Concentraciones plasmáticas medias (*± S.E.) *de progesterona (P4) desde el día del retiro del progestágeno hasta el día del sacrificio (día 5 tras el celo), en ovejas raza Rasa Aragonesa alimentadas durante 45 días de periodo post-parto con una dieta lactancia para un solo cordero, y tras el destete alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto, durante la estación reproductiva.* (Día 0=celo). En el día 5, letras diferentes (a, b) indican P=0,03.

3.3. Efecto de los tratamientos sobre los perfiles de glucosa, ácidos grasos no esterificados y β-hidroxibutirato

La melatonina exógena tendió a mejorar las concentración media de glucosa en las ovejas que consumieron la dieta control durante todo el periodo experimental; por lo tanto, de manera global y a lo largo de la experiencia, el grupo C+MEL presentó una mayor concentración media que las ovejas C-MEL (4,3±0,05 vs. 3,8±0,05 mmol/L respectivamente; P<0,001). A excepción del grupo L-MEL, los demás grupos presentaron la aparición de un pico leve en la secreción de glucosa el día previo a la aparición del estro (Figura 16a). Además, se observó un efecto del tipo de dieta

consumida sobre la concentración global de la glucosa, siendo las dietas C las que presentaron mayores niveles de este metabolito (C: 4,0±0,04 vs. B: 3,7±0,04 mmol/L, P<0,001).

Con respecto a la secreción de AGNE, y coincidiendo con las pérdidas de peso vivo observadas, las ovejas de los grupos B-MEL y B+MEL presentaron concentraciones de entre 7 y 8 veces superiores que las observadas en los grupos C-MEL y C+MEL durante todo el periodo del tratamiento nutricional (Figura 16b), lo cual demostró un marcado efecto del tipo de dieta sobre la secreción de este metabolito (P<0,001). Por el contrario, no se observó ningún efecto de la melatonina exógena.

Al evaluar el perfil de secreción de BHB, se observó que las ovejas que consumieron la dieta C presentaron una menor concentración de este metabolito respecto a las ovejas subnutridas (C: 0,33±0,009 vs. B: 0,37±0,008; P<0,005). Por el contrario, no hubo efecto alguno de la melatonina exógena sobre la secreción de BHB. Es de destacar que, en todos los grupos se observó la aparición de un pico leve de secreción el día previo a la aparición del celo (día 15 del tratamiento nutricional), seguido de un descenso de las concentraciones hacia el día 5 tras el celo (Figura 16c).

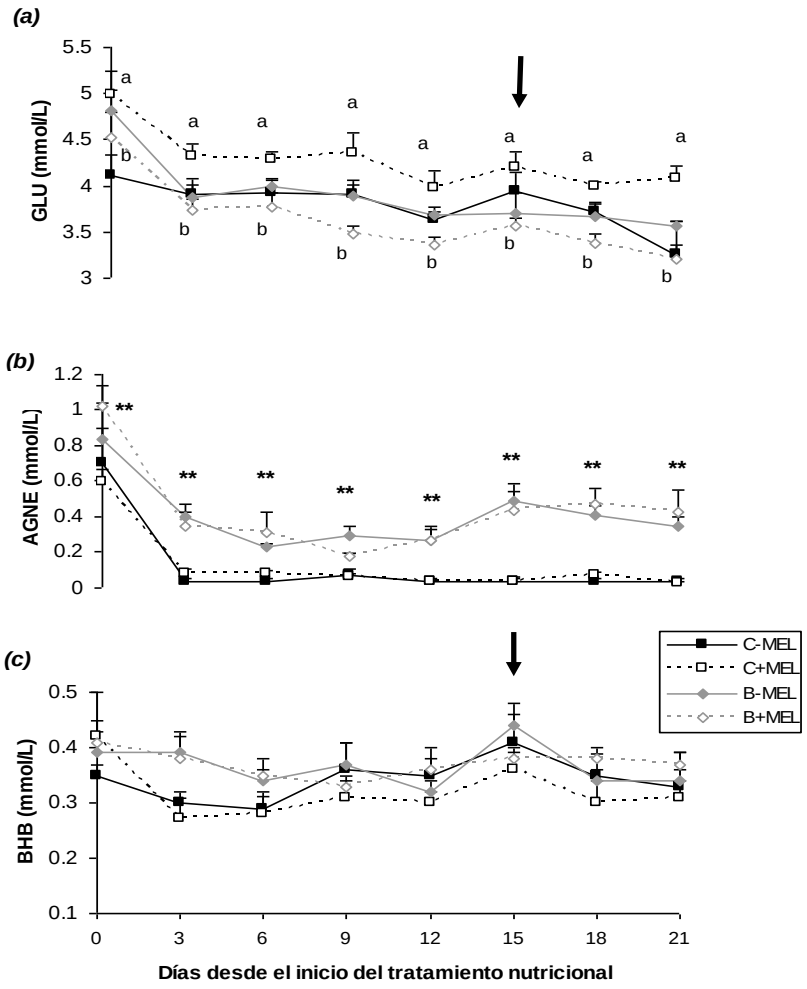


Figura 16. *Concentraciones plasmáticas medias (*± S.E.) *de glucosa* ***(a)****, ácidos grasos no esterificados* ***(b)*** *y β-hidroxibutirato* ***(c)*** *en ovejas raza Rasa Aragonesa alimentadas durante 45 días de periodo post-parto con una dieta lactancia para un solo cordero, y tras el destete alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto, durante la estación reproductiva.* La flecha señala el día previo al celo. Letras diferentes (a, b) en glucosa indican P<0,001 entre grupos C+MEL y B+MEL. Asteriscos indican P<0,01 entre grupos C y B.

3.4. Efectos de los tratamientos sobre la recuperación de embriones obtenidos in vivo

No hubo efecto de la dieta ni de la melatonina sobre la tasa de ovulación o sobre el número de embriones recuperados por CL (Tabla 1), pero se observó que la presencia de melatonina exógena tendió a mejorar el número de estructuras recuperadas por oveja (-MEL: 0,75±0,3 vs. +MEL: 1,50±0,3; P=0,09). Además, se observó que las ovejas subnutridas implantadas con melatonina presentaron un mayor número de embriones viables/CL y mayores tasas de viabilidad y de gestación a día 5 tras el celo que aquellas ovejas no implantadas (Tabla 6, P<0,05).

Tabla 6. *Respuesta ovárica y producción de embriones en la estación reproductiva de ovejas raza Rasa Aragonesa con 45 días de periodo post-parto, alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto (70 d antes de la recuperación de los embriones), durante la estación reproductiva (medias* ± S.E.)

	GRUPOS			
	C-MEL	**C+MEL**	**B-MEL**	**B+MEL**
No. de ovejas	6	6	6	6
No. de ovejas en celo	6/6	6/6/	6/6	6/6
Tasa de ovulación	2,17±0,3	2,17±0,3	1,50±0,3	2,0±0,3
No. de estructuras recuperadas/CL	0,30±0,2	0,60±0,3	0,50±0,2	0,83±0,2
No. de embriones totales/CL	0,30±0,2	0,30±0,2 [c]	0,50±0,2	0,75±0,2 [d]
Tasa de fecundación (%)	100 [c]	50 [d]	100	90
No. de embriones viables/CL	0,30±0,2	0,20±0,2	0,12±0,1 [a]	0,50±0,2 [b]
Tasa de viabilidad (%)	100 [a, c]	37,5 [d]	25 [b]	65 [a]
Tasa de desarrollo *in vitro* (%)	80	100	75	87,5
Tasa de gestación (%)*	50,0 (3/6)	33,3 (2/6)	16,6 (1/6) **[a]**	66,6 (4/6) **[b]**

Diferentes superíndices (a, b) en la misma fila indican diferencias significativas (P<0.05).
Diferentes superíndices (c, d) en la misma fila indican diferencias significativas (P<0.1).
*Porcentaje de ovejas con embriones viables en el Día 5 tras el celo.

3.5. Efecto de los tratamientos sobre la población de oocitos como fuente de embriones in vitro

No hubo efecto del nivel nutricional ni del tratamiento con melatonina sobre el número de oocitos recuperados; tampoco se observaron diferencias sobre la tasa de oocitos aptos para MIV ni sobre la tasa de blastocistos (Tabla 6). Sin embargo, en las ovejas que consumieron la dieta control hubo una tendencia a aumentar el número de embriones divididos (C: 8,1±0,9 vs. B: 5,4±0,9; P=0,09) y, en consecuencia, también se observó una tendencia a un mayor número de blastocistos obtenidos tras el cultivo *in vitro* (C: 3,5±0,7 vs. B: 1,1±0,7; P=0,09). En las ovejas subnutridas, se observó un efecto positivo de los implantes de melatonina sobre el número de embriones divididos (Tabla 7, P<0,05).

Tabla 7. *Producción in vitro de embriones con ovarios recogidos de ovejas raza Rasa Aragonesa con 45 días de periodo post-parto, alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto (70 d antes de la recuperación de los embriones) durante la estación reproductiva (medias* ± S.E.)

	C-MEL	**C+MEL**	**B-MEL**	**B+MEL**
No. de ovejas con CL funcional	6	6	6	6
Tasa de ovulación	2,2 ± 0,3	2,2 ± 0,3	1,50 ± 0,3	2,0 ± 0,3
No. de oocitos recuperados	25,3 ± 5,5	25,5 ± 5,5	21,2 ± 5,4	27,3 ± 5,4
No. de oocitos aptos para MIV	15,8 ± 3,4	13,5 ± 3,4	14,3 ± 3,4	15,0 ± 3,4
Tasa de oocitos aptos (%)	62,5	52,9	67,5	54,9
No. de oocitos no-aptos para MIV	9,5 ± 2,8	12,0 ± 2,8	6,8 ± 2,8	12,3 ± 2,8
No. de embriones divididos	8,2 ± 1,3 c	8,0 ± 1,3 c	3,9 ± 1,3 d	6,8 ± 1,3 c
Tasa de división (%)	51,9	59,3	27,3	45,3
No. de blastocistos	3,6 ± 1,0	3,3 ± 1,0	1,5 ± 1,0	1,1 ± 1,0
Tasa de blastocistos (%)	43,9	41,3	38,5	16,2

Todos los valores están expresados por oveja, CL=cuerpo lúteo. Superíndices (a, b) en la misma fila indican diferencias significativas de P<0,05 y superíndices (c, d) indican P<0,1.

3.6. Efecto de los tratamientos sobre la expresión de los receptores esteroideos (RP y REα) en el endometrio

No se observaron efectos de los tratamientos de nutrición y de melatonina, así como tampoco de la interacción entre ellos sobre el porcentaje de células positivas para REα. Tampoco hubo efecto de la nutrición sobre el porcentaje de células positivas a RP. Sin embargo, el tratamiento con melatonina aumentó el porcentaje de células positivas a RP en el epitelio glandular profundo, independientemente del nivel nutricional (P=0,1). No se observaron efectos de la nutrición o de la melatonina sobre la intensidad de tinción de RP o REα. Para RP, se observó una interacción entre la nutrición y la melatonina mostró efectos solamente en el epitelio glandular profundo (P<0,05). Así las ovejas subnutridas implantadas con melatonina experimentaron una mayor intensidad de PR en EGp que las ovejas subnutridas sin implantar, mientras que no se observaron cambios en los grupos control (Figura 17). En cambio, se observó que la melatonina aumentó la expresión de REα en el estroma superficial del grupo control (P<0,01), mientras que las ovejas subnutridas implantadas tendieron a disminuir la expresión del mismo en el estroma profundo respecto a las subnutridas sin implantar (P<0,01).

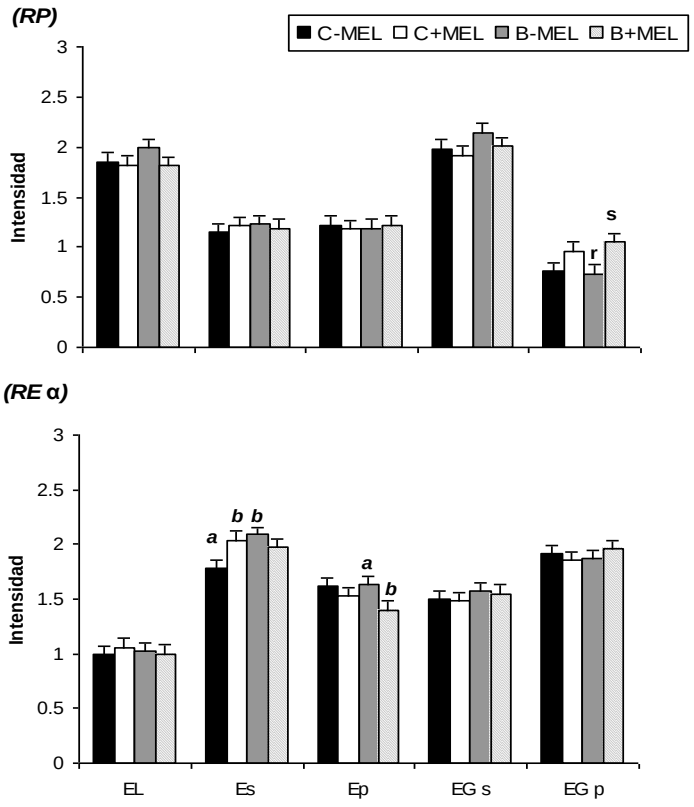
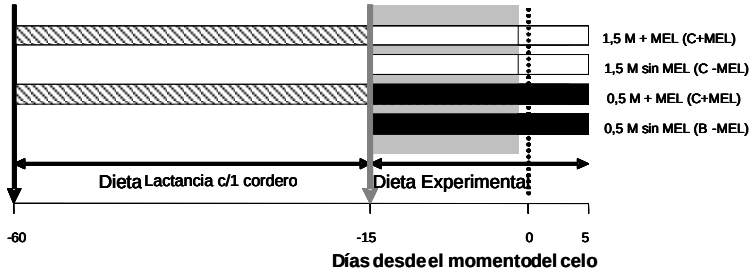


Figura 17. *Intensidad de tinción de RP (arriba) y REα (abajo) en el endometrio de ovejas raza Rasa Aragonesa con 45 días de periodo post-parto, alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto, durante la estación reproductiva.* Dentro de cada tipo celular, letras diferentes indican P<0,01 (a, b) y P=0.1 (r, s).

***EXPERIMENTO 4. Ovejas post-parto, Anoestro estacional* (Artículo III).**



4.1. Efecto de los tratamientos sobre el peso vivo y condición corporal

Todos los grupos presentaron una pérdida significativa de PV durante el intervalo parto-inicio del tratamiento nutricional (Figura 18a), aunque durante el período de consumo de las dietas experimentales (20 días después del destete) los grupos C-MEL y C+MEL mantuvieron los PV mientras que los grupos B-MEL y B+MEL experimentaron una pérdida significativa de 4,2 y 6,0 kg de PV, respectivamente ($P<0,01$). Las CC no presentaron diferencias significativas entre grupos durante todo el período experimental (Figura 18b).

4.2. Efecto de los tratamientos sobre los perfiles hormonales

4.2.1. Melatonina

El día 45 posterior a la colocación de los implantes, todas las ovejas implantadas con melatonina presentaron altos niveles plasmáticos de la hormna pineal durante las horas del día (abril: 81,0 ± 14,9 pg/ml), lo cual indicó que los implantes liberaron la hormona de manera apropiada.

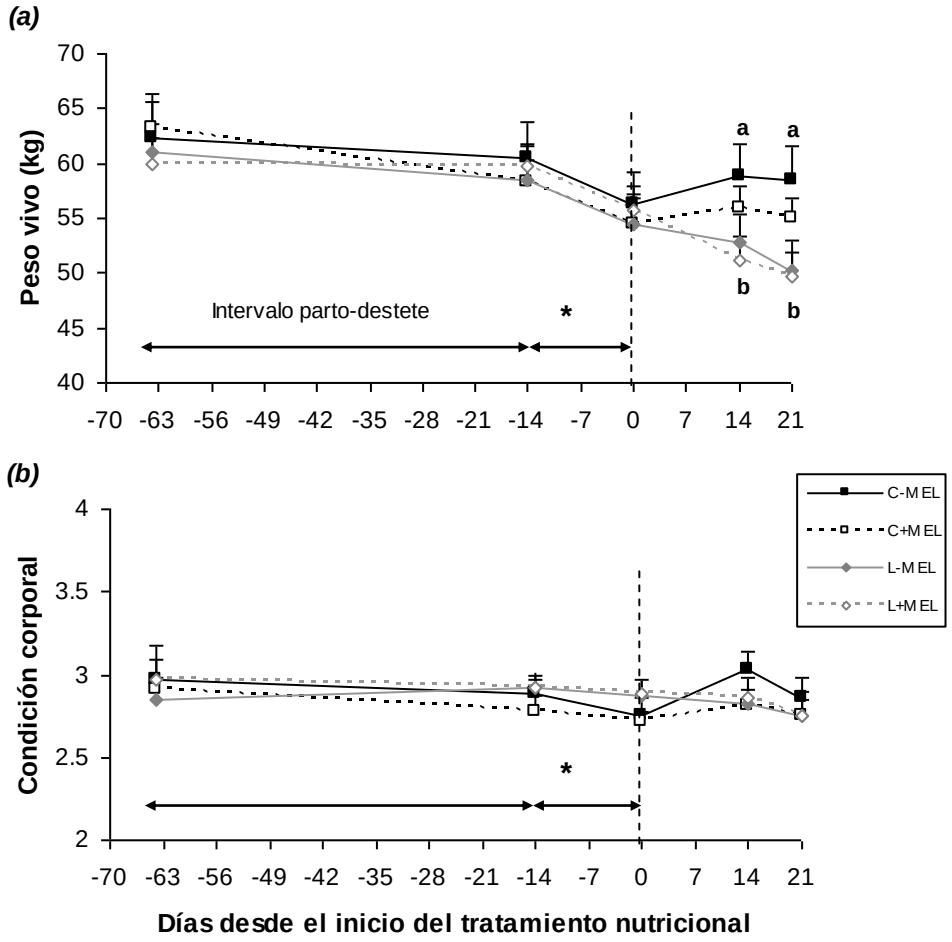


Figura 18. *Medias (± S.E.) de peso vivo* ***(a)*** *y de CC* ***(b)*** *en ovejas raza Rasa Aragonesa alimentadas durante 45 días de periodo post-parto con una dieta lactancia para un solo cordero, y tras el destete alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto, durante el anoestro.* Letras diferentes (a, b) entre grupos que consumieron dietas C y B indican P<0,01.

4.2.2. Progesterona

Los niveles de P4 aumentaron gradualmente desde el momento del celo hasta el día del sacrificio en todas las ovejas, indicando que todas ellas exhibían un CL funcional. En la Figura 19 se observa que las concentraciones plasmáticas de P4 el día del sacrificio para cada grupo fueron de: 2,6±0,5 ng/ml (C-MEL); 2,8±0,3 ng/ml (C+MEL), 3,02±0,5 ng/ml (B-MEL) y 4,1±0,7 ng/ml (B+MEL). Aunque no hubo diferencias significativas entre grupos, se observó una tendencia a que las ovejas que consumieron la dieta B presentaran niveles mayores de P4 que aquéllas que consumieron la dieta C en el día 5 tras el celo (B: 3,6±0,3 vs. C: 2,7±0,4 ng/ml; P<0,1). Además también se observó que las ovejas implantadas con melatonina (+MEL) tuvieron mayores niveles de P4 que aquellas ovejas sin implantar (+MEL: 3,5±0,3 vs. –MEL: 2,8±0,4 ng/ml; P<0,1).

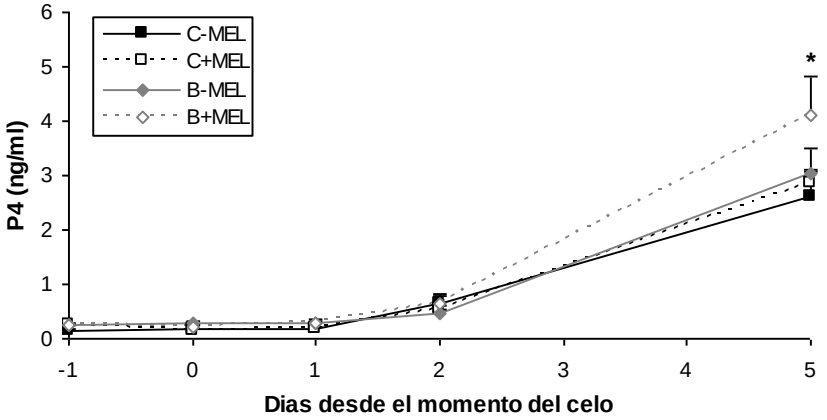


Figura 19. *Concentraciones plasmáticas medias (*± S.E.) *de progesterona (P4) desde el día del retiro del progestágeno hasta el día del sacrificio (día 5 tras el celo), en ovejas raza Rasa Aragonesa alimentadas durante 45 días de periodo post-parto con una dieta lactancia para un solo cordero, y tras el destete alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto, durante el anoestro.* (Día 0=celo). El asterisco indica P<0,05 entre grupos B-MEL y B+MEL, así como también entre C+MEL y B+MEL.

4.3. Efecto de los tratamientos sobre los perfiles de glucosa, ácidos grasos no esterificados y β-hidroxibutirato

De manera global durante todo el periodo del tratamiento nutricional (21 días), la media de la concentración plasmática de glucosa fue mayor en los grupos que consumieron la dieta C que en aquellas ovejas de los grupos B (C: 3,54±0,02 vs. B: 3,40±0,02 mmol/L; P<0,01); así mismo, se observaron diferencias significativas entre grupos durante los días 15, 18 y 21 después de iniciadas las dietas experimentales (P<0,01, Figura 20a). Un hallazgo interesante fue que, en todos los grupos, las concentraciones de glucosa presentaron un pico leve previo a la presentación del celo (día -1), que solamente en los grupos B-MEL y B+MEL fue seguido de un descenso gradual de los mismos (Figura 20a). No hubo efecto de la melatonina exógena sobre los niveles plasmáticos de glucosa.

Acompañando las pérdidas de peso vivo observadas en los grupos de ovejas subnutridas (B-MEL y B+MEL), se observó un rápido incremento de los niveles plasmáticos de AGNE; de esta manera, ambos grupos B presentaron concentraciones de este metabolito entre 5 a 6 veces mayores que las observadas en los grupos C-MEL y C+MEL (C: 0,05±0,01; B: 0,30±0,01 mmol/L, P<0.01) (Figura 20b). No se observó un efecto significativo de la melatonina sobre los niveles plasmáticos de AGNE.

Con respecto a los niveles plasmáticos de BHB, se observó una interacción significativa entre el nivel nutricional y la melatonina sobre este parámetro (P<0.01), siendo los niveles plasmáticos mayores en los grupos implantados con melatonina que en los no implantados, y en las dietas C comparadas con las dietas B (C-MEL: 0,27±0,005; C+MEL: 0,26±0,007; B-MEL: 0,24±0,006 y B+MEL: 0,28±0,007 mmol/L; P<0,001) (Figura 20c).

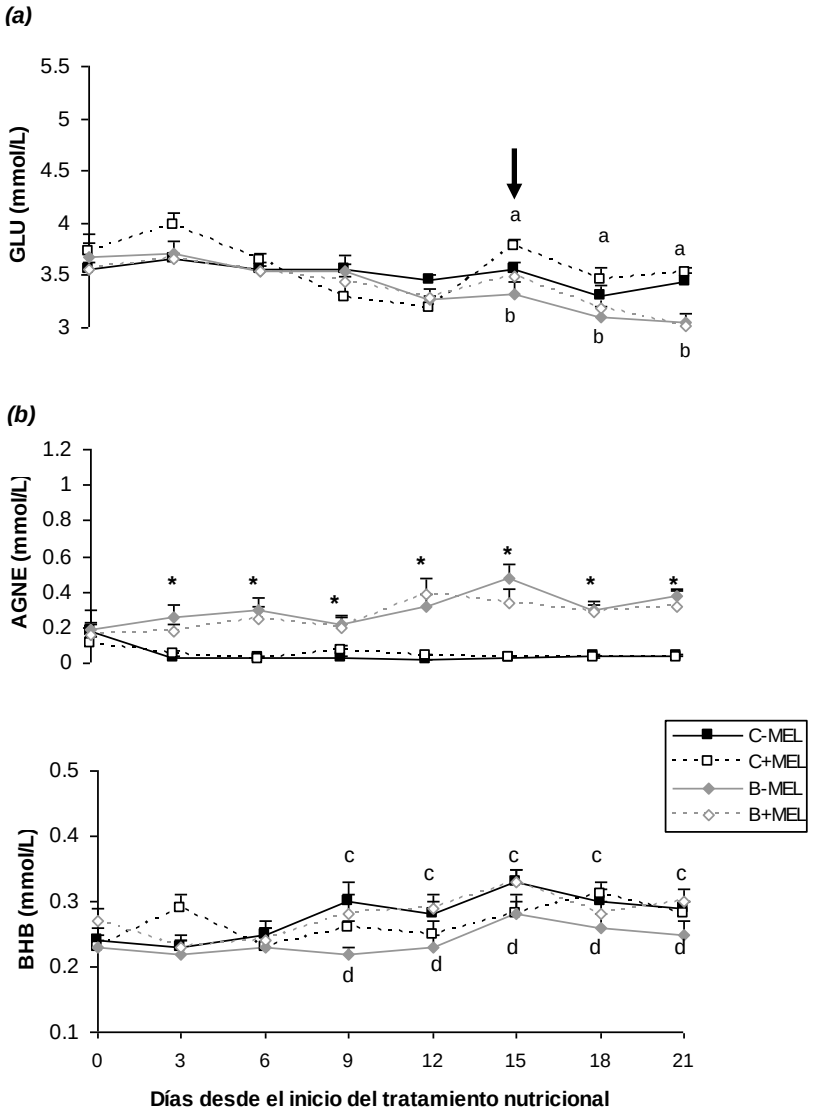


Figura 20. *Concentraciones plasmáticas medias (*± S.E.) *de glucosa* ***(a)****, ácidos grasos no esterificados* ***(b)*** *y β-hidroxibutirato* ***(c)*** *en ovejas raza Rasa Aragonesa alimentadas durante 45 días de periodo post-parto con una dieta lactancia para un solo cordero, y tras el destete alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto, durante el anoestro.* Flecha señala el día previo al celo. Letras diferentes (a, b) en glucosa indican P<0,01 entre grupos C+MEL y B+MEL. Letras diferentes (c, d) en BHB indican P<0,01 entre grupos C-MEL y B-MEL. Asteriscos indican P<0,01 entre grupos C y B.

4.4. Efectos de los tratamientos sobre la recuperación de embriones obtenidos in vivo

La tasa de ovulación no presentó diferencias significativas entre grupos (Tabla 8). La melatonina exógena tendió a incrementar el número de embriones recuperados por CL (+MEL: 0,62±0,1 vs. –MEL: 0,35±0,1; P=0,08), siendo su efecto significativo en el incremento del número de embriones viables por CL (+MEL: 0,62±0,1 vs. –MEL: 0,23±0,1; P<0,01), la tasa de viabilidad (+MEL: 83,9% vs. –MEL: 46,6%; P>0,05) y la tasa de gestación (+MEL: 76,5% vs. –MEL: 26,3%; P>0,05). Además, es importante evidenciar que el efecto de la melatonina exógena fue particularmente evidente en las ovejas de los grupos subnutridos, de manera que las ovejas subnutridas e implantadas con melatonina tuvieron un mayor número de embriones viables (B+MEL: 0,6±0,1 vs. B–MEL: 0,2±0,1; P>0,05), y en consecuencia una superior tasa de viabilidad (B+MEL: 100% vs. B–MEL: 40%; P>0,01) (Tabla 8). No hubo efecto significativo del tratamiento nutricional o de la interacción de los tratamientos de melatonina y de nutrición en ninguno de los parámetros evaluados.

4.5. Efecto de los tratamientos sobre la población de oocitos como fuente de embriones in vitro

En la Tabla 9 se observa que no hubo efecto de la nutrición, de la melatonina exógena ni de la interacción entre ambos tratamientos sobre ninguno de los parámetros evaluados. Así mismo, tampoco se observo un efecto significativo del tratamiento nutricional y/o de la melatonina exógena sobre la competencia de los oocitos. El número de embriones divididos y de blastocistos fue similar en los diferentes grupos experimentales.

Tabla 8. *Respuesta ovárica y producción de embriones en el anoestro de ovejas raza Rasa Aragonesa con 45 días de periodo post-parto, alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto (70 d antes de la recuperación de los embriones) (medias*±S.E.)

	GRUPOS			
	C-MEL	**C+MEL**	**L-MEL**	**L+MEL**
No. de ovejas	9	8	10	9
No. de ovejas en estro	9/9	8/8	10/10	9/9
Tasa de ovulación	1,8±0,3	1,6±0,2	1,4±0,2	1,9±0,4
No. de estructuras recuperadas/CL	0,4±0,1 [c]	0,8±0,1 [d]	0,5±0,1	0,6±0,1
No. de embriones recuperados/CL	0,3±0,1 [c]	0,6±0,1 [d]	0,4±0,1	0,6±0,1
Tasa de fecundación (%)	75,0	75,0	80,0	100
No. de embriones viables/CL	0,3±0,1 [c]	0,6±0,1 [d]	0,2±0,1 [a]	0,6±0,1 [b]
Tasa de viabilidad (%)	75,0	75,0	40,0 [a]	100 [b]
Tasa de gestación (%)*	33,3 (3/9)[a]	75,0 (6/8) [b]	20,0 (2/10) [a]	77,7 (7/9) [b]

En la misma fila, diferentes superíndices indican P<0.05 (a, b) o P<0,1 (c, d). Estructuras recuperadas=oocitos + embriones. *Porcentaje de ovejas con embriones viables en el Día 5 tras el celo.

Tabla 9. *Producción in vitro de embriones con ovarios provenientes de ovejas raza Rasa Aragonesa con 45 días de periodo post-parto, alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto (70 d antes de la recuperación de los embriones), durante el anoestro estacional (medias* ± S.E.)

	C-MEL	**C+MEL**	**B-MEL**	**B+MEL**
No. de ovejas con CL funcional (Día 5)	9	8	10	9
Tasa de ovulación	1,8 ± 0,3	1,6 ± 0,2	1,4 ± 0,2	1,9 ± 0,4
No. de oocitos recuperados	23,3 ± 3,5	23,7 ± 3,7	24,5 ± 3,4	22,2 ± 3,5
No. de oocitos aptos para MIV	15,2 ± 2,2	14,4 ± 2,4	16,5 ± 2,1	12,2 ± 2,2
Tasa de oocitos aptos (%)	65,7	62,8	67,9	56,3
No. de oocitos no-aptos para MIV	8,1 ± 1,8	9,4 ± 1,9	8,0 ± 1,8	10,0 ± 1,9
No. de embriones divididos	9,4 ± 1,9	9,0 ± 1,9	9,4 ± 1,9	8,8 ± 1,9
Tasa de división (%)	61,8	62,5	57,0	72,2
No. de blastocistos	2,1 ± 1,2	1,9 ± 1,2	3,2 ± 1,2	2,0 ± 1,2
Tasa de blastocistos (%)	21,8	21,1	33,0	22,7

Todos los valores están expresados por oveja, CL=cuerpo lúteo. Superíndices (a, b) en la misma fila indican diferencias significativas de $P<0,05$ y superíndices (c, d) indican $P<0,1$.

4.6. Efecto de los tratamientos sobre la expresión de los receptores esteroideos (RP y REα) en el endometrio

No se observaron efectos de la nutrición, ni de la melatonina o la interacción entre ambas sobre el porcentaje de células positivas para RP y REα. Respecto a la intensidad en la expresión de RP o REα no se observaron efectos de la nutrición o de la melatonina, pero la interacción de ambos produjo cambios en algunos tipos celulares (Figura 21). En el epitelio glandular y en el estroma (tanto superficiales como profundos) de las ovejas subnutridas, el tratamiento con melatonina disminuyó la intensidad de tinción de RP ($P<0,01$). Sin embargo, este efecto no se observó en las ovejas controles, sino que por el contrario, la melatonina aumentó los RP en el EGp ($P<0,01$). En cuanto a los REα, en el EGp también se observó una interacción entre nutrición y melatonina: el tratamiento con melatonina disminuyó la expresión de RE en las ovejas subnutridas, mientras que la aumentó en las ovejas controles ($P<0,01$).

Efecto de la época del año. Experimentos 3 y 4

Efecto de la época del año sobre los parámetros estudiados

En la Tabla 10 se resumen los principales efectos observados sobre los parámetros estudiados en los experimentos 3 y 4, siguiendo el diseño factorial 2x2x2 como hemos mencionado anteriormente.

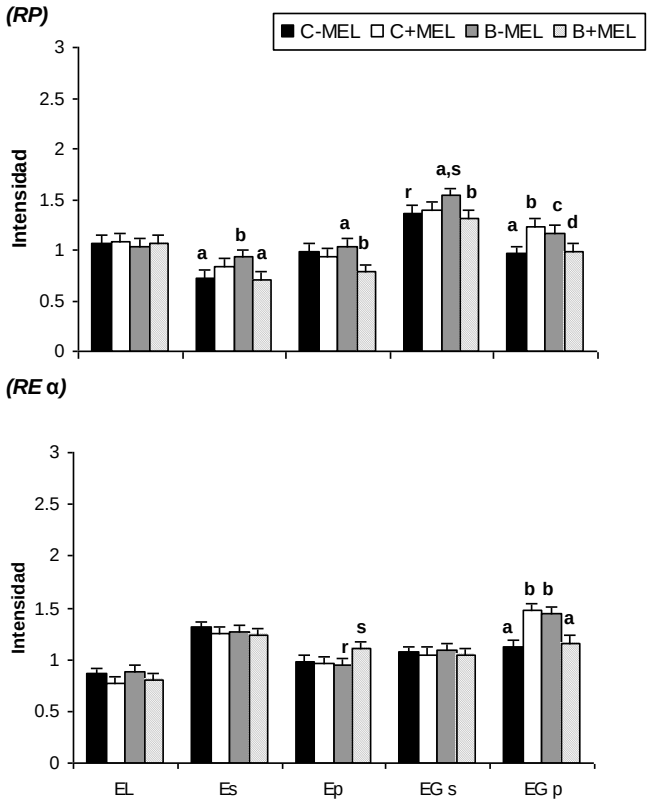


Figura 21. *Intensidad de tinción de RP (arriba) y REα (abajo) en el endometrio de ovejas raza Rasa Aragonesa con 45 días de periodo post-parto, alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto, durante la estación reproductiva.* Dentro de cada tipo celular, letras diferentes indican P<0,01 (a, b, c, d) y P=0.1 (r, s).

Tabla 10. *Efectos de la estación del año sobre las principales variables estudiadas en los experimentos 3 y 4 con ovejas raza Rasa Aragonesa con 45 días de periodo post-parto, alimentadas con una dieta 1,5 M (C) o 0,5 M (B) veces sus necesidades de mantenimiento y tratadas (+MEL) o no (-MEL) con melatonina exógena en el momento del parto.*

	ESTACIÓN	NUTRICIÓN	MELATONINA	INTERACCIÓN (A)
PESO VIVO	**	**	ns	ns
CC	ns	*	ns	ns
Metabolitos				
GLUCOSA	**	**	*	**
AGNE	**	**	ns	ns
BHB	**	P<0,1	ns	**
PROGESTERONA	*	ns	ns	P<0,1
Parámetros de la recuperación de embriones in vivo				
TASA DE OVULACIÓN	ns	ns	ns	ns
No. DE EMBRIONES TOTALES	ns	P=0,08	P=0,09	ns
TASA DE FECUNDACIÓN	ns	P<0,1	ns	P=0,09 (estac x mel)
No. DE EMBRIONES VIABLES	ns	ns	**	ns
TASA DE VIABILIDAD	ns	ns	ns	** (nut x mel)
TASA DE PREÑEZ (a día 5 tras el celo)	ns	ns	**	P<0,1 (nut x mel)
Parámetros de la producción in vitro de embriones				
No. OOCITOS RECUPERADOS	ns	ns	ns	ns
No. OOCITOS APTOS PARA MIV	ns	ns	ns	ns
No. EMBRIONES DIVIDIDOS	P=0,09	ns	ns	ns
TASA DE DIVISIÓN	*	ns	ns	ns
No. DE BLASTOCISTOS	ns	ns	ns	ns
TASA DE BLASTOCISTOS	P<0,1	ns	ns	ns
Receptores de hormonas esteroideas				
Receptor de Progesterona (RP)	**	ns	ns	(A) P<0,1 y (B) es **
Receptor de Estrógenos (REα)	**	ns	ns	(A) ns y (B) es **

Referencias, (**) indica P< 0,01 y (*) indica P<0,05. (A)=Interacción se consideró como la intervención de los tres efecto fijos (estación, nutrición y melatonina), excepto en las aclaraciones que figuran en la tabla. (B)= Interacción entre los sgtes. efectos estación, nutrición, melatonina, tipo y ubicación celular.

DISCUSIÓN

La presente tesis investigó los efectos de la melatonina exógena tanto sobre la competencia oocitaria y la viabilidad embrionaria en situaciones de subnutrición en la oveja, evaluando ambas *in vivo* e *in vitro,* como también el efecto sobre la población de receptores esteroideos en el endometrio, durante la estación reproductiva y el anoestro estacional y sobre hembras en diferentes estados fisiológicos (vacías y a los 45 días post-parto).

Efectos sobre el PV, la CC y la secreción de metabolitos

En los 4 experimentos realizados, las ovejas subnutridas mostraron una disminución significativa en el PV y la CC durante el periodo en el que se les ofrecieron las dietas experimentales. Aunque las pérdidas de PV y CC no fueron similares en los diferentes experimentos, se asumió que el tratamiento nutricional para los grupos subnutridos fue efectivo dado que en todos los ensayos el PV final fueron menor al inicial, observándose por lo tanto diferencias significativas en los PV de las ovejas de los grupos B y C en el momento del sacrificio. Además, se observó que las ovejas subnutridas, tanto las vacías como las post-parto, exhibieron un incremento en su actividad lipolítica durante ambas estaciones, evidenciado por un incremento de los niveles de AGNE paralelo al descenso de PV. Sin embargo, en las ovejas vacías del grupo B-MEL este incremento no se constató durante el anoestro. El perfil de secreción de BHB aunque estuvo influenciado por el nivel nutricional de la dieta en todos los experimentos, no mostró diferencias tan evidentes entre ovejas de los grupos B y C como lo hicieron los niveles de AGNE. Clarke (2001) observó una regulación estacional

del consumo de alimento en ovejas, describiendo un aumento del consumo voluntario de alimento durante condiciones de días largos, lo que podría explicar las diferencias en el comportamiento del PV encontradas en ambas estaciones. En estudios previos desarrollados en la misma raza, se observó que periodos de 3-4 semanas de subnutrición con niveles semejantes a los del presente trabajo indujeron una reducción significativa en el PV y la CC de las ovejas Rasa Aragonesa durante la estación reproductiva y durante el anoestro (Abecia et al., 1997, 1999; Sosa et al., 2008).

En todos los experimentos se observó que las ovejas subnutridas presentaron una concentración plasmática de glucosa significativamente inferior respecto a las ovejas control. Sin embargo, se observó un pico en los niveles de glucosa que coincidió con el muestreo del día previo a la aparición del celo, siendo más pronunciado en las ovejas vacías que en las ovejas post-parto y a su vez en los grupos control respecto de los subnutridos. Resultados previos en nuestro laboratorio han evidenciado unos patrones de secreción de glucosa y AGNE similares a los del presente estudio, observándose además el mismo incremento puntual en los perfiles de insulina e IGF-I (Sosa et al., 2006). Parece probable que dichos incrementos puedan deberse a una respuesta frente al posible estrés inducido por las prácticas de manejo inherentes a la detección de los celos (retiro de esponjas, introducción de machos, aumento del número de personas en contacto con los animales), que podría provocar una liberación refleja de insulina y glucagón, así como también una resistencia periférica a la insulina, reduciendo la incorporación de glucosa a las células (Elsasser et al, 2000). Sin embargo, otra posible explicación fisiológica podría asociarse al incremento notable de las necesidades de glucosa a nivel hipofisiario durante la fase folicular del ciclo estral, siendo entonces necesaria para aumentar las concentraciones basales de LH en respuesta al aumento de

los niveles de estradiol producidos por el folículo dominante (retroacción positiva), a fin de lograr el pico preovulatorio de LH y en consecuencia, la ovulación (I'Anson et al., 1991).

Efectos sobre la ovulación y la secreción de P4

Los resultados del presente estudio parecen avalar las observaciones de que tratamientos de subnutrición a corto plazo asociados a la utilización de progestágenos en los protocolos de inducción/sincronización de celos, no parecen influir en la tasa de ovulación durante la estación reproductiva o el anestro (Abecia et al., 1997, 1999; Borowczyk et al., 2006; Sosa et al., 2006).

Además, tampoco se observaron diferencias significativas entre grupos sobre la tasa ovulatoria en los experimentos con ovejas paridas, lo que podría deberse a que las dietas suministradas durante el periodo de lactancia lograron mantener el peso vivo desde el momento del parto hasta el destete en casi todos los grupos experimentales, principalmente en las ovejas paridas en anoestro. Al respecto, Abecia et al. (1993) demostraron que en ovejas Rasa Aragonesa destetadas en primavera existe una alta correlación positiva y significativa entre el peso al momento del destete y la tasa de ovulación. Sin embargo, estos autores evidenciaron una correlación negativa entre las pérdidas de peso desde el parto hasta el destete y la tasa de ovulación, indicando que a menores pérdidas de peso vivo en dicho intervalo parto-destete, mayor era la tasa de ovulación observada tras el primer celo detectado una vez destetados los animales.

Respecto a la secreción de P4, es consistente en la literatura el hallazgo de que las ovejas subnutridas presentan mayores concentraciones de dicha hormona que las ovejas controles entre los días 10 y 14 del ciclo sexual (Rhind et al., 1989c; Lozano et al., 1998; O'Callaghan et al., 2000; Sosa et al., 2006). No obstante, en nuestros

experimentos no encontramos diferencias en la secreción de P4 el día 5 tras el celo en función del tratamiento nutricional aplicado, exceptuando los resultados del experimento 4 (ovejas post-parto y en anoestro estacional), que mostraron una tendencia a que las ovejas subnutridas presentaran mayores niveles de P4 respecto a aquéllas con la dieta control. Es evidente que hasta el día 5 el CL está iniciando su secreción de P4, con lo que los factores que hacen que existan diferencias en sus niveles plasmáticos en momentos más avanzados de la fase luteal (a partir del día 10) todavía no han ejercido su influencia, sino mas bien se encuentran consolidando la secreción (y su metabolización) de P4.

Efectos sobre los embriones recuperados in vivo

Un hallazgo inesperado del presente estudio por sus diferencias estacionales en las ovejas vacías, ha sido la reducida viabilidad embrionaria encontrada el día 5 tras el celo en las ovejas subnutridas implantadas con melatonina durante la estación reproductiva, no sucediendo lo mismo durante el anoestro, donde dicha hormona pareció mejorar las tasas de fecundación y de viabilidad embrionaria en dichos animales. No hubo efecto de la estación sobre el número de embriones viables ni sobre la tasa de viabilidad en los diferentes grupos de ovejas vacías. Durante el anoestro e independientemente del nivel nutricional, se observaron menores tasas de fecundación y de viabilidad en las ovejas que no recibieron implantes de melatonina. Diferentes estudios han mostrado que la suplementación con melatonina durante el anoestro no mejora la tasa de fertilidad de los embriones recuperados en ovejas superovuladas (McEvoy et al., 1998; Forcada et al., 2006) o procedentes de oocitos recuperados en ovejas superovuladas y procesados por FIV (Luther et al., 2005). A su vez, resultados previos de nuestro equipo de trabajo han demostrado que la suplementación con

melatonina reduce significativamente el número y la tasa de embriones no viables (degenerados y retardados) en ovejas superovuladas durante el anoestro (Forcada et al., 2006).

Aunque el tratamiento nutricional no tuvo un efecto significativo sobre la mayoría de los parámetros estudiados en las ovejas post-parto, la melatonina exógena mejoró significativamente la viabilidad *in vivo* de los embriones recuperados en las ovejas subnutridas el día 5 tras el celo, en ambas estaciones. Además, independientemente del nivel nutricional, las tasas de gestación fueron significativamente mayores en las ovejas implantadas con melatonina que en las no implantadas, si bien en la estación reproductiva este efecto benéfico de la melatonina sólo se observó en las ovejas subnutridas.

Los mecanismos involucrados en la mejora de la viabilidad embrionaria por la melatonina no son totalmente conocidos, si bien podrían ser explicados en parte por el efecto luteotrófico de la hormona pineal, observado tanto *in vivo* (Durotoye et al., 1997) como *in vitro* (Abecia et al., 2002), así como por sus efectos a nivel del eje hipotálamo-hipofisiario ya descriptos hace años (Malpaux et al., 1997), y también por sus propiedades antioxidantes y de captación de radicales libres (Chetsawang et al., 2006). En nuestros resultados en las ovejas post-parto, hemos encontrado una evidencia adicional del efecto luteotrófico de la melatonina, ya que las hembras implantadas exhibieron niveles plasmáticos de P4 mayores a los de aquéllas no implantadas, tanto durante el anoestro como en la estación reproductiva. Este hecho podría por tanto haber influido en la mejora de las tasas de viabilidad y de gestación encontradas en los grupos implantados. Sin embargo, esta manifestación del efecto luteotrófico de la melatonina no pudo ser evidenciada en las ovejas vacías de nuestros experimentos 1 y 2, ya que no encontramos diferencias significativas entre grupos ni entre estaciones del año en los

niveles de P4 el día 5 tras el celo. En conjunto y teniendo en cuenta los resultados del presente estudio, se puede sugerir que los efectos de la melatonina exógena sobre la viabilidad embrionaria podrían ser debidos a diferentes mecanismos en función del estado fisiológico en que se encuentre la oveja.

Efectos sobre la producción in vitro de embriones

Aunque la melatonina exógena no tuvo efecto significativo en la mayoría de los parámetros *in vitro* evaluados en nuestros experimentos con ovejas vacías, el tratamiento con esta hormona durante el anoestro mejoró significativamente la tasa de fecundación de los oocitos procedentes de las ovejas subnutridas, así como la tasa de blastocistos obtenida, en este caso independientemente del nivel de nutrición. Por el contrario, en nuestros experimentos con ovejas post-parto se observó que la melatonina exógena tuvo un efecto positivo sobre la competencia de los oocitos de las ovejas subnutridas únicamente durante la estación reproductiva, mejorando la división y el desarrollo *in vitro* de los embriones; por el contrario, no se observó efecto alguno de la hormona pineal sobre los parámetros evaluados *in vitro* durante el anoestro estacional. Algunos estudios tampoco han encontrado efectos positivos de la melatonina sobre el número de folículos visibles o sobre el número de oocitos recuperados de ovejas (Luther et al., 2005) y cabras (Berlinguer et al., 2007) superovuladas con FSH durante el anoestro, lo cierto es que los tratamientos con melatonina exógena durante el anoestro parecen mejorar la competencia para el desarrollo *in vitro* de oocitos obtenidos de ovejas sincronizadas con progestágenos y gonadotropina coriónica equina (Valasi et al., 2006), así como las tasas de divididos y de blastocistos obtenidas por FIV en cabras (Berlinguer et al., 2007).

El anoestro es un periodo de presentación de pulsos de LH de baja frecuencia,

mientras que durante la estación reproductiva la frecuencia y amplitud de los pulsos de LH es la adecuada para controlar la presentación y sucesión de las fases luteal y folicular del ciclo estral (Goodman, 1988). Por su parte, Oussaid et al. (1999) demostró que una supresión temporal de la descarga de LH durante la fase folicular perjudicaba la competencia de los oocitos en ovejas cíclicas. Además, el anoestro post-parto es un periodo caracterizado por una baja frecuencia de los pulsos de la LH (Goodman, 1988). Por lo tanto y en conjunto, nuestros resultados *in vitro* principalmente en las ovejas vacías, son coherentes con lo anteriormente mencionado en el sentido de que el anoestro tuvo un efecto negativo sobre el número de oocitos recuperados por oveja, la competencia de los mismos y las tasas de fecundación. Del mismo modo, Stenback et al. (2001) señalaron que los oocitos procedentes de ovejas superovuladas tuvieron una mejor respuesta a la fecundación *in vitro* durante la estación reproductiva que durante el anoestro. No obstante, en el presente estudio estas diferencias estacionales no fueron tan evidentes en las ovejas post-parto como en las ovejas vacías. Además, se ha observado que la estación influye en la tasa de fecundación *in vivo,* y algunos autores han demostrado la existencia de una mayor proporción de oocitos no fecundados y/o degenerados en ovejas superovuladas e inseminadas durante el periodo de anoestro, así como también que un mayor número de folículos pequeños fueron inducidos a ovular en dicha estación, la mayoría de los cuales contenían oocitos inmaduros al momento de la ovulación (Mitchell et al., 2002; Gónzalez-Bulnes et al., 2003), comprometiendo por tanto la fecundación o el desarrollo posterior. Estas diferencias se podrían atribuir, al menos en parte, a cambios estacionales en la calidad del semen; sin embargo, se han observado pocos cambios en el volumen o calidad del semen procedente de eyaculados de machos Rasa Aragonesa a lo largo del año (Martí et al., 2007), lo que sugiere que la mayor tasa de fertilidad durante la estación reproductiva podría deberse principalmente

a una mayor competencia del oocito en el momento de la ovulación.

Efectos sobre los receptores esteroideos en el endometrio

En general, en nuestros experimentos tanto en las ovejas vacías como en las ovejas en post-parto, no se observaron diferencias en la expresión de ambos receptores esteroideos con respecto al tratamiento nutricional. Esto está en desacuerdo con el trabajo de Sosa et al. (2004) donde se encontró en el día 5 tras el celo una disminución en la expresión de RP y ningún cambio de REα en la mayoría de los tipos celulares evaluados en las ovejas subnutridas. Sin embargo, los mismos autores en un trabajo posterior (Sosa et al., 2006) tampoco encontraron diferencias en la expresión de RP y REα debidas al tratamiento nutricional, de manera similar a lo observado en nuestros experimentos. A pesar de que la subnutrición a la que se sometió a los animales es muy similar entre los diferentes experimentos, las diferencias encontradas entre estudios podrían explicarse en base a las diferentes CC alcanzadas por los animales. Por otro lado, no puede descartarse que en nuestros experimentos existan efectos que la subnutrición pudiera ejercer a otro nivel, como por ejemplo a nivel del mRNA o de su capacidad de unión.

Según nuestro conocimiento y hasta el momento de la realización de la presente tesis, no existe en la literatura otro trabajo que haga referencia a los efectos de la melatonina exógena sobre la expresión de los receptores esteroideos en el endometrio ovino y mucho menos sobre los efectos de su interacción con la nutrición. Tampoco se han encontrado trabajos en otras especies con las que poder comparar nuestras observaciones. En general, observando sólo las ovejas controles, la melatonina no tuvo efecto sobre la expresión de RP o REα, excepto por un aumento en la expresión de ambos receptores en las glándulas profundas de las ovejas en post-parto durante el

anoestro estacional. Este hallazgo implica una mayor sensibilidad a la P4 y los E en las glándulas de las ovejas cuando no están en una situación óptima desde el punto de vista reproductivo (e.g. anoestro estacional más periodo post-parto).

En este sentido, es interesante observar que los efectos de la melatonina exógena sobre la expresión de RP fueron dependientes de la nutrición, de la estación del año y del estado fisiológico de las ovejas. De esta manera, durante la estación reproductiva se observó una disminución de RP en los epitelios glandulares (superficial y profundo) de las ovejas vacías subnutridas implantadas respecto de las subnutridas sin implantar. Mientras que, fue durante el anoestro de las ovejas post-parto donde el grupo de ovejas subnutridas implantadas presentó una disminución de RP en los epitelios glandulares y estromas (superficiales y profundos) respecto de las subnutridas sin implantar. No nos queda claro cual podría ser la causa del efecto de la melatonina sobre la expresión de RP en las ovejas vacías durante la estación reproductiva. Tal vez, es posible que una sobreexposición a la melatonina durante la estación reproductiva de estas ovejas hubiera perjudicado el ambiente uterino. La interacción entre la melatonina y el estado nutricional fue especialmente llamativa en las ovejas en post-parto durante el anoestro estacional, mientras que en las ovejas controles la melatonina mejoró los niveles de ambos receptores, en las ovejas subnutridas este efecto no se observó o incluso se invirtió. La disminución en la expresión de RP en las ovejas subnutridas de este experimento (Exp. 4) podría atribuirse a los mayores niveles de P4 observados en estos animales, ya que como hemos mencionado anteriormente es la misma P4 la encargada de regular la expresión de su propio receptor.

Los mecanismos por los cuales la melatonina puede influir en la expresión de los receptores esteroideos uterinos, y por ende, en el ambiente uterino, no son fáciles de dilucidar. La posibilidad de una acción directa a nivel uterino, a través de receptores

específicos no puede descartarse ya que hasta el momento no se ha investigado en el tracto reproductivo de la oveja ni de ninguna otra especie animal. Sin embargo, se ha demostrado su presencia en el ovario, describiéndose sitios de unión específicos para esta hormona en las células de la granulosa en humanos y en ratas (Yie et al., 1995; Clemmens et al., 2001). Por otro lado, su efecto a nivel uterino podría ser mediado indirectamente por vías endocrinas sobre los receptores esteroideos endometriales.

La técnica de inmunohistoquímica se puede considerar una técnica única en el sentido de que permite conocer la localización tisular de las proteínas medidas. Como era de esperarse, la tinción de RP y REα se observó solamente en los núcleos celulares, ya que estos receptores forman parte de una familia de receptores nucleares donde actúan como factores de transcripción (Tsai y O´Malley, 1994). Debido a que sus hormonas dianas (P4 y E) son de naturaleza lipídica, son capaces de atravesar la membrana plasmática y llegar al núcleo de las células. En ambas estaciones del año estudiadas y tanto en ovejas vacías como en post-parto, un hallazgo consistente fue la observación de una mayor tinción para REα en las glándulas profundas. A su vez, RP presentó una mayor tinción en las glándulas superficiales únicamente en las ovejas post-parto. Al respecto, Sosa et al. (2004, 2006) observaron el día 5 tras el celo, que ambos receptores esteroideos se expresaban de diferente manera en las glándulas, encontrando una mayor tinción de RP y una menor tinción de REα en las glándulas superficiales. La expresión de los receptores en los diferentes tipos celulares está lógicamente asociada a las diferentes funciones de las hormonas esteroideas: los E estimulan la proliferación del epitelio uterino, mientras que la P4 estimula la diferenciación celular e inhibe la proliferación inducida por los E. Además los E actúan sobre células que no se están dividiendo y que se sitúan en la base de las glándulas, mientras que la P4 actúa en células en división (fase activa del ciclo celular) y que se localizan más cerca de la luz

uterina (Conti et al., 1984). Por otro lado, y a diferencia de lo descrito por Sosa et al (2004, 2006), nosotros hemos observado que ambos receptores tendieron a expresarse de manera similar tanto en el estroma superficial como en el profundo, haciendo la salvedad de que REα tuvo una mayor expresión en el estroma superficial únicamente en el experimento 4 (ovejas post-parto y en anoestro).

Efectos de la nutrición sobre la funcionalidad ovárica y el desarrollo embrionario temprano

En conjunto, experimentos del presente estudio han mostrado que la subnutrición no pareció influir significativamente en la competencia de los oocitos para su desarrollo *in vitro* o *in vivo* durante la estación reproductiva. Sin embargo, las ovejas subnutridas presentaron un reducido número de oocitos recuperados y de embriones divididos durante el anoestro, lo que indicaría que, al menos en las razas mediterráneas, una adecuada nutrición puede compensar parcialmente los efectos adversos de la estacionalidad sobre el eje hipotálamo-hipofisario-ovárico (Forcada et al., 1992, Lindsay, 1996). Algunos autores han observado que la competencia oocitaria está alterada en ovejas subnutridas superovuladas (Papadopoulos et al., 2001; Borowicz et al., 2006). Al respecto, la subnutrición ha sido asociada a un aumento de la secreción de estradiol durante la fase folicular temprana, lo que aumentaría la retroacción negativa del esteroide a nivel hipotálamo-hipófisario, perjudicando por tanto la competencia folicular. Así mismo, la subnutrición conlleva una reducción de la sensibilidad ovárica y endometrial a los esteroides ováricos (Sosa et al., 2006 y 2008), lo que compromete el desarrollo embrionario temprano.

Por otra parte, la subnutrición, además de ejercer distintos efectos adversos sobre la reproducción, ha sido asociada a fallos en la competencia folicular (Scaramuzzi et al.,

2006), así como también con una baja sensibilidad a los esteroides ováricos a nivel de oviductos y de endometrio (Sosa et al., 2006 y 2008); todo ello estaría de alguna manera comprometiendo el desarrollo embrionario temprano y la posibilidad de llevar a término la gestación. En nuestros resultados, la ausencia de efectos de la subnutrición sobre los parámetros de FIV durante el anoestro estacional de las ovejas paridas nos sorprendió, de manera que no resulta fácil explicar las posibles causas. Tal vez la condición fisiológica de las ovejas podría darnos alguna pista, debido a que en las ovejas vacías sí se observaron efectos como ya hemos mencionado. Durante el anoestro post-parto, el estado metabólico puede modificar la distribución de los nutrientes y la utilización de los mismos, priorizando la energía para cubrir las demandas fisiológicas específicas (Bauman et al., 1980). Además, es claro que la reactivación de la actividad ovárica cuando el periodo postparto tiene lugar durante el anoestro es más lenta, e incluso aunque exista un tratamiento de inducción y sincronización de celos, la calidad de los oocitos se resiente si no ha habido una fase luteal previa (Abecia et al., 1996), que es lo que pareció suceder en el experimento realizado en periodo de anoestro. La reactivación de la actividad ovárica tras el parto podría explicar las diferencias estacionales observadas. Así, durante la estación reproductiva hemos observado un efecto adverso de la subnutrición sobre el número de embriones divididos y de blastocistos obtenidos como consecuencia de que probablemente los animales ya estaban cíclicos en el momento del tratamiento de sincronización y por tanto con un adecuado potencial de fecundación, el cual se puede ver notablemente alterado por un periodo de subnutrición.

CONCLUSIONES

En las condiciones en que se han desarrollado los presentes experimentos puede concluirse que:

1- El efecto de la melatonina exógena sobre los parámetros reproductivos estudiados en la presente Tesis en ovejas subnutridas mostró una regulación diferente dependiendo del estado fisiológico (vacía o con más de 45 días post-parto) y de la época del año (estación reproductiva o anoestro estacional) en la que se encontraba la hembra.

2- En las ovejas vacías, la melatonina exógena mejoró la competencia oocitaria y la viabilidad embrionaria (tanto *in vivo* como *in vitro*) únicamente durante el anoestro estacional. Por el contrario, la viabilidad se vio perjudicada en los embriones provenientes de ovejas subnutridas implantadas con melatonina durante la estación reproductiva

3- En ovejas con más de 45 días post-parto la melatonina exógena tuvo efectos benéficos sobre la viabilidad *in vivo* de los embriones obtenidos de ovejas subnutridas durante ambas épocas del año (estación reproductiva y anoestro estacional), pero no tuvo efecto sobre la competencia oocitaria y el desarrollo embrionario *in vitro*.

4- La melatonina exógena produjo cambios en la expresión de los receptores esteroideos (RP y REα) en el endometrio de las ovejas vacías así como también de las ovejas post-parto, cambios que fueron diferentes según la época del año. Así, en las ovejas subnutridas vacías disminuyó la expresión de RP en algunos tipos celulares durante la estación reproductiva aunque no tuvo efecto alguno durante el anoestro estacional, mientras que en las ovejas subnutridas post-parto la expresión de RP disminuyó en algunos tipos celulares durante el anoestro estacional y aumentó solo en el epitelio glandular profundo durante la estación reproductiva. En general, no se observaron cambios en la expresión de REα entre los tratamientos.

BIBLIOGRAFÍA

Abecia, J.A., Forcada, F., Zarazaga, L. (1993). Variación del peso vivo durante La lactación: efecto sobre la reactivación cíclica y ováricaen ovejas paridas en anoestro estacionario. ITEA 89A, 78-89.

Abecia, J.A., Rhind, S.M., Bramley, T.A., McMillen, S.R. (1995). Steroid production and LH receptor concentrations of ovarian follicles and corpora lutea and associated rates of ova wastage in ewes given high and low levels of food intake before and after mating. Animal Science 61, 57-62.

Abecia, J.A., Rhind, S.M., Goddard, P.J., Mcmillen, S.R., Ahmadi, S., Elston, D.A. (1996). Jugular and ovarian venous profiles of progesterone and associated endometrial progesterone concentrations in pregnant and non-pregnant ewes. Animal Science 63, 229-234.

Abecia, J.A., Lozano, J.M., Forcada, F., Zarazaga, L. (1997). Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Anim. Reprod. Sci. 48, 209-218.

Abecia, J.A., Forcada, F., Lozano, J.M. (1999). A preliminary report on the effect of dietary energy on prostaglandin F2α production in vitro, interferon-tau synthesis by the conceptus, endometrial progesterone concentration on days 9 and 15 of pregnancy and associated rates of embryo wastage in ewe. Theriogenology 52(7), 1203-1213.

Abecia, J.A., Forcada, F., Zuñiga, O. (2002). The effect of melatonin on the secretion of progesterone in sheep and on the development of ovine embryos in vitro. Vet. Res. Commun. 26, 151-158.

Abecia, J.A., Sosa, C., Forcada, F., Meikle, A. (2006). The effect of undernutrition on the establishment of pregnancy in the ewe. Reprod. Nutr. Dev. 46, 367-378.

Abecia, J.A., Valares, J.A., Forcada, F., Palacin, I., Martin, S., Martino, A. (2007). The effect of melatonin on the reproductive performance of three sheep breeds in Spain. Small Rum. Res. 69, 10-16.

Agricultural and Food Research Council (1993). Energy and protein requirements of ruminants. An advisor manual prepared by the AFRC Technical Committee on responses to nutrients. CAB International, Wallingford, United Kingdom.

Ashworth, C.J. (1995). Maternal and conceptus factors affecting histotrophic nutrition and survival of embryos. Livestock Production Science 44, 99-105.

Berlinguer, F., Spezzigu, A., Succu, S., Bebbere, D., Madeddu, M., Tedde, A., Satta, V., Leoni, G.G., Naitana, S. (2007). Melatonin treatment during anestrus enhances oocyte developmental competence in FSH-treated goats. Proceedings of 23rd Annual Meeting A.E.T.E, ed. by European Embryo Transfer Association. Italy, pp. 136.

Bister, J.L., Noël, B., Perrad, B., Mandiki, S.N.M., Mbayahaga, J., Paquay, R. (1999). Control of ovarian follicles activity in the ewe. Dom. Anim. Endocrin. 17, 315-328.

Boland, M.P., Lonergan, P., O´Callaghan, D. (2001). Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology 55, 1323-1340.

Boos, A., Meyer, W., Schwarz, R., Grunert, E. (1996). Immunohistochemical assessment of oestrogen receptor and progesterone receptor distribution in biopsy samples of the bovine endometrium collected throughout the oestrous cycle. Anim. Reprod. Sci. 44, 11-21.

Borowczyk, E., Caton, J.S., Redmer, D.A., Bilski, J.J., Weigl, R.M., Vonnahme, K.A., Borowicz, P.P., Kirsch, J.D., Kraft, K.C., Reynolds, L.P., Grazul-Bilska, A.T. (2006): Effects of plane of nutrition on in vitro fertilization and early embryonic development in sheep. J. Anim. Sci. 84(6), 1593–1599.

Buhi, W.C., Alvarez, I.M., Kouba, A.J. (1997). Oviductal regulation of fertilization and early embryonic development. J. Reprod. Fertil. Suppl. 52, 285-300.

Buhi, W.C. (2002). Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. Reproduction 123, 355-62.

Chemineau, P., Martin, G.B., Saumande, J., Normant, E. (1988). Seasonal and hormonal control of pulsatile LH secretion in the dairy goat (Capra hircus). J. Reprod. Fertil. 83, 91-98.

Chemineau, P., Malpaux, B., Pelletier, J., Leboeuf, B., Delgadillo, J.A., Deletang, F., Pobel, T., Brice, G. (1996). Emploi des implants de mélatonine at des traitements photopériodiques pour maîtriser la reproduction saissonière chez les ovins et les caprins (use of melatonin implantsand photoperiodic tratments to resuce seasonal reproduction in sheep and goats). INRA Prod. Anim. 9, 45-60.

Chetsawang, B., Putthaprasart, C., Phansuwan-Pujito, P., Govitrapong, P. (2006). Melatonin protects against hydrogen peroxide-induced cell death signaling in SH-SY5Y cultured cells: involvement of nuclear factor kappa B, Bax and Bcl-2. J. Pineal Res. 41, 116-123.

Chilliard, Y., Bocquier, F., Doreau, M. (1998). Digestive and metabolic adaptations of ruminants to undernutrition, and consequences on reproduction. Reprod. Nutr. Dev. 38, 131-152.

Clark, J.H., Schrader, W.T., O'malley, B.W. (1992) Mechanisms of steroid hormones action. In "Textbook of Endocrinology", pp. 35-90. Eds Wilson, J.D. y Foster, D.W. (W B Saunders: Philadelphia).

Clark, J.H., Mani, S.K. (1994) Actions of ovarian steroid hormones. In "The Physiology of Reproduction". pp. 1011-1046. Eds Knobil, E. y Neill, J.D. (Raven Press: New York).

Clarke, I.J. (2001). Sex and season are major determinants of voluntary food intake in sheep. Reprod. Fertil. Dev. 13, 577-582.

Clemmens, J.W., Jarzunka, M.J., Witt-Enderby, P.A. (2001). Down-regulation of mt1 melatonin receptors in rat ovary following estrogen exposure. Life Sciences 69, 27-35.

Conti, C.J., Gimenez-Conti, I.B., Conner, E.A., Lehman, J.M., Gerschenson, L.E. (1984). Estrogen and progesterone regulation of proliferation, migration, and loss in different target cells of rabbit uterine epithelium. Endocrinology 114, 345-51.

Cooper, D. A,Carver, D. A., Vieneuve, P., Silvia W., Weep, E.K.. (1991). Effects of progestogen treatment on concentrations of prostaglandins and oxytocin in plasma from posterior vena cava of post-partum beef cows. J. Reprod. Fed. 91, 411.

Cox, J.F., Saravia, F. (1992): Use of multiple sperm penetration assay in ovines. In: Proceedings of the XVII Annual Meeting of Chilean Society of Animal Production, Chile.

Cunha, G.R., Young, P., Brody, J.R. (1989). Role of uterine epithelium in the development of myometrial smooth muscle cells. Biology of Reproduction 40, 861- 871.

Durotoye, L.A., Webley, G.E., Rodway, R.G. (1997). Stimulation of the production of progesterone by the corpus luteum of the ewe by the perfusion of melatonin in vivo and by treatment of granulosa cells with melatonin in vitro. Res. Vet. Sci. 62, 87-91.

Dziuk, P.J. y Bellows, R. A. (1983) Management of reproduction of beef cattle, sheep and pigs. J. Anim. Sci. (Suppl. 2):355-379.

Elsasser, T.H., Klasing, K.C., Filipov, N., Thompson, F. (2000) The metabolic consequences of stress: targets for stress and priorities of nutrient use. In "The biology of animal stress: basic principles and implications for animal welfare", pp. 77-110. Eds Moberg, G.P. y Mench, J.A. (CAB International: Oxon, UK).

Fawcett, D.W. (1988) Sistema reproductor femenino. In "Tratado de histología", pp. 909-912. (Interamericana-McGraw Hill: Madrid).

Fiske, V.M., Parker, K.L., Ulmer, A., Seng, H. O., Aziz, N. (1984). Effects of melatonin alone or in combination with human chorionic gonadotropin or ovine luteinizing hormone on the in vitro secretion of oestrogen or progesterone by granulosa cells of rats. Endocrinology 114, 407-410.

Forcada, F., Abecia, J.A., Sierra, I. (1992). Seasonal changes in oestrus activity and ovulation rate in Rasa Aragonesa ewes maintained at two different body condition levels. Small Rum. Res. 8(4), 313-324.

Forcada, F., Zarazaga, L., Abecia, J.A. (1995). Effect of exogenous melatonin and plane of nutrition after weaning on estrous activity, endocrine status and ovulation rate in Salz ewes lambing in the seasonal anestrus. Theriogenology 43(7), 1179-1193.

Forcada, F. Abecia, J.A., Zúñiga, O., Lozano, J.M. (2002). Variation in the ability of melatonin implants inserted at two different times after the winter solstice to restore reproductive activity in reduced seasonality ewes. Australian Journal of Agricultural Research 53, 167-173.

Forcada, F., Abecia, J.A. (2006). The effect of nutrition on the seasonality of reproduction in ewes. Reprod. Nutr. Dev. 46(4), 355-365.

Forcada, F., Abecia, J.A., Cebrián-Perez, J.A., Muiño-Blanco, T., Valares, J.A., Palacín, I., Casao, A. (2006). The effect of melatonin implants during the seasonal anoestrus on embryo production after superovulation in aged high-prolificacy Rasa Aragonesa ewes. Theriogenology 65(2), 356–365.

Foster, D.L., Ebling, F.J., Micka, A.F., Vannerson, L.A., Bucholtz, D.C., Wood, R.I.,Suttie, J.M., Fenner, D.E. (1989). Metabolic interfaces between growth and reproduction. I. Nutritional modulation of gonadotropin, prolactin, and growth hormone secretion in the growth-limited female lamb. Endocrinology 125, 342-350.

Fraser, H.M., Jeffcoate, S.L., Gunn, A., Holland, D.T. (1975). Effect of active immunization to luteinizing hormone releasing hormone on gonadotrophin levels in ovariectomized rats. J. Endocr. 64, 191-192.

Fray, M.D., Lamming, G.E., Haresign, W. (1995). Inter-breed variation in the postpartum response to continuous low dose infusion of GnRH. Theriogenology 45, 1047-1064.

Geisert, R.D., Morgan, G.L., Short, E.C., Jr., Zavy, M.T. (1992). Endocrine events associated with endometrial function and conceptus development in cattle. Reprod Fertil Dev 4, 301-5.

González-Bulnes, A., García-García, R.M., Santiago-Moreno, J., Domínguez, V., López-Sebastian, A., Cocero, M.J. (2003). Reproductive season affects inhibitory effects from large follicles on the response to superovulatory FSH treatments in ewes. Theriogenology 60(2), 281-288.

Goodman, R.L., Karsch, E.J. (1980). Pulsatile secretion of luteinizing hormone: differential suppression by ovarian steroids. Endocrinology 107:1286-1291.

Goodman, R.L. (1988): Neuroendocrine control of the ovine estrous cycle. In "The Physiology of Reproduction", pp. 1929-1968. Eds. E. Knobil and J. Neil. (Raven Press, New York).

Goodman, R.L. (1994). Neuroendocrine control of the ovine estrous cycle. In "The Physiology of Reproduction", pp. 406-412. Eds. Knobil, E. and Neill, J.D. (Raven Press, New York, vol 2).

Gordon, I. 1997. Controlled reproduction in sheep and goats. From Controlled reproduction in farm animals series, Vol. 2. Ed. Cab International, New York, USA.

Graham, J.D., Clarke, C.L. (1997). Physiological Action of Progesterone in Target Tissues. Endocr. Rev. 18, 502-519.

Gray, C.A., Bartol, F.F., Tarleton, B.J., Wiley, A.A., Johnson, G.A., Bazer, F.W., Spencer, T.E. (2001). Developmental biology of uterine glands. Biol. Reprod. 65, 1311-23.

Hafez, E.S.E. (1989). Reproducción e inseminación artificial en animales. (Ed. McGraw Hill Interamericana, España).

Hafez, E.S. (1996). Anatomía del aparato reproductor femenino. In "Reproducción e inseminación artificial en animales", pp. 20-52. (Interamericana-McGraw Hill: Madrid).

Haresign, W., Peters, A.R., Staples, L.D. (1990). The effect of melatonin implants on breeding activity and litter size in commercial sheep flocks in the UK. Anim. Prod. 50, 111-121.

Hopper, H.W., Silcox, R.W., Byerley, D.J., Kiser, T.E. (1993). Follicular development in prepubertal heifers. Anim. Reprod. Sci. 31, 7-12.

Huneau, D., Crozet, N., Ahmed-Ali, M. (1994). Estrous sheep serum as a potent agent for ovine IVF: Effect on cholesterol efflux from spermatozoa and the acrosome reaction. Theriogenology 42, 1017-1028.

Jenkin, G., Heap, R.B., Symons, D.B.A. (1977). Pituitary responsiveness to synthetic LH-RH and pituitary LH content at various reproductive stages in the sheep. J. Reprod. Fertil. 49, 207-214.

Kakar, M.A., Maddocks, S., Lorimer, M.F., Kleemann, D.O., Rudiger, S.R., Hartwich, K.M., Walker, S.K. (2005). The effect of peri-conception nutrition on embryo quality in the superovulated ewe. Theriogenology 64, 1090-1103.

Karsch, F.J., Bittman, E.L., Foster, D.L., Goodman, R.L., Legan, S.J., Robinson, J.E. (1984). Neuroendocrine basis of seasonal reproduction. Recent Prog. Horm. Res. 40, 185-232.

Lamming, L.E. (1978). Reproduction during lactation. In Control of Ovulation, p 345. Eds. D.B. Crighton, N.B. Haynes, G.R. Foxcroft, G.E. Lamming. (Butterworth, London).

Lamming, G.E., Walters, D.C. y Peters, A.R. (1981). Endocrine patterns of the postpartum cow. J. Reprod. Fert. (Suppl. 30):155-169.

Li, F., Pi, W.H., Zhu, H.Z., Zhang, S.S., Liu, S.R., Xue, J.L. (2006). The effect of estrous ewe serum and heparin on in vitro fertilization and subsequent embryonic development in sheep. Small Rumin. Res. 63, 226-232.

Lindsay, D., Martin, G., Williams, I. (1993). Nutrition and reproduction. In "Reproduction in Domesticated Animals", pp. 459-491. (Elsevier Science, Amsterdam).

Lindsay, D.R. (1996). Environment and reproductive behaviour. Anim Reprod Sci 42, 1-12.
López-Sebastián, A., Cognié, Y., Cocero, M.J., De La Fuente, J., Poulin, N., 1990. Effect of season and duration of FSH treatment on embryo production in sheep. Theriogenology 34(1), 175-180.

Lozano, J.M., Abecia, J.A., Forcada, F., Zarazaga, L., Alfaro, B. (1998). Effect of undernutrition on the distribution of progesterone in the uterus of ewes during the luteal phase of the estrous cycle. Theriogenology 49, 539-546.

Lozano, J.M., Lonergan, P., Boland, M.P., O'Callaghan, D. (2003). Influence of nutrition on the effectiveness of superovulation programmes in ewes: effect on oocyte quality and post-fertilization development. Reproduction 125, 543-553.

Lucy, M.C., Beck, J., Staples, C.R., Head, H.H., de la Sota, R.L., Thatcher, W.W. (1992). Follicular dynamics, plasma metabolites, hormones and insulin-like growth factor I (IGF-I) in lactating cows with positive or negative energy balance during the preovulatory period. Reprod. Nutr. Dev. 32, 331-341.

Luther, J.S., Redmer, D.A., Reynolds, L.P., Choi, J.T., Pant, D., Navanukraw, C., Arnold, D.R., Scheaffer, A.N., Borowicz, P., Kirsch, J.D., Weigl, R.M., Kraft, K.C., Grazul-Bilska, A.T. (2005). Ovarian follicular development and oocyte quality in anestrous ewes treated with melatonin, a controlled internal drug release (CIDR) device and follicle stimulating hormone. Theriogenology 63, 2136–2146.

Malpaux, B., Viguie, C., Skinner, D.C., Thiéry, J.C., Chemineau, P. (1997). Control of the circannual rhythm of reproduction by melatonin in the ewe. Brain Research Bulletin 44(4), 431-438.

Martal, J., Chene, N., Camous, S., Huynh, L., Lantier, F., Hermier, P., L'haridon, R., Charpigny, G., Charlier, M., Chaouat, G. (1997). Recent developments and potentialities for reducing embryo mortality in ruminants: the role of IFN-tau and other cytokines in early pregnancy. Reprod. Fertil. Dev. 9, 355-80.

Martí, E., Mara, L., Marti, J.I., Muino-Blanco, T., Cebrian-Perez, J.A. (2007). Seasonal variations in antioxidant enzyme activity in ram seminal plasma. Theriogenology 67, 1446-1454.

McEvoy, T.G., Robinson, J.J., Aitken, R.P., Robertson, I.S. (1998). Melatonin treatment of embryo donor and recipient ewes during anestrus affects their endocrine status, but not ovulation rate, embryo survival or pregnancy. Theriogenology 49, 943-955.

Mcneilly, A.S., Jonassen, J.A., Rhind, S.M. (1987). Reduced ovarian follicular development as a consequence of low body condition in ewes. Acta Endocrinol (Copenh) 115, 75-83.

McNeilly, A.S. (1988). Suckling and the control of gonadotropin secretion. In "The Physiology of Reproduction", p 2414. Eds. E. Knobil, J.D. Neill (Raven Press, New York).

Meikle, A., Tasende, C., Rodriguez, M., Garofalo, E.G. (1997). Effects of estradiol and progesterone on the reproductive tract and on uterine sex steroid receptors in female lambs. Theriogenology 48, 1105-1113.

Meikle, A., Tasende, C., Sosa, C., Garofalo, E.G. (2004). The role of sex steroid receptors in sheep female reproductive physiology. Reprod. Fertil. Dev. 16, 385-94.

Miller, B.G., Murphy, L., Stone, G.M. (1977). Hormone receptor levels and hormone, RNA and protein metabolism in the genital tract during the oestrous cycle of the ewe. Journal of Endocrinology 73, 91-98.

Mitchell, L.M., King, M.E., Aitken, R.P., Gebbie, F.E., Wallace, J.M. (1999). Ovulation, fertilization and lambing rates, and peripheral progesterone concentrations, in ewes inseminated at a natural oestrus during November or February. J Reprod. Fertil. 115(1), 133-140.

Mitchell, L.M., Dingwall, W.S., Mylneb, M.J.A., Hunton, J., Matthews, K., Gebbie, F.E., McCallum, G.J., McEvoy, T.G. (2002). Season affects characteristics of the pre-ovulatory LH surge and embryo viability in superovulated ewes. Anim. Reprod. Sci. 74, 163–174.

Moss, G.E., Adams, T.E., Niswender, G.D., Nett, T.M. (1980). Effects of parturition and suckling on concentrations of pituitary gonadotrophin, hypothalamic GnRh and pituitary responsiveness to GnRH in ewes. J. Anim. Sci. 50, 496-501.

Moss, G.E., Crowder, M.E., Nett, T.M. (1981). GnRH-receptor interaction. VI. Effect of progesterone and estradiol on hypophyseal receptors for GnRH and serum and hypophyseal concentrations of gonadotropins in ovariectomized ewes. Biol. Reprod. 25, 938-944.

Murray, M.K. (1995). Epithelial lining of the sheep ampulla oviduct undergoes pregnancy-associated morphological changes in secretory status and cell height. Biol. Reprod. 53, 653-663.

Nancarrow, C.D., Hill, J.L. (1995). Oviduct proteins in fertilization and early embryo development. J Reprod Fertil Suppl 49, 3-13.

Nett, T.M. 1987. Function of the hypothalamic-hypophyseal axis during the postpartum period in ewes and cows. J. Reprod. Fert. Suppl. 34, 201-220.

Niswender, G.D.; Suter, D.E.; Sawyer, H.R. (1981). Factors regulating receptors for LH on ovine luteal cells. J. Reprod. Fertil. Suppl. 30, 183-189.

Niswender, G.D., Nett, T.M. (1994) Corpus luteum and its control in infraprimate species. In "The Physiology of Reproduction", pp. 781- 816. Eds Knobil, E. y Neill, J.D. (Raven Press, New York).

Noël, B., Mandiki, S.M.N., Perrad, B., Bister, J.L., Paquay, R. (1999). Terminal follicular growth, ovulation rate and hormonal secretion after melatonin pre-treatment prior to FGA_PMSG synchronisation in Suffolk ewes at the onset of breeding season. Small Rumin. Res. 32, 269-277.

O´Callagan, D., Karsch, F.J., Boland, M.P., Roche, J.F. (1991). What photoperiodic signal is provided by a continuous melatonin implant?. Biol. Reprod. 45, 927-933.

O'Callaghan, D., Boland, M.P. (1999). Nutritional effects on ovulation, embryo development and the establishment of pregnancy in ruminants. Animal Science 68, 299-314.

O'Callaghan, D., Yaakub, H., Hyttel, P., Spicer, L.J., Boland, M.P. (2000). Effect of nutrition and superovulation on oocyte morphology, follicular fluid composition and systemic hormone concentrations in ewes. J. Reprod. Fertil. 118, 303-313.

Oussaid, B., Mariana, J.C., Poulin, N., Fontaine, J., Lonergan, P., Beckers, J.F., Cognié, Y. (1999). Reduction of the developmental competence of sheep oocytes by inhibition of LH pulses during the follicular phase with a GnRH antagonist. J. Reprod. Fertil. 117, 71-77.

Papadopoulos, S., Lonergan, P., Gath, V., Quinn, K.M., Evans, A.C.O., O'Callaghan, D., Boland, M.P. (2001). Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. Theriogenology 55(5), 1059-1069.

Parr, R.A., Davis, I.F., Fairclough, R.J., Miles, M.A. (1987). Overfeeding during early pregnancy reduces peripheral progesterone concentration and pregnancy rate in sheep. J. Reprod. Fertil. 80, 317-320.

Parr, R.A. (1992). Nutrition-progesterone interactions during early pregnancy in sheep. J. Fertil. Dev. 4, 297-300.

Pelletier, J., Thimonier, J. (1975). Interactions between ovarian steroids or progestagens and LH release. Ann. Biol. Anim. Biochim.Biophis. 15, 131-146.

Randel, R.D. (1990). Nutrition and postpartum rebreeding in cattle. J. Anim. Sci. 68:853-862.

Rhind, S.M., Martin, G.B., Mcmillen, S., Tsonis, C.G., Mcneilly, A.S. (1989a). Effect of level of food intake of ewes on the secretion of LH and FSH and on the pituitary response to gonadotrophin-releasing hormone in ovariectomized ewes. J. Endocrinol. 121, 325-30.

Rhind, S.M., Mckelvey, W.A.C., Mcmillen, S., Gunn, R.G., Elston, D.A. (1989b). Effect of restricted food-intake, before and or after mating, on the reproductive performance of greyface ewes. Animal Production 48, 149-155.

Rhind, S.M., Mcmillen, S., Wetherill, G.Z., Mckelvey, W.A.C., Gunn, R.G. (1989c). Effects of low levels of food intake before and/or after mating on gonadotrophin and progesterone profiles in Greyface ewes. Animal Production 49, 267-273.

Rhind, S.M. (1992). Nutrition: its effect on reproductive performance and its control in female sheep and goats. In "Progress in sheep and goat research", pp. 25-52. Ed. Speedy, A.W. (CAB International, Wallingford).

Robinson, J.E., Radford, H.M., Karsch, F.J. (1985). Seasonal changes in pulsatile luteinizing hormone (LH) secretion in the ewe: relationship of frequency of LH pulses to day length and response to estradiol negative feedback. Biol. Reprod. 33(2), 324-334.

Robinson, J.J., Wigzell, S., Aitken, R.P., Wallace, J.M., Ireland, S., Robertson, I.S. (1991) The modifying effects of melatonin, ram exposure and plane of nutrition on the onset of ovarian activity, ovulation rate and the endocrine status of ewes. Anim. Reprod. Sci. 26, 73-91.

Robinson, J.J. (1996). Nutrition and Reproduction. Anim. Reprod. Sci. 42(1-4), 25-34.

Roche, J.F., Boland, M.P., Mcgeady, T.A. (1981). Reproductive wastage following artificial insemination of heifers. Vet. Rec. 109, 401-404.

Rondon, Z., Forcada, F., Zarazaga, L., Abecia, J.A., Lozano, J.M. (1996). Oestrous activity, ovulation rate and plasma melatonin concentrations in Rasa Aragonesa ewes maintained at two different and constant body condition score levels and implanted or reimplanted with melatonin. Anim. Reprod. Sci. 41, 225-236.

SAS Institute Inc. (1999). SAS/STAT. Software Version 8. SAS Institute Inc., Cary, NC, USA.

Scaramuzzi, R.J., Campbell, B.K., Downing, J.A., Kendall, N.R., Khalid, M., Munoz-Gutierrez, M., Somchit, A. (2006). A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. Reprod. Nutr. Dev. 46, 339-54.

Schillo, K.K. (1992). Effects of dietary energy on control of luteinizing hormone secretion in cattle and sheep. J. Anim. Sci. 70, 1271-1282.

Senger, P.L. (1997). Pathways to Pregnancy and Parturition. (Ed. Current Conceptions, Inc., Pullman, Washington, USA).

Short, R.V. (1969) Implantation and the maternal recognition of pregnancy. In "Ciba Foundation Symposium on Foetal Autonomy", pp. 2-26. Eds Wolstenholm, G.E.W. y O'Connor, M.. (Churchill Livingston, London).

Short, R.E., Bellows, R.A., Staigmiller, R.B., Berardinelli, J.G. y Custer, E.E. (1990). Physiological mechanisms controlling anestrus and infertility in postpartum beef cattle. J. Anim. Sci. 68:799-816.

Sosa, C., Lozano, J.M., Viñoles, C., Acuña, S., Abecia, J.A., Forcada, F., Forsberg, M., Meikle, A. (2004). Effect of plane of nutrition on endometrial sex steroid receptor expression in ewes. Anim. Reprod. Sci. 84, 337-348.

Sosa, C., Abecia, J.A., Forcada, F., Viñoles, C., Tasende, C., Valares, J.A., Palacin, I., Martin, G.B., Meikle, A. (2006). Effect of undernutrition on uterine progesterone and oestrogen-receptors and on endocrine profiles during the ovine oestrous cycle. Reprod. Fertil. Dev. 18, 447–458.

Sosa, C., Abecia, J.A., Forcada, F., Meikle, A. (2008). Undernutrition reduces the oviductal mRNA expression of progesterone and oestrogen receptors in sheep. Vet. J. 175 (3), 413-415.

Spencer, T.E., Burghardt, R.C., Johnson, G.A., Bazer, F.W. (2004). Conceptus signals for establishment and maintenance of pregnancy. Anim. Reprod. Sci. 82-83, 537-50.

Stenbak, T.K., Redmer, D.A., Berginski, H.R., Erickson, A.S., Navanukraw, C., Toutges, M.J. (2001). Effects of follicle stimulating hormone (FSH) on follicular development, oocyte retrieval, and in vitro fertilization (IVF) in ewes during breeding season and seasonal anestrus. Theriogenology 56, 51–64.

Tasende, C., Meikle, A., Rodriguez-Piñon, M., Forsberg, M., Garófalo, E.G. (1996). Restoration of estrogen and progesterone uterine receptors during the ovine postpartum period. Theriogenology 45(8), 1545-1551.

Tervit, H.R., Whittingham, D.G. (1972). Successful culture in vitro of sheep and cattle ova. J. Reprod. Fert. 30 493-497.

Tervit, H.R., Smith, H.F. y Kaltenbach, C.C. (1977). Postpartum anestrus in beef cattle: a review. Proc. N. Zeal. Soc. Anim. Prod. 37:109-121.

Thatcher, W.W., Guzeloglu, A., Meikle, A., Kamimura, S., Bilby, T., Kowalski, A.A., Badinga, L., Pershing, R., Bartolome, J., Santos, J.E. (2003). Regulation of embryo survival in cattle. Reprod. Suppl. 61, 253-66.

Thomas, G.B., Mercer, J.E., Karalis, T., Rao, A., Cummins, J.T., Clarke, I.J. (1990). Effect of restricted feeding on the concentrations of growth hormone (GH),gonadotropins, and prolactin (PRL) in plasma, and on the amounts of messenger ribonucleic acid for GH, gonadotropin subunits, and PRL in the pituitary glands of adult ovariectomized ewes. Endocrinology 126, 1361-1367.

Tsai, M.J., O'Malley, B.W. (1994). Molecular mechanisms of actions of steroid/thyroid receptor superfamily members. Annual Review of Biochemistry 63, 451-486.

Valasi, F., Tsiligianni, T., Papanikolaou, T., Dimitriadis, I., Vainas, E., Samartzi, F., Amiridis, G.S. (2006). Melatonin improves the developmental competence of sheep oocytes. Reprod. Domest. Anim. 41(4), 341-341.

Viñoles, C., Forsberg, M., Banchero, G., Rubianes, E. (2002). Ovarian follicular dynamics and endocrine profiles in Polwarth ewes with high and low body condition. Animal Science 74, 539-545.

Walker, S.K., Hill, J.L., Kleemann, D.O., Nancarrow, C.D. (1996). Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. Biol Reprod 55, 703–708.

Wallace, J.M., Robinson, J.J., Wigzell, S., Aitken, R.P. (1988). The effect of melatonin on the peripheral concentrations of LH and progesterone postestrus and on conception rate in ewes. Anim Prod 46, 499.

Wallace, J.M., Robinson,J.J., McKelvey, W.A.C.,, Aitken, R.P. (1989). Studies on increasing breeding frequency in the ewe. II. The endocrine status of lactating ewes induced to ovulate 28, 35 or 42 days post-partum. Anim. Reprod. Sci. 18, 271-283.

Wani, N.A., Wani, G.M., Khan, M.Z., Sidiqi, M.A. (1999). Effect of different factors on the recovery rate of oocytes for in vitro maturation and in vitro fertilisation procedures in sheep. Small Rumin. Res. 34(1), 71-76.

Wani, N.A., Wani, G.M., Khan, M.Z., Salahudin, S. (2000). Effect of oocyte harvesting techniques on in vitro maturation and in vitro fertilization in sheep. Small Rumin. Res. 36(1), 63-67.

Wathes, D.C., Lamming, G.E. (1995). The oxytocin receptor, luteolysis and the maintenance of pregnancy. J Reprod Fertil Suppl 49, 53-67.

Watson, A.J., Westhusin, M.E., Winger, Q.A. (1999). IGF paracrine and autocrine interactions between conceptus and oviduct. J. Reprod. Fertil. Suppl. 54, 303-15.

Whiterman, J.V., Zollinger, W.A., Thrift, F.A., Gould, M.B. (1972). Post-partum mating performance of ewes involved in a twice yearly lambing program. J. Anim. Sci. 35, 836-842.

Williams, G.L. (1990). Suckling as a regulator of postpartum rebreeding in cattle: a review. J. Anim. Sci. 68:831-852.

Wintenberger-Torres, S., Flechon, J.E. (1974). Ultrastructural evolution of the trophoblast cells of the pre-implantation sheep blastocyst from day 8 to day 18. J. Anat. 118, 143-53.

Winterberger-Torres, S., Sevellec, C. (1987). Atlas of the early development of the sheep embryo. INRA, Paris, France.

Wise, M.E., Glass, J.D., Nett, T.M. (1986). Changes in the concentrations of hypothalamic and hypophyseal receptors for estradiol in pregnant and postpartum ewes. J. Anim. Sci. 62, 1021-1028.

Yie, S.M., Brown, G.M., Liu, G.Y., Collins, J. A., Daya, S., Hughes, E.G., Foster, W.G., Younglai, E.V. (1995). Melatonin and steroids in human pre-ovulatory follicular fluid: seasonal variations and granulosa cell steroid production. Hum. Reprod. 10(1), 50-55.

Zhao, H., Poon, A.M., Pang, S.F. (2000). Pharmacological characterization, molecular subtyping, and autoradiographic localization of putative melatonin receptorsin uterine endometrium of estrous rats. Life Sciences 66, 1581-1591.

Zúñiga, O., Abecia, J.A., Forcada, F., Valares, J.A. (2001) Efecto de la melatonina y la condición corporal sobre la respuesta al efecto macho de ovejas de raza Rasa Aragonesa. ITEA Vol Extra nº 22 (II), 733-735.

Zuñiga, O., Forcada, F., Abecia, J.A. (2002). The effect of melatonin implants on the response to the male effect and on the subsequent cyclicity of Rasa Aragonesa ewes implanted in April. Anim. Reprod. Sci. 72(3-4), 165-174.

APÉNDICE

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Effects of melatonin and undernutrition on the viability of ovine embryos during anestrus and the breeding season

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Abstract

This study examined the effects of melatonin and level of nutrition on embryo yield during anestrous and breeding season. Adult Rasa Aragonesa ewes were assigned randomly to one of the four treatment groups in two experiments using a $2 \times 2 \times 2$ factorial design. Individuals were treated (+MEL) or not treated (−MEL) with a subcutaneous implant of melatonin for 42 d (Melovine®, CEVA) and fed 1.5 (control, C) or 0.5 (low, L) times the daily maintenance requirements for 20 d. Ewes were mated at oestrus (Day = 0) and embryos were recovered on Day 5. Level of nutrition and melatonin supplements did not have a significant effect on ovulation rate or the number of recovered ova per ewe in the Reproductive Season (RS) and the Anestrous Season (AS). During the RS, undernutrition reduced the number of viable embryos per ewe (C: 1.1 ± 0.2; L: 0.6 ± 0.2; $P < 0.05$); however, the number of viable embryos per ewe in the L+MEL group (0.2 ± 0.15) was significantly lower than it was in the L, C+MEL and C groups (0.9 ± 0.3, 1.2 ± 0.3, 1.0 ± 0.4, respectively; $P < 0.05$). In the AS, nutrition did not have a significant effect on the number of viable embryos per ewe, although melatonin supplements might have improved rates slightly. Embryo viability rate (% viable embryos/embryos recovered) was unaffected by melatonin supplements or level of nutrition in the RS and the AS. Season had a strong effect on the number of viable embryos per functional corpus luteum among ewes in the L+MEL group, only (RS: 0.2 ± 0.1; AS: 0.6 ± 0.2; $P < 0.05$). In conclusion, undernutrition impaired the viability of sheep embryos in the RS, particularly among ewes that were given melatonin supplements subcutaneously, but melatonin appeared to improve embryo quality in the AS, which suggests

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that the mechanisms involved in the interactive effects of melatonin and nutrition on embryo development are influenced by season.

Keywords: Sheep; Embryo; Melatonin; Nutrition; Anestrus; Breeding season

1. Introduction

Embryo viability in sheep can be affected by several factors, such as nutrition, seasonality, melatonin treatment, among others. It is well documented that undernutrition affects reproductive function at multiple levels of the hypothalamus–pituitary–gonadal axis in ruminants (for a review, see Robinson, 1996; Boland et al., 2001; Forcada and Abecia, 2006b). In sheep, subnutrition increases embryo mortality and reduces pregnancy rates (Rhind et al., 1989; Abecia et al., 1995, 1999; Lozano et al., 2003) because of an inadequate oocyte quality and embryo development (for a review, see Abecia et al., 2006). Low dietary intake alters oocyte morphology in cattle (Yaakub et al., 1999) and reduces the number of high quality oocytes and embryos in superovulated ewes (Lozano et al., 2003).

On the other hand, season can also exert a significant effect on embryo viability of sheep. Mitchell et al. (2002) observed that some of the characteristics of the embryos, such as the number of fertilized ova, the number of embryos with <16 cells, and embryo quality were higher in the reproductive season than it was in the anestrous season. Season did not influence the number of embryos recovered after natural estrus (Mitchell et al., 1999) or superovulatory treatment (López-Sebastián et al., 1990; González-Bulnes et al., 2003).

Seasonality in sheep is mediated by photoperiod, which is conveyed to the reproductive-neuroendocrine axis by melatonin (Bittman et al., 1983). Melatonin is released at night and acts in the mediobasal hypothalamus to modulate the pulsatile secretion of GnRH (Karsch et al., 1984; Robinson et al., 1985). Subcutaneous implants of melatonin are used widely to advance the breeding season and to improve reproductive performance during anestrus both in highly seasonal (Haresign et al., 1990) and in Mediterranean ewes (Chemineau et al., 1996; Zuñiga et al., 2002). Subcutaneous implants cause a short-day length-like response without suppressing endogenous secretion (O'Callagan et al., 1991; Malpaux et al., 1997). Melatonin treatment is an effective method for inducing estrous cycles, increasing ovulation and lambing rates during anestrus (Haresign et al., 1990; Robinson et al., 1991; Haresign, 1992; Bister et al., 1999; Luther et al., 2005). In general, melatonin treatment increases fertility and prolificacy in ewes (Forcada et al., 1995; Chemineau et al., 1996; Zuñiga et al., 2002; Abecia et al., 2007). Melatonin treatments can improve the viability of embryos in ewes after superovulation in the anestrous period (Forcada et al., 2006a); however, McEvoy et al. (1998) did not observe significant differences in the number and quality of embryos from superovulated melatonin-treated and untreated donor ewes in the AS. For the reasons above mentioned, melatonin appears to be beneficial to embryo survival (Durotoye et al., 1997; Abecia et al., 2002; Forcada et al., 2006a); thus we hypothesize that melatonin can override the detrimental effects of undernutrition on the same parameter. Furthermore, the effects of undernutrition and melatonin treatments on oocyte and embryo quality might vary seasonally because the endogenous secretion of melatonin and the voluntary intake vary significantly throughout the year (Chilliard et al., 1998). In this study, we investigated the effects of melatonin and level of nutrition on embryo viability in the reproductive and anestrous seasons.

2. Materials and methods

2.1. Animals

The experiment was conducted using 42 adult, cycling, non-pregnant Rasa Aragonesa ewes at the Experimental Farm of the University of Zaragoza, Spain (41°41′N) and in compliance with the requirements of the European Union for Scientific Procedure Establishments and the Ethics Committee of the University of Zaragoza, Spain.

2.2. Reproductive season (RS)

In the RS, at the beginning of the experiment, the 42 adults, cycling, and non-pregnant Rasa Aragonesa ewes had a mean (±S.E.M.) live weight (LW) of 64.1 ± 1.7 kg and a mean body condition (BC) score (Russell et al., 1969) of 3.1 ± 0.1. To confirm ovarian cyclicity, at 7 and 14 d before the application of the melatonin implants, blood samples were collected and plasma progesterone concentrations were measured. All of the ewes had a progesterone concentration >1 ng/ml in at least one of the samples. On 8 December, ewes were randomly assigned to either a group that would receive a subcutaneous melatonin implant (18 mg melatonin, Melovine®, CEVA Salud Animal, Barcelona, Spain) at the base of the ear ($n = 21$) or to a group that would not receive an implant ($n = 21$). Although date of implantation could be considered late in the breeding season, it should be noted that Forcada et al. (2002) have previously reported the efficacy of melatonin implants on reproductive parameters of Rasa Aragonesa ewes implanted as late as in mid January. Therefore, the photorefractoriness to short days in this Mediterranean breed would appear not earlier than in mid February.

In mid-January, 42 d after the addition of the melatonin implants, individuals in the two groups were synchronized using a 14-d treatment with intravaginal progestagen pessaries (30 mg Fluorogestone Acetate; Sincropart®, Ceva Salud Animal S.A., Barcelona, Spain). When pessaries were removed, the ewes were injected i.v. with 400 IU of equine chorionic gonadotrophin (eCG) (Sincropart® PMSG, Ceva Salud Animal S.A.). Fig. 1 summarizes the experimental design.

2.3. Anestrus (AS)

At 7 and 14 d before the start of the experiment, blood samples were collected from 42 ewes and their plasma progesterone concentrations were measured. To create a group of anestrous ewes, those that had progesterone levels >1 ng/ml in at least one of their samples were considered cyclic, and were excluded from the study. Consequently, in the AS, the experiment included 31 adults, non-cycling, non-pregnant Rasa Aragonesa ewes that had a mean (±S.E.M.) LW of 57.2 ± 1.2 kg and a BC of 2.9 ± 0.04. On 26 March, 16 of those ewes received a subcutaneous implant of melatonin (18 mg melatonin, Melovine®, CEVA Salud Animal, Barcelona, Spain) at the base of the ear and 15 ewes were not treated and served as a control group. On 6 May, 42 d after the melatonin implants were inserted, the ewes were synchronized using a 14-d treatment with intravaginal progestagen pessaries (30 mg Fluorogestone Acetate; Sincropart®, Ceva Salud Animal S.A., Barcelona, Spain). Ewes were injected i.v. with 480 IU eCG (Sincropart® PMSG, Ceva Salud Animal S.A.) at pessary removal.

In both of the experiments (Fig. 1), after the insertion of the pessaries and until slaughter (Day 5 post-estrus), the ewes were offered diets that differed in the level of nutrition they provided. The control (C) and low (L) groups were fed diets that provided 1.5 and 0.5 times the daily maintenance

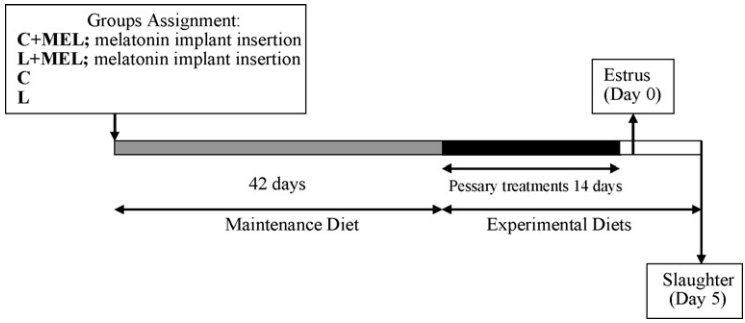

Fig. 1. Experimental design for both experiments.

requirements, respectively (Agricultural and Food Research Council, 1993), and had unlimited access to water. The 1.5 M diet ensures the maintenance of LW and BC, whereas the 0.5 M diet leads to a 12% reduction in LW and BC after 20 d (Abecia et al., 1995, 1997; Lozano et al., 1998; Sosa et al., 2004; Sosa et al., 2006).

Thus, four groups were considered: (1) ewes that were offered the C diet and did not receive a melatonin implant (C), (2) ewes that were offered the C diet and received a melatonin (C+MEL), (3) ewes that were offered the L diet and did not receive a melatonin implant (L), and (4) ewes that were offered the L diet and received a melatonin implant (L+MEL). Ewes were randomly assigned to each group in both seasons. These groups were replicated in each season.

Ewe fed the C diet received 0.60 kg of pellets and 1 kg of barley straw per day, which provided 12.4 MJ of metabolizable energy (ME) and 9.3% crude protein (CP). The L diet comprised 0.20 kg of pellets and 0.35 kg of barley straw per ewe per day (4.1 MJ ME and 9.1% CP). The pelleted diet consisted of barley (73%), soybean (22%), and a mineral supplement (5%). Live weight and BC were measured at the time of pessary insertion, pessary withdrawal, and at slaughter.

Every 8 h from 24 h after pessary withdrawal, estrus (Day 0) was monitored using intact rams wearing harnesses with marking crayons. To quantify plasma progesterone concentrations, at Days 0, 3, and 5 (slaughter), jugular blood samples were collected in evacuated heparins tubes. To test the effectiveness of the implants, at 45 d after implantation one daytime blood sample was collected from the melatonin-implanted ewes. The samples were centrifuged within 15 min (1000 g for10 min) and the plasma was stored at −20 °C.

On Day 5, 20 d after the start of the experimental diets, embryos were collected by mid-ventral laparotomy. Ewes were anesthetized using an i.m. injection of 0.4 ml 2% xylazine (Xilagesic 2%®, Calier, Barcelona, Spain), and 10 ml of sodium thiopental (20 mg/ml) (Thiobarbital Braun Medical, Jaén, Spain) administered by i.v. injection 5 min later. The ovarian response was quantified using the number of corpora lutea that were morphologically sound and congruent with an active luteal phase. Uterine horns were exposed and flushed using a Foley catheter and pre-warmed (36 °C) phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) and antibiotics (penicillin and streptomycin). Ewes were euthanized using an i.v. injection of sodium thiopental (T-61®; Intervet, Salamanca Spain). Ova and embryos were examined under a stereomicroscope (20–40× magnification) and classified based on their stage of development and morphology (Winterberger-Torres and Sevellec, 1987). Morulae and compacted morulae were considered viable embryos, according to the day of pregnancy on which the embryos were recovered. The in vitro viability of embryos was assessed based on the proportion of embryos that were capable of developing into expanded or hatched blastocysts after

48 h of culture at 39 °C with 5% CO_2. The culture medium consisted of synthetic oviductal fluid (SOF) without glucose, BME essential amino acids, MEM non-essential amino acids (Walker et al., 1996), 10% (v/v) of foetal bovine serum, 1 mM of L-glutamine, 100 UI/ml of penicillin-G, and 100 μg/ml of streptomycin sulphate.

For each ewe, the following variables were recorded: number of corpora lutea, number of recovered ova (oocytes + embryos), recovery rate (number of ova recovered/number of corpora lutea), number of fertilized embryos, number of viable embryos (as compacted morulae), and number of viable embryos after 48 h in culture (as expanded and hatched blastocysts). Fertilization rate was the number of embryos divided by the number of ova recovered. Viability rate was the number of viable embryos divided by the number of ova recovered. Ewes that did not provide recovered ova were excluded from the calculations of fertilization and viability rates.

2.4. Hormone assays

Plasma melatonin concentrations were measured by a direct, solid-phase radioimmunoassay (RIA) using a commercially available kit (Bühlmann RK-MDI; Bühlmann Lab; Switzerland), within a single assay. The sensitivity of the assay was 1.3 pg/mL and the intra-assay CV was 10.9% for low control concentrations (3.3 pg/mL) and 4.9% for high control concentrations (20.6 pg/mL).

Plasma progesterone concentrations were measured by a direct, solid-phase RIA using commercially available kits (Count-A-Count TKPG; DPC) (Meikle et al., 1997). The RIA had a sensitivity of 0.02 ng/mL. The intra-assay CV was 14% for low control concentrations (3 ng/mL), 8.5% for medium concentrations (15 ng/mL), and 7.5% for high concentrations (30 ng/mL). The interassay CV was <15% for all of the standard concentrations.

2.5. Statistical analyses

The experimental was based on a $2 \times 2 \times 2$ factorial design, with nutritional level, melatonin treatment, and season as fixed effects. The effects of the treatments on the development and quality of oocytes and embryos were evaluated using the PROC GEN MOD (Statistical Analysis System; SAS Institute, Cary, NC, USA) with the Poisson distribution specified in a model that included Season (reproductive or anestrus), Nutrition Level (low or control), and Melatonin Treatment (with or without melatonin implant) and their interactions. The values expressed as percentages were arcsine-transformed before being subjected to statistical analyses. The probability level for statistical significance was set to $P < 0.05$ and the results are expressed as mean ± S.E.M.

3. Results

3.1. Live weight and body condition

In the RS, the LW of the ewes in the L and L+MEL groups dropped significantly (from 61.8 ± 3.5 to 54.3 ± 3.2 kg and 64.1 ± 2.8 to 54.8 ± 2.5 kg, respectively; $P < 0.001$), but not those in the C and C+MEL groups (from 63.8 ± 3.7 to 64.6 ± 3.6 kg and 64.7 ± 3.7 to 64.4 ± 3.5 kg, respectively). Ewes in the L and L+MEL groups experienced a significant reduction in BC (from 2.8 ± 0.1 to 2.5 ± 0.1 and from 3.0 ± 0.1 to 2.5 ± 0.1, respectively; $P < 0.001$), but ewes in the C and C+MEL groups did not (2.9 ± 0.1 to 3.0 ± 0.1 and 2.9 ± 0.2 to 2.8 ± 0.1, respectively).

In the AS, ewes in the C and C+MEL groups maintained their LW (from 62.5 ± 2.4 to 63.5 ± 2.2 kg and 54.6 ± 0.9 to 53.3 ± 0.9 kg, respectively) and BC (from 3.0 ± 0.6 to 3.0 ± 0.7

and from 2.9 ± 0.1 to 2.9 ± 0.1, respectively); but those in the L and L+MEL groups experienced a significant reduction in LW (from 54.6 ± 3.2 to 52.8 ± 3.2 kg and 56.6 ± 2.2 to 51.8 ± 1.9 kg, respectively; $P < 0.001$) and BC (from 2.9 ± 0.1 to 3.0 ± 0.1 and from 2.75 ± 0.06 to 2.8 ± 0.1, respectively). In the RS and AS, after pessary withdrawal (14 d after the onset of nutritional treatments), the LW and BC of ewes in the L group were significantly lower than were those of the control ewes ($P < 0.01$).

3.2. Circulating hormones

In both seasons, when measured during daylight on Day 45, the concentrations of plasma melatonin in all of the ewes that received a melatonin implant were high, which confirmed that the implants functioned properly (mean ± S.E.M.): 53.6 ± 4.2 and 62.6 ± 4.9 pg/ml in January and May, respectively.

In both seasons and all of the groups, progesterone concentrations increased gradually after estrus. In the RS, nutrition level and supplemental melatonin did not have a significant effect on plasma progesterone concentrations at estrus, at Day 3, and at slaughter (Day 5). Plasma progesterone concentrations on Day 5 were 3.7 ± 0.5 ng/mL (C), 3.8 ± 0.5 ng/mL (C+MEL), 4.7 ± 0.7 ng/mL (L), and 4.0 ± 0.8 ng/mL (L+MEL). In the AS, the interaction effect of melatonin × nutrition on plasma progesterone concentrations at estrus presented a trend to significance (C: 0.30 ± 0.09 ng/mL, C+MEL: 0.11 ± 0.09 ng/mL, L: 0.19 ± 0.11 ng/mL, L+MEL: 0.35 ± 0.11 ng/mL; $P < 0.09$), but nutrition or melatonin treatments had no significant effects on plasma concentrations on Day 3 and at slaughter (Day 5). On Day 5, mean plasma progesterone concentrations were 3.6 ± 0.5 ng/mL (C), 3.8 ± 0.5 ng/mL (C+MEL), 2.6 ± 0.6 ng/mL (L), and 3.4 ± 0.6 ng/mL (L+MEL).

3.3. Ovulation rate

Ovulation rates did not differ among groups or between seasons (Tables 1 and 2).

3.4. Embryo recovery

3.4.1. Reproductive season

Nutrition level had a significant effect on the number of viable embryos (1.1 ± 0.2 vs. 0.6 ± 0.2 in C and L ewes, respectively; $P < 0.05$), and undernutrition reduced the number of viable embryos in ewes treated with melatonin ($P < 0.05$) (Table 1). Melatonin treatment had a detrimental effect on fertilization rate ($P < 0.05$) and, consequently, the numbers of fertilized and viable embryos were consistently lower in the undernourished, melatonin-implanted group ewes than they were in the other groups. Ewes in the L+MEL group exhibited the lowest in vitro viability (40.0%), and differed significantly from the ewes in the C and C+MEL groups (77.8% and 80.0%, respectively; $P < 0.05$) and, to a lesser extent, from the ewes in the L group (66.7%; $P = 0.07$).

3.4.2. Anestrous season

Only one ewe in the L+MEL group did not exhibit estrous behaviour after pessary withdrawal. Two of the ewes in group L ($n = 5$) and two in group L+MEL ($n = 6$) were excluded from the analysis of the AS because they did not exhibit normal corpora lutea at slaughter. On Day 5 after estrus, the groups did not differ significantly in the number of fertilized and viable embryos,

Table 1
Ovarian response and embryo production in the reproductive season by Rasa Aragonesa ewes fed either 1.5 (C) or 0.5 (L) times the maintenance requirements and treated (MEL) or not treated with melatonin 62 d before embryo recovery

	GROUP			
	C	C+MEL	L	L+MEL
No. of ewes	10	11	11	10
No. of ewes in estrus	8/10	9/11	9/11	9/10
Ovulation rate	1.9 ± 0.4	2.2 ± 0.3	2.2 ± 0.4	1.8 ± 0.4
No. of recovered ova	1.1 ± 0.3	1.7 ± 0.3	1.1 ± 0.2	0.9 ± 0.4
Recovery rate (%)	60	75	50	50
No. of fertilized embryos	1.1 ± 0.3^{c}	1.3 ± 0.3^{c}	1.1 ± 0.3^{c}	0.5 ± 0.3^{d}
Fertilization rate (%)	100^{a}	87.5 ± 9.5^{b}	100^{a}	70 ± 12^{b}
No. of viable embryos	1 ± 0.3^{a}	1.2 ± 0.3^{a}	0.9 ± 0.3^{a}	0.2 ± 0.3^{b}
Viability rate (%)	83.3 ± 17^{c}	81.3 ± 15.6^{c}	75 ± 15.6^{c}	40 ± 19.8^{d}
Pregnancy rate (%)[a]	75 (6/8)	77.7 (7/9)	88.8 (8/9)	44.4 (4/9)

Different superscript letters (a, b) in the same row indicate significant differences ($P < 0.05$). Different superscript letters (c, d) in the same row indicate differences of $P < 0.1$. Ova = oocytes + embryos.
[a] Percentage of ewes with viable embryos on Day 5.

which indicates that undernutrition and supplemental melatonin did not have a significant effect on these parameters in the AS (Table 2). Supplemental melatonin and undernutrition did not have a significant effect on the in vitro viability of recovered embryos in the AS; thus, the percentages of embryos that were capable of developing into expanded or hatched blastocysts were 87.5% (C), 83.3% (C+MEL and L), and 88.9% (L+MEL).

The interaction between season × melatonin treatments had a significant effect on fertilization rate ($P < 0.05$), and tended to have an effect on embryo viability ($P < 0.08$). In general, unlike in the RS, melatonin had a positive effect on fertilization rate and embryo viability in the AS (Table 2). In the RS, the effect of this interaction was particularly pronounced among the undernourished ewes, in which supplemental melatonin significantly ($P < 0.05$) impaired the number of viable

Table 2
Ovarian response and embryo production in the anestrous season by Rasa Aragonesa ewes fed either 1.5 (C) or 0.5 (L) times the maintenance requirements and treated (MEL) or not treated with melatonin 62 d before embryo recovery

	GROUP			
	C	C+MEL	L	L+MEL
No. of ewes	8	7	5	6
No. of ewes in estrus	8/8	7/7	5/5	5/6
Ovulation rate	2.4 ± 0.4	2.1 ± 0.3	2.0 ± 0.6	1.8 ± 0.2
No. of recovered ova	1.0 ± 0.3	1.6 ± 0.3	1.2 ± 0.4	1.4 ± 0.4
Recovery rate (%)	42^{a}	73.3^{b}	60^{b}	78^{b}
No. of fertilized embryos	0.6 ± 0.3^{c}	1.3 ± 0.3^{d}	0.8 ± 0.4^{c}	1.2 ± 0.4^{d}
Fertilization rate (%)	66.7 ± 18	83.3 ± 18	75 ± 22	87.5 ± 22
No. of viable embryos	0.6 ± 0.3^{c}	1.1 ± 0.3^{d}	0.8 ± 0.4^{c}	1.2 ± 0.4^{d}
Viability rate (%)	66.7 ± 18.1	75 ± 18.1	75 ± 22.2	87.5 ± 22.2
Pregnancy rate (%)[a]	50 (4/8)	71.4 (5/7)	60 (3/5)	80 (4/5)

Different superscript letters (a, b) in the same row indicate significant differences ($P < 0.05$). Different superscripts letters (c, d) in the same row indicate differences of $P < 0.1$. Ova = oocytes + embryos.
[a] Percentage of ewes with viable embryos on Day 5.

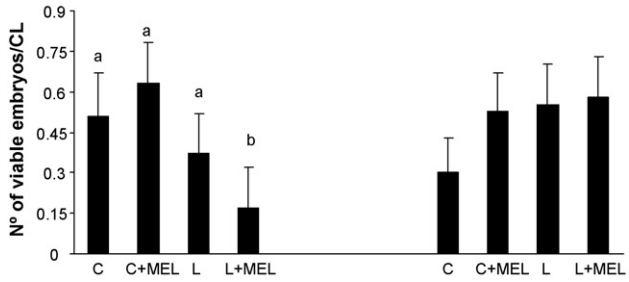

Fig. 2. Number of viable embryos per corpus luteum (CL) in the reproductive season (left panel) and the anestrous season (right panel) in Rasa Aragonesa ewes fed either 1.5 (C) or 0.5 (L) times the LW maintenance requirements and treated (MEL) or not treated with melatonin 62 d before embryo recovery. Mean ± S.E.M. Within the same panel, bars that have different superscripts are significantly different ($P \leq 0.05$).

embryos, whereas, in the AS, embryo viability tended ($P < 0.1$) to improve. The mean number of viable embryos per functional corpus luteum in each group is shown in Fig. 2.

4. Discussion

This study investigated the effects of exogenous melatonin and level of nutrition on embryo viability in Rasa Aragonesa ewes in the anestrous and the reproductive seasons in northern Spain. We hypothesized that the known beneficial effects of exogenous melatonin on embryo survival would override the negative effects of undernutrition at any time of year; however, in this study, the effects of melatonin implants on embryo development differed between the reproductive or anestrous seasons.

In our experiments, nutritional restrictions reduced significantly the mean live weights and body condition of ewes. Although the rate of live weight decrease of undernourished ewes were not similar in both seasons, the effectiveness of the nutritional treatments can be assumed because of the significant differences observed between C and L live weight at slaughter time ($P < 0.01$). Several factors could explain this situation, such as nutritional status of the ewes before the onset of the experiment, metabolic and hormonal factors interacting with season, nutrition and reproduction, environment factors and others. In addition, Clarke (2001) observed a seasonal regulation of food intake in sheep, observing an increment of voluntary food intake under long-day conditions, which in turn, could provoke live weight differences. Our previous studies (Abecia et al., 1997, 1999; Sosa et al., 2006) demonstrated that mature ewes subjected to a degree of undernutrition similar to that of the present experiment for 3–4 weeks exhibited a significant reduction in LW and BC. In addition, underfed ewes exhibited an increase in lipolytic activity (Sosa et al., 2006). However, such short-term undernutrition associated with a progestagen-synchronized estrus does not appear to impair ovulation rate in the reproductive season (Abecia et al., 1997, 1999; Borowczyk et al., 2006) or the anestrous season (Sosa et al., 2006).

It was unexpected that undernutrition impaired embryo viability on Day 5 after the onset of estrus in melatonin-implanted ewes in the reproductive season, but not in the anestrous season, when the melatonin treatment seemed to improve embryo fertilization and viability. Season did not have a significant effect on the number of viable embryos or viability rate. Regardless of the level of nutrition, seasonal anestrus seemed to reduce the fertilization rate and viability rate of the ewes that did not receive melatonin implants. Seasonal differences might be due in part to differences in semen quality; however, low seasonal variations in the volume and quality of

the ejaculates from Rasa Aragonesa rams (Martí et al., 2007) suggest that the higher fertilization rate in the reproductive season might be mainly due to higher oocyte quality at the time of ovulation. Mitchell et al. (2002) reported a higher proportion of unfertilized or degenerated ova in the anestrous period following superovulatory treatments and artificial insemination, and a larger number of small follicles were induced to ovulate in April than in October, and most of them contained immature oocytes at the time of ovulation. In vitro fertilization rates of oocytes recovered from superovulating ewes can be lower in the anestrous season than in the reproductive season (Stenbak et al., 2001). The presence of a corpus luteum before gonadotrophin treatment is beneficial to embryo development (Gonzalez-Bulnes et al., 2002; González-Bulnes et al., 2003), which might be due to the inability of progestagen protocols to suppress LH to the level achieved in the luteal phase (Kojima et al., 1992), thereby inducing inadequate follicular development that can lead to abnormalities in fertilization and embryo development. In our experiment, all of the ewes that were induced to ovulate in anestrus were non-cyclic at the time of sponge insertion.

In our study, four undernourished ewes failed to exhibit functional corpora lutea after ovulation in the anestrous season. Undernutrition is associated with a low uterine expression of progesterone receptors on Day 5 after estrus in ewes (Sosa et al., 2004; Sosa et al., 2006). In addition, progesterone can modulate the endometrial $PGF_{2\alpha}$ secretion by down-regulating the concentration of oxytocin receptor and delaying the time of luteolysis (McCracken et al., 1999). Given that undernutrition during early embryo development in sheep increases uterine in vitro production of $PGF_{2\alpha}$ after an induced estrus (Abecia et al., 1999) or following superovulation (Lozano et al., 2003), undernutrition might lead to a poor uterine environment that compromises the development of embryos. Furthermore, undernutrition can influence earlier embryo development, by reducing oviductal sensitivity to ovarian steroids (Sosa et al., 2008) or by altering oocyte quality (Borowczyk et al., 2006).

In the present experiments, melatonin implants had a significant detrimental effect on fertilization rate in the reproductive season, but not in the anestrous season. Elsewhere, studies have shown that supplemental melatonin in anestrus does not improve fertilization rate in terms of embryos recovered from superovulated ewes (McEvoy et al., 1998; Forcada et al., 2006a) or after IVF of oocytes recovered from superovulatory treatment (Luther et al., 2005). Although McEvoy et al. (1998) found that melatonin implants did not have a significant effect on embryo production and survival after superovulation during anestrus. Our recent studies have shown that supplemental melatonin can reduce significantly the number and rate of non-viable (degenerated and retarded) embryos in superovulated ewes during anestrus (Forcada et al., 2006a). The luteotrophic effect of the pineal hormone observed in vivo or in vitro (Durotoye et al., 1997; Abecia et al., 2002) and the effects of melatonin at the hypothalamic–hypophyseal level (Malpaux et al., 1997) might be involved in the melatonin-induced improvement in embryo viability during anestrus. In our study, it is unclear why melatonin implants had a detrimental effect on fertilization and viability rates in Rasa Aragonesa ewes in the reproductive season. In humans and rats, granulosa cells express specific melatonin binding sites (Yie et al., 1995; Clemmens et al., 2001). In addition, in rats, chronic exposure to estrogen can down-regulate melatonin receptors in the ovaries and, therefore, reduce their expression (Clemmens et al., 2001). Thus, it is possible that over exposure to melatonin during the reproductive season in sheep might be detrimental at the ovarian level and associated with high concentrations of estradiol, particularly around the estrus.

In conclusion, undernutrition impaired the viability of embryos in the RS, particularly in the ewes that were given melatonin implants. In the AS, supplemental melatonin appeared to improve embryo quality. Those results suggest that the mechanisms underlying the interaction between

exogenous melatonin and level of nutrition on embryo development are seasonally regulated, but the nutrition–melatonin interactions and their mechanisms require further study.

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References

Abecia, J.A., Rhind, S.M., Bramley, T.A., McMillen, S.R., 1995. Steroid production and LH receptor concentrations of ovarian follicles and corpora lutea and associated rates of ova wastage in ewes given high and low levels of food intake before and after mating. Anim. Sci. 61, 57–62.

Abecia, J.A., Lozano, J.M., Forcada, F., Zarazaga, L., 1997. Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Anim. Reprod. Sci. 48, 209–218.

Abecia, J.A., Forcada, F., Lozano, J.M., 1999. A preliminary report on the effect of dietary energy on prostaglandin F2α production in vitro, interferon-tau synthesis by the conceptus, endometrial progesterone concentration on days 9 and 15 of pregnancy and associated rates of embryo wastage in ewe. Theriogenology 52 (7), 1203–1213.

Abecia, J.A., Forcada, F., Zuñiga, O., 2002. The effect of melatonin on the secretion of progesterone in sheep and on the development of ovine embryos in vitro. Vet. Res. Commun. 26, 151–158.

Abecia, J.A., Sosa, C., Forcada, F., Meikle, A., 2006. The effect of undernutrition on the establishment of pregnancy in the ewe. Reprod. Nutr. Dev. 46, 367–378.

Abecia, J.A., Valares, J.A., Forcada, F., Palacin, I., Martin, S., Martino, A., 2007. The effect of melatonin on the reproductive performance of three sheep breeds in Spain. Small Ruminant Res. 69, 10–16.

Agricultural and Food Research Council, 1993. Energy and protein requirements of ruminants. An Advisor Manual Prepared by the AFRC Technical Committee on Responses to Nutrients. CAB International, Wallingford, United Kingdom.

Bister, J.L., Noel, B., Perrad, B., Mandiki, S.N.M., Mbayahaga, J., Paquay, R., 1999. Control of ovarian follicles activity in the ewe. Domest. Anim. Endocrin. 17, 315–328.

Bittman, E.L., Dempsey, R.J., Karsch, F.J., 1983. Pineal melatonin secretion drives the reproductive response to day length in the ewe. Endocrinology 113, 2276–2283.

Boland, M.P., Lonergan, P., O'Callaghan, D., 2001. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology 55, 1323–1340.

Borowczyk, E., Caton, J.S., Redmer, D.A., Bilski, J.J., Weigl, R.M., Vonnahme, K.A., Borowicz, P.P., Kirsch, J.D., Kraft, K.C., Reynolds, L.P., Grazul-Bilska, A.T., 2006. Effects of plane of nutrition on in vitro fertilization and early embryonic development in sheep. J. Anim. Sci. 84 (6), 1593–1599.

Clarke, I.J., 2001. Sex and season are major determinants of voluntary food intake in sheep. Reprod. Fertil. Dev. 13, 577–582.

Clemmens, J.W., Jarzunka, M.J., Witt-Enderby, P.A., 2001. Down-regulation of mt1 melatonin receptors in rat ovary following estrogen exposure. Life Sci. 69, 27–35.

Chemineau, P., Malpaux, B., Pelletier, J., Leboeuf, B., Delgadillo, J.A., Deletang, F., Pobel, T., Brice, G., 1996. Emploi des implants de mélatonine et des traitements photopériodiques pour maîtriser la reproduction saissonière chez les ovins et les caprins (use of melatonin implants and photoperiodic treatments to control seasonal reproduction on sheep and goats). INRA Prod. Anim. 9, 45–60.

Chilliard, Y., Bocquier, F., Doreau, M., 1998. Digestive and metabolic adaptations of ruminants to undernutrition, and consequences on reproduction. Reprod. Nutr. Dev. 38, 131–152.

Durotoye, L.A., Webley, G.E., Rodway, R.G., 1997. Stimulation of the production of progesterone by the corpus luteum of the ewe by the perfusion of melatonin in vivo and by treatment of granulosa cells with melatonin in vitro. Res. Vet. Sci. 62, 87–91.

Forcada, F., Zarazaga, L., Abecia, J.A., 1995. Effect of exogenous melatonin and plane of nutrition after weaning on estrous activity, endocrine status and ovulation rate in Salz ewes lambing in the seasonal anestrus. Theriogenology 43 (7), 1179–1193.

Forcada, F., Abecia, J.A., Zúñiga, O., Lozano, J.M., 2002. Variation in the ability of melatonin implants inserted at two different times after the winter solstice to restores reproductiveactivity in reduced seasonality ewes. Aust. J. Agric. Res. 53, 167–173.

Forcada, F., Abecia, J.A., Cebrián-Perez, J.A., Muiño-Blanco, T., Valares, J.A., Palacin, I., Casao, A., 2006a. The effect of melatonin implants during the seasonal anestrus on embryo production after superovulation in aged high-prolificacy Rasa Aragonesa ewes. Theriogenology 65 (2), 356–365.

Forcada, F., Abecia, J.A., 2006b. The effect of nutrition on the seasonality of reproduction in ewes. Reprod. Nutr. Dev. 46 (4), 355–365.

Gonzalez-Bulnes, A., Garcia-Garcia, R.M., Santiago-Moreno, J., Lopez-Sebastian, A., Cocero, M.J., 2002. Effect of follicular status on superovulatory response in ewes is influenced by presence of corpus luteum at first FSH dose. Theriogenology 58, 1607–1614.

González-Bulnes, A., García-García, R.M., Santiago-Moreno, J., Domínguez, V., López-Sebastian, A., Cocero, M.J., 2003. Reproductive season affects inhibitory effects from large follicles on the response to superovulatory FSH treatments in ewes. Theriogenology 60 (2), 281–288.

Haresign, W., Peters, A.R., Staples, L.D., 1990. The effect of melatonin implants on breeding activity and litter size in commercial sheep flocks in the UK. Anim. Prod. 50, 111–121.

Haresign, W., 1992. The effect of implantation of lowland ewes with melatonin on the time of mating and reproductive performance. Anim. Prod. 54, 31–39.

Karsch, F.J., Bittman, E.L., Foster, D.L., Goodman, R.L., Legan, S.J., Robinson, J.E., 1984. Neuroendocrine basis of seasonal reproduction. Recent Prog. Horm. Res. 40, 185–232.

Kojima, F.N., Stumpf, T.T., Cupp, A.S., Werth, L.A., Robertson, M.S., Wolfe, M.W., Kittok, R.J., Kinder, J.E., 1992. Exogenous progesterone and progestins as used in estrous synchrony regimens do not mimic the corpus luteum in regulation of luteinizing-hormone and 17β-estradiol in circulation of cows. Biol. Reprod. 47, 1009–1017.

López-Sebastián, A., Cognié, Y., Cocero, M.J., De La Fuente, J., Poulin, N., 1990. Effect of season and duration of FSH treatment on embryo production in sheep. Theriogenology 34 (1), 175–180.

Lozano, J.M., Abecia, J.A., Forcada, F., Zarazaga, L., Alfaro, B., 1998. Effect of undernutrition on the distribution of progesterone in the uterus of ewes during the luteal phase of the estrous cycle. Theriogenology 49, 539–546.

Lozano, J.M., Lonergan, P., Boland, M.P., O'Callaghan, D., 2003. Influence of nutrition on the effectiveness of superovulation programmes in ewes: effect on oocyte quality and post-fertilization development. Reproduction 125, 543–553.

Luther, J.S., Redmer, D.A., Reynolds, L.P., Choi, J.T., Pant, D., Navanukraw, C., Arnold, D.R., Scheaffer, A.N., Borowicz, P., Kirsch, J.D., Weigl, R.M., Kraft, K.C., Grazul-Bilska, A.T., 2005. Ovarian follicular development and oocyte quality in anestrous ewes treated with melatonin, a controlled internal drug release (CIDR) device and follicle stimulating hormone. Theriogenology 63, 2136–2146.

Malpaux, B., Viguie, C., Skinner, D.C., Thiéry, J.C., Chemineau, P., 1997. Control of the circannual rhythm of reproduction by melatonin in the ewe. Brain Res. Bull. 44 (4), 431–438.

Martí, E., Mara, L., Marti, J.I., Muino-Blanco, T., Cebrian-Perez, J.A., 2007. Seasonal variations in antioxidant enzyme activity in ram seminal plasma. Theriogenology 67, 1446–1454.

McCracken, J.A., Custer, E.E., Lamsa, J.C., 1999. Luteolysis: a neuroendocrine-mediated event. Physiol. Rev. 79, 263–323.

McEvoy, T.G., Robinson, J.J., Aitken, R.P., Robertson, I.S., 1998. Melatonin treatment of embryo donor and recipient ewes during anestrus affects their endocrine status, but not ovulation rate, embryo survival or pregnancy. Theriogenology 49, 943–955.

Meikle, A., Tasende, C., Rodriguez, M., Garofalo, E.G., 1997. Effects of estradiol and progesterone on the reproductive tract and on uterine sex steroid receptors in female lambs. Theriogenology 48, 1105–1113.

Mitchell, L.M., King, M.E., Aitken, R.P., Gebbie, F.E., Wallace, J.M., 1999. Ovulation, fertilization and lambing rates, and peripheral progesterone concentrations, in ewes inseminated at a natural oestrus during November or February. J. Reprod. Fertil. 115 (1), 133–140.

Mitchell, L.M., Dingwall, W.S., Mylneb, M.J.A., Hunton, J., Matthews, K., Gebbie, F.E., McCallum, G.J., McEvoy, T.G., 2002. Season affects characteristics of the pre-ovulatory LH surge and embryo viability in superovulated ewes. Anim. Reprod. Sci. 74, 163–174.

O'Callagan, D., Karsch, F.J., Boland, M.P., Roche, J.F., 1991. What photoperiodic signal is provided by a continuous melatonin implant? Biol. Reprod. 45, 927–933.

Rhind, S.M., McMillen, S., Wetherill, G.Z., McKelvey, W.A.C., Gunn, R.G., 1989. Effects of low levels of food intake before and/or after mating on gonadotrophin and progesterone profiles in Greyface ewes. Anim. Prod. 49, 267–273.

Robinson, J.E., Radford, H.M., Karsch, F.J., 1985. Seasonal changes in pulsatile luteinizing hormone (LH) secretion in the ewe: relationship of frequency of LH pulses to day length and response to estradiol negative feedback. Biol. Reprod. 33 (2), 324–334.

Robinson, J.J., Wigzell, S., Aitken, R.P., Wallace, J.M., Ireland, S., Robertson, I.S., 1991. The modifying effects of melatonin, ram exposure and plane of nutrition on the onset of ovarian activity, ovulation rate and the endocrine status of ewes. Anim. Reprod. Sci. 26, 73–91.

Robinson, J.J., 1996. Nutrition and Reproduction. Anim. Reprod. Sci. 42 (1–4), 25–34.

Russell, A.J.F., Doney, J.M., Gunn, R.G., 1969. Subjective assessment of body fat in live sheep. J. Agric. Sci. Camb. 72, 451–454.

SAS Institute Inc., 1999. SAS/STAT. Software Version 8. SAS Institute Inc., Cary, NC, USA.

Sosa, C., Lozano, J.M., Viñoles, C., Acuña, S., Abecia, J.A., Forcada, F., Forsberg, M., Meikle, A., 2004. Effect of plane of nutrition on endometrial sex steroid receptor expression in ewes. Anim. Reprod. Sci. 84, 337–348.

Sosa, C., Abecia, J.A., Forcada, F., Viñoles, C., Tasende, C., Valares, J.A., Palacin, I., Martin, G.B., Meikle, A., 2006. Effect of undernutrition on uterine progesterone and oestrogen-receptors and on endocrine profiles during the ovine oestrous cycle. Reprod. Fert. Dev. 18, 447–458.

Sosa, C., Abecia, J.A., Forcada, F., Meikle, A., 2008. Undernutrition reduces the oviductal mRNA expression of progesterone and oestrogen receptors in sheep. Vet. J. 175 (3), 413–415.

Stenbak, T.K., Redmer, D.A., Berginski, H.R., Erickson, A.S., Navanukraw, C., Toutges, M.J., 2001. Effects of follicle stimulating hormone (FSH) on follicular development, oocyte retrieval, and in vitro fertilization (IVF) in ewes during breeding season and seasonal anestrus. Theriogenology 56, 51–64.

Walker, S.K., Hill, J.L., Kleemann, D.O., Nancarrow, C.D., 1996. Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. Biol. Reprod. 55, 703–708.

Winterberger-Torres, S., Sevellec, C., 1987. Atlas of the Early Development of the Sheep Embryo. INRA, Paris, France.

Yaakub, H., O'Callaghan, D., Boland, M.P., 1999. Effect of type and quantity of concentrates on superovulation and embryo yield in beef heifers. Theriogenology 51, 1259–1266.

Yie, S.M., Brown, G.M., Liu, G.Y., Collins, J.A., Daya, S., Hughes, E.G., Foster, W.G., Younglai, E.V., 1995. Melatonin and steroids in human pre-ovulatory follicular fluid: seasonal variations and granulosa cell steroid production. Hum. Reprod. 10 (1), 50–55.

Zuñiga, O., Forcada, F., Abecia, J.A., 2002. The effect of melatonin implants on the response to the male effect and on the subsequent cyclicity of Rasa Aragonesa ewes implanted in April. Anim. Reprod. Sci. 72 (3–4), 165–174.

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Undernutrition and Exogenous Melatonin Can Affect the *In Vitro* Developmental Competence of Ovine Oocytes on a Seasonal Basis

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Contents

This study evaluated the effects of exogenous melatonin and level of nutrition on oocyte competence, *in vitro* fertilization (IVF), and early embryonic development in sheep during seasonal anoestrus (SA) and the reproductive season (RS). Adult Rasa Aragonesa ewes were assigned randomly to one of four treatment groups in two experiments based on a $2 \times 2 \times 2$ factorial design. Individuals were treated (+MEL) or not treated (−MEL) with a subcutaneous implant of melatonin for 42 days and then were fed 1.5 (Control, C) or 0.5 (Low, L) times the daily maintenance requirements for 20 days. Ewes were synchronized and mated at oestrus (Day = 0). On Day 5, ovaries were collected and oocytes were used for IVF. Season had a significant ($p < 0.01$) effect on the number of oocytes recovered (RS: 19.6 ± 1.0; SA: 14.5 ± 1.0) and the number of healthy oocytes (RS: 13.9 ± 0.7; SA: 9.0 ± 0.7). In the RS, neither nutrition nor melatonin had a significant effect on the evaluated oocytes quality parameters although melatonin implants appeared to reduce the number of unhealthy oocytes in the undernourished group ($p < 0.05$). During SA, in undernourished ewes exogenous melatonin tended to increase the number of healthy (L+MEL: 9.4 ± 1.0, L−MEL: 7.6 ± 1.4; $p < 0.1$), and significantly improved both cleaved oocytes (L+MEL: 7.0 ± 0.7, L−MEL: 4.1 ± 0.9; $p < 0.05$) and blastocyst rate (L+MEL: 37.2, L−MEL: 21.9%; $p < 0.05$). In conclusion, oocyte competence in ewes was affected by season, and melatonin implants appeared to improve developmental competence in the seasonal anoestrous period, particularly in experimentally undernourished ewes.

Introduction

Season and nutritional condition can have a significant effect on reproduction in sheep, particularly in the Mediterranean region, where the availability of nutritional resources are highly seasonally variable. Embryo production and viability are the main factors that reflect female reproductive success, and both are affected by follicular development and oocyte quality. Season appears to influence the fertilization and viability rates of sheep embryos (Vázquez et al. 2008), and can affect some of the characteristics of the embryos (Mitchell et al. 2002). Mitchell et al. (2002) observed that the numbers of fertilized ova and embryos that had <16 cells impacted embryo quality, which was lower during anoestrus than during the reproductive season (RS). In addition, Stenbak et al. (2001) found the *in vitro* fertilization (IVF) rates of oocytes recovered from superovulated ewes were higher in the RS than they were during anoestrus.

Melatonin implants can be an effective method of inducing oestrous cycles, increasing lambing rates, and prolificacy during anoestrus (Haresign et al. 1990; Robinson et al. 1991; Haresign 1992; Abecia et al. 2007). Abecia et al. (2008) summarized the effects of exogenous melatonin on the ovary and early embryos in ewes. Although melatonin treatments during anoestrus did not improve fertilization rates in superovulated ewes (McEvoy et al. 1998; Forcada et al. 2006), this pineal hormone can improve the viability of embryos (Forcada et al. 2006). Although information about the effects of melatonin on oocyte quality and IVF during anoestrus is limited, Luther et al. (2005) did not observe a significant difference between melatonin-treated and untreated ewes in their fertilization rates after IVF of the oocytes recovered from superovulated ewes in the non-reproductive season.

Nutrition can have a significant effect on numerous aspects of reproduction, including hormone release, fertilization, and early embryonic development (Boland et al. 2001). Nutritional condition can be correlated with embryo survival and is a key factor in the efficiency of assisted reproductive technologies (Webb et al. 2004). Borowczyk et al. (2006) found that the oocytes derived from underfed ewes yielded fewer blastocysts and had lower rates of cleavage and blastocyst formation than did control ewes. Lozano et al. (2003) observed lower cleavage rates in underfed ewes than in overfed ewes; in addition, undernutrition can impair embryo viability (Vázquez et al. 2008). To our knowledge, yet, there are no published studies on the competence of oocytes recovered from the ovaries of undernourished ewes.

As melatonin can increase embryo survival and IVF rates (Valasi et al. 2006), we hypothesized that this hormone might override the effects of undernutrition and season on oocyte competence, which is the ability of an oocyte to be fertilized and develop to the blastocyst stage. Thus, the aim of this study was to evaluate the effects of melatonin and undernutrition on oocyte competence, IVF and early embryonic development during the anoestrus and the RS in ewes.

Materials and Methods

All procedures used in this study were approved by the Ethics Committee of the University of Zaragoza, Spain, performed at the Experimental Farm of the University of Zaragoza, Spain (41°41′N), and met the requirements of the European Union for Scientific Procedure Establishments.

Animals and experimental design

The study included multiparous, non-pregnant Rasa Aragonesa ewes. In the RS, 42 cycling ewes with a mean (± SEM) live weight (LW) of 64.1 ± 1.7 kg and mean body condition (BC) (Russell et al. 1969) of 3.1 ± 0.1 were used. To confirm ovarian cyclicity, 7 days and 14 days before the insertion of melatonin implants, blood samples were collected and plasma progesterone concentrations were measured. All of the ewes had progesterone concentrations >1 ng/ml in at least one of the samples, which indicated ovarian activity. On 8th December, 21 ewes received a subcutaneous melatonin implant (18 mg melatonin, Melovine®, CEVA Salud Animal S.A., Barcelona, Spain) at the base of the ear and 21 others did not. Forty-two days after implantation, all of the ewes were synchronized after a 14-day treatment with intravaginal progestagen (30 mg Flourogestone Acetate; Sincropart®, Ceva Salud Animal S.A.). When the pessaries were removed, the ewes were treated i.v. with 400 IU of equine chorionic gonadotrophin (eCG) (Sincropart® PMSG, Ceva Salud Animal S.A.).

During the seasonal anoestrus (SA), at 7 and 14 days before the start of the experiment, blood samples were collected from 42 ewes and their plasma progesterone concentrations were measured. To create a group of anoestrous ewes, those that had progesterone levels >1 ng/ml in at least one of their samples were considered cyclic, and were excluded from the study. Consequently, the experiment included 36 adult, non-cycling, non-pregnant Rasa Aragonesa ewes that had a mean (± SEM) LW of 59.2 ± 1.3 kg and BC of 3.2 ± 0.06. On 26 March, 17 ewes received a subcutaneous implant of melatonin, while another 19 did not. Synchronization with intravaginal progestagen pessaries occurred 42 days after implantation. When the pessaries were removed, the ewes were treated i.v. with 480 IU of equine chorionic gonadotrophin (eCG).

In both seasons, from the date of insertion of the pessaries until slaughter (Day 5 post-oestrus), the ewes were fed with a diet that provided either 1.5× M (control, C groups) or 0.5× M (low, L groups) the daily maintenance requirements (Agricultural and Food Research Council 1993) and had unlimited access to water. Ewes fed the C diet received 0.60 kg of pellets and 1 kg of barley straw per day, which provided 12.4 MJ of metabolizable energy (ME) and 9.3% crude protein (CP). The L diet comprised 0.20 kg of pellets and 0.35 kg of barley straw per day, which provided 4.1 MJ of ME and 9.1% CP. The pellets consisted of barley (73%), soybean (22%), and a mineral supplement (5%). Live weight and BC were recorded at the time of pessary insertion, pessary withdrawal, and at slaughter. A 1.5× M diet ensures the maintenance of LW and BC, whereas a 0.5× M diet leads to a 12% reduction in LW and BC after 20 days. Thus, the four groups in the study were: group C−MEL, ewes that were offered the C diet and did not receive a melatonin implant; group C + MEL, ewes that were offered the C diet and received a melatonin implant; group L−MEL, ewes that were offered the L diet and did not receive a melatonin implant; and group L + MEL, ewes that were offered the L diet and received a melatonin implant.

From 24 h after pessary withdrawal, every 8 h intact rams wearing harnesses with marking crayons were used to monitor oestrus (Day 0). To verify the functionality of the corpora lutea at Day 5 (slaughter), jugular blood samples were collected in evacuated heparinized tubes. To test the effectiveness of the melatonin implants, one diurnal blood sample was collected from each of the melatonin-implanted ewes 45 days after implantation. The samples were centrifuged within 15 min ($1000 \times g$ for 10 min) and the plasma was stored at −20°C.

Collection and *in vitro* maturation of oocytes

On Day 5, 20 days after the start of the experimental diets, ewes were anaesthetized using an i.m. injection of 0.4 ml 2% xylazine (Xilagesic 2%®, Calier, Barcelona, Spain), and 10 ml of sodium thiopental (20 mg/ml) (Thiobarbital Braun Medical, Jaén, Spain) administered by i.v. injection 5 min later. Uterine horns were flushed with pre-warmed (36°C) phosphate-buffered saline (PBS) and embryos were collected by mid-ventral laparotomy. The effects of exogenous melatonin and level of nutrition on the viability of embryos are described elsewhere (Vázquez et al. 2008).

After embryo collection, ewes were euthanized using an i.v. injection of sodium thiopental (T-61®; Intervet, Salamanca, Spain). Ovaries were collected and placed in PBS supplemented with 100 IU/ml of penicillin-G and 100 μg/ml of streptomycin sulphate at 39°C until they were processed. Except where indicated otherwise, all of the reagents were from Sigma-Aldrich Co., St. Louis, MO, USA. A combination of puncture and slicing techniques (Wani et al. 1999) were used to collect oocytes in a Petri dish and partially covered with a handling medium (Hepes-buffered TCM-199 supplemented with 0.1% polyvinyl alcohol (PVA), 0.04% sodium bicarbonate, 25 IU/ml of heparin, 100 IU/ml of penicillin-G, and 100 μg/ml of streptomycin sulphate). Following Wani et al. (2000), oocytes were classified, based on their cumulus cells and cytoplasm morphology, as: Good, included all oocytes with a lot of complete layers of granulose cells and homogeneous cytoplasm; Fair, included all oocytes with few or incomplete layers of granulose cells and homogeneous cytoplasm; and Poor, included oocytes with few or absence of granulose cells and non-homogeneous cytoplasm. Only the oocytes that had several layers of cumulus cells and a uniform cytoplasm (called healthy oocytes) were selected for *in vitro* maturation (IVM).

Healthy oocytes were transferred into a maturation medium that contained bicarbonate-buffered TCM-199 supplemented with 10% (v : v) oestrous sheep serum, 0.1 μg/ml each of FSH and LH, 100 μM of cysteamine, 0.3 μM of sodium pyruvate, 100 IU/ml of penicillin G, and 100 μg/ml of streptomycin sulphate, which was covered with mineral oil and incubated at 39°C under 5% CO_2 and saturated humidity for 24 h.

In vitro fertilization (IVF) and embryo cultures

At the end of IVM, oocytes were freed from the cumulus cells and transferred to the fertilization medium, which

consisted of synthetic oviductal fluid (SOF) without glucose (Tervit and Whittingham 1972) and supplemented with 2% (v : v) of oestrous sheep serum (Hopper et al. 1993; Huneau et al. 1994; Li et al. 2006), 10 μg/ml of heparin (Cox and Saravia 1992), and 1 μg/ml of hypotaurine.

On the same day as fertilization, semen was collected from four Rasa Aragonesa rams, pooled, diluted 1:10 in a saline medium with 0.25 mol/l of sucrose, 10 mmol/l of Hepes, 2 mmol/l of potassium hydroxide, 5 mmol/l of glucose, 0.5 mol/l of sodium phosphate monobasic, and 100 mmol/l of ethylene glycol tetra-acetic acid (EGTA), and kept at 15°C until fertilization. Highly motile spermatozoa were selected using the swim-up technique (Wani et al. 2000; Luther et al. 2005). The fertilization dose was 1×10^{-6} spermatozoa/ml, sperm were added to the fertilization medium that contained the oocytes, covered with mineral oil, and incubated for 24 h at 39°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

At 24 h and 36 h after fertilization, presumptive zygotes were placed in a culture medium that contained SOF supplemented with essential and non-essential amino acids at oviductal concentration (Walker et al. 1996), 0.4% bovine serum albumine (wt/vol), 1 mM of L-glutamine, 100 IU/ml of penicillin G, and 100 μg/ml of streptomycin sulphate, and covered with mineral oil and kept at 39°C in a maximally humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 8 days, to blastocyst stage.

The cleaved embryos were added to the embryo wells, and uncleaved oocytes were examined under a stereomicroscope to assess their stage of maturation. Oocytes that exhibited the first polar body were considered mature, and oocytes that had two polar bodies were considered fertilized, but not cleaved.

The following information was recorded for each animal in each experimental group: ovulation rate (expressed as the number of corpora lutea observed at Day 5 per ewe), number of recovered oocytes (included all recovered oocytes), number of healthy oocytes (included only such of the recovered oocytes that were rated as good and fair), healthy oocytes rate (number of healthy oocytes/number of recovered oocytes), number of non-healthy oocytes, number of cleaved oocytes, cleavage rate (number of cleaved oocytes/number of healthy oocytes), number of blastocysts, and blastocyst rate (number of blastocysts/number of cleaved oocytes). All values were expressed per ewe and all rates were expressed as percentages.

Hormone assays

Plasma melatonin concentrations were measured by a direct, solid-phase radioimmunoassay (RIA) using a commercially available kit (Bühlmann RK-MDI; Bühlmann Lab, Schönenbuch, Switzerland) within a single assay. The sensitivity of the assay was 1.3 pg/ml and the intra-assay CV was 10.9% for low control concentrations (3.3 pg/ml) and 4.9% for high control concentrations (20.6 pg/ml).

Plasma progesterone concentrations were measured by a direct, solid-phase RIA using commercially available kits (Count-A-Count TKPG; DPC) (Meikle et al. 1997). The RIA had a sensitivity of 0.02 ng/ml. The intra-assay CV was 14% for low control concentrations (3 ng/ml), 8.5% for medium concentrations (15 ng/ml), and 7.5% for high concentrations (30 ng/ml). The interassay CV was <15% for all of the standard concentrations.

Statistical analyses

The experimental was based on a $2 \times 2 \times 2$ factorial design in which nutritional level, melatonin treatment, and season were fixed effects. The effects of the treatments on the development and quality of oocytes and blastocyst were evaluated statistically using the PROC GEN MOD (SAS 1999) with the Poisson distribution specified in a model that included season (reproductive or anoestrus), nutrition level (low or control), and melatonin treatment (with or without melatonin implant), and their interactions. The values expressed as percentages were arcsine-transformed before being compared using the chi-squared test. The probability level for statistical significance was set to $p < 0.05$ and the results are expressed as mean ± SEM.

Results

Live weight and body condition of Rasa Aragonesa ewes

In the RS, during the period of the experimental diets (20 days), the LW of ewes in groups C−MEL and C + MEL did not change significantly, but ewes in groups L−MEL and L + MEL experienced significant ($p < 0.001$) weight loss (on average, in the two groups, 7.5 and 9.3 kg, respectively) (Fig. 1). In addition, ewes in groups L−MEL and L + MEL experienced significant ($p < 0.001$) reductions in BC (on average, in the two groups, 0.3 and 0.5 respectively), but ewes in groups C−MEL and C + MEL did not (Fig. 1). In the SA, ewes in groups C−MEL and C + MEL maintained their LW and BC, but the ewes in groups L−MEL and L + MEL experienced significant ($p < 0.001$) reductions in LW (in the two groups, average losses of 2.5 and 3.8 kg, respectively) and BC (average losses of 0.2 and 0.3, respectively; $p < 0.05$) in the 20-day period of the experimental diets (Fig. 1). In both seasons, after pessary withdrawal (14 days after the start of the experimental diets), mean LW and BC were significantly ($p < 0.01$) lower among the ewes in the Low groups than among the ewes in the Control groups.

Circulating hormones

In both seasons, all melatonin implants induced high plasma melatonin concentrations during daylight hours at Day 45 after implantation (mean = 53.6 ± 4.2 pg/ml and 62.6 ± 4.9 pg/ml in January and May, respectively), which indicated that the implants released melatonin properly. Plasma progesterone concentrations indicated that all of the ewes exhibited functional corpora lutea at slaughter on Day 5 after oestrus. During the RS, mean progesterone levels ranged from 3.7 ± 0.5 ng/ml (group C−MEL) to 4.7 ± 0.7 ng/ml (group L−MEL) and, during the SA, they ranged from

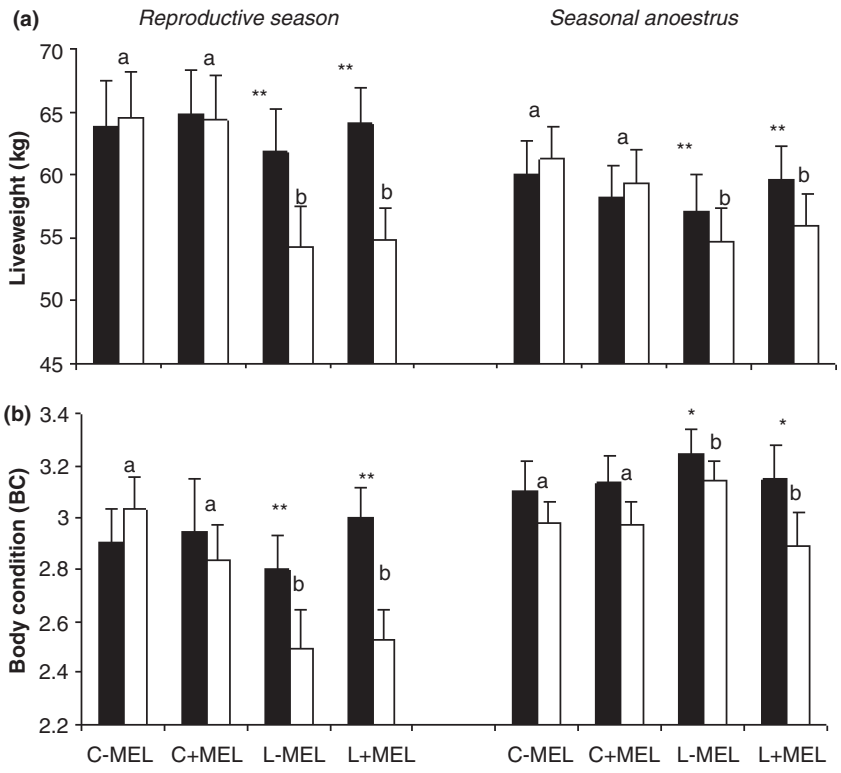

Fig. 1. Mean (±SEM) live weight (a) and body condition (b) of Rasa Aragonesa ewes fed either 1.5× (C) or 0.5× (L) maintenance requirements and treated (+MEL) or not (−MEL) with melatonin implants at the beginning (black bars) and the end (open bars) of the 20-days experimental diet period in the reproductive (left panel) and anoestrous (right panel) seasons. Bars with different superscripts (a, b) among groups differ at $p < 0.01$. Asterisks within groups indicate $p < 0.001$ (**) or $p < 0.05$ (*)

2.6 ± 0.6 ng/ml (group L−MEL) to 3.8 ± 0.5 ng/ml (group C+MEL).

Ovulation rate

Ovulation rates did not differ significantly among treatments or between seasons (Tables 1 and 2).

Oocyte quality and IVF

The number of recovered oocytes per ewe was significantly ($p < 0.01$) higher in the RS (19.6 ± 1.0) than it was in the SA (14.5 ± 1.0). In addition, the competence of the oocytes was influenced by season; the number of healthy oocytes that could be used for IVF and the healthy oocyte rate were significantly ($p < 0.01$) higher in the RS than they were in the SA (13.9 ± 0.7 and 71.4% vs 9.0 ± 0.7 and 62.3% respectively). In contrast, cleavage rate tended ($p < 0.1$) to be higher in the SA (65.0%) than in the RS (54.2%).

Reproductive season

Neither nutrition nor treatment with melatonin implants appeared to have a significant effect on the number of recovered, healthy, or cleaved oocytes, or on the healthy oocytes and cleavage rates (Table 1); yet, diet and supplemental melatonin had a significant ($p < 0.05$) interaction effect on the number of non-healthy oocytes, which suggests that melatonin implants had a negative effect on this parameter in the C+MEL group and a positive effect in the L+MEL group.

Anoestrous season

Undernutrition tended ($p < 0.1$) to reduce the number of recovered oocytes per ewe (C: 15.6 ± 1.4, L: 13.3 ± 1.8) (Table 2). In the groups that did not receive melatonin implants, undernutrition induced a significant ($p < 0.05$) decrease in the number of cleaved oocytes per ewe (C−MEL: 7.2 ± 0.8, L−MEL: 4.1 ± 0.9). In

Reproductive season	C−MEL	C+MEL	L−MEL	L+MEL
No. of ovulating ewes with functional corpus luteum	8/10	9/11	9/11	9/10
Ovulation rate	1.9 ± 0.4	2.2 ± 0.3	2.2 ± 0.4	1.8 ± 0.4
No. of recovered oocytes	17.1 ± 2.3[c]	22.2 ± 2.2[d]	21.3 ± 2.2[d]	17.8 ± 2.2
No. of healthy oocytes used for IVF	13.3 ± 1.4	15.3 ± 1.3	14.1 ± 1.0	13.0 ± 1.6
Healthy oocytes rate (%)	77.7[c]	68.9[d]	66.2	73.0
No. of non-healthy oocytes	3.9 ± 1.2[a]	6.9 ± 1.1[b]	7.2 ± 1.1[b]	4.8 ± 1.1[a]
No. of oocytes cleaved	6.1 ± 0.9	9.3 ± 1.2	8.1 ± 1.0	6.8 ± 1.3
Cleavage rate (%)	45.8[c]	60.8[d]	57.5	52.3
No. of blastocysts	0.8 ± 0.5	1.2 ± 0.3	2.6 ± 0.6	0.8 ± 0.5
Rate of blastocysts (%)	13.1[d]	12.9	32.0[c]	11.8[d]

Table 1. Results of the *in vitro* fertilization (IVF) of oocytes collected from Rasa Aragonesa ewes that were fed to provide 1.5× (C) or 0.5× (L) the maintenance requirements and treated (+MEL) or not treated (−MEL) with melatonin during the reproductive season

All values are expressed per ewe.
Different superscripts (a, b) in the same row mean indicate significant differences ($p < 0.05$).
Different superscripts (c, d) in the same row indicate significant differences ($p < 0.1$) .

Table 2. Results of the *in vitro* fertilization (IVF) of oocytes collected from Rasa Aragonesa ewes that were fed to provide 1.5× (C) or 0.5× (L) the maintenance requirements and treated (+MEL) or not treated (−MEL) with melatonin during the anoestrus

Anoestrus	C−MEL	C+MEL	L−MEL	L+MEL
No. of ovulating ewes with functional corpus luteum	11	9	8	8
Ovulation rate	2.3 ± 0.4	2.4 ± 0.4	2.5 ± 0.5	1.9 ± 0.5
No. of recovered oocytes	16.1 ± 1.8	15.2 ± 1.9	12.0 ± 2.1	14.6 ± 2.1
No. of healthy oocytes used for IVF	9.6 ± 0.4[d]	9.4 ± 0.8	7.6 ± 1.4[e]	9.4 ± 1.0[d]
Healthy oocytes rate (%)	59.6	61.8	63.3	64.4
No. of non-healthy oocytes	6.5 ± 0.9	5.8 ± 1.0	4.4 ± 1.1	5.2 ± 1.1
No. of oocytes cleaved	7.2 ± 0.8[a]	5.3 ± 0.9	4.1 ± 0.9[b]	7.0 ± 0.7[a]
Cleavage rate (%)	75.0	56.4	53.9[d]	74.5[e]
No. of blastocysts	0.8 ± 0.4	1.4 ± 0.5	0.9 ± 0.6	2.6 ± 0.8
Rate of blastocysts (%)	11.3[a]	26.4[b,d]	21.9[b]	37.2[c,e]

All values are expressed per ewe.
Different superscripts (a, b, c) in the same row mean indicate significant differences ($p < 0.05$).
Different superscripts (d, e) in the same row indicate significant differences ($p < 0.1$).

contrast, none of the parameters evaluated were significantly affected by exogenous melatonin or the interaction between nutrition and melatonin treatment; yet, among the undernourished ewes, supplemental melatonin appeared to increase the number of healthy oocytes (L+MEL: 9.4 ± 1.0, L−MEL: 7.6 ± 1.4; $p < 0.1$), and significantly improved the number of cleaved oocytes (L+MEL: 7.0 ± 0.7, L−MEL: 4.1 ± 0.9; $p < 0.05$) and the blastocyst rate (L+MEL: 37.2%, L−MEL: 21.9%; $p < 0.05$). In addition, melatonin treatment significantly ($p < 0.05$) improved the blastocyst rate among C ewes (C+MEL: 26.4%, C−MEL: 11.3%).

Discussion

This study investigated the effects of exogenous melatonin and undernutrition on oocyte quality, IVF, and early embryonic development in Rasa Aragonesa ewes in the reproductive and anoestrous seasons. We hypothesized that the known beneficial effects of exogenous melatonin – both *in vivo* and even *in vitro* – on embryo survival would compensate for the negative effects of undernutrition and season on oocyte developmental competence.

In our experiments, undernourished ewes exhibited a significant decrease in LW and BC. Although the extent of the decrease in the LW of ewes in the L groups was lower during the seasonal anestrous period, the nutritional treatments appeared to be effective because of the significant ($p < 0.01$) differences in the LW and BC of the ewes in the control groups and the low groups at slaughter. In previous studies, we found that a level of undernutrition similar to that of the present study for 3–4 weeks induced a significant reduction in the LW and BC of Rasa Aragonesa ewes in the breeding season (Abecia et al. 1997, 1999) and during anoestrus (Sosa et al. 2006). In addition, underfed ewes exhibited increased lipolytic activity (Sosa et al. 2006). The results of this study are consistent with the observations that short-term undernutrition associated with a progestagen-synchronized oestrus does not appear to impair ovulation rate in the RS (Abecia et al. 1997, 1999; Borowczyk et al. 2006) or the anoestrous season (Sosa et al. 2006).

In our study, SA had a significant detrimental effect on the number of recovered oocytes per ewe, the competence of the oocytes, and the fertilization rate. Anoestrus is a period of infrequent pulses of LH, whereas, in the breeding season, the frequency and amplitude of LH release is appropriate for controlling the luteal and follicular phases of the oestrous cycle (Goodman 1988). Oussaid et al. (1999) showed that a temporary suppression of LH release during the follicular phase impaired oocyte developmental competence in cyclic ewes. Even in humans, reduced pre-ovulatory levels of LH are associated with impaired *in vitro* oocyte fertilization (Verpoest et al. 2000). Stenbak et al. (2001) reported that IVF of oocytes recovered from superovulated ewes was higher in the breeding season than during the anoestrus. In addition, season influences *in vivo* fertilization rate, and some studies have shown a greater number of fertilized oocytes recovered from superovulated ewes in the breeding season than during the anoestrus (Mitchell et al. 2002; González-Bulnes et al. 2003). Changes in semen quality might contribute to the seasonal differences in IVF, but low seasonal variations in the volume and quality of the ejaculates from Rasa Aragonesa rams (Martí et al. 2007) suggest that the lower cleavage rates in the anoestrous season might be mainly caused by lower oocyte quality.

Although exogenous melatonin did not have a significant effect on most of the parameters evaluated in our study, treatment with this pineal hormone during anoestrus significantly improved the number of oocytes cleaved in undernourished ewes and also the blastocyst rate independently of the level of nutrition. In some studies, no significant positive effect of melatonin was found either on the number of visible follicles or the number of oocytes recovered from both anoestrous FSH-superovulated ewes (Luther et al. 2005) and goats (Berlinguer et al. 2007); yet, treatment with exogenous melatonin in anoestrus seems to improve the developmental competence (IVF) of oocytes recovered from progestagen and eCG-treated ewes (Valasi et al. 2006) and the cleavage and blastocyst rates after IVF of oocytes collected from superovulated goats (Berlinguer et al. 2007). Our recent experiments have shown that supplemental melatonin can reduce significantly the number and rate of non-viable (degenerated and retarded) recovered embryos from superovulated ewes during anoestrus (Forcada et al. 2006). The luteotrophic effect of the hormone *in vivo* (Durotoye et al. 1997) and *in vitro* (Abecia et al. 2002), and the effects of melatonin

at the hypothalamic-hypophyseal level (Malpaux et al. 1997) might be involved in the melatonin-induced improvement in embryo viability during anoestrus. Furthermore, in humans and rats, granulose cells express specific melatonin-binding sites (Yie et al. 1995; Clemmens et al. 2001) but there are no reports in sheep. On the other hand, the radical scavenger properties of melatonin could have exerted a beneficial effect on the early embryo development, protecting embryonic cells from oxidative stress during *in vitro* maturation, as reported by Chetsawang et al. (2006). During the IVF, high concentrations of spermatozoa in the small volumes of IVF medium result in increased levels of free radicals but toxic effects caused by the oxidative stress were reduced with the protective radical scavenger properties of melatonin (Tamura et al. 2008). However, the mechanism by which exogenous melatonin improves the developmental competence of oocytes is still unclear, although it might be exerted both at ovarian level and at early cleavage stages.

In our study, although undernutrition did not influence significantly oocyte competence during the RS, undernourished ewes showed a reduced number of recovered and cleaved oocytes in the SA, which indicated that, in Mediterranean breeds of sheep, nutrition can, at least, partially compensate for the negative effects of season on the hypothalamic-hypophyseal-ovarian axis (Forcada et al. 1992; Lindsay 1996). Impaired *in vitro* developmental competence of oocytes in undernourished ewes has been observed in superovulated ewes (Papadopoulos et al. 2001; Borowczyk et al. 2006). Scaramuzzi et al. (2006) summarized the effects of nutrition on the regulation of folliculogenesis in sheep. Undernutrition is associated with an increase in the secretion of oestradiol in the early follicular phase, which enhances the negative feedback at the hypothalamic-hypophyseal level and, therefore, impairs follicular competence. The effect of undernutrition on the release of oestradiol is maintained during the 2 weeks after oestrus (Sosa et al. 2006) and, in our experiment, probably affected the follicular population and in consequence the oocyte quality at Day 5. In addition, undernutrition is associated with a reduction in ovarian and endometrial sensitivity to ovarian steroids (Sosa et al. 2006, 2008), which compromises early embryo development (Vázquez et al. 2008).

In our study, the melatonin implants appeared to improve the low developmental competence of oocytes induced by undernutrition during anoestrus, and the beneficial effect of melatonin on nutrition-impaired folliculogenesis during anoestrus has been observed elsewhere (Robinson et al. 1991; Forcada et al. 1995). These studies clearly showed that the ability of exogenous melatonin to improve ovulation rates was more pronounced in ewes that were on a low, rather than a high, level of feed intake; although the mechanisms involved in that response remain to be elucidated.

In conclusion, the results of our study indicate that seasonal anoestrus and undernutrition can impair the quality of oocytes recovered from Rasa Aragonesa, a Mediterranean breed of sheep. Furthermore, supplemental melatonin appeared to improve oocyte developmental competence during the seasonal anestrous period, especially in undernourished ewes. Those results underscore the importance of understanding the nature of nutrition-melatonin interactions and their mechanisms, particularly at the ovarian and early embryo development levels and during the non-reproductive season.

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Author contributions

Drs Forcada and Abecia have made contributions on the main aspects of this publication (as research design, analysis and interpretation of data and writing, drafting and revising the manuscript critically). Drs Sosa and Palacin participated in the acquisition of data; Dr Casao was the responsible for the IVF laboratory. Miss Vazquez was involved in the acquisition, analysis and interpretation of data and writing, drafting and revising the manuscript.

References

Abecia JA, Lozano JM, Forcada F, Zarazaga L, 1997: Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Anim Reprod Sci **48,** 209–218.

Abecia JA, Forcada F, Lozano JM, 1999: A preliminary report on the effect of dietary energy on prostaglandin F2 production *in vitro*, interferon-tau synthesis by the conceptus, endometrial progesterone concentration on days 9 and 15 of pregnancy and associated rates of embryo wastage in ewe. Theriogenology **52,** 1203–1213.

Abecia JA, Forcada F, Zuñiga O, 2002: The effect of melatonin on the secretion of progesterone in sheep and on the development of ovine embryos *in vitro*. Vet Res Commun **26,** 151–158.

Abecia JA, Valares JA, Forcada F, Palacín I, Martin S, Martino A, 2007: The effect of melatonin on the reproductive performance of three sheep breeds in Spain. Small Rumin Res **69,** 10–16.

Abecia JA, Forcada F, Casao A, Palacín I, 2008: Effect of exogenous melatonin on the ovary, the embryo and the establishment of pregnancy in sheep. Animal **2,** 399–404.

Agricultural and Food Research Council, 1993: Energy and protein requirements of ruminants. An Advisor Manual Prepared by the AFRC Technical Committee on Responses to Nutrients. CAB International, Wallingford, United Kingdom.

Berlinguer F, Spezzigu A, Succu S, Bebbere D, Madeddu M, Tedde A, Satta V, Leoni GG, Naitana S, 2007: Melatonin Treatment During Anestrus Enhances Oocyte Developmental Competence in FSH-Treated Goats. Proceedings of 23rd Annual Meeting A.E.T.E, ed. by European Embryo Transfer Association, Italy, pp. 136.

Boland MP, Lonergan P, O'Callaghan D, 2001: Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology **55,** 1323–1340.

Borowczyk E, Caton JS, Redmer DA, Bilski JJ, Weigl RM, Vonnahme KA, Borowicz PP, Kirsch JD, Kraft KC, Reynolds LP, Grazul-Bilska AT, 2006: Effects of plane of nutrition on *in vitro* fertilization and early embryonic development in sheep. J Anim Sci **84,** 1593–1599.

Chetsawang B, Putthaprasart C, Phansuwan-Pujito P, Govitrapong P, 2006: Melatonin protects against hydrogen peroxide-induced cell death signaling in SH-SY5Y cultured cells: involvement of nuclear factor kappa B, Bax and Bcl-2. J Pineal Res **41,** 116–123.

Clemmens JW, Jarzunka MJ, Witt-Enderby PA, 2001: Down-regulation of mt1 melatonin receptors in rat ovary following estrogen exposure. Life Sci **69,** 27–35.

Cox JF, Saravia F 1992: Use of multiple sperm penetration assay in ovines. Proceedings of the XVII Annual Meeting of Chilean Society of Animal Production, Chillán, Chile.

Durotoye LA, Webley GE, Rodway RG, 1997: Stimulation of the production of progesterone by the corpus luteum of the ewe by the perfusion of melatonin *in vivo* and by treatment of granulosa cells with melatonin *in vitro*. Res Vet Sci **62,** 87–91.

Forcada F, Abecia JA, Sierra I, 1992: Seasonal changes in oestrus activity and ovulation rate in Rasa Aragonesa ewes maintained at two different body condition levels. Small Rumin Res **8,** 313–324.

Forcada F, Zarazaga L, Abecia JA, 1995: Effect of exogenous melatonin and plane of nutrition after weaning on oestrous activity, endocrine status and ovulation rate in Salz ewes lambing in the seasonal anoestrus. Theriogenology **43,** 1179–1193.

Forcada F, Abecia JA, Cebrián-Perez JA, Muiño-Blanco T, Valares JA, Palacín I, Casao A, 2006: The effect of melatonin implants during the seasonal anoestrus on embryo production after superovulation in aged high-prolificacy Rasa Aragonesa ewes. Theriogenology **65,** 356–365.

González-Bulnes A, García-García RM, Santiago-Moreno J, Domínguez V, López-Sebastian A, Cocero MJ, 2003: Reproductive season affects inhibitory effects from large follicles on the response to superovulatory FSH treatments in ewes. Theriogenology **60,** 281–288.

Goodman RL, 1988: Neuroendocrine control of the ovine estrous cycle. In: Knobil E, Neil J (eds), The Physiology of Reproduction. Raven Press, New York, pp. 1929–1968.

Haresign W, 1992: The effect of implantation of lowland ewes with melatonin on the time of mating and reproductive performance. Anim Prod **54,** 31–39.

Haresign W, Peters AR, Staples LD, 1990: The effect of melatonin implants on breeding activity and litter size in commercial sheep flocks in the UK. Anim Prod **50,** 111–121.

Hopper HW, Silcox RW, Byerley DJ, Kiser TE, 1993: Follicular development in prepubertal heifers. Anim Reprod Sci **31,** 7–12.

Huneau D, Crozet N, Ahmed-Ali M, 1994: Estrous sheep serum as a potent agent for ovine IVF: effect on cholesterol efflux from spermatozoa and the acrosome reaction. Theriogenology **42,** 1017–1028.

Li F, Pi WH, Zhu HZ, Zhang SS, Liu SR, Xue JL, 2006: The effect of estrous ewe serum and heparin on *in vitro* fertilization and subsequent embryonic development in sheep. Small Rumin Res **63,** 226–232.

Lindsay DR, 1996: Environment and reproductive behaviour. Anim Reprod Sci **42,** 1–12.

Lozano JM, Lonergan P, Boland MP, O'Callaghan D, 2003: Influence of nutrition on the effectiveness of superovulation programmes in ewes: effect on oocyte quality and post-fertilization development. Reproduction **125,** 543–553.

Luther JS, Redmer DA, Reynolds LP, Choi JT, Pant D, Navanukraw C, Arnold DR, Scheaffer AN, Borowicz P, Kirsch JD, Weigl RM, Kraft KC, Grazul-Bilska AT, 2005: Ovarian follicular development and oocyte quality in anestrous ewes treated with melatonin, a controlled internal drug release (CIDR) device and follicle stimulating hormone. Theriogenology **63,** 2136–2146.

Malpaux B, Viguié C, Skinner DC, Thiéry JC, Chemineau P, 1997: Control of the circannual rhythm of reproduction y melatonin in the ewe. Brain Res Bull **44,** 431–438.

Martí E, Mara L, Martí JI, Muiño-Blanco T, Cebrián-Perez JA, 2007: Seasonal variations in antioxidant enzyme activity in ram seminal plasma. Theriogenology **67,** 1446–1454.

McEvoy TG, Robinson JJ, Aitken RP, Robertson IS, 1998: Melatonin treatment of embryo donor and recipient ewes during anestrus affects their endocrine status, but not ovulation rate, embryo survival or pregnancy. Theriogenology **49,** 943–955.

Meikle A, Tasende C, Rodriguez M, Garofalo EG, 1997: Effects of estradiol and progesterone on the reproductive tract and on uterine sex steroid receptors in female lambs. Theriogenology **48,** 1105–1113.

Mitchell LM, Dingwall WS, Mylneb MJA, Hunton J, Matthews K, Gebbie FE, McCallum GJ, McEvoy TG, 2002: Season affects characteristics of the pre-ovulatory LH surge and embryo viability in superovulated ewes. Anim Reprod Sci **74,** 163–174.

Oussaid B, Mariana JC, Poulin N, Fontaine J, Lonergan P, Beckers JF, Cognié Y, 1999: Reduction of the developmental competence of sheep oocytes by inhibition of LH pulses during the follicular phase with a GnRH antagonist. J Reprod Fertil **117,** 71–77.

Papadopoulos S, Lonergan P, Gath V, Quinn KM, Evans ACO, O'Callaghan D, Boland MP, 2001: Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. Theriogenology **55,** 1059–1069.

Robinson JJ, Wigzell S, Aitken RP, Wallace JM, Ireland S, Robertson IS, 1991: The modifying effects of melatonin, ram exposure and plane of nutrition on the onset of ovarian activity, ovulation rate and the endocrine status of ewes. Anim Reprod Sci **26,** 73–91.

Russell AJF, Doney JM, Gunn RG, 1969: Subjective assessment of body fat in live sheep. J Agric Sci Camb **72,** 451–454.

SAS, 1999: User's Guide, Statistics. SAS/STAT, Software Version 8. SAS Institute Inc., Cary, NC, USA.

Scaramuzzi RJ, Campbell BK, Downing JA, Kendall NR, Khalid M, Munoz-Gutierrez M, Somchit A, 2006: A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. Reprod Nutr Dev **46,** 339–354.

Sosa C, Abecia JA, Forcada F, Viñoles C, Tasende C, Valares JA, Palacin I, Martin GB, Meikle A, 2006: Effect of undernutrition on uterine progesterone and oestrogen-receptors and on endocrine profiles during the ovine oestrous cycle. Reprod Fertil Dev **18,** 447–458.

Sosa C, Abecia JA, Forcada F, Meikle A, 2008: Undernutrition reduces the oviductal mRNA expression of progesterone and oestrogen receptors in sheep. Vet J **175,** 413–415.

Stenbak TK, Redmer DA, Berginski HR, Erickson AS, Navanukraw C, Toutges MJ, 2001: Effects of follicle stimulating hormone (FSH) on follicular development, oocyte retrieval, and *in vitro* fertilization (IVF) in ewes during breeding season and seasonal anestrus. Theriogenology **56,** 51–64.

Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, Taketani T, Matsuoka A, Yamagata Y, Shimamura K, Morioka H, Ishikawa H, Reiter RJ, Sugino N, 2008: Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. J Pineal Res **44,** 280–287.

Tervit HR, Whittingham DG, 1972: Successful culture *in vitro* of sheep and cattle ova. J Reprod Fert **30,** 493–497.

Valasi F, Tsiligianni T, Papanikolaou T, Dimitriadis I, Vainas E, Samartzi F, Amiridis GS, 2006: Melatonin improves the

developmental competence of sheep oocytes. Reprod Domest Anim **41,** 341.

Vázquez MI, Forcada F, Casao A, Sosa C, Palacín I, Abecia JA, 2008: Effects of melatonin and undernutrition on the viability of ovine embryos during anoestrus and the breeding season. Anim Reprod Sci doi:10.1016/j.anireprosci.2008.04.004.

Verpoest WM, Cahill DJ, Harlow CR, Hull MG, 2000: Relationship between midcycle luteinizing hormone surge quality and oocyte fertilization. Fertil Steril **73,** 75–77.

Walker SK, Hill JL, Kleemann DO, Nancarrow CD, 1996: Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. Biol Reprod **55,** 703–708.

Wani NA, Wani GM, Khan MZ, Sidiqi MA, 1999: Effect of different factors on the recovery rate of oocytes for *in vitro* maturation and *in vitro* fertilisation procedures in sheep. Small Rumin Res **34,** 71–76.

Wani NA, Wani GM, Khan MZ, Salahudin S, 2000: Effect of oocyte harvesting techniques on *in vitro* maturation and *in vitro* fertilization in sheep. Small Rumin Res **36,** 63–67.

Webb R, Garnsworthy PC, Gong JG, Armstrong DG, 2004: Control of follicular growth: local interactions and nutritional influences. J Anim Sci **82,** 63–74.

Yie SM, Brown GM, Liu GY, Collins JA, Daya S, Hughes EG, Foster WG, Younglai EV, 1995: Melatonin and steroids in human pre-ovulatory follicular fluid: seasonal variations and granulosa cell steroid production. Hum Reprod **10,** 50–55.

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Abstract: This study investigated the effects of exogenous melatonin on embryo viability and oocyte competence in post-partum undernourished ewes during the seasonal anestrus. At parturition (mid-Feb), 36 adult Rasa Aragonesa ewes were assigned to one of two groups: treated (+MEL) or not treated (-MEL) with a subcutaneous implant of melatonin (Melovine®, CEVA) on the day of lambing. After 45 d of suckling, lambs were weaned, ewes were synchronized using intravaginal pessaries and fed to provide 1.5x (Control, C) or 0.5x (Low, L) times daily maintenance requirements. Thus, ewes were divided into four groups: C-MEL, C+MEL, L-MEL, and L+MEL. At estrus (Day=0), ewes were mated. At Day 5 after estrus, embryos were recovered by mid-ventral laparotomy and classified based on their developmental stage and morphology. After embryo collection, ovaries were recovered and oocytes were classified and selected for use in in vitro fertilization (IVF). Neither diet nor melatonin treatment had a significant effect on ovulation rate and on the number of ova recovered per ewe. Melatonin treatment improved significantly the number of fertilized embryos/corpus luteum (CL) (-MEL: 0.35±0.1, +MEL: 0.62±0.1; $P=0.08$), number of viable embryos/CL (-MEL: 0.23±0.1, +MEL: 0.62±0.1; $P<0.01$), viability rate (-MEL: 46.6%, +MEL: 83.9%; $P<0.05$), and pregnancy rate (-MEL: 26.3%, +MEL: 76.5%; $P<0.05$). In particular, exogenous melatonin improved embryo viability in undernourished ewes (L-MEL: 40%, L+MEL: 100%, $P<0.01$). Neither nutrition nor exogenous melatonin treatments significantly influenced the competence of oocytes during IVF. Treatment groups did not differ significantly in the number of healthy oocytes used for IVF, number of cleaved embryos, or number of blastocysts and, consequently, the groups had similar cleavage and blastocyst rates. In conclusion, melatonin treatments improved ovine embryo viability during anestrus, particularly in undernourished post-partum ewes, although the effects of melatonin did not appear to be mediated at the oocyte competence level.

EFFECTS OF EXOGENOUS MELATONIN ON IN VIVO EMBRYO VIABILITY AND OOCYTE COMPETENCE OF UNDERNOURISHED EWES AFTER WEANING DURING THE SEASONAL ANESTRUS

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Abstract

This study investigated the effects of exogenous melatonin on embryo viability and oocyte competence in post-partum undernourished ewes during the seasonal anestrus. At parturition (mid-Feb), 36 adult Rasa Aragonesa ewes were assigned to one of two groups: treated (+MEL) or not treated (-MEL) with a subcutaneous implant of melatonin (Melovine®, CEVA) on the day of lambing. After 45 d of suckling, lambs were weaned, ewes were synchronized using intravaginal pessaries and fed to provide 1.5x (Control, C) or 0.5x (Low, L) times daily maintenance requirements. Thus, ewes were divided into four groups: C-MEL, C+MEL, L-MEL, and L+MEL. At estrus (Day=0), ewes were mated. At Day 5 after estrus, embryos were recovered by mid-ventral laparotomy and classified based on their developmental stage and morphology. After embryo collection, ovaries were recovered and oocytes were classified and selected for use in *in vitro* fertilization (IVF). Neither diet nor melatonin treatment had a significant effect on ovulation rate and on the number of ova recovered per ewe. Melatonin treatment improved significantly the number of fertilized embryos/corpus luteum (CL) (-MEL: 0.35±0.1, +MEL: 0.62±0.1; P=0.08), number of viable embryos/CL (-MEL: 0.23±0.1, +MEL: 0.62±0.1; P<0.01), viability rate (-MEL: 46.6%, +MEL: 83.9%; P<0.05), and pregnancy rate (-MEL: 26.3%, +MEL: 76.5%; P<0.05). In particular, exogenous melatonin improved embryo viability in undernourished ewes (L-MEL: 40%, L+MEL: 100%, P<0.01). Neither nutrition nor exogenous melatonin treatments significantly influenced the competence of oocytes during IVF. Treatment groups did not differ significantly in the number of healthy oocytes used for IVF, number of cleaved embryos, or number of blastocysts and, consequently, the groups had similar cleavage and blastocyst rates. In conclusion, melatonin treatments improved ovine embryo viability during anestrus, particularly in undernourished post-partum ewes, although the effects of melatonin did not appear to be mediated at the oocyte competence level.

Keywords: embryo, oocyte competence, melatonin, undernutrition, post-partum ewes, anestrus.

1. *Introduction*

As in other livestock, sheep production is strongly conditioned by embryo growth and development, which is an important factor that reflects productive success. Typically, embryo production *in vivo* is used to increase the number of lambs produced by individual females, which often is combined with multiple ovulation and embryo transfer (MOET) programs. Embryo production *in vitro* is an alternative method that provides an inexpensive, abundant source of oocytes (collected in vivo or from ovaries recovered post-mortem at a slaughterhouse) and, subsequently, zygotes and embryos for research in developmental physiology and the commercial application of new biotechniques [1, 2]. Moreover, it increases the capacity for individual uses to produce more embryos when MOET cannot be applied [3, 4].

Reproductive seasonality and nutritional condition are the main factors that influence embryo production in sheep, particularly in the Mediterranean region, where the availability of food is highly seasonal. Nutrition has a significant effect on several aspects of reproduction including hormone production, fertilization, and early embryonic development [5, 6]. The relationship between ewe nutrition and embryo outcomes has not been established conclusively, but undernutrition can compromise follicle-oocyte competence [7], luteal function [8, 9], embryo development [for review, 10], and the sensitivity of the endometrium to progesterone [9].

The luteotrophic effect of exogenous melatonin can have a positive effect on embryo survival in ewes [11, 12] even after superovulation [13]; however, the effects of exogenous melatonin on the in vitro maturation (IVM) and fertilization (IVF) of oocytes is less well established. Stenbak, et al. [14] reported that the fertilization rates of oocytes recovered from superovulated ewes were higher in the reproductive season than they were in anestrus. Abecia et al. [15] summarized the effects of exogenous melatonin on the ovary and early embryos in ewes but many of the effects remain to be characterized.

Reproductive seasonality limits reproductive efficiency in sheep production systems [16] and the resumption of ovarian activity after lambing is influenced by numerous factors, particularly season of parturition [17, 18] and nutrition [19]. In temperate areas, if lambing occurs near the end of the reproductive period, the postpartum anestrus will overlap the seasonal anestrus [20]; however, exogenous melatonin can advance the breeding season [21] and improved nutrition can increase ovulation rates after the resumption of ovarian activity early in the breeding season [22]. Rasa Aragonesa, a Spanish genotype that has a short seasonal anestrous period (<100 d) between May and Jul [23], is well adapted for intensive production systems and an accelerated lambing rate (typically, three lambings in two years). Conception rate and prolificacy in the seasonal anestrous period, 2-3 months after lambing, are important to the profitability of sheep farms. In this study, we evaluated the effect of exogenous melatonin on embryo viability and oocyte competence in post-partum undernourished Rasa Aragonesa ewes during the early anestrous season.

2. *Materials and methods*

The experiment was conducted at the Experimental Farm of the University of Zaragoza, Spain (41º 41' N). All procedures were approved by the in-house Ethics Committee for Animal Experiments, University of Zaragoza. The care and use of the animals followed the Spanish Policy for Animal Protection RD1201/05, which meets the requirements of the European Union Directive 86/609 on the Protection of Animals used for Experimental and Other Scientific Purposes.

2.1. *Animals and experimental design*

The experiment involved 36 adult, non-cycling, post-partum Rasa Aragonesa ewes mean (±SEM) live weight (LW) = 61.0 ± 1.4 kg; mean body condition (BC) score [24] =2.9 ± 0.07. At parturition (mid-Feb), ewes were assigned either to a group that received a subcutaneous melatonin implant (18 mg melatonin, Melovine®, CEVA Salud Animal, Barcelona, Spain) (+MEL, n=17) or to a non-implanted group (-MEL, n=19). Ewes suckled single lambs for 45 d and, during lactation, all of the

ewes received a diet that provided the daily requirements for one lamb [25]. In mid-Apr, 55 d after melatonin implantation, ewes received intravaginal progestagen pessaries for 14 d and 440 IU eCG (Sincropart® PMSG, Ceva Salud Animal S.A.) at pessary withdrawal. From pessary insertion to slaughter (Day 5 after estrus), ewes were offered one of two diets: 1.5 (control, C) or 0.5 (low, L) times the daily maintenance requirements [25] and unrestricted access to water. To monitor estrous behavior, from 24 h after pessary withdrawal, intact rams wearing harnesses fitted with marking crayons were introduced once every 8 h (Day 0). Thus, the four groups were C-MEL (C diet, non-implanted); C+MEL (C diet, implanted); L-MEL (L diet, non implanted), and L+MEL (L diet, implanted).

To confirm anestrous, we measured plasma progesterone concentrations in blood samples collected from each of the ewes at 7 and 14 d before weaning. Ewes that had progesterone levels >1 ng/mL in at least one of their blood samples were considered cyclic and were excluded from the experiment. To quantify, metabolite and progesterone concentrations, jugular blood from the non-cycling ewes (n=36) was collected in heparinised tubes on Days 0, 3, and 5. In addition, to test the effectiveness of the melatonin implants, one diurnal (noon) blood sample was collected from each of the melatonin-implanted ewes at 40 d after implantation. Blood samples were centrifuged within 15 min of collection (1000G, 10 min) and plasma was stored at -20ºC until analysis.

From parturition until weaning, ewes fed a lactation diet each received 0.8 kg of commercial pellets (Ovinanta-Triplex®, Nanta, España), 1 kg of alfalfa hay, and 0.3 kg of barley straw per day, which provided 4.0 Mcal of metabolizable energy (ME). From the end of weaning and the beginning of the experimental diets, ewes received 0.45 kg of pellets and 0.55 kg of barley straw per day, which provided 2.0 Mcal of ME and 12% crude protein (CP). During the experimental diet period, the C diet provided 0.7 kg of pellets and 0.85 kg of barley straw per ewe per day, which provided 3.0 Mcal of ME and 12% CP. The L diet comprised 0.25 kg of pellets and 0.30 kg of barley straw per ewe per day (1.0 Mcal of ME and 11% CP). Pellets contained barley (65%), soybean (30%), and a

121 mineral supplement (5%). Live weight and BC were recorded at parturition, weaning, pessary
122 insertion, pessary withdrawal, and slaughter.
123
124 2.2. *Embryo Recovery*
125
126 On Day 5, 20 d after the start of the experimental diets, ewes were anaesthetized using an i.m.
127 injection of 0.4 mL 2% xylazine (Xilagesic 2%®, Calier, Barcelona, Spain) and, 5 min later, an iv
128 injection of 10 mL of sodium thiopental (20 mg⁄mL) (Thiobarbital®, Braun Medical, Jaén, Spain).
129 After a mid-ventral laparotomy, the uterine horns were flushed with pre-warmed (36 ºC)
130 phosphate-buffered saline (PBS) to collect the embryos. The ovarian response was quantified using
131 the number of corpora lutea (CL) that were morphologically sound and consistent with an active
132 luteal phase. Ova and embryos were examined and classified [26]. Morulae and compacted
133 morulae were considered viable embryos, depending on the day of pregnancy on which the
134 embryos were recovered [26]. For each ewe, we recorded the following: number of corpora lutea
135 (CL), number of recovered ova (oocytes + embryos), number of total embryos per CL, and number
136 of viable embryos per CL. Fertilization rate is the number of embryos divided by the number of ova
137 recovered. Viability rate is the number of viable embryos divided by the number of ova recovered.
138 All rates are expressed as proportions (%).
139
140 2.3. *Collection and in vitro maturation of oocytes*
141
142 After embryos were collected, ewes were euthanized using an iv injection of sodium thiopental (T-
143 61®; Intervet, Salamanca, Spain). Ovaries were recovered and placed in PBS supplemented with
144 100 IU⁄mL of penicillin-G and 100 µg⁄mL of streptomycin sulphate at 39 ºC until they were
145 analyzed. Except where indicated otherwise, all of the reagents were from Sigma-Aldrich Co., St.
146 Louis, MO, USA. A combination of puncture and slicing techniques [27] were used to collect
147 oocytes into a Petri dish that contained a handling medium (Hepes-buffered TCM-199
148 supplemented with 0.1 % polyvinyl alcohol (PVA), 0.04 % sodium bicarbonate, 25 IU⁄mL of

heparin, 100 IU⁄mL of penicillin-G, and 100 µg⁄mL of streptomycin sulphate). Following Wani et al. [28], oocytes were classified based on their cumulus cells and cytoplasm morphology, as follows: Good: oocytes with many complete layers of granulose cells and homogeneous cytoplasm; Fair: oocytes with few or incomplete layers of granulose cells and homogeneous cytoplasm; Poor: oocytes with few or an absence of granulose cells and non-homogeneous cytoplasm. Only oocytes that had several layers of cumulus cells and a uniform cytoplasm (Good and Fair, called healthy oocytes) were selected for in vitro maturation (IVM). Healthy oocytes were transferred into a maturation medium that contained bicarbonate-buffered TCM-199 supplemented with 10% (v:v) estrous sheep serum, 0.1 µg⁄mL each of FSH and LH, 100 µM of cysteamine, 0.3 µM of sodium pyruvate, 100 IU⁄mL of penicillin G, and 100 µg/mL of streptomycin sulphate, which was covered with mineral oil and incubated at 39 ºC under 5% CO2 and saturated humidity for 24 h.

2.4. *In vitro fertilization (IVF) and embryo cultures*

At the end of IVM, oocytes were freed from the cumulus cells and transferred to the fertilization medium, which consisted of synthetic oviductal fluid (SOF) without glucose [29] and supplemented with 2% (v:v) of estrous sheep serum, 10 µg⁄mL of heparin, and 1 µg⁄mL of hypotaurine. On the same day as fertilization, semen was collected from four Rasa Aragonesa rams, pooled, diluted 1:10 in a saline medium with 0.25 mol⁄L of sucrose, 10 mmol⁄L of Hepes, 2 mmol⁄L of potassium hydroxide, 5 mmol⁄L of glucose, 0.5 mol⁄L of sodium phosphate monobasic, and 100 mmol⁄L of ethylene glycol tetra-acetic acid (EGTA), and kept at 15 ºC until fertilization. Highly motile spermatozoa were selected using the swim-up technique [28, 30]. The fertilization dose was $1x10^{-6}$ spermatozoa⁄mL, and the sperm were added to the fertilization medium that contained the oocytes, covered with mineral oil, and incubated for 24 h at 39 ºC in an atmosphere of 5% CO2, 5% O2 and 90% N2. At 24 h and 36 h after fertilization, cleaved embryos were placed in a culture medium that contained SOF supplemented with essential and non-essential amino acids at oviductal concentrations [31], 0.4% bovine serum albumin (wt⁄vol), 1 mM of L-glutamine, 100 IU⁄mL of penicillin G, and 100 µg⁄mL of streptomycin sulphate, and covered with mineral oil and

kept at 39 C in a maximally humidified atmosphere of 5% CO2, 5% O2 and 90% N2 for 8 d, until the blastocyst stage. After fertilization, to assess their stage of maturation, the uncleaved oocytes were examined under a stereomicroscope. Oocytes that exhibited the first polar body were considered mature, and oocytes that had two polar bodies were considered fertilized, but not cleaved. The following information was recorded for each animal in each experimental group: ovulation rate (expressed as the number of corpora lutea observed at Day 5 per ewe), number of recovered oocytes (including all recovered oocytes), number of healthy oocytes (including only those recovered oocytes that were rated as Good or Fair), healthy oocyte rate (number of healthy oocytes divided by the number of recovered oocytes), number of non-healthy oocytes, number of cleaved embryos, cleavage rate (number of cleaved embryos divided by the number of healthy oocytes), number of blastocysts, and blastocyst rate (number of blastocysts divided by the number of cleaved embryos). All values are expressed per ewe and all rates are expressed as proportions (%).

2.5. *Metabolite assays*

From the start of the experimental diets until slaughter, plasma samples were collected every 2 d. Plasma glucose and non-esterified fatty acids (NEFA) were determined using an autoanalyzer (Gernonstar®, Transasia, Bombay, India). Glucose was analyzed using an enzymatic AA kit (Gernon®, RAL for Laboratories Techniques, Barcelona, Spain). The intra- and inter-assay coefficients of variation (CV) were 2.2% and 3.1%, respectively. Non-esterified fatty acids were quantified using commercially available kits (NEFA®, Randox Laboratories Ltd., Crumlin, UK). The intra- and inter-assay CVs were 11.7% and 13.0%, respectively.

2.6. *Hormone assays*

Plasma melatonin concentrations were measured using a direct, solid-phase radioimmunoassay (RIA) and a commercially available kit (Melatonin direct RIA; IBL International GMBH,

Hamburg, Germany) within a single assay. The sensitivity of the assay was 0.9 pg⁄mL and the intra-assay CV was 5.5% for low control concentrations (9.0 pg⁄mL) and 9.5% for high control concentrations (110.0 pg⁄mL).

Plasma progesterone concentrations were measured using a direct, solid-phase RIA and a commercially available kit (Count-A-Count TKPG; Siemens, Los Angeles, USA). The RIA had a sensitivity of 0.02 ng/mL. The intra-assay CV was 9.3% for low control concentrations (3 ng⁄mL), 5.8% for medium concentrations (15 ng⁄mL), and 4.2% for high concentrations (30 ng⁄mL). For each of the standard concentrations, the interassay CV was <10%.

2.7. *Statistical analyses*

The experiment was based on a 2x2 factorial design in which nutritional level and melatonin treatment were fixed effects. The effects of the treatments on the development and quality of *in vivo* embryos and oocytes and blastocysts were evaluated statistically using the PROC GEN MOD [32] with the Poisson distribution specified in a model that included nutrition level (low or control), melatonin treatment (with or without melatonin implant), and their interactions. Statistical comparisons of proportional values were based on the Chi-squared Test. The results are expressed as mean ± SEM, and the level for statistical significance was set to $P < 0.05$.

3. *Results*

3.1. *Live weight and body condition*

In the period of the experimental diets (21 d), the mean LW of ewes in the C groups did not change significantly (C-MEL: 56.3 ±2.9 to 57.5 ±3.0 kg; C+MEL: 54.4 ±2.4 to 55.0 ±1.9, NS), but, ewes in the L groups lost, on average >5 kg (L-MEL: 54.4 ±2.9 to 50.2 ±2.7 kg, L+MEL: 55.8 ±2.2 to 49.8 ±2.2; $P<0.01$) (Figure 1). In addition, L ewes experienced a significant reduction in their BC

(P<0.01). After pessary withdrawal (14 d after the onset of the experimental diets), the mean LW and BC of L ewes were significantly lower than those of the C ewes (*P*<0.01).

Appropriate position for Figure 1.

3.2. *Circulating metabolites*

Overall mean plasma glucose concentration was significantly (P<0.01) higher in the C groups (64.4 ±0.5 mg/dL) than in the L groups (61.8 ±0.5 mg/dL), and significant (P<0.01) differences between groups were apparent at Days 15, 18, and 21 of the experimental diets (Figure 2A). In all of the groups, glucose concentrations were highest just before estrus at Day -1 (Day 0=estrus) and decreased gradually until Day 5 in the L-MEL and L+MEL groups, only (Figure 1A). Exogenous melatonin did not have a noticeable effect on glucose levels.

In parallel with the reductions in live weight among ewes in the undernourished groups, in the course of the experimental diets, plasma NEFA concentrations increased rapidly in the L groups, which were five to six times higher than they were in the C-MEL and C+MEL groups (C: 0.05±0.01 vs. L: 0.30±0.01 mmol/L; P<0.01) (Figure 2B). Exogenous melatonin did not have a noticeable effect on plasma NEFA levels.

Appropriate position for Figure 2.

3.3. *Circulating progesterone and ovulation rate*

In the four groups of ewes, plasma progesterone concentrations increased gradually after estrus, which confirmed that all CL on Day 5 (slaughter) were reflective of an active luteal phase. In addition, P4 concentrations at Day 5 tended to be higher in the L ewes than in the C ewes (L diets: 3.6 ±0.33, C diets: 2.7 ±0.4 ng/mL; P<0.1) and higher in the melatonin-implanted ewes than in the

non-implanted ewes (+MEL: 3.48 ±0.34, -MEL: 2.81 ±0.36 ng/mL; P<0.1) (Figure 3). Ovulation rate did not differ significantly among treatments (Table 1).

Appropriate position for Figure 3.

3.4. *Embryo recovery*

Exogenous melatonin tended to increase the number of total embryos per CL (-MEL: 0.35±0.1, +MEL: 0.62±0.1; P=0.08), which was essential for a significant increase in the number of viable embryos per CL (-MEL: 0.23±0.1, +MEL: 0.62±0.1; P<0.01), viability rate (-MEL: 46.6, +MEL: 83.9%; P<0.05), and pregnancy rate (-MEL: 26.3, +MEL: 76.5%; P<0.05). The effect was particularly evident in undernourished ewes, where melatonin implants increased significantly the number of viable embryos (L-MEL: 0.2±0.1, L+MEL: 0.6±0.1; P<0.05) and viability rate (L-MEL: 40, L+MEL: 100%, P<0.01) (Table 1). Neither nutrition treatment nor the interaction between nutrition and melatonin treatment had a significant effect on any of the parameters evaluated.

Appropriate position for Table 1.

3.5. *In vitro* development of oocytes (IVM and IVF)

Neither nutrition, exogenous melatonin, nor the interaction between nutrition and melatonin treatments had a significant effect on any of the parameters evaluated (Table 2). Nor did nutrition or melatonin treatments significantly influence oocyte competence. The numbers of cleaved embryos and blastocysts were similar among treatment groups.

Appropriate position for Table 2.

4. ***Discussion***

This study investigated the effects of exogenous melatonin on *in vivo* embryo viability and *in vitro* oocyte competence of undernourished Rasa Aragonesa ewes lambing in early anestrus. Exogenous melatonin had a beneficial effect on *in vivo* embryo viability, particularly in undernourished ewes, but did not have a significant effect on *in vitro* oocyte competence.

In our experiment, among undernourished ewes, live weight and body condition decreased and NEFA concentrations increased significantly, which reflected an increase in lipolytic activity. Research has shown that mature ewes subject to energetic restrictions similar to those in our experiment for 3-4 weeks exhibited a significant reduction in LW and BC [33, 34] and had higher lipolytic activity than did ewes in control groups [35]; however, short-term undernutrition associated with a progestagen-synchronized estrus, did not appear to impair ovulation rate in either the reproductive season [33, 34, 36] or in the seasonal anestrous period [35, 37].

Although nutritional treatments did not have a significant effect on most of the parameters evaluated in our study, exogenous melatonin significantly improved the viability of *in vivo* embryos on Day 5 after estrus. Thus, despite differences in nutritional levels, pregnancy rates on Day 5 after estrus were significantly higher in the melatonin-implanted groups than they were in the non-implanted groups. Previous studies have shown that supplemental melatonin in the seasonal anestrus can improve embryo viability in superovulated [13] and non-superovulated ewes [37]. The luteotrophic effect of the pineal hormone observed *in vivo* [11] and *in vitro* [12], and the effects of melatonin at the hypothalamic-hypophyseal level [38] might be involved in the melatonin-induced improvement in embryo viability during anestrus. Our study appears to provide additional evidence of those luteotrophic effects because melatonin-implanted ewes exhibited a trend toward having P4 concentrations that were higher than those in non-implanted animals. The high P4 levels at Day 5 after estrus might be responsible, at least in part, for the greater embryo viability and pregnancy rates in the melatonin-implanted ewes.

316 Although undernutrition did not significantly influence in vivo embryo viability, the beneficial
317 effects of melatonin were relatively greater in the undernourished ewes. Undernutrition is common
318 in extensive pastoral and intensive-accelerated reproductive sheep farms, and undernutrition
319 impairs the expression of endometrial steroid receptors and binding capacity in the early luteal
320 phase, which compromises embryo survival [9, 35]. In addition to increasing progesterone
321 production, exogenous melatonin might improve uterine expression of progesterone receptors or
322 their binding capacity, which would result in higher reproductive efficiency in undernourished
323 ewes during the seasonal anestrous period.
324
325 In this study, glucose concentrations were significantly lower in undernourished ewes than in
326 control ewes. Moreover, plasma glucose concentrations increased coincident with the sampling just
327 before estrous, which was more pronounced in the control groups and less pronounced in the
328 underfed groups. Sosa et al. [35] found that the patterns of secretion of glucose, insulin, and IGF-I
329 were similar, which they interpreted as a response to stress induced by the estrous detection
330 procedures, which provokes a cortisol release and a peripheral insulin resistance that leads to a
331 reduction in glucose uptake [39]. Alternatively, however, the pattern of secretion might be
332 associated with an increase in glucose uptake at the level of the pituitary, which is necessary in the
333 follicular phase of the cycle to increase basal LH concentrations and the pituitary response to
334 oestradiol, which is responsible for the subsequent preovulatory LH surge and ovulation [40].
335
336 We expected that exogenous melatonin or nutritional treatment would affect *in vitro* embryo
337 production in Rasa Aragonesa ewes. Some studies did not find that exogenous melatonin had a
338 significant positive effect on the number of oocytes recovered from anoestrus FSH-superovulated
339 ewes [30] and goats [41], but treatments with exogenous melatonin seemed to improve the
340 developmental competence for the IVM-IVF of oocytes recovered from progestagen and eCG-
341 treated ewes [42] and superovulated goats [41, 43]. In addition, our studies with a similar
342 experimental design with non-pregnant Rasa Aragonesa ewes showed that supplemental melatonin

during the anoestrus increased the number of cleaved embryos in undernourished ewes and blastocyst rates, independently of the level of nutrition [44].

Several studies have reported impaired in vitro developmental competence of oocytes from superovulated [45, 46] and non-superovulated undernourished ewes [44]; thus, it is surprising that, in our study, undernutrition did not impair oocyte IVF competence. Undernutrition is associated with impaired follicular competence [47] and lower oviductal and endometrial sensitivity to ovarian steroids [35, 48], which compromises early embryo development. In our study, the absence of effects of undernutrition on the IVF parameters might be partly due to the physiological condition of the ewes. During the post-partum period, metabolic state can influence the repartitioning of nutrients and their use in various pathways through homeorhesis or the prioritization of the distribution of energy to meet specific physiological demands [49].

In summary, our study demonstrated that treatment with exogenous melatonin at lambing during the anestrous period improves the viability of ovine embryos, particularly after a period of undernutrition, although the effect of melatonin seems not to be mediated at the oocyte competence level.

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References

[1] O'Brien JK, Hollinshead FK, Maxwell WMC. In vivo development capacity of in vitro-produced embryos derived from sex-sorted and re-cryopreserved frozen.thawed ram sperm. Reprod Fertil Dev 2004; 16:286.

[2] Baldasarre H, Wang B, Kafidi N, Keefer C, Lazaris A, Karatzas CN. Advances in the production and propagation of transgenic goats using laparoscopic folliculocentesis: alternative gonadotropin treatments for stimulation of oocyte donors. Theriogenology 2002; 57:275-284.

[3] González-Bulnes A, Baird DT, Campbell BK, Cocero MJ, García-García RM, Inskeep EK, López-Sebastián A, McNeilly AS, Santiago-Moreno J, Souza CJH, Veiga-López A. Multiple factors affecting the efficiency of multiple ovulation and embryo transfer in sheep and goats. Reprod Fertil Dev 2004; 16:421-435.

[4] Cognié Y, Poulin N, Locatelli Y, Mermillod P. State-of-the-art production, conservation and transfer of in-vitro-produced embryos in small ruminants. Reprod Fertil Dev 2004; 16:437-445.

[5] Boland MP, Lonergan P, O'Callaghan D. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology 2001; 55:1323-1340.

[6] Boland MP, Lonergan P. Effects of nutrition on fertility on dairy cows. Adv Dairy Techn 2005; 15:19-33.

[7] O'Callaghan D, Yaakub H, Hytell P, Spicer LJ, Boland MP. Effect of nutrition and superovulation on oocyte morphology, follicular fluid composition and systemic hormone concentrations in ewes. J. Reprod Fertil 2000; 18:303-313.

[8] Jabbour HN, Ryan JP, Evans G, Maxwell WMC. Effect of season, GnRH administration and lupin supplementation on the ovarian and endocrine responses of merino ewes treated with PMSG and FSH-P to induce superovulation. Reprod Fertil Dev 1991; 3:699-707.

[9] Sosa C, Lozano JM, Viñoles C, Acuña S, Abecia JA, Forcada F, Forsberg M, Meikle A. Effect of plane of nutrition on endometrial sex steroid receptor expression in ewes. Anim Reprod Sci 2004; 84:337–348.

[10] Abecia JA, Sosa C, Forcada F, Meikle A. The effect of undernutrition on the establishment of pregnancy in the ewe. Reprod Nutr Dev 2006; 46:367-378.

396 [11] Durotoye LA, Webley GE, Rodway RG. Stimulation of the production of progesterone by
397 the corpus luteum of the ewe by the perfusion of melatonin in vivo and by treatment of granulosa
398 cells with melatonin in vitro. Res Vet Sci 1997; 62:87-91.
399 [12] Abecia JA, Forcada F, Zuñiga O. The effect of melatonin on the secretion of progesterone
400 in sheep and on the development of ovine embryos in vitro. Vet Res Commun 2002; 26:151-158.
401 [13] Forcada F, Abecia JA, Cebrián-Perez JA, Muiño-Blanco T, Valares JA, Palacin I, Casao A.
402 The effect of melatonin implants during the seasonal anestrus on embryo production after
403 superovulation in aged high-prolificacy Rasa Aragonesa ewes. Theriogenology 2006; 65:356-365.
404 [14] Stenbak TK, Redmer DA, Berginski HR, Erickson AS, Navanukraw C, Toutges MJ.
405 Effects of follicle stimulating hormone (FSH) on follicular development, oocyte retrieval, and in
406 vitro fertilization (IVF) in ewes during breeding season and seasonal anestrus. Theriogenology
407 2001; 56:51–64.
408 [15] Abecia JA, Forcada F, Casao A, Palacín I. Effect of exogenous melatonin on the ovary, the
409 embryo and the establishment of pregnancy in sheep. Animal 2008; 2:399-404.
410 [16] Martin GB, Rodger J, Blache D. Nutritional and environmental effects on reproduction in
411 small ruminants. Reprod Fertil Dev 2004; 16:491-501.
412 [17] Pope WF, McClure KE, Hogue DE, Day ML. Effect of season and lactation on postpartum
413 fertility of Polipay, Dorset, St. Croix and Targhee ewes. J Anim Sci 1989; 67:1167–1174.
414 [18] Delgadillo JA, Flores JA, Villarreal O, Flores MJ, Hoyos G, Chemineau P, Malpaux B.
415 Length of postpartum anestrus in goats in subtropical Mexico: effect of season of parturition and
416 duration of nursing. Theriogenology 1998; 49:1209–1218.
417 [19] Mbayahaga J, Mandiki SNM, Bister JL, Paquay R. Body weight oestrous and ovarian
418 activity in local Burundian ewes and goats after parturition in the dry season. Anim Reprod Sci
419 1998; 51:289–300.
420 [20] Fitzgerald BP, Cunningham FJ. Effect of removal of lambs or treatment with bromocriptine
421 on plasma concentrations of prolactin and FSH during the post-partum period in ewes lambing at
422 different times during the breeding season. J Reprod Fertil 1981; 61:141–148.

[21] Forcada F, Zarazaga L, Abecia JA. Effect of exogenous melatonin and plane of nutrition after weaning on estrous activity, endocrine status and ovulation rate in Salz ewes lambing in the seasonal anestrus. Theriogenology 1995; 43:1179–1193.

[22] Abecia JA, Forcada F, Zarazaga L, Lozano JM. Effect of plane of protein after weaning on resumption of reproductive activity in Rasa Aragonesa ewes lambing in late spring. Theriogenology 1993; 39:463-473.

[23] Forcada F, Abecia JA, Sierra I. Seasonal changes in oestrus activity and ovulation rate in Rasa Aragonesa ewes maintained at two different body condition levels. Small Ruminant Research 1992; 8:313-324.

[24] Russell AJF, Doney JM, Gunn RG. Subjective assessment of body fat in live sheep. J Agric Sci Camb 1969; 72:451-454.

[25] Agricultural and Food Research Council, 1993. Energy and protein requirements of ruminants by the AFRC Technical Committee. CAB International, Wallingford, United Kingdom.

[26] Winterberger-Torres, S., Sevellec, C., 1987. Atlas of the Early Development of the Sheep Embryo. INRA, Paris, France.

[27] Wani NA, Wani GM, Khan MZ, Sidiqi MA. Effect of different factors on the recovery rate of oocytes for in vitro maturation and in vitro fertilization procedures in sheep. Small Rum Res 1999; 34:71-76.

[28] Wani NA, Wani GM, Khan MZ, Salahudin S. Effect of oocyte harvesting techniques on in vitro maturation and in vitro fertilization in sheep. Small Rum Res 2000; 36:63-67.

[29] Tervit, H.R., Whittingham, D.G., 1972. Successful culture in vitro of sheep and cattle ova. J. Reprod. Fert. 30, 493-497.

[30] Luther JS, Redmer DA, Reynolds LP, Choi JT, Pant D, Navanukraw C, Arnold DR, Scheaffer AN, Borowicz P, Kirsch JD, Weigl RM, Kraft KC, Grazul-Bilska AT. Ovarian follicular development and oocyte quality in anestrous ewes treated with melatonin, a controlled internal drug release (CIDR) device and follicle stimulating hormone. Theriogenology 2005; 63:2136–2146.

450 [31] Walker SK, Hill JL, Kleemann DO, Nancarrow CD. Development of ovine embryos in
451 synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. Biol Reprod
452 1996; 55:703–708.
453 [32] SAS Institute Inc., 1999. SAS/STAT. Software Version 8. SAS Institute Inc., Cary, NC,
454 USA.
455 [33] Abecia JA, Lozano JM, Forcada F, Zarazaga L. Effect of level of dietary energy and protein
456 on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa
457 ewes. Anim Reprod Sci 1997; 48:209–218.
458 [34] Abecia JA, Forcada F, Lozano JM. A preliminary report on the effect of dietary energy on
459 prostaglandin F2 production in vitro, interferon-tau synthesis by the conceptus, endometrial
460 progesterone concentration on days 9 and 15 of pregnancy and associated rates of embryo wastage
461 in ewe. Theriogenology 1999; 52:1203–1213.
462 [35] Sosa C, Abecia JA, Forcada F, Viñoles C, Tasende C, Valares JA, Palacin I, Martin GB,
463 Meikle A. Effect of undernutrition on uterine progesterone and oestrogen-receptors and on
464 endocrine profiles during the ovine oestrous cycle. Reprod Fert Dev 2006; 18:447–458.
465 [36] Borowczyk E, Caton JS, Redmer DA, Bilski JJ, Weigl RM, Vonnahme KA, Borowicz PP,
466 Kirsch JD, Kraft KC, Reynolds LP, Grazul-Bilska AT. Effects of plane of nutrition on in vitro
467 fertilization and early embryonic development in sheep. J Anim Sci 2006; 84:1593–1599.
468 [37] Vázquez MI, Forcada, F, Casao A, Sosa C, Palacín I, Abecia JA. Effects of melatonin and
469 undernutrition on the viability of ovine embryos during anestrus and the breeding season. Anim
470 Reprod Sci 2009; 112:83-94.
471 [38] Malpaux B, Viguié C, Skinner DC, Thiéry JC, Chemineau P. Control of the circannual
472 rhythm of reproduction by melatonin in the ewe. Brain Res. Bull. 1997; 44:431-438.
473 [39] Elsasser TH, Klasing KC, Filipov N, Thompson F. The metabolic consequences of stress
474 and priorities of nutrient use. In: The biology of animal stress: Basic principles and implications for
475 animal welfare, Moberg GP, Mench JA (Eds.), CAB International, Oxon, UK, 2000, pp. 77-110.
476 [40] I'anson H, Foster DL, Foxcroft GR, Booth PJ. Nutrition and reproduction. Oxford Reviews
477 of Reproductive Biology 1991; 13:239-311.

[41] Berlinguer F, Gonzalez-Bulnes A, Succu S, Leoni G, Mossa F, Bebbere D, Ariznavarreta C, Tresguerres JAF, Veiga-Lopez A, Naitana S. Effects of progestagens on follicular growth and oocyte developmental competence in FSH-treated ewes. Dom Anim Endocrin 2007; 32:303-314.

[42] Valasi F, Tsiligianni T, Papanikolaou T, Dimitriadis I, Vainas E, Samartzi F, Amiridis GS. Melatonin improves the developmental competence of sheep oocytes. Reprod Dom Anim 2006; 41:341.

[43] Berlinguer F, Leoni G, Succu S, Spezzigu A, Madeddu M, Satta V, Bebbere D, Contreras-Solis I, Gonzalez-Bulnes A, Naitana S. Exogenous melatonin positively influences follicular dynamics, oocyte developmental competence and blastocyst output in a goat model. J Pineal Res 2009; 46:383-391.

[44] Vázquez MI, Forcada F, Casao A, Abecia JA, Sosa C, Palacín I. Undernutrition and Exogenous Melatonin Can Affect the In Vitro Developmental Competence of Ovine Oocytes on a Seasonal Basis. Reprod Dom Anim 2009; doi: 10.1111/j.1439-0531.2008.01329.

[45] Papadopoulos S, Lonergan P, Gath V, Quinn KM, Evans ACO, O'Callaghan D, Boland MP. Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. Theriogenology 2001; 55:1059-1069.

[46] Borowczyk E, Caton JS, Redmer DA, Bilski JJ, Weigl RM, Vonnahme KA, Borowicz PP, Kirsch JD, Kraft KC, Reynolds LP, Grazul-Bilska AT. Effects of plane of nutrition on in vitro fertilization and early embryonic development in sheep. J Anim Sci 2006; 84:1593–1599.

[47] Scaramuzzi RJ, Campbell BK, Downing JA, Kendall NR, Khalid M, Munoz-Gutierrez M, Somchit A. A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. Reprod Nutr Dev 2006; 46:339-354.

[48] Sosa C, Abecia JA, Forcada F, Meikle A. Undernutrition reduces the oviductal mRNA expression of progesterone and oestrogen receptors in sheep. Vet J 2008; 175:413–415.

[49] Bauman DE, Curie WB. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. J Dairy Sci 1980; 63:1514-1529.

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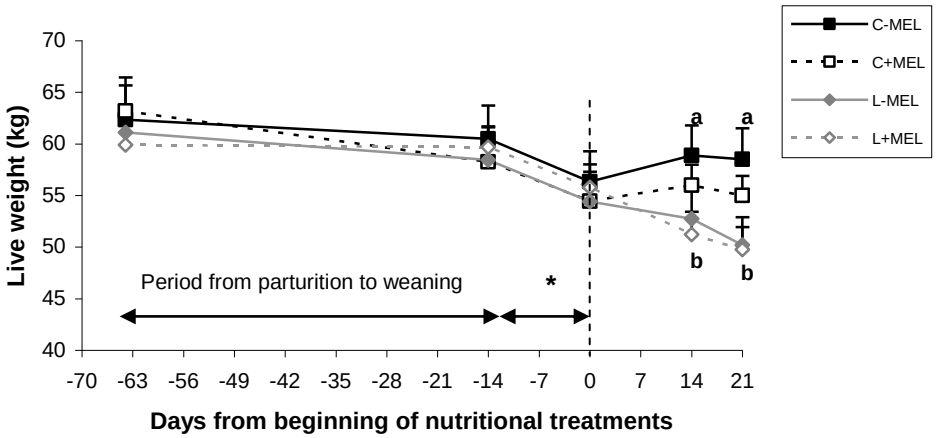
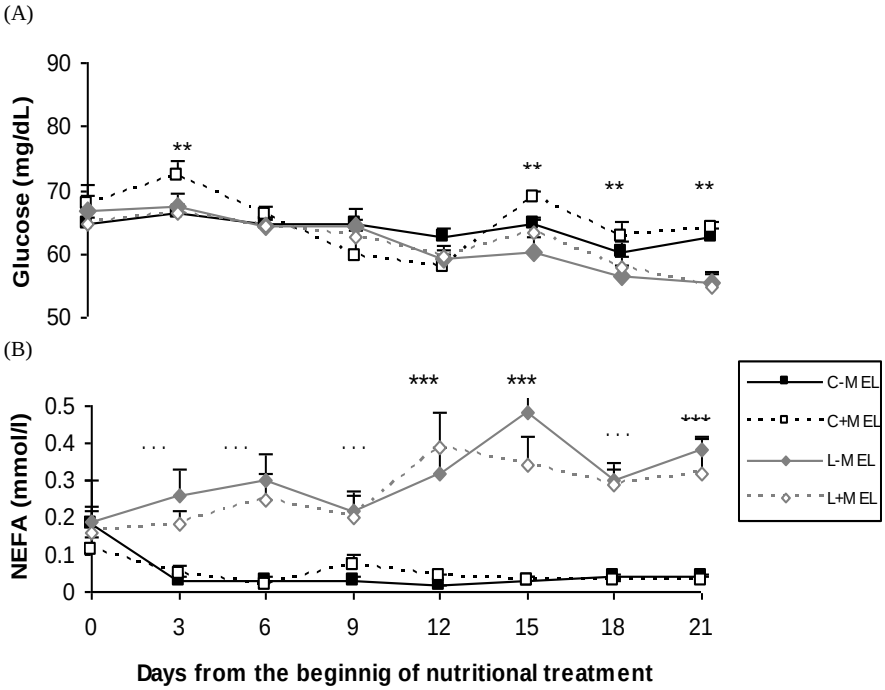


507

508 **Figure 1.** Development of live weight in the postpartum period of Rasa Aragonesa ewes
509 lambing in early anestrus, fed either 1.5x (C) and 0.5x (L) the maintenance requirements and
510 treated (+MEL) or not (-MEL) with melatonin implant during the anestrus.
511 Vertical dotted line indicates the beginning of nutritional treatment (Day 0). (*) indicate transitional
512 period from weaning to nutritional treatment. (a-b) indicate significant differences between C and L
513 groups ($P<0.01$).

514



515

516 **Figure 2.** Plasma concentrations of glucose (A) and non-esterified fatty acids (B) in
517 postpartum Rasa Aragonesa ewes fed either 1.5x (C) and 0.5x (L) the maintenance
518 requirements and treated (+MEL) or not (-MEL) with melatonin implant during the anestrus.
519 (**) $P<0.001$ and (***) $P<0.0001$ between C and L groups.

520

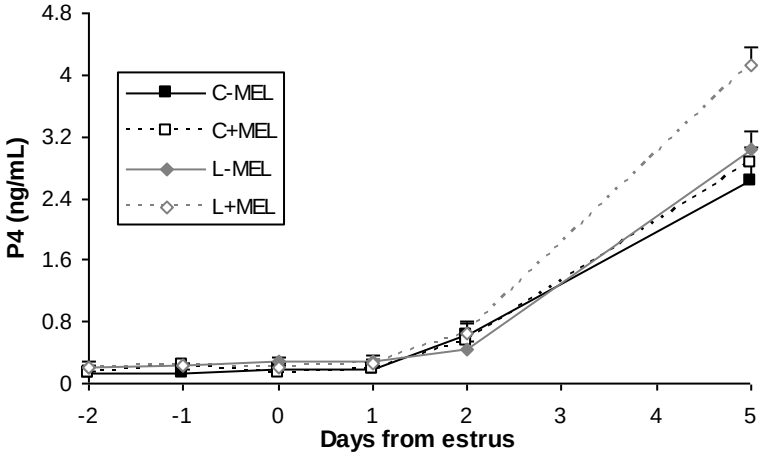


Figure 3. Plasma progesterone (P4) concentrations from pessary withdrawal (Day -2) to embryo collection (Day 5) in post-partum Rasa Aragonesa ewes fed either 1.5x (C) and 0.5x (L) the maintenance requirements and treated (+MEL) or not with melatonin implant during the anestrus. On Day 5, significant differences were observed between L-MEL and L+MEL; and between C+MEL and L+MEL ($P<0.05$).

Table 1. Ovarian response and embryo production of anestrous post-partum Rasa Aragonesa ewes fed either 1.5x (C) or 0.5x (L) times the maintenance requirements and treated (+MEL) or not treated (-MEL) with melatonin at lambing, 74 d before embryo recovery.

	GROUP			
	C-MEL	**C+MEL**	**L-MEL**	**L+MEL**
No. of ewes	9	8	10	9
No. of ewes in estrus	9/9	8/8	10/10	9/9
Ovulation rate	1.8±0.3	1.6±0.2	1.4±0.2	1.9±0.4
No. of recovered ova/CL	0.4±0.1 [c]	0.8±0.1 [d]	0.5±0.1	0.6±0.1
No. of total embryos/CL	0.3±0.1 [c]	0.6±0.1 [d]	0.4±0.1	0.6±0.1
Fertilization rate (%)	75.0	75.0	80.0	100
No. of viable embryos/CL	0.3±0.1 [c]	0.6±0.1 [d]	0.2±0.1 [a]	0.6±0.1 [b]
Viability rate (%)	75.0	75.0	40.0 [a]	100 [b]
Pregnancy rate (%)*	33.3 (3/9)[a]	75.0 (6/8) [b]	20.0 (2/10) [a]	77.7 (7/9) [b]

*Different superscripts (a, b) in the same row indicate significant differences (P<0.05). Different superscripts (c, d) in the same row indicate differences of P<0.1. *Percentage of ewes with viable embryos on Day 5.*

Table 2. Results of the in vitro fertilization (IVF) of oocytes collected from anestrous post partum Rasa Aragonesa ewes that were fed to provide 1.5x (C) or 0.5x (L) the maintenance requirements and treated (+MEL) or not (-MEL) with melatonin during 74 d before ovary collection.

	C-MEL	**C+MEL**	**L-MEL**	**L+MEL**
No. of ovulating ewes with functional CL	9	8	10	9
Ovulation rate	1.8±0.3	1.6±0.2	1.4±0.2	1.9±0.4
No. of recovered oocytes	23.3±3.5	23.7±3.7	24.5±3.4	22.2±3.5
No. of healthy oocytes used for IVF	15.2±2.2	14.4±2.4	16.5±2.1	12.2±2.2
Healthy oocytes rate (%)	65.7±3.9	62.8±4.1	67.9±3.7 [a]	56.3±3.9 [b]
No. of non-healthy oocytes	8.1±1.8	9.4±1.9	8.0±1.8	10.0±1.9
No. of cleaved embryos	9.4±1.9	9.0±1.9	9.4±1.9	8.8±1.9
Cleavage rate (%)	61.8	62.5	57.0	72.2
No. of blastocysts	2.1±1.2	1.9±1.2	3.2±1.2	2.0±1.2
Rate of blastocysts (%)	21.8	21.1	33.0	22.7

All values are expressed per ewe. CL=corpus luteum. Different superscripts (a, b) in the same row mean significant differences (P<0.05). *Different superscripts (c, d) in the same row indicate P*<0.1.

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Effects of treatment with a prostaglandin analogue on developmental dynamics and functionality of induced corpora lutea in goats

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ABSTRACT

The aim of this study was to compare morphological and functional features of spontaneous and induced corpora lutea (CLs) in goats. Fourteen adult and cycling Anglo Nubian goats (Argentina) were randomly allocated to two groups: Group N ($n = 7$) included goats with natural spontaneous oestrus and Group PG ($n = 7$) included does in which oestrus was synchronized by the administration of two i.m. cloprostenol doses, 10 days apart. In both groups, oestrous behaviour was checked twice daily (Day of oestrus = Day 0) and daily transrectal ultrasonographies were performed for evaluating CLs and follicles dynamics through the complete subsequent oestrous cycle; the luteal activity was determined directly, in terms of progesterone (P4) secretion, and indirectly, by assessing effects of CL on follicular dynamics. All goats exhibited oestrous behaviour and ovulation without differences in ovulation rate (N: 1.67 ± 0.2, PG: 2.0 ± 0.1). The total luteal tissue area showed linear growth from Day 4 to Day 15 of oestrous cycle in all goats, but the developmental dynamics differed between groups, treated goats had larger area ($P < 0.01$). Plasma P4 concentrations also increased from Day 0 to Day 15 in all the does; however, from Day 5 to Day 15, treated does had a lower concentrations than the untreated group ($P < 0.001$). There were differences in the development of follicular waves between groups; assessment of size-distribution showed that treated group had a higher number of small and larger follicles ($P < 0.05$). The largest follicles recorded in treated goats had a higher maximum diameter both at the first (PG: 7.6 ± 0.8 mm; N: 4.9 ± 0.7 mm, $P < 0.05$) and second follicular waves (PG: 6.3 ± 1.4 mm; N: 5.0 ± 0.4 mm, $P < 0.05$) and a longer growth phase during the second wave (PG: 6.5 ± 1.7 days; N: 4.6 ± 0.7 days, $P < 0.05$), coincident with the period of maximal luteal secretion. In conclusion, synchronization of oestrus and ovulation by the administration of a prostaglandin analogue causes differences in developmental dynamics and functionality of induced corpora lutea when compared to natural spontaneous ovulation.

1. Introduction

The management of the productive cycles by programming reproduction through synchronization of ovulation is the basis for animal production, in goats as in other species. Synchronization is usually accomplished by using hormonal treatments mimicking the activity of the corpus luteum (progesterone or progestagen-based treatments; Corteel et al., 1988), due to their availability and the possi-

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bility of being used in breeding and non-breeding season. On the other hand, the use of progestagens is limited as a result of considerations related to economy (expensive cost and limited availability in some countries) and, mainly, to animal welfare (by inducing problems like vaginitis and sponge retention) and to food safety (by producing biochemical residues), which are contrary to consumer demands for "clean, green and ethical" products (Martin et al., 2004). The latter has caused the prohibition of progestagen use in farm animals (USA) and a higher restriction in the maximum residue limit allowed in European Union countries.

Thus, the alternative for synchronization of oestrus and ovulation, in cycling does, is the induction of a controlled luteolysis and a subsequent follicular phase by administering two doses of luteolytic agents like prostaglandin $F_{2\alpha}$ or its analogues, 9–11 days apart (Greyling, 1996). PGF2α treatments are easily applied by intramuscular injection, avoiding problems of intravaginal devices, rapidly metabolized, and avoiding chemical residues in animal products. However, in goats, fertility at first service after PGF2α treatments is reduced when compared with progestagen sponges (67% vs. 80%, Kusina et al., 2000). Possible causes for such decreased conception rate remain unclear. A previous study in goats pointed to alterations in the functionality of preovulatory follicles (Fernandez-Moro et al., 2008).

We hypothesize that such defective follicles may give way to defective corpora lutea unable to maintain pregnancy. However, to our knowledge, there are no previous studies comparing the functionality of corpora lutea from oestrus induced with prostaglandin analogues and natural oestrus in goats. Thus, the objective of the current experiment was to compare the characteristics of spontaneous and induced corpora lutea both by a direct evaluation, determining their tisular and endocrine features during a complete oestrous cycle, and by indirect assessment, evaluating the follicular population in the ovaries. Deleterious effect of the presence of an active corpus luteum on dynamics and lifespan of follicles in ruminants is well known (Adams, 1999); thus, evaluation of the follicle population may be likely to be used as an indirect index of the quality of the corpora lutea.

2. Materials and methods

2.1. Animals and experimental design

Fourteen Anglo Nubian adult goats were used. The does were maintained, under natural day-length and temperature, at the experimental farm of the National University of Río Cuarto, Argentina, latitude 33°07′S, which meets the requirements of the Ethic Committee of the University. The study was conducted in May, during the period of breeding season, and all the females were confirmed to be cycling at starting the experimental procedure by ultrasonography and evaluation of plasma progesterone concentrations. All the procedures were approved by the Committee for Scientific Procedures of the University.

Does were randomly allocated to two groups (7 goats per group). The first group included does with natural spontaneous oestrus (Group N); the second included does treated with a prostaglandin analogue (Group PG), in which oestrus was synchronized by the administration of two i.m. injections of 1 mL of a prostaglandin analogue (263 μg of cloprostenol, Estrumate®, Schering-Plough Animal Health Corporation, NJ, USA), 10 days apart.

In both groups, oestrous behaviour was checked with a vasectomized buck, twice a day for 30 min (8:00 a.m. and 6:00 p.m.), throughout the experimental period for detecting oestrus (considered as Day 0 for experimental purposes) and establishing the inter-oestrous intervals.

Each goat was observed through the complete oestrous cycle subsequent to oestrous detection, in both groups. The variables evaluated were corpora lutea and follicular dynamics, by ultrasonography, and luteal functionality, by analysis of plasma progesterone concentration. Ovaries were evaluated again at Day 7 after the second oestrus for determination of ovulation rate.

2.2. Ultrasonographic evaluation of follicular and luteal development

The number, position and diameter of all follicles ≥2 mm in size, and the position and size of corpora lutea (CL) were determined by transrectal daily ultrasonographies, conducted by a single operator, by using an Aloka SSD-500 (Aloka, USA) fitted with a 7.5 MHz linear array transducer, as previously described (Ginther and Kot, 1994). Data were recorded in a diagram of the ovaries to evaluate their development in successive observations. For measuring corpora lutea with cavity, the cavity area was subtracted from corpus luteum area to obtain the total area of luteal tissue.

For every oestrous cycle, ultrasonographic data were summarized to characterize patterns of ovarian follicular development. First, the subordinate, dominant and ovulatory follicles were characterized retrospectively. Follicular waves were characterized by adapting the methodology of Ginther and Kot (1994) and Gonzalez-Bulnes et al. (1999) for goats: (a) wave onset (emergence): day in which follicles were firstly detected at 3 mm, growing to at least 4 mm at the following day; (b) growth phase: time taken by a single follicle to grow from 3 mm to its maximum diameter; (c) regression phase (atresia): time taken by a single follicle to regress from its maximum size until the day that it reach its smallest size; (d) end of the wave: day when the dominant follicle ending its regressing phase and/or ovulate (last wave).

Thereafter, follicles present in the ovaries were classified by their diameter. Four groups were categorized: total follicles ≥3 mm in size, large (≥5.5 mm), medium (3.5–5.4 mm) and small follicles (3–3.4 mm). After this, during the follicular phase, the number of new follicles (not previously detected), growing follicles (those that increased in size with respect to the previous day), and decreasing follicles (those that decreased in diameter with respect to the previous day or disappeared) were also considered.

2.3. Hormonal evaluation of luteal activity

The luteal activity was evaluated in terms of progesterone secretion by drawing jugular blood samples coincidentally with ultrasound scannings. Blood sampling

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was performed by jugular venopuncture, using heparinized blood tubes, and plasma was separated by centrifugation at 3000 × *g* for 20 min and stored at −20 °C until assayed. Plasma progesterone concentrations were measured using a commercially available direct solid-phase RIA kit (Coat-A-Count®, Diagnostic Products Corp., Los Angeles, CA, USA), within a single assay. Sensitivity for progesterone was 0.02 ng/mL and the intra-assay variation coefficients were 12.5% for low control concentrations (3 ng/mL) and 2.8% for high control concentrations (15 ng/mL).

2.4. Statistical analyses

For each goat, the interval from the first to the second oestrus was defined as inter-oestrus interval and used for representing total oestrous cycle length. The day of ovulation was detected by the collapse of a large follicle, followed by the development of the subsequent CL; the interval from oestrus to ovulation was defined as oestrus–ovulation interval.

Effect of group, and interactions with effects from day of oestrous cycle and wave of follicular development, on oestrous cycle length, number and characteristics of dominant and remaining follicles, and number and size of corpora lutea were assessed by ANOVA (SAS Institute, NC, USA; 1999). Pearson correlation analyses were used to determine the correlation between CL diameter, luteal area and plasma progesterone levels. All results are expressed as mean ± s.e.m. and considered to be statistically significant from $P < 0.05$.

3. Results

3.1. Effects of a prostaglandin analogue on characteristics of oestrous cycles

All goats from both groups exhibited oestrous behaviour. Two animals in the prostaglandin group (Group PG) showed a shorter inter-oestrous interval (4.0 ± 0.5 days) and one goat from the untreated group (Group N) showed a persistent CL; these animals were excluded from subsequent analysis. In the remaining does, the mean length of the oestrous cycles and the mean ovulation rate (N: 1.67 ± 0.2; PG: 2.0 ± 0.1) were similar for both groups (Table 1); there were no observed luteinized anovulatory follicles in any of the goats. On the other hand, the interval from oestrus to ovulation was around 7 h longer in Group N ($P < 0.05$).

3.2. Effects of prostaglandin analogue on characteristics of induced corpora lutea

The mean day of first detection of corpora lutea by ultrasound was the Day 4.5 ± 0.3, having a mean area of 2.3 ± 0.2 cm^2 when all the animals were evaluated. Corpora lutea revealed a linear growth from Day 4 to Day 15 in both groups; the day of oestrous cycle was positively correlated with individual luteal area (N: $r = 0.243$, $P < 0.008$ and PG, $r = 0.643$, $P < 0.0001$). The same was found when considering total luteal tissue in goats having single and multiple ovulation (N: $r = 0.572$, $P < 0.0001$ and PG: $r = 0.580$, $P < 0.0001$). However, corpora lutea from PG goats had a higher area than N does ($P < 0.01$; Fig. 1A).

Table 1
Characteristics of the oestrous cycle in female goats treated with prostaglandin analogues, 10 days apart (PG, $n = 5$) and non-treated (N, $n = 6$).

	Groups	
	PG	N
Length of interoestrous interval (d)	19.8 ± 0.5	19.0 ± 0.3
Oestrous to ovulation interval (h)	28.0 ± 2.5[a]	34.7 ± 1.9[b]
2nd Prostaglandin injection to oestrous interval (h)	52.8 ± 2.9	–
2nd Prostaglandin injection to ovulation interval (h)	78.4 ± 4.6	–

Different superscripts (a and b) in the same row indicate significant differences ($P < 0.05$).

In both groups, analysis of luteal function in terms of progesterone secretion also showed an increase from Day 0 to Day 15 (N: $r = 0.782$, $P < 0.0001$; PG, $r = 0.822$, $P < 0.0001$); however, the plasma progesterone concentration was lower in Group PG than in Group N throughout all the luteal phase ($P < 0.001$; Fig. 1B). Changes in the values of progesterone concentrations were correlated, in both groups, with total luteal tissue area in the ovaries ($r = 0.645$; $P < 0.0001$) and individual size of corpora lutea; this last relationship being stronger in the PG than in the N Group (N: $r = 0.645$, $P < 0.0001$ and PG: $r = 0.857$, $P < 0.0001$).

The analysis of the rate of change in progesterone concentration and total luteal tissue, as an index of efficiency of luteal tissue for secreting progesterone, showed again significant differences between groups; goats from Group PG showed a lower progesterone secretion than does in the Group N during all the luteal phase of the oestrous cycle studied ($P < 0.001$; Fig. 1C).

3.3. Effects of prostaglandin analogue on follicular dynamics during the induced oestrous cycle

The mean number of total follicles developing in each cycle did not differ significantly between groups (N: 53.2 ± 3.7 vs. PG: 46.6 ± 3.4). Assessment of size-distribution of follicles showed that the Group PG had a higher number of small (PG: 34.0 ± 2.0 vs. N: 28.0 ± 1.8, $P < 0.05$) and large follicles (PG: 7.8 ± 1.0 vs. N: 4.2 ± 0.9, $P < 0.05$). On the other hand, there were no significant differences between groups in the number of medium follicles (PG: 11.4 ± 1.9 vs. N: 15.2 ± 1.8).

Assessment of follicle growth patterns during the oestrous cycle showed that, in the Group N, two goats (33.3%) had two main follicular waves and the remaining four does (66.6%) had three waves. In Group PG, one goat (20%) had two waves, three (60%) had three waves and one (20%) had four waves; therefore, oestrous cycles with three waves were the most frequent pattern in both groups. There was no relationship between oestrous cycle length and number of follicular waves; cycles with two, three and four waves had 19.3 ± 0.3, 19.1 ± 0.3 and 21 days of inter-oestrus interval, respectively.

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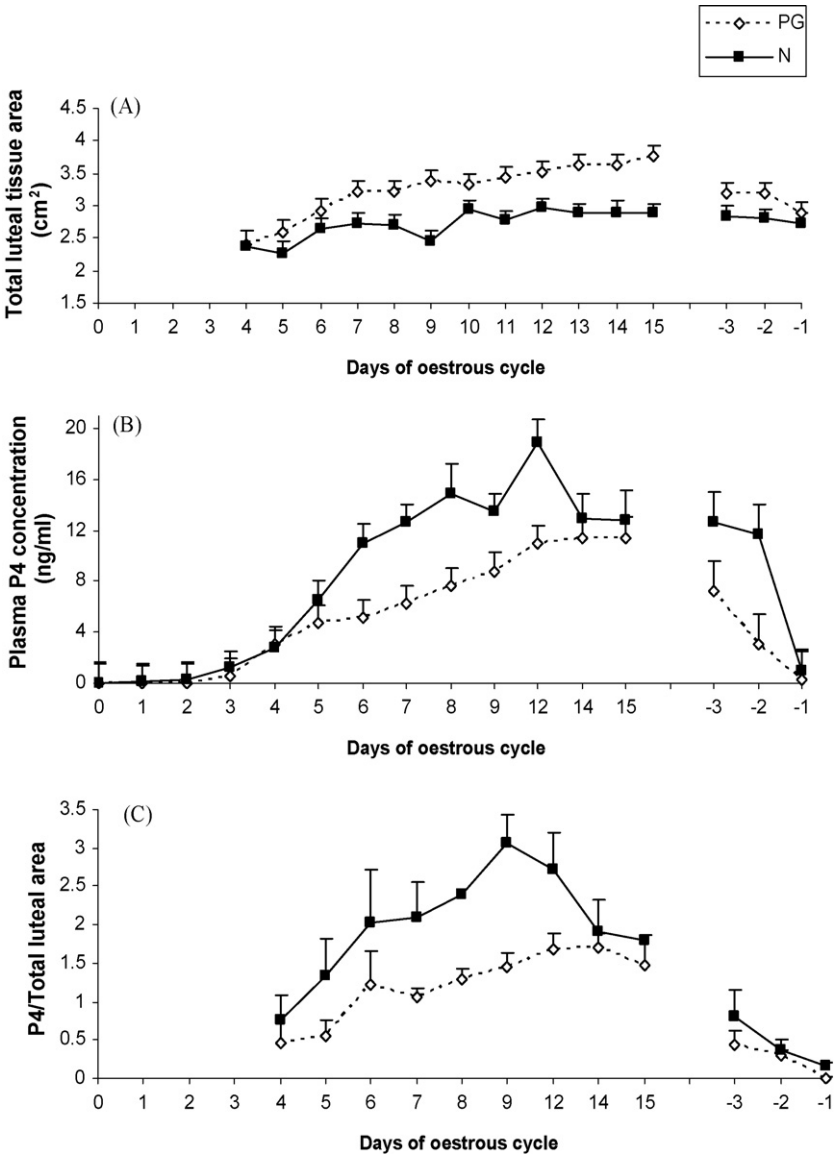

Fig. 1. Mean (±s.e.m.) total luteal tissue area (A), mean (±s.e.m.) plasma progesterone (P4) concentrations (B) and relationship between plasma P4 concentration and total luteal tissue area (C) in prostaglandin-treated (PG, white rhombus) and non-treated (N, black square) goats during the oestrous cycle. All the variables showed significant differences between groups from Day 5 to Day −3 ($P < 0.01$).

The analysis of effects of the luteal phase of the oestrous cycle on follicular development was restricted to cycles with three follicular waves, due to their higher frequency in both groups. There were differences in the development of follicular waves between groups (Table 2). The large follicles recorded in Group PG had a higher maximum diameter both at the first (N: 4.9 ± 0.7 mm; PG: 7.6 ± 0.8 mm, $P < 0.05$) and second follicular waves (N: 5.0 ± 0.4 mm; PG: 6.3 ± 1.4 mm, $P < 0.01$) and longer growth phase during the second wave (N: 4.6 ± 0.7 days; PG: 6.5 ± 1.7 days, $P < 0.05$), coincidentally with the period of maximal luteal secretion; plateau phase of these follicles was also longer but differences did not reach statistical significance (N: 3.0 ± 0.8 days; PG: 4.0 ± 0.9 days).

Ovulatory waves emerged at Day 12.9 ± 0.9 and Day 13.0 ± 1.1 of the oestrous cycle in goats from Group PG and Group N, respectively; data of ovulatory follicles dynamics are shown in Table 3. In Group PG, all goats (100%, 5/5) had double ovulations; but in Group N, only 67% of goats (4/6) had double ovulations and the rest of them (33%, 2/6) had a single ovulation. There were no significant differences in the day of emergence of the ovulatory follicle in females with single ovulation (Day 10.6 ± 1.9) and in the day of emergence of the first detected ovulatory follicle in does with double ovulation (Day 13.3 ± 0.7). In does with double ovulations, the follicles that became ovulatory emerged as part of different waves in 2 of 5 goats (40.0%) in Group PG and in 1 of 6 goats (16.6%) in Group N.

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Table 2
Highlights of the characteristics of the follicular dynamics in prostaglandin-treated (PG) and non-treated (N) goats with three follicular waves per oestrous cycle.

		PG (n = 3)	N (n = 4)
Emergence of the follicular wave (d)	Wave 1	1.2 ± 0.4	1.4 ± 0.3
	Wave 2	7.3 ± 0.7	8.3 ± 0.5
	Ovulatory wave	15.1 ± 0.7	15.4 ± 0.5
Maximum diameter of the largest follicle (mm)	Wave 1	7.6 ± 0.8[a]	4.9 ± 0.7[b]
	Wave 2	6.3 ± 1.4[c]	5.0 ± 0.4[d]
	Ovulatory wave	6.3 ± 0.6	5.8 ± 0.4
Duration of the growth phase of the largest follicle (d)	Wave 1	5.7 ± 1.5	6.7 ± 1.8
	Wave 2	6.5 ± 1.7[a]	4.6 ± 0.7[b]
	Ovulatory wave	5.5 ± 0.8	5.9 ± 0.8
Duration of the plateau phase of the largest follicle (d)	Wave 1	2.2 ± 0.6	3.5 ± 1.2
	Wave 2	4.0 ± 0.9	3.0 ± 0.8
	Ovulatory wave	–	–
Duration of the decreasing phase of the largest follicle (d)	Wave 1	3.0 ± 0.7	3.2 ± 0.8
	Wave 2	1.0 ± 0.7[c]	3.3 ± 0.4[d]
	Ovulatory wave	–	–

Different superscripts in the same row means $P < 0.05$ (a and b) and $P < 0.1$ (c and d).

4. Discussion

The main important findings in the present study revealed deficiencies in the growth and functionality of luteal tissue in goats treated with prostaglandin analogue. First, around 30% of the animals had short-length cycles. Secondly, corpora lutea of treated-does with regular-length cycles were larger in size than those of untreated-goats, but showed a lower secretion of progesterone and a weakened effect on dynamics and lifespan of dominant follicles growing during the mid-luteal phase.

In both groups, the ultrasonographic image and size of corpora lutea changed throughout the oestrous cycle as previously described (Ginther and Kot, 1994); changes in total luteal tissue area were correlated with day of cycle and plasma progesterone concentrations. However, functionality of corpora lutea was depressed in the prostaglandin-treated group; the rate between progesterone secretion and luteal tissue was lower than in untreated goats. Such dysfunction of induced corpora lutea may be related to defective preovulatory follicle development. In sheep, it has been found that inadequate follicular development leads to subnormal corpus luteum formation (White et al., 1987; Keisler and Keisler, 1989) causing low progesterone secretion. Secretion of progesterone in goats, like in other mammals, during the first days after ovulation plays an important role for early embryo development (Watson et al., 1999). Fertility was not studied in current study but the results suggest that the lower plasma progesterone concentrations found in prostaglandin-treated goats would explain the decrease in fertility rates previously described after such treatment (Kusina et al., 2000). The evaluation of the follicle population during the luteal phase, as an indirect index of the quality of luteal structures, confirmed these direct evidences based on tisular and endocrine features of the corpora lutea.

Table 3
Characteristics of the ovulatory wave in prostaglandin-treated (PG) and non-treated (N) goats with three follicular waves per oestrous cycle.

	PG (n = 3)	N (n = 4)
Emergence of the follicular wave (d)	12.8 ± 1.6	14.0 ± 1.6
Initial diameter of the ovulatory follicle (mm)	2.9 ± 0.3	3.0 ± 0.3
Maximum diameter of the ovulatory follicle (mm)	8.3 ± 0.4[a]	7.2 ± 0.4[b]
Duration of the ovulatory follicle (d)	5.0 ± 0.9	5.6 ± 0.8
Rate of growth (mm/d)	1.2 ± 0.2[c]	0.7 ± 0.2[d]

Different superscripts in the same row means $P < 0.05$ (a and b) and $P < 0.1$ (c and d).

In goats, follicular development is characterized by wavelike patterns, with a range from 2 to 4 waves per oestrous cycle (Ginther and Kot, 1994; Adams, 1999; de Castro et al., 1999; Evans et al., 2000; Cueto et al., 2006). On the basis of hypothesis of Adams (1999), confirmed later and currently accepted (Evans et al., 2000; Rubianes and Menchaca, 2003; Gonzalez-Bulnes et al., 2005), follicular waves in goats and ewes may be characterized as major waves during early and late luteal phases, with a clearly discernible dominant follicle, and minor waves, with no clear dominant follicle, particularly during mid luteal phase. Thus, the corpus luteum would be the dominant structure in the ovary; its progesterone secretion during mid-luteal phase would modify pituitary LH secretion and would affect the development of dominant follicles (Campbell et al., 1995; Adams, 1999; Gonzalez-Bulnes et al., 1999).

This was the scenario in control goats with spontaneous cycles in this study. However, the goats treated with a prostaglandin analogue, when compared to control groups, had dominant follicles of mid-luteal phase with higher maximum diameter and longer permanence. These characteristics are coincidental with previously described for persistent follicles and are related to low progesterone levels found in the cloprostenol-treated goats; a consequence of low levels of progesterone is an increase of LH pulse frequency, which is associated with an increment in number, size and permanence of largest follicles (Menchaca and Rubianes, 2004; Gonzalez-Bulnes et al., 2004). The increase in number of large follicles, besides increases in size and duration, was also found in this study.

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Altogether, both direct and indirect analyses of luteal function in prostaglandin-treated goats indicate a weakened functionality of the induced corpora lutea that can be related to a previous inadequate preovulatory follicle development, as discussed above (White et al., 1987; Keisler and Keisler, 1989). Having in mind previous information, such possible alteration in follicle development can be an effect of the timing of prostaglandin treatment. The administration of a double dose of prostaglandins is commonly performed in practice, as in this study, 10–12 days apart. Such interval assures that most of the animals should be in mid-luteal phase at second dose and, therefore, assures the presence of an active corpus luteum responding to the treatment. Stage of the cycle at second prostaglandin dose was not assessed in the current study, but the interval from oestrus to ovulation was shorter in treated than in control does; it is known that intervals from treatment-oestrus-ovulation are shorter when animals are treated earlier in the cycle (Gonzalez-Bulnes et al., 2005), so it seems adequate to hypothesize that animals were in the beginning of mid-luteal phase.

The mid-luteal phase is the optimal moment to find a corpus luteum responding to prostaglandins; however, it is the worst moment from the point of view of follicle dynamics since large follicles coexisting with corpora lutea during mid-luteal phase have compromised functionality (Gonzalez-Bulnes et al., 2005). To our knowledge, there is no previous information in goats, but, in cattle, the ovulation of defective follicles has been related to alterations in the developmental competence of their oocytes (Revah and Butler, 1996; Mihm, 1999), in the oviduct secretory patterns (Binelli et al., 1999), and in the processes of fertilization and early embryo development (Greve et al., 1995). Thus, our current results suggest the necessity for further studies into intrinsic mechanism affecting fertility after treatment with prostaglandin analogues and/or alternative protocols for enhancing the yields obtained. Maybe, a possible way is the adaptation of protocols used in sheep, species in which prostaglandin administration is effective from Day 3 after ovulation (Rubianes et al., 2003). Therefore, two doses of prostaglandin 7 days apart are efficient to induce fertile oestrus (Menchaca and Rubianes, 2004), with a high synchronization (80% of the ewes in oestrus between 24 and 48 h after second dose; Menchaca et al., 2004).

In conclusion, synchronization of oestrus and ovulation by the administration of prostaglandin analogue 10–12 days apart causes differences in developmental dynamics and functionality of induced corpora lutea when compared to natural spontaneous oestrus and ovulation. The issues limiting the use of progestagens may favour the use of prostaglandins for application of assisted reproductive techniques, but protocols based in these hormones still need to be improved.

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References

Adams, G.P., 1999. Comparative patterns of follicle development and selection in ruminants. J. Reprod. Fertil. Suppl. 54, 17–32.
Binelli, M., Hampton, B.W.C., Thatcher, W.W., 1999. Persistent dominat follicle alters pattern of oviductal secretory proteins from cows at estrus. Biol. Reprod. 61, 127–134.
Campbell, B.K., Scaramuzzi, R.J., Webb, R., 1995. Control of the antral follicle development and selection in sheep and cattle. J. Reprod. Fertil. Suppl. 49, 335–350.
Corteel, J.M., Baril, G., Leboeuf, B., 1988. Artificial breeding of goats and kids induced to ovulate with hormones outside the breeding season. Small Rumin. Res. 1, 19–35.
Cueto, M., Gibbons, A., Alberio, R., Taddeo, H., Gonzalez-Bulnes, A., 2006. Timing of emergence of ovulatory follicles in polyovulatory goats. Anim. Reprod. Sci. 91, 275–284.
de Castro, T., Rubianes, E., Menchaca, A., Rivero, A., 1999. Ovarian dynamics, serum estradiol and progesterone concentrations during the interovulatory interval in goats. Theriogenology 52, 399–411.
Evans, A.C.O., Duffy, P., Hynes, N., Boland, M.P., 2000. Waves of follicles development during the estrous cycles in sheep. Theriogenology 53, 699–715.
Fernandez-Moro, D., Veiga-Lopez, A., Ariznavarreta, C., Tresguerres, J.A.F., Encinas, T., Gonzalez-Bulnes, A., 2008. Preovulatory follicle development in goats following oestrous synchronization with progestagens or prostaglandins. Reprod. Dom. Anim. 43, 9–14.
Ginther, O.J., Kot, K., 1994. Follicular dynamics during the ovulatory season in goats. Theriogenology 42, 987–1001.
Gonzalez-Bulnes, A., Santiago Moreno, J., Gomez Brunet, A., Inskeep, E.K., Townsend, E.M., Lopez Sebastián, A., 1999. Follicular dynamics during the oestrous cycle in dairy goats. Anim. Sci. 68, 547–554.
Gonzalez-Bulnes, A., Souza, C.J., Campbell, B.K., Baird, D.T., 2004. Systemic and intraovarian effects of dominant follicles on ovine follicular growth. Anim. Reprod. Sci. 84, 107–119.
Gonzalez-Bulnes, A., Veiga-Lopez, A., García, P., García-García, R.M., Ariznavarratea, C., Sánchez, M.A., Tresguerres, J.A., Cocero, M.J., Flores, J.M., 2005. Effects of progestagens and protaglandin analogues on ovarian function and embryo viability in sheep. Theriogenology 63, 2523–2534.
Greve, T., Callesen, H., Hyttel, P., Hoier, R., Assey, R., 1995. The effects of exogenous gonadotropins on oocyte and embryo quality in cattle. Theriogenology 43, 41–50.
Greyling, J.P.C., 1996. The effect of dose of prostaglandin in the synchronization of oestrus in the Boer goat doe. In: Proceedings of the Sixth International Conference in Goats, International Academic Publisher, Beijing, China, p. 826 (Abstract).
Keisler, D.H., Keisler, L.W., 1989. Formation and function of GnRH induced subnormal corpora lutea in cyclic ewes. J. Reprod. Fertil. 87, 267–273.
Kusina, N.T., Tarwirei, H., Hamudikuwanda, H., Agumba, G., Mukwena, J., 2000. A comparison of the effects of progesterone sponges and ear implants, PGF2a, and their combination on efficacy of estrus synchronization and fertility of Mashona goat does. Theriogenology 53, 1567–1580.
Martin, G., Milton, J., Davidson, R., Banchero Hunzicker, G., Lindsay, D., Blache, D., 2004. Natural methods for increasing reproductive efficiency in small ruminants. Anim. Reprod. Sci., 231–245.
Menchaca, A., Miller, V., Gil, J., Pinczak, A., Laca, M., Rubianes, E., 2004. Prostaglandin F2a treatment associated with timed artificial insemination in ewes. Reprod. Domest. Anim. 39, 352–355.
Menchaca, A., Rubianes, E., 2004. New treatments associated with timed artificial insemination in small ruminants. Reprod. Fertil. Dev. 16, 403–414.
Mihm, M., 1999. Delayed resumption of cyclicity in postpartum dairy and beef cows. Reprod. Domest. Anim. 34, 277–284.
Revah, I., Butler, W.R., 1996. Prolonged dominance of follicles and reduced viability of bovine oocytes. J. Reprod. Fertil. 106, 39–47.
Rubianes, E., Menchaca, A., 2003. The pattern and manipulation of ovarian follicular growth in goats. Anim. Reprod. Sci. 78, 271–287.
Rubianes, E., Menchaca, A., Carbajal, B., 2003. Response of the 1–5 day-aged ovine corpus luteum to prostaglandin F2 alpha. Anim. Reprod. Sci. 78, 47–55.
SAS Institute Inc., 1999. SAS/STAT. Software Version 8. SAS Institute Inc., Cary, NC, USA.
Watson, A.J., Westhusin, M.E., Winger, Q.A., 1999. IGF paracrine and autocrine interactions between conceptus and oviduct. J. Reprod. Fertil. Suppl. 54, 303–315.
White, L.M., Keisler, D.H., Dailey, R.A., Inskeep, E.K., 1987. Characterization of ovine follicles destined to form subfunctional corpora lutea. J. Anim. Sci. 65, 1595–1601.

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Effect of undernutrition on the uterine environment during maternal recognition of pregnancy in sheep

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Abstract. The effects of pregnancy and undernutrition on endometrial gene expression were investigated in ewes fed all or half their maintenance requirements and killed on Day 14 of pregnancy or of the oestrous cycle. The endometrial expression of progesterone, oestrogen, oxytocin and interferon receptors (PR, ERα, OXTR and IFNAR, respectively), cyclo-oxygenase (COX)-2, proliferating cell nuclear antigen (PCNA), insulin-like growth factors (IGF)-I and -II, and IGF-1 receptor (IGF-1R) was studied by immunohistochemistry or real-time reverse transcription–polymerase chain reaction. The luminal epithelium of cyclic control ewes was devoid of PR staining and had relatively high levels of ERα, OXTR, COX-2 and IFNAR2. The presence of a conceptus decreased the *in vitro* uterine secretion of prostaglandin (PG) $F_{2\alpha}$ and the expression of IFNAR2 in most cell types, and increased the gene expression of IGF-I and IGF-II. Undernutrition tended to increase ERα protein and gene, but decreased *in vitro* uterine secretion of PGE_2 and the gene expression of IFNAR2 in cyclic ewes. There was no effect of undernutrition on pregnancy rates or the number of conceptuses recovered. Consistent with this, undernutrition of pregnant ewes did not have any effect on uterine gene expression. Moreover, in cases where changes were observed in cyclic ewes, these changes were negated when a conceptus was present.

Introduction

The establishment of pregnancy depends on successful impairment by the conceptus of lysis of the corpus luteum (CL). The lifespan of the CL depends on the release of oxytocin-induced pulses of prostaglandin (PG) $F_{2\alpha}$, which acts to cause the functional regression of the CL. The luteolytic mechanism, which takes place at around Day 14 of the ovine oestrous cycle in the endometrial luminal epithelium and superficial glandular epithelium, likely involves an interplay between progesterone, oestrogen and oxytocin acting through their receptors (PR, ERα and OXTR, respectively; McCracken *et al*. 1999). At around Day 10 of pregnancy in sheep, the conceptus starts to synthesise an antiluteolytic signal, namely interferon (IFN)-τ, and elongates, thereby ensuring the delivery of IFN-τ along the uterine horn. After binding to endometrial Type I IFN receptors (IFNAR), IFN-τ silences ERα gene transcription subsequent to downregulation of the PR, which prevents the induction of OXTR gene expression and the uterine release of oxytocin-induced luteolytic pulses of $PGF_{2\alpha}$, thereby maintaining the CL and progesterone production. This mechanism is known as the maternal recognition of pregnancy and occurs around Day 14 of pregnancy in sheep (for a review, see Spencer *et al*. 2004). Cyclo-oxygenase (COX)-2 is an enzyme common to the biosynthetic pathway of both $PGF_{2\alpha}$ and PGE_2 that converts arachidonic acid to PGH_2 (Goff 2004). The endometrial production of COX-2 in sheep has been reported to increase, to be reduced or to remain unchanged with pregnancy (Charpigny *et al*. 1997, 1999; Chen *et al*. 2006).

There are many reports on the role of various hormones, growth factors and their receptors in the development of embryos during the preimplantation period (Kaye 1997). Insulin-like growth factors (IGF)-I and -II are produced locally in many organs of the body, including the reproductive tract of cyclic and pregnant ewes (Stevenson *et al*. 1994*a*). In the uterus, both IGF-I and -II stimulate cell proliferation and differentiation of the early embryo and of endometrial cells; these growth-promoting effects are considered to be mediated primarily by IGF-1 receptor (IGF-1R) and modulated by IGF binding proteins (IGFBPs), which further increases the complexity of this system (Kaye 1997; Wathes *et al*. 1998).

Taking into account the relevance of a precisely timed uterine environment for the establishment of pregnancy and the development of an early conceptus, any event that disrupts endometrial gene expression around maternal recognition of pregnancy (on Day 14 in the sheep) could be responsible for embryo loss. It is well known that feeding sheep at levels below the nutritional

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requirements for maintenance decreases pregnancy rates and affects embryo quality and development (Rhind *et al*. 1989; Abecia *et al*. 1997). Although we have recovered the same number of embryos from underfed ewes and control ewes on Days 8–9 after mating (Abecia *et al*. 1997, 1999), reduced pregnancy rates were observed as pregnancy progressed, specifically on Days 14–15 (Abecia *et al*. 1995, 1999). In addition, Rhind *et al*. (1989) reported lower pregnancy rates on Days 21–26 in ewes fed under similar nutritional regimens. Previously, we demonstrated that cyclic ewes subjected to a short period (20 days) of undernutrition have reduced endometrial sensitivity to oestrogens and progesterone (in terms of specific receptor expression) early during the luteal phase (Day 5), whereas no effect was observed later in the luteal phase (Day 10) or at the time of maternal recognition of pregnancy (Day 14; Sosa *et al*. 2004, 2006). These results may be due to the action of metabolic mediators, such as IGF-I and leptin, which may act in either an endocrine or paracrine/autocrine manner on the uterine tissue (Thissen *et al*. 1994; Barash *et al*. 1996; Sosa *et al*. 2004, 2006). To our knowledge, there are no studies reported in the literature of the effects of undernutrition on OXTR, COX-2 and IFNAR, all of which are involved in the luteolytic cascade, that could explain the embryonic losses observed. Moreover, previous studies have been performed in cyclic ewes and have assessed maternal effects only, but it remains to be determined whether the effects of the conceptus on uterine gene expression are dependent on nutritional status.

Thus, in the present study, we have investigated the effects of undernutrition and the presence of the conceptus on the endometrial secretion of $PGF_{2\alpha}$ and PGE_2, as well as the abundance and/or expression of the transcripts involved in the maternal recognition of pregnancy (i.e. PR, ERα, OXTR, COX-2 and IFNAR2) and in preparing the uterine environment for pregnancy (i.e. IGF-I, IGF-II and IGF-1R). In addition, because the presence of ERα in glands has been related to endometrial preparation to support pregnancy (Kimmin and MacLaren 2001), we also determined levels of proliferating cell nuclear antigen (PCNA).

Materials and methods

Animals and treatments

Experiments were performed at the experimental farm of the University of Zaragoza (Zaragova, Spain; latitude 41°41′N), under supervision of the Ethics Committee of the University of Zaragoza and according to the requirements of the European Union for Scientific Procedure Establishments.

During the breeding season, 46 adult multiparous Rasa Aragonesa ewes (*Ovis aries*), with a mean (±s.e.m.) bodyweight 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× liveweight maintenance requirements (Agricultural and Food Research Council 1993). The diet, offered for 1 month prior to the beginning of the experimental procedures to allow for adaptation, was comprised of 0.42 kg pellets and 0.70 kg barley straw per day, providing 7.8 MJ of metabolisable energy per ewe. The pellets consisted of barley (85%) and soybean (15%). Ewes had unrestricted access to water and mineral supplement. The oestrous cycles were synchronised using intravaginal sponges containing 40 mg progestagen (Fluorogestone acetate; Intervet, Salamanca, Spain), inserted 1 month after ewes had been started on the nutritional regimen and withdrawn 12 days later. At the time of sponge insertion, ewes were allocated to one of two nutritional treatment groups: (*1*) a control group ($n = 21$); and (*2*) the low group ($n = 25$), fed 0.5-fold the daily requirements for maintenance. The control group continued to receive the same diet, whereas the low group was offered half the daily quantity of pellet and straw to provide 3.9 MJ of metabolisable energy per ewe. These dietary regimens were maintained 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 oestrus (Day 0) was checked every 8 h. Thirteen control ewes and 18 ewes in the low group were mated to intact rams to establish a cyclic and pregnant group in each nutritional group. Jugular blood was sampled 1 h before feeding every 2 days, starting 1 week before the starting the different nutritional regimens and continuing until the end of the experiment. Samples were centrifuged within 15 min of collection (1000*g*, 10 min) and plasma was stored at −20°C until analysis. Bodyweight and BCS were recorded at the time of sponge insertion, at sponge withdrawal and when ewes were killed (Days −13, −1 and 14).

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 and one cyclic ewe from the low group 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 further study, so the final treatment groups consisted of five cyclic and six pregnant control ewes, and six cyclic and seven pregnant ewes in the low group. On Day 14 of the oestrous cycle or pregnancy, ewes were anaesthetised with injection of 10 mL sodium thiopental (20 mg kg^{-1}; Tiobarbital; Braun Medical, Jaen, Spain) before being killed by injection of 5–10 mL 50 kg^{-1} T-61 (Intervet). 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. Three sections of uterine tissue (including the endometrium and myometrium) from the middle one-third of the uterine horn ipsilateral to the CL were dissected from each ewe. One section was fixed in 4% w/v paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin for immunohistochemistry; another section was incubated *in vitro* to determine endometrial prostaglandin synthesis; and the third section was snap frozen in liquid N_2 and stored at −80°C until real-time polymerase chain reaction (PCR) assays.

Plasma progesterone concentrations

Plasma progesterone concentrations were determined from Day −1 (where Day 0 = oestrus) to Day 14 (when ewes were killed) using a direct solid-phase commercial radioimmunoassay (Coat-A-Count; DPC, Los Angeles, CA, USA) that had been previously described for sheep (Meikle *et al*. 1997). The

sensitivity of the assay was 0.06 nmol L^{-1}. The intra-assay coefficient of variation (CV) for low (10 nmol L^{-1}), medium (47.7 nmol L^{-1}) and high (95.4 nmol L^{-1}) concentrations of progesterone were 6.5%, 5.2% and 5.6%, respectively. The corresponding interassay CVs were 7.8%, 6.7% and 5.3%.

Endometrial $PGF_{2\alpha}$ and PGE_2 concentrations

Explants of intercaruncular uterine tissue (including the endometrium and myometrium) were rinsed with culture medium and cut into 3–5 mm^3 cubes. Approximately 100 mg tissue was placed (in duplicate) into CellStar 24-well culture plates (Greiner Bio-One, Frickenhausen, Germany) containing 500 μL cell culture medium and incubated for 24 h at 39°C in an atmosphere of 5% CO_2 in air. The cell culture medium consisted of Ham's F-12 medium supplemented with penicillin–streptomycin, L-glutamine, insulin, transferrin and selenium, as described by Thibodeaux *et al*. (1994). After culture, the media were collected and stored at −20°C until analysis.

Culture media from wells were analysed for $PGF_{2\alpha}$ and PGE_2 concentrations using commercial ELISA kits (Cayman Chemical, Ann Arbor, MI, USA). The limit of detection of the assay was 9 pg mL^{-1} for $PGF_{2\alpha}$ and 15 pg mL^{-1} for PGE_2. For each variable, samples were run in duplicate in a single assay with a CV of 9.2% for $PGF_{2\alpha}$ and 7.0% for PGE_2. The concentrations of PG were then corrected against the amount of tissue (in mg) incubated for each ewe and expressed as ng PG mg^{-1} tissue.

Protein localisation and abundance

Immunoreactive PR, ERα, OXTR, IFNAR2, COX-2 and PCNA were visualised in transverse 5-μm sections from uterine horns ipsilateral to the CL using an avidin–biotin–peroxidase immunohistochemical technique (Meikle *et al*. 2000). The primary antibodies used were mouse monoclonal anti-PR (Zymed, South San Francisco, CA, USA), anti-ERα and anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-OXTR (Rohto Pharmaceutical, Osaka, Japan) diluted 1 : 100, 1 : 25, 1 : 100 and 1 : 75 in PBS, respectively, and rabbit polyclonal anti-IFNAR2 (Santa Cruz) and anti-COX-2 (Cayman Chemical) diluted 1 : 100 and 1 : 200 in PBS, respectively. Negative controls for each receptor were obtained by replacing the primary antibody with a homologous non-immune IgG at equivalent concentrations (Santa Cruz). After primary antibody binding, sections were incubated with a biotinylated secondary antibody (horse anti-mouse or goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA, USA) diluted 1 : 200 in normal serum from horse or goat, respectively. A Vectastain ABC anti-rabbit or anti-mouse kit (Vector Laboratories) was used for the detection of all proteins. The location of the bound enzyme was visualised by 3,3′-diaminobenzidine in H_2O_2 (DAB kit; Vector Laboratories) and the sections were counterstained with haematoxylin and dehydrated before they were mounted. For each receptor, all samples were analysed in the same immunohistochemical assay.

Image analysis

The amount of immunoreactive proteins in the different cell types was estimated subjectively by two independent observers who were blinded to the treatment group (Sosa *et al*. 2004). Ten fields were analysed for each cell type at a magnification of ×1000 in all ewes. The staining of the nuclei was scored as negative (−), faint (+), moderate (++) or intense (+++) and the extent of staining of each cell type was expressed over a scale of 0–10 (Thatcher *et al*. 2003), where 0 is the absence of staining and 10 is the maximum staining intensity. The average staining intensity was calculated as $1 \times n_1 + 2 \times n_2 + 3 \times n_3$, where n is the number of cells in each field exhibiting faint (n_1), moderate (n_2) and intense (n_3) staining (Boos *et al*. 1996).

Isolation and reverse transcription of RNA

Total RNA was extracted from the uterus 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 ($A_{260/280}$) and the integrity of the RNA was determined by electrophoresis (on a 1% agarose gel). All samples presented $A_{260/280}$ ratios between 1.8 and 2.1. For each sample, cDNA was synthesised by reverse transcription (RT) using the SuperScript III First-Strand Synthesis System Kit (Invitrogen) with random primers and 1 μg total RNA added as a template.

Quantitative real-time PCR

Primers to specifically amplify the cDNA of PR, OXTR, COX-2 and IFNAR2 were designed on the basis of ovine nucleotide sequences available from NCBI (http://www.ncbi.nlm.nih.gov/, accessed March 2009). Primer sequences for ERα, IGF-I, IGF-II and IGF-1R were obtained from the literature (Pfaffl *et al*. 2002; Schams *et al*. 2003; Wu *et al*. 2004). The level of expression of ovine ribosomal protein L19 (RPL19) was used as an endogenous control, using the primer sequences of Chen *et al*. (2006). The expression of the *RPL19* gene was tested in a pilot study under our conditions and it proved to be a good housekeeping gene because it exhibited minimum variation across individuals and treatments. The primer sequences and expected product lengths are listed in Table 1. Amplification reactions were performed in a final volume of 20 μL containing 10 μL SYBR Green master mix (Quantimix EASY SYG kit; Biotools BandM Laboratories, Madrid, Spain), 500 nM forward and reverse primers (Operon Biotechnologies, Cologne, Germany) and 3 μL diluted cDNA (1 : 7.5 in RNase/DNase-free water). Samples were analysed in duplicate in a 72-disk Rotor-Gene™ 6000 (Corbett Life Sciences, Sydney, NSW, Australia). Standard amplification conditions were 10 min at 95°C and 40 cycles of 15 s at 95°C, 60 s at 60°C and 20 s at 72°C. For quantification, standard curves were generated by amplifying serial dilutions of each amplicon according to Fenwick *et al*. (2008). To standardise quantitative gene expression measurements for differences in cellular input, RNA quality and RT efficiency among samples, data for gene expression were analysed by the $2^{-\Delta\Delta CT}$ method, which implies normalisation against the endogenous control (*RPL19*; Livak and Schmittgen 2001; Sosa *et al*. 2009).

Statistical analysis

Data were analysed in a complete randomised design with a 2×2 factorial arrangement of nutritional treatment and reproductive status. All variables were subjected to analysis of variance using a mixed model, including in every case the nutritional treatment (low or control), reproductive status (cyclic or pregnant) and their interaction as fixed effects. Pregnancy rates and conceptus recovery rate were compared by Chi-square test. For analysis of repeated measurements (bodyweight, BCS and progesterone), values obtained on days prior to the start of the nutritional treatment were included in the model as covariables and the covariance structure was modelled to consider the correlation between sequential observations in the same animal (Littell *et al*. 2000). For these variables, the day of the cycle/pregnancy was included in the statistical model. In addition to the effects of nutrition and reproductive status, the model studying immunohistochemical data also included the fixed effects of cell type (luminal epithelium, glandular epithelium and stroma), location (superficial and deep) and their interactions. Gene expression data were analysed in a randomised block design with the day of RNA extraction as a random effect. Pearson correlation coefficients and linear regression coefficients were used to describe relationships between variables. Data are presented as the least square mean ± pooled s.e.m. Statistical analyses were performed using the SAS program (SAS Institute, Cary, NC, USA). The level of significance was set at $P < 0.05$.

Results

Bodyweight and body condition

Results regarding bodyweight and body condition have been published recently elsewhere (Sosa *et al*. 2009). Briefly, bodyweight in the low group decreased progressively from 61.2 ± 0.4 to 56.3 ± 0.4 kg ($P < 0.0001$) over the duration of the experiment, whereas bodyweight in the control group was maintained (from 61.2 ± 0.4 to 60.5 ± 0.4 kg). In addition, there was a significant decrease in BCS in the low group over the duration of the experiment (from 3.4 ± 0.1 to 2.9 ± 0.1; $P < 0.0001$), but not in the control group (from 3.4 ± 0.1 to 3.2 ± 0.1).

Ovulation and pregnancy rates

The ovulation rate did not differ between ewes in the control and low groups (2.0 ± 0.6 *v.* 2.2 ± 0.8 CL, respectively). In addition, there were no significant differences in the pregnancy rate between the control (6/11 ewes pregnant; 55%) and low (7/16 ewes pregnant; 44%) group. The conceptus recovery rate (i.e. the number of conceptuses recovered/the number of CL) did not differ between the control (7/11; 64%) and low (9/16; 56%) groups. The ewes that were not pregnant where they were killed were not considered further.

Plasma progesterone concentrations

Three cyclic ewes (two in the control and one in the low group) that had shown oestrus behaviour but did not have progesterone luteal levels were excluded from the study. There was no effect of nutritional treatment or reproductive condition on plasma progesterone concentrations. However, the day of the oestrous cycle did have a significant effect on plasma progesterone levels ($P < 0.0001$). Progesterone concentrations increased progressively from Day 3 to reach a maximum on Day 13 of the cycle (Fig. 1).

Endometrial $PGF_{2\alpha}$ and PGE_2 concentrations

Endometrial $PGF_{2\alpha}$ concentrations were affected by reproductive status ($P < 0.001$), but not by the nutritional treatment or their interaction. Pregnancy significantly decreased *in vitro*

Table 1. Primer sequences and expected amplicon sizes used for real-time reverse transcription–polymerase chain reaction assays
PR, progesterone receptor; ERα, oestrogen receptor α; OXTR, oxytocin receptor; COX-2, cyclo-oxygenase-2; IFNAR2, subunit 2 of Type 1 interferon receptor; IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; RPL19, ribosomal protein L19. *Multiple species primers. Referenced primer sequences: IGF-I (Wu *et al*. 2004); IGF-II and IGF-1R (Pfaffl *et al*. 2002); and RPL19 (Chen *et al*. 2006)

Gene	Accession no.	Primer sequence	Length (bp)
PR	Z66555	Forward: GACAGCACTTTCTAAGGCGACAT Reverse: TGTGCTGGAAGAAACGATTGC	79
ERα	AY033393	Forward: AGGGAAGCTCCTATTTGCTCC Reverse: CGGTGGATGTGGTCCTTCTCT	234
OXTR	NM_001009752	Forward: ACGCTCGCCGTCTACATTGT Reverse: GCCGTAAGTTCTGCCAGATCTT	85
COX-2	NM_001009432	Forward: CCAGGGCACAAATCTGATGTT Reverse: TGGTCCTCGTTCAATATCTGTCT	82
IFNAR2	NM_001009342	Forward: ACATTCAGCAGGGTTCATAGCA Reverse: TTTCTGTGGCTTTTCTGGTCTTC	89
IGF-I	NM_001077828	Forward: CCAGACAGGAATCGTGGATG Reverse: ACTTGGCGGGCTTGAGAG	89
IGF-II	*	Forward: GACCGCGGCTTCTACTTCAG Reverse: AAGAACTTGCCCACGGGGTAT	205
IGF-1R	*	Forward: TTAAAATGGCCAGAACCTGAG Reverse: ATTATAACCAAGCCTCCCAC	314
RPL19	AY158223	Forward: CCCCAATGAGACCAATGAAATC Reverse: CAGCCCATCTTTGATCAGCTT	119

$PGF_{2\alpha}$ secretion in both the control and low groups ($P < 0.05$; Fig. 2). Endometrial PGE_2 concentrations tended ($P = 0.06$) to be affected by nutritional treatment, with undernutrition resulting in a decreased concentration of PGE_2 in cyclic ewes ($P = 0.05$), but no differences observed in pregnant ewes in the different nutritional groups (Fig. 2).

Proteins localisation and abundance

All proteins studied were evaluated in five endometrial compartments, namely the luminal epithelium (LE), glandular epithelium (arbitrarily divided in two portions, the superficial glandular epithelium (SGE), next to the uterine lumen, and the deep glandular epithelium (DGE), next to the myometrium) and stroma (classified as superficial (SS) and deep (DS) using the same criteria). The PR, ERα and PCNA were localised in the nuclei of cells, whereas IFNAR2, OXTR and COX-2 were located in the cytoplasm (Fig. 3). When specific antibodies were substituted with a non-immune IgG, the absence of staining confirmed the high specificity of immunostaining (Fig. 3*f*). The effects of nutritional treatment, reproductive status and their interaction on each variable are listed in Table 2.

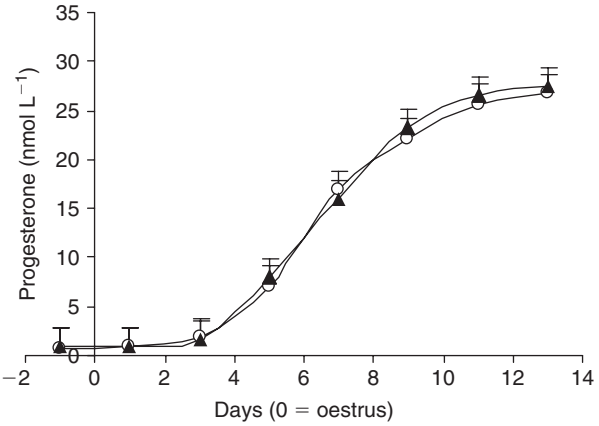

Fig. 1. Plasma concentrations of progesterone in ewes fed 0.5× (Low; ○) or 1× (Control; ▲) the maintenance requirements. Data from cyclic and pregnant ewes have been pooled. Data are the least square mean ± pooled s.e.m.

Overall, pregnancy decreased immunostaining for the PR ($P < 0.05$). In undernourished cyclic ewes, 12% of LE cells were stained for PR with a low intensity of staining, but in the remaining groups the LE was devoid of cells positive for PR. Similarly, in cyclic ewes undernutrition tended ($P < 0.1$) to increase staining intensity for PR in the DGE (Fig. 4). The most intense staining for PR was observed in the deep glands, whereas very weak staining was observed in the LE, SGE and DS.

In control ewes, pregnancy tended ($P < 0.1$) to decrease ERα immunostaining in the DS, whereas in the low group the same trend was observed in the LE. Undernutrition tended ($P < 0.1$) to increase ERα in the LE in cyclic ewes and in the SS in pregnant ewes (Fig. 4). Overall, there was less ERα in the DS ($P < 0.05$) than in the remaining cell types.

In general, pregnancy increased ($P < 0.05$) OXTR protein, but this was evident only in the DS of the control group (Fig. 4). In the DS of pregnant ewes, undernutrition decreased OXTR ($P < 0.01$). The staining intensity of OXTR was greater ($P < 0.05$) in the stroma than in the epithelia.

Specific staining for COX-2 was observed almost exclusively in the LE and with a very high proportion of stained cells (>97%). In this cell type, no main effects (nutrition, pregnancy or interaction) were observed (data not shown). There were very few COX-2-positive cells in the SGE (<8%), with very faint staining in all groups.

The staining intensity for IFNAR2 was affected by reproductive status, because IFNAR2 expression was significantly decreased by pregnancy ($P < 0.01$). There was a significant ($P < 0.05$) interaction between nutritional treatment and reproductive status on IFNAR2 expression: in control ewes, pregnancy decreased ($P < 0.05$) IFNAR2 staining in all cell types except in the LE, whereas in the low group pregnancy increased IFNAR2 only in the LE ($P < 0.05$; Fig. 5). Undernutrition decreased ($P < 0.05$) IFNAR2 staining intensity in the DS of cyclic ewes. The LE showed the greatest IFNAR2 expression and the stroma the least.

Overall, the intensity of staining for PCNA was not affected by nutritional treatment, reproductive status or their interaction. Nevertheless, an increase ($P < 0.05$) in PCNA protein staining was observed in the LE of control pregnant ewes (Fig. 5).

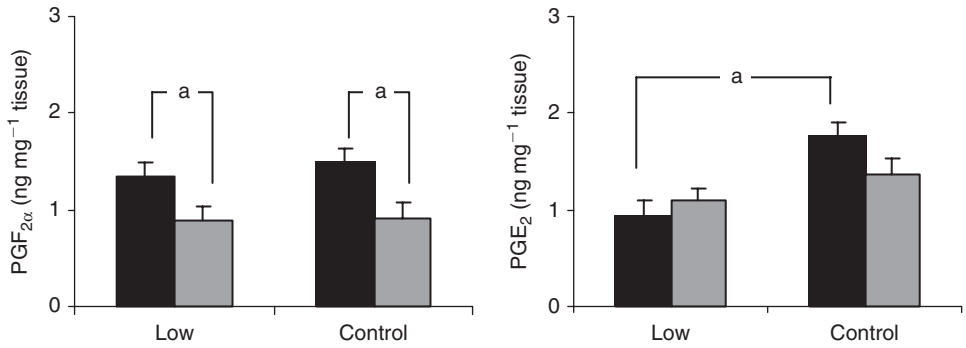

Fig. 2. Prostaglandin (PG) $F_{2\alpha}$ and PGE_2 concentrations on Day 14 (where Day 0 = oestrus) in the endometrium of cyclic (grey bars) or pregnant (black bars) ewes fed 0.5× (Low) or 1× (Control) the maintenance requirements. Data are the least square mean ± pooled s.e.m. [a] $P < 0.05$.

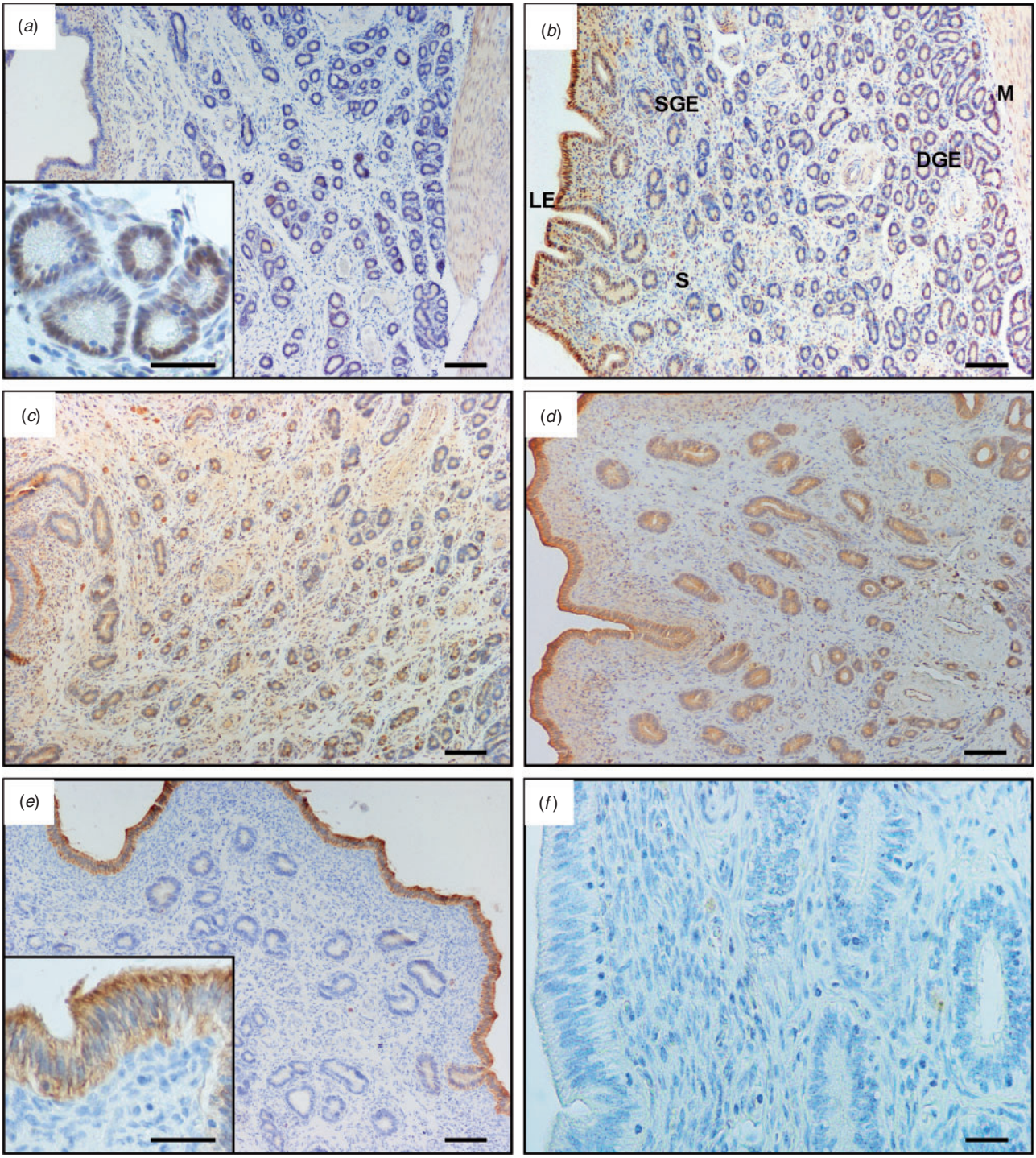

Fig. 3. Examples of immunohistochemical localisation of (*a*) progesterone receptor (PR), (*b*) oestrogen receptor α (ERα), (*c*) oxytocin receptor (OXTR), (*d*) subunit 2 of Type 1 interferon receptor (IFNAR2) and (*e*) cyclo-oxygenase (COX)-2 on Day 14 (where Day 0 = oestrus) in the endometrium of ewes fed 0.5× (Low) or 1× (Control) the maintenance requirements. (*f*) Negative control. Inset in (*a*): nuclear staining; inset in (*e*): cytoplasmic staining. Scale bars = 100 μm.

Endometrial expression of transcripts

The effects of nutritional treatment, reproductive status and their interaction on each variable are given in Table 2 and the levels of expression of the target genes are shown in Fig. 6.

There was a trend ($P = 0.07$) for an interaction between nutritional treatment and reproductive status on *PR* gene expression, although no significant differences were found between groups when statistical comparisons were performed.

Table 2. Effects of nutritional treatment (0.5× and 1× of maintenance requirements), reproductive status (pregnant and cyclic) and their two-way interaction on endometrial protein abundance (staining intensity), mRNA expression and uterine secretion of variables studied in Day 14 sheep uterus

PR, progesterone receptor; ERα, oestrogen receptor α; OXTR, oxytocin receptor; COX-2, cyclo-oxygenase-2; IFNAR2, subunit 2 of Type 1 interferon receptor; IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; PG, prostaglandin; NS, not significant. [a]$P < 0.05$; [b]$P < 0.1$. Arrows indicate significant increases (↑) or decreases (↓)

	Undernutrition	Pregnancy	Two-way interaction
Protein			
PR	NS	↓	NS
ERα	↑[b]	NS	NS
OXTR	NS	↑	NS
COX-2	NS	NS	NS
IFNAR2	NS	↓	a
PCNA	NS	NS	NS
mRNA			
PR	NS	NS	b
ERα	↑[b]	NS	NS
OXTR	NS	NS	NS
COX-2	↓	NS	NS
IFNAR2	NS	↑	NS
IGF-I	NS	↑	NS
IGF-II	NS	NS	a
IGF-1R	NS	NS	NS
Uterine secretion			
$PGF_{2\alpha}$	NS	↓	NS
PGE_2	↓[b]	NS	NS

Undernutrition tended ($P = 0.08$) to increase the expression of the *ERα* gene, with this effect being evident in cyclic ewes.

The expression of *OXTR* mRNA was not affected by nutritional treatment or reproductive status. Levels of *OXTR* mRNA expression were the highest, along with *COX-2* mRNA expression. Overall, undernutrition reduced *COX-2* gene expression by 49% ($P = 0.05$). In the low group, pregnancy increased *IFNAR2* gene expression ($P < 0.05$), but no changes were observed in *IFNAR2* gene expression in control ewes.

Pregnancy increased ($P < 0.05$) the expression of *IGF-I* mRNA in ewes from both nutritional groups and no effect of nutritional treatment was observed. The expression of the *IGF-II* gene was 10-fold greater than that of *IGF-I* and *IGF-1R* gene expression. A significant ($P < 0.05$) interaction between nutritional treatment and reproductive status was found; pregnancy increased ($P < 0.05$) *IGF-II* mRNA in the control group, whereas no effect was observed in the low group. Undernutrition increased ($P < 0.05$) *IGF-II* mRNA expression in cyclic ewes, but no differences were observed between pregnant ewes. The level of *IGF-1R* mRNA expression was not affected by nutritional treatment, reproductive status or their interaction.

Discussion

The present study investigated the effects of pregnancy and undernutrition on uterine gene expression on Day 14 (of pregnancy or of the oestrous cycle) in adult sheep.

Effects of reproductive status on PG secretion and on uterine gene expression

In cyclic ewes, continuous exposure of the uterus to progesterone downregulates PR in the luminal and glandular epithelia at the end of the luteal phase, allowing increases in ERα followed by OXTR, which triggers the release of oxytocin-induced pulses of $PGF_{2\alpha}$ that cause regression of the CL (for a review, see Spencer *et al*. 2004). The results of the present study are consistent with the triggering of luteolysis: the LE of cyclic control ewes was totally devoid of PR and had relatively high levels of ERα, OXTR and COX-2 proteins, along with a high uterine secretion of $PGF_{2\alpha}$ *in vitro*. These findings are further supported by findings regarding transcript expression: *PR* expression was approximately 10-, 20- or 40-fold lower than that of the *ERα*, *OXTR* and *COX-2* genes, respectively.

The high ERα expression observed in cyclic ewes coincides with other reports in that most cell types express the receptor on or around Day 14 of the oestrous cycle (Cherny *et al*. 1991; Spencer and Bazer 1995), and is consistent with the high levels of OXTR expression because oestrogens induce the formation of the OXTR protein (Spencer *et al*. 2004). In the present study, OXTR protein and gene expression was detected in all cell types studied. Similarly, in cyclic ewes, high levels of OXTR protein and gene have been observed in the endometrial epithelia during the late luteal phase (Wathes and Hamon 1993; Stevenson *et al*. 1994*b*; Spencer *et al*. 1998). Although the present study detected some COX-2-positive cells in the superficial glands (8% positive cells with very low staining intensity in cyclic control ewes), COX-2 was localised almost exclusively to the LE, in agreement with other reports (Charpigny *et al*. 1997).

When a conceptus is present in the uterus, it must prevent luteolysis to ensure continuous secretion of progesterone. It does so by secreting IFN-τ, which binds to IFNAR on the endometrial luminal and superficial glandular epithelia, thus preventing $PGF_{2\alpha}$ secretion (Spencer *et al*. 2004), as was observed *in vitro* in the present study. The highest expression of IFNAR2 was observed in the LE and glands, in agreement with the immunohistochemical studies of Rosenfeld *et al*. (2002), who demonstrated that these cells are targeted by IFN-τ during pregnancy. Although no differences were found in *IFNAR2* gene expression between cyclic and pregnant control ewes, it is interesting that pregnancy affected IFNAR2 protein expression in a cell type-specific manner: a clear pregnancy induced downregulation of IFNAR2 occurred in the glands and stroma regardless of their location (superficial or deep), but not in the LE, which is the cell type most intimately related to the conceptus.

In pregnant ewes, the PR remained low or absent in the LE, which is in agreement with the lack of sensitivity to progesterone in this cell type during early pregnancy in ruminants (Wathes and Hamon 1993; Spencer and Bazer 1995; Kimmin and MacLaren 2001). Nevertheless, results regarding the effects of pregnancy on ERα expression are contradictory. Although a total loss of ERα staining has been reported from luminal and superficial glands epithelia from Day 13 of early pregnancy in sheep (Wathes and Hamon 1993; Spencer and Bazer 1995), in the present study ERα was visualised in all cell types studied. The apparent discrepancy may arise from differences in antibody selection and their different epitopes. PCNA is an accessory

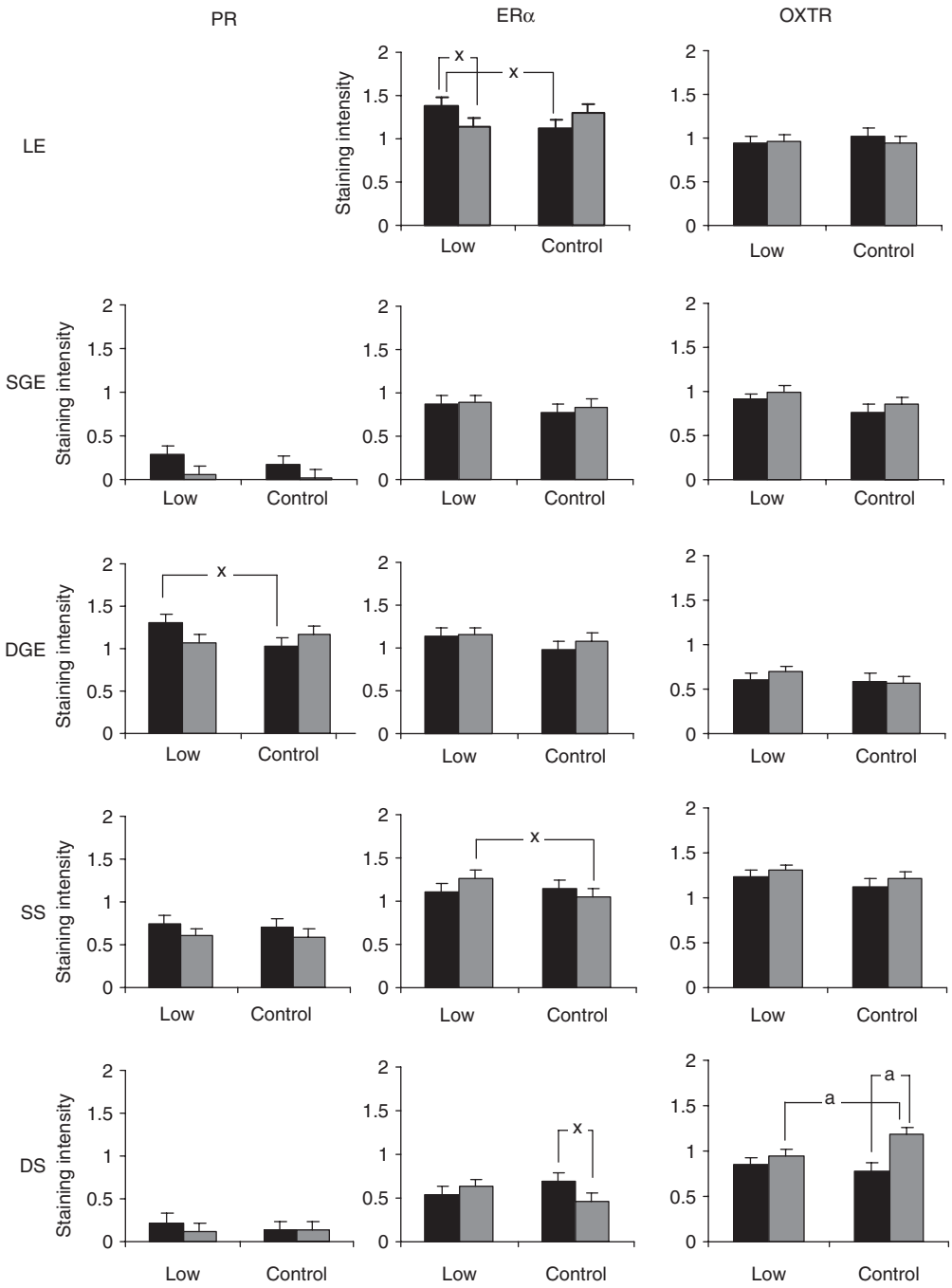

Fig. 4. Staining intensity for progesterone receptor (PR), oestrogen receptor α (ERα) and oxytocin receptor (OXTR) on Day 14 (where Day 0 = oestrus) in the endometrium of cyclic (grey bars) or pregnant (black bars) ewes fed 0.5× (Low) or 1× (Control) the maintenance requirements. LE, luminal epithelium; SGE, superficial glandular epithelium; DGE, deep glandular epithelium; SS, superficial stroma; DS, deep stroma. Data are the least square mean ± pooled s.e.m. $^{a}P < 0.05$; $^{x}P < 0.1$.

protein of DNA polymerase and its expression is correlated with cellular proliferation (Waseem and Lane 1990). The vast and homogeneous expression of PCNA observed throughout the endometrium does not seem to indicate increased activity of specific cell types, as observed in the neonatal ovine uterus (Taylor *et al*. 2000), but extensive proliferation to sustain the function of an adult tissue that undergoes regular changes. The increase in PCNA expression observed in the LE of pregnant

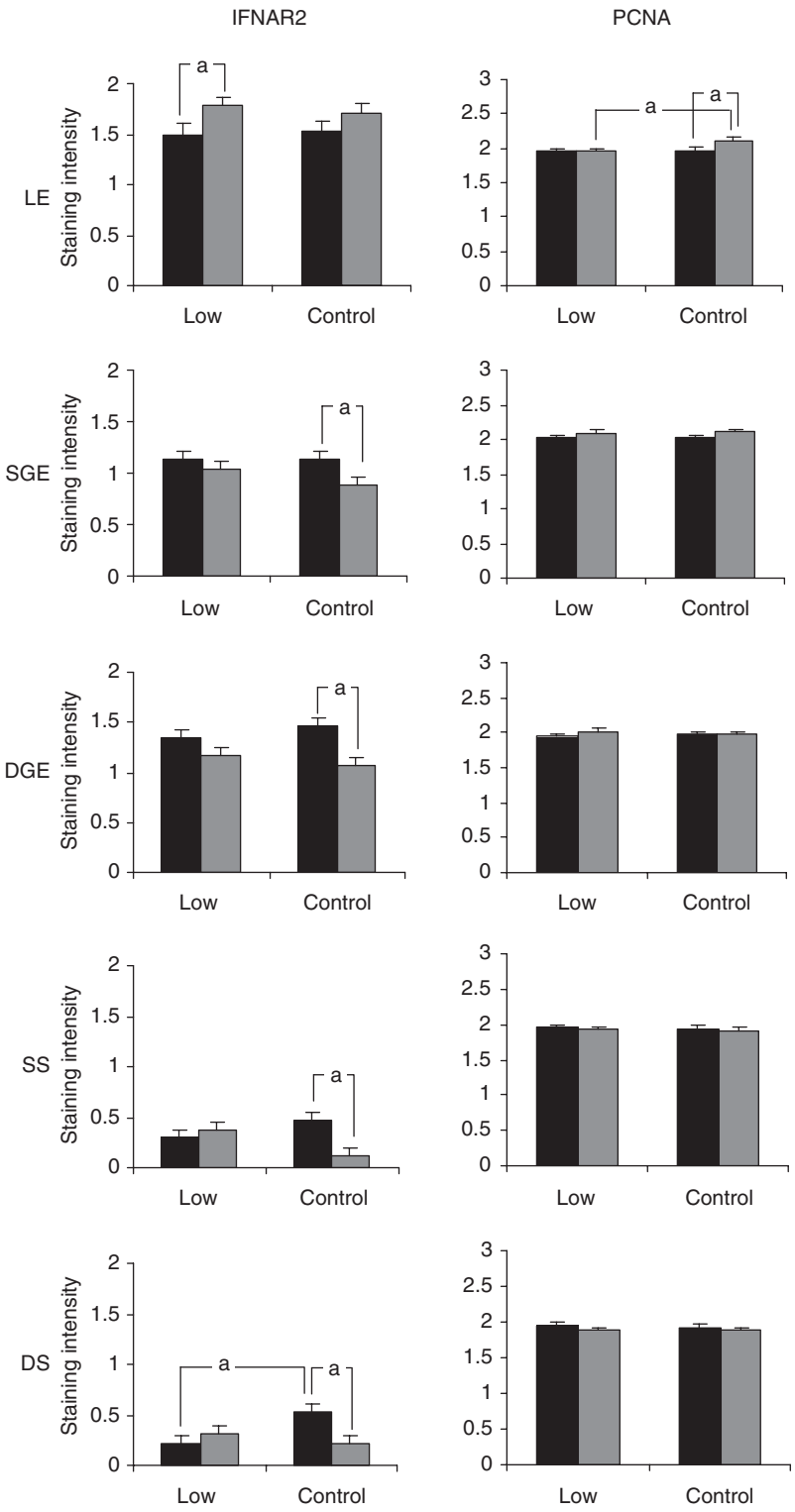

Fig. 5. Staining intensity for Type 1 interferon receptor (IFNAR2) and proliferating cell nuclear antigen (PCNA) on Day 14 (where Day 0 = oestrus) in the endometrium of cyclic (grey bars) or pregnant (black bars) ewes fed 0.5× (Low) or 1× (Control) the maintenance requirements. LE, luminal epithelium; SGE, superficial glandular epithelium; DGE, deep glandular epithelium; SS, superficial stroma; DS, deep stroma. Data are the least square mean ± pooled s.e.m. [a] $P < 0.05$.

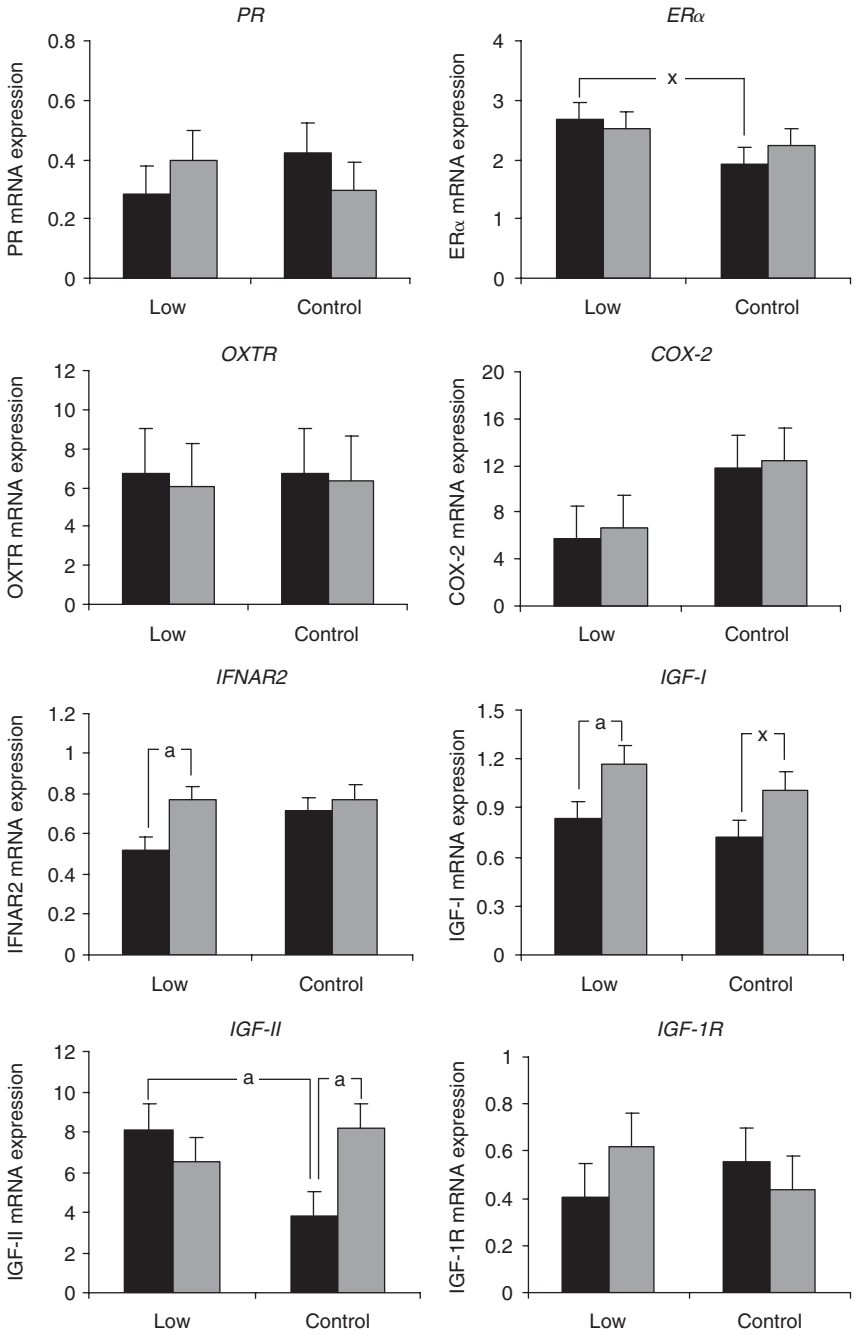

Fig. 6. Levels of expression (normalised against *RPL19*) of progesterone receptor (PR), oestrogen receptor α (ERα), oxytocin receptor (OXTR), cyclo-oxygenase (COX)-2, subunit 2 of Type 1 interferon receptor (IFNAR2), insulin-like growth factor (IGF)-I, IGF-II and IGF-1 receptor (IGF-1R) transcripts on Day 14 (where Day 0 = oestrus) in the uterus of cyclic (grey bars) or pregnant (black bars) ewes fed 0.5× (Low) or 1× (Control) the maintenance requirements. Data are the least square means ± pooled s.e.m. $^{a}P < 0.05$; $^{x}P < 0.1$.

control ewes could represent a resistance of the uterus against the invasion of a foreign tissue, the trophoblast, as suggested for human endometrium (Demir *et al*. 2002).

During the establishment of pregnancy, inhibition of the increase in *OXTR*, and the concomitant reduction in $PGF_{2\alpha}$ secretion, seems to be the determining event for prolonged CL life (Spencer *et al*. 2004). Nevertheless, in the present study, expression of *OXTR* gene and protein did not differ between cyclic and pregnant ewes. Because the presence of the receptor protein does not necessarily confer responsiveness to the tissue,

it is still feasible that the binding capacity of the receptors could have been altered by pregnancy in the present study or that the level of OXTR expression is not a determining factor in the initiation of luteolysis or maternal recognition of pregnancy. Indeed, it has been suggested that luteolytic $PGF_{2\alpha}$ secretion in cattle is not directly dependent upon ovarian oxytocin (Kotwica *et al*. 1998). Conversely, the expected reduction of the endometrial secretion of $PGF_{2\alpha}$ was observed, so it could be assumed that luteolysis was successfully prevented. Moreover, the uterine secretion of PGE_2, which has been postulated to be involved in the maternal recognition of pregnancy as a temporary luteotropic signal in ruminants (Arosh *et al*. 2002), remained high in pregnant ewes. The expression of *COX-2* mRNA in the uterus of pregnant ewes was not altered by pregnancy. Charpigny *et al*. (1999) found pregnancy had no effect on the secretion of $PGF_{2\alpha}$ in sheep and the endometrial expression of COX-2 has also been reported to remain unchanged with pregnancy (Charpigny *et al*. 1997, 1999) or to decrease after the administration of IFN-τ (Chen *et al*. 2006). IFN-τ reduces the expression of PGF synthase, an enzyme downstream of COX-2 in the synthesis of $PGF_{2\alpha}$, whereas it has no effect on PGE synthase (Arosh *et al*. 2004); this could be a possible explanation for the decreased concentrations of $PGF2_{\alpha}$, without any concomitant changes in PGE_2 and COX-2, in pregnant ewes in the present study.

It has been demonstrated that IGF-I and -II, acting mostly through IGF-1R, promote the growth and differentiation of embryonic and endometrial cells (Kaye 1997; Wathes *et al*. 1998). Consistently, pregnancy increased the uterine expression of *IGF-I* and *IGF-II* mRNA in control ewes, with *IGF-II* mRNA expression being quantitatively greater than that of *IGF-I* mRNA (almost 10-fold higher), which agrees with *in situ* hybridisation studies in the ovine (Wathes *et al*. 1998) and with the higher concentrations of IGF-II found in uterine fluids (Ko *et al*. 1991). However, Stevenson *et al*. (1994*a*) reported no effect of pregnancy on Days 14–15 on *IGF-II* mRNA expression, as determined by *in situ* hybridisation.

Effects of the nutritional treatment on PG secretion and on uterine gene expression

Undernutrition affected the uterine expression of certain genes in cyclic but not pregnant ewes. Cyclic undernourished ewes were the only group in which some faintly immunoreactive cells to PR were detected in the LE. The PR is considered a marker of oestrogen action and, consistently, this group showed a trend for greater levels of ERα protein and mRNA in the LE. In addition, undernutrition decreased the secretion of the luteoprotective PGE_2 and expression of *IFNAR2* mRNA in cyclic ewes. Taken together, these differences seem to indicate a uterine environment more prone to the advent of luteolysis in undernourished ewes. Because the plasma concentrations and the hepatic synthesis of IGF-I are decreased by undernutrition (Thissen *et al*. 1994; Sosa *et al*. 2006, 2009), it was expected that the endometrial expression of and/or sensitivity to would be altered by undernutrition also. Undernutrition did not affect the endometrial expression of IGF-I or IGF-1R but, in cyclic ewes, it doubled *IGF-II* mRNA expression. Changes in IFNAR2 protein expression were also observed: in control ewes, pregnancy decreased the expression of IFNAR2 in the glands and stroma without affecting levels in the LE, whereas in undernourished ewes differences in IFNAR2 expression depending on reproductive status were observed only in the LE. Overall, the expression of *COX-2* mRNA was strongly reduced by undernutrition (by almost 50%), but when the interaction between reproductive status and nutritional treatment was considered, no significant differences were observed between the groups, probably due to the high variation within each group. Nevertheless, the diminished expression in undernourished ewes is consistent with the decreased endometrial secretion of PGE_2 observed in underfed cyclic ewes.

It has been widely reported that plasma progesterone concentrations vary inversely with the level of food intake due to changes in the hepatic metabolic rate (for a review, see Abecia *et al*. 2006). Nevertheless, no such effect of nutritional level was observed in the present study. Different BCS could account for the discrepancies between the present and previous studies. However, no differences were observed in progesterone concentrations between pregnant and cyclic ewes and these results are consistent with previous findings, in which pregnant ewes presented similar jugular concentrations of progesterone, but higher concentrations in both the ovarian vein and endometrial tissue than cyclic animals (Abecia *et al*. 1996).

Nutritional restriction was effective in terms of reductions in BCS and bodyweight (10% and 15% respectively). The extent of the loss of body reserves was similar to that reported in previous studies (Lozano *et al*. 1998; Sosa *et al*. 2006) and was reflected in the endocrine profiles (Sosa *et al*. 2009). Conversely, the initial BCS and bodyweight of the ewes in the present study was higher than in those previous studies, which, in turn, resulted in a relatively high final BCS and bodyweight in the undernourished group. Thus, it is possible that this greater level of body reserve was not low enough to translate into notable deleterious effects on uterine physiology. Nevertheless, as discussed above, it seems to have been sufficient to modify the expression of some of the genes studied in the cyclic ewes, so it is surprising that the endometrial gene expression of pregnant ewes was not affected by undernutrition. Conceptuses present in the uterus of undernourished mothers managed to evoke effects similar to those in well-fed pregnant ewes. Even in those cases in which undernutrition changed gene expression in cyclic ewes (e.g. *IFNAR2* and *IGF-II* or the secretion of PGE_2), these differences were somehow negated when a conceptus was present in the uterus. Overall, these observations suggest an adaptive capacity of the embryo–maternal system to adverse changes in metabolic status. However, the potential and extent of this adaptive capacity, as well as the underlying mechanisms, remain to be evaluated.

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References

Abecia, J. A., Rhind, S. M., Bramley, T. A., and McMillen, S. R. (1995). Steroid production and LH receptor concentrations of ovarian follicles and corpora lutea and associated rates of ova wastage in ewes given high and low levels of food intake before and after mating. *Anim. Sci.* **61**, 57–62.

Abecia, J. A., Rhind, S. M., Goddard, P. J., McMillen, S. R., Ahmadi, S., and Elston, D. A. (1996). Jugular and ovarian venous profiles of progesterone and associated endometrial progesterone concentrations in pregnant and non-pregnant ewes. *Anim. Sci.* **63**, 229–234.

Abecia, J. A., Lozano, J. M., Forcada, F., and Zarazaga, L. (1997). Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. *Anim. Reprod. Sci.* **48**, 209–218. doi:10.1016/S0378-4320(97)00021-3

Abecia, J. A., Forcada, F., and Lozano, J. M. (1999). A preliminary report on the effect of dietary energy on prostaglandin F2 alpha production *in vitro*, interferon-tau synthesis by the conceptus, endometrial progesterone concentration on Days 9 and 15 of pregnancy and associated rates of embryo wastage in ewes. *Theriogenology* **52**, 1203–1213. doi:10.1016/S0093-691X(99)00212-5

Abecia, J. A., Sosa, C., Forcada, F., and Meikle, A. (2006). The effect of undernutrition on the establishment of pregnancy in the ewe. *Reprod. Nutr. Dev.* **46**, 367–378. doi:10.1051/RND:2006018

Agricultural and Food Research Council (1993). 'Energy and Protein Requeriments of Ruminants. An Advisor Manual Prepared by the AFRC Technical Committee on Responses to Nutrients.' (CAB International: Wallingford, UK.)

Arosh, J. A., Parent, J., Chapdelaine, P., Sirois, J., and Fortier, M. A. (2002). Expression of cyclooxygenases 1 and 2 and prostaglandin E synthase in bovine endometrial tissue during the estrous cycle. *Biol. Reprod.* **67**, 161–169. doi:10.1095/BIOLREPROD67.1.161

Arosh, J. A., Banu, S. K., Kimmins, S., Chapdelaine, P., Maclaren, L. A., and Fortier, M. A. (2004). Effect of interferon-tau on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: evidence of polycrine actions of prostaglandin E_2. *Endocrinology* **145**, 5280–5293. doi:10.1210/EN.2004-0587

Barash, I. A., Cheung, C. C., Weigle, D. S., Ren, H., Kabigting, E. B., Kuijper, J. L., Clifton, D. K., and Steiner, R. A. (1996). Leptin is a metabolic signal to the reproductive system. *Endocrinology* **137**, 3144–3147. doi:10.1210/EN.137.7.3144

Boos, A., Meyer, W., Schwarz, R., and Grunert, E. (1996). Immunohistochemical assessment of oestrogen receptor and progesterone receptor distribution in biopsy samples of the bovine endometrium collected throughout the oestrous cycle. *Anim. Reprod. Sci.* **44**, 11–21. doi:10.1016/0378-4320(96)01492-3

Charpigny, G., Reinaud, P., Tamby, J. P., Creminon, C., Martal, J., Maclouf, J., and Guillomot, M. (1997). Expression of cyclooxygenase-1 and -2 in ovine endometrium during the estrous cycle and early pregnancy. *Endocrinology* **138**, 2163–2171. doi:10.1210/EN.138.5.2163

Charpigny, G., Reinaud, P., Creminon, C., and Tamby, J. P. (1999). Correlation of increased concentration of ovine endometrial cyclooxygenase 2 with the increase in PGE_2 and PGD_2 in the late luteal phase. *J. Reprod. Fertil.* **117**, 315–324. doi:10.1530/JRF.0.1170315

Chen, Y., Green, J. A., Antoniou, E., Ealy, A. D., Mathialagan, N., Walker, A. M., Avalle, M. P., Rosenfeld, C. S., Hearne, L. B., and Roberts, R. M. (2006). Effect of interferon-τ administration on endometrium of nonpregnant ewes: a comparison with pregnant ewes. *Endocrinology* **147**, 2127–2137. doi:10.1210/EN.2005-1310

Cherny, R. A., Salamonsen, L. A., and Findlay, J. K. (1991). Immunocytochemical localization of oestrogen receptors in the endometrium of the ewe. *Reprod. Fertil. Dev.* **3**, 321–331. doi:10.1071/RD9910321

Demir, R., Kayisli, U. A., Celik-Ozenci, C., Korgun, E. T., Demir-Weusten, A. Y., and Arici, A. (2002). Structural differentiation of human uterine luminal and glandular epithelium during early pregnancy: an ultrastructural and immunohistochemical study. *Placenta* **23**, 672–684. doi:10.1053/PLAC.2002.0841

Fenwick, M. A., Fitzpatrick, R., Kenny, D. A., Diskin, M. G., Patton, J., Murphy, J. J., and Wathes, D. C. (2008). Interrelationships between negative energy balance (NEB) and IGF regulation in liver of lactating dairy cows. *Domest. Anim. Endocrinol.* **34**, 31–44. doi:10.1016/J.DOMANIEND.2006.10.002

Goff, A. K. (2004). Steroid hormone modulation of prostaglandin secretion in the ruminant endometrium during the estrous cycle. *Biol. Reprod.* **71**, 11–16. doi:10.1095/BIOLREPROD.103.025890

Kaye, P. L. (1997). Preimplantation growth factor physiology. *Rev. Reprod.* **2**, 121–127. doi:10.1530/ROR.0.0020121

Kimmins, S., and MacLaren, L. A. (2001). Oestrous cycle and pregnancy effects on the distribution of oestrogen and progesterone receptors in bovine endometrium. *Placenta* **22**, 742–748. doi:10.1053/PLAC.2001.0708

Ko, Y., Lee, C. Y., Ott, T. L., Davis, M. A., Simmen, R. C., Bazer, F. W., and Simmen, F. A. (1991). Insulin-like growth factors in sheep uterine fluids: concentrations and relationship to ovine trophoblast protein-1 production during early pregnancy. *Biol. Reprod.* **45**, 135–142. doi:10.1095/BIOLREPROD45.1.135

Kotwica, J., Skarzynski, D., Jaroszewski, J., Williams, G. L., and Bogacki, M. (1998). Uterine secretion of prostaglandin F2alpha stimulated by different doses of oxytocin and released spontaneously during luteolysis in cattle. *Reprod. Nutr. Dev.* **38**, 217–226. doi:10.1051/RND:19980301

Littell, R. C., Pendergast, J., and Natarajan, R. (2000). Modelling covariance structure in the analysis of repeated measures data. *Stat. Med.* **19**, 1793–1819. doi:10.1002/1097-0258(20000715)19:13<1793::AID-SIM482>3.0.CO;2-Q

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408. doi:10.1006/METH.2001.1262

Lozano, J. M., Abecia, J. A., Forcada, F., Zarazaga, L., and Alfaro, B. (1998). Effect of undernutrition on the distribution of progesterone in the uterus of ewes during the luteal phase of the estrous cycle. *Theriogenology* **49**, 539–546. doi:10.1016/S0093-691X(98)00005-3

McCracken, J. A., Custer, E. E., and Lamsa, J. C. (1999). Luteolysis: a neuroendocrine-mediated event. *Physiol. Rev.* **79**, 263–323.

Meikle, A., Tasende, C., Rodriguez, M., and Garofalo, E. G. (1997). Effects of estradiol and progesterone on the reproductive tract and on uterine sex steroid receptors in female lambs. *Theriogenology* **48**, 1105–1113. doi:10.1016/S0093-691X(97)00343-9

Meikle, A., Bielli, A., Masironi, B., Pedrana, G., Wang, H., Forsberg, M., and Sahlin, L. (2000). An immunohistochemical study on the regulation of estrogen receptor alpha by estradiol in the endometrium of the immature ewe. *Reprod. Nutr. Dev.* **40**, 587–596. doi:10.1051/RND:2000102

Naderi, A., Ahmed, A. A., Barbosa-Morais, N. L., Aparicio, S., Brenton, J. D., and Caldas, C. (2004). Expression microarray reproducibility is improved by optimising purification steps in RNA amplification and labelling. *BMC Genomics* **5**, 9. doi:10.1186/1471-2164-5-9

Pfaffl, M. W., Georgieva, T. M., Georgiev, I. P., Ontsouka, E., Hageleit, M., and Blum, J. W. (2002). Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3 in the bovine species. *Domest. Anim. Endocrinol.* **22**, 91–102. doi:10.1016/S0739-7240(01)00128-X

Rhind, S. M., McKelvey, W. A. C., McMillen, S., Gunn, R. G., and Elston, D. A. (1989). Effect of restricted food-intake, before and or after mating, on the reproductive performance of greyface ewes. *Anim. Prod.* **48**, 149–155.

Rosenfeld, C. S., Han, C. S., Alexenko, A. P., Spencer, T. E., and Roberts, R. M. (2002). Expression of interferon receptor subunits,

IFNAR1 and IFNAR2, in the ovine uterus. *Biol. Reprod.* **67**, 847–853. doi:10.1095/BIOLREPROD.102.004267

Russel, A. J. F., Doney, J. M., and Gunn, R. G. (1969). Subjective assessment of body fat in live sheep. *J. Agric. Sci.* **72**, 451–454. doi:10.1017/S0021859600024874

Schams, D., Kohlenberg, S., Amselgruber, W., Berisha, B., Pfaffl, M. W., and Sinowatz, F. (2003). Expression and localisation of oestrogen and progesterone receptors in the bovine mammary gland during development, function and involution. *J. Endocrinol.* **177**, 305–317. doi:10.1677/JOE.0.1770305

Sosa, C., Lozano, J. M., Vinoles, C., Acuna, S., Abecia, J. A., Forcada, F., Forsberg, M., and Meikle, A. (2004). Effect of plane of nutrition on endometrial sex steroid receptor expression in ewes. *Anim. Reprod. Sci.* **84**, 337–348. doi:10.1016/J.ANIREPROSCI.2003.12.011

Sosa, C., Abecia, J. A., Forcada, F., Vinoles, C., Tasende, C., Valares, J. A., Palacin, I., Martin, G. B., and Meikle, A. (2006). Effect of undernutrition on uterine progesterone and oestrogen receptors and on endocrine profiles during the ovine oestrous cycle. *Reprod. Fertil. Dev.* **18**, 447–458. doi:10.1071/RD05138

Sosa, C., Abecia, J. A., Carriquiry, M., Forcada, F., Martin, G. B., Palacin, I., and Meikle, A. (2009). Early pregnancy alters the metabolic responses to restricted nutrition in sheep. *Domest. Anim. Endocrinol.* **36**, 13–23. doi:10.1016/J.DOMANIEND.2008.08.003

Spencer, T. E., and Bazer, F. W. (1995). Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. *Biol. Reprod.* **53**, 1527–1543. doi:10.1095/BIOLREPROD53.6.1527

Spencer, T. E., Ott, T. L., and Bazer, F. W. (1998). Expression of interferon regulatory factors one and two in the ovine endometrium: effects of pregnancy and ovine interferon tau. *Biol. Reprod.* **58**, 1154–1162. doi:10.1095/BIOLREPROD58.5.1154

Spencer, T. E., Burghardt, R. C., Johnson, G. A., and Bazer, F. W. (2004). Conceptus signals for establishment and maintenance of pregnancy. *Anim. Reprod. Sci.* **82–83**, 537–550. doi:10.1016/J.ANIREPROSCI.2004.04.014

Stevenson, K. R., Gilmour, R. S., and Wathes, D. C. (1994*a*). Localization of insulin-like growth factor-I (IGF-I) and -II messenger ribonucleic acid and type 1 IGF receptors in the ovine uterus during the estrous cycle and early pregnancy. *Endocrinology* **134**, 1655–1664. doi:10.1210/EN.134.4.1655

Stevenson, K. R., Riley, P. R., Stewart, H. J., Flint, A. P., and Wathes, D. C. (1994*b*). Localization of oxytocin receptor mRNA in the ovine uterus during the oestrous cycle and early pregnancy. *J. Mol. Endocrinol.* **12**, 93–105. doi:10.1677/JME.0.0120093

Taylor, K. M., Gray, C. A., Joyce, M. M., Stewart, M. D., Bazer, F. W., and Spencer, T. E. (2000). Neonatal ovine uterine development involves alterations in expression of receptors for estrogen, progesterone, and prolactin. *Biol. Reprod.* **63**, 1192–1204. doi:10.1095/BIOLREPROD63.4.1192

Thatcher, W. W., Guzeloglu, A., Meikle, A., Kamimura, S., Bilby, T., Kowalski, A. A., Badinga, L., Pershing, R., Bartolome, J., and Santos, J. E. P. (2003). Regulation of embryo survival in cattle. *Reprod. Suppl.* **61**, 253–266.

Thibodeaux, J. K., Broussard, J. R., Godke, R. A., and Hansel, W. (1994). Stimulation of progesterone production in bovine luteal cells by co-incubation with bovine blastocyst-stage embryos or trophoblastic vesicles. *J. Reprod. Fertil.* **101**, 657–662. doi:10.1530/JRF.0.1010657

Thissen, J. P., Ketelslegers, J. M., and Underwood, L. E. (1994). Nutritional regulation of the insulin-like growth factors. *Endocr. Rev.* **15**, 80–101.

Waseem, N. H., and Lane, D. P. (1990). Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. *J. Cell Sci.* **96**, 121–129.

Wathes, D. C., and Hamon, M. (1993). Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrous cycle and early pregnancy of the ewe. *J. Endocrinol.* **138**, 479–491. doi:10.1677/JOE.0.1380479

Wathes, D. C., Reynolds, T. S., Robinson, R. S., and Stevenson, K. R. (1998). Role of the insulin-like growth factor system in uterine function and placental development in ruminants. *J. Dairy Sci.* **81**, 1778–1789.

Wu, S. H., Weber, W. J., Chester-Jones, H., Hansen, L. B., and Crooker, B. A. (2004). Effects of intake, selection for milk yield, and bST administration on hepatic expression of insulin receptors and components of the somatotropic axis in growing Holstein heifers. *J. Dairy Sci.* **87**(Suppl. 1), 364. [Abstract]

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The effects of melatonin on *in vitro* oocyte competence and embryo development in sheep

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Abstract

The aim of this study was to assess the effects of melatonin on the *in vitro* maturation and fertilization of sheep oocytes, and on the *in vitro* culture of the embryos. Oocytes from sheep ovaries collected at the slaughterhouse were divided into four groups, two of which were treated with either 10^{-5} M (M5) or 10^{-6} M (M6) melatonin, while the other two groups served as untreated controls (C5 and C6). After *in vitro* fertilization with fresh ram semen, the embryos produced in each group were divided into two sets, one cultured with melatonin (M5M, C5M, M6M and C6M), and the other without melatonin (M5C, C5C, M6C, and C6C). A melatonin concentration of 10^{-6} M increased maturation rate (82.5% *vs*. 73.7% for M6 and C6, respectively; $P<0.05$) and tended to improve cleavage rate (79.4% *vs*. 72.6% for M6 and C6, respectively, $P=0.08$). A higher melatonin concentration (10^{-5} M) did not have significant effects on those parameters. Blastocyst rates on Day 8 did not differ significantly among groups.

Additional keywords: I*n vitro* fertilization, *in vitro* maturation .

Resumen

Efecto de la melatonina en la competencia del oocito y el desarrollo embrionario ovino *in vitro*

El objetivo de este estudio es determinar el efecto de la melatonina sobre la maduración y fecundación *in vitro* de ovocitos ovinos, y sobre el cultivo *in vitro* de los embriones obtenidos. Ovocitos extraídos de ovarios de ovejas obtenidos en matadero se dividieron en cuatro grupos, de los cuales dos fueron tratados con melatonina 10^{-5} M (M5) ó 10^{-6} M (M6), mientras que los otros dos sirvieron como grupos control (C5 y C6). Tras su fecundación *in vitro* con semen fresco de morueco, los embriones obtenidos en cada grupo se dividieron a su vez en otros dos grupos, de forma que la mitad se cultivaron con melatonina (M5M, C5M, M6M and C6M), y la otra mitad sin ella (M5C, C5C, M6C, and C6C). La concentración 10^{-6} M de melatonina aumentó el porcentaje de maduración de los ovocitos (82,5% *vs*. 73,7% en los grupos M6 y C6, respectivamente; $P<0,05$) mientras que el porcentaje de embriones divididos 36 horas tras la fecundación mostró una tendencia a la significación (79,4% *vs*. 72,6% en los grupos M6 y C6, respectivamente; $P=0,08$). Una mayor concentración de melatonina (10^{-5} M) no tuvo efecto significativo sobre estos parámetros. No se encontraron diferencias significativas en los porcentajes de blastocistos tras ocho días de cultivo.

Palabras clave adicionales: Fecundación *in vitro* maduración *in vitro*,

Abbreviations used: DMSO (dimethyl sulfoxide), FSH (follicle-stimulating hormone), IVF (in vitro fertilization), IVM (in vitro maturation), LH (luteinizing hormone), PBS (phosphate buffered saline), SOF (synthetic oviductal fluid)

Introduction

The use of melatonin implants to improve reproduction in sheep is widely spread in some Mediterranean countries. In the last few years some reports have revealed that melatonin not only modifies the perception of photoperiod by ewes at hypothalamic level but also can exert its influence at other targets of the reproductive system (Abecia *et al.*, 2008). Melatonin, an endogenous hormone produced by several organs, particularly the pineal gland

(Huether, 1993), is essential for sleep and temperature regulation, circadian and seasonal rhythmic cycles, and reproductive physiology (Arendt, 1998; Arendt, 2003). Melatonin is used widely to advance the breeding season in sheep and is an effective method of inducing oestrous cycles, increasing lambing rates and prolificacy during the seasonal anoestrous (Haresign *et al.*, 1990; Haresign, 1992; Abecia *et al.*, 2007). Those effects are associated with an improvement in ovulation rate (Zúñiga *et al.*, 2002), luteal function (Durotoye *et al.*, 1997; Abecia *et al.*, 2002) and embryo viability (Forcada *et al.*, 2006). For a summary of the effects of exogenous melatonin on the ovary and early embryos in ewes, see Abecia *et al.* (2008).

The high amphilicity of melatonin promotes its rapid transfer into organs and fluids, and this pineal hormone can rapidly pass through cellular membranes. Reported increases in prolificacy subsequent to the administration of melatonin in sheep might be due to the direct effect of exogenous melatonin on the ovine oocyte, which has been observed in other species (Ishizuka *et al.*, 2000; Na *et al.*, 2005; Parka *et al.*, 2006). Several studies have reported the presence of melatonin in human pre-ovulatory follicular fluid and the seasonal variation of its contents (Brzezinski *et al.*, 1987; Yie *et al.*, 1995), and melatonin receptors have been found on rat, mouse, and porcine ovarian cells (Soares *et al.*, 2003; Na *et al.*, 2005; Kang *et al.*, 2008), which suggests that melatonin has a direct effect on oocyte competence.

In sheep, some studies have suggested that seasonal anoestrous can have a significant detrimental effect on the number of recovered oocytes per female, the competence of the oocytes, and the *in vitro* fertilization rate (Stenbak *et al.*, 2001; Vázquez *et al.*, 2009); however, information about the effects of melatonin treatments on oocyte quality and *in vitro* fertilization (IVF) during anoestrous is limited. In one study, after IVF of the oocytes recovered from superovulated ewes in the non-reproductive season, melatonin-treated and untreated ewes did not differ significantly in their fertilization rates (Luther *et al.*, 2005). In another study, developmental competence of oocytes collected from ewes during the anoestrous was significantly improved by supplemental exogenous melatonin (Valasi *et al.*, 2006). Although there are reports of the positive effects of adding melatonin in *in vitro* maturation (IVM) (Dimitriadis *et al.*, 2005) and embryo culture media (Ishizuka *et al.*, 2000; Na *et al.*, 2005), the effect of melatonin in the IVM and IVF media on the developmental competence of ovine oocytes has not been evaluated thoroughly, which is needed to improve the efficiency of IVM, IVF, and *in vitro* embryo culture procedures.

The objective of this study was to evaluate the effects of two different melatonin concentrations on the *in vitro* maturation and fertilization of oocytes from sheep ovaries collected at the slaughterhouse, and on the *in vitro* culture of the embryos.

Material and methods

Oocyte collection and in vitro maturation

From November to February, the ovaries of mature Rasa Aragonesa ewes were collected at the slaughterhouse and transported to the laboratory in phosphate-buffered saline solution (PBS) supplemented with 100 IU mL^{-1} of penicillin-G and 100 µg mL^{-1} of streptomycin sulphate at 35-37ºC. Except where indicated otherwise, all of the reagents for the preparation of the media were purchased from Sigma-Aldrich Co.

Oocytes were collected using a combination of puncture and slicing techniques (Wani *et al.*, 1999) in a Petri dish that contained handling medium (Hepes-buffered TCM-199 supplemented with 0.1% of polyvinyl alcohol, 0.04% of sodium bicarbonate, 25 IU mL^{-1} of heparin, 100 IU mL^{-1} of penicillin-G, and 100 µg mL^{-1} of streptomycin sulphate). Following Wani *et al*. (2000), oocytes were categorized based on their cumulus cells and cytoplasm morphology; only the oocytes that had several layers of cumulus cells and a uniform cytoplasm were selected for *in vitro* maturation. Those oocytes were randomly assigned either to one of two melatonin-treatment groups [10^{-5} M (M5) or 10^{-6} M (M6)] or to one of two untreated (control) groups (C5 and C6) and transferred into a maturation medium that contained bicarbonate-buffered TCM-199, supplemented with 10% (v/v) oestrus sheep serum, 10 µg mL^{-1} each of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), 100 µM of cysteamine, 0.3 mM of sodium piruvate, 100 IU mL^{-1} of penicillin G, and 100 µg mL^{-1} of streptomycin sulphate, covered with mineral oil, and incubated for 24 h at 39ºC under 5% CO_2 and a saturated humidity.

Melatonin was dissolved in dimethyl sulfoxide (DMSO) and PBS to produce concentrations of 10^{-5} or 10^{-6} M, which was administered to the oocytes in the M5 and M6 groups, respectively. The final concentration of DMSO in the media was 0,01 vol/vol, with no effect on embryo development, as stated by Ishizuka *et al.* (2000).

In vitro fertilization and embryo culture

After the end of incubation, oocytes were freed from the cumulus cells and transferred to a fertilization medium that consisted of synthetic oviductal fluid (SOF) without glucose (Tervit and Whittingham, 1972), supplemented with 2% (vol/vol) of oestrous sheep serum, 10

µg mL^{-1} of heparin, and 1 µg mL^{-1} of hypotaurine. Again, melatonin was added to the fertilization medium of the M5 and M6 groups in the concentrations used in the maturation medium.

On the day of fertilization, fresh semen was collected from four Rasa Aragonesa rams, pooled, diluted 1:10 in a saline medium that contained 0.25 M sucrose, 10 mM Hepes, 2 mM KOH, 5 mM glucose, 0.5 M sodium phosphate monobasic, and 100 mM ethylene glycol tetraacetic acid (EGTA), and kept at 15ºC until needed for fertilization. Highly motile spermatozoa were selected using the Swim-up Technique, added to the fertilization medium (350 µL) containing the oocytes at a final concentration of 1 x 10^6 spermatozoa mL^{-1}, covered with mineral oil, and incubated for 24 h, at 39ºC in 5% CO_2.

After 24 and 36 h, the cleaved embryos in each group were randomly divided into two sets. Half of the embryos in each group were cultured in a media that contained melatonin at either a 10^{-5} M concentration (embryos from the M5 and C5 oocytes) or a 10^{-6} M concentration (embryos from the M6 and C6 oocytes), and the other half remained as untreated controls. The embryos from the eight groups (M5M, M5C, C5M, C5C, M6M, M6C, C6M, and C6C) were placed in a culture medium that contained SOF supplemented with essential and non-essential amino acids at oviductal concentrations (Walker *et al.*, 1996), 0.4% BSA (w/v), 1 mM of L-glutamine, 100 IU mL^{-1} of penicillin G, and 100 µg mL^{-1} of streptomycin sulphate, covered with mineral oil, and kept at 39ºC in a maximally humidified atmosphere, with 5% CO_2, 5% O_2, and 90% N_2 until the blastocyst stage (Day 8). The number and developmental stage of blastocysts (young, expanded, hatching and hatched blastocysts) were recorded at 6, 7, and 8 days after fertilization.

Non-cleaved oocytes were observed under stereomicroscope to assess their maturation stage. Ooocytes showing the first polar body were considered matured, and oocytes with two polar bodies were considered fertilized, but not cleaved.

Statistical analysis

Maturation and cleavage rates were calculated over the number of selected oocytes, whereas fertilization rate was calculated over the number of matured oocytes, and blastocysts rates were calculated over the number of cleaved embryos.

Maturation, cleavage, and blastocyst rates of the experimental groups were compared by means of chi-square test (SPSS Software, v.14.0). When at least one value had an expected frecuency less than 5, Yates' correction for continuity was used.

Results and discussion

In this study, the lower of the two melatonin concentrations (10^{-6} M) increased significantly the maturation rate of Rasa Aragonesa oocytes ($P<0.05$) and tended to increase the cleavage rate ($P<0.1$); however, a 10^{-5} M concentration of melatonin in the media did not influence significantly the maturation, fertilization or cleavage (Table 1). The addition of melatonin to IVM and IVF media can improve *in vitro* oocyte maturation and cleavage rates in bovids (Dimitriadis *et al*., 2005), mice (Na *et al*., 2005) and humans (Parka *et al*., 2006).

High concentrations of melatonin in follicular fluid (Brzezinski *et al*., 1987), and the presence of receptors in granulose cells (Soares *et al*., 2003; Na *et al*., 2005; Kang *et al*., 2008), suggest that melatonin might be important to ovarian function. At night, the melatonin concentrations in physiological plasma in Rasa Aragonesa ewes are about 4×10^{-7} M (Forcada *et al*., 1995). Studies have shown that physiological melatonin concentrations in follicular fluid are almost three times higher than they are in the serum (Brzezinski *et al*., 1987; Ronnberg *et al*., 1990). Thus, the melatonin concentration of 10^{-6} M used in our study is similar to that found in human follicular fluid, which could provide a basis for evaluating the benefits of exogenous melatonin at the gamete and early embryo stages. Furthermore, the beneficial effect of melatonin on early embryo development might be mediated through the reactive oxygen species (ROS) scavenger properties of the pineal hormone (Reiter *et al*., 2000). As a ROS scavenger, melatonin might be very valuable shortly after IVF because high concentrations of spermatozoa in small volumes of IVF medium lead to an increase in free radicals. Oxidative stress has toxic effects and melatonin provides protection against the oxidative stress (Tamura *et al*., 2008).

These results and those of others (Adriaens *et al*., 2006) suggest that the effect of melatonin on oocyte maturation is dose-dependent. Although melatonin toxicity is reported to be extremely low, oocyte maturation in female mice was significantly impaired by melatonin concentrations of 10^{-3} M or higher. In the early stages of maturation, melatonin has an even greater effect on the *in vitro* maturation of oocytes (Dimitriadis *et al*., 2005).

In this study, blastocyst rates did not differ significantly among groups at either of the two melatonin concentrations used (Table 1) but the highest dose added to the culture medium of the control group seemed to impair embryo development. When data were analyzed by culture day and blastocyst type, significant differences were found on hatching blastocyst rate in day 8 between 10^{-5} M treated and control groups, and on young blastocyst rate in day 8

derived from oocytes matured with or without 10^{-6} M melatonin and cultured without the pineal hormone (Table 2). Elsewhere, we demonstrated a negative effect of melatonin on blastocyst rate when it was added to the maturation medium at 10^{-5} M concentration (Casao *et al*., 2007), but a concentration halfway between 10^{-6} and 10^{-5} M ($0.43 \cdot 10^{-5}$ M) in the culture medium appeared to increase the hatching rate of thawed ovine blastocysts (Abecia *et al.*, 2002).

In general, the effects of melatonin on *in vitro* preimplantational embryo development are unclear. Several studies have shown that enriching the culture medium with melatonin can improve embryo development, but blastocyst rates can vary widely depending on the melatonin concentrations (Ishizuka *et al*., 2000; Danilova *et al*., 2004; Manjunatha *et al*., 2008). When embryos are subjected to melatonin concentrations of 10^{-3} M or higher, cleavage rates can be impaired (Rodriguez-Osorio *et al*., 2007), but other studies covering a wide range of concentrations suggest that melatonin treatments do not influence the preimplantation development of embryos (McElhinny *et al*., 1996; Tsantarliotou *et al*., 2007).

In addition to its benefits to *in vitro* maturation and early embryo development, melatonin might have a beneficial effect on embryo preimplantation development because of its capacity as a radical scavenger (Chetsawang *et al*., 2006), to protect embryonic cells from oxidative stress. High levels of reactive oxygen species can damage spermatozoa, oocytes, and embryos (Agarwal *et al*., 2003), and *in vitro* melatonin treatments might protect them against oxidative stress. In a recent study, Papis *et al.* (2007) showed that the beneficial effects of melatonin on bovine embryo development were greater when embryos were cultured in the presence of high concentrations of atmospheric oxygen (20%) rather than at physiological concentrations (7%). In addition, melatonin can increase blastocyst rates and blastocyst total cell number concomitant to a significant decrease of apoptotic nuclei rate in preimplantational parthenogenetic porcine embryos (Choi *et al*., 2008).

In conclusion, our study demonstrated that adding 10^{-6} M melatonin to IVM and IVF media increased *in vitro* maturation and early cleavage rates of sheep oocytes. Although a higher concentration (10^{-5} M) of melatonin did not have the same effect, additional studies are needed to optimize the use of melatonin in IVM, IVF, and *in vitro* embryo culture procedures.

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References

ABECIA J.A., FORCADA F., CASAO A., PALACIN I., 2008. Effect of exogenous melatonin on the ovary, the embryo and the establishment of pregnancy in sheep. Animal 2, 399-404.

ABECIA J.A., FORCADA F., ZUÑIGA O., 2002. The effect of melatonin on the secretion of progesterone in sheep and on the development of ovine embryos in vitro. Vet Res Commun 26, 151 - 158.

ABECIA J.A., VALARES J.A., FORCADA F., PALACIN I., MARTIN S., MARTINO A., 2007. The effect of melatonin on the reproductive performance of three sheep breeds in Spain. Small Rumin Res 69, 10-16.

ADRIAENS I., JACQUET P., CORTVRINDT R., JANSSEN K., SMITZ J., 2006. Melatonin has dose-dependent effects on folliculogenesis, oocyte maturation capacity and steroidogenesis. Toxicology 228, 333-343.

AGARWAL A., SALEH R.A., BEDAIWY M.A., 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 79, 829-843.

ARENDT J., 1998. Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. Rev Reprod 3, 13-22.

ARENDT J., 2003. Importance and relevance of melatonin to human biological rhythms. J Neuroendocrinol 15, 427-431.

BRZEZINSKI A., SEIBEL M.M., LYNCH H.J., DENG M.H., WURTMAN R.J., 1987. Melatonin in human preovulatory follicular fluid. J Clin Endocrinol Metab 64, 865-867.

CASAO A., YANIZ J., FORCADA F., ABECIA J.A., CEBRIAN-PEREZ J.A., MUINO-BLANCO T., 2007. Effect of melatonin in the IVM and IVF process in ovine species. In: XII Jornadas sobre Producción Animal, AIDA, Zaragoza, Spain, pp. 36-38.

CHETSAWANG B., PUTTHAPRASART C., PHANSUWAN-PUJITO P., GOVITRAPONG P., 2006. Melatonin protects against hydrogen peroxide-induced cell death signaling in

SH-SY5Y cultured cells: involvement of nuclear factor kappa B, Bax and Bcl-2. J Pineal Res 41, 116-123.

CHOI J., PARK S.M., LEE E., KIM J.H., JEONG Y.I., LEE J.Y., PARK S.W., KIM H.S., HOSSEIN M.S., JEONG Y.W., KIM S., HYUN S.H., HWANG W.S., 2008. Anti-apoptotic effect of melatonin on preimplantation development of porcine parthenogenetic embryos. Mol Reprod Dev 75, 1127-1135.

DANILOVA N., KRUPNIK V.E., SUGDEN D., ZHDANOVA I.V., 2004. Melatonin stimulates cell proliferation in zebrafish embryo and accelerates its development. FASEB J 18, 751-753.

DIMITRIADIS I., PAPANIKOLAU T., VAINAS E., AMIRIDIS G.S., VALASI I., SAMARTZI F., REKKAS C.A., 2005. Effects of melatonin on in vitro maturation of bovine oocytes. In: ESDAR (Annual Conference of The European Society for Domestic Animal Reproduction), Murcia, Spain, pp. 397-397.

DUROTOYE L.A., WEBLEY G.E., RODWAY R.G., 1997. Stimulation of the production of progesterone by the corpus luteum of the ewe by the perfusion of melatonin in vivo and by treatment of granulosa cells with melatonin in vitro. Res Vet Sci 62, 87-91.

FORCADA F., ABECIA J.A., CEBRIAN-PEREZ J.A., MUINO-BLANCO T., VALARES J.A., PALACIN I., CASAO A., 2006. The effect of melatonin implants during the seasonal anestrus on embryo production after superovulation in aged high-prolificacy Rasa Aragonesa ewes. Theriogenology 65, 356-365.

FORCADA F., ZARAZAGA L., ABECIA J.A., 1995. Effect of exogenous melatonin and plane of nutrition after weaning on estrous activity, endocrine status and ovulation rate in Salz ewes lambing in the seasonal anestrus. Theriogenology 43, 1179-1193.

HARESIGN W., 1992. Manipulation of reproduction in sheep. J Reprod Fertil Suppl 45, 127-139.

HARESIGN W., PETERS A.R., STAPLES L.D., 1990. The effect of melatonin implants on breeding activity and litter size in commercial sheep flocks in the Uk. Animal Production 50, 111-121.

290 HUETHER G., 1993. The contribution of extrapineal sites of melatonin synthesis to
291 circulating melatonin levels in higher vertebrates. Experientia 49, 665-670.

292 ISHIZUKA B., KURIBAYASHI Y., MURAI K., AMEMIYA A., ITOH M.T., 2000. The
293 effect of melatonin on in vitro fertilization and embryo development in mice. J Pineal
294 Res 28, 48–51.

295 KANG J.T., KOO O.J., KWON D.K., PARK H.J., JANG G., KANG S.K., LEE B.C., 2008.
296 Effects of melatonin on in vitro maturation of porcine oocyte and expression of
297 melatonin receptor RNA in cumulus and granulosa cells. J Pineal Res 46, 22-28.

298 LUTHER J.S., REDMER D.A., REYNOLDS L.P., CHOI J.T., PANT D., NAVANUKRAW
299 C., ARNOLD D.R., SCHEAFFER A.N., BOROWICZ P., KIRSCH J.D., WEIGL R.M.,
300 KRAFT K.C., GRAZUL-BILSKA A.T., 2005. Ovarian follicular development and
301 oocyte quality in anestrous ewes treated with melatonin, a controlled internal drug
302 release (CIDR) device and follicle stimulating hormone. Theriogenology 63, 2136–
303 2146.

304 MANJUNATHA B.M., DEVARAJ M., GUPTA P.S.P., RAVINDRA J.P., NANDI S., 2008.
305 Effect of taurine and melatonin in the culture medium on buffalo "in vitro" embryo
306 development. Reprod Domest Anim 44, 12-16.

307 MCELHINNY A.S., DAVIS F.C., WARNER C.M., 1996. The effect of melatonin on
308 cleavage rate of C57BL/6 and CBA/Ca preimplantation embryos cultured in vitro. J
309 Pineal Res 21, 44-48.

310 NA K., KIM J., LEE J., YOON T., CHA K., LEE D., 2005. Effect of melatonin on the
311 maturation of mouse GV oocytes and apoptosis of cumulus cells in vitro. Fertil Steril
312 84, S103-S103.

313 PAPIS K., POLESZCZUK O., WENTA-MUCHALSKA E., MODLINSKI J.A., 2007.
314 Melatonin effect on bovine embryo development in vitro in relation to oxygen
315 concentration. J Pineal Res 43, 321-326.

316 PARKA E., LEEA D., CHOA J., HANA J., CHAA K., YOONA T., 2006. Addition of
317 melatonin in in vitro maturation (IVM) medium increases maturation and fertilization of
318 immature human oocytes. Fertil Steril 86, S168.

REITER R.J., TAN D.X., OSUNA C., GITTO E., 2000. Actions of melatonin in the reduction of oxidative stress - A review. J Biomed Sci 7, 444-458.

RODRIGUEZ-OSORIO N., KIM I.J., WANG H., KAYA A., MEMILI E., 2007. Melatonin increases cleavage rate of porcine preimplantation embryos in vitro. J Pineal Res 43, 283-288.

RONNBERG L., KAUPPILA A., LEPPALUOTO J., MARTIKAINEN H., VAKKURI O., 1990. Circadian and seasonal variation in human preovulatory follicular fluid melatonin concentration. J Clin Endocrinol Metab 71, 492-496.

SOARES J.M.J., MASANA M.I., ERSAHIN C., DUBOCOVICH M.L., 2003. Functional melatonin receptors in rat ovaries at various stages of the estrous cycle. J Pharmacol Exp Ther 306, 694-702.

STENBAK T.K., REDMER D.A., BERGINSKI H.R., ERICKSON A.S., NAVANUKRAW C., TOUTGES M.J., BILSKI J.J., KIRSCH J.D., KRAFT K.C., REYNOLDS L.P., GRAZUL-BILSKA A.T., 2001. Effects of follicle stimulating hormone (FSH) on follicular development, oocyte retrieval, and in vitro fertilization (IVF) in ewes during breeding season and seasonal anestrus. Theriogenology 56, 51-64.

TAMURA H., TAKASAKI A., MIWA I., TANIGUCHI K., MAEKAWA R., ASADA H., TAKETANI T., MATSUOKA A., YAMAGATA Y., SHIMAMURA K., MORIOKA H., ISHIKAWA H., REITER R.J., SUGINO N., 2008. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. J Pineal Res 44, 280-287.

TERVIT H.R., WHITTINGHAM D.G., 1972. Succesful culture in vitro of sheep and cattle ova. J. Reprod. Fert. 30, 493 - 497.

TSANTARLIOTOU M.P., ATTANASIO L., DE ROSA A., BOCCIA L., PELLERANO G., GASPARRINI B., 2007. The effect of melatonin on bovine in vitro embryo development. In: 17th Congress of the Scientific-Association-of-Animal-Production, Alghero, ITALY, pp. 488-489.

VALASI F., TSILIGIANNI T., PAPANIKOLAOU T., DIMITRIADIS I., VAINAS E., SAMARTZI F., AMIRIDIS G.S., 2006. Melatonin improves the developmental

Table 1: Maturation, fertilization, cleavage and blastocyst rate of oocytes from Rasa Aragonesa ewes matured and / or cultured with or without 10^{-5} or 10^{-6} mol/L melatonin. Within columns, different letters (a, b) indicate differences of $P<0.05$ at the same treatment dose. Within columns, different letters (c, d) indicate differences of $P = 0.08$ at the same treatment dose.

Oocyte group	Maturation:	Fertilization:	Cleavage:	Embryo culture group	Blastocyst rate
M5	151/186 (81.2%)	146/151 (96.7%)	144/186 (77.4%)	M5M	23/69 (33.3%)
				M5C	20/75 (26.7%)
C5	147/191 (77.0%)	144/147 (98.0%)	144/191 (75.4%)	C5M	21/74 (28.4%)
				C5C	26/70 (37.1%)
M6	156/189 (82.5%) [a]	150/156 (96.2%)	150/189 (79.4%) [c]	M6M	27/74 (36.5%)
				M6C	29/76 (38.2%)
C6	132/179 (73.3%) [b]	131/132 (99.2%)	130/179 (72.6%) [d]	C6M	22/64 (34.4%)
				C6C	20/66 (30.3%)

Table 2: Blastocyst type rate of oocytes from Rasa Aragonesa ewes matured and/or cultured with or without 10^{-5} or 10^{-6} mol/L melatonin, on days 6, 7 and 8 after oocyte fertilization. Within columns, different letters (a, b) indicate differences of $P<0.05$ at the same treatment group.

	Day 6		**Day 7**				**Day 8**			
	Young	Expanded	Young	Expanded	Hatching	Hatched	Young	Expanded	Hatching	Hatche
M5M	7/69 (10.1%)	1/69 (1.4%)	11/69 (15.9%)[a]	9/69 (13.0%)	2/69 (2.9%)	1/69 (1.4%)	3/69 (4.3%)	12/69 (17.4%)	5/69 (7.2%)	3/69 (4.3%)
M5C	6/75 (8.0%)	1/75 (1.3%)	4/75 (5.3%)[b]	11/75 (14.6%)	3/75 (4.0%)	1/75 (1.3%)	3/75 (4.0 %)	8/75 (10.6%)	1/75 (1.3%)[b]	8/75 (10.6%
C5M	10/74 (13.6%)	2/74 (2.7%)	5/74 (6.8%)	11/74 (14.9%)	3/74 (4.1%)	2/74 (2.7%)	1/74 (1.4%)	10/74 (13.6%)	2/74 (2.7%)[b]	8/74 (10.8%
C5C	7/70 (10.0%)	6/70 (8.6%)	6/70 (8.6%)	11/70 (15.7%)	6/70 (8.6%)	0/70 (0.0%)	7/70 (10.0%)	7/70 (10.0%)	10/70 (14.3%)[a]	2/70 (2.9%)
M6M	7/74 (9.5%)	4/74 (5.4%)	9/74 (12.2%)	11/74 (14.9%)	4/74 (5.4%)	0/69 (0.0%)	5/74 (6.8%)	11/74 (14.9%)	3/74 (4.1%)	8/69 (10.8%
M6C	5/76 (6.6%)	6/76 (7.9%)	7/76 (9.2%)	15/76 (19.7%)	0/75 (0.0%)	3/76 (3.9%)	7/76 (9.2%)[a]	10/76 (13.2%)	3/76 (3.9%)	9/76 (11.8%
C6M	4/64 (6.2%)	3/64 (3.1%)	9/64 (14.1%)	6/64 (9.4%)	1/64 (1.6%)	2/64 (3.1%)	5/64 (7.8%)	11/64 (17.2%)	1/64 (1.6%)	5/64 (7.8%)
C6C	4/66 (6.1%)	5/66 (7.6%)	7/66 (10.6%)	9/66 (13.6%)	4/66 (6.1%)	0/66 (0.0%)	0/66 (0.0%)[b]	7/66 (10.6%)	7/66 (10.6%)	6/66 (9.1%)

Table 2: Blastocyst type rate of oocytes from Rasa Aragonesa ewes matured and/or cultured with or without 10^{-5} or 10^{-6} mol/L melatonin, on days 6, 7 and 8 after oocyte fertilization. Within columns, different letters (a, b) indicate differences of $P<0.05$ at the same treatment group.

	Day 6		**Day 7**				**Day 8**			
	Young	Expanded	Young	Expanded	Hatching	Hatched	Young	Expanded	Hatching	Hatched
M5M	7/69 (10.1%)	1/69 (1.4%)	11/69 (15.9%)[a]	9/69 (13.0%)	2/69 (2.9%)	1/69 (1.4%)	3/69 (4.3%)	12/69 (17.4%)	5/69 (7.2%)	3/69 (4.3%)
M5C	6/75 (8.0%)	1/75 (1.3%)	4/75 (5.3%)[b]	11/75 (14.6%)	3/75 (4.0%)	1/75 (1.3%)	3/75 (4.0 %)	8/75 (10.6%)	1/75 (1.3%)[b]	8/75 (10.6%
C5M	10/74 (13.6%)	2/74 (2.7%)	5/74 (6.8%)	11/74 (14.9%)	3/74 (4.1%)	2/74 (2.7%)	1/74 (1.4%)	10/74 (13.6%)	2/74 (2.7%)[b]	8/74 (10.8%
C5C	7/70 (10.0%)	6/70 (8.6%)	6/70 (8.6%)	11/70 (15.7%)	6/70 (8.6%)	0/70 (0.0%)	7/70 (10.0%)	7/70 (10.0%)	10/70 (14.3%)[a]	2/70 (2.9%)
M6M	7/74 (9.5%)	4/74 (5.4%)	9/74 (12.2%)	11/74 (14.9%)	4/74 (5.4%)	0/69 (0.0%)	5/74 (6.8%)	11/74 (14.9%)	3/74 (4.1%)	8/69 (10.8%
M6C	5/76 (6.6%)	6/76 (7.9%)	7/76 (9.2%)	15/76 (19.7%)	0/75 (0.0%)	3/76 (3.9%)	7/76 (9.2%)[a]	10/76 (13.2%)	3/76 (3.9%)	9/76 (11.8%
C6M	4/64 (6.2%)	3/64 (3.1%)	9/64 (14.1%)	6/64 (9.4%)	1/64 (1.6%)	2/64 (3.1%)	5/64 (7.8%)	11/64 (17.2%)	1/64 (1.6%)	5/64 (7.8%)
C6C	4/66 (6.1%)	5/66 (7.6%)	7/66 (10.6%)	9/66 (13.6%)	4/66 (6.1%)	0/66 (0.0%)	0/66 (0.0%)[b]	7/66 (10.6%)	7/66 (10.6%)	6/66 (9.1%)