UNIVERSIDAD DE LA REPÚBLICA
FACULTAD DE AGRONOMÍA

IATF CERVICAL EN OVINOS: PROTOCOLOS DE SINCRONIZACIÓN CON INTERVALO CORTO, MEDIO Y LARGO ENTRE DOSIS DE PROSTAGLANDINAS

por

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TESIS presentada como uno de los requisitos para obtener el título de Doctor en Ciencias Agrarias

MONTEVIDEO
URUGUAY
Diciembre 2017

Tesis aprobada por el tribunal integrado por: DMV. PhD. Alejo Menchaca (presidente), DMV. PhD. Alejandro Gibbons (relator), DMV. PhD. Antonio González-Bulnes (relator), DMV. PhD. Raquel Pérez (vocal), Ing. Agr. PhD. Daniel Fernández Abella (vocal), el 14 de diciembre de 2017.

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Dedico este trabajo a una persona que desde que me inicié en el camino de la investigación me ha apoyado, ha estado junto a mi desde la planificación a través del estudio y el intercambio franco de ideas, la búsqueda de financiación, la ejecución de los trabajos, discusión de los resultados, publicación…. Pero más importante considero yo, es que ha creído en mí y me ha ayudado siempre solidariamente, sin búsqueda de retribuciones, haciendo el bien por el hecho de hacer el bien, basándose en virtudes como el compromiso, la lealtad, el ser recto y justo y valorizando el trabajo en equipo. Tutor de mi tesis de Grado, co-tutor de mi tesis de Maestría, tutor de esta tesis, jefe, compañero, amigo, "mi padre del norte", con quien hemos compartido más de 10 años de ganar, perder y empatar. Las dualidades de la vida nunca nos han separado, sino que nos han fortalecido en conocernos a nosotros mismos. Este trabajo entonces está dedicado al muy querido Dr. Julio Olivera-Muzante ... simplemente "al Pájaro".

AGRADECIMIENTOS

El logro de culminar esta tesis no podría haber sido posible sin la ayuda y compromiso de las siguientes personas e instituciones, por lo tanto mí sentido agradecimiento a mi tutor Dr. PhD. Julio Olivera-Muzante y co-tutora Dra. PhD. Carolina Viñoles Gil por su continuo apoyo durante el desarrollo de este trabajo; y al Dr. PhD. Jorge Gil, Dr. Mauro Minteguiaga e Ing. Agr. MSc. Martín Claramunt por su ayuda en el trabajo de campo. A los estudiantes tesistas de Facultad de Veterinaria hoy Doctores en Ciencias Veterinarias: Eliana Bentancor, Carolina Bonino, Diego Donadio, Ramiro Dutra da Silveira, Josefina Ferrari, Valentina González, Sofía Grela, Graciana Hourcade, Cindy Pechi, Juan Manuel Vizcaino, y a los estudiantes de Trabajo Final de la Escuela Agraria La Carolina, hoy Técnicos en Sistemas Intensivos de Producción Animal: Johana Braga y Federico Soca; a todos por su trabajo y compromiso en el apoyo de la realización de los trabajos de campo. Aprendimos y nos divertimos… qué más se puede pedir? A la Escuela Agraria La Carolina, sus docentes "Salvito" García Pintos y Ana González, a todos los estudiantes de grado que participaron en las tareas de campo y a la Estación Experimental Dr. Mario A. Cassinoni (EEMAC) y los funcionarios de la Sección de Ovinos, sin su ayuda esto no hubiera sido posible. A la Facultad de Veterinaria por las oportunidades de formación técnico – académicas y a las funcionarias de la Hemeroteca de Facultad de Veterinaria por sus invalorables aportes en la búsqueda de artículos científicos. Al Secretariado Uruguayo de la Lana (SUL), institución de la cual formo parte actualmente y me ha concedido el tiempo solicitado para culminar con mi formación de doctorado. A Inés, Agustín y Micaela "que viene llegando", quienes me han acompañado en todos "mis viajes".

La financiación de estos trabajos fue realizada a través de la Beca doctoral de la Agencia Nacional de Investigación e Innovación (ANII, 2013-2014; POS_NAC_2012_1_9449) y del proyecto INIA - FPTA Perfil 315 "Validación de la inseminación artificial a tiempo fijo (IATF) en ovinos considerando aspectos del bienestar humano-ambiental-animal: uso de análogos de prostaglandina y de la vía cervical de inseminación".

TABLA DE CONTENIDO

RESUMEN

El objetivo del trabajo de tesis fue el de generar información respecto a protocolos de sincronización de estros y ovulaciones en ovejas, basados en dos dosis de prostaglandina (PG) administradas a intervalos de duración corta (7 o 10 días), media (12 o 13 días) o larga (14, 15 o 16 días), para validar su uso en inseminación a tiempo fijo (IATF) utilizando semen fresco por la vía cervical, y compararlos con un protocolo basado en progestágenos y eCG (P4-eCG). La hipótesis general fue que intervalos de mayor duración en días entre dosis de PG determinarían diferencias en respuesta estral, desarrollo folicular ovárico y niveles plasmáticos de progesterona y estradiol, determinando mejores resultados reproductivos y similares a un protocolo de P4-eCG. Se realizaron cuatro experimentos utilizando un total de 966 ovejas Corriedale durante la estación reproductiva. Los protocolos de 10 y 12 días fueron asociados a una mayor sincronía en la respuesta estral. El desarrollo folicular ovárico, los intervalos PG-estro, PG-ovulación y la tasa ovulatoria fueron similares entre los protocolos de PG, siendo la tasa ovulatoria superior en el protocolo de P4-eCG. La administración de dos dosis de PG a diferentes intervalos, determinó diferente duración de la fase luteal y niveles plasmáticos de progesterona, lo cual fue asociado positivamente con la concentración plasmática de estradiol. Los intervalos de 15 y 16 días entre las dosis de PG obtuvieron mejor tasa de no retorno al estro, fertilidad y fecundidad, respecto a intervalos de 7, 10, 12 o 13 días, pero similares al protocolo en base a P4-eCG, sin diferencias en prolificidad. Se concluye que, en nuestras condiciones de experimentación, los protocolos de intervalos largos entre dosis de PG (15 y 16 días), obtendrían mejores resultados reproductivos en programas de IATF vía cervical con semen fresco que los protocolos cortos o medios y similares a los obtenidos con un protocolo en base a P4-eCG. Los hallazgos de esta tesis generan una nueva alternativa para el manejo reproductivo en ovinos durante la estación de cría.

Palabras clave: sincronización de estros, prostaglandina, inseminación a tiempo fijo, ovinos.

CERVICAL TIMED ARTIFICIAL INSEMINATION IN EWES: SYNCRONIZATION PROTOCOLS WITH SHORT, MID AND LONG INTERVALS BETWEEN PROSTAGLANDIN DOSES

SUMMARY

The aim of this work was to generate information on protocols to synchronise estrous and ovulation using prostaglandin (PG), where the injections were administered at short (7 or 10 days), mid (12 or 13 days) or long intervals (14, 15 or 16 days). To assess its use for cervical timed artificial insemination (TAI) with fresh semen they were compared to a progestagen-eCG based protocol (P4-eCG). The hypothesis was that longer intervals (days) between PG injections will determine differences in estrus response, ovarian follicular growth and progesterone and estradiol plasma concentrations, improving the reproductive outcome, resulting in reproductive results comparable with a P4-eCG protocol. Four experiments were performed during the breeding season using 966 adult Corriedale ewes. Protocols with 10 or 12 day-intervals were associated to a higher estrous synchrony. The ovarian follicular growth, the interval PG-estrus, PG-ovulation and the ovulation rate were similar among PG protocols; however the ovulation rate was higher using the P4-eCG protocol. The administration of PG at different intervals determined differences in the duration of the luteal phase and progesterone levels that was positively correlated with plasma estradiol concentrations. Intervals of 15 and 16 days between PG injections determined greater rates of non-return to service, fertility and fecundity compared to 7, 10, 12 or 13 intervals, but similar to the P4-eCG protocol. It was concluded that under the conditions of these experiments, long intervals between PG injections (15 or 16 days) resulted in higher reproductive outcomes using cervical TAI with fresh semen, compared to short or mid intervals and similar to a P4-eCG protocol. The results obtained in this work lead to the development of a new alternative for the reproductive management of the ewe during breeding season.

Keywords: estrus synchronization, prostaglandin, timed artificial insemination, ewe.

1. INTRODUCCIÓN

La sincronización de estros en ovinos asociada a la inseminación artificial (IA), es una herramienta de manejo reproductivo que permite concentrar los servicios de la majada en períodos de tiempo más reducidos respecto al uso del estro espontáneo ("celo natural"). Esta biotecnología, facilita el uso de carneros genéticamente superiores adquiridos asociativamente, mediante el transporte del reproductor por un corto período de tiempo hasta los establecimientos o del semen preservado de los mismos para inseminar las hembras seleccionadas. Además, permite disminuir la cantidad de días en que la majada es llevada a los corrales, evitando la pérdida de estado corporal y estatus sanitario que genera el manejo de una IA tradicional (Olivera y Gil, 2005). A su vez, facilita un uso más eficiente de una alimentación focalizada en momentos de alto impacto productivo como es el pre-servicio y el pre-parto (Blache et al., 2008, Martin et al., 2004), ya que al estar las ovejas en etapas del ciclo estral similares y conocidas, permite aplicar la nutrición en momentos más precisos, disminuyendo los días efectivos de suplementación y por ende los costos. Finalmente, determina una época de partos más concentrada permitiendo una atención más adecuada en ese momento.

Otra alternativa de manejo reproductivo que potencia las características previamente expresadas es la IA a tiempo fijo (IATF), la cual disminuye el manejo diario de la majada en las inseminaciones ya que no necesita de la detección de estros (Olivera y Gil, 2005, Menchaca y Rubianes, 2004). La aplicación de la IATF requiere de una alta sincronía de los estros y ovulaciones, de manera que la inseminación sea realizada en el momento óptimo (Menchaca y Rubianes, 2004). Para lograr esta sincronía es necesario el uso de protocolos hormonales, siendo los más utilizados los protocolos en base a progestágenos (P4) asociados a la gonadotrofina coriónica equina (eCG), o en base a análogos sintéticos de prostaglandina -PG- (Fierro et al., 2013, Abecia et al., 2012, Menchaca y Rubianes, 2004, Thimonier, 1979).

El uso de PG para la sincronización de estros ha sido extensamente estudiado desde su descubrimiento como un potente agente luteolítico (McCracken et al., 1970), y en nuestro conocimiento se ha utilizado para protocolos de IATF desde el

año 1978 (Fairnie et al., 1978). Su fácil aplicación mediante inyección intramuscular (Abecia et al., 2012), buena respuesta estral en ovejas en estación reproductiva (Fierro et al., 2013), la menor generación de residuos ambientales y una rápida metabolización en su pasaje por los pulmones no acumulándose en los tejidos (Davis et al., 1980, Piper et al., 1970), la identifican como una alternativa más "limpia y verde", lo cual es bien valorado actualmente por los consumidores de productos animales en muchas zonas del mundo (Martin y Ferasyi, 2016, Martin et al., 2004). Además, su significativo menor costo respecto a los protocolos de P4-eCG, la transforman en una alternativa interesante a validar para el manejo reproductivo de la majada durante la estación de cría.

Los protocolos tradicionales de sincronización de estros con PG basados en dos dosis administradas con intervalos entre 9 y 12 días de separación, han determinado una alta respuesta estral, pero una gran variabilidad en el momento de ocurrencia del estro y la ovulación (Viñoles y Rubianes, 1998, Houghton et al., 1995, Loubser y van Niekerk, 1981, Acritopoulou et al., 1978), lo cual ha desaconsejado su uso para IATF (Menchaca y Rubianes, 2004). Basado en la sensibilidad del cuerpo lúteo (CL) ovino de tan solo tres días de edad a la PG (Rubianes et al., 2003), fue desarrollado un protocolo de 7 días de intervalo entre las dosis de PG (protocolo “corto”), el cual genera una onda “1” de desarrollo folicular muy sincronizada, determinando además que los CLs sean sensibles a la PG al momento de aplicación de la segunda dosis (protocolo Synchrovine®; Rubianes et al., 2004). Este protocolo genera una muy buena sincronización de estros y ovulaciones, pero bajos resultados reproductivos luego de la IATF (Fierro et al., 2011, Fierro, 2010, Menchaca et al., 2004, Rubianes et al., 2003). Diferentes alternativas han sido evaluadas para intentar mejorar la fertilidad obtenida con este protocolo (30 a 50% de las hembras servidas), sin embargo ninguna ha logrado incrementar sus bajos resultados (Vilariño et al., 2017, Fierro et al., 2014, Olivera-Muzante et al., 2013, 2011a, 2011b, Contreras-Solis et al., 2009). Los magros resultados reproductivos obtenidos han sido asociados a menores concentraciones plasmáticas de progesterona, con un perfil alterado durante la fase de crecimiento de los folículos pre-ovulatorios, lo cual determinaría la menor fertilidad, tasa ovulatoria (TO) y prolificidad observada respecto a un estro

espontáneo (Fierro et al., 2011). En base a estos reportes, a una revisión exhaustiva del uso de la PG para el control del ciclo estral en los ovinos (Fierro et al., 2013), y a trabajos internacionales previos donde intervalos de más días entre las dosis de PG demostraban la posibilidad de mejorar los resultados obtenidos (Gibbons et al., 2010, Loubser y van Niekerk, 1981, Greyling y van der Westhuysen, 1980a, Fairnie et al., 1978, 1977, 1976), es que se sugiere que protocolos de intervalos aún más extensos entre las dosis podrían generar un ambiente hormonal más adecuado, y por ende mejores resultados reproductivos a la IATF. Sin embargo, no se conoce con profundidad, bajo similares condiciones experimentales, la respuesta estral, ovárica y/o reproductiva de ovejas sincronizadas con protocolos con diferentes intervalos entre dosis de PG (cortos, medios y largos), en majadas donde no se conoce el momento del ciclo estral cuando es administrada la primera PG.

Finalmente, a pesar de que reportes previos sugieren que la PG sería una mejor opción a los protocolos basados en P4-eCG, debido a su efecto negativo sobre el folículo ovulatorio (Letelier et al., 2011, Berlinguer et al., 2007, Gonzalez-Bulnes et al., 2005), la mayoría de los resultados reproductivos obtenidos en programas de IATF utilizando PG no han confirmado esa hipótesis (Olivera- Muzante et al., 2011b, Viñoles et al., 2011, Greyling y van der Westhuysen, 1980a, Boland et al., 1978a, 1978b). La posibilidad de generar nuevos protocolos de IATF en base a PG, alternativos a los tradicionales de P4-eCG, con menores implicancias prácticas y de costos (Viñoles et al., 2011, Contreras-Solis et al., 2009, Gonzalez-Bulnes et al., 2005), es de gran interés para el manejo del ciclo reproductivo ovino.

1.1. HIPÓTESIS

La hipótesis general de este trabajo fue que un mayor intervalo de separación en días entre las dosis de PG determinaría diferencias en la respuesta estral, desarrollo folicular ovárico, y niveles plasmáticos de progesterona y estradiol, conllevando a obtener mejores resultados reproductivos que intervalos de menor duración, y similares a un protocolo en base a P4-eCG.

1.2. OBJETIVO GENERAL Y ESPECÍFICOS

El objetivo general de la tesis fue el de generar información respecto a protocolos de sincronización de estros y ovulaciones en ovejas, basados en dos dosis de PG administradas a intervalos de duración corta (7 y 10 días), media (12 y 13 días), o larga (14, 15 y 16 días), para su uso en IATF en estación reproductiva utilizando la vía cervical con semen fresco.

Los objetivos específicos fueron: 1- para protocolos de PG con intervalos de 10, 12, 14 y 16 días entre las dosis: determinar la respuesta estral (en porcentaje y dispersión), el desarrollo final de los folículos ováricos, los niveles plasmáticos de hormonas esteroideas (progesterona y estradiol) durante el desarrollo del o de los folículos pre-ovulatorios y posteriores al servicio, los intervalos de tiempo entre la administración de la segunda dosis de PG y el inicio del estro (intervalo PG-estro) y la ovulación (intervalo PG-ovulación); y 2- para protocolos de PG con intervalos de 7, 10, 12, 13, 14, 15 y 16 días entre las dosis: evaluar la TO, el no retorno al estro entre el Día 13 y 21 pos-servicio (NRR-21), la fertilidad, prolificidad y fecundidad y compararlos con un protocolo basado en P4-eCG.

1.3. ESQUEMA GENERAL DE LA TESIS

La estructura general de la tesis se basa en un marco teórico (revisión publicada), metodología y resultados (contenida en los artículos científicos publicados), una discusión general, conclusiones, implicancias prácticas y anexos. Para testar la hipótesis planteada, se realizaron cuatro experimentos durante la estación reproductiva, estando todos los procedimientos aprobados por la CUEA - Facultad de Veterinaria, comprendidos en tres artículos científicos publicados en revistas internacionales de alto impacto, a saber:

a. "Concentrations of steroid hormones, estrous, ovarian and reproductive responses in sheep estrous synchronized with different prostaglandin-based protocols" (Fierro et al., 2016. Animal Reproduction Science, 167: 74 - 82), donde se plantean dos experimentos con el objetivo de evaluar la respuesta estral, el desarrollo folicular ovárico (diámetro máximo y final -mm-, tasa de crecimiento

folicular -mm/día-), la concentración de progesterona y estradiol, los intervalos PG-estro y PG-ovulación (horas), la TO (total de CLs/ovejas que ovularon), la concepción (ovejas gestantes/ovejas inseminadas*100) y la fertilidad (ovejas gestantes/ovejas ofrecidas al servicio*100) a estro detectado de ovejas sincronizadas con dos dosis de PG administradas a intervalos de duración corta (10 días), media (12 días) o larga (14 y 16 días) e inseminadas por vía cervical con semen fresco.

b. *"Long term prostaglandin based-protocols improve the reproductive performance after timed artificial insemination in sheep" (Fierro et al., 2017. Theriogenology, 90: 109 - 113)*, donde se evaluó la respuesta ovulatoria (ovejas que ovularon) y la TO, el NRR-21, la fertilidad y prolificidad (cantidad de fetos/ovejas gestantes), luego de una IATF vía cervical con semen fresco de los protocolos evaluados en el 1er artículo (10, 12, 14 o 16 días entre dosis de PG), comparándolos además con un protocolo de 7 días de intervalo entre las dosis de PG.

c. *"Long interval prostaglandin as an alternative to progesterone-eCG based protocols for timed AI in sheep" (Fierro y Olivera-Muzante, 2017. Animal Reproduction Science, 180: 78 - 84)*, en el cual fue evaluada la fertilidad, prolificidad y fecundidad (cantidad de fetos/ovejas ofrecidas al servicio*100) de protocolos de mediana (12 o 13 días) y larga (14, 15 o 16 días) duración de intervalo entre las dosis de PG, y comparados con un protocolo basado en P4-eCG.

2. MARCO TEÓRICO: THE USE OF PROSTAGLANDINS IN CONTROLLING ESTROUS CYCLE OF THE EWE: A REVIEW

RESUMEN

Esta revisión resume información respecto al uso de la prostaglandina F2 alfa y sus análogos sintéticos (PG) para el control del ciclo estral ovino. Se revisan y discuten aspectos como la fase del ciclo estral en la cual es utilizada, las dosis de PG (cantidad de inyecciones y concentración), el patrón de desarrollo folicular ovárico, formación del CL y síntesis de progesterona, la TO, el transporte espermático, la calidad embrionaria y la fertilidad obtenida luego de su aplicación. Además se presentan diferentes protocolos de sincronización basados en el uso de PG y los resultados reproductivos asociados a IATF. Basado en la información presentada, se resume que el CL ovino es refractario a la PG hasta el día 2 pos-ovulación. La respuesta folicular luego de administrar una PG es dependiente de la fase del ciclo estral al momento de la aplicación. Todos los análogos de prostaglandina son efectivos cuando es utilizada la concentración de dosis adecuada, existiendo una asociación positiva entre las dosis administradas (cantidad y concentración) y la proporción de ovejas que son detectadas en estro. De esta manera, cuando la primera dosis de PG es administrada sin conocer la etapa del ciclo estral al momento de su aplicación, una segunda inyección de PG incrementa la respuesta estral del protocolo. Generalmente el uso de la PG se asocia con alteración en el transporte espermático y baja fertilidad. Sin embargo, la información respecto a la alteración en la capacidad esteroidogénica de los folículos pre-ovulatorios, TO y de recolección de embriones, calidad embrionaria y prolificidad es controversial. La duración del ciclo estral posterior a la administración de PG es normal. Los protocolos en base a PG para IATF obtienen bajos resultados reproductivos, pero el incremento en días en el intervalo entre las dosis de PG podría mejorar la fertilidad final obtenida. Las alternativas para mejorar los resultados reproductivos han sido dirigidas a generar un incremento sincronizado de LH, al uso de diferentes vías de inseminación (cervical o intra-uterina), diferentes dosis e incremento leve de intervalo entre las dosis de PG. Finalmente se presentan las perspectivas del uso de PG en el control reproductivo ovino.

Palabras clave: sincronización de estros, prostaglandina, tasa ovulatoria, fertilidad, inseminación artificial a tiempo fijo, oveja.

Theriogenology 79 (2013) 399–408

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Review

The use of prostaglandins in controlling estrous cycle of the ewe: A review

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ARTICLE INFO

Article history:
Received 9 December 2011
Received in revised form 17 August 2012
Accepted 28 October 2012

Keywords:
Ovine estrus synchronization
Prostaglandin
Ovulation rate
Fertility
Timed artificial insemination
Sheep

ABSTRACT

This review considers the use of prostaglandin $F_{2\alpha}$ and its synthetic analogues (PG) for controlling the estrous cycle of the ewe. Aspects such as phase of the estrus cycle, PG analogues, PG doses, ovarian follicle development pattern, CL formation, progesterone synthesis, ovulation rate, sperm transport, embryo quality, and fertility rates after PG administration are reviewed. Furthermore, protocols for estrus synchronization and their success in timed AI programs are discussed. Based on available information, the ovine CL is refractory to PG treatment for up to 2 days after ovulation. All PG analogues are effective when an appropriate dose is given; in that regard, there is a positive association between the dose administered and the proportion of ewes detected in estrus. Follicular response after PG is dependent on the phase of the estrous cycle at treatment. Altered sperm transport and low pregnancy rates are generally reported. However, reports on alteration of the steroidogenic capacity of preovulatory follicles, ovulation rate, embryo quality, recovery rates, and prolificacy, are controversial. Although various PG-based protocols can be used for estrus synchronization, a second PG injection improves estrus response when the stage of the estrous cycle at the first injection is unknown. The estrus cycle after PG administration has a normal length. Prostaglandin-based protocols for timed AI achieved poor reproductive outcomes, but increasing the interval between PG injections might increase pregnancy rates. Attempts to improve reproductive outcomes have been directed to provide a synchronized LH surge: use of different routes of AI (cervical or intrauterine), different PG doses, and increased intervals between PG injections. Finally we present our point of view regarding future perspectives on the use of PG in programs of controlled sheep reproduction.

1. Introduction

Prostaglandin $F_{2\alpha}$ and its synthetic analogues (PG) have been studied extensively since its discovery in 1970 as a powerful luteolytic agent [1]. Although progestagen-based protocols are preferred by technicians and farmers to manage reproduction of the flock [2], they have the potential for environmental contamination because of residual progesterone (P4) in used devices and the addition of antibiotic agents to avoid vaginitis [3,4]. Because consumers demand foods produced by "clean, green, and ethical" methods [5], PG are a good alternative, because they are rapidly metabolized in the lung and therefore not accumulated in tissues [6,7].

The use of unsynchronized estrous behavior requires considerable labor in large flocks, complicating the use of other assisted reproductive techniques under extensive

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0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2012.10.022

production systems. Therefore, PG as an alternative to progestagen-based protocols is certainly a possibility for reproductive management of flocks [8].

This review considers the use of PG in the control of the ewe estrous cycle. Historical and present information regarding the phase of the estrus cycle, PG analogues, doses, follicle development pattern, CL formation, P4 synthesis, ovulation rate (OR), sperm transport, embryo quality, and fertility rates after PG administration are reviewed. Furthermore, protocols for estrus synchronization and their success in timed AI (TAI) programs are discussed. Finally, we present our point of view regarding future perspectives of PG in controlled sheep reproduction programs.

2. Responsiveness of the ovine CL to a PG injection

It is well known that administration of PG (ICI 80996 im) between Days 5 and 14 of the estrus cycle (Day 0 = estrus) induces luteolysis (rapid luteal regression), followed by estrus and ovulation [9,10]. Acritopoulou and Haresign [10] reported that 50% of the ewes treated on Day 3 showed estrus (2/4), and they suggested that the ewes that responded to the treatment were in a more advanced stage of luteal development compared with those that failed to respond to treatment. Similarly, there were earlier reports [11] that the CL of the ewe is sensitive to prostaglandin $F2_{\alpha}$ (10 mg) given on Days 3 and 4 (2/8) of the cycle. In addition, Rubianes et al. [12] reported that the refractoriness of a recently formed ovine CL to PG might be restricted to the first 2 days after ovulation. These findings were subsequently confirmed by Contreras-Solís et al. [13]. Furthermore, Pope and Cárdenas [14] induced luteolysis in 20% of ewes treated on Day 3.5 using 10 mg of prostaglandin $F2_{\alpha}$. A possible explanation for the relative refractoriness of a young CL (Day 4) might be the greater capability for catabolism of PG, because of enhanced activity of the enzyme 15-hydroxyprostaglandin dehydrogenase compared with a mature CL (Day 13) [15].

To summarize, based on the information available, refractoriness of the ovine CL to a PG dose occurs up to Day 2 postovulation. Therefore, higher doses are required to promote luteolysis during the early luteal phase.

3. Prostaglandin synthetic analogues and dose

Various synthetic analogues were developed with the aim to delay the rapid metabolic degradation of natural $PGF_{2\alpha}$ (reviewed in [16]). For example, the synthetic analogue of prostaglandin 15-[RS]-methyl-13,14-dihydro-$PGF_{2\alpha}$ (ONO 453) described by Hughes et al. [17], is a potent luteolytic agent in cyclic ewes, effective in doses of 2 mg (minimum luteolytic dose) when it was administered after Day 3. The increased potency of this compound on the reproductive tract is associated with an increase in its biological activity on other tissues (smooth muscle of the vascular system, and gastrointestinal tract), undesirable effects that limited its use in medical and veterinary practice [16]. In addition, the use of another synthetic analogue of PG, ICI 79939, was reported in sheep by Hearnshaw et al. [18]. These authors studied the effects of various doses (15.6, 31.2, 62.5, and 125 μg), and all except the lowest dose promoted luteolysis followed by estrus behavior in ewes.

The most widely used synthetic analogue has been 16-aryloxyprostaglandin (ICI 80996; Cloprostenol) [19], which is 100-fold more potent than $PGF_{2\alpha}$, and with more selective biological properties. The luteolytic effect of ICI 80996 administered im was identical to that produced by a local ovarian infusion via the ovarian artery using $PGF_{2\alpha}$ [16]. Its effectiveness was in part because of the most selective action of this compound on the CL [20] and to its longer life span [16]. An injection of 100 μg of Cloprostenol resulted in a high degree of synchrony in the return to estrus and the timing of the LH peak [9]. Other researchers suggested that the appropriate dose of this analogue was 125 μg [21]. However, doses as low as 50 μg were reported to be effective to induce luteolysis in the ewe [16]. Two active isomers (D and L) and a racemic mixture, DL, of Cloprostenol are commercially available, but only the D-isomer binds to the PG receptors of the bovine CL and myometrial cells, allowing for its luteolytic activity. In addition, because D-Cloprostenol is 10-fold more potent than DL-Cloprostenol [22], a lower dose of the isomer D is effective.

Another analogue used in sheep reproduction is ONO 1052 (Delprostenate). Bonifacino and Aragunde [23] reported that the lowest effective dose in a single injection regimen was 40 μg; however, when a double injection regimen was applied, the effective dose may be decreased to 35 μg. Despite the promising results using this prostaglandin analogue, few articles have reported its use in sheep reproduction [12,24–28].

Loubser and van Niekerk [29] used two doses of Dinoprost 11 days apart, achieving promising results with 10 mg per dose compared with 5 mg. Other researchers used this analogue to study the effects of PG on uterine motility and sperm transport [30–35]. The interval between two doses on reproductive results was studied in other reports [36] with acceptable reproductive results.

To conclude, considering the effective doses for each analogue, all products described above could be used for estrus synchronization in sheep. However, there is a positive association between the dose administered and the percentage of ewes that respond to the treatment by showing estrous behavior [14,29,37].

4. Progesterone decrease, follicle development, steroidogenic function, OR, and CL life span, after PG administration

The decrease in plasma P4 concentrations is more pronounced after luteolysis induced by PG compared with natural luteolysis [38,39]. Complete luteal regression is achieved from 6 to 24 hours versus 72 hours (induced vs. natural luteolysis, respectively [40–42]).

Early reports indicated that characteristics of the LH discharge after PG administration were similar to control untreated ewes [9,40,43]. However, a second smaller discharge of LH occurred 7 to 8 hours [41] or 10.5 hours [44] after PG injection. Probably, the initial peak of LH released during PG administration stimulated the ovary to secrete

estradiol which in turn stimulated a secondary release of LH (positive feedback mechanism) [44].

Houghton et al. [45] reported smaller follicular diameter in ewes given PG compared with untreated ewes, and larger diameters when follicles were induced to ovulate during the early versus late luteal phase of the estrous cycle. Similarly, the preovulatory follicle (POF) was larger after PG-induced luteolysis during the early luteal phase of the estrous cycle compared with follicle size after natural luteolysis. It was noteworthy that the larger diameter of the POF was associated with a faster follicular growth rate [26]. Conversely, Nephew et al. [46] and Letelier et al. [47] did not detect an effect of PG on follicular diameter.

Follicles induced to ovulate after a PG treatment have fewer granulosa cells, resulting in lower P4 production between Days 3 to 6 after estrus [42,46]. This is in agreement with White et al. [48] who described development of a CL with a shorter life span that secreted lower quantities of P4 after PG-induced ovulation (because of alterations in the POF compared with a CL derived from a spontaneous ovulation). Altered steroidogenic capacity of the POF was recently confirmed [47]. However, this remains controversial, because other researchers observed similar steroidogenic capacity of the POF, allowing development of a CL with normal P4 production after PG-induced luteolysis compared with natural luteolysis [26], and to progestagen-induced estrus [49]. Therefore, an estrous cycle of normal duration after PG injection has been frequently reported [17,18,26,41,50–52].

Data regarding OR and prolificacy after the use of PG are contradictory. No detrimental effect on OR has been reported when PG was given during the mid [41,45] or early luteal phase [24]. However, Fierro et al. [26] and Forichi et al. [53] reported a decrease in OR (1.37 vs. 1.61; $P < 0.06$) and prolificacy (1.18 vs. 1.39, $P < 0.05$) when PG was given during the early luteal phase compared with a naturally occurring estrus. Additionally, there was an increase in prolificacy (1.28 vs. 1.13, $P < 0.07$) when 8.0 μg of GnRH was given at the time of AI using a two PG 7-days interval (Delprostenate) [28,54]. Perhaps there is a detrimental effect of PG on prolificacy. An increase in OR occurred when PG was administered in the mid luteal phase; this could be explained by development of a "less dominant" POF with a lower steroidogenic capacity, that maintained FSH concentrations above the threshold to stimulate selection of multiple ovulatory follicles in a single follicular wave [47]. Alternatively, POFs might be derived from two consecutive follicular waves (i.e., the penultimate and ultimate waves of the cycle [55,56]).

Some researchers suggested that PG-induced luteolysis could alter follicular recruitment [57]. The sharp increase in plasma estradiol concentrations after PG-induced luteolysis could promote an altered endogenous FSH pattern, affecting recruited follicles and associated with accelerated ovulatory rupture [58]. In that regard, Davies et al. [59] reported disappearance ("ovulation") of follicles that were not linked to behavioral estrous or an LH peak; therefore, they failed to develop into a normal CL. The authors claimed inadequate luteogenesis originated from development of an inadequate POF [60]. The altered steroidogenic capacity of the POF could affect the estradiol–LH feedback, which is needed for ovulation and normal luteogenesis [61].

A prolonged FSH interpeak interval occurs between the first and second FSH surges when serum P4 concentrations are increasing during CL formation [55]. Furthermore, PG administration at the expected time of the FSH surge at midcycle delays the increase in serum FSH concentrations [58]. A recent report provides further support implicating P4 as the key regulator of circulating FSH concentrations in the ewe [62]. These authors hypothesized that under the influence of luteal P4, the response of FSH to GnRH might be increased, resulting in higher secretion of FSH from the pituitary gland. They also suggested that lower P4 concentrations increase the less acidic isoforms of FSH in the circulation and its faster clearance from the blood, that results in a late FSH concentration increase. These reports could account for the alteration in OR when ewes were synchronized with a double PG protocol with a 7-day interval [26].

To summarize, the response to PG is dependent on the phase of the estrous cycle at administration [63], because dynamic changes in follicle growth occur during the luteal phase [3] because of dynamic changes in P4, FSH, and LH [64]. However, alterations of the steroidogenic function of POF, OR [26], and prolificacy after PG administration are controversial.

5. Sperm transport, embryo quality, and fertility rates after PG injection

Pregnancy rate is generally lower in ewes bred at a synchronized estrus with PG or P4 compared with untreated ewes [2,26]. Furthermore, pregnancy rates are lower when comparing PG with P4-eCG treated ewes [27,36]. However, some researchers reported similar pregnancy rate of ewes mated after a PG treatment compared with those mated at natural estrus [28,40].

Reproductive failure might occur at various moments after a PG-induced estrus (Fig. 1). The lower pregnancy rate obtained after PG synchronization has been associated with alterations in myometrial contractions [59], in which a decreased number of uterine contractions toward the oviduct resulted in fewer sperm reaching the fertilization site [32,33,60]. Other authors reported alterations of the vaginal mucus impairing sperm transport from the cervix to the uterus [63]. Immobilized and dead sperm were present on the anterior third of the cervix and in the uterine body in PG-treated ewes, probably as a result of the presence of a spermicidal factor, or because of the absence of substances that protect the semen [34]. Conversely, Fukui and Roberts [35] reported no differences in the numbers of sperm recovered from any parts of the reproductive tract of ewes treated or untreated with PG. Alterations in steroidogenic capacity of the POF have been reported [48], thereby altering gamete transport, because steroids prepare the oviduct and the uterus for fertilization and embryo transport, including inducing the appropriate muscular contractions to increase chances of reproductive success [65,66].

Similar embryo recovery rates were reported in ewes treated with PG compared with those treated with P4

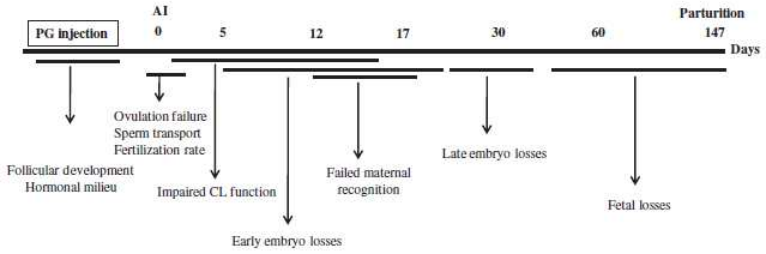

Fig. 1. Schematic representation of potential causes of reproductive looses when a synthetic analogue of prostaglandin is used for estrus synchronization in ewes. PG injection indicates injection of a prostaglandin dose, and Day 0 indicates day of AI.

[3,67], but recovery rates were lower in PG-treated ewes compared with ewes in spontaneous estrus [26,68]. Fierro et al. [26] reported similar quality of the embryos collected from PG-treated ewes compared with those collected after spontaneous estrus. Furthermore, Gonzalez-Bulnes et al. [3] had improved embryo viability in PG- compared with P4-treated ewes.

Fertilization rates were also similar when PG-treated ewes were compared with ewes in spontaneous estrus [26,31], or P4-treated ewes [3,31]. However, a low fertilization rate was reported by Boland et al. [69] when ewes were synchronized with PG compared with progestagens (7% vs. 69%). However, too few embryos were evaluated to draw definitive conclusions.

In conclusion, the use of PG altered sperm transport and, in general, reduced pregnancy rates. Embryo recovery rate was lower, but embryo quality was similar in PG-treated ewes compared with those with spontaneous cycles.

6. The use of PGs in estrus synchronization protocols

The use of PG for estrus synchronization is restricted to the breeding season in breeds of temperate climates, because of the luteolytic effect of the hormone [1]. However, in tropical sheep, it might be applied throughout the entire year [13,49,70]. Regardless, treated animals should not be in lactational or nutritional anestrous [71], or in suboptimal health.

Various PG-based protocols are acceptable to synchronize estrus in the flock. The selection of a particular protocol depends on the technician's opinion and the management possibilities of the farmers. A single PG injection is the simplest protocol for reproductive management of the flock [9]. When PG is administered at random stages of the estrous cycle, luteal regression occurs in most ewes, resulting in estrus and ovulation [10,40]. However, the onset of estrus, the preovulatory LH surge and the interval from the PG treatment to ovulation differs among ewes, depending on their stage of the estrous cycle at treatment [10,29,72].

The use of transrectal ultrasonography facilitated the determination that the interval from PG treatment to estrus was related to CL age and the stage of follicular development at the time of treatment [45,73]. When PG is administered during the midluteal phase, plasma P4 concentrations slowly decline to subluteal values; therefore, estrous behavior and ovulation are delayed [45]. Furthermore, the follicular population of individual ewes affects the interval from PG to the onset of estrus [73]. If a dominant follicle in its growing phase is present when PG is administered, estrus and ovulation occur relatively rapidly; however, if the dominant follicle is already regressing, a new follicle needs to emerge and grow, and therefore estrus and ovulation are delayed.

When PG is administered to a group of cycling ewes, luteal regression is induced in 66% of the ewes with the subsequent induction of estrus (37.7 ± 1.6 hours) [10]. The administration of a second injection of PG induces estrus in most ewes when there is no reference to the stage of the estrous cycle at the time of the first injection [10,74]. For instance, a second dose of PG, given 9 days after the first dose, improved the synchrony of estrus, because 95% of treated ewes were in estrus within 72 hours after the last injection [51].

Onset of estrus and ovulation differ according to differences in the intervals between PG treatments (Table 1). When a single PG was administered between Days 8 to 11 (estrus = Day 0), the proportion of ewes in estrus (21/25) and its onset (46.3 ± 1.32 hours) were similar to that obtained with a double PG regimen given 10 days apart at random (22/25 and 51.6 ± 2.4; [76]). If estrus detection is feasible, the first option would be less expensive.

The mean interval from treatment to ovulation was similar after two doses of ICI 80996 administered at an 8-day interval compared with a 14-day interval (79 and 78

Table 1
Onset of estrus and ovulation reported after the second PG injection in ewes synchronized with a double injection PG regimen with various intervals between treatments.

Reference	Interval between PG treatments	Onset of estrus (h)	Ovulation (h)
Rubianes et al. [12]	7 d	40.6 ± 0.5	60.8 ± 1.8
Contreras-Solís et al. [13]	7 d	-	61.1 ± 1.1
Acritopoulou et al. [74]	9 d	38.8 ± 1.3	73.1 ± 1.6
Haresign and Acritopoulou [40]	9 d	38.6 ± 0.8	72.9 ± 1.5
Acritopoulou [75]	9 d	43.5 ± 6.0[a]	-
	9 d	49.9 ± 3.7[b]	-
Godfrey et al. [70]	10 d	69.6 ± 9.6	-
Das et al. [76]	10 d	51.6 ± 2.4	-
Oyediji et al. [52]	11 d	41.7 ± 2.2	-

Abbreviation: PG, prostaglandin.
[a] Mid breeding season.
[b] Late breeding season.

hours respectively); however, ovulations occurred over a longer interval in the former treatment (43 to 100 hours vs. 67 to 89 hours, respectively) [77]. Perhaps giving the second PG treatment 8 days after the first injection induces ovulation of follicles from the first follicular wave in some ewes (resulting in earlier ovulations), or from the second in others (delayed ovulations) [12,28]. Conversely, when treatments are given 14 days apart, most of the ovulatory follicles originate from the last wave of the cycle, with CL that are highly sensitive to PG action, thus reducing intervals to estrus and ovulation (Fig. 2). In addition, at least some of the variability among reports in the intervals to ovulation presented above might be because of the method used (laparoscopy or ultrasonography), and the frequency of observations to confirm ovulations.

A recent study linked the use of PG treatment (two injections administered 10 days apart) with the male effect during the breeding season [78]. The author concluded that introduction of vasectomized rams concurrently with the second PG injection advanced the onset of estrus and increased the number of responding ewes compared with a double PG regimen alone. With regard to attempts to reduce the use of hormones in synchronization protocols, the introduction of rams 13 days after a single PG injection gave poorer results compared with a double PG regimen [78].

Another alternative is to presynchronize the flock with a single or double PG regimen and start checking estrus behavior 15 days after the last injection. This approach would allow the use AI or natural mating in a spontaneous and synchronized estrus that results in a high pregnancy rate [23,26].

Although various PG-based protocols could be used for estrus synchronization, it is important to consider that the administration of a second PG injection improves estrus response when there is no reference to the stage of the estrous cycle at the time of the first treatment.

7. Prostaglandin-based protocols for timed AI

Timed AI represents a practical tool in genetic programs, allowing a more efficient use of superior males, decreasing the workload because it eliminates the need for estrus detection, thereby minimizing sanitary risks [79]. Furthermore, ewes in estrus that teasers fail to detect would still get bred, because all sheep are inseminated. Ewes receiving TAI on a single day lambed over a 13-day interval, with 86% of the lambs born from 6 to 11 days after the first lamb was born (Fierro et al., unpublished).

To our knowledge, the first report regarding the use of PG for TAI in ewes was that of Loubser and van Niekerk [29]. However, the variable pregnancy rates reported rendered PG protocols impracticable [4,25–29,51,80] (Table 2). Loubser and van Niekerk [29] used a protocol based on two treatments of PG given 11 days apart and performed a double insemination (20 and 32 hours after 10% of ewes showed estrous behavior). There was an acceptable conception rate after TAI (assessed as nonreturn rates) that was significantly better when the PG dose was increased from 5 to 10 mg of Dinoprost (Table 2). Using the same interval between PG injections and a single TAI at 60 or 72 hours, or a double insemination at 60 and 72 hours after the last injection, Hackett et al. [80] obtained similar pregnancy rates between groups (note the different breeds and low numbers of animals used; Table 2).

Other researchers [51] used a protocol based on two PG treatments given 12 days apart and inseminated once at 56, 60, or 66 hours, or inseminated twice at 56 and 66 hours after the second treatment. Double AI resulted in a similar lambing rate compared with a single AI at 56 hours, and both were higher ($P < 0.05$) than a single AI at 60 hours or 66 hours (Table 2).

Although a high proportion of ewes were detected in estrus after the second injection in these protocols, this

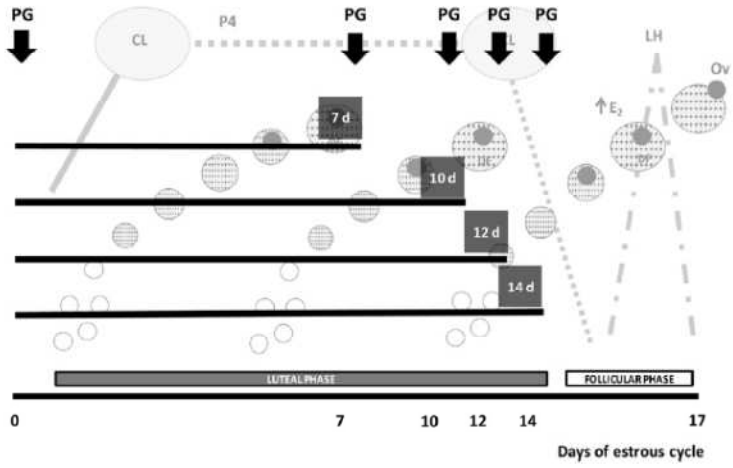

Fig. 2. Schematic representation of the expected impact of the second prostaglandin (PG) administration at 7-, 10-, 12-, and 14-day intervals on the dominant follicle of the developing wave (assuming a 3-wave cycle). Arrows indicate first and second PG injections. Day 0 is defined as day of the first PG administration. DF, dominant follicle; E_2, estradiol; Ov, ovulation; P4, progesterone.

Table 2
Pregnancy rates after timed AI in ewes synchronized with a double injection prostaglandin (PG) regimen (with various intervals between the two PG treatments).

Reference	Protocol	AI	Pregnancy (%)
Loubser and van Niekerk [29]	2 PG treatments 11 d apart (5 mg per injection)	20 and 32 h	59.1[*]
	2 PG treatments 11 d apart (10 mg per injection)		75.3[†]
Hackett et al. [80]	2 PG treatments 11 d apart	60 h	35
		72 h	52
		60 and 72 h	52
Acritopoulou-Fourcroy et al. [51]	2 PG treatments 12 d apart	56 h	54.8[*]
		60 h	37.5[†]
		66 h	30.8[†]
		56 and 66 h	61.9[*]
	MAP and eCG	55 h	37.8[†]
Menchaca et al. [25]	2 PG treatments 7 d apart	42 h	36.8[*]
		48 h	25.8[*,†]
		54 h	22.6[†]
Fierro et al. [26]	2 PG treatments 7 d apart	IU 48 h	63[†]
	Spontaneous estrus	IU 12–24 h[a]	88[*]
Olivera-Muzante et al. [27]	2 PG treatments 7 d apart	42 h	27[†]
		48 h	31[†]
		54 h	26[†]
	MAP and eCG	54 h	48[*]
Viñoles et al. [4]	3 PG treatments 7 d apart	IU 53 h	47[*]
	FGA 14 d and eCG	IU 53 h	85[†]

Within a report, pregnancy rates without a common superscript symbols differed ($P < 0.05$).
Abbreviations: FGA, fluorogestone acetate; IU, intrauterine AI; MAP, medroxiprogesterone acetate.
[a] Ewes in the group with spontaneous estrus were checked for estrous behavior every 12 h, and AI was performed 12 to 24 h after onset of estrus.

occurred over a 4-day period [29]. When the second PG injection was applied during the midluteal phase, the asynchrony among follicular waves determined the presence of follicles at different stages of growth (growing, static, or regressing phases), causing two different scenarios: (1) an extended life span of the ovulatory follicle; and (2) ovulation of newly emerged follicles [45,57,73] (Fig. 2). These different scenarios limit the possibility of a single optimal time for AI and might explain the low reproductive outcome of long-term PG-based protocols for TAI.

The poor results obtained with the use of PG for TAI protocols limited research until recently [12,24,79]. The

Table 3
Pregnancy rates obtained with prostaglandin-based protocols plus various other approaches (eCG, ME), time of AI, AI method, injection of GnRH to improve reproductive outcome.

Reference	Protocol	Type of service	Pregnancy (%)
Boland et al. [36]	2 PG treatments 9 d apart	Natural service	65
	2 PG treatments 9 d apart and eCG		53.8
	2 PG treatments 14 d apart		42.1
	2 PG treatments 14 d apart and eCG		71.4
	MAP at 14 d		72.7
	MAP at 14 d and eCG		85.7
Contreras-Solís et al. [13]	2 PG treatments 7 d apart and ME	Cervical TAI 48 h	62.5
	2 PG treatments 7 d apart and ME	Cervical TAI 55 h	44
	FGA for 12 d	Cervical TAI 55 h	47.4
Olivera-Muzante et al. [27][a]	2 PG treatments 7 d apart	Cervical TAI 51	12[*]
		Cervical TAI 57	14[*]
	MAP and eCG	Cervical TAI 54	28[†]
	2PG 7 d apart	Intrauterine TAI 51	43[‡]
		Intrauterine TAI 57	51[‡]
	MAP at 13 d and eCG	Intrauterine TAI 54	71[§]
Olivera-Muzante et al. [28] (Experiment 1)	2 PG treatments 7 d apart and HD	Cervical TAI 42 h	42[*]
	2 PG treatments 7 d apart and LD	Cervical TAI 42 h	24[†]
Olivera-Muzante et al. [28] (Experiment 2)	2 PG treatments 7 d apart	Cervical TAI 42 h	45[*]
		Cervical TAI 48 h	51[*]
	2 PG treatments 8 d apart	Cervical TAI 42 h	33[†]
		Cervical TAI 48 h	29[†]
Olivera-Muzante et al. [28] (Experiment 3)	2 PG treatments 7 d apart	Cervical TAI 42 h	50
	2 PG treatments 7 d apart and GnRH	Cervical TAI 42 h	38

Within a report, pregnancy rates without a common superscript symbols differed ($P < 0.05$).
Abbreviations: FGA, fluorogestone acetate; GnRH, 8 μg of GnRH at the time of TAI; HD, 160 μg Delprostenate; LD, 80 μg Delprostenate; MAP, medroxiprogesterone acetate; ME, male effect; PG, prostaglandin; TAI, timed AI.
[a] Experiments were conducted using chilled ram semen.

finding that a 3-day-old CL is sensitive to PG [12], was the impetus to test the hypothesis that shortening the interval between PG injections to 7 days would induce a highly synchronous estrus and ovulation of the first follicular wave (Synchrovine protocol, MIEM - Cámara Nacional de Registros, Montevideo, Uruguay) [24,79]. The rationale was that the second PG treatment applied in the early luteal phase (during the growing phase of the first dominant follicle of the cycle) would reduce the interval to ovulation in all ewes [25,73]. Although this protocol induces a consistently synchronized interval from treatment to ovulation (Table 1) [12,13], pregnancy rates after TAI are still low [25–28], similar to previous reports under different conditions (Table 2).

Recently, Olivera-Muzante et al. [27] compared reproductive performance of the Synchrovine protocol versus a P4-eCG based protocol in an experiment involving 1297 multiparous Merino ewes. Independent of semen preservation method, time of AI after the second PG, or deposition site of the AI dose, the Synchrovine protocol yielded a lower reproductive outcome than a conventional P4-eCG based protocol [27] (Tables 2 and 3). Similarly, Viñoles et al. [4] reported lower pregnancy rates in PG (three treatments given 7 days apart) versus P4-treated ewes (Table 2). Whether the low pregnancy rate is because of ovulation failure, lack of fertilization, development of an abnormal CL that cannot sustain embryo development, or death of the embryo before or after maternal recognition of pregnancy, remains to be elucidated (Fig. 1). Conversely, Acritopoulou-Fourcroy et al. [51] reported better lambing rates with PG 12 days apart versus P4-eCG protocols, according to the interval from the last PG treatment to TAI (Table 2).

Research to identify reproductive losses when ewes are synchronized with the 7-day interval PG protocol was recently conducted [26]. The low reproductive outcome achieved after TAI of PG treated ewes compared with ewes in spontaneous estrus was linked to the endocrine environment, whereas development of the POF (Fig. 3) was associated with a lower OR, conception rate, prolificacy, and fecundity.

Although this protocol consistently induces synchronized intervals from treatment to ovulation, with potential for improved reproductive outcomes after TAI, it ultimately yielded results similar to previous protocols. Lower fertilization rates (based on recovered embryos on Day 3 after AI, and blastocysts on Day 10) were reported when the interval between injections was reduced from 14 to 8 days [77]. Shortening the interval between PGs decreased the length of the luteal phase, which modifies the concentrations and profile of P4 before the ovulation induced by the PG protocol [26] (Fig. 3). An extra P4 source, provided by an intravaginal impregnated device 8 days before the PG injection [29], increased the number of ewes in estrus (93.4% vs. 82.0%), and pregnancy rates (84.9% vs. 75.3%) compared with untreated ewes.

Therefore, PG-based protocols generally achieve poor reproductive outcomes after TAI. Perhaps longer intervals between PG injections could yield better pregnancy rates. Regardless, further research and validations under similar experimental conditions are required to determine the best protocol for TAI.

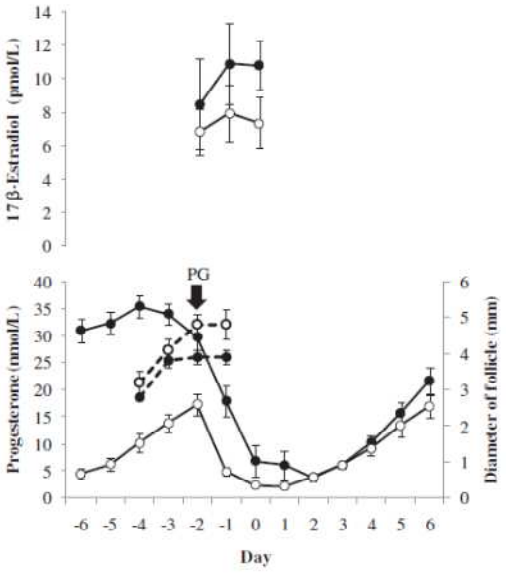

Fig. 3. Plasma progesterone concentrations (solid line) from Days −6 to 6, growth of the ovulatory follicle (dashed line) and plasma estradiol concentrations from 48 hours before to AI (Day 0), in ewes synchronized with DL-Cloprostenol (solid line, open circles; prostaglandin [PG] group; N = 15) or spontaneous estrus (solid line, filled circles; control group; N = 15). Untransformed data are presented as means ± SEM. Plasma progesterone concentrations from Days −6 to 1, and growth of the ovulatory follicle on Days −2 and −1 differed ($P < 0.05$) [26].

8. Attempts to improve the reproductive outcomes of PG-based protocols

Efforts to improve the reproductive outcomes of PG protocols (Table 3) were based on: (1) promoting a surge of LH to induce (and synchronize) ovulation; (2) using different AI pathways; (3) decreasing PG dose; and (4) increasing intervals between PG injections [13,27,28,36,69].

The introduction of rams increased LH concentrations of ewes under a double PG regimen 7 days apart, resulting in a similar pregnancy rate compared with a progestagen-based protocol [13] (Table 3). Although a control group of ewes in spontaneous estrus was missing in that study, these observations and others, including ovulation failure [57] and lower OR [26], strengthened the hypothesis of altered LH patterns causing ovulation failure in ewes synchronized with PG.

In ewes synchronized with two PG treatments given 14 days apart, the use of 500 IU of eCG im concurrent with the second PG treatment improved the estrous response (77.8%), conception rate (71.4%), and litter size (2.1) compared with synchronized ewes without eCG (73.0%, 42.1%, and 1.7, respectively). However, eCG had no effect when PG were administered 9 days apart (54.2%, 53.8%, and 1.6 vs. 76.9%, 65%, and 1.5, with and without eCG respectively) using natural mating [36]. Furthermore, the same dose of eCG did not improve fertilization rate when ewes were synchronized with two PG injections 11 days apart and TAI at 56 hours [69].

Based on the same premise, GnRH at TAI was tested as a means to increase LH concentrations, yielding better prolificacy but decreasing pregnancy rate. However, the authors suggested that the GnRH dose was given too late to improve results [28] (Table 3). In ewes synchronized with a double PG protocol with injections 7 days apart, intrauterine AI improved reproductive outcomes compared with cervical AI using chilled semen [27] (Table 3).

Decreasing the PG dose reduced the number of ewes showing estrous behavior from 25 to 48 hours after the second PG treatment ($P < 0.07$), and significantly reduced pregnancy rates obtained after the Synchrovine protocol and TAI with fresh semen [28] (Table 3). Similarly, a lower pregnancy rate was obtained when the interval between PG treatments was extended from 7 to 8 days. This was probably because of a larger proportion of ovulatory follicles originating from the second follicular wave of the cycle, resulting in less synchronous ovulation that reduced the probability of conception when TAI was performed 42 or 48 hours after the last PG injection [28] (Table 3).

9. Future perspectives

The use of PG in sheep reproduction has some practical advantages, including simple application (im injection), reduced cost, and less environmental contamination compared with progestagen intravaginal devices. However, more research is needed to improve the reproductive outcome when PG is used in TAI programs under field conditions. Based on the information presented, extending the interval between doses (12 to 14 days) could yield better reproductive outcomes after TAI compared with shorter intervals (7 to 8 days), because the POF develops under high P4 concentrations [26]. The LH pattern (frequency and amplitude) in long versus short PG intervals needs to be studied, assessing the hormonal environment under which the follicle develops, oocyte quality, and the interval to ovulation after PG treatments.

The use of GnRH or the male effect in PG-based protocols for TAI needs basic and field research to determine the optimal time of GnRH administration and male introduction. The use of focus feeding also has potential to improve follicle quality and increase OR, because short-term increases in nutrient influx can stimulate folliculogenesis in sheep [81]. Nutritional supplementation with high energy and protein, e.g., Lupin grain feed for 6 days [82], or corn grain plus soybean meal for 7 days, or the use of improved pastures (e.g., *Lotus corniculatus*) for 12 days [83], increased the number of 3 mm follicles, OR by 14%, and the number of twin lambs born.

To date, PG has been recommended to presynchronize and concentrate the estrous behavior of the flock, and to breed (AI or natural service) ewes in spontaneous estrus at least 15 days after the last PG treatment [23,26,83], thereby allowing strategies to increase lamb survival, e.g., short-term nutrition [5]. Although a decrease in the PG dose reduces costs, less synchrony is achieved. High doses are recommended when double PG protocols at short intervals between injections are used. The use of one, two, or more PG injections for flock synchronization depends on the level of synchrony desired, the economics, and the ability of each farmer to manage the flock during a highly concentrated breeding and lambing period.

Acknowledgments

The authors thank the library staff of Facultad de Veterinaria (UDELAR) for assistance with literature, and Valeria Fagundez for language revision.

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3. CONCENTRATIONS OF STEROID HORMONES, ESTROUS, OVARIAN AND REPRODUCTIVE RESPONSES IN SHEEP ESTROUS SYNCHRONIZED WITH DIFFERENT PROSTAGLANDIN-BASED PROTOCOLS

RESUMEN

Con el objetivo de determinar la respuesta estral, ovárica y reproductiva luego de la aplicación de diferentes protocolos en base a PG, se conformaron cuatro grupos: PG10, PG12, PG14 o PG16 (dos inyecciones de PG administradas a intervalos de 10, 12, 14 o 16 días respectivamente). El Experimento I evaluó la respuesta estral, TO, concepción y fertilidad a estro detectado, mientras que en el Experimento II se evaluó el crecimiento folicular ovárico, la concentración de hormonas esteroideas (progesterona y estradiol) y los intervalos desde la segunda PG hasta el inicio del estro (PG-estro) y hasta la ovulación (PG-ovulación). La respuesta estral fue menor en el grupo PG16 ($P<0,05$) y la sincronía de los estros fue mayor en los grupos PG10 y PG12. El desarrollo folicular ovárico, los intervalos PG-estro, PG-ovulación y la TO fueron similares entre los grupos ($P>0,05$). Las concentraciones de progesterona entre los días 8 a 4 previo al estro, fueron superiores en los grupos PG14 y PG16 comparado con los grupos PG10 y PG12 ($P<0,05$). Existieron más días con progesterona con niveles superiores a 3,18 nmol/L en los grupos PG14 y PG16 respecto a los grupos PG10 y PG12 ($P<0,05$). Los niveles de estradiol al estro y 12 horas posteriores, fueron superiores con el uso de los grupos PG14 y PG16 comparados con los grupos PG10 y PG12. Se observó una correlación positiva entre la duración de la fase luteal y la concentración máxima de estradiol, y entre la duración de la fase luteal y los días con estradiol con valores superiores a 10 pmol/L. La concepción y la fertilidad al estro detectado fueron mayores con el uso de PG14 comparado con PG10 y PG12 ($P<0,05$). Se concluye que la administración de dos inyecciones de PG separadas 10, 12, 14 o 16 días, generaría diferencias en la duración de las fases luteales, en la respuesta estral, en la sincronía de los estros y en la respuesta reproductiva. Sin embargo no incidiría en las variables PG-estro, PG-ovulación, o en la TO de los diferentes grupos.

Palabras clave: sincronización de estros, prostaglandina, hormonas esteroideas, inseminación artificial a tiempo fijo, oveja.

Animal Reproduction Science 167 (2016) 74–82

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Concentrations of steroid hormones, estrous, ovarian and reproductive responses in sheep estrous synchronized with different prostaglandin-based protocols

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ARTICLE INFO

Article history:
Received 14 August 2015
Received in revised form 7 February 2016
Accepted 8 February 2016
Available online 10 February 2016

Keywords:
Estrous synchronization
Prostaglandin
Steroid hormones
Timed artificial insemination
Ewe

ABSTRACT

To determine estrous, ovarian and reproductive responses after different prostaglandin (PG)-based protocols, ewes were assigned to groups PG10, PG12, PG14 or PG16 (two PG injections administered 10, 12, 14 or 16 days apart; respectively). Experiment I ($n = 132$) was conducted to evaluate the estrous response, ovulation rate (OR), conception and fertility. Experiment II ($n = 24$) was conducted to evaluate ovarian follicle growth, steroid concentrations and the interval from the second PG injection to estrus (PG-estrus) and ovulation (PG-ovulation). Estrous response was less with the PG16 ($P < 0.05$) treatment, and the extent of estrous synchrony was greater with the PG10 and PG12 treatments. Ovarian follicle growth and the intervals for the variables PG-estrus, PG-ovulation and OR were similar among groups ($P > 0.05$). From 8 to 4 days before estrus, progesterone (P4) concentrations were greater for the PG14 and PG16 than for the PG10 and PG12 ($P < 0.05$) groups. There were more days where concentrations of P4 were above 3.18 nmol/L with the PG14 and PG16 than PG10 and PG12 ($P < 0.05$) treatments. Use of the PG14 and PG16 treatments resulted in greater estradiol (E2) at estrus and 12 h later than use of the PG10 and PG12 treatments. A positive correlation was observed between the duration of the luteal phase and maximum E2 concentrations, and between duration of the luteal phase and days with E2 concentrations above 10 pmol/L. Conception and fertility were greater with use of the PG14 compared with PG10 and PG12 ($P < 0.05$) treatments. The administration of two PG injections 10, 12, 14 or 16 days apart resulted in different durations of the luteal phase that were positively associated with E2 concentrations and the reproductive outcome. The shorter luteal phases were associated with greater synchrony in time of estrus. The intervals for the variables PG-estrus, PG-ovulation and OR were similar among groups.

1. Introduction

Timed artificial insemination (TAI) represents a practical tool in genetic programs, but requires hormonal treatments that ensure a synchronized time of ovulation and acceptable pregnancy rates (Menchaca and Rubianes, 2004). Progestagen-based protocols are the

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http://dx.doi.org/10.1016/j.anireprosci.2016.02.009
0378-4320/

preferred option by technicians and farmers to manage flock reproduction (Gordon, 1999) even though there are potentially environmental and tissue contamination risks due to residues of progestagen devices, as well as risks with use of eCG, or the addition of antibiotics to avoid vaginitis (Gonzalez-Bulnes et al., 2005; Viñoles et al., 2011). Also, progestagen based estrous synchronization protocols have been associated with alterations in oocyte quality that can result in lesser fertilization rates and impaired embryo development (Gonzalez-Bulnes et al., 2005; Berlinguer et al., 2007). Because consumers demand foods produced by "clean, green and ethical" guidelines (Martin et al., 2004), prostaglandins became a desirable alternative because lungs rapidly metabolize the drug hence it does not accumulate in tissues of treated animals (Piper et al., 1970; Davis et al., 1980). Furthermore, prostaglandin-based protocols are easily applied by intramuscular injection, thus improving animal management and welfare (Abecia et al., 2012), and are more economically feasible compared with intravaginal devices plus eCG in sheep production enterprises.

Prostaglandin $F_{2\alpha}$ and its synthetic analogues (PG) have been widely studied since its discovery in 1970 as a powerful luteolytic agent (McCracken et al., 1970). Different alternatives of PG-based protocols have been used to synchronize time of estrus in sheep for TAI (rev: Fierro et al., 2013). However, use of most of these treatments is associated with lesser pregnancy rates compared with use of progesterone-eCG based protocols (Boland et al., 1978; Olivera-Muzante et al., 2011a; Viñoles et al., 2011).

Traditional PG-based protocols consist of two PG injections administered 9–14 days apart (Fierro et al., 2013), however, there is considerable variability in timing of estrous onset and ovulation (Acritopoulou et al., 1978; Loubser and van Niekerk, 1981; Houghton et al., 1995; Viñoles and Rubianes, 1998), that limit the practicality of use of these protocols for TAI programs (Menchaca and Rubianes, 2004). When a PG-based protocol of two injections given 7 days apart is used (Rubianes et al., 2004), a highly synchronized time of estrus and timing of ovulation are observed (Rubianes et al., 2003; Menchaca et al., 2004), but undesirable pregnancy rates are often achieved that are related to an altered profile and lesser progesterone (P4) concentrations that in turn result in a lesser ovulation rate (OR), fertility and prolificacy compared with what occurs when inseminations occur as a result of spontaneous estrus (Fierro et al., 2011). Attempts to develop alternatives to improve this protocol (two injections given 7 days apart) have not been successful (Olivera-Muzante et al., 2011b, 2013; Fierro et al., 2013, 2014).

Lesser fertilization rates were reported when the interval between PG injections was reduced from 14 to 8 days (Fairnie et al., 1977). Furthermore, treatment with a supplemental P4 source provided via an intra-vaginal impregnated device that is inserted 8 days before the PG injection increased the number of ewes in estrus (93.4% compared with 82.0%), and pregnancy rate (84.9% compared with 75.3%) than occurred with untreated ewes (Loubser and van Niekerk, 1981). The interval between PG-injections has been defined as important by Fairnie and Wales (1980), and other reports indicated that the interval between PG injections should not be reduced to less than 11 days (Greyling and van der Westhuysen, 1980) or 13 and 14 days (Fairnie et al., 1978). Similarly, Fierro et al. (2013) suggested that the extension of the period between PG injections (with the second PG applied during the mid-to-late luteal phase) may contribute to developing a protocol to prolong the exposure of follicles from which ovulation occurs to adequate P4 concentrations during the growth phase of these developing follicles. The evaluation of longer periods between PG injections is necessary to understand the physiologic processes (estrous response, hormonal profile, time of ovulation) when these protocols are applied.

To the best of our knowledge, there are no previous studies that compared the concentrations of steroid hormones, as well as estrous, ovarian and reproductive responses in sheep where time of estrus was synchronized with use of different PG-based protocols under the same conditions (breed, photoperiod, nutrition, and health management). In the present study, the working hypothesis was that the administration of two PG injections 10, 12, 14 or 16 days apart would allow for development of an estrous synchronization protocol that resulted in desirable pregnancy rates in ewes. The aim of these experiments was to study ovarian follicular growth, concentrations of steroid hormones, estrous response, ovulation time, OR, conception and fertility rates after estrous detection when different PG-based protocols were used for sheep estrous synchronization. This information is necessary to identify which of these protocols are most effective if used in TAI programs.

2. Materials and methods

Experiment I was conducted at Escuela Agraria "La Carolina" (33° S–57° W; Flores, Uruguay), and Experiment II at Estación Experimental "Dr. Mario A. Cassinoni" (32° S–58° W; Paysandú, Uruguay) during the breeding season for ewes at these locations (March to April). The experimental procedures were approved by the Universidad de la República's Animal Ethics Committee (CUEA-Universidad de la República, Facultad de Veterinaria, Exp: 111400-000079-12).

2.1. Animals and management

2.1.1. Experiment I

Multiparous Corriedale ewes (older than 2.5 y old, $n = 132$), in a moderate body condition (3.2 ± 0.4, scale 0–5; Russel et al., 1969) and weighing 51.5 ± 6.2 kg were used. The flock used had an OR, based on previous records for this flock, that ranged from 1.20 to 1.61 (Fierro et al., 2011; 2014). Ewes grazed pastures that are typically used for sheep production in the region where the studies were conducted with more than 600 kg of dry matter (DM) available per hectare (8% CP and 8.5 MJ ME/kg DM), and water was available *ad libitum*.

2.1.2. Experiment II

Multiparous (older than 2.5 y old, $n = 21$) and nuliparous (1.5 y old, $n = 3$) Corriedale ewes, in a moderate body condition (3.1 ± 0.4 and 2.6 ± 0.1), and weighing 51.7 ± 5.3 kg

and 41.0 ± 2.4 kg, respectively, were used. Ewes were maintained under field conditions, grazing pastures that are typically used for sheep production in the region where the studies were conducted (500 kg DM available per hectare, 90.6% DM, 13.8% CP and 9.2 MJ ME/kg DM), 0.4 kg/ewe/day of supplement (91.9% DM, 20.5% CP and 11.3 MJ ME/kg DM), and water was provided *ad libitum*.

2.2. Experimental design

Two experiments were conducted with the objective to determine estrous, OR and reproductive response (Experiment I; $n = 132$), concentrations of steroid hormones, ovarian follicular growth and time of ovulation (Experiment II; $n = 24$) in ewes in which stage of the estrous cycle was synchronized with different PG-based protocols. Ewes were assigned by body condition and weight (Experiments I and II), and by age (Experiment II) to four groups: time of estrus was synchronized with two injections of Delprostenate i.m. (160 μg per injection, Glandinex®, Universal Lab, Montevideo, Uruguay), administered 10, 12, 14 or 16 days apart (PG10, PG12, PG14 or PG16, respectively; $n = 33$ and $n = 6$ in each group, Experiment I or II, respectively; Fig. 1).

2.3. Estrous detection

Estrous detection was performed using Corriedale androgenized wethers (given 100 mg of Ciclopentilpropionate, on three occasions, 7 days apart; Testosterona Ultra Fuerte®, Laboratorio Dispert, Uruguay), with marker paint at a rate of six wethers/100 ewes. In Experiment I, estrous response (ewes in estrus/total ewes) was evaluated every 24 h from Days-4 (−96 h) to 0 (day of second PG injection; Hour 0), and then every 12 h until 120 h. In Experiment II, estrous detection was performed every 12 h after the second PG injection until ovulation, considering the intervals from PG administration to estrous detection (PG-estrus) and PG administration to the time of ovulation (PG-ovulation).

2.4. Semen collection, evaluation and dilution

Semen from five adult Corriedale rams, assessed for breeding soundness, was collected using an artificial vagina and assessments occurred as described by Evans and Maxwell (1987a). Two consecutive ejaculates from each ram were collected, evaluated and pooled. Soon after pooling, the semen was extended with UHT skim milk and antibiotics (250 mg of enrofloxacyn/1 L of extender; Baytril®), and assessed for progressive sperm motility (80%) before its use.

2.5. Artificial insemination

Cervical AI was performed using a speculum equipped with a light source and an insemination device (Walmur® Veterinary Instruments, Montevideo, Uruguay) after estrous detection, as described by Evans and Maxwell (1987b). The insemination dose was 0.08 mL and contained 100×10^6 sperm cells, slowly released into the cervix at the time of insemination. Extended semen was maintained at room temperature and protected from sunlight until AI.

2.6. Ultrasonograhy evaluation

In both experiments, the ewes' estrous cyclicity (presence of a corpus luteum −CL-) was evaluated before the first PG injection (Viñoles et al., 2004). In Experiment I, OR (CL/ewes having ovulations) was evaluated 10 days after estrous detection in ewes that showed estrous behavior after the second PG injection ($n = 32$, 33, 31 and 24, PG10, PG12, PG14 and PG16 respectively). Conception (pregnant ewes/inseminated ewes × 100) and fertility rates (pregnant ewes/estrous synchronized ewes × 100) were evaluated 35 days after estrous detection. Values for all variables were determined by trans-rectal ultrasonography using a 7.5 MHz rigid linear array transducer (ALOKA SSD-500, Overseas Monitor Corp., Ltd., Richmond, BC, Japan) using the methodology described by Viñoles et al. (2010).

In Experiment II, pre-ovulatory follicle growth was evaluated every 24 h from the day of the second PG injection (Day 0, Hour 0) to the time of estrous detection, and time of ovulation was assessed every 12 h after estrous detection until the disappearance of the largest follicle (ovulation) by trans-rectal ultrasonography. The number, diameter and relative position of all ovarian follicles with a diameter of ≥2 mm and CL on both ovaries were mapped in each ultrasonography session. Follicle definitions used in this study were: the maximum diameter was the largest diameter of the pre-ovulatory follicle (mm), final diameter was the diameter detected the day before ovulation (mm), rate of follicular growth was calculated as the size difference from the first ultrasonic evaluation to ovulation, divided by the number of days it took to attain the maximum diameter (mm/d).

2.7. Blood collection and hormone concentrations

Jugular blood was collected into glass tubes with heparin using disposable needles. Blood samples were collected once a day (in the morning after a 12 h fasting period) from Day-10 to estrous detection, each 12 h after estrus to ovulation, and at 5 and 12 days after estrous detection (Experiment II; Fig. 1).

Blood samples were stored at 5 °C until centrifugation at 2100g for 15 min; plasma was pipetted and stored at −20 °C until hormonal concentrations were analyzed. Progesterone and 17-β estradiol concentrations (E2) were quantified by radioimmunoassay (RIA) as described by Meikle et al. (1997). Progesterone concentrations were determined by a direct solid-phase RIA using Siemens kits (Siemens, Los Angeles, CA, 90045 USA). The RIA had a sensitivity of 0.32 nmol/L. The intra-assay coefficients of variation for the lesser (1.59 nmol/L) and greater (31.8 nmol/L) concentration control samples were 5.7% and 3.4%; respectively. The inter-assay coefficients of variation for the lesser and greater concentration control samples were 5.6% and 3.7%; respectively. Estradiol 17β concentrations were determined by a liquid phase RIA (DPC kits; Diagnostic Product Co., Los Angeles, CA, USA). Samples were analyzed in duplicate in the same assay. The sensitiv-

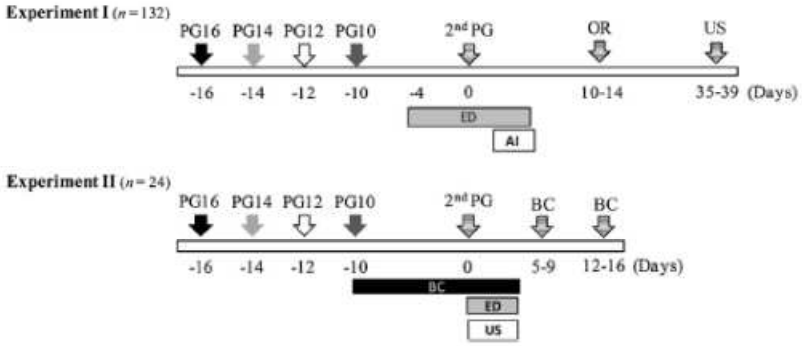

Fig. 1. Schematic representation of the experimental design.
PG16, PG14, PG12, PG10: first PG injection of the protocol 16, 14, 12 or 10 days apart; 2nd PG: second PG injection in all synchronized groups (Day 0). ED: estrous detection; AI: cervical artificial insemination; BC: blood collection (between Day-10 to estrous detection, and 5 and 12 days after estrus); US: trans-rectal ultrasonography examination to determine follicular growth and ovulation (Experiment II), conception and fertility rate 35 days after estrous detection (Experiment I); OR: ovulation rate evaluated by trans-rectal examination 10 days after estrous detection

ity of the assay was 3.8 pmol/L. The intra-assay coefficients of variation were 17% for the lesser (45 pmol/L) and 14% for the greater concentration control (75 pmol/L) samples.

2.8. Statistical analyses

All analyses were performed using the Statistical Analysis System (SAS, version 9.1.3, 2004). Differences in the variance in time of estrus, OR, conception and fertility were analyzed by GENMOD. Follicle growth and the intervals for the variables PG-estrus and PG-ovulation, P4 and E2 concentrations in plasma were compared by analysis of variance using PROC Mixed. The model included the fixed effects of the group, ewe age, day and the interactions of these variables. Ewe within group was considered as the random effect. The covariance was modelled to consider the correlation between successive measurements of the same animal, with the option autoregressive order 1 (AR(1)). The duration of the luteal phase was analyzed using PROC GLM, including in the model the age of ewe, group and the interaction as fixed effects. Differences were considered significant if $P < 0.05$ and trends if $P < 0.1$ and > 0.05.

3. Results

3.1. Experiment I

Fig. 2 depicts data for estrous behavior observed from −48 to 96 h after the second PG injection. Overall, the estrous response from 0 to 96 h after the second PG injection was similar for the PG10, PG12 and PG14 (97%, 100%, 94%; $P > 0.05$) groups, but greater compared to the PG16 group (73%; $P < 0.05$). More ewes from the PG16 group were in estrus 48 and 24 h prior to the second PG than the other groups ($P < 0.05$). Estrous response was greater in the PG14 group at 24 h, PG10 group at 48 h, and PG12 group at 72 h ($P < 0.05$). No differences were detected in OR among groups ($P > 0.05$, Table 1). Conception and fertility rates for the PG14 group were greater compared with

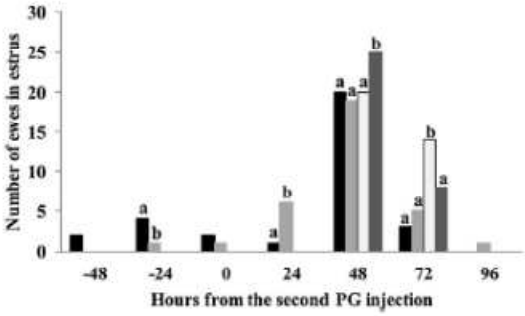

Fig. 2. Estrous response (number of ewes in estrus) previous or after second PG injection (Hour 0), in ewes estrous synchronized with two PG injections administered 10 (PG10–■), 12 (PG12–□), 14 (PG14–■) or 16 (PG16–■) days apart; [a] compared with [b]: $P < 0.05$.

Table 1
Ovulation, conception and fertility rates in ewes that were estrous synchronized with two PG injections administered at 10 (PG10), 12 (PG12), 14 (PG14) or 16 (PG16) day intervals and inseminated with fresh semen after estrous detection.

Group	Ovulation rate	Conception rate (%)	Fertility rate (%)
PG10 ($n = 33$)	1.44 ± 0.50^{a}	22.6^{a}	21.9^{a}
PG12 ($n = 33$)	1.36 ± 0.49^{a}	21.2^{a}	21.2^{a}
PG14 ($n = 33$)	1.27 ± 0.45^{a}	54.8^{b}	51.5^{bx}
PG16 ($n = 33$)	1.32 ± 0.48^{a}	41.7^{ab}	30.3^{aby}

Ovulation rate (ovulations/ovulated ewe) measurement by trans-rectal ultrasonography 10 days after cervical artificial insemination; Conception (pregnant ewes/inseminated ewes × 100) and fertility rate (pregnant ewes/synchronized ewes × 100), were evaluated by trans-rectal ultrasonography 35 days after artificial insemination; Data are presented as means ± SD. [a] compared with [b] in the same column: $P < 0.05$, [x] compared with [y] in the same column: $P = 0.08$.

the PG10 and PG12 groups ($P < 0.05$), but similar to the PG16 group (except that fertility tended to be less for the PG16 than PG14 group, $P = 0.08$). The PG10, PG12 and PG16 groups had similar conception and fertility rates ($P > 0.05$, Table 1).

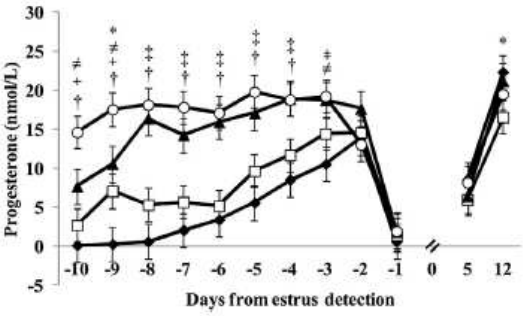

Fig. 3. Plasma progesterone concentrations relative to estrus (Day 0) in ewes estrous synchronized with two injections of PG (Delprostenate) administered 10 (PG10-◆-), 12 (PG12-□-), 14 (PG14-▲-) or 16 (PG16-○-) days apart (n = 6 ewes per group); Data are presented as least square means ± SEM. †: $P < 0.05$ between PG16 compared with PG12 and PG10; +: $P < 0.05$ between PG16 compared with PG14; ≠: $P < 0.05$ between PG14 compared with PG10; ‡: $P < 0.05$ between PG16 compared with PG10; ‡: $P < 0.05$ between PG14 compared with PG12 and PG10; *: $P < 0.05$ between PG10 compared with PG12.

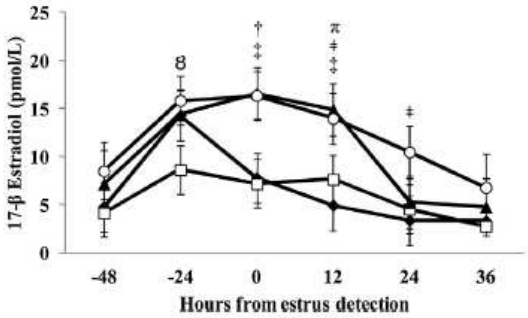

Fig. 4. Plasma 17-β Estradiol concentrations relative to estrus (Hour 0) in ewes estrous synchronized with two injections of Delprostenate administered 10 (PG10-◆-), 12 (PG12-□-), 14 (PG14-▲-), or 16 (PG16-○-) days apart (n = 6 ewes per group); Data are presented as least square means ± SEM. 8: $P < 0.05$ between PG16 compared with PG12; †: $P < 0.05$ between PG16 compared with PG12 and PG10; ‡: $P < 0.05$ between PG14 compared with PG12 and PG10; +: $P < 0.05$ between PG16 compared with PG10; π: $P = 0.08$ between PG16 compared with PG12.

3.2. Experiment II

Data for P4 concentrations relative to time of estrous detection are presented in Fig. 3. The effects of group, day and the interaction were significant ($P < 0.05$), but not ewe age ($P > 0.05$). Progesterone concentrations were greater in the PG14 and PG16 groups from 8 to 4 days prior to estrus compared to ewes from the PG10 and PG12 groups ($P < 0.05$; Fig. 3). However, the decrease in P4 concentrations after the second PG injection and concentrations at 5 and 12 days after estrus were similar among groups ($P > 0.05$) except that ewes from the PG10 group had greater concentrations than those from the PG12 group on Day 12 ($P < 0.05$). The number of days with P4 above 3.18 nmol/L was similar between the PG14 (10.6 ± 1.3 d) and PG16 groups (12.5 ± 0.8 d; $P > 0.05$), and both were greater compared with the PG10 (4.3 ± 1.6 d) and PG12 groups (6.8 ± 2.6 d; $P < 0.05$).

Data for E2 concentrations from −48 to 36 h after estrus are depicted in Fig. 4. The effects of group and day were significant ($P < 0.05$), but not that of ewe age and the interaction of group and day ($P > 0.05$). At estrus and 12 h later, E2 concentrations were greater in ewes from the PG14 and PG16 groups compared with ewes from the PG10 and PG12 groups ($P < 0.05$, Fig. 4). Ewes from the PG14 and PG16 groups had more days with E2 concentrations greater than 10 pmol/L (1.40 ± 0.89 and 2.83 ± 0.75 d, PG14 and PG16 groups, respectively), compared with ewes from the PG10 and PG12 groups (0.83 ± 0.75 d; $P < 0.05$). A positive correlation was observed between the duration of the luteal phase and maximum E2 concentrations ($r = 0.5$; $P < 0.01$) and between the duration of the luteal phase and the number of days with E2 concentrations greater than 10 pmol/L ($r = 0.78$; $P < 0.001$).

Data on the intervals for the variables PG-estrus and PG-ovulation, final and maximum diameter of the ovulatory follicle, and follicular growth rate are presented in Table 2. No significant differences were detected among groups for any of these variables ($P > 0.05$).

4. Discussion

The working hypothesis in the present research that the administration of two PG injections administered 10, 12, 14 or 16 days apart affected differences in time of estrous response, ovarian response, concentrations of steroid hormones and reproductive performance in ewes was supported by some of the results from these studies. The use of the protocols evaluated resulted in differences in P4 and E2 concentrations, estrous response up to 96 h after the second PG administration, and in conception and fertility. However, the morphological changes in the preovulatory follicle, and intervals for the variables PG-estrus and PG-ovulation, and OR were similar among groups.

The PG-based protocols evaluated in the present experiments were associated with different estrous responses until 96 h after the second PG administration. The intervals between PG injections determined that at the moment of the second PG administration the age of the CLs (in ewes that responded to the first PG injection) varied from 7 to 14 days (mid to late luteal phases) so they were all sensitive to the luteolytic effect of PG (Houghton et al., 1995; Rubianes et al., 2003; Contreras-Solís et al., 2009). Estrous response variables were reported previously using the two PG injection protocols with an interval of 9 days (95%, Acritopoulou-Fourcroy et al., 1982; 76.9%, Boland et al., 1978; 100%, Haresing and Acritopoulou, 1978); 10 days (71.4%, Godfrey et al., 1997; 88%, Das et al., 1999); 11 days (82%, Loubser and van Niekerk, 1981; 100%, Oyediji et al., 1990); and 14 days (73%, Boland et al., 1978). Nevertheless, the use of two PG injections administered 16 days apart (PG16 group) resulted in a lesser overall estrous response compared with the values for the PG10, PG12 and PG14 groups. This could be explained by some ewes in the PG16 group, expressing estrous behavior 48 h prior

Table 2
Intervals from the second prostaglandin administration to estrus (PG-estrus) and ovulation (PG-ovulation), and morphological characteristics of the pre-ovulatory follicle (maximum diameter, final diameter and growth rate) in ewes in which time of estrus was synchronized with two PG injections administered at 10 (PG10), 12 (PG12), 14 (PG14) or 16 (PG16) day intervals ($n = 6$ ewes per group).

Group	PG-estrus (h)	PG-ovulation (h)	Maximum diameter (mm)	Final diameter (mm)	Growth rate (mm/d)
PG10	50.0 ± 6.0[a]	72.0 ± 6.6[a]	6.3 ± 0.5[a]	6.0 ± 0.9[a]	0.7 ± 0.4[a]
PG12	44.0 ± 4.9[a]	70.0 ± 6.2[a]	6.5 ± 0.8[a]	6.3 ± 0.8[a]	1.0 ± 0.5[a]
PG14	37.2 ± 23.4[a]	58.8 ± 21.8[a]	6.2 ± 0.4[a]	6.0 ± 0.7[a]	1.1 ± 0.7[a]
PG16	44.0 ± 15.9[a]	64.0 ± 9.0[a]	6.5 ± 1.2[a]	6.3 ± 1.0[a]	1.3 ± 0.5[a]

Maximum diameter: the largest diameter reached by the pre-ovulatory follicle; Final diameter: the diameter reached before ovulation; Growth rate: calculated as the size difference from the first ultrasonic evaluation to ovulation, divided by the number of days it took to reach the maximum diameter; Data are presented as LS means ± pooled SEM. [a]In the same column: $P > 0.05$.

to the administration of the second PG injection because natural luteolysis had already occurred (Rubianes et al., 2003). The PG16 group was, nevertheless, initially included in the present experiment because the interval between PG injections was expected to facilitate the induction of a physiologic luteal phase (duration and hormonal profile), and be associated with an enhanced reproductive performance. Most of the ewes from the PG10 and PG12 groups expressed estrous behaviuor between 48 to 72 h after the second PG injection; demonstrating a concentrated estrous response when these protocols are applied, a result that is very important for TAI programs. In summary, under the conditions of the present study, all the protocols were determined to provide for an acceptable estrous response after treatment; however, the use of the PG10, PG12 and PG14 protocols resulted in a greater number of ewes in estrus than use of the PG16 protocol and the PG10 and PG12 groups had a greater synchrony in time of estrus than the other groups.

The intervals for the PG-estrus and PG-ovulation variables were similar among groups, although the variation was greater for these variables with the PG14 and PG16 groups. These results were unexpected because the interval PG-estrus variable has been associated with the age of the CL (Houghton et al., 1995) and with the follicular status of each ewe at the time of the PG administration (Viñoles and Rubianes, 1998). Nevertheless, this result was supported by a similar rate of P4 decrease for all groups in the present study after PG administration. There are previous reports for the PG-estrus interval variable under different experimental conditions with results for 7 day (48 ± 2.3 h, Rubianes et al., 2003; 36.6 ± 3.6 h, Contreras-Solís et al., 2009); 9 day (38.8 ± 1.3 h, Acritopoulou et al., 1978; 38.6 ± 0.8 h, Haresign and Acritopoulou, 1978; 43.5 ± 6 h, Acritopoulou, 1979; 45.8 ± 1.1 h, Acritopoulou-Fourcroy et al., 1982); 10 day (51.6 ± 2.4 h, Das et al., 1999); and 11 day (41.7 ± 2.2 h, Oyediji et al., 1990) intervals between PG injections being reported, however, longer intervals (14 and 16 d) between injections were not evaluated in previous studies and the comparisons have not been made under the same experimental conditions as occurred in the present study. The interval between when basal P4 concentrations are achieved to estrous detection (Wiley et al., 1997), and between estrous onset and LH peak to ovulation (Cumming et al., 1973) has been reported to be similar. Fairnie et al. (1977) observed a similar mean time from treatment to ovulation after two PG injections were administered at 8 compared with 14 day intervals. Even though there were non-significant differences among PG-ovulation interval variables of the groups in the present study, the differences observed with some groups may need to be considered in determining the optimal AI time in TAI programs, mainly when chilled or frozen semen are used, due the shorter lifespan of sperm cells stored in these conditions compared with use of fresh semen (Salamon and Maxwell, 2000). Furthermore, it is possible that the experimental design in the present study was not appropriate to detect differences among groups in these variables due to the extended time between estrous observations (12 h).

Follicular growth rate, as well as final and maximum follicle diameter were similar among groups. These results were not expected, because the final stage of growth of a follicle is related to LH pulse frequency that is down-regulated by P4 (Ginther et al., 1995). In the present study, LH pulse frequency was not assessed but there were differences in P4 concentrations detected and in the number of days with P4 above 3.18 nmol/L (longer in PG14 and PG16 groups). An altered P4 profile was associated with altered ovarian follicular dynamics and poor reproductive performance when a short PG-based protocol was used (Fierro et al., 2011). Furthermore, similar follicular diameters were related to different E2 concentrations among groups. Ewes from the PG14 and PG16 groups had greater E2 concentrations compared with ewes from the PG10 and PG12 groups around the time of estrus. It is well known that the secretion of E2 by the follicles is associated with the LH pulse frequency (Sirois and Fortune, 1990; Stock and Fortune, 1993; Viñoles et al., 1999), reinforcing the existence of physiological differences in the dominant follicles among groups. There was a similar diameter for pre-ovulatory follicles in all groups, and the lesser steroidogenic function of the follicles of ewes in the PG10 and PG12 groups may indicate that these ewes had an impaired steroidogenic capacity (White et al., 1987) even though there was greater estrous synchrony for these groups. An altered steroidogenic function of follicles has been described as a consequence of an inadequate P4 priming (Coleman and Dailey, 1983), and to lesser numbers of LH receptors in granulosa cells (McNatty et al., 1984). Furthermore, a premature exposure to LH inhibited the potential of human granulosa cells to secrete steroids *in vitro* (McNatty and Sawers, 1975). Moreover, Deaver et al. (1986) reported that differences in the patterns of gonadotropin secretion before the pre-ovulatory surge of LH might be caused by differences in P4 or the P4-E2 ratio when luteal regression is induced on different days of the estrous cycle.

Conception and fertility rates were related to the hormonal profiles in the different groups in the present study. The ewes of the PG14 group had greater conception and fertility rates after estrous detection compared with the PG10 and PG12 groups. The greater concentrations and numbers of days with elevated P4 concentrations observed in the PG14 group prior to mating may be responsible for the greater conception and fertility rates for this group (Folman et al., 1973; Fairnie et al., 1977; Loubser and van Niekerk, 1981). The alteration of the steroidogenesis, as observed in ewes of the PG10 and PG12 groups (lesser P4 and E2 concentrations compared to groups with longer periods between PG injections) have been reported to affect oocyte development and transport, fertilization rate and early embryo development in cattle (Gustafsson and Plöen, 1986; Greve et al., 1995). Estradiol induces the development of the secretory cells in the oviduct, which affects fertilization and early embryo development (Murray, 1992; Nancarrow and Hill, 1995), and lesser E2 concentrations have been associated with a decreased fertilization rate in estrous synchronized sheep (Gonzalez-Bulnes et al., 2005). Furthermore, E2 and P4 concentrations regulate the expression of the E2 and P4 receptor genes in the uterus (Clark and Mani, 1994; Ing et al., 1996; Ing and Ott, 1999), and it was suggested that reproductive failure in P4 estrous-synchronized ewes may be related to a decrease in the expression of E2 and P4 receptor genes (García-Palencia et al., 2007). It is possible that these altered physiological functions when P4 was used to synchronize the time of estrus may have contributed to the reproductive performance achieved by ewes of the PG10 and PG12 groups. Detailed morphological and functional studies of the follicles, oocytes and embryo quality are needed to determine the reasons of this altered steroidogenic function when protocols with different intervals between PG injections are used. In general, the use of protocols with longer periods between PG injections resulted in enhanced reproductive performance after estrous detection, although conception and fertility of the PG16 group in the present study was intermediate. The tendency for a lesser fertility in the PG16 compared with the PG14 group could be due to the lesser number of ewes that expressed estrous behavior and were inseminated after the second PG injection in this group.

Regarding the P4 profile after mating, there was a similar P4 profile and concentrations at 5 and 12 days after estrous detection in all groups, except the PG10 group had greater concentrations than PG12 group on Day 12 subsequent to ovulation. This may indicate a normal lifespan of the CL after PG administration (Fierro et al., 2011, 2013) even when there were differences in follicle functionality observed during the follicular phase. Moreover, findings of the present study reinforce the importance of the hormonal milieu during the follicular phase preceding ovulation on oocyte quality and the uterine environment so as to support early embryo development (Gustafsson and Plöen, 1986; García-Palencia et al., 2007). This effect cannot be overcome by having greater P4 concentrations during the mid-luteal phase following ovulation. This rationale is supported by the lesser conception and fertility rates in PG10 and PG12 groups of the present study, even though there were greater P4 concentrations on Day 12 in ewes from the PG10 group.

Ovulation rate is a variable associated with inconsistent results when PG-based estrous synchronization protocols are used (Fierro et al., 2013). No differences were detected for OR among groups in the present study and this was unexpected because an increase in OR had been reported when PG was administered in the mid-luteal phase of the estrous cycle probably because of the development of a "less dominant" pre-ovulatory follicle with a lesser steroidogenic capacity that contributed to maintaining FSH concentrations above the threshold so as to stimulate the selection of multiple ovulatory follicles from a single follicular (Letelier et al., 2011), or from two consecutive follicular waves (Bartlewski et al., 1999; Gibbons et al., 1999). Considering the findings with the present study on the E2 production by the dominant follicles in the different groups, it is speculated that the development of follicles with a longer dominant phase occurred in PG14 and PG16 groups compared with the PG10 and PG12 groups. This is inconsistent with results when PG was administered in the mid-luteal phase (Letelier et al., 2011) where with this treatment it would be expected to result in a greater OR. To the best of our knowledge, this is the first report where OR was compared with use of different PG-based protocols for estrous synchronization in sheep under the same conditions. However, it is important to note the small number of ewes used to evaluate this variable, thus, more research is needed to determine whether the findings of the present study can be confirmed.

The experimental designs used in the present study allowed for the enhanced understanding of the effects of different PG-based protocols for estrous synchronization in sheep under similar conditions. In brief, the PG14 treatment resulted in a desirable estrous response, lesser time to estrous synchrony, and greater P4, and E2 concentrations as well as reproductive performance after estrous detection compared with the results with use of the PG10 and PG12 treatment protocols. Ewes of the PG16 group have a lesser estrous response and intermediate reproductive outcome, but similar hormonal profiles as the PG14 group. It is important to note that the more desirable reproductive performance of the PG14 group may not necessarily indicate a greater fertility after AI at fixed time. When a TAI protocol is applied, a greater estrous response and synchrony are important for desirable reproductive outcomes. Future trials need to be performed to determine the reproductive success with use of the protocols applied in the present study in TAI programs.

5. Conclusion

It is concluded that the administration of two PG injections 10, 12, 14 or 16 days apart contributed to the different durations of the luteal phase that were positively associated with E2 production by the pre-ovulatory follicles and the reproductive outcome in the present study. However, the shorter intervals between PG injections in the present study were associated with an enhanced estrous synchrony which is a requisite for TAI programs. There were no differ-

ences in the variables of intervals PG-estrus, PG-ovulation and OR among groups in the present study.

Conflict of interest

None.

All authors have no financial or personal relationship with organizations or people that could influence or bias the study.

Acknowledgements

Sergio Fierro was a PhD student scholarship from Agencia Nacional de Investigación e Innovación (ANII) during the research that gives rise to the results presented in this publication (2013–2014; POS_NAC_2012_1_9449). The authors thank Jorge Gil, Carolina Bonino, Johana Braga, Ramiro Dutra da Silveira, Sofía Grela, Graciana Hourcade, Cindy Pechy, Federico Soca, and Dolores Soler for the help in the laboratory and field work. We also are grateful to the directorate of Escuela Agraria UTU "La Carolina", Estación Experimental "Dr. Mario A. Cassinoni" and Facultad de Veterinaria in Paysandú, for providing the facilities and animals to perform this experiment, and to students and staff of these Institutions for valuable support. This work was funded by Instituto Nacional de Investigación Agropecuaria—INIA Uruguay, FPTA No. 315 project (2012).

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4. LONG TERM PROSTAGLANDIN BASED-PROTOCOLS IMPROVE THE REPRODUCTIVE PERFORMANCE AFTER TIMED ARTIFICIAL INSEMINATION IN SHEEP

RESUMEN

El objetivo de este experimento fue evaluar la respuesta reproductiva de ovejas sincronizadas con dos dosis de PG administradas con diferentes intervalos e inseminadas artificialmente a tiempo fijo. Durante la estación reproductiva (abril a junio), 370 ovejas Corriedale multíparas fueron asignadas a cinco grupos de acuerdo al estado corporal y peso vivo, y sincronizadas con dos dosis de PG administradas a intervalos de 7, 10, 12, 14 o 16 días (grupos PG7, PG10, PG12, PG14, PG16 respectivamente). La inseminación (Día 0) fue realizada vía cervical a tiempo fijo a las 48 ± 1,0 h (PG7) o 56 ± 1,0 h (PG10, PG12, PG14 y PG16) luego de administrada la segunda PG, utilizando semen fresco pool de seis carneros Corriedale adultos. Se evaluó el porcentaje de ovejas que ovularon luego de la segunda PG y la TO al Día 10 mediante ecografía trans-rectal. El NRR-21 fue evaluado mediante el uso de retarjos pintados en el pecho. La fertilidad y la prolificidad fueron determinadas el Día 60 mediante ultrasonografía trans-abdominal. El NRR-21 y la fertilidad fueron superiores en los grupos PG12 (46,0, 46,0), PG14 (59,7, 56,9), y PG16 (58,7, 56,0) comparados con los grupos PG7 (30,1, 28,8) y PG10 (30,3, 30,3, NRR-21 y fertilidad respectivamente; $P<0,05$). El porcentaje de ovejas que ovularon, la TO y la prolificidad fueron similares entre los grupos ($P>0,05$). Bajo las condiciones de este experimento, el uso de protocolos de PG con intervalo entre las dosis de 12, 14 o 16 días incrementó la fertilidad respecto a 7 y 10 días, en ovejas inseminadas vía cervical a tiempo fijo con semen fresco.

Palabras clave: prostaglandina, sincronización de estros, inseminación artificial a tiempo fijo, fertilidad, tasa ovulatoria, oveja.

Theriogenology 90 (2017) 109–113

Contents lists available at ScienceDirect

Theriogenology

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Long term prostaglandin based-protocols improve the reproductive performance after timed artificial insemination in sheep

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ARTICLE INFO

Article history:
Received 25 April 2016
Received in revised form
28 September 2016
Accepted 30 November 2016
Available online 2 December 2016

Keywords:
Prostaglandin
Oestrus synchronization
Timed artificial insemination
Fertility
Ovulation rate
Ewe

ABSTRACT

The aim of the experiment was to evaluate the reproductive performance of ewes synchronized with two doses of prostaglandin $F_{2\alpha}$ (PG) at different intervals and inseminated at a fixed time. During the breeding season (April to June), 370 multiparous Corriedale ewes were assigned to five groups according to body condition score and body weight, and synchronized with two doses of PG administered 7, 10, 12, 14 or 16 days apart (groups PG7, PG10, PG12, PG14 or PG16; n = 73, 76, 74, 72, 75; respectively). Cervical timed artificial insemination (Day 0) was performed at 48 ± 1.0 h (group PG7) or 56 ± 1.0 h (groups PG10, PG12, PG14 and PG16) after the second PG injection, with diluted fresh semen pooled from six adult rams. The percentage of ovulating ewes after the second PG injection and the ovulation rate (number of corpus luteum/ovulating ewes) were assessed on Day 10 by trans-rectal ultrasonography. The rate of non return to service (ewes not returning to service/inseminated ewes × 100; NRR-21) was evaluated using painted vasectomized rams. Pregnancy rate (pregnant ewes/inseminated ewes × 100) and prolificacy (foetuses/pregnant ewes) were determined on Day 60 by trans-abdominal ultrasonography. Higher NRR-21 and pregnancy rates was observed in groups PG12 (46.0%, 46.0%), PG14 (59.7%, 56.9%) and PG16 (58.7%, 56.0%) compared to PG7 (30.1%, 28.8%) and PG10 (30.3%, 30.3%; respectively $P < 0.05$). No significant differences were observed in the percentage of ovulating ewes, ovulation rate and prolificacy among groups ($P > 0.05$). Under the condition of this trial, 12, 14 or 16 days interval between PG injections enhances the pregnancy rate of ewes at cervical timed artificial insemination with fresh semen.

1. Introduction

The use of prostaglandin $F_{2\alpha}$ and its synthetic analogues (PG) in sheep reproduction programs have been studied extensively since its discovery in 1970 as a powerful luteolytic agent [1] and, to our knowledge, PG has been used to synchronize ovulation associated to timed artificial insemination (TAI) since 1978 [2]. Due to its rapid rate of lung metabolization [3,4], PG represents a better alternative for the reproductive management of the flock than sponges impregnated with progestagens [5–7].

In spite of the high oestrus response achieved following a PG synchronization protocol, variable fertility rates have been reported under different experimental conditions in ewes artificially inseminated at a fixed time [8–15]. Alterations in the contractions of the myometrium [16], sperm transport [17–20], and variability in the timing of onset of oestrus and ovulation [9,21–23] have been reported as the sources of the reproductive failure when using of PG. After finding that a 3 days old ovine corpus luteum is sensitive to PG [24], a new protocol based on two PG injections administered 7 days apart was developed (Synchrovine® protocol; [25,26]). The second PG injection applied in the early luteal phase (during the growing phase of the dominant follicle of the first wave of the cycle), induced a consistently synchronized interval from the end of the treatment to ovulation [7,24], but the pregnancy rates were low [11–13]. These poor results were associated with an altered progesterone profile that determined lower ovulation rate, fertility and prolificacy compared to a spontaneous oestrus [12]. Alternatives to improve this protocol based on promoting a surge of LH to induce and synchronize ovulation (the use of male effect or GnRH injection), different AI pathways (cervical or intrauterine), decreasing

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http://dx.doi.org/10.1016/j.theriogenology.2016.11.031
0093-691X/

the PG dose or increasing intervals between PG injections (7–8 days apart) [7,14,27–29] were used, but they have been unsuccessful.

It has been suggested that the interval between PG injections should not be less than 11 to 14 days [2,30,31]. Furthermore, a decrease in fertilization rate was reported when the interval between PG injections was reduced from 14 to 8 days [32]. Administration of two PG injections 10, 12, 14 or 16 days apart lead to different durations of the luteal phase and progesterone concentrations prior to service, which were positively associated with oestradiol production by the pre-ovulatory follicles and with the pregnancy rate obtained following AI with fresh semen after oestrus detection [33]. On the other hand, the shorter PG intervals (10 and 12 days) were associated with improved oestrus response and synchrony [33], which is very important for TAI programs. Therefore, it is necessary to determine the reproductive performance achieved when these protocols are used in a TAI program.

The aim of this study was to determine the percentage of ovulating ewes, ovulation rate, rate of non return to service, pregnancy rate and prolificacy after TAI of ewes synchronized with two PG injections applied at different moments during the luteal phase under the same experimental conditions (breed, photoperiod, nutrition and health management). We hypothesized that despite the lesser oestrus response and synchrony, longer PG intervals would be associated to a greater reproductive performance after TAI. To our knowledge, this is the first study that compares the reproductive performance of different PG based protocols for TAI applying the PG injections at different moments during the luteal phase in randomly cycling ewes.

2. Materials and methods

The experiment was carried out at Escuela Agraria "La Carolina", Flores, Uruguay (33° S-57° W), during the breeding season (April to June). The experimental procedures were approved by the Universidad de la República's Animal Ethics Committee (CUEA-Universidad de la República, Facultad de Veterinaria, Exp: 111400-000079-12).

2.1. Animals and nutrition management

Multiparous (older than 2.5 year old, and at least one parturition; n = 370) Corriedale ewes with a moderate body condition score (mean ± SD, 3.2 ± 0.4; scale 0–5, [34]) and weighing 54.5 ± 6.3 kg were used. Ewes were maintained under field conditions grazing native pasture with more than 600 kg of DM available per hectare (8% CP and 8.5 MJ ME/kg DM) and water available *ad libitum*.

2.2. Experimental design

Ewes were stratified by body condition score and body weight, then assigned randomly to five groups synchronized with two injections of Delprostenate im (160 μg per injection, Glandinex®, Universal Lab, Montevideo, Uruguay), administered 7, 10, 12, 14 or 16 days apart (PG7, PG10, PG12, PG14 or PG16 respectively; n = 73, 76, 74, 72 and 75 ewes respectively). The average body condition score of each group was 3.2 ± 0.4, 3.2 ± 0.4, 3.2 ± 0.4, 3.2 ± 0.4 and 3.2 ± 0.3 (PG7, PG10, PG12, PG14 and PG16 respectively). Each group received the first injection of PG on a different day in order to perform the second PG injection and TAI of all groups on the same day (a schematic representation of the experimental design is shown in Fig. 1). In order to reach the adequate insemination time, using the same semen and made the insemination randomly between groups, the ewes of PG7 group received the second PG injection 8 h later to the remains groups.

2.3. Semen collection, evaluation and dilution

Semen from six adult Corriedale rams, checked for normal breeding soundness, was collected using an artificial vagina and assessed as described by Evans and Maxwell [35]. Two consecutive ejaculates from each ram were collected, evaluated and pooled according to the individual sperm concentration, so that each ram contributed with similar number of spermatozoa to the pool. Soon after pooling, the semen was extended to the final sperm concentration with UHT skim milk supplemented with 5% of egg yolk (v/v) and antibiotics (100.000 IU sodium penicillin and 100 mg of dihydrostreptomycin/100 mL of extender). Diluted semen with a minimum of 80% of progressive motility was used for TAI.

2.4. Artificial insemination

Cervical AI was performed using a speculum equipped with a light source and an insemination device (Walmur® Veterinary Instruments, Montevideo, Uruguay) as described by Evans and Maxwell [36], by two teams of technicians. Ewes were inseminated randomly among PG groups by one of the two teams of technicians that were not aware on the treatments. The insemination dose per ewe was 0.15 mL containing 160 × 10^6 spermatozoa, slowly released as deep as possible into the cervix. Extended semen was maintained at room temperature and protected from sunlight until AI. On the basis of previous reports describing ovulation and insemination time of PG protocols, ewes were inseminated at 48 ± 1.0 h after the second PG injection in group PG7 [11,13] and at 56 ± 1.0 h in groups PG10, PG12, PG14 and PG16 [10,33].

2.5. Ultrasound evaluation and non return rates

The percentage of ovulating ewes (ewes that ovulated after second PG injection/total ewes × 100), and ovulation rate (number of corpus luteum/ovulating ewes) were evaluated on Day 10 by trans-rectal ultrasonography with a 7.5 MHz linear array transducer (ALOKA SSD-500, Overseas Monitor Corp. Ltd., Tokyo, Japan) as described by Viñoles et al. [37]. In order to calculate the rate of non return to service up to Day 21 (ewes not returning to service/inseminated ewes × 100; NRR-21) oestrus was detected daily (from 18:00–08:00 h the following morning) from Day 13 to 21, with painted Corriedale vasectomized rams at a rate of 3 rams/100 ewes. In order to calculate pregnancy rate (pregnant ewes/inseminated ewes × 100) and prolificacy (foetuses/pregnant ewes) pregnancy was diagnosed and foetuses counted on Day 60 by ultrasonography with a 3.5 MHz convex array trans-abdominal transducer (ALOKA, Tokyo, Japan).

2.6. Statistical analyses

Data were analyzed by logistic regression using the Genmode procedure in SAS (SAS 9.2V; SAS Institute Inc., Cary, NC, USA), considering a binary response (0 and 1 or ≥1) and treatment as the explanatory variable. Ovulation rate and prolificacy are presented as means ± SD, ovulating ewes, NRR-21 and pregnancy are presented as percentage. Differences were considered significant if $P < 0.05$.

3. Results

The rates of non return to service up to Day 21 and pregnancy rate of groups PG12, PG14 and PG16 were higher than groups PG7 and PG10 ($P < 0.05$; Table 1). No differences were found in these

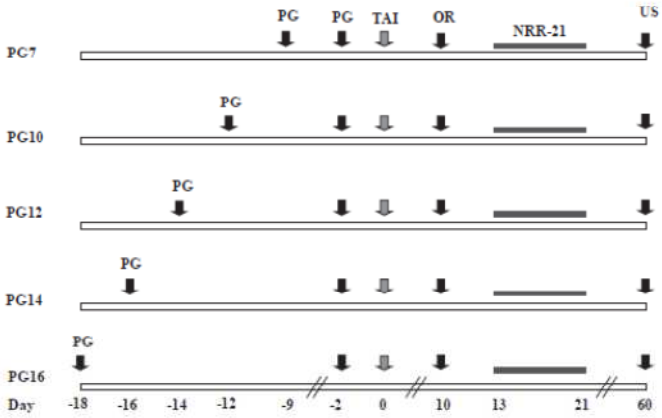

Fig. 1. Schematic representation of the experimental design. Groups PG7, PG10, PG12, PG14 and PG16: ewes synchronized with two injections of Delprostenate im (160 μg per injection) given 7, 10, 12, 14 or 16 days apart respectively, starting 18 days before the timed artificial insemination (Day 0). The second PG in PG7 group was administered 8 h later to the other groups. TAI: day of cervical artificial insemination at fixed time with fresh pooled semen. OR: ovulation rate measurement by trans-rectal ultrasonography on Day 10. NRR-21: non return rate assessed by painted vasectomized rams between Days 13 to 21. US: pregnancy and prolificacy evaluated by trans-abdominal ultrasonography on Day 60.

variables between groups PG12, PG14 and PG16, and between groups PG7 and PG10 ($P > 0.05$; Table 1). The percentage of ovulating ewes, ovulation rate and prolificacy were similar among groups ($P > 0.05$; Table 1).

4. Discussion

Longer intervals between PG injections improved the reproductive performance at TAI in this study. Ewes from groups PG12, PG14 and PG16 reached greater NRR-21 and pregnancy rate after TAI compared with ewes from the groups PG7 and PG10. Longer periods of higher progesterone levels prior to mating, higher oestradiol plasma levels around the onset of oestrus [33] and a higher fertilization rate [32], were reported when longer intervals between PG injections were applied. Previous reports showed that longer intervals between PG injections improved fertility after TAI, however, these studies were performed under different experimental conditions and studied different intervals between PG doses compared to ours [2,30,31,38]. Apart from the different methodological approaches, the positive relationship between longer PG intervals and reproductive performance was observed.

Despite the high oestrus response and synchrony of ovulation observed previously [11,12,24,33], shorter intervals between PG injections decrease the reproductive performance at TAI. Low progesterone concentrations during the luteal phase of the oestrus cycle prior to mating have been associated with altered steroidogenic function of the pre-ovulatory follicles [33,39], which affects oocyte development and transport, fertilization rate and early embryo development [12,40–44], decreasing fertility and fecundity [12,33,40]. The altered progesterone profile previously reported in groups PG7 and PG10 [12,33] may affects the oocyte and embryo development and therefore the final pregnancy rate. In this sense, progesterone supplementation during the follicular development of Wave 1 improves oocyte viability and embryo quality at embryo transfer programs in sheep [45]. In summary, the altered progesterone profiles observed when a short interval PG protocol is used in TAI programs, affects the overall outcome negatively.

It is important to note that ewes synchronized with a 12-day interval between PG had a pregnancy rate numerically lower (10.9 and 10%), but not significantly different compared to 14 and 16-day intervals between PG, which was unexpected. Under similar experimental conditions, but inseminating after oestrus detection, Fierro et al. [33] obtained similar pregnancy rates after 10 and 12-day intervals between PG, but both were lower compared to the PG14 group. Similarly, Fairnie et al. [2] reported a lower lambing rate with a 12-day interval (14%) compared to a 14-day PG interval (55%). It is not clear why the lower steroid production of ovarian structures in the PG12 group [33] had no effect on pregnancy rate in this study; perhaps due to the insufficient number of involved ewes, necessary to express statistical differences. A similar pregnancy rate (54.8%) was reported by Acritopoulou-Fourcroy et al. [10] using a 12-day interval and TAI at 56 h after the second PG. Taking into consideration the similar results achieved by PG12 compared to PG14 and PG16 groups, may indicate that a minimum of 4 to 7 days with high levels of progesterone [33] may be necessary for adequate follicle and embryo development, and this would be achieved with a minimum of 12 days between PG injections.

Table 1
Reproductive performance in multiparous Corriedale ewes synchronized with different PG-based protocols for cervical timed artificial insemination with fresh semen.

Group	OE	OR	NRR-21	Pregnancy	Prolificacy
PG7 (n = 73)	91.8[a]	1.39 ± 0.52[a]	30.1[a]	28.8[a]	1.24 ± 0.44[a]
PG10 (n = 76)	93.4[a]	1.48 ± 0.53[a]	30.3[a]	30.3[a]	1.26 ± 0.45[a]
PG12 (n = 74)	94.6[a]	1.40 ± 0.52[a]	46.0[b]	46.0[b]	1.35 ± 0.49[a]
PG14 (n = 72)	97.2[a]	1.59 ± 0.52[a]	59.7[b]	56.9[b]	1.39 ± 0.54[a]
PG16 (n = 75)	97.3[a]	1.51 ± 0.53[a]	58.7[b]	56.0[b]	1.31 ± 0.47[a]

Groups PG7, PG10, PG12, PG14 and PG16: ewes synchronized with two injections of Delprostenate im (160 μg per injection) given 7, 10, 12, 14 or 16 days apart respectively. OE: percentage of ovulating ewes (ewes that ovulated after second PG injection/total ewes × 100) measurement by trans-rectal ultrasonography on Day 10 (Day 0 = cervical timed artificial insemination). OR: ovulation rate (number of corpus luteum/ovulating ewes) evaluated by trans-rectal ultrasonography on Day 10. NRR-21: non return rate to service assessed by painted vasectomized rams (ewes no returning to service/inseminated ewes × 100). Pregnancy rate (pregnant ewes/inseminated ewes × 100) and prolificacy (foetuses/pregnant ewes) were evaluated on Day 60 by trans-abdominal ultrasonography. Data of OR and prolificacy are presented as means ± SD, OE, NNR-21 and pregnancy are presented as percentage. [a] vs. [b] in the same column: $P < 0.05$.

Previous reports indicated less synchrony of oestrus after 16 compared to 10, 12 and 14-day intervals between PG injections due to some ewes showed spontaneous oestrus behavior during the 48 h prior to the administration of the second PG in the PG16 group [33]. However, in this study PG16 reached similar NRR-21 and pregnancy rates as PG12 and PG14 groups, an unexpected result considering our previous work [33]. This might be due to the higher fertility of spontaneous oestrus occurring in some ewes in the PG16 group.

The percentage of ovulating ewes, ovulation rate and prolificacy were similar among groups. These results are in agreement with Fairnie and Wales [30], who reported that 94% to 97% of the ewes ovulated after a double PG protocol, and with Fierro et al. [33] who reported a similar ovulation rates among ewes synchronized with PG at 10, 12, 14 and 16-day intervals. The effect of PG on ovulation rate and prolificacy is controversial, with reports showing no change, a decrease, and an increase in ovulation rate [27].

In summary, the findings of this study indicate that the use of PG-based protocols for cervical TAI with fresh semen, is a feasible reproductive management alternative for the flock during the breading season. We suggest that the shortest interval between PG injections should be 12 days, which ensures the minimum length of the luteal phase prior to insemination necessary for acceptable reproductive results. Future research is needed to compare these PG intervals with the traditional progesterone-eCG protocols for TAI programs.

5. Conclusion

Under the condition of this trial, 12, 14 or 16 days interval between PG injections enhances the pregnancy rate of ewes at cervical TAI with fresh semen.

Conflict of interest statement

None.

Funding

All authors have no financial or personal relationship with organizations or people that could influence or bias the study.

Acknowledgements

Sergio Fierro was a PhD student scholarship from Agencia Nacional de Investigación e Innovación (ANII) during the development of this research (2013-2014; POS_NAC_2012_1_9449). The authors thank Jorge Gil, Carolina Bonino, Johana Braga, Ramiro Dutra da Silveira, Sofía Grela, Federico Soca, and Dolores Soler for the help with the field work, and Elize Van Lier for the English revision. We also are grateful to the directorate of Escuela Agraria UTU "La Carolina" and Facultad de Veterinaria in Paysandú, for providing the facilities and animals to perform this experiment, and to students and staff of these Institutions for valuable support. This work was funded by Instituto Nacional de Investigación Agropecuaria - INIA Uruguay, FPTA N° 315 project (2012).

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5. LONG INTERVAL PROSTAGLANDIN AS AN ALTERNATIVE TO PROGESTERONE-ECG BASED PROTOCOLS FOR TIMED AI IN SHEEP

RESUMEN

El objetivo de este trabajo fue comparar el resultado reproductivo a la IATF de ovejas sincronizadas con protocolos basados en dos dosis de PG administradas a intervalos de media (12 y 13 días) y larga duración (14, 15 y 16 días), o con un protocolo basado en P4-eCG. Para ello 440 ovejas Corriedale multíparas fueron sincronizadas con dos inyecciones de PG administradas a intervalos de 12 a 16 días (PG12, PG13, PG14, PG15, PG16) o con esponjas intra-vaginales impregnadas con medroxi-progesterona, colocadas durante 14 días con la adición de una dosis de eCG (380 UI) al momento del retiro de las mismas. Se realizó IATF (Día 0) vía cervical con semen fresco pool de nueve carneros. Se evaluó el porcentaje de ovejas que ovularon pos-tratamiento y la TO mediante ultrasonografía trans-rectal (Día 8), el NRR-21 mediante el uso de carneros pintados y la fertilidad, prolificidad y fecundidad al Día 60 mediante ultrasonografía trans-abdominal. No se observaron diferencias en el porcentaje de ovejas que ovularon luego de los tratamientos ($P>0,05$), pero el grupo P4-eCG presentó mayor TO respecto a los grupos sincronizados con PG ($P<0,05$), sin diferencias entre ellos ($P>0,05$). El NRR-21, la fertilidad y fecundidad fueron similares entre los grupos PG15 (64,3, 62,9 y 84,3), PG16 (59,7, 59,7 y 77,8) y P4-eCG (70,3, 66,2 y 95,9), pero superiores a los grupos PG12 (42,5, 39,7 y 52,1) y PG13 (44,0, 40,0 y 48, NRR-21, fertilidad y fecundidad respectivamente; $P<0,05$). El grupo PG14 obtuvo resultados intermedios comparado con todos los grupos. No se observaron diferencias en prolificidad entre los grupos ($P>0,05$), excepto el grupo PG13 que fue inferior comparado con P4-eCG ($P<0,05$). Se concluye que intervalos de larga duración entre las inyecciones de PG (15 o 16 días) determinaron mejores resultados reproductivos que intervalos de duración media (12 o 13 días), y resultados similares al protocolo P4-eCG luego de una IATF vía cervical con semen fresco en ovejas durante la estación reproductiva.

Palabras clave: prostaglandina, progestágenos, inseminación artificial a tiempo fijo, fertilidad, oveja.

Animal Reproduction Science 180 (2017) 78–84

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Long interval prostaglandin as an alternative to progesterone-eCG based protocols for timed AI in sheep

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ARTICLE INFO

Article history:
Received 14 January 2017
Received in revised form 24 February 2017
Accepted 11 March 2017
Available online 15 March 2017

Keywords:
Prostaglandin
Progestagens
Timed artificial insemination
Fertility
Ewe

ABSTRACT

To compare the reproductive performance after TAI in ewes synchronized with mid (12 or 13) or long (14–16 d) interval prostaglandin (PG) or progesterone plus eCG (P4-eCG) based protocols, 440 multiparous Corriedale ewes were synchronized with two PG injections administered 12–16 d apart (PG12, PG13, PG14, PG15 and PG16 respectively), or P4-eCG (MAP sponges 14 d and eCG). Cervical TAI (Day 0) was performed with fresh semen. It was evaluated the ovulated ewes (OE, %) and the ovulation rate (OR) on Day 8 by *trans*-rectal ultrasonography, the rate of non-return to service between Days 13 and 21 by painted rams, and the pregnancy rate, prolificacy and fecundity on Day 60 by *trans*-abdominal ultrasonography. No significant differences were found in OE among groups ($P > 0.05$), but P4-eCG achieved higher OR ($P < 0.05$) compared to PG protocols, without differences among them ($P > 0.05$). Similar NRR-21, pregnancy and fecundity were observed among PG15 (64.3, 62.9 and 84.3), PG16 (59.7, 59.7 and 77.8) and P4-eCG (70.3, 66.2 and 95.9), but higher compared to PG12 (42.5, 39.7 and 52.1) and PG13 group (44.0, 40.0 and 48.0, respectively; $P < 0.05$). PG14 achieved intermediate results compared to other groups. No differences were found in prolificacy among groups ($P > 0.05$), except PG13 that was lower compared to P4-eCG ($P < 0.05$). In conclusion, long interval between PG injections (15 or 16 d) determined better reproductive outcome that mid interval (12 or 13 d), equating the P4-eCG based protocol after cervical TAI with fresh semen during the breeding season in sheep.

1. Introduction

Timed artificial insemination (TAI) represents a practical tool in genetic programs, allowing synchronized inseminations and more efficient use of superior males, but requires hormonal treatments that ensure synchronized ovulation and acceptable pregnancy rates (Menchaca and Rubianes, 2004). Nowadays, the application of these biotechnologies under commercial farming conditions requires easy implementation procedures, acceptable pregnancy rates and low environmental impact (Martin and Ferasyi, 2016).

Conventional TAI protocols involve intravaginal devices impregnated with progestagens (P4) maintained during 12–14 d, in conjunction or not with equine chorionic gonadotrophin at device withdrawal (P4-eCG), yielding acceptable pregnancy rates within and outside the physiologic breeding season (Gordon, 1983; Abecia et al., 2012). However, P4-eCG based protocols have been associated to alterations in oocyte quality, which determine lower fertilization rates and impaired embryo development (González-Bulnes et al., 2005; Berlinguer et al., 2007).

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http://dx.doi.org/10.1016/j.anireprosci.2017.03.004
0378-4320/

Furthermore, the repeated use of eCG has been associated to a humoral immune response in ewes and development of follicular cysts (Roy et al., 1999), followed by low pregnancy rates. On the other hand, P4-eCG based protocols are potentially environmental and tissue contaminants due to residues of devices, eCG, or the addition of antibiotics to avoid vaginitis (González-Bulnes et al., 2005; Contreras-Solís et al., 2009; Viñoles et al., 2011). Nowadays, consumers worldwide are beginning to demand products that are "clean, green and ethical" (Martin and Kadokawa, 2006; Martin and Ferasyi, 2016 Martin and Ferasyi, 2016), and due to its rapid rate of metabolization (Piper et al., 1970; Davis et al., 1980), and easiness of application, prostaglandin represents another option to P4 impregnated sponges for the reproductive management in sheep (Contreras-Solís et al., 2009; Abecia et al., 2012; Fierro et al., 2013).

Prostaglandin F2α and its synthetic analogues (PG) are potent luteolytic agents in ruminants (McCracken et al., 1970), proposed for TAI previously (Fierro et al., 2013). Despite of some authors suggest that PG could be better than P4 based protocols due to the negative effects of P4 on the ovulatory follicle (González-Bulnes et al., 2005; Berlinguer et al., 2007; Letelier et al., 2011), reproductive outcomes when PG based protocols are used in TAI programs do not support this idea (Menchaca and Rubianes, 2004; Fierro et al., 2013). A short interval PG based protocol (two PG injections 7 d apart); that achieved highly synchronized oestrus and ovulation time was proposed for its use in TAI programs (Rubianes et al., 2003, 2004). However, low reproductive performance was obtained when this protocol was applied and lower results were achieved compared with P4-eCG based protocols (Menchaca et al., 2004; Olivera-Muzante et al., 2011a,b; Viñoles et al., 2011; Vilariño et al., 2017). This has been associated with an altered hormonal profile during the early luteal phase, that affect the follicular growth, determining lower ovulation rate (OR), fertility and prolificacy (Fierro et al., 2011). Different alternatives to improve this short-interval TAI protocol such as reduction in the dose of PG, an increase in the interval between PG injections (7–8 d), inseminating only the ewes that showed oestrus behaviour after PG treatment (Olivera-Muzante et al., 2011b), the inclusion of a GnRH dose at AI moment (Olivera-Muzante et al., 2013), applying a focus feeding between PG injection (Fierro et al., 2014), or the inclusion of eCG at the second PG (Vilariño et al., 2017) were used, however, they have not been successful, evidencing the needs for more research.

Based on the hypothesis that the extension of the interval between PG injections may increase the time that follicles destined to ovulate are exposed to adequate progesterone levels during its growth phase, improving the reproductive performance of these PG based protocols (Fierro et al., 2013), recent studies reported that the administration of two PG 10, 12, 14 or 16 d apart, determined different oestrus response and duration of the luteal phase, that affected the functionality of the follicles. The protocols based on two PG injections administered 14 or 16 d apart (long interval) presented lower oestrus synchronicity but better hormonal profile compared to 10 or 12 d (short or mid interval respectively; Fierro et al., 2016). Furthermore, the pregnancy rate after TAI was enhanced when 12, 14 or 16 d compared to 7 or 10 d interval PG protocol was used (Fierro et al., 2017), possibly by the longer extension of the luteal phase prior to mating, that was associated with a better reproductive outcomes (Folman et al., 1973; Fairnie et al., 1977; Loubser and van Niekerk, 1981).

The aim of this study was to compare the ovarian and reproductive response in randomly cycling ewes synchronized with mid (12 or 13) or long (14, 15 or 16 d) interval between PG and to a conventional P4-eCG based protocol, after cervical TAI during breeding season. We hypothesize that long PG interval determine a better reproductive performance than mid PG interval, and similar than the conventional P4-eCG based protocol. To the best of our knowledge this is the first report that proposes this comparison under the same experimental conditions.

2. Materials and methods

The experiment was carried out at Escuela Agraria "La Carolina", Flores, Uruguay (33° S – 57° W), during the breeding season (April to June). The experimental procedures were approved by the Animal Ethics Committee of the Facultad de Veterinaria – Universidad de la República (CUEA, Exp: 111400-000079-12).

2.1. Animals and nutrition management

Multiparous (older than 2.5 y old, and at least one parturition; n = 440) Corriedale ewes were used. Ewes had a moderate body condition score (3.1 ± 0.4, mean ± SD; scale 0–5, Russel et al., 1969), and weighed 54.2 ± 5.5 kg. Ewes were maintained under field conditions grazing natural pastures with 1956 kg DM available per hectare (6.7% CP and 2.3 Mcal ME/kg DM) and with water *ad libitum*.

2.2. Experimental design

Ewes were balanced with regard to body condition score and body weight, assigned randomly to six groups, and then synchronized with two injections of PG (Delprostenate im, 160 μg per injection, Glandinex®, Universal Lab, Montevideo, Uruguay) administered 12, 13, 14, 15 or 16 d apart (PG12, PG13, PG14, PG15 and PG16 respectively) or with Medroxi-progesterone acetate impregnated intravaginal sponges (MAP 60 mg; Syntex Lab., Uruguay) take in place for 14 d, with an im injection of eCG (380 IU; Novormon 5000®; Syntex Lab., Uruguay) at sponge withdrawal (P4-eCG). In order to reach the adequate AI time, using the same semen and making the insemination randomly between groups, each PG group received the first injection on a different correlative d and the second PG at the same d and time. The sponge withdrawal in ewes of P4-eCG group was done 6 h later from second PG injection of the PG based protocols (a schematic representation of the experimental design is shown in Fig. 1).

2.3. Semen collection, evaluation and dilution

Semen was collected from nine Corriedale adult rams (checked for normal breeding soundness) using an artifi-

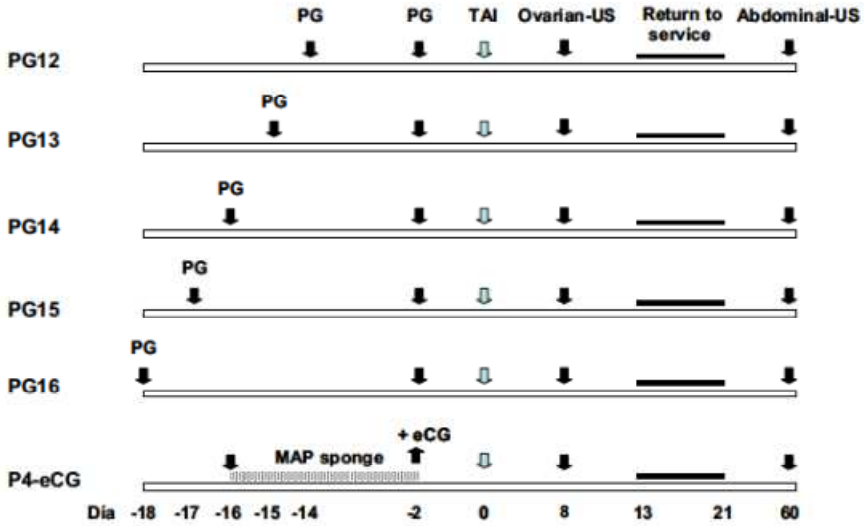

Fig. 1. Experimental design. PG12, PG13, PG14, PG15 and PG16: ewes synchronized with two doses of prostaglandin (PG: Delprostenate im 160 μg per dose) given 12, 13, 14, 15 or 16 d apart respectively. P4-eCG: ewes synchronized with Medroxi-progesterone acetate impregnated sponges (MAP 60 mg) inserted intravaginally for 14 d and eCG (380 IU im) after sponge withdrawal. TAI (Day 0): cervical artificial insemination at fixed time with fresh pooled semen. Ovarian-US: ovarian *trans*-rectal ultrasonography on Day 8 to evaluate ovulated ewes and ovulation rate. Return to service: assessed by painted rams from Day 13–21. Abdominal-US: *trans*-abdominal ultrasonography on Day 60 to evaluate pregnancy and prolificacy.

cial vagina and assessed as described by Evans and Maxwell (1987a). Two consecutive ejaculates from each ram were collected, evaluated for approval and pooled according to the individual sperm concentration, so that each ram contributed with similar number of spermatozoa to the pool. Soon after pooling, the semen pool was extended with UHT skim milk with antibiotics (Enrofloxacin 250 mg/L) to the final sperm concentration. Semen was assessed for progressive motility (80%) for approval for its use.

2.4. Artificial insemination

Cervical AI (Day 0) was performed using a speculum equipped with a light source and an insemination device (Walmur® Veterinary Instruments, Montevideo, Uruguay) by two teams of technicians, as described by Evans and Maxwell (1987b). Ewes were inseminated randomly between groups and technicians. The insemination dose was 0.15 cc and contained 170×10^6 spermatozoa, slowly released as deep as possible into the cervix. Extended semen was maintained at room temperature and protected from sunlight until AI. On the basis of previous reports describing ovulation and AI time, ewes were inseminated at 56 ± 1.5 h after second PG injection in all PG groups (Acritopoulou-Fourcroy et al., 1982; Fierro et al., 2016, 2017) and 50 ± 1.5 h after sponge withdrawal (Evans and Maxwell, 1987c).

2.5. Ovarian response, non-return and fertility rates

The percentage of ovulated ewes (ewes that ovulated after treatment/total ewes x 100; OE), and OR (number of corpus luteum/ovulated ewe) were evaluated on Day 8 by *trans*-rectal ultrasonography (7.5 MHz linear array, ALOKA SSD-500, Overseas Monitor Corp. Ltd., Tokyo, Japan), as described by Viñoles et al. (2010). The non-return rate to service at Day 21 (ewes no returning to service/total ewes × 100; NRR-21) was detected from Day 13–21 with Corriedale painted rams (3 rams/100 ewes). Pregnancy rate (pregnant ewes/total ewes*100), prolificacy (foetuses/pregnant ewes), and fecundity (foetuses/total ewes × 100) were evaluated on Day 60 by ultrasonography with a 3.5 MHz convex array *trans*-abdominal transducer (ALOKA, Tokyo, Japan).

2.6. Statistical analyses

Data were analysed by logistic regression using the Genmode procedure in SAS (SAS 9.2 V; SAS Institute Inc., Cary, NC, USA), considering a binary response (0 and 1 or ≥1) and protocol of synchronization as the explanatory variable. Data of OR and prolificacy are presented as means ± SD; OE, NRR-21, pregnancy and fecundity are presented as percentage. Differences were considered significant if $P < 0.05$.

3. Results

Similar percentage of OE were observed among groups (97.2, 100, 97.4, 98.6, 100 and 98.6%; PG12, PG13, PG14, PG15, PG16 and P4-eCG respectively; $P > 0.05$), however, P4-eCG achieved higher OR ($P < 0.05$) compared to PG based protocols, without differences among them ($P > 0.05$). The NNR-21, pregnancy and fecundity of PG15, PG16 and P4-eCG were higher than PG12 and PG13 groups ($P < 0.05$; Table 1). No differences were found in these variables among PG15, PG16 and P4-eCG or between PG12 and

Table 1
Reproductive performance in multiparous Corriedale ewes synchronized with two prostaglandin injections administered 12 or 13 d (mid interval) or 14–16 d apart (long interval protocols), or progestagens plus eCG based protocol for cervical timed AI with fresh pooled semen.

Group (n)	OR	NRR-21	Pregnancy	Prolificacy	Fecundity
PG12 (73)	1.54 ± 0.65^{a}	42.5^{a}	39.7^{a}	1.31 ± 0.54^{ab}	52.1^{a}
PG13 (75)	1.50 ± 0.60^{a}	44.0^{a}	40.0^{a}	1.20 ± 0.41^{a}	48.0^{a}
PG14 (76)	1.65 ± 0.63^{a}	50.0^{ab}	48.7^{ab}	1.40 ± 0.50^{ab}	68.4^{ab}
PG15 (70)	1.58 ± 0.65^{a}	64.3^{bc}	62.9^{bc}	1.34 ± 0.48^{ab}	84.3^{bc}
PG16 (72)	1.57 ± 0.50^{a}	59.7^{bc}	59.7^{bc}	1.30 ± 0.46^{ab}	77.8^{bc}
P4-eCG (74)	1.88 ± 0.67^{b}	70.3^{cd}	66.2^{cd}	1.45 ± 0.54^{bc}	95.9^{cd}

Groups PG12, PG13, PG14, PG15 and PG16: ewes synchronized with two doses of prostaglandin (PG; Delprostenate im 160 μg per dose) given 12, 13, 14, 15 or 16 d apart respectively, and timed AI at 56 ± 1.5 h after second PG dose. P4-eCG: ewes synchronized with Medroxi-progesterone acetate impregnated intravaginal sponges (MAP 60 mg for 14 d) plus eCG (380 IU im) at sponge withdrawal, and then inseminated at 50 ± 1.5 h. OR: ovulation rate (number of corpus luteum/ovulated ewe) measurement by *trans*-rectal ultrasonography on Day 8 (Day 0 = cervical timed AI). NRR-21: non-return rate to service between Days 13 and 21 assessed by painted rams (ewes no returning to service/total ewes × 100). Pregnancy rate (pregnant ewes/total ewes × 100), Prolificacy (foetuses/pregnant ewes), and Fecundity (foetuses/total ewes × 100) evaluated on Day 60 by *trans*-abdominal ultrasonography. Data of OR and Prolificacy are presented as means ± SD; OE, NRR-21, Pregnancy and Fecundity are presented as percentage. [a] vs. [b] in the same column: $P < 0.05$.

PG13 groups ($P > 0.05$). Group PG14 achieved intermediate results compared to the other groups ($P > 0.05$). No differences were found on prolificacy among groups, except PG13 who was lower compared to P4-eCG group ($P < 0.05$).

4. Discussion

The hypothesis that long PG intervals determine better reproductive performance in ewes after cervical TAI than mid interval, and similar as P4-eCG based protocol was partially accepted. Long PG interval determined higher NRR-21, pregnancy and fecundity compared to mid interval, and similar reproductive rates compared to P4-eCG. However, OR was higher in P4-eCG treated ewes, but no significant differences in OE or prolificacy were observed among groups. In our knowledge, this is the first report that validates the use of PG based protocols as a feasible alternative to P4-eCG for cervical TAI in sheep, with acceptable reproductive outcomes.

Long PG interval (15 and 16 d) determined a higher NRR-21, pregnancy and fecundity rates compared to mid interval protocols (12 and 13 d). The differences in fertility between intervals are already evident at Day 21, therefore were generate prior to or at the time of maternal recognition. These results supported the hypothesis suggested by Fierro et al. (2013) that increasing the interval between PG injections may be a possibility to enhance the time that follicles destined to ovulate are exposed to adequate progesterone levels during its growth phase, improving the reproductive performance of short and mid interval PG based protocol. Higher fertilization rates were reported when the interval between PG injections was increased from 8 to 14 d (Fairnie et al., 1977) and from 9 to 11 d (Greyling and van der Westhuysen, 1980a). Furthermore, an extra P4 source provided by an intravaginal impregnated device 8 d before the PG injection increased the number of ewes in oestrus and the pregnancy rate compared with untreated ewes (Loubser and van Niekerk, 1981). In this sense, Cuadro et al. (2016) improved oocyte viability and embryo quality when a 7 d PG protocol was supplemented with progesterone during the follicular development of the Wave 1, at embryo transfer programs in sheep. On the other hand, Hawk and Cooper (1977) found that the number of spermatozoa in the uterine horn was increased in ewes treated with a 16 d in comparison with a 10 d PG interval, also suggesting alterations in the sperm transport. The interval between PG injections has been defined as critical by Fairnie and Wales (1980) and other reports indicated that it should not be reduced to less than 11 d (Greyling and van der Westhuysen, 1980a), or 13 and 14 d (Fairnie et al., 1976, 1978), if satisfactory fertility at TAI want to be assured. Nevertheless, all these reports take into consideration the estrus cycle phase of the ewes when the first PG injection was given, making sure to injected ewes in the luteal phase, and not randomly cycling ewes as in our experiment, which seems somewhat more common to occur under field conditions. In previous reports of our group, the use of protocols with 14 or 16 d between PG injections (long interval), showed lower estrus synchronicity but better hormonal profile compared to short (10 d) or mid (12 d) PG interval (Fierro et al., 2016), and reached better NRR-21, pregnancy and fecundity at cervical TAI compared to short interval (7 or 10 d; Fierro et al., 2017). The higher levels and numbers of d with elevated progesterone observed in long PG interval prior to mating, but not after this moment (Fierro et al., 2016), may be responsible for the greater conception and fertility rates observed (Folman et al., 1973; Fairnie et al., 1977; Loubser and van Niekerk, 1981). The alteration of the steroidogenesis in ewes from short and mid PG interval (lower progesterone and estradiol levels compared to long interval PG; Fierro et al., 2016), affects oocyte development and transport, fertilization rate and early embryo development in sheep and cattle, reinforcing the importance of the hormonal milieu during the follicular phase to support early embryo development (Gustafsson and Plöen, 1986; Greve et al., 1995; González-Bulnes et al., 2005; García-Palencia et al., 2007; Sosa et al., 2008). Finally, it is possible that the second PG in long intervals (15–16 d apart) may be apply in some ewes that are having a natural luteolysis and oestrus, biasing the results. However, this should not be seen as something to avoid, because it might increase the pregnancy at TAI. In summary, the progesterone and estradiol profiles observed when a long interval (no lower than 15 d) is used in a TAI program improve the overall reproductive outcome of the PG based protocols.

Long PG interval (15 and 16 d) determined similar reproductive outcome at TAI compared to P4-eCG

based protocol, achieving these protocols acceptable results considering the insemination way used (Le Roux, 1976; Gordon, 1983; Abecia et al., 2012). Different studies reported information about the reproductive rates obtained following PG or P4 based protocols in sheep (Fierro et al., 2013). Most of the reports apply the first PG injection during the luteal phase, not using randomly cycling ewes as in our experiment. Boland et al. (1978a) using a 9 or 14 d PG interval with natural mating, reached a pregnancy rate numerically lower, but not significantly different compared to a P4 based protocol (with or without eCG in both protocols). Greyling and van der Westhuysen (1980b,c), inseminating after detected estrus, obtained lower or similar pregnancy and fecundity rate using a 9 or 10 d PG interval compared to the P4 based protocol (without eCG). With TAI, a 10 d PG interval had a similar fertility, prolificacy and fecundity than a P4 based protocol (without eCG; Greyling and van der Westhuysen, 1980c); but 7 (Olivera-Muzante et al., 2011a; Viñoles et al., 2011) or 11 d interval PG had lower reproductive outcome than a P4-eCG based protocol (Boland et al., 1978b). However, Acritopoulou-Fourcroy et al. (1982) using a 12 d PG interval and inseminating at fixed time 56 h after second PG injection, reach higher fertility and similar fecundity than P4-eCG based protocol. To sum up, when the stage of the estrous cycle at the moment of the first PG in unknown, the use of a long PG interval (as least 15 d between PG injections) is necessary to equate the reproductive outcome of P4-eCG based protocol after cervical TAI with fresh semen.

Finally, without differences in OE, OR was higher in P4-eCG based protocol, but no significant differences in prolificacy were observed between PG or P4-eCG based protocols. The OE was high in all groups, validating the protocols used to induce ovulation (Abecia et al., 2012; Fierro et al., 2017). The moment when the second PG injection was given in our experiment (mid or late luteal phase) does not appear to have an effect on the OR or in prolificacy. Some authors argue that follicular dominance during the mid luteal phase is lower than observed in the late luteal phase, probably explained by development of a pre-ovulatory follicle with a lower steroidogenic capacity, which maintained FSH concentrations above the threshold to stimulate the selection of multiple ovulatory follicles from a single follicular wave (Letelier et al., 2011). However, in a previous report with similar experimental conditions, no differences in OR and prolificacy were observed in ewes induced to ovulate during early, mid or late luteal phase (Fierro et al., 2017), confirming the present results. On the other hand, the inclusion of an average dose of eCG (380 UI) at the end of the P4 based protocol, with its FSH and LH activity (Gordon, 1999; Abecia et al., 2012), determines the growth and ovulation of follicles of more than one ovulatory wave. Consequently, a higher OR with the P4 protocol and this eCG dose is expected (Boland et al., 1979). In spite of this, similar prolificacy was obtained in P4-eCG and PG based protocols, with similar percentage of reproductive failure or embryo-foetus losses between protocols. The high molecular weight and long half-life in blood of eCG (Menchaca and Rubianes, 2004) could lead to an increased estradiol production of the ovulatory follicles (Barret et al., 2002), improve the development of follicular cysts (Viñoles et al., 2001), and achieved a higher percentage of unfertilized or degenerate embryonic structures in embryo transfer programs (Kelly et al., 1997; Olivera et al., 2001). In addition, the possibility of ovulation of aged follicles when long P4 protocols are used (Ungerfeld and Rubianes, 1999; Viñoles et al., 2001), could lead to the development of oocytes and embryos with lower quality, determining higher reproductive failures, which could explained the higher OR but the similar prolificacy observed in the present study.

5. Conclusion

Under this trial conditions, long interval between PG injections (15 or 16 d) determined better reproductive outcome that mid interval (12 or 13 d), equating the P4-eCG based protocol after cervical TAI with fresh semen during the breeding season in sheep.

Acknowledgements

The authors thank Eliana Bentancor, Diego Donadio, Valentina González, Josefina Ferrari, Mauro Minteguiaga, and Juan Manuel Vizcaíno for the help with the field work. We also are grateful to the directorate of Escuela Agraria UTU "La Carolina" and Facultad de Veterinaria in Paysandú, for providing the facilities and animals to perform this experiment, and to students and staff of these Institutions for valuable support. This work was funded by Instituto Nacional de Investigación Agropecuaria – INIA Uruguay, FPTA N° 315 project (2012).

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6. DISCUSIÓN GENERAL

La hipótesis general planteada de que intervalos de mayor duración entre las dosis de PG determinarían diferencias en la respuesta estral, desarrollo folicular ovárico, y niveles plasmáticos de progesterona y estradiol, conllevando a obtener mejores resultados reproductivos que intervalos de menor duración, y similares a un protocolo basado en P4-eCG, fue parcialmente aceptada. La administración de dos dosis de PG a intervalos cortos (10 días), medios (12 días) o largos (14 y 16 días) determinó diferencias entre ellos en la concentración de progesterona y estradiol, y en la respuesta estral hasta las 96 horas luego de administrada la segunda dosis de PG. El NRR-21, la fertilidad y la fecundidad a la IATF de protocolos de intervalos de larga duración entre dosis de PG (15 y 16 días) fueron mayores respecto a intervalos cortos (7 y 10 días) o medios (12 y 13 días), logrando resultados similares a un protocolo de P4-eCG. Sin embargo, los cambios morfológicos de los folículos pre-ovulatorios, los intervalos PG-estro y PG-ovulación, la TO y la prolificidad fueron similares en los protocolos de PG evaluados.

Intervalos más prolongados entre la administración de las dosis de PG determinaron un incremento en la fertilidad y fecundidad en servicios de IATF, respecto a protocolos de menos días de separación entre dosis (Fierro et al., 2017, Fierro y Olivera-Muzante, 2017). La fertilidad obtenida estuvo relacionada a los perfiles hormonales observados (Fierro et al., 2016). La mayor concentración de progesterona y la cantidad de días con progesterona alta previo al servicio en los protocolos de 14 y 16 días entre dosis de PG (Fierro et al., 2016), podrían ser los responsables de la mayor fertilidad observada con estos intervalos largos (Loubser y van Niekerk, 1981, Fairnie et al., 1977, Folman et al., 1973). A pesar de la alta respuesta estral y sincronía del momento de ovulación (Fierro et al., 2016, 2011, Menchaca et al., 2004, Rubianes et al., 2003), el uso de intervalos cortos (7 y 10 días) resultó en una fertilidad inferior a la IATF (Fierro et al., 2017). Bajas concentraciones de progesterona durante la fase luteal del ciclo estral previo al servicio han sido asociados a alteraciones en la función esteroidogénica de los folículos pre-ovulatorios (Fierro et al., 2016, Coleman y Dailey, 1983), lo cual podría afectar el desarrollo y transporte de los ovocitos, la tasa de fertilización y el

desarrollo embrionario temprano (Fierro et al., 2011, Gonzalez-Bulnes et al., 2005, Greve et al., 1995, Ashworth et al., 1989, Gustafsson y Plöen, 1986, Folman et al., 1973), disminuyendo la fertilidad y la fecundidad (Fierro et al., 2017, 2016, 2011, Folman et al., 1973).

A pesar de no observarse diámetros foliculares finales significativamente diferentes entre los folículos pre-ovulatorios de los intervalos de PG comparados, se registraron diferentes concentraciones de estradiol secretadas por ellos. Los folículos pre-ovulatorios de las ovejas sincronizadas con protocolos de mayor extensión entre las dosis, produjeron mayor cantidad de estradiol que los folículos de los protocolos de intervalo medio (Fierro et al., 2016). El crecimiento final del folículo está relacionado con la frecuencia de pulsos de LH, la cual es negativamente regulada por la progesterona (Ginther et al., 1995). A pesar de que la frecuencia de pulsos de LH no fue evaluada, se observaron diferencias en las concentraciones de progesterona y en el número de días con niveles superiores a 3,18 nmol/L (a favor de los protocolos largos), por tanto se esperaría en ellos menor frecuencia de pulsos de LH y pon ende diferente crecimiento folicular. La secreción de estradiol por los folículos está asociada a la frecuencia de pulsos de LH (Viñoles et al., 1999, Stock y Fortune, 1993, Sirois y Fortune, 1990), reforzando esto la existencia de diferencias fisiológicas en los folículos dominantes entre los grupos comparados, sin poder determinarse diferencias morfológicas mediante ultrasonografía. Los diámetros similares de los folículos pre-ovulatorios observados entre protocolos, y la menor producción de estradiol de los folículos de ovejas de los protocolos de 10 y 12 días de intervalo, podrían indicar una capacidad esteroidogénica alterada (White et al., 1987). Esto ha sido descrito como consecuencia de inadecuados niveles previos de progesterona (Deaver et al., 1986, Coleman y Dailey, 1983), y de una menor cantidad de receptores a LH en las células de la granulosa (McNatty et al., 1984).

La alteración de la esteroidogénesis observada en los grupos de 10 y 12 días de intervalo entre las PG (menores concentraciones de progesterona y estradiol comparado con grupos de mayores intervalos), ha sido previamente relacionada a alteraciones en el desarrollo y transporte del ovocito, la tasa de fertilización y el desarrollo embrionario temprano en vacas (Greve et al., 1995, Gustafsson y Plöen,

1986). El estradiol induce el desarrollo de las células secretoras del oviducto, lo cual afectaría la fertilización y el desarrollo embrionario temprano (Nancarrow y Hill, 1995, Murray, 1992), y menores concentraciones de estradiol han sido asociadas a una disminución en la tasa de fertilización en ovejas con estros sincronizados (Gonzalez-Bulnes et al., 2005). Además, las concentraciones de progesterona y estradiol regulan la expresión génica de sus receptores en el útero (Ing y Ott, 1999, Ing et al., 1996, Clark y Mani, 1994), y previamente ha sido sugerido que las fallas reproductivas en ovejas sincronizadas con progestágenos, podrían estar relacionadas a una disminución en la expresión génica de receptores de progesterona y estradiol (García-Palencia et al., 2007). Es posible que estas alteraciones observadas cuando los progestágenos son utilizados para la sincronización de estros, hayan contribuido a los resultados reproductivos obtenidos por las ovejas de los protocolos de corta y media duración entre las dosis de PG (Fierro et al., 2017, 2016). Sin embargo, para determinar las causas de esta alteración de la función esteroidogénica de los folículos, deberían realizarse estudios morfológicos y funcionales de los mismos, así como de calidad de los ovocitos y los embriones generados.

Reportes previos han demostrado una mejora de la fertilidad cuando se incrementa el intervalo entre las dosis de PG (Fairnie y Wales, 1980, Greyling y van der Westhuysen, 1980b, Fairnie et al., 1978, Greyling, 1978), sin embargo esos trabajos evaluaron diferentes intervalos entre las dosis, aplicando la primera dosis en ovejas en fase luteal definida, utilizaron diferentes métodos y momentos de servicio (monta natural, estro detectado y/o IATF), y diferentes formas de evaluar la fertilidad obtenida (tasa de fertilización, no retorno al estro, desarrollo de ubre), respecto a la metodología utilizada en la presente tesis. A pesar de las diferencias metodológicas mencionadas, la relación positiva entre un mayor intervalo en días entre las dosis de PG y la mejora de los resultados reproductivos, fue observada nuevamente. El intervalo entre las dosis de PG ha sido reportado como crítico por Fairnie y Wales (1980), y otros autores han indicado que no debería ser menor a 11 días (Greyling y van der Westhuysen, 1980b) o a 13 - 14 días (Fairnie et al., 1978, 1976), para alcanzar adecuados resultados reproductivos. En resumen, el uso de protocolos de mayor duración entre las dosis de PG determina una mejora de los resultados

reproductivos luego de la detección de estros (Fierro et al., 2016), o a la IATF (Fierro et al., 2017, Fierro y Olivera-Muzante, 2017), relacionado esto a un ambiente hormonal más favorable durante el desarrollo folicular. Cuando no se conoce el momento del ciclo estral de las ovejas al aplicar la primera dosis de PG, el menor intervalo entre las dosis para utilizar el estro inducido hormonalmente con éxito sería de 15 días, lo cual aseguraría una duración mínima necesaria de la fase luteal previa al servicio, como para generar niveles y perfiles de progesterona adecuados, y obtener resultados reproductivos aceptables.

El uso de protocolos de PG con intervalos largos entre las dosis determinó una menor concentración de los estros, observándose estros previos a la administración de la segunda PG (Fierro et al., 2016). Esto seguramente es debido a lo extenso del intervalo entre las dosis, lo cual podría estar determinando que algunas ovejas presenten luteólisis natural. A pesar de ello, cuando el servicio es realizado mediante IATF (sin detección de estros), los resultados reproductivos de los protocolos de 15 y 16 días fueron superiores a los intervalos medios y cortos, y similares al protocolo P4-eCG. Esto indicaría una mayor fertilidad en los mencionados protocolos, que podría estar determinado también por una mayor proporción de ovejas en estro "espontáneo", a pesar de estar inseminando menos ovejas en el momento óptimo de inseminación. En suma, el extenso intervalo entre dosis de PG (15 y 16 días) podría llevar a la ocurrencia de luteólisis natural en un porcentaje de ovejas con la consiguiente mejora de la fertilidad a la IATF.

El intervalo entre la administración de la PG y el inicio del estro ha sido asociado con la edad del CL (Houghton et al., 1995) y con el estatus folicular de cada oveja al momento de la administración de la PG (Viñoles y Rubianes, 1998). Sin embargo, los intervalos PG-estro y PG-ovulación fueron similares entre los protocolos comparados (Fierro et al., 2016), respaldados por una similar tasa de disminución de progesterona observada en todos los grupos luego de la administración de la segunda PG (Fierro et al., 2016). Una duración similar en el intervalo PG-ovulación luego de la administración de dos dosis de PG separadas 8 o 14 días también ha sido reportada previamente (Fairnie et al., 1977). Existen diversos reportes, bajo diferentes condiciones experimentales, que han evaluado el intervalo

PG-estro aplicando dos dosis de PG separadas 7, 9, 10 u 11 días (Fierro et al., 2013), no obstante, intervalos de mayor duración como por ejemplo de 14 o 16 días entre dosis, no han sido evaluados bajo las mismas condiciones experimentales como en el actual estudio, de allí el interés de incluirlos. Sin embargo, es posible que el diseño experimental planteado en el presente trabajo no fuera el más apropiado para detectar diferencias entre los grupos en estas variables, debido al intervalo de tiempo en que fueron realizadas las observaciones (cada 12 horas). A pesar de ello, los resultados obtenidos en esta tesis permitieron definir un momento adecuado de inseminación con semen fresco (56 horas pos segunda PG; Fierro et al., 2016) para los grupos evaluados a la IATF (Fierro et al., 2017, Fierro y Olivera-Muzante, 2017). Estas posibles diferencias en los intervalos PG-estro y PG-ovulación parecerían no ser inconveniente si es utilizado semen fresco en la IA, sin embargo podrían ser factor de disminución de fertilidad en caso de uso de semen preservado debido a su menor vida media en el tracto reproductivo (Salamon y Maxwell, 2000), lo cual conllevaría a la necesidad de realizar más estudios para determinar horas de inseminación óptimas para semen preservado.

Los intervalos medios y largos utilizados en la presente tesis determinaron que al momento de la segunda PG, la edad del CL de las ovejas que respondieron a la primera dosis varíe entre 7 y 14 días (fase luteal media o tardía), siendo sensibles a la acción luteolítica de la PG (Contreras-Solis et al., 2009, Rubianes et al., 2003, Houghton et al., 1995). Esto conllevó a que todos los protocolos en base a PG evaluados presentaran un similar y elevado porcentaje de ovejas que ovularon pos tratamiento (Fierro et al., 2017, 2016). Aun existiendo diferencias en la funcionalidad folicular durante la fase folicular previa, el o los CL generados tuvieron una vida media normal luego de la administración de la segunda PG en todos los intervalos comparados (Fierro et al., 2016, 2011). Estos resultados, sumados a la mejor fertilidad obtenida por los intervalos largos, refuerzan la importancia del ambiente hormonal durante la fase folicular previa a la ovulación sobre la calidad del ovocito y sobre el ambiente uterino, así como para el desarrollo embrionario temprano (García-Palencia et al., 2007, Gustafsson y Plöen, 1986). La deficiencia esteroidogénica pre-servicio de protocolos cortos y medios no pudo ser revertida por la presencia de

niveles fisiológicos de progesterona durante la fase luteal media posterior a la ovulación.

En cuanto a la TO y la prolificidad, ambas son variables con resultados contradictorios en ovinos cuando se utilizan protocolos en base a PG (Fierro et al., 2013). En los presentes trabajos no se detectaron diferencias en TO entre los protocolos comparados, resultados diferentes a los esperados, ya que ha sido reportado un incremento en la TO cuando la PG es administrada en la fase luteal media (Letelier et al., 2011, Bartlewski et al., 1999, Gibbons et al., 1999). El número de ovejas utilizadas para evaluar esta variable, podría ser una limitante para emitir conclusiones contundentes de la misma.

Respecto a la comparación de los protocolos de PG con el de P4-eCG, los protocolos con 15 o 16 días de intervalo entre las dosis de PG obtuvieron, a excepción de la TO, resultados reproductivos similares al protocolo P4-eCG (Fierro y Olivera-Muzante, 2017). No fue posible encontrar en la literatura comparaciones de protocolos con 15 o 16 días de separación entre las dosis de PG y un protocolo en base a P4-eCG. En términos generales, y teniendo en cuenta las diferencias metodológicas utilizadas, los reportes previos existentes de comparación entre protocolos en base a PG y P4-eCG señalan mejores resultados con el uso de P4-eCG, independientemente del intervalo entre dosis de PG utilizado (Olivera-Muzante et al., 2011b, Viñoles et al., 2011, Boland et al., 1978a, 1978b). Ha sido sugerido un efecto nocivo de los tratamientos largos con progestágenos sobre los folículos pre-ovulatorios (Viñoles et al., 2001, Ungerfeld y Rubianes, 1999), con alteraciones en la calidad de los ovocitos y en el desarrollo embrionario (Berlinguer et al., 2007, Gonzalez-Bulnes et al., 2005). También se han descrito efectos negativos del uso de la eCG sobre la calidad de los folículos, ovocitos y tasa de fertilización (Olivera et al., 2001, Kelly et al., 1997), conllevando al desarrollo de ovocitos y embriones de inadecuada calidad y por ende a un deterioro de la fertilidad y prolificidad, lo cual podría explicar la TO superior pero la prolificidad similar del protocolo P4-eCG respecto a los protocolos de PG empleados. Los presentes trabajos indican que los protocolos largos de PG (intervalos de 15 y 16 días) permitirían al menos igualar los resultados de fertilidad, prolificidad y fecundidad de un protocolo de 14 días de P4

con adición de eCG, generando una alternativa más práctica en su implementación a campo, menos costosa y con menos residuos ambientales o tiempo de espera.

Los diseños experimentales utilizados en esta tesis, permitieron una mayor comprensión de los efectos causados por diferentes intervalos entre dosis de PG, en ovejas bajo similares condiciones experimentales. En concreto, todos los protocolos de PG determinaron una buena respuesta estral, generando los de menor intervalo entre las dosis una presentación de estros más sincrónica. Los protocolos de mayor intervalo entre las dosis de PG (15 y 16 días) generaron ambientes hormonales más favorables, conllevando a mejores resultados reproductivos, y equiparando los obtenidos con un protocolo en base a P4-eCG. Los resultados de esta tesis indican que el uso de protocolos en base a dos dosis de PG para IATF con semen fresco, es una alternativa válida de aplicación durante la estación reproductiva en nuestros sistemas de producción. Se sugiere que cuando no se conoce el momento del ciclo estral de las ovejas al aplicar la primera dosis de PG, el menor intervalo entre las dosis de PG para utilizar el estro inducido hormonalmente sea de 15 días, el cual aseguraría una duración mínima necesaria de la fase luteal previa a la inseminación, como para generar niveles y perfiles de progesterona adecuados y obtener aceptables resultados reproductivos.

7. CONCLUSIÓN

En nuestras condiciones de experimentación los protocolos de intervalos largos (15 y 16 días), obtendrían mejores resultados reproductivos en programas de IATF vía cervical con semen fresco, respecto a intervalos cortos o medios entre las dosis de PG, e igualarían reproductivamente a un protocolo en base a P4-eCG.

8. IMPLICANCIAS PRÁCTICAS

En nuestro conocimiento estos trabajos son los primeros reportes que logran validar el uso de protocolos en base a PG para IATF en ovinos, obteniendo resultados que equiparan a los tradicionales protocolos de P4-eCG. Esta información genera una nueva opción para el manejo reproductivo de la majada durante la estación reproductiva, siendo más económica y con menos implicancias prácticas. Genera además nuevas opciones para el uso del estro inducido por las PG (pre-sincronizaciones y sincronizaciones), y su asociación con servicios a campo o IA a estro detectado. Es importante remarcar que el éxito de estas herramientas de manejo reproductivo se basa en su aplicación en animales aptos para la reproducción, con manejos nutricionales (estado corporal, suplementación pre-servicio y pre-parto), sanitarios (control de parasitosis y afecciones podales) y aplicación de recomendaciones adecuadas (correcta elección de época de servicio, uso de la información aportada por la ecografía, abrigos a la parición), que potencien los beneficios de la concentración de estros y por ende de las pariciones.

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10. ANEXOS: EFFECTS OF PROSTAGLANDIN ADMINISTRATION ON FOLLICULAR DYNAMICS, CONCEPTION, PROLIFICACY AND FECUNDITY IN SHEEP

RESUMEN

Dos experimentos fueron realizados para determinar los efectos de la administración de PG sobre la dinámica folicular, concepción, prolificidad y fecundidad en ovejas. Durante la estación reproductiva, ovejas Corriedale multíparas fueron asignadas aleatoriamente a dos grupos: 1) Grupo PG (n= 15 y 135, Experimento I y II respectivamente): sincronizadas con dos inyecciones de DL-Cloprostenol (125 ug) administradas a un intervalo de 7 días e inseminadas a tiempo fijo (Día 0), 48 horas luego de la segunda PG; y 2) Grupo Control (n= 15 y 73, Experimento I y II respectivamente): ovejas en estro espontáneo inseminadas a estro detectado. La IA fue realizada vía laparoscópica con una dosis seminal conteniendo 100 x 10^6 espermatozoides. Se evaluó el crecimiento del folículo pre-ovulatorio, la TO, concepción y prolificidad al Día 30 y 60 mediante ultrasonografía. Las ovejas del grupo PG presentaron un folículo pre-ovulatorio de mayor tamaño (4,8 ± 0,5 mm, media ± SEM; P<0,05) con mayor tasa de crecimiento (1,2 ± 0,3 mm/día; P= 0,08) y menor TO (1,37 ± 0,1; P<0,05) comparadas con el grupo Control (3,9 ± 0,2 mm, 0,7 ± 0,2 mm/día y 1,61 ± 0,1 respectivamente). Las concentraciones plasmáticas de progesterona entre los Días -6 a 1 fue menor en el grupo PG (P<0,05), pero la concentración de estradiol fue similar entre grupos (P> 0,05). La concentración de progesterona fue similar entre grupos durante la fase luteal temprana y en los Días 12 y 17 (P>0,05). La tasa de recolección embrionaria (Día 7) tendió a ser menor en el grupo PG (39 *vs* 64%; P=0,08), pero la calidad embrionaria fue similar entre grupos. La concepción, prolificidad y fecundidad fueron menores en el grupo PG respecto al grupo Control (P<0,05). Las pérdidas reproductivas acumuladas fueron similares entre grupos, pero una mayor cantidad de gestaciones dobles se perdieron en el grupo PG (P<0,05). Se concluyó que en ovejas sincronizadas con dos dosis de PG separadas 7 días, los menores resultados reproductivos estuvieron asociados a bajas concentraciones de progesterona que determinó un mayor crecimiento del folículo pre-ovulatorio, y esto fue asociado a menor TO, concepción, prolificidad y fecundidad.

Palabras clave: folículo ovulatorio, inseminación artificial a tiempo fijo, pérdidas embrionarias, fertilidad, oveja.

Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 76 (2011) 630–639
Theriogenology
www.theriojournal.com

Effects of prostaglandin administration on ovarian follicular dynamics, conception, prolificacy, and fecundity in sheep

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Received 23 August 2010; received in revised form 17 March 2011; accepted 18 March 2011

Abstract

Two experiments were conducted to determine the effects of prostaglandin administration on ovarian follicular dynamics, conception, prolificacy, and fecundity in sheep. During the breeding season, multiparous Corriedale ewes were randomly allocated to two groups: 1) PG group (n = 15 and n = 135 in Experiments I and II, respectively): synchronized with two injections of DL-Cloprostenol (125 μg) given 7 d apart, and inseminated at a fixed time (Day 0), 48 h after the second injection; and 2) Control group (n = 15 and n = 73 in Experiments I and II): ewes in spontaneous estrus inseminated at detected estrus. Ewes received 100 × 10^6 sperm by intrauterine AI. Ultrasonography was used to evaluate growth of the ovulatory follicle, ovulation rate (OR), conception rate, and prolificacy on Days 30 and 60. Ewes from the group PG had a larger (4.8 ± 0.5 mm, mean ± SEM; $P < 0.05$) ovulatory follicle that grew faster (1.2 ± 0.3 mm/d, $P = 0.08$), and a lower OR (1.37 ± 0.1, $P < 0.05$), compared to ewes from the Control group (3.9 ± 0.2 mm, 0.7 ± 0.2 mm/d, and 1.61 ± 0.1 respectively). Plasma progesterone concentrations from Days −6 to 1 were lower in the PG group ($P < 0.05$), but plasma estradiol concentrations were similar between groups ($P > 0.05$). Progesterone concentrations were similar between groups during the early luteal phase and on Days 12 and 17 ($P > 0.05$). The embryo recovery rate (Day 7) tended to be lower in the PG group (39 vs 64%, $P = 0.08$), but embryo quality did not differ between groups. Conception, prolificacy and fecundity, were lower in the PG than in the Control group ($P < 0.05$). Cumulative reproductive losses were similar between groups, but more twins were lost in the PG group ($P < 0.05$). We concluded that in ewes synchronized with $PGF_{2\alpha}$ given twice, 7 d apart, lower reproductive performance was associated with an environment dominated by lower progesterone concentrations that stimulated the preovulatory follicle to grow faster and become larger; this was associated with lower rates of ovulation, conception, prolificacy, and fecundity.

Keywords: Ovulatory follicle; Timed artificial insemination; Embryo loss; Reproductive performance; Ewe

1. Introduction

Timed artificial insemination (TAI) is a practical tool in genetic programs, but requires hormonal treatments to ensure synchronized ovulation and acceptable pregnancy rates. Until the development of a synchronization protocol that included two injections of prostaglandin ($PGF_{2\alpha}$) 7 d apart (Synchrovine®), TAI of ewes with $PGF_{2\alpha}$ protocols was not viable [1,2]. Although this protocol induced good synchrony of estrus and ovulation, fertility was poor [3]. Despite substantial research to identify reproductive losses in ewes syn-

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0093-691X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2011.03.016

chronized with $PGF_{2\alpha}$, the cause and timing of loss remained unclear [4–8]. The poor fertility in $PGF_{2\alpha}$-synchronized ewes was associated with alterations in the steroidogenic capacity of the ovulatory follicle [9]. Steroids prepare the oviducts and uterus for fertilization and embryo transport, inducing appropriate muscular contractions to improve fertility [10,11].

Follicles induced to ovulate following $PGF_{2\alpha}$ treatment developed into a CL that secreted less progesterone (P4) [9], which may be insufficient to stimulate embryo development and interferon tau production, thereby compromising maternal recognition of pregnancy [12]. Conception and fertilization rates were often decreased in ewes synchronized with $PGF_{2\alpha}$, compared to the classical protocol of progestagens plus eCG [4,5,13,14]; however, in other studies, fertilization rates were not affected [15,16]. Data on the number of embryos recovered and their quality were contradictory. Mutiga and Baker [13] and Gonzalez-Bulnes et al [16] reported similar recovery rates, conversely Schiewe et al [17] reported reduced recovery rates in $PGF_{2\alpha}$-treated ewes. Hawk [15] found no differences in embryo quality (number of cells), whereas Gonzalez Bulnes et al [16] reported a tendency for better viability when ewes were synchronized with cloprostenol compared to progestagens. It is noteworthy that consumers worldwide are beginning to demand products that are "clean, green and ethical" [18]; due to its rapid metabolism, $PGF_{2\alpha}$ represented a better option than P4-impregnated sponges for reproductive management of sheep [19]. To promote its use, it is important to determine the causes of poor reproductive performance when $PGF_{2\alpha}$ is used in TAI programs.

In the present study, we tested the hypothesis that synchronization with a $PGF_{2\alpha}$ analogue affects the steroidogenic capacity of the ovulatory follicle, inducing the development of a CL with reduced capacity to produce P4. Decreased P4 during the early luteal phase would reduce embryo quality and conception rate. Therefore, the objective of this study was to evaluate the effects of prostaglandin administration on ovarian follicular dynamics, conception, prolificacy, and fecundity in sheep.

2. Materials and methods

Two experiments were carried out at Escuela Agraria La Carolina, Flores, Uruguay (33 S-57 W), during the breeding season (March to April, 2009). These experiments were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine.

2.1. Animals

Multiparous Corriedale ewes (> 2.5 y), in moderate body condition (3.1 ± 0.06 and 3.2 ± 0.02, Experiments I and II respectively; scale 1 to 5 [20]) and weighing 45.7 ± 0.9 and 47 ± 0.3 kg were used in Experiments I and II, respectively.

2.2. Management

In both experiments, ewes grazed natural pastures with > 1,000 kg dry matter available per hectare (8% CP and 8.5 MJ ME/Kg dry matter). In Experiment I, ewes were housed indoors at night with water available *ad libitum*, to allow for a fasting period before collecting the blood samples, thus avoiding the decrease in circulating progesterone concentrations induced by feed intake [21], which could have varied among animals, depending on their grazing patterns.

2.3. Experimental design

Two experiments were conducted. In Experiment I, with the objective to determine reproductive losses from the beginning of the Synchrovine® protocol to Day 7 (Day 0 = day of AI; Fig. 1), plasma P4 concentrations (from Days −6 to 6), plasma estradiol concentrations (E2; 48 h before to the beginning of estrus), ovulation rate (OR), and embryo quality (Day 7) were assessed. Experiment II evaluated reproductive losses between Day 0 and Day 60 of pregnancy (Fig. 1) by measuring: plasma progesterone concentrations until Day 17, OR, conception, prolificacy, and fecundity at Days 30 and 60. In both experiments, ewes were randomly assigned to a Control group (n = 15 in Experiment I and n = 73 in Experiment II) and a prostaglandin group (n = 15 in Experiment I and n = 135 in Experiment II). In the Control group, ewes were pre-synchronized with two injections of DL–Cloprostenol im (125 μg each; Sincron®, Laboratorio, Uruguay S.A, Montevideo, Uruguay), given 8 d apart, starting 27 d before AI (Fig. 1), with the objective to increase the number of ewes in spontaneous estrus at Day 0. In the prostaglandin group (PG), ewes were synchronized with two injections of DL–Cloprostenol im (125 μg each), given 7 d apart (Synchrovine® protocol), starting on Day −9 (Fig. 1).

Estrus was detected in all ewes every 12 h, using Corriedale androgenized wethers (given 100 mg cyclopentylpropionate, on three occasions, 7 d apart; Testosterona Ultra Fuerte®, Laboratorio Dispert S.A, Montevideo, Uruguay), with marker paint, at a rate of six wethers/100 ewes.

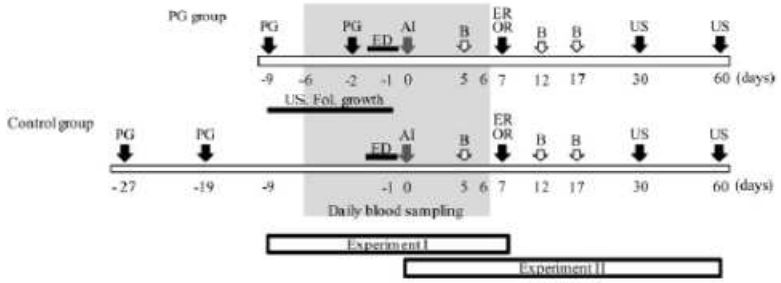

Fig. 1. Overview of the experimental design. Control group: ewes pre-synchronized with two injections of 125 μg DL–Cloprostenol (PG) im, given 8 d apart, 27 d before AI (Day 0). PG group: ewes were synchronized with two injections of DL–Cloprostenol im (125 μg), given 7 d apart (Days −9 and −2). US Fol. growth: follicle growth assessed by transrectal ultrasonography. ED, estrus detection; AI, day of intrauterine AI; ER, embryo recovery by uterine flushing; OR, ovulation rate measurement by ultrasonography on Day 7; US 30, US 60, conception, prolificacy and fecundity evaluated by ultrasonography on Days 30 and 60; Shadow, blood samples (B) taken daily from Days −6 to 6 (Experiment I) and on Days 0, 5, 12, and 17 (Experiment II).

2.4. Blood collection and hormone assays

Blood sample were collected (into glass tubes with heparin) by venipuncture of the jugular vein. In Experiment I, blood samples were collected once daily (morning) from Days −6 to 6 (Fig. 1). In Experiment II, blood samples were collected on Days 0 (25 ewes/group), 5, 12, and 17 (57 ewes/group, Fig. 1). Ewes selected for blood collection in the PG group in Experiment II, were those that demonstrated estrous behavior on the day of TAI.

Blood samples were kept at 5 °C until centrifuged at 2100 g for 15 min; plasma was separated and stored at −20 °C, pending assays. Plasma P4 and E2 concentrations were determined by radioimmunoassay (RIA), as described [22]. Progesterone concentrations were determined by a direct solid-phase RIA (Diagnostic Products Corporation, Los Angeles, CA, USA), with a sensitivity of 0.3 nmol/L. In Assay I (Experiment I), the intra-assay coefficients of variation for low (2.5 nmol/L), medium (6.4 nmol/L) and high (23.8 nmol/L) controls, were 13.5, 5.4, and 6.2%, respectively. In Assay II (Experiment II), the intra-assay coefficients of variation for low (2.5 nmol/L), medium (6.7 nmol/L) and high (24.2 nmol/L) controls, were 13.4, 4.7, and 6.2%. Concentrations of 17β estradiol were determined by a liquid phase RIA (DPC) in samples collected from 48 h before to the beginning of estrus, and were analyzed in duplicate in the same assay. The sensitivity of the assay was 3.6 pmol/L. The intra-assay coefficients of variation for low (6.0 pmol/L) and high (21 pmol/L) controls, were 22.3 and 3.7%, respectively.

2.5. Collection, evaluation, and dilution of semen

Three Corriedale adults rams (verified breeding sound) were used. Semen was collected using an artificial vagina, and evaluated as described [23]. Two consecutive ejaculates from each ram were collected, evaluated and pooled according to the individual sperm concentration, so each ram contributed similar numbers of sperm. The pool was extended with UHT skim milk and 5% egg yolk with antibiotics. The dilution rate was 1 to 5 (volume of semen to extender).

2.6. Artificial insemination

Intrauterine AI was performed by laparoscopy (Medit, Manitoba, Canada; and Karl Storz®, Hopkins, Tuttlingen, Germany) by two technicians, as described [23]. Ewes were inseminated randomly between groups and technicians. The insemination dose was 0.2 mL and contained 100×10^6 sperm. Semen was maintained at room temperature and protected from sunlight prior to AI. Ewes of the Control group were inseminated at detected estrus, whereas ewes in the PG group were inseminated at a fixed time, 48 h after the second $PGF_{2\alpha}$ injection (87% of ewes in the PG group were detected in estrus prior to AI).

2.7. Uterine status

During AI, uterine tone was recorded for each ewe as: 1) poor (low contractility, that made it difficult to stab the uterine horns with the aspic®); 2) normal (normal contractility, the uterine horns were stabbed with less difficulty); and 3) high (high contractility, the uterine horns were easily stabbed).

2.8. Embryo recovery and classification

In Experiment I, embryos were collected by flushing the uterus on Day 7. During the procedure, ewes were maintained under general anesthesia using a combination of acepromazine maleate (Prequillan®, Laboratorio Fatro, Uruguay, 10 mg/mL) and xylazine im (Seton®, Laboratorio Calier, Uruguay, 20 mg/mL), and acepromazine maleate - ketamine iv (Vetanarcol®, Laboratorio König, Uruguay, 50 mg/mL).

An endoscopic surgical technique was used to flush the uterus from the utero-tubal junction to the uterine horns [24]. The number and location of corpora lutea were evaluated by laparoscopy. Then, an Adbocat® was inserted near the utero-tubal junction, kept fixed by the operator's fingers to avoid backflow, and 20 to 25 mL of flushing medium were infused into each horn. A 9 FG Foley® catheter was introduced into the uterine lumen near the bifurcation of the horns and the medium collected directly into a Petri dish. Embryos were recovered and maintained in PBS-BSA 0.4% (Emcare® ICPbio NZ, Laboratorio Syntex) at 38 °C. Embryos were visualized with a stereomicroscope (Arcano 600® × 20–40), and maintained separately by ewe in NUNC® dishes (Bioniche®, Ontario, Canada) with DMPBS-BSA 0.4% (Laboratorio Nutricell) at room temperature, pending evaluation and classification. The stage of embryo development and its quality were determined as described [25,26]. The scale used to classify the embryos was: 1) excellent; 2) good; 3) poor; 4) degenerated, oocytes or 1-cell embryos (non-viable).

2.9. Ultrasonography

In Experiment I, ovulatory follicle growth was assessed (both groups) by daily transrectal ultrasonography from Days −9 to −1, using a 7.5 MHz linear array transducer designed for examination of the human prostate (ALOKA SSD-500, Overseas Monitor Corp. Ltd., Richmond, BC, Canada), using the methodology described by Viñoles et al [27]. The number, diameter and relative position of all ovarian follicles with a diameter of ≥ 2 mm and CL on both ovaries were recorded daily. The following follicle definitions were used in this study: the day of emergence of the ovulatory follicle was the day it was retrospectively identified at 2–3 mm in diameter; the diameter at emergence was defined as the diameter (mm) measured at the day of emergence; and the day of maximum diameter was the day after emergence that the ovulatory follicle reached its maximum diameter. The growth rate was calculated as the size difference from emergence to maximum size, divided by the numbers of days it took to reach the maximum size (mm/d). The lifespan of the ovulatory follicle was calculated as the number of days from emergence to the last measurement, the day before AI.

Ultrasonographic examinations were done with ewes restrained (standing position) in a metal cradle. Feces were removed from the rectum and a lubricant gel was introduced, to avoid damage to the mucosa, and improve contact between the transducer and the mucosa [27].

In Experiment II, ovulation rate (OR = CL/total cyclic ewes) was evaluated on Day 7 in a sub-sample of ewes from each group (n = 57) by transrectal ultrasonography, using a 7.5 MHz linear-array transducer. Ewes selected for evaluation of OR in the PG group, were those that demonstrated estrous behavior at AI. For evaluation of embryo losses, only Control ewes that started estrous behavior the same day than ewes in the PG group were used.

Conception (pregnant ewes/inseminated ewes*100), prolificacy (embryos/pregnant ewe) and fecundity (embryos/inseminated ewe) on Days 30 and 60 was evaluated by ultrasonography, using all ewes of Experiment II (n = 73 in the Control group and n = 135 in the PG group). On Day 30, ultrasonography was performed transrectally, using a 7.5 MHz linear-array transducer, whereas on Day 60, it was done by transabdominal ultrasonography using a 3.5 MHz, convex-array transducer.

2.10. Statistical analyses

Continuous data with repeated measurements, e.g., follicle growth and plasma concentrations of P4 and E2, were compared by ANOVA, using the mixed procedure in SAS [28]. The model included the fixed effects of group, day and their interactions. Ewe within group was considered as the random effect. The covariance was modeled to consider the correlation between successive measurements of the same animal, with the option autoregressive order 1 (AR(1)). Progesterone and E2 were analysed after log and log-1 transformation of the data, respectively.

Categorical data were analysed using the genmod procedure in SAS. Ovulation rate (1, 2, or 3), conception (0 or 1), prolificacy (1, 2, or 3) and fecundity (0 or 1) were analysed after log transformation of the data and assuming a binomial distribution. The fixed effects in the model were group (PG and Control) and Day (7, 30, and 60) and their interactions. The effect of inseminating technician was included as a random effect. To analyze embryo losses, ewes were allocated into 10 categories, based on their reproductive outcome: 1) ewes with OR = 1, prolificacy on Day 30 (P30) = 0 and prolificacy on Day (P60) = 0; 2) OR = 1, P30 =

1, P60 = 0; 3) OR = 1, P30 = 1, P60 = 1; 4) OR = 2, P30 = 0, P60 = 0; 5) OR = 2, P30 = 1, P60 = 0; 6) OR = 2, P30 = 1, P60 = 1; 7) OR = 2, P30 = 2, P60 = 0; 8) OR = 2, P30 = 2, P60 = 1; 9) OR = 2, P30 = 2, P60 = 2; 10) OR = 3, P30 = 3, P60 = 3. Reproductive looses were calculated as ewes that had no losses (Total retention -TR- = equal OR as embryos on Day 30 and 60), partial embryo losses (PL = higher OR than embryos at either Day 30 -PL30- and 60 -PL60-) or total embryo losses (TL = OR of 1 or 2 and absence of embryos on Day 30 -TL30- or 60 -TL60-). The model included the fixed effects of category, group and their interactions. All values are presented as mean ± SEM. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Hormonal concentrations and follicle growth

In Experiment I, plasma P4 concentrations were lower ($P < 0.05$) in PG than in the Control ewes from Day −6 to Day 1 but there were no significant differences during the early luteal phase (Days 2 to 6; Fig. 2).

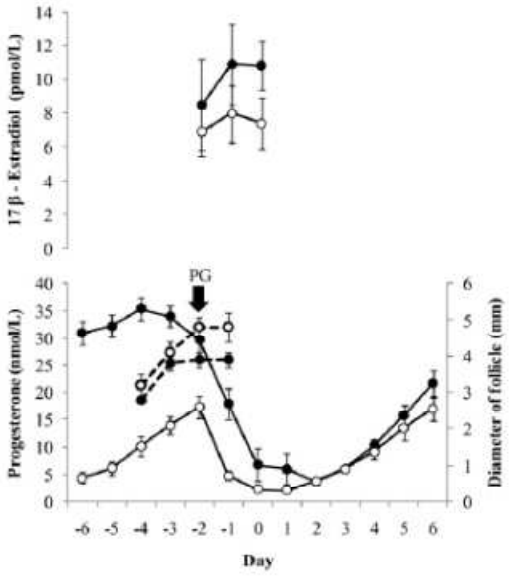

Fig. 2. Plasma progesterone concentrations (——) from Days −6 to 6, ovulatory follicle growth (– – –), and plasma estradiol concentrations relative to AI (Day 0), in ewes synchronized with DL-Cloprostenol (-○- -PG group- n = 15) or spontaneous estrus (-●- -Control group- n = 15). Untransformed data are presented as means ± SEM. Plasma progesterone concentrations from Days −6 to 1, and ovulatory follicle growth on Days −2 and −1: $P < 0.05$.

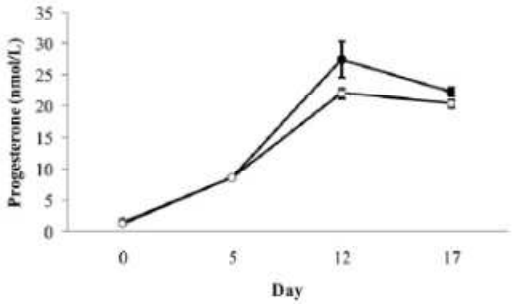

Fig. 3. Plasma progesterone concentrations in sheep synchronized with DL-Cloprostenol (-○- -PG group-) or in spontaneous estrus (-●- -Control group-). Day 0= day of artificial insemination (n = 25/group). Days 5, 12, and 17 (n = 57/group). Untransformed data are presented as means ± SEM.

In Experiment II, plasma P4 concentrations were similar ($P > 0.05$) between groups on Days 0, 5, 12, and 17 (Fig. 3).

Ewes from the PG group had a larger ovulatory follicle at the last ultrasonographic examination ($P < 0.05$), that reached a higher maximum diameter ($P < 0.05$), because it tended to grow at a faster rate ($P = 0.08$) compared to follicles in the Control group (Table 1). Differences in ovulatory follicle diameter in ewes from the PG group were not associated with a greater steroidogenic capacity ($P > 0.05$; Fig. 2).

3.2. Ovulation rate, recovery rate, fertilization rate and embryo quality

Ovulation rate was lower in ewes synchronized with $PGF_{2\alpha}$ (1.37 ± 0.1) compared to Control ewes (1.61 ± 0.1; $P < 0.05$) in Experiment II. However, it was similar between groups in Experiment I (1.53 ± 0.2 vs 1.79 ± 0.1, PG vs Control respectively). The rate of embryo recovery tended to be lower in the PG group (39%) compared to the Control group (64%; $P = 0.08$), but the fertilization rate of the structures recovered were similar between groups (93 vs 93%, $P > 0.05$). The majority (80%) of the structures collected were morulae and blastocysts (4.5 to 6.5 d old), whereas there were fewer underdeveloped or degenerated embryos (20%). Although embryo quality was poorer in the PG group compared to the Control group (Table 2), the difference was not significant ($P = 0.2$).

3.3. Conception, prolificacy, and fecundity

Conception, prolificacy, and fecundity on Days 30 and 60 were lower in ewes from the PG group compared to ewes from the Control group ($P < 0.05$, Table

Table 1
Characteristics of the ovulatory follicle in ewes synchronized with DL-Cloprostenol (two injections, 7 d apart; PG group, n = 15), and ewes in spontaneous estrus (Control group, n = 15).

Ovulatory follicle	PG	Control	P
Day of emergence	−3.0 ± 0.5	−3.0 ± 0.4	0.96
Diameter at emergence (mm)	2.8 ± 0.1	2.7 ± 0.1	0.26
Day of maximum diameter	1.8 ± 0.3	1.8 ± 0.3	0.98
Maximum diameter (mm)	5.2 ± 0.7[a]	4.0 ± 0.3[b]	0.053
Diameter in the last ultrasound examination (mm)	4.8 ± 0.5[a]	3.9 ± 0.2[b]	0.04
Growth rate (mm/d)	1.2 ± 0.3	0.7 ± 0.2	0.08
Lifespan (d)	3.0 ± 0.5	2.6 ± 0.4	0.42

The day of emergence was retrospectively analysed from the day of AI (Day 0). Data are presented as means ± SEM.

3), and differences remained unchanged until Day 60. The decrease in conception rate between Days 30 and 60 was similar between groups (1.6% vs 3.4%, PG vs Control group, respectively). Ewes from the PG group lost more twins at Day 30 (8/23 vs 4/41, PG and Control group respectively, $P < 0.05$) and there was a tendency for a higher total loss of embryos at Day 30 compared to the Control ewes (Table 4).

The cumulative embryo losses were similar between groups ($P > 0.05$; Table 4). The percentage of ewes that had high P4 concentrations on Day 17 but were not pregnant on Day 30 was similar between groups (2.2 vs 4.1%, PG vs Control respectively; $P > 0.05$).

3.4. Uterine tone

Uterine tone was lower in ewes from the PG group (2.1 ± 0.04) than in the ewes from the Control group (2.3 ± 0.04; $P < 0.05$).

4. Discussion

Our hypothesis that synchronization with a $PGF_{2\alpha}$ analogue would affect the steroidogenic capacity of the ovulatory follicle and further induce the development of a CL with reduced capacity to produce P4, thus reducing embryo quality, was not supported. However, estrus synchronization with two injections of $PGF_{2\alpha}$, 7 d apart (Synchrovine® protocol), induced a reduction in P4 concentrations during development of the ovulatory follicle, that affected its growth rate and size, which induced a reduction in OR. Moreover, $PGF_{2\alpha}$-synchronized ewes had lower rates of conception, prolificacy, and fecundity compared to ewes inseminated at spontaneous estrus.

In this study, ewes synchronized with $PGF_{2\alpha}$ had a lower OR compared to ewes in spontaneous estrus, a result that was not expected. Our finding was opposite to previous reports [8] suggesting that OR was not affected by a single $PGF_{2\alpha}$ regime (two injections, 3 h apart). It has been reported that the endogenous FSH secretion could be altered after $PGF_{2\alpha}$-induced luteolysis [29,30], which may reduce the number of follicles recruited into the ovulatory wave. Barrett et al [29] reported that 30% of the follicles induced to ovulate after a $PGF_{2\alpha}$ protocol would not form a new CL. Rubianes et al [1] confirmed the ovulation by the disappearance of the ovulatory follicles; however they did not evaluate OR as the number of CL that subsequently developed. In the present study, OR was determined by evaluation of the number of CL 7 d after AI, and ovulatory follicles were retrospectively tracked to the day of their emergence. Although we cannot exclude the possibility of an altered FSH pattern, that would have affected follicle recruitment, or an altered LH surge that may have altered ovulation and the CL formation, we can relate our findings to the growth profile of the ovulatory follicle.

Table 2
Quality of embryos collected from ewes synchronized with a double injection of DL-Cloprostenol (7 d apart, PG group, n = 15) and Control group (spontaneous estrus, n = 15).

	Embryo Quality Score				Total embryos
	1	2	3	4	
Control	7	3	4	2	16
PG	1	2	5	1	9

The scale used to classify embryos was: 1) excellent; 2) good; 3) poor; 4) degenerated or underdeveloped. Data are presented as means.

Table 3
Conception (pregnant ewes/inseminated ewes*100), prolificacy (number of embryos/number of pregnant ewes), and fecundity (number of embryos/total number of ewes), evaluated by ultrasonography (US) in ewes synchronized with DL-Cloprostenol (two injections, 7 d apart; PG group), and ewes in spontaneous estrus (Control group) on Days 30 and 60 after AI.

	US Day 30		US Day 60	
	Control	PG	Control	PG
Conception (%)	88[a]	63[b]	85[a]	62[b]
Prolificacy	1.58[a]	1.27[b]	1.50[a]	1.25[b]
Fecundity	1.4[a]	0.8[b]	1.3[a]	0.8[b]

Ewes in the PG group (n = 135) were inseminated at fixed timed, whereas Control ewes (n = 73) were inseminated at detected spontaneous estrus. Data on prolificacy and fecundity are presented as means.

[a,b] Within a row and day, means without a common superscript differed ($P < 0.05$).

Table 4
Reproductive losses in frequency and percentage (in brackets), of ewes synchronized with two injections of DL-Cloprostenol 7 d apart (PG, n = 57) and ewes in spontaneous estrus (Control, n = 57).

	PL 30	PL 30 - TL60	PL 60	TL30	TL60	TR	Total loss
Control	11 (19)	1 (2)	4 (7)	8 (14)[x]	0 (0)	33 (58)	24/57 (42)
PG	7 (12)	0 (0)	1 (2)	18 (32)[y]	1 (2)	30 (53)	27/57 (47)

[x] vs [y]; P = 0.08. PL (partial embryo losses), higher OR than embryos at either Days 30 and 60, or a decrease in the numbers of embryos from Days 30 to 60 (PL 30 - TL60); TL (total embryo losses), OR of 1, 2, or 3 and absence of embryos on Days 30 or 60; TR (Total retention), equal OR as embryos on Days 30 and 60.

The ovulatory follicle had a faster growth rate and larger size in $PGF_{2\alpha}$-synchronized than in Control ewes. These results contrasted previous reports describing similar [31] or smaller ovulatory follicle in ewes treated with $PGF_{2\alpha}$ [8]. A larger follicle produced more E2, with greater suppression of FSH, which reduced the probability of other follicles of the wave to ovulate [32–34]. The final stages of growth of a follicle depend on the LH pulse frequency that is down-regulated by P4 concentrations. Although we did not measure LH pulse frequency, we inferred that low P4 concentrations promoted development of a larger follicle in $PGF_{2\alpha}$-treated ewes.

Ewes synchronized with $PGF_{2\alpha}$ had lesser P4 concentrations during the growing phase of the ovulatory follicle, and the P4 profile was opposite between PG and Control ewes. It is well known that the first follicular wave emerges when P4 concentrations are rising [35–37]. In contrast, the last wave of the cycle emerges in the late luteal phase, when P4 concentrations are declining [36,37]. Increased progesterone concentrations suppressed LH pulse frequency, which is relevant to final growth and maturation of the follicle [38–40]. Perhaps reduced P4 concentrations induced by $PGF_{2\alpha}$ synchronization favored development of a single follicle, in contrast to the situation in the Control ewes.

Although PG ewes developed a larger follicle, this was not associated with a higher steroidogenic capacity. There results seemed contradictory with previous observations reporting follicles that developed under decreased P4 concentrations were larger, had a longer lifespan, and produced more E2 [41–45]. It also differed with previous reports that follicles induced to ovulate after $PGF_{2\alpha}$ treatment produced less E2 [9]. However, the present results were in agreement with earlier observations reporting that plasma estradiol concentrations following prostaglandin-induced luteolysis were similar to those occurring at the end of a physiologic cycle [46–48]. In the present study, ewes from the PG group had less uterine tone than Control ewes at AI. White et al [9] reported lower E2 concentrations produced by follicles after $PGF_{2\alpha}$ treatment; in our study, the similar plasma E2 concentrations between groups of ewes were not consistent with the decreased uterine tone observed. Perhaps decreased uterine tone was due to decreased uterine sensitivity to E2, associated with a reduction in the concentration of E2 receptors. Since blood sampling was not frequent enough to provide a comprehensive assessment of E2 production, the hypothesis that lower E2 induced the decrease in uterine contractility cannot be excluded. Therefore, in future experiments, serial plasma samples should be collected (e.g., every 10–15 min) to determine whether plasma E2 concentrations were altered when the Synchrovine® protocol was applied.

The present results confirmed that the follicle induced to ovulate after the second $PGF_{2\alpha}$ injection resulted in formation of a CL with a normal lifespan that had the capacity to synthesize normal P4 concentrations. This was in agreement with the results reported by Acritopoulou et al [38], but opposite to those published by others [9,30]. However, it has been suggested that a normal CL could coexist with an altered CL in the same or contralateral ovary [49–51]. If plasma P4 concentration was a good indicator of fertility, this may not be cause of reproductive failure in ewes synchronized with $PGF_{2\alpha}$.

Conception and prolificacy were lower in the ewes synchronized with $PGF_{2\alpha}$ compared to Control ewes (spontaneous estrus). Low P4 concentrations during the luteal phase of the estrus cycle prior to mating and around the time of ovulation are associated with decreased fertility [52,53], consistent with hormonal profiles in the present study. The lower prolificacy obtained in the ewes from the PG group on Day 30 and 60 were in agreement with other reports [54], and was explained by their lower OR and greater loss of twins. Since plasma P4 concentrations in the periovulatory period (Day 0 to 1) were positively associated with embryo survival [53], reduced concentrations in the PG ewes may have contributed to additional embryo losses. In cattle, follicles that develop under a low P4 environment released an oocyte capable of being fertilized, but

embryo development was altered resulting in lower pregnancy rates by Day 35 of pregnancy [55].

In this study, embryo quality assessed on Day 7 was not affected by $PGF_{2\alpha}$ treatment, consistent with Trounson et al [56], who compared ewes given $PGF_{2\alpha}$ plus eCG versus those only given eCG. However, the numbers of embryos collected were low, reducing the chances to detect significant differences. Moreover, perhaps capacity of the embryo to continue development after Day 7 may have been altered, in spite of appropriate P4 concentrations on Days 12 and 17, thus failing to induce maternal recognition of pregnancy [57].

The rate of embryo recovery tended to be lower in the PG group ($P = 0.08$) compared to the Control group. Schiewe et al [17] associated the decrease in the rate of embryo recovery to a shortened CL lifespan induced by $PGF_{2\alpha}$ treatment. However, this is not the case in our study, since the lifespan and P4 production of the CL were similar between groups. However, decreased uterine tone in $PGF_{2\alpha}$-treated ewes may have altered oocyte, sperm and/or embryo transport, thus decreasing the chances of reproductive success.

In conclusion ewes synchronized with two $PGF_{2\alpha}$ injections, 7 d apart, poorer reproductive performance was associated with an environment dominated by lower P4 concentrations that resulted in a preovulatory follicle with a faster growth rate, and a larger maximal diameter. Furthemore, there were lower rates of ovulation, conception, prolificacy, and fecundity.

Acknowledgments

The authors thank Laboratorio Uruguay for donation of $PGF_{2\alpha}$; Mariana Carriquiry for assistance with statistical analyses; Mario Martinicorena and Virginia López for laboratory and field work; and Gabriel Durán for intrauterine AI. We also are grateful to the directorate of Escuela Agraria La Carolina, UTU, for providing the facilities and animals to perform this experiment, and to students and staff of his institution for their valuable support. This work was funded by the Universidad de la República (CSIC 600/6015) and INIA.

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