Universitat Autònoma de Barcelona

PAPEL DEL PLASMA SEMINAL EN EL CONTROL DE LA INFLAMACIÓN ENDOMETRIAL POSTINSEMINACIÓN EN ÉQUIDOS

Henar Marín Estruch

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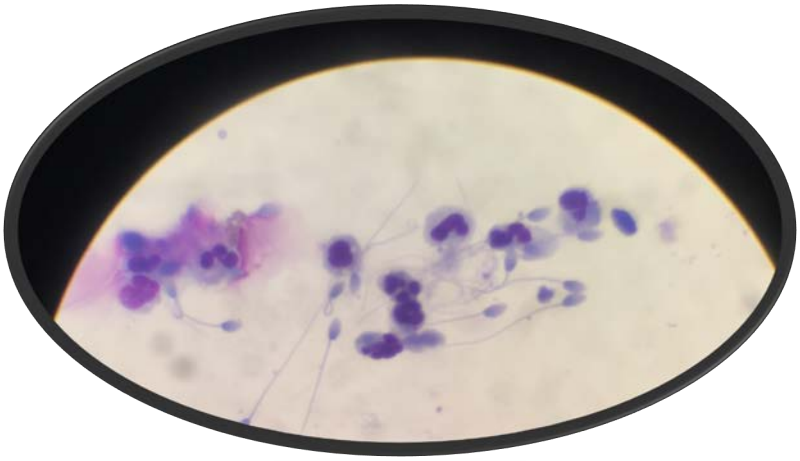

TESIS DOCTORAL

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PAPEL DEL PLASMA SEMINAL EN EL CONTROL DE LA INFLAMACIÓN ENDOMETRIAL POSTINSEMINACIÓN EN ÉQUIDOS

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Programa de doctorado en Medicina y Sanidad Animal

Departamento de Medicina y Cirugía de Animales

Facultad de Veterinaria

2020

Universitat Autònoma de Barcelona

Jordi Miró Roig, Professor Titular del Departament de Medicina i Cirurgia Animal de la Universitat Autònoma de Barcelona,

CERTIFICA

Que la Tesi titulada "Paper del plasma seminal en el control de la inflamació endometrial postinseminació en èquids" presentada per Henar Marín Estruch per optar al grau de Doctor en Medicina i Sanitat Animals per la Universitat Autònoma de Barcelona s'ha realitzat sota la seva direcció i, considerant-la acabada, autoritza la seva presentació perquè sigui jutjada per la comissió corresponent.

I per què així consti als efectes que correspongui, signo el present document a Bellaterra (Cerdanyola del Vallès), a 17 de juliol de 2020.

A ti Papá, espero que te sientas orgulloso allí donde estés. A ti Mamá por tu fuerza. A ti Santi, por tu apoyo. A mis bebés, porque ahora todo es por vosotros.

Platero es pequeño, peludo, suave; tan blando por fuera, que se diría todo de algodón, que no lleva huesos. Sólo los espejos de azabache de sus ojos son duros cual dos escarabajos de cristal negro. Lo dejo suelto y se va al prado y acaricia tibiamente con su hocico, rozándolas apenas, las florecillas rosas, celestes y gualdas... Lo llamo dulcemente: ¿Platero?, y viene a mí con un trotecillo alegre, que parece que se ríe, en no sé qué cascabeleo ideal...

Juan Ramón Jiménez

"Como no sabíamos que era imposible, lo hicimos"

Siempre en la pizarra de Iñaki Marín

Agradecimientos

A mi madre, Aniana, porque soy lo que soy y estoy donde estoy gracias a ella.

A mi marido, Santi, porque desde que estamos juntos siempre me ha apoyado para que hiciese aquello que yo considerara importante y que me hiciera feliz, aunque igual no fuese el momento más oportuno.

Al Dr. Jordi Miró, por enseñarme todo lo que sé dentro del mundo de la reproducción equina. Por darme la oportunidad y los medios para realizar esta investigación. Por confiar en mí y dejarme libertad para probar las distintas metodologías que se han acabado utilizando, o no, para el desarrollo de los experimentales. Por esta Tesis.

Al Dr. Marc Yeste, porque sin su ayuda no habría sido posible.

Al Dr. José Álvaro Cebrián (Pepín) por sus conocimientos y esos días diferentes en la Facultad de Veterinaria de Zaragoza.

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No quiero olvidarme de Álex Peña, Gaia Lafortezza, Anderson Ledezma, Efren Estrada, Marta Cittone, Sebastián Bonilla, Federico Noto y Marion Papas, personas que han estado presentes en algún momento durante el desarrollo de esta Tesis y con los que he pasado grandes momentos, por lo que también tiene un poco de ellos.

Y a ellos, estos magníficos animales muchas veces ensombrecidos por sus "primos" los caballos y sin los cuales no habría Tesis.

A todos, GRACIAS.

Rovelló y Lluquet (UAB, 2015)

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Introducción

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Listado de abreviaturas

- A.C.: antes de Cristo
- ACP: Àcid phosphatase
- ADN: ácido desoxiribonucleico
- AI: Artificial insemination
- ALH: Amplitude of lateral head displacement
- BCF: Frequency of head displacement
- CASA: Computer-Assisted Sperm Analysis
- CASMA: anàlisis computarizado de la morfometria espermàtica
- CAT: catalasa
- CL-CSA: CL cross-sectional area
- CL: cuerpo lúteo
- COX-2: ciclooxigenasa-2
- CRISP-3: proteínas secretoras ricas en cisteína 3
- CRISP: proteínas secretoras ricas en cisteínas
- DMSO: Dimethyl sulfoxide
- DNA: deoxyribonucleic acid
- DT: dinoprost thromethamine
- EDTA: Ethylenediaminetetraacetic acid
- ELA: Elastase
- ERK: regulada por señal extracelular
- FMLP: Formyl-methionyl-leucyl-phenylalanine
- Fn-2: fibrinectina tipo II
- FSH: hormona folículo estimulante
- GnRH: hormona liberadora de gonadotropina
- GOT: Glutamic oxalacétic transaminase
- GPT: Glutàmic pyruvic transaminase
- GPX: glutation peroxidasa
- GSR: glutation reductasa
- hCG: gonadotropina coriónica humana
- HSP: horse seminal proteins
- IP: índice de pulsatibilidad
- ISAS: Integrated Semen Analysis System
- kDa: kilo Dalton
- LDH: lactato deshidrogenasa
- LH: hormona luteinizante
- LIN: Linearity
- MADabs: Absolute angular mean displacement
- MADalg: Algebraic angular mean displacement
- mL: mililitros
- MPO: Myeloperoxidase
- NETs: Neutrophil Extracellular Traps
- PBS: Phosphate-buffered saline
- PCA: principal component analysis
- PFA: Paraformaldehyde
- $PGF_{2\alpha}$: prostaglandina $F_{2\alpha}$
- PMN: neutrófilos polimorfonucleares

- ROS: especies reactivas de oxigeno
- SD: Standard deviation
- SEM: Standard error of the mean
- SOD: supoeroxido dismutasa
- SP: seminal plasma
- Sp1:subpopulation 1
- Sp2:subpopulation 2
- Sp3:subpopulation 3
- Sp4:subpopulation 4
- Spz: espermatozoide
- spz/ml: espermatozoides por mililitro
- STR: Straightness
- UI: unidades Internacionales
- VAP: Average path velocity
- VCL: curvilinear velocity
- VSL: linear velocity
- WOB: wobble coefficient

Resumen

Los burros (*Equus asinus),* son una especie de origen salvaje, seguramente la primera en ser domesticada por el hombre. Durante siglos fue indispensable en las tareas del campo, como animal de tracción y para la producción de híbridos mulares. Sin embargo, la mecanización lo llevó casi a la desaparición en las zonas desarrolladas. Hoy en día, la importancia de esta especie ha vuelto a crecer gracias al interés de su uso hacia nuevos mercados, la producción de leche, de carne o de piel, el agroturismo, la asinoterapia, la silvicultura o el mantenimiento de razas en peligro de extinción. Como consecuencia el interés científico por el burro ha aumentado significativamente en los últimos años y, en especial, el interés por el conocimiento en la fisiología y la aplicación de tecnologías reproductivas.

La inducción de ovulaciones fértiles es fundamental para la aplicación de dichas tecnologías reproductivas y la mejora de su eficacia. El uso de las prostaglandinas como agente luteolítico es el método más usado en los équidos. En el primer trabajo de la presente Tesis se plantea utilizar la ecografía Doppler Color como instrumento para controlar la vascularización del cuerpo lúteo (CL) de la burra y así poder determinar el mejor momento para administrar $PGF_{2\alpha}$ e inducir la luteólisis. Se ha observado que la luteólisis se produce entre las 3 y 5 horas tras la aplicación de una prostaglandina y es el resultado de una isquemia del cuerpo lúteo con la consiguiente pérdida de funcionalidad de este y bajada de los niveles plasmáticos de progesterona.

La inseminación artificial (AI) de burras con semen congelado-descongelado de burro se asocia con bajas tasas de fertilidad. La adición de plasma seminal (SP), eliminando durante el proceso de congelación, o el aumento de la concentración espermática, han mostrado un cierto incremento de dichas tasas de preñez. Por otro lado, en la burra se observó una exagerada respuesta inflamatoria pocas horas después de la inseminación con posible relación con la baja fertilidad. En estudios previos se analizó esta respuesta mediante la recuperación de neutrófilos polimorfonucleares (PMN) de lavados uterinos

post-AI. No obstante, la repetición de estos lavados puede afectar negativamente al endometrio y provocar problemas de fertilidad. Por esta razón, en el segundo artículo se ha realizado un conjunto de experimentos para poder establecer un modelo *in vitro* mediante la recolección de PMN de la sangre periférica de las burras. Además, se ha probado como afecta la concentración de espermatozoides a su interacción con los PMN con cuatro concentraciones diferentes ($100x10^6$, $200x10^6$, $500x10^6$ y $1000x10^6$ espermatozoides/mL). Tras los distintos experimentales, se ha obtenido un modelo válido para poder recrear *in vitro* las condiciones fisiológicas que se dan *in vivo*. Se ha concluido que el SP tiene capacidad por sí solo para activar los PMN. Por otro lado, se ha observado una escasa fagocitosis de espermatozoides mientras la mayor parte de estos aparecen unidos a la superficie de los PMN o en un halo circundante. Así mismo, buena parte de estos se mantienen vivos, con un intenso movimiento de cola y, en las concentraciones superiores a $500x10^6$ algunos pueden liberarse con una excelente movilidad tras 3h de incubación a 37ºC. Solo estas concentraciones presentan espermatozoides libres mótiles tras 3h de incubación mostrando un claro efecto de la concentración espermática.

El papel de SP parece ser muy importante en la modulación de esta inflamación fisiológica de las burras. El SP es un fluido complejo con un elevado contenido de proteínas. El estudio de todas las proteínas y su posible papel seria un trabajo difícil y costosísimo. Para facilitar dicho estudio, el objetivo del tercer experimental fue fraccionar el plasma seminal de burro basándose en el peso molecular de las proteínas y analizar el efecto sobre la motilidad spz y la interacción PMN:spz. Del SP de cinco burros se obtuvieron 6 fracciones (<3 / 3–10 / 10–30 / 30–50 / 50–100 /> 10 kDa) que se pusieron en contacto con una solución de PMN:spz y se incubaron a 37ºC. A 1h, 2h, 3h, 4h se evaluó la motilidad espermática mediante el sistema CASA (ISAS®), la viabilidad de los espermatozoides mediante una tinción eosina-nigrosina y la interacción PMN:spz mediante una triple tinción Diff-Quik^R.

Las fracciones de SP que incluyen proteínas entre 30-50 kDa y 50-100kDa mostraron el mejor mantenimiento de las subpoblaciones móviles de spz de burro. Por otro lado, ambas fracciones están involucradas en el control de la respuesta inflamatoria post-AI de la burra, mostrando un porcentaje significativamente mayor de espermatozoides no unidos viables después de 3h de incubación que los otros tratamientos. Varias proteínas, descritas en SP de caballo o de burro e involucradas en el control de la endometritis post-AI, en el mantenimiento de la motilidad espermática y en el control del estrés oxidativo, tienen un peso molecular entre 30 y 100 kDa. Se necesitan más estudios para evaluar un posible efecto *in vivo* de estas fracciones y para definir las proteínas incluidas y su papel específico en la estrategia de reproducción de burros.

Palabras clave: reproducción burros, inseminación artificial, luteólisis inducida, endometritis fisiológica, plasma seminal, concentración espermática, fracciones proteicas.

Resum

Els rucs *(Equus asinus),* són una espècie d'origen salvatge, segurament la primera en ser domesticada per l'home. Durant segles va ser indispensable a les feines del camp, com a animal de tracció i per a la producció d'híbrids mulars. No obstant això, la mecanització el va portar quasi a la desaparició a les zones més desenvolupades. Avui dia, la importància d'aquesta espècie ha tornat a créixer gràcies a l'interès del seu ús cap a nous mercats, la producció de llet, de carn o de pell, l'agroturisme, la asinoteràpia, la silvicultura o el manteniment de races en perill d'extinció. Com a conseqüència, l'interès científic per l'ase ha augmentat significativament als últims anys i, en especial, l'interès pel coneixement de la fisiologia i l'aplicació de tecnologies reproductives.

La inducció d'ovulacions fèrtils és fonamental per a l'aplicació d'aquestes tecnologies reproductives i la millora de la seva eficàcia. L'ús de les prostaglandines com a agent luteolític és el mètode més utilitzat en èquids. Al primer treball de la present Tesi es planteja utilitzar l'ecografia Doppler Color com a instrument per controlar la vascularització del cos luti (CL) de la burra i així poder determinar el millor moment per administrar $PGF_{2\alpha}$ i induir la luteòlisi. S'ha observat que la luteòlisi es produeix entre les 3 i 5 hores després de l'aplicació d'una prostaglandina i és el resultat d'una isquèmia del cos luti amb la conseqüent pèrdua de funcionalitat d'aquest i baixada dels nivells plasmàtics de progesterona.

La inseminació artificial (AI) de someres amb semen congelat-descongelat de ruc s'associa amb baixes taxes de fertilitat. L'addició de plasma seminal (SP), eliminant durant el procés de congelació, o l'augment de la concentració espermàtica, han mostrat un cert increment d'aquestes taxes de gestació. D'altra banda, a la burra es va observar una exagerada resposta inflamatòria poques hores després de la inseminació amb possible relació amb la baixa fertilitat. En estudis previs es va analitzar aquesta resposta mitjançant la recuperació de neutròfils polimorfonuclears (PMN) de rentats uterins post-AI.

No obstant això, la repetició d'aquests rentats pot afectar negativament a l'endometri i provocar problemes de fertilitat. Per aquesta raó, en el segon article s'ha realitzat un conjunt d'experiments per poder establir un model *in vitro* mitjançant la recol·lecció de PMN de la sang perifèrica de les burres. A més, s'ha provat com afecta la concentració d'espermatozoides a la seva interacció amb els PMN amb quatre concentracions diferents ($100x10^6$, $200x10^6$, $500x10^6$ i $1000x10^6$ espermatozoides/ml). Després dels diferents experimentals, s'ha obtingut un model vàlid per poder recrear *in vitro* les condicions fisiològiques que es donen *in vivo*. S'ha conclòs que el SP té capacitat, per si sol, d'activar els PMN. D'altra banda, s'ha observat una escassa fagocitosi d'espermatozoides mentre la major part d'aquests apareixen units a la superfície dels PMN o en un halo circumdant. Així mateix, bona part d'aquests es mantenen vius, amb un intens moviment de cua i, en les concentracions superiors a $500x10^6$ alguns poden alliberar-se amb una excel·lent mobilitat després de 3h d'incubació a 37ºC. Només aquestes concentracions presenten espermatozoides lliures mòtils després de 3h d'incubació mostrant un clar efecte de la concentració espermàtica.

El paper de SP sembla ser molt important en la modulació d'aquesta inflamació fisiològica de les someres. El SP és un fluid complex amb un elevat contingut de proteïnes. L'estudi de totes les proteïnes i el seu possible paper seria un treball difícil i costosíssim. Per facilitar aquest estudi, l'objectiu del tercer experimental va ser fraccionar el plasma seminal de ruc basant-se en el pes molecular de les proteïnes i analitzar l'efecte sobre la motilitat spz i la interacció PMN:spz. Del SP de cinc rucs es van obtenir 6 fraccions (<3 / 3-10 / 10-30 / 30-50 / 50-100 /> 10 kDa) que es van posar en contacte amb una solució de PMN:spz i es van incubar a 37ºC. A 1h, 2h, 3h, 4 h es va avaluar la motilitat espermàtica mitjançant el sistema CASA (ISAS®), la viabilitat dels espermatozoides mitjançant una tinció eosina-nigrosina i la interacció PMN:spz mitjançant una triple tinció Diff- QuikR.

Les fraccions de SP que inclouen proteïnes entre 30-50 kDa i 50-100kDa van mostrar el millor manteniment de les subpoblacions mòbils de spz de ruc.

D'altra banda, les dues fraccions estan involucrades en el control de la resposta inflamatòria post-AI de la burra, mostrant un percentatge significativament major d'espermatozoides no units viables després de 3h d'incubació que els altres tractaments. Diverses proteïnes, descrites en SP de cavall o de ruc i involucrades en el control de la endometritis post-AI, en el manteniment de la motilitat espermàtica i en el control de l'estrès oxidatiu, tenen un pes molecular entre 30 i 100 kDa. Calen més estudis per avaluar un possible efecte *in vivo* d'aquestes fraccions i per definir les proteïnes incloses i el seu paper específic en l'estratègia de reproducció de rucs.

Paraules clau: reproducció rucs, inseminació artificial, luteòlisi induïda, endometritis fisiològica, plasma seminal, concentració espermàtica, fraccions proteiques.

Abstract

Donkey (*Equus asinus*), is a wild origin specie, surely the first to be domesticated by man. For centuries it was indispensable in the field tasks, as a traction animal and for the production of mule hybrids. However, mechanization almost led to its disappearance in developed areas. Today, the importance of this species has grown again thanks to the interest of its use towards new markets, milk, meat or skin production, agrotourism, asynotherapy, forestry or the maintenance of endangered breeds. As a consequence, the scientific interest in the donkey has increased significantly in recent years and, especially, the interest in knowledge in the physiology and application of reproductive technologies.

The induction of fertile ovulations is essential for the application of these reproductive technologies and the improvement of their effectiveness. The use of prostaglandins as a luteolytic agent is the most widely used method in equidae. In the first work of this Thesis, it is proposed to use Color Doppler ultrasound as an instrument to control the vascularization of the jennies' corpus luteum (CL) and thus be able to determine the best time to administer $PGF_{2\alpha}$ and induce luteolysis. Luteolysis has been observed to occur between 3 and 5 hours after the application of a prostaglandin and is the result of ischemia of the corpus luteum with the consequent loss of functionality of the corpus luteum and a decrease in plasma progesterone levels.

Artificial insemination (AI) of jennies with frozen-thawed donkey semen is associated with low fertility rates. The addition of seminal plasma (SP), eliminating during the freezing process, or the increase in sperm concentration, have shown a certain increase in these pregnancy rates. On the other hand, in the jenny, an exaggerated inflammatory response was observed a few hours after insemination, possibly related to low fertility. Previous studies have analyzed this response by recovering polymorphonuclear neutrophils (PMN) from post-AI uterine lavages. However, repetition of these washes can adversely affect the endometrium and lead to fertility problems.

For this reason, in the second article, a set of experiments has been carried out to establish an *in vitro* model by collecting PMN from the peripheral blood of jennies. Furthermore, it has been proven how sperm concentration affects its interaction with PMNs with four different concentrations ($100x10^6$, $200x10^6$, $500x10^6$ and $1000x10^6$ sperm/mL). After the different experiments, a valid model has been obtained to be able to recreate *in vitro* the physiological conditions that occur *in vivo*. It has been concluded that the PS *per se* has the capacity to activate the PMNs. On the other hand, scarce sperm phagocytosis has been observed while most of these appear attached to the surface of the PMN or in a surrounding halo. Likewise, a good part of these are kept alive, with an intense tail movement and, in concentrations above $500x10^6$, some can be released with excellent mobility after 3h incubation at 37ºC. Only these concentrations showed motile free sperm after 3h of incubation showing a clear effect of the sperm concentration.

The role of SP appears to be very important in modulating this physiological inflammation of jennies. SP is a complex fluid with a high protein content. The study of all proteins and their possible role would be difficult and very expensive work. To facilitate this study, the objective of the third experiment was to fractionate donkey seminal plasma based on the molecular weight of the proteins and to analyze the effect on spz motility and PMN:spz interaction. From the SP of five donkeys, 6 fractions were obtained (<3 / 3–10 / 10–30 / 30–50 / 50–100 /> 10 kDa) that were contacted with a PMN:spz solution and incubated at 3 7ºC. At 1h, 2h, 3h, 4h, sperm motility was assessed using a CASA system (ISAS®), sperm viability by eosin-nigrosin staining, and PMN:spz interaction by triple staining, Diff-Quik®.

SP fractions including proteins between 30-50 kDa and 50-100kDa showed the best maintenance of the mobile sub-populations of donkey spz. On the other hand, both fractions are involved in the control of the jennies' post-AI inflammatory response, showing a significantly higher percentage of viable unbound sperm after 3h of incubation than the other treatments. Several proteins, described in horse or donkey SP and involved in the control of post-

AI endometritis, in the maintenance of sperm motility and in the control of oxidative stress, have a molecular weight between 30 and 100 kDa. Further studies are needed to assess a possible *in vivo* effect of these fractions and to define the included proteins and their specific role in the donkey breeding strategy.

Key words: donkey reproduction, artificial insemination, induced luteolysis, physiological endometritis, seminal plasma, sperm concentration, protein fractions.

1. INTRODUCCIÓN

1. Interés actual por la especie

Los burros (*Equus asinus*), son una especie de origen salvaje, seguramente la primera en ser domesticada por el hombre. Existen evidencias zooarqueológicas de restos de esqueletos de asnos cerca de tres tumbas egipcias en Abydos que datan del 3000 A.C., hecho que aportó mucha información acerca los inicios del uso y la domesticación del burro (Rossel *et al.* 2008). En un inicio se utilizaron para la agricultura y transporte y posteriormente en la guerra. A medida que aumentó su uso, se planteó crear individuos más grandes y veloces por lo que se empezaron a cruzar con caballos (*Equus ferus caballus*), su especie más cercana, para crear híbridos mulares (*Equus mulus mulus*).

A raíz de la mecanización de la agricultura y la industralización, empezó a bajar su uso y a quedar en un segundo plano su crianza en los países más desarrollados, de forma que en Europa la mayor parte de razas se encuentra en peligro de extinción. En España, por ejemplo, existen 6 razas reconocidas, Catalán, Zamorano-Leonés, Anadaluz, Balear, Majorero y Asno de las Encartaciones. Todas ellas en peligro de extinción. Si bien es cierto que los asnos se siguen utilizando como transporte o soporte de la agricultura en África, Asia y Sudamérica (McLean *et al.,* 2018), con unas poblaciones asnales muy importantes.

En los últimos años se ha observado un creciente interés por el burro con la recuperación de antiguos usos y la aparición de nuevas posibilidades.

El uso de la leche de burra en cosmética es antiguo. Son bien conocidos los famosos baños en leche de burra de Cleopatra, moda que se fue extendiendo entre grandes personalidades a lo largo de la historia. Aunque prácticamente había desaparecido, en la actualidad ha experimentado un fuerte aumento con la producción de jabones, lociones corporales o cremas hidratantes.

El consumo de leche de burra representa una importante alternativa para niños con intolerancia a la leche materna (Vita *et al.,* 2007). Su bajo contenido en grasas, su composición proteica y sus niveles de lactosa la convierten en una leche hipoalergénica, pero a su vez con un gran parecido a la leche humana (Salimei *et al.,* 2004). Así mismo, estas propiedades la hacen un interesante producto nutracéutico para adultos con problemas inflamatorios y/o tumorales del digestivo (Maó *et al.,* 2009; Martini, 2018). No obstante, la leche de burra mantiene sus propiedades durante un corto periodo de tiempo. Siendo este un importante campo de investigación actual.

La producción de derivados de la leche de burra, fermentados, quesos u otros, con una buena digestibilidad, ha despertado también un importante interés. Debido a su composición, la leche de burra da grandes problemas en la producción de quesos. Si embargo, existe en el mercado un queso leche de burra, producido por Slodovan Simic en la Reserva Natural Especial de Zaravica en Servia, considerado el queso más caro del mundo (Vucovic, 2016).

En el mundo desarrollado el uso del burro y sus híbridos mulares en el turismo rural está creciendo (McLean *et al.* 2018; Toribio, 2019). Las características de comportamiento y movimiento del asno lo hacen especialmente apto para la terapia de niños con problemas psicológicos (autismo, síndrome de Down…), la llamada asinoterapia (Heinemann, 2009). Algunos estudios recientes demuestran que el asno puede tener un interesante papel en la gestión de bosques para la prevención de grandes incendios (silvicultura) (Bartolomé *et al.,* 2020), o la producción de mulares para ocio o deporte tiene un importante mercado en USA (McLean *et al.,* 2018) que, poco a poco, parece extenderse también a Europa.

Pero la gran demanda mundial de burros tiene relación con su piel, usada en China para la obtención de una gelatina, llamada Ejiao. Se trata de un producto de la medicina tradicional china, cuyo uso data de hace más de 2500 años, pero que últimamente está en auge. A este producto se le atribuyen propiedades como mejora de la anemia, ayuda a dormir o prevenir el cáncer (Jin *et al.,* 2017).

Las tecnologías reproductivas, control de ciclos sexuales de las burras, inducción de celos y/o ovulaciones, producción y conservación de semen, inseminación artificial, transferencia embrionaria, etcétera, son instrumentos básicos para salvar las razas en peligro de extinción o para las nuevas explotaciones asininas para la producción de leche, de piel o incluso de carne.

Por otro lado, el control de poblaciones salvajes de burros, mediante control reproductivo, en distintas partes del mundo (USA, Argentina, México, Argentina, Chile, Australia, isla de la Asinara en Italia…) es también un problema actual (McLean *et al.,* 2018)

Como resultado de todo lo expuesto anteriormente, existe un interés creciente por esta especie. En los últimos años ha aumentado significativamente el número de trabajos científicos publicados, especialmente en el mundo de la reproducción asinina (Camilo *et al.,* 2018).

2. Contexto reproductivo

Tradicionalmente se han usado en el burro los conocimientos y tecnologías utilizadas en el caballo. Pero, aunque caballo y burro son especies filogenéticamente cercanas existen grandes diferencias reproductivas entre ambas. Aunque, como se citaba anteriormente, el conocimiento reproductivo de los asnos ha mejorado significativamente en los últimos años, existen aun muchos aspectos desconocidos.

2.1. Particularidades anatómicas

2.1.1. La burra

El aparato genital de la burra es parecido al de la yegua, aunque existen diferencias importantes entre ellas con una incidencia importante en la estrategia reproductiva.

La vulva de la yegua presenta solo labios menores, mientras que la burra presenta labios menores y, parcialmente, más o menos desarrollados labios mayores (Canisso *et al.,* 2019). Proporcionalmente el clítoris de la burra es mayor que el de la yegua. En la yegua aproximadamente dos tercios de la apertura vulvar se encuentran por debajo del suelo de la pelvis, mientras que en la burra el 100% debe estar por debajo. Este hecho implica que la inclinación del vestíbulo vaginal de la burra es superior y que el esfínter vestíbulo-vaginal cierre de forma más significativa el acceso a las zonas más craneales del tracto reproductor. Las lesiones, consecuencia de un parto, que modifiquen la conformación del vestíbulo vaginal originan en la burra grandes problemas de fertilidad (Climent *et al.,* 2012). Por otro lado, el microbismo vaginal (por delante del esfínter vestíbulo-vaginal) en la burra sana es muy inferior al que se observa en la yegua, siendo escaso y prácticamente igual al intrauterino (Maschio *et al.,* 2017).

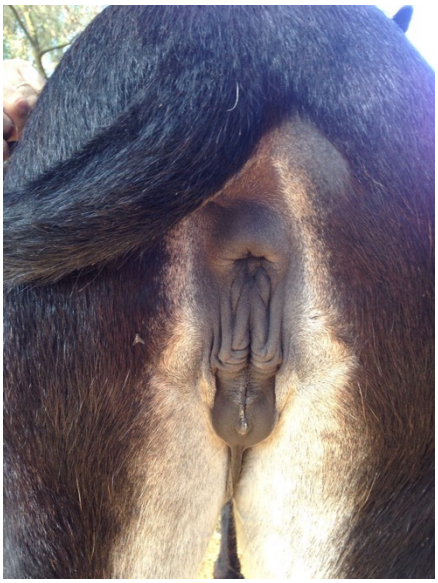

Fig 1. Vulva de burra con labios menores y parcialmente labios mayores (Miró, 2020).

El cuello uterino de la yegua es corto y sin accidentes anatómicos. Sin embargo, el cérvix de la burra es largo y tortuoso, proyectándose caudalmente en la vagina. Esta protrusión vaginal suele estar fijada dorsal y ventralmente por sendos pliegues de mucosa que limitan su desplazamiento (Renner Martín, 2009; Canisso *et al.,* 2019). La complejidad de este cérvix hace difícil su paso, especialmente en burras jóvenes y por personal inexperto (Miró, 2020).

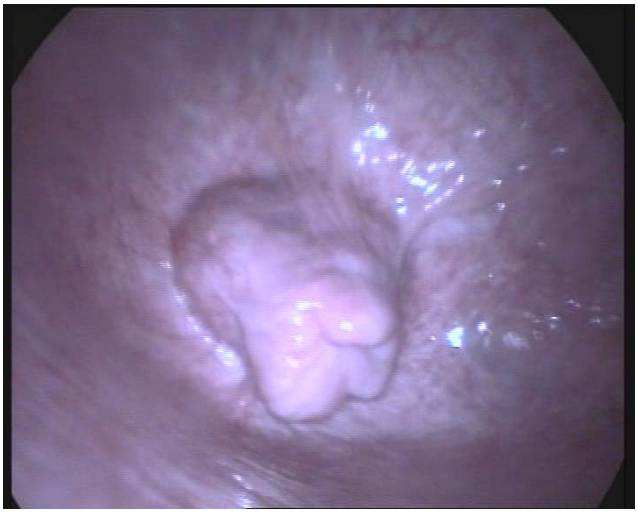

Fig 2. Cérvix de Burra desde el fondo de vagina (Miró, 2020).

El útero de la burra tiene una disposición en Y al igual que en la yegua. En el extremo de los cuernos uterinos se encuentran los ovarios, relativamente más craneales que en la yegua (Renner Martín, 2009, Canisso *et al.,* 2019). Los ovarios, como en la yegua son arriñonados, aunque ligeramente más aplanados. Al igual que en la yegua, y a diferencia de otras especies, folículos y cuerpos lúteos son internos. Los folículos deben ovular hacia la fosa de ovulación y, tanto folículos como cuerpos lúteos sólo pueden explorarse correctamente mediante ecografía.

2.1.2. El burro o garañón

El pene del burro es anatómicamente similar al del caballo, aunque algo más largo. En el prepucio podemos observar 2 prominencias, los pezones, que en el caballo son mucho más pequeños y los localizamos más caudalmente, a nivel inguinal (Canisso *et al.,* 2019).

Los burros poseen un escroto péndulo, en general más descolgado que el caballo. La piel del escroto es fina con abundantes glándulas sudoríparas que contribuyen en la regulación térmica del testículo. Los testículos son relativamente más grandes que en el caballo, elípticos y con una ligera inclinación cráneo-caudal. A

diferencia del caballo, en extremo caudal, se observa externamente una gran cola del epidídimo (Canisso *et al.,* 2019).

El burro, con unos testículos de gran tamaño posee una elevada eficiencia espermatogénica y una duración relativamente corta de la espermatogénesis, siendo considerado el mamífero doméstico con mayor eficiencia en la producción de semen (Neves *et al.,* 2002).

El parénquima testicular ecográficamente presenta una imagen gris, ecogénica, granular y homogénea. La vena central, difícilmente observable en el caballo, es fácil de identificar en el burro (Canisso *et al.,* 2019).

El burro posee un importante cordón espermático con el músculo cremáster lateral, el conducto deferente y el paquete vascular, el plexo pampiniforme. La arteria testicular es una arteria de gran calibre que serpentea junto a la vena testicular a lo largo del cordón espermático constituyendo el citado plexo pampiniforme. Un estudio reciente demuestra que el burro posee un flujo sanguíneo arterial significativamente mayor al de los caballos, posiblemente relacionado con la mayor eficiencia espermatogénica. Este flujo sanguíneo en la arteria testicular disminuye de forma significativa desde el abdomen, con sucesivas ondas bifásicas (sístole-diástole), hasta el testículo con una onda monofásica, debido a la práctica desaparición del pico sistólico. La disminución del flujo sanguíneo supone la disminución de la temperatura (4-5ºC) y del aporte de oxígeno, ambos hechos muy importantes para la espermatogénesis. El índice de pulsatibilidad (IP) de la arteria testicular valorado con ecografía Doppler pulsado presenta una correlación negativa con el número de espermatozoides del eyaculado y la velocidad espermática evaluada mediante un sistema CASA (Gacem *et al.,* 2020).

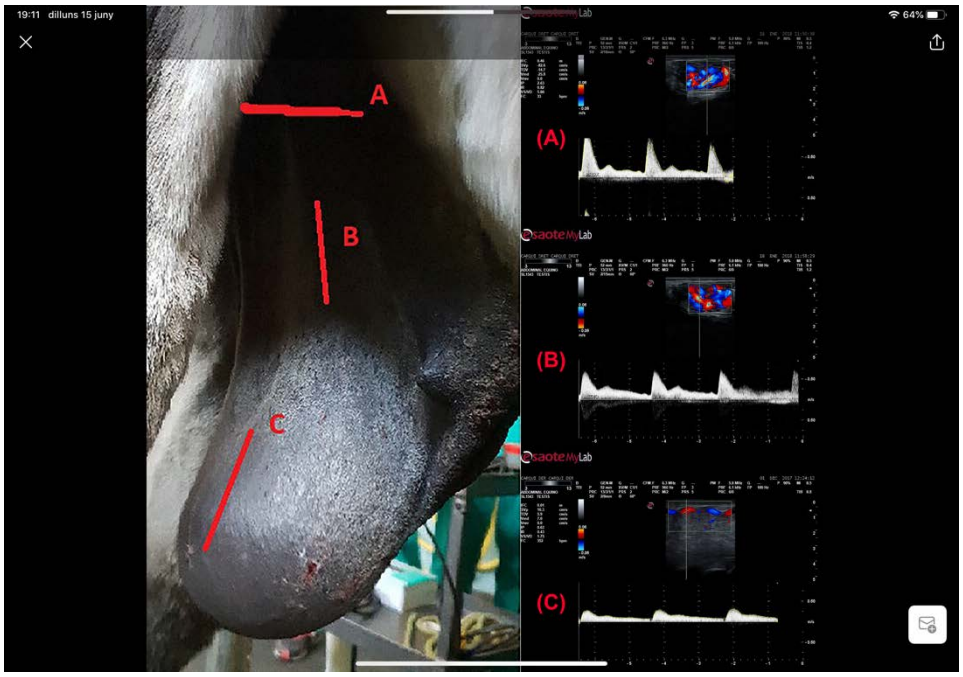

Fig 3. Anàlisis del flujo sanguíneo en distintos puntos de la arteria testicular mediante ecografía Doppler pulsado (Gacem *et al.,* 2020).

Los conductos deferentes entran en el abdomen por el anillo inguinal, uno por cada lado, y progresan dorsalmente hasta la uretra. En su desembocadura en la uretra los conductos deferentes se dilatan de forma significativa en lo que se conoce como las ampulas o ampollas de los conductos deferentes. El diámetro de las ampulas de los burros es hasta tres veces superior al de las ampulas de los caballos. Ecográficamente las ampulas de los caballos son grises y homogéneas, mientras que las ampulas de los burros muestras un aspecto glandular con espacios anecogénicos que tienden a dirigirse hacia la luz central. En la desembocadura de las ampulas, caudalmente se encuentra la próstata, significativamente mayor a la del caballo. La próstata del burro normalmente a la ecografía transrectal muestra espacios anecogénicos mientras que la del caballo solo los muestra tras el estímulo sexual. Lateralmente a las ampulas y desembocando en la uretra junto a la próstata, encontramos las vesículas seminales o glándulas vesiculares. Las vesículas seminales del burro son finas, alargadas, con un diámetro inferior a las del caballo. Caudalmente, próximas a la zona perineal, cerca del ano, encontramos las 2 glándulas bulbouretrales,

elípticas, más o menos simétricas y, también, significativamente mayores que en el caballo (Kandiel & El Shafey, 2017; Gacem *et al.,* 2020). La contribución de cada una de estas glándulas en el plasma seminal del burro es un tema importante aun sin conocer.

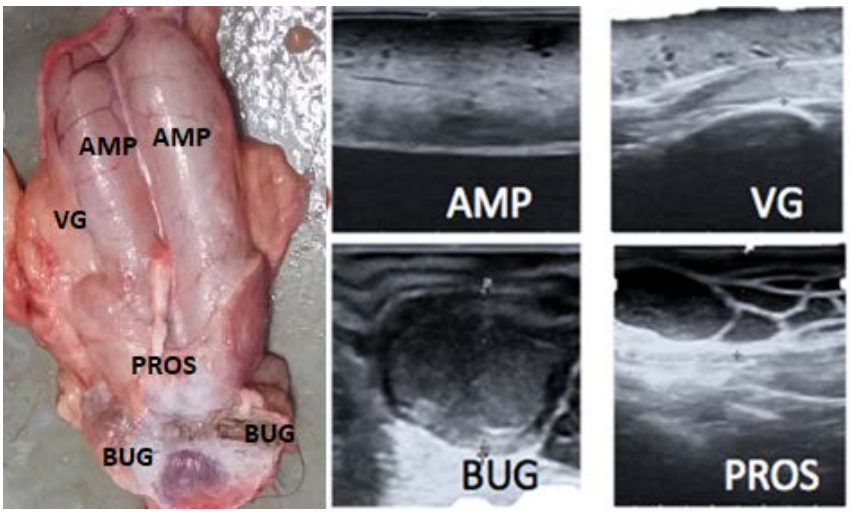

Fig 4. Glándulas accesorias del aparato genital del burro. Imagen real e imágenes de ecografía transrectal (Gacem *et al.,* 2020).

2.2. Comportamiento sexual

Conocer el comportamiento sexual y reproductivo de cada especie en libertad es básico para poder reproducir su ciclo en cautividad. Según McDonnell (1992) en lo équidos la mayoría de errores cometidos a la hora de reproducir las condiciones de monta y cría, se deben a un desconocimiento del manejo y del comportamiento animal en estado salvaje por parte de los criadores.

Caballos y burros, aunque filogenéticamente cercanos, presentan una estrategia de apareamiento opuesta (Herny *et al.,* 1998; Canisso, 2008).

El caballo tiene un comportamiento social, y forma harenes. Grupos de yeguas con un semental. Las hembras en celo atraen a los machos por las feromonas presentes tanto en la orina como en las descargas vaginales. El cortejo de los

caballos es más elegante y "seductor". Los machos miran a la hembra en celo de manera insistente, caminan cerca de ella y relinchan constantemente para llamar su atención. La yegua en celo busca al semental, le ofrece la grupa, levanta la cola, arquea el dorso y orina repetidamente, abriendo y cerrando constantemente la vulva a la vez que expone el clítoris. Cuando la hembra le deja acercarse, el caballo olfatea y lame el cuello de la yegua, así como el vientre, los flancos, los cuartos posteriores y la zona genital, y hace el reflejo de Flehmen. Generalmente, en erección el caballo va a cubrir la yegua con facilidad (Crowell-Davis, 2007). En la monta dirigida, la interacción caballo-yegua es reducida, el caballo suele estimularse fácilmente, montar a la yegua y copular normalmente rápido y tras 4-6 golpes de riñón eyacula.

Por el contrario, el burro es un macho territorial, que permanece en un área restringida siempre y cuando las condiciones tanto de agua como de alimento sean óptimas. Estos suelen ser pastos muy agradables que acaban por atraer a las burras. Las burras forman pequeños grupos de 2 a 5 individuos que tienen un comportamiento migratorio y al pasar por este pasto, las asnas que se encuentren en estro podrán ser cortejadas y cubiertas por el asno del territorio, e incluso permanecer allí durante todo el periodo de celo (McDonnell, 1998). El asno del territorio tiene un comportamiento de macho alfa por lo que luchará contra otros asnos que intenten entrar en él y como mucho permitirá la entrada de otros individuos siempre y cuando adquieran una actitud subordinada. En alguna ocasión este macho podrá efectuar alguna monta después del macho dominante, por lo que tiene alguna opción de reproducirse. Esta estrategia suele ser utilizada por machos más débiles para intentar reproducirse y es aceptada por los machos dominantes ya que así se obtiene ayuda a la hora de poder defender su territorio (Henry *et al.,* 1991, 1998).

En lo que hace referencia al cortejo sexual de los burros, la interacción macho-hembra va a ser mucho más importante que en el caballo. Las burras en celo van a mostrar, como la yegua, signos heterosexuales, pero a diferencia de esta también mostraran signos heterosexuales. En un grupo de burras es normal la sincronización de celos. Las burras en celo van a mostrar Flehmen como los

machos, ante otras burras en celo. Y, al igual que las vacas, van a montar a otras burras que seguramente también estarán en celo. Ante el macho, la sintomatología de celo de la burra es muy evidente, posiblemente para llamar más su atención. La burra extiende el cuello, coloca las orejas hacia atrás sobre el cuello y masca (realiza repetidos movimientos de masticación) y en algunas ocasiones, al igual que la yegua, levanta la cola, orina y abre y cierra la vulva repetidamente (Clayton *et al.,* 1981; Taberner *et al.,* 2008). Ante una burra en celo los garañones suelen rebuznar intensamente y mostrar un comportamiento inicial un tanto agresivo. Es normal que realicen una primera monta sin erección mordiendo intensamente el cuello de la burra. Una monta corta de entre 10 y 30 segundos. Una vez desciende puede mostrar interés por la zona perineal, flancos, presentar Flehmen, morder los corvejones y especialmente las extremidades anteriores de la burra, haciéndola claudicar como señal de dominancia. Durante el periodo precópula el burro puede montar a la burra entre 3 y 6 veces sin erección. Y mostrar fases de aparente desinterés. Esta fase es muy variable y puede durar desde 5 minutos a más de media hora. En una de las fases de aparente desinterés el burro manifestará la erección y cubrirá a la burra. Sin embargo, la fase de cópula del burro será corta, relativamente más rápida que en el caballo (Canisso *et al.,* 2008, Canisso *et al.,* 2019).

Fig 5. Burro realizando Flehmen durante el cortejo precópula.

Fig 6. Burra en celo, con movimientos masticatorios y orejas hacia atrás ante el macho.

2.3. Pubertad y ciclo sexual de la burra.

Una burra sana con las necesidades nutritivas cubiertas suele alcanzar la pubertad entre el primer el segundo año de edad, aunque deberían cubrir se hasta pasado s los tres años de edad (Miró, 2020). Las burras adultas suelen presentar actividad cíclica continua, durante todo el año, solo burras con males condiciones corporales, o en postpartos de otoño-invierno, pueden mostrar fases de anestro (Taberner *et al.,* 2008; Contri *et al.,* 2014; Canisso *et al.*, 2019).

El ciclo sexual de la burra suele durar unos 24 días de media, frente a los 21 de la yegua. El estro o celo, fase receptiva al macho, dura entre 4-10 días, siendo el último el día en el que ocurre la ovulación. Los celos tienden a ser más cortos en primavera-verano que en otoño-invierno (Blanchard *et al.* 1999, Taberner *et al.,* 2008).

Durante el celo, el folículo dominante tiene un ritmo de crecimiento de 0,3-0,4 mm diarios, para que se produzca una ovulación con un tamaño folicular de entre 3,7-4,5 cm de diámetro (Taberner *et al.*, 2008; Díaz-Durán e*t al.,* 2017; Canisso *et al.,* 2019).

En algunas razas, como la burra Catalana o razas derivadas de esta (Balear, Mammoth o Kentucky, Martina Franca, Ragusano, Pantesco), el porcentaje de ovulaciones dobles es importante, superando el 40% (Taberner *et al.,* 2008).

Ecográficamente, una burra sana en celo mostrará, a diferencia de la yegua, un edema uterino escaso o nulo, (Abd-Elnaeim, 2008; Taberner *et al.,* 2008; Canisso *et al.,* 2019).

2.4. Inducción y sincronización de celo y ovulación.

La utilización de progestágenos por vía vaginal, usados en otras especies, parecen ser un buen método para inducir el celo en burras. No obstante, y posiblemente ligado a la anatomía del tracto genital de la burra descrita anteriormente, esta vía origina una importante vaginitis, con descargas vaginales y escasa fertilidad (Miró *et al.,* 2010).

La inducción de la luteólisis, con la aparición de un celo fértil de manera controlada mediante las prostaglandinas $F_{2\alpha}$ ($PGF_{2\alpha}$) o sus análogos, suele ser el método más empleado. Carluccio *et al.* (2006) afirman que en burras esta respuesta se daría a partir del tercer día postovulación. No obstante, según Canisso *et al.* (2019), la experiencia en el campo demuestra que la respuesta puede no darse hasta el quinto día.

El cuerpo lúteo, como glándula que es, necesita estar muy vascularizado para poder llevar a cabo su actividad y drenar su producción hormonal (Miró *et al.,* 2015; Panzani *et al.*, 2018). Una elevada vascularización rodeando el cuerpo lúteo suele coincidir con niveles elevados de progesterona en sangre y una mayor sensibilidad del cuerpo lúteo a la $PGF_{2\alpha}$ (Miró, 2020).

La ecografía Doppler Color es un instrumento importante para valorar la vascularización del cuerpo lúteo y, por tanto, su actividad y posible sensibilidad a las prostaglandinas. Un cuerpo lúteo activo mostrará una periferia ricamente vascularizada, con un alto porcentaje de color. La luteólisis será el resultado de una isquemia del cuerpo lúteo y la pérdida de su actividad. En el caso de las burras, la luteólisis inducida se va a producir entre 3 y 5 horas después de la aplicación de la $PGF_{2\alpha}$, coincidiendo con un descenso de los niveles plasmáticos de progesterona. Sin embargo, la ovulación variará entre los 2-11 días postinducción, dependiendo de la dinámica folicular en el momento de la luteólisis inducida (Miró *et al.* 2015).

Para poder aplicar técnicas de reproducción asistida de manera efectiva, es imprescindible poder determinar el momento de la ovulación. Así pues, la inseminación artificial con semen congelado debe ajustarse lo máximo posible al momento de ovulación. El seguimiento ecográfico exhaustivo durante el celo es una tarea tediosa. Para reducir el tiempo de exploración se han utilizado sustancias como la hCG (gonadotropina coriónica humana) (2500UI IV) o como agonistas de GnRH (gonadotropin-realising hormone) que ofrecen buenos resultados en una inducción de la ovulación en unas 40h (Carluccio *et al.,* 2007; Canisso *et al.,* 2019).

Como sucede en otras especies de producción, el creciente interés por la producción de leche de burra en Europa o de piel y otros derivados en China han suscitado recientemente el interés por protocolos de inducción y sincronización de celos e inseminación es a tiempo fijo. Se han propuesto distintos tratamientos con $PGF_{2\alpha}$ o sus análogos, en combinación, o no, con progestágenos, y/o GnRH (Zhou *et al.,* 2018; Fanelli *et al.,* 2019).

2.5. Colecta de semen

El semen de los garañones puede obtenerse de forma similar al de los sementales. No obstante, debido a su comportamiento fisiológico, va a requerir más tiempo y paciencia. Los asnos son fáciles de colectar y responden bien a las mismas vaginas

artificiales que los caballos. Pueden ser entrenados a maniquí o puede colectarse sobre burra o incluso yegua si están acostumbrados a esta (Canisso *et al.,* 2019).

2.5.1. Análisis seminal

El semen de los burros suele poseer escaso gel y mostrar un color más grisáceo que el de caballo. El volumen seminal puede oscilar entre 40 y 90 mL y la calidad suele ser excelente, mejor de media que en caballo. La concentración espermática es normalmente elevada, 300 a 400 millones de espermatozoides/ml, el porcentaje de anomalías espermáticas bajo, menor al 20% y la motilidad espermática excelente (Miró *et al.* 2005). Analizada la motilidad espermática mediante un sistema computarizado (CASA) los espermatozoides de burro muestras una alta linealidad y una velocidad significativamente superior a los de caballos (Quintero-Moreno *et al.,* 2003; Miró *et al.,* 2005; Flores *et al.,* 2008).

Por otro lado, el análisis computarizado de la morfometría espermática (CASMA) evidencia que la cabeza del espermatozoide de burro es ligeramente más pequeña que la de caballo, pero la pieza intermedia es significativamente mayor (Miró *et al.,* 2006). Esta mayor pieza intermedia está, seguramente, relacionada con una mayor actividad mitocondrial evaluada por citometría de flujo mediante sonda JC-1 (Papas *et al.,* 2020) y a su vez con la citada mayor velocidad (Miró *et al.,* 2005). Fruto de su elevada actividad metabólica el espermatozoide de burro va a producir grandes cantidades de radícales libres de oxígeno (ROS) (Papas *et al.,* 2019) y de ácido láctico (Miró *et al.,* 2005).

2.5.2. Plasma seminal

El plasma seminal es la fracción líquida del semen. Es el resultado de los fluidos procedentes del testículo, epidídimo y, fundamentalmente de las glándulas accesorias del aparato genital del macho, sobre todo antes y durante la eyaculación. El plasma seminal es un fluido complejo que no solo va a transportar a los espermatozoides, sino que va a ser vital para su fisiología, nutrición,

metabolismo e interacción con el tracto genital femenino, por lo tanto, va a tener un papel fundamental en la fertilidad (Juyena & Stelleta, 2012).

El plasma seminal es un fluido complejo, con azúcares, lípidos, minerales y una importante cantidad de proteinas. El SP de burro posee niveles de glucosa, proteinas, lípidos, colesterol, calcio o fósforo significativamente superiores al caballo (Tabla 1) (Talluri *et al.,* 2017).

Parámetros	Caballo	Burro
Glucosa (mg/dl)	24.31±2.92	25.94±1.28
Proteinas totales (g/dl)	0.95±0.04	4.33±0.12
Lípidos totales (mg/dl)	54.07±4.42	132.49±4.51
Colesterol (mg/dl)	5.45±0.49	23.08±1.02
GOT (U/l)	215.31±21.46	218.46±22.18
GPT (U/l)	11.70±0.36	20.65±1.85
LDH (U/l)	782.63±54.55	2082.23±49.14
ALP (U/l)	18.03±2.66	11.84±0.61
ACP (U/l)	4.93±0.50	5.60±0.33
Calcio (mg/dl)	13.66±1.13	18.07±1.16
Fósforo (mg/dl)	8.73±0.016	11.92±0.59

Tabla1. Media ± error estándar de distintos parámetros evaluados en plasma seminal de Caballos (Marwari) y de burros (Poitou) sementales (Talluri *et al.,* 2017).

Los niveles de proteínas del plasma seminal de burro son entre 4 y 5 veces superiores a los del caballo (Talluri *et al.,* 2017; Papas *et al.,* 2019).

Las proteínas integrantes y su función son aún en buena parte desconocidas en las distintas especies y juegan un papel fundamental en las estrategias reproductivas especie-específicas.

Entre estas proteínas encontramos enzimas como la LDH (lactato deshidrogenasa) con niveles de casi 3 veces en el burro que en el caballo (Tabla 2). Posiblemente estos valores tengan relación con el control las grandes cantidades de ácido láctico producido por el espermatozoide de burro (Miró *et al.,* 2005). Talluri *et al.*, (2017) observan una correlación positiva entre el volumen seminal y la concentración espermática y GOT (*glutamic oxalacétic transaminase*), GPT (*glutàmic pyruvic tranasminase*) o ACP (*àcid phosphatase*). Existen grupos de proteinas ligadas al control del ROS como SOD *(supoeroxide dismutase),* CAT (*catalase*), GPX (*glutatione peroxidase*) o GSR (*glutatione reductase*). Todas ellas con valores muy superiores en el plasma de burro respecto al caballo y correlacionadas con la motilidad espermática en el burro (Papas *et al.,* 2019). Así mismo, la actividad de la SOD en el plasma seminal muestra una correlación positiva con la criotolerancia de los espermatozoides de burro (Papas *et al.,* 2020).

	PROTEINAS TOTALES (mg/mL)	SOD (U/mg)	GPx (U/g)	GSR (U/g)	CAT (U/g)
BURRO	48.00±28.49	49.65±26.56	14.02±4.08	0.91±0.67	194.84±184.83
CABALLO	8.70±4.81	25.34±10.20	25.06±4.77	1.69±1.20	76.25±75.83

Tabla 2. Proteinas totales y actividad enzimática de SOD (*superóxido dismutasa*), GPX (*glutation peroxidasa*), GSR (*glutation reductasa*) y Cat (*catalasa)* en el plasma seminal de burro y de caballo (Papas *et al.,* 2019).

En mamíferos ungulados, entre los que se encuentran los burros, la mayoría de proteínas del plasma seminal son portadoras de fibronectina de tipo II (Fn-2), espermadhesinas o proteínas secretoras ricas en cisteínas (CRISP), cuya función es fundamental para una correcta fecundación del ovocito. En la mayoría de especies estas proteínas provienen de las vesículas seminales o del epitelio epididimario, y se ha observado que tienen un gran poder inmunoestimulador tanto *in vivo* como *in vitro*, que estará relacionado con citosinas específicas (Rodríguez- Martínez *et al.,* 2011). Este tipo de proteínas en el caballo se denominaron inicialmente *horse seminal proteins* (HSP).

El plasma seminal en el caballo reduce la llegada fisiológica de neutrófilos polimorfonucleares al útero tras la deposición de semen (Alghamdi *et al.,* 2009). La proteína secretora rica en cisteina 3 (CRISP-3) aislada del plasma seminal parece proteger a los espermatozoides de la unión a los neutrófilos, mientras una DNAsa seminal podría liberar a los espermatozoides de las NETs (trampas extracelulares de neutrófilos (Alghamdi & Foster, 2005).

Está claro que el SP juega ciertos roles como modular la capacidad de los spz de interactuar con los epitelios y secreciones del tracto femenino o ser portadores de señales particulares (Rodríguez- Martínez *et al.* 2011). También se ha observado que hay ciertos componentes que intervienen en la viabilidad de los spz, como el bicarbonato, que modula la motilidad de los spz y desestabiliza el plasmalema durante su capacitación (Rodríguez- Martínez *et al.* 2011) o como el zinc, modulador de la cromatina (Björndahl y Kvist, 2010).

2.6. La inseminación artificial

La inseminación artificial en la burra requiere experiencia y habilidad para atravesar el cuello uterino descrito anteriormente. La inseminación con semen fresco o diluido y refrigerado ofrece buenos resultados, pero con semen congelado los resultados son escasos (Miró, 2020).

Los resultados de viabilidad y motilidad espermática al descongelar semen congelado de burro son muy buenos, posiblemente mejores de media que en el caballo (Flores *et al.,* 2008).

Este semen congelado-descongelado de burro es capaz de penetrar ovocitos bovinos denudados *in vitro* formando pronúcleos masculinos (Taberner *et al.,* 2010). *In vivo,* cuando este semen se utiliza para inseminar yeguas los índices de preñez son buenos, no obstante, cuando se utiliza en burras, la misma especie, los porcentajes de gestación son muy malos (Trimeche *et al.,* 1998; Oliveira *et al.,* 2006; Vidament *et al.,* 2009; Canisso *et al.,* 2011; Rota *et al.,* 2012).

La inseminación en la burra produce una gran inflamación uterina, endometritis, significativamente mayor que en la yegua (Rota *et al.,* 2008; Miró *et al.,* 2011). Esta inflamación va a estar modulada por el plasma seminal (Miró *et al.,* 2011; Vilés *et al.,* 2013b). No obstante, el plasma seminal tiene un efecto negativo en la conservación del semen de burro y eliminarse necesariamente en el proceso de congelación (Miró *et al.,* 2009)

2.7. Endometritis postinseminación y posible control

En las especies de deposición de semen vaginal por monta natural, como la vaca, la oveja, la perra o la mujer, el cuello uterino y las mucinas secretadas en este van a tener un papel fundamental en la selección espermática (López-Gatius *et al.,* 1993). Miles de millones de espermatozoides son depositados en el fondo de vagina, miles acceden al útero, cientos al oviducto y uno va a fecundar (Larson & Larson, 1985).

Sin embargo, en las especies de deposición intrauterina, como la cerda, la yegua o la burra, el cérvix es obviado. En estas especies se produce una endometritis fisiológica, con la movilización de una gran cantidad de neutrófilos polimorfonucleares (PMN) hacia la luz uterina (Miró & Papas, 2017). Estos PMN van a ejercer un papel de limpieza de suciedad y bacterias, pero, fundamentalmente de selección espermática (Kotilainen *et al.,* 1994; Troedson *et al.,* 2001). De esta forma, miles de millones de espermatozoides son depositados en el interior del útero, solo miles escaparán a los neutrófilos, solo cientos llegarán al oviducto y solo uno fecundará un ovocito.

En la yegua en celo los niveles elevados de estrógenos, junto a bajos niveles de progesterona, producirán un importante incremento del aporte sanguíneo al útero. Este aumento de llegada de sangre producirá una extravasación de líquido entre los pliegues del endometrio que, a la exploración ecográfica puede observarse como el edema de celo. Este puede considerarse un estado preinflamatorio, una "preinstalación". Cuando se produce la llegada del semen se

va a producir un gran aflujo de PMN hacia la luz uterina originado por la llegada de espermatozoides más que por la llegada de posibles contaminaciones bacterianes con el semen (Troedson *et al.,* 2001).

En la burra, la inflamación postmonta o inseminación va a ser exagerada, con una gran cantidad de PMN y algún eosinófilo observados en la luz del útero 6h postinseminación mediante citologia uterina (Fig 7) (Rota *et al.,* 2008; Miró *et al.,* 2011).

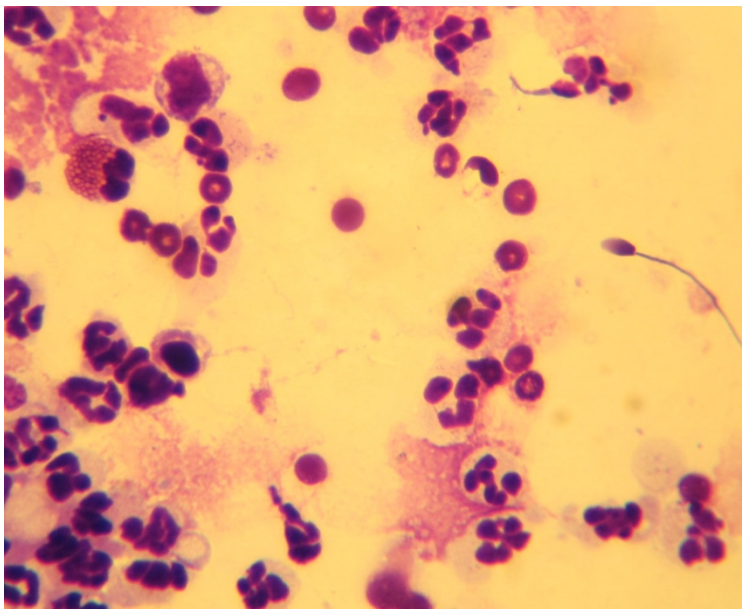

Fig 7. Gran reacción inflamatoria endometrial 6h postinseminación en la burra. Abundantes neutrófilos polimorfonucleares (PMN) y algún eosinófilo.

Esta reacción inflamatoria puede observarse también histológicamente mediante biopsias uterinas de burras en celo. Poco después de la inseminación podremos observar una importante inflamación celular con migración de PMN, así como la presencia de eosinófilos en todos los casos, a diferencia de la yegua. Por otro lado, mediante inmunohistoquímica puede observarse una importante presencia de COX-2 (cicloxigenasa), mediador de la inflamación humoral (Fig 8) (Vilés *et al.,* 2013a).

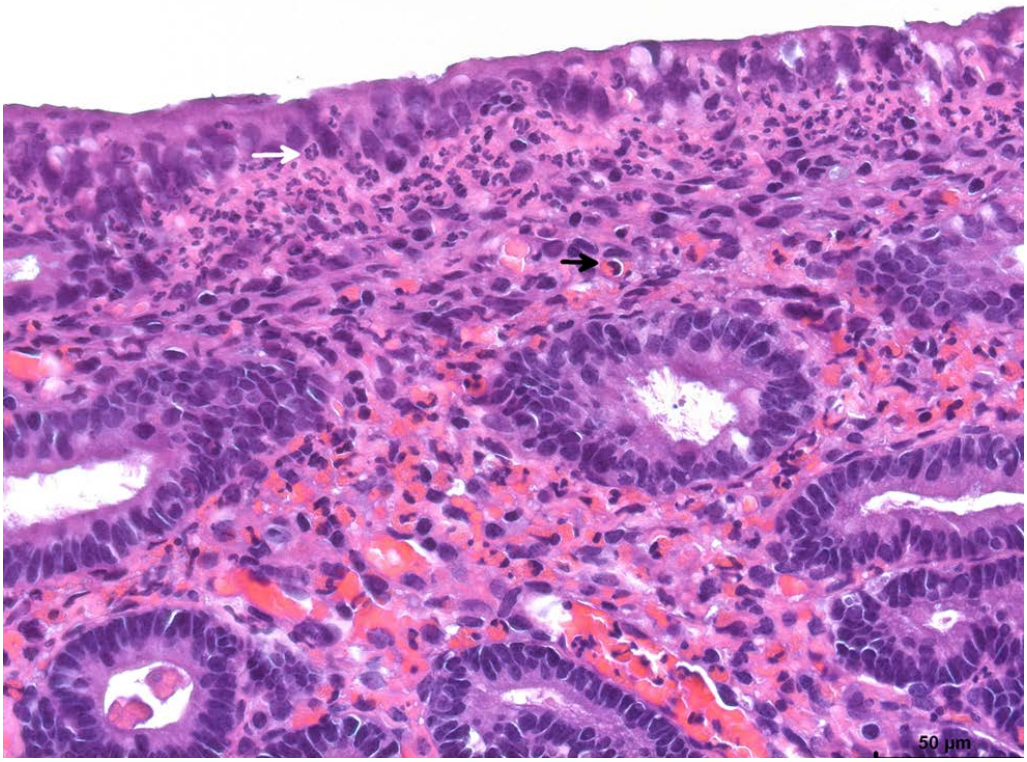

Fig 8. Biopsia uterina de burra postinseminación con gran movilización de PMN (flecha blanca) hacia la luz uterina y la presencia de eosinófilos (flecha negra).

Los PMN tienen dos mecanismos de acción, la fagocitosis, y otro descrito recientemente, la producción de NETs (neutrophil extracelular traps) (Brinkmann *et al.,* 2004; Rebordão *et al.,* 2014; Nakazawa *et al.,* 2017). En el momento en que los PMN entran en contacto con bacterias y/o espermatozoides muchos PMN se activan, degranulan, mueren y expulsan gran cantidad de proteínas y su ADN formando una compleja red que va a atrapar a bacterias o espermatozoides (trampas extracelulares de neutrófilos).

Para intentar reducir la respuesta inflamatoria postinseminación con semen congelado se han utilizado drogas como la dexametasona, aunque los resultados generan una importante controversia (Stout & Ruitjter-Villani, 2012). El uso de drogas no esteroideas, como el vedaprofen, ofrecen resultados interesantes en la yegua (Aurich *et al.,* 2010). No obstante, la vía de administración del vedaprofen es oral y su dosificación es difícil.

Un fármaco de la misma familia, el ketoprofen, se utilizó en la burra, pudiéndose administrar por vía parenteral, con un mejor control de la dosificación. El ketoprofen produce una disminución significativa en la expresión de COX-2 (inflamación humoral) mediante inmunohistoquímica. No obstante, no se observó ningún descenso en la movilización de PMN y eosinófilos (Vilés *et al.,* 2013a).

Vistos estos resultados se planteó el uso del plasma seminal eliminado durante el proceso de congelación seminal. El plasma seminal se asocia con procesos inmunosupresivos en varias especies, incluyendo el caballo (Schopf *et al.,* 1984; Troedsson *et al.,* 2001; Rozeboom *et al.,* 2001). También se ha demostrado que el plasma seminal tiene efectos favorables sobre la motilidad espermática y la supervivencia de las células espermáticas en el útero, evitando el daño oxidativo (Alghamdi *et al*., 2004; Rota e*t al*., 2008; Aloé *et al*., 2012).

Alghamdi *et al.* (2004) observaron un incremento en las ratios de preñez en yeguas inseminadas con semen de fertilidad comprobada diluido en SP. Alghamdi *et al.* (2004) demostraron que el SP reduce significativamente la proporción de spz fagocitados por los PMN's en el caballo. Posteriormente, observaron que una DNAsa seminal podría liberar a los espermatozoides de las NETs (Alghamdi & Foster, 2005).

En burras, estudios *in vitro* han mostrado efectos favorables del SP sobre la motilidad espermática (Rota *et al.,* 2008). Posteriormente un estudio *in vivo* del mismo grupo sobre el uso de plasma seminal como diluyente de semen de burro descongelado mostró una tendencia hacia la mejora de la fertilidad (Rota *et al.,* 2012).

En otro estudio *in vivo* sobre el efecto del plasma seminal para controlar la inflamación post AI, se observó que el plasma seminal reduce la expresión de COX-2 post inseminación, también se observó una reducción el número de los eosinófilos en las biopsias endometriales, con un significado aun desconocido,

pero no se observaron diferencias significativas en el número de PMN con o sin plasma seminal (Vilés et *al*., 2013b).

Miró *et al.* (2013) realizaron lavados uterinos de burras 6h postinseminación. Estos lavados eran ricos en PMN que se cuantificaban mediante citometría de flujo. Posteriormente se realizaba una experiencia *in vitro*, los PMN eran puestos en contacto con semen completo, semen diluido o semen congelado, es decir con todo, parte o nada de plasma seminal. Tras la incubación en baño maría a 37ºC se observaba claramente que el plasma seminal modula la interacción PMN:espematozoides. En las muestras con semen congelado la unión de espermatozoides a PMN era máxima siendo, significativamente muy inferior, con semen total, y significativamente menor, pero en una situación intermedia para el semen diluido.

Parece pues que el plasma seminal es capaz de controlar la inflamación humoral, reducir en parte la inflamación celular, con una disminución de los eosinófilos, aunque no de los PMN. Pero si modulará la interacción entre PMN y espermatozoides.

Como se citó anteriormente la gran actividad metabólica del espermatozoide de burro, produce una gran cantidad de radicales libres de oxígeno (ROS). Por otro lado, la actividad de lo neutrófilos, fagocitosis y en especial la producción de NETs, produce una cantidad significativamente mayor de ROS, de hasta 100 veces (Aitken *et al.,* 1990; Ford *et al.,* 1997).

Los ROS van a ser importantes en el proceso de capacitación. Una de las vías capacitadoras es la de la quinasa, regulada por señal extracelular (ERK), que se activa mediante la unión a los receptores de membrana y mediante la activación intracelular por especies reactivas de oxígeno (ROS). Muchas investigaciones han indicado que la producción de especies reactivas de oxígeno (ROS) se expresan en el período inicial de capacitación (Shi-Kai y Whan-Xi, 2017).

Sin embargo, los ROS en exceso producen en la célula, el espermatozoide, un estrés oxidativo, con daños celulares irreparables, peroxidación de la membrana, penetración y daño de estructuras internas (Jones *et al.,* 1979; Jain *et al.,* 2010; Aitken, 1989, Aitken *et al.,* 2012) y fragmentación del ADN (Cicaré *et al.,* 2015). Estos daños evidentemente afectan a la funcionalidad espermática y a la fertilidad en distintas especies de mamíferos (Collins *et al.,* 2008; Aitken *et al.,* 2012; Peña, 2019).

El control del ROS por parte del plasma seminal va a ser también fundamental. Anteriormente hemos citado el papel de enzimas (CAT, SOD, GPX o GSR) con un papel primordial, pero debemos tener en cuenta otros componentes no enzimáticos del plasma seminal con capacidad antioxidante como el ácido ascórbico, el glutatión reducido o la melatonina (González-Arto *et al.*, 2016).

Por otro lado, la media de concentración espermática y la velocidad es mayor en burros que en caballos. El rol de estos parámetros en la interacción PMN:spz es también desconocido.

Oliveira *et al.* (2016) estudiaron cuál podría ser la dosis más recomendable para realizar inseminaciones artificiales de eyaculados frescos en burras o yeguas. Llegaron a la conclusión de que al incrementar la dosis a 10^9 spz/ml mejora la ratio de fertilidad en burras, siendo similar a la obtenida en yeguas, realizando un total de cuatro inseminaciones por ciclo. También probaron que la mejor técnica para mejorar la fertilidad de las burras con semen congelado/descongelado es la inseminación profunda en cuerno uterino, realizando cuatro inseminaciones por ciclo utilizando dosis de 10^9 spz/ml.

Un mayor conocimiento de la reacción endometrial postinseminación, de la producción de ROS, del papel del plasma seminal y sus componentes y del posible papel de la concentración espermática son necesarios para mejorar los resultados obtenidos mediante inseminación artificial con semen congelado en asnos. El estudio del papel de las proteínas del plasma seminal en el control de la respuesta

inflamatoria postinseminación es de gran complejidad debido a la gran cantidad de proteínas existentes en el plasma seminal de burro.

Así mismo, el conocimiento de estrategias reproductivas especie-específicas puede contribuir en el conocimiento de otras y el tratamiento de problemas de fertilidad.

No obstante, la repetición de los lavados uterinos para estudios *in vitro* puede producir daños significativos en el endometrio, así como problemas de fertilidad, hecho especialmente grave cuando se trabaja con razas en peligro de extinción.

2. OBJETIVOS

Objetivo general

Mejorar el conocimiento sobre la reacción endometrial postinseminación artificial en la burra y del papel del plasma seminal en su control.

Objetivos específicos:

- Estudio de un método efectivo para la inducción de celos fértiles y obtención de muestras en celo o postinseminación

- Desarrollo de un modelo *in vitro* para estudiar la reacción inflamatoria postinseminación artificial en burra.

- Analizar el efecto de la concentración espermática sobre la respuesta inflamatoria post-inseminación artificial en burra.

- Observar los efectos de las diferentes fracciones proteicas del plasma seminal sobre la interacción PMN:espermatozoide.

- Observar los efectos de las diferentes fracciones proteicas del plasma seminal sobre la formación de ROS (*Reactive Oxygen Species)*

3. CAPÍTULOS

3.1. Artículo 1

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Color Doppler provides a reliable and rapid means of monitoring luteolysis in female donkeys

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ABSTRACT

When artificial reproduction technologies designed for use with horses are used with donkeys, success is dependent on awareness of the physiological differences between these species, yet little information is available on many aspects of donkey reproduction. The present work examines the activity of the CL in Catalonian jennies after induced luteolysis. Plasma progesterone concentration, luteal blood flow (determined by color Doppler), and CL cross-sectional area (CL-CSA; determined by B-mode ultrasound examination) were assessed after a single dose (5 mg intramuscular) of dinoprost thromethamine (DT, a PGF2α analog) on Day 10 after ovulation in two experiments. In experiment 1, a preliminary experiment, data were collected daily for 4 days after DT administration. Values for all the measured variables decreased over this period. In experiment 2, data were collected during the first 24 hours after DT administration because in experiment 1, most luteolytic activity occurred during this time. An increase in luteal blood flow was seen between 0 and 3 hours, followed by a progressive reduction, whereas the values for plasma progesterone and CL-CSA gradually decreased from 0 hours onward. In both studies, negative correlations were seen between all variables and the time of sampling. In contrast, positive correlations were seen between plasma progesterone, CL-CSA, uterine tone, and luteal blood flow. Indeed, a strong correlation was recorded between plasma progesterone and luteal blood flow ($r = 0.70$; $P < 0.0001$). In conclusion, plasma progesterone and CL-CSA both become reduced after induced luteolysis in Catalonian jennies. Unlike in mares, an increase in luteal blood flow occurs soon after induced luteolysis, rather like that seen in the cow. The luteal blood flow, as evaluated here by color Doppler, was also closely related to the plasma progesterone concentration. Color Doppler would appear therefore to offer a rapid and easy means of examining the state of luteolysis.

1. Introduction

Little information is available on many aspects of donkey reproduction. This could reduce the success of artificial reproduction programs when techniques originally developed for use with mares are used.

The CL is a temporary endocrine gland formed from fibroblasts and the remaining cells of the ovulated follicle's granulose layer. It produces progesterone and maintains the uterus lining during early pregnancy [1,2]. When pregnancy does not occur, the CL naturally involutes in response to the release of PGF2α from the nonpregnant endometrium. This occurs from Days 13 to 16 after ovulation in the mare [3–5]. Sequential pulses of PGF2α, on average separated by 9 hours, lead to a reduced plasma

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progesterone concentration by 3 to 4 hours after the first pulse. Estrous is achieved when plasma progesterone drops to 1 to 2 ng/mL [5–7]. The exogenous administration of prostaglandins is commonly used to shorten the estrous cycle in domestic farm animals and has been used to control postbreeding endometritis in mares [4,8–10]. A single high-dose bolus can be administered to induce luteolysis. Natural rather than synthetic PGF2α analogs are usually used in mares and jennies and at lower doses than those needed for ruminants (5 vs. 25 mg), given the greater sensitivity of their luteal cells to prostaglandins [11–13]. However, luteolysis has been little studied in the jenny [37], and understanding CL physiology and morphology after induced luteolysis is important if reproductive technologies are to be successfully used in this species.

Luteal vascularization plays an important role in the physiology of the CL [14]. Intense angiogenesis occurs during luteinization in many species [15–17], but curiously, in the cow, increased luteal vascularization also occurs before an intense reduction of the same just before luteolysis [18,19]. Until about 10 years ago, changes in the CL during the estrus cycle, and after induced luteolysis, in mares were usually monitored by B-mode ultrasound [1,20] and by following changes in the plasma progesterone concentration [21–23]. In humans, however, color Doppler ultrasound was already being used to examine blood circulation in patients presenting with CL development problems [24]. Later, Bollwein et al. [25] showed that color Doppler monitoring offered valuable information on CL activity and vascularization in the mare and that the technique was a useful noninvasive tool for examining luteal blood flow. Color Doppler assessment of the CL in mares [26,27] and heifers [28] has been increasing over recent years, and in the latter, it has recently been shown to better assess luteal blood flow than CL cross-sectional area (CL-CSA) or plasma progesterone measurements [29,30].

In the bovine CL, the luteal blood flow increases temporarily (0.5 hours) before the fall of the plasma progesterone concentration at 1 hour after physiological [31–33] and induced luteolysis [31,34]. However, in the mare, luteal blood flow does not increase during early luteolysis, and plasma progesterone decreases before significant reductions in luteal blood flow are detected [32,35,36]. Moreover, a reduction in angiogenesis in the CL of mares has been described during the late luteal phase induced by PGF2α [15]. The literature, however, contains no information on what happens in jennies.

The aim of the present study was to examine the changes in the CL, luteal blood flow, and plasma progesterone after the administration of a single intramuscular dose of a PGF2α analog to Catalonian jennies on Day 10 after ovulation.

2. Materials and methods

The animals examined were seven clinically healthy and normally cycling Catalonian jennies (*Equus asinus*) aged 4 to 13 years. All were kept together outdoors and fed grain forage, straw, hay, and water *ad libitum*. All were monitored daily from estrus to ovulation *via* transrectal palpation and real-time ultrasound (MyLabTM30 VET; Esaote, Genoa, Italy) using a 5-MHz linear transducer. After detecting an active CL, 5 mg of dinoprost thromethamine (DT; a natural PGF2α analog; Dinolytic, Pfizer Animal Health, Belgium) was administered intramuscularly on Day 10 after ovulation to induce luteolysis.

2.1. Experiment 1: CL activity over the 4 days after induced luteolysis

Twelve active CL involving three jennies were detected. Corpus luteum activity in these jennies was examined daily by measuring, as described in the following, the CL-CSA, luteal blood flow, and plasma progesterone from Days 0 to 4 after DT administration. Corpus luteum echogenicity and uterine tone were also recorded as previously described [38].

The CL-CSA (cm^2) was determined by B-mode ultrasound analysis. Cross-sectional images of the identified CL at their maximum size were stored, and the CL-CSA was determined using the software provided with the ultrasound equipment. The color Doppler function was then activated to examine the luteal blood flow [27]. Four transverse sections were recorded at maximum color pixel density. Images were later analyzed using a computer image analyzer running analySIS 2.1 software (Soft Imaging System GmbH, Münster, Germany). The percentage area of the CL-CSA with color Doppler signals returned by the blood flow was determined as previously described [25,39].

At the same time, blood samples from the jugular vein were collected in vacutainers and centrifuged (10 minutes, × 1500*g*). Plasma was decanted and frozen at −20 °C until being assayed for progesterone using a progesterone radioimmunoassay kit (Immunotech SAS, Marseille, France).

Given the results obtained, a second experiment was performed involving closer monitoring over the first 24 hours after induced luteolyis.

2.2. Experiment 2: CL activity over the first 24 hours after induced luteolysis

Ten active CL involving four jennies were detected. Corpus luteum activity was monitored by measuring the CL-CSA, the luteal blood flow, and plasma progesterone as described in experiment 1. Readings were taken every hour from 0 to 7 hours after DT administration and then at 10, 12, and 24 hours.

2.3. Statistical analysis

The Kolmogorov–Smirnov test was used to test the normality of distribution of the results. Analysis of variance (general linear model and univariate procedure) was performed to detect differences between variables at different experimental times. When significant differences were detected, the least significant difference test was performed to examine variables that showed homogeneity of variance (plasma progesterone and CL-CSA), and the Tamhane test was used to examine those that did not (blood flow as determined by color Doppler). Multiple linear regression analysis was performed to detect relationships between variables and between variables and experimental time. When double ovulations were detected, the mean

CL-CSA and luteal blood flow (as determined by color Doppler) were calculated to test their correlation with plasma progesterone. All data were expressed as mean ± standard error of the mean. Significance was set at $P < 0.05$. All calculations were performed using IBM SPSS 19.0 for Windows (Statistical Package for the Social Sciences Inc., IBM Company, Chicago, IL, USA) software.

3. Results

All the CL examined were highly echogenic and showed a central hyperechogenic area (B mode). Color Doppler detected strong blood movement around the CL. The plasma progesterone concentration was greater than 20 ng/mL on Day 10 after ovulation (Day 0 of the experiments), indicating the presence of a functional CL. Plasma progesterone correlated positively with uterine tone ($r = 0.53$; $P < 0.05$) in both experiments. All the jennies ovulated 4 to 7 days after DT treatment.

3.1. Experiment 1: CL activity over the 4 days after induced luteolysis

Significant differences (up to $P < 0.0001$) in luteal blood flow, CL-CSA, and plasma progesterone were seen between Day 0 and the other days (Figs. 1 and 2).

A strong positive correlation was seen between luteal blood flow as determined by color Doppler and plasma progesterone ($r = 0.70$; $P < 0.0001$) and between the CL-CSA and plasma progesterone ($r = 0.62$; $P < 0.05$). Uterine tone was maximum on Day 0 and decreased over time after DT administration ($r = -0.75$; $P < 0.0001$). Positive correlations were seen between uterine tone and plasma progesterone concentration ($r = 0.37$; $P < 0.05$), luteal blood flow ($r = 0.44$; $P < 0.05$), and the CL-CSA ($r = 0.44$; $P < 0.05$). Negative correlations were recorded between the day of experiment and luteal blood flow, CL-CSA, and plasma progesterone ($P < 0.0001$; $r = -0.58$, -0.68, and -0.79, respectively).

3.2. Experiment 2: CL activity over the first 24 hours after induced luteolysis

The plasma progesterone concentration and CL-CSA decreased over time, with significant differences observed

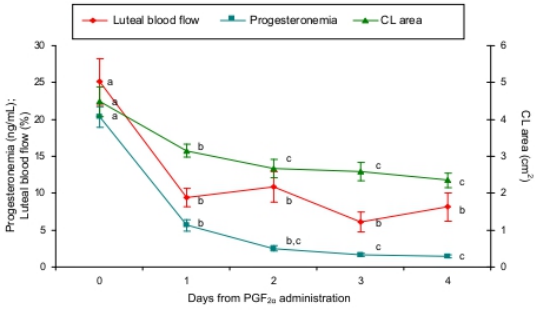

Fig. 1. Daily mean ± standard error of the mean values of progesteronemia, CL area, and luteal blood flow after PGF2α administration on Day 10 of ovulation. Means with different letters (a, b, and c) within a day are significantly different. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

at 7 and 10 hours after DT administration, respectively. Luteal blood flow tended to increase between 0 and 3 hours after DT administration; it then progressively decreased until 24 hours, with significant differences observed between 3 and 10 hours and between 10 and 24 hours (Figs. 3 and 4; Table 1).

A negative correlation ($r = -0.71$, $P < 0.001$) was seen between the time of sampling and plasma progesterone, luteal blood flow, and CL-CSA. Plasma progesterone showed a positive correlation with CL-CSA ($r = 0.33$; $P < 0.01$) but no correlation with luteal blood flow. However, a positive correlation between CL-CSA and luteal blood flow ($r = 0.48$; $P < 0.001$) was observed.

4. Discussion

Exogenous prostaglandins are commonly used in large farm animal reproduction programs, although gaps exist in our understanding of the luteolytic process in some species. In the present study, plasma progesterone, luteal blood flow, and CL-CSA were examined to assess the activity of the CL after induced luteolysis in the jenny. Corpus luteum echogenicity and uterine tone were also monitored.

All the CL detected showed strong echogenicity and a hyperechogenic central area [40]. In contrast, an active CL in the mare is normally characterized by a uniform echogenic pattern with a hypoechogenic central area [20].

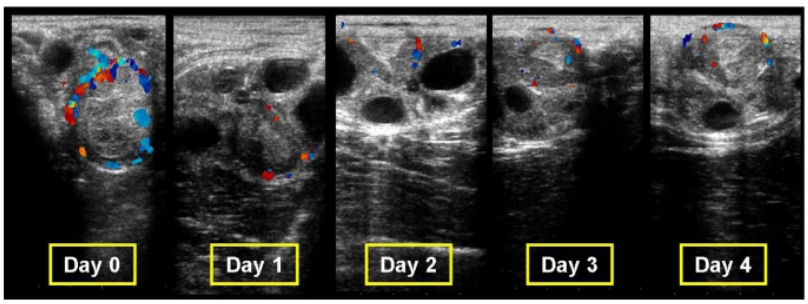

Fig. 2. Representative images of the luteal blood flow assessed by color Doppler ultrasound before PGF2α administration (Day 0) and daily over the next 4 days. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

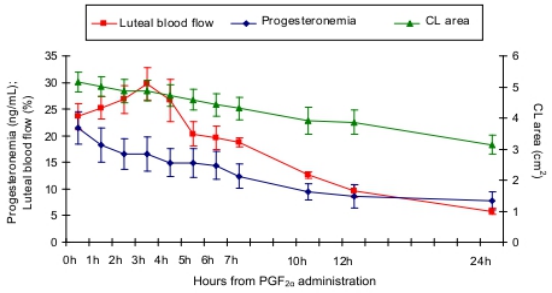

Fig. 3. Mean ± standard error of the mean values of progesteronemia, CL area, and luteal blood flow during the first 24 hours of PGF2α administration on Day 10 of ovulation. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Echogenicity and uterine tone are generally used to predict ovulation in equine veterinary practice [38]. In the present jennies, echogenicity was found to be positively correlated with the plasma progesterone concentration, but the uterine tone tended to decrease in the presence of this hormone.

Starting from peak plasma progesterone values of greater than 20 ng/mL, reductions were seen in the present jennies as soon as 1 hour after DT administration. However, even by Day 3, they had not fallen below 2 ng/mL. Similar results have been reported for Martina Franca jennies, although lower plasma progesterone concentrations (8–10 ng/mL) were observed before PGF2α treatment on Day 3 of ovulation [37]. In the mare, however, rapid luteolytic activity occurs with plasma progesterone falling to less than 1 ng/mL by 10 hours after PGF2α treatment [41]. Similar reductions in plasma progesterone are seen by 30 minutes in the cow [31,32]. A temporary increase in plasma progesterone, along with LH, FSH, and cortisol, has been described in mares, with a peak at 10 minutes after PGF2α treatment [42]. No such behavior, however, was seen in the present jennies.

An increase in luteal blood flow appears to be a universal phenomenon in mares whether luteolysis is natural or induced [34,36,43–46]. A transient increase in luteal blood flow between 1 and 3 hours was also seen in the present jennies. In cows too, an early increase (at 0–0.5 hours) in luteal blood flow occurs after natural and induced luteolysis, remaining elevated for up to 2 hours, at which time CL vascularization begins to decrease [34,47]. Interestingly, a lack of intraluteal vascular changes in response to PGF2α treatment appears to be responsible for the refractory luteolytic action seen in cows before Day 5 after ovulation [31].

Prostaglandin synthesis from arachidonic acid and the expression of COX-2 in the late luteal phase have been reported in cows and ewes [48,49]. In mares, an increase in cortisol has been observed after the administration of a single bolus of PGF2α [42]. Thus, although more studies are needed, luteolysis should be understood as a physiological process of inflammation involving a release of prostaglandins, other hormones, and cytokines, which results in ischemia-induced necrosis of the luteal tissue [14]. As described in the cow, an increase in luteal blood flow may be triggered by vasodilatory prostaglandin I2 or prostacyclin and nitrogen oxide release from arterioles surrounding the CL; this probably also causes vasodilatation in the active CL jenny after induced luteolysis. The secretion of angiotensin II and endothelin 1 is thus stimulated from microcapillary vessels in the CL, resulting in vasoconstriction of the arterioles and, eventually, luteolysis [18,31,34,43]. Recently, Korzewa et al. [50] described cytotoxic and proapoptotic effects for PGF2α *in vitro* that caused luteal cell death in the cow. In addition, the effect of DT (also used in the present study) was found to be more similar to natural luteolysis than that induced by synthetic prostaglandins. In cows, plasma progesterone decreases by 0.5 hours after PGF2α administration, but luteal blood flow remains unchanged until 8 hours, coinciding with the initiation of structural luteolysis as reflected by a significant reduction in CL volume [31]. In mares, CL-CSA decreases while luteal tissue echogenicity increases after induced luteolysis [40]. In the present jennies, the CL-CSA decreased slowly over the first 48 hours compared with the luteal blood flow and plasma progesterone, suggesting that functional luteolysis manifests itself before structural luteolysis. However, this needs to be further studied.

In both experiments, the time of sampling was negatively correlated with all variables, with positive correlations seen between all the latter. Similar results were described when a whole estrus cycle was monitored in the ewe [2]. In addition, a highly significant positive correlation was detected between plasma progesterone and luteal blood flow, although this did not reach significance in experiment 2 because of the initial increase in luteal blood flow before the progressive reduction starting 3 hours after the DT treatment. Daily plasma progesterone levels in the

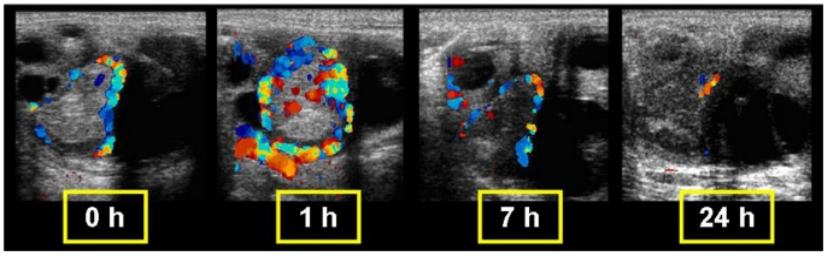

Fig. 4. Representative images of the luteal blood flow assessed by color Doppler ultrasound before (Day 0) and after 1 hour, 7 hours, and 24 hours of PGF2α administration. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Table 1
Mean ± standard error of the mean values of progesteronemia and luteal blood flow by color Doppler and CL area during the first 24 hours of PGF2α administration.

Hours of PGF2α administration (h)	Progesteronemia (ng/mL)	Luteal blood flow (%)	CL area (cm^2)
0	21.5 ± 3.08^{a}	23.65 ± 2.46^{a,d}	5.16 ± 0.31^{a}
1	18.26 ± 3.15^{a,c}	25.27 ± 2.12^{a}	5.02 ± 0.32^{a,b}
2	16.52 ± 2.84^{a,b}	26.82 ± 2.56^{a}	4.87 ± 0.37^{a,b}
3	16.55 ± 3.16^{a,b}	29.70 ± 3.05^{a}	4.87 ± 0.33^{a,b}
4	14.96 ± 2.69^{a,b}	26.71 ± 3.97^{a,e}	4.72 ± 0.34^{a,b}
5	14.88 ± 2.66^{a,b}	20.37 ± 2.27^{a,e}	4.59 ± 0.34^{a,b}
6	14.45 ± 2.57^{a,b}	19.56 ± 2.21^{a,e}	4.42 ± 0.36^{a,b}
7	12.41 ± 2.21^{b,c}	18.80 ± 0.80^{a,f}	4.31 ± 0.36^{a,b}
10	9.44 ± 1.56^{b,c}	12.62 ± 0.55^{b,d,e}	3.92 ± 0.44^{b,c}
12	8.66 ± 2.16^{b}	9.62 ± 1.24^{c,e,f}	3.86 ± 0.39^{b,c}
24	7.76 ± 1.66^{b}	5.80 ± 0.60^{c}	3.14 ± 0.31^{c}

$^{a-f}$Different superscripts in the same column indicate significant differences.

jenny might therefore be reliably predicted by color Doppler assessment of the luteal blood flow, making it a useful noninvasive tool for rapidly evaluating CL function, as described in other species [25,28].

In conclusion, exogenous administration of DT to jennies on Day 10 of ovulation induced a progressive reduction in the plasma progesterone concentration and CL-CSA. However, plasma progesterone did not fall to less than 2 ng/mL until after Day 3 after DT administration, indicating it to have a slower luteolytic effect than that in mares and cows. The luteal blood flow increased over the first 3 hours after the induction of luteolysis, after which time, it continued to decrease as in the cow but not as in the mare. The very strong correlation seen between the luteal blood flow and the plasma progesterone concentration suggests color Doppler to be an excellent means of monitoring induced luteolysis in jennies.

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3.2. Artículo 2

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Article

Seminal Plasma, Sperm Concentration, and Sperm-PMN Interaction in the Donkey: An In Vitro Model to Study Endometrial Inflammation at Post-Insemination

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Abstract: In the donkey, artificial insemination (AI) with frozen-thawed semen is associated with low fertility rates, which could be partially augmented through adding seminal plasma (SP) and increasing sperm concentration. On the other hand, post-AI endometrial inflammation in the jenny is significantly higher than in the mare. While previous studies analyzed this response through recovering Polymorphonuclear Neutrophils (PMN) from uterine washings, successive lavages can detrimentally impact the endometrium, leading to fertility issues. For this reason, the first set of experiments in this work intended to set an in vitro model through harvesting PMN from the peripheral blood of jennies. Thereafter, how PMN, which require a triggering agent like formyl-methionyl-leucyl-phenylalanine (FMLP) to be activated, are affected by donkey semen was interrogated. Finally, we tested how four concentrations of spermatozoa (100×10^6, 200×10^6, 500×10^6 and 1000×10^6 spermatozoa/mL) affected their interaction with PMN. We observed that semen, which consists of sperm and SP, is able to activate PMN. Whereas there was a reduced percentage of spermatozoa phagocytosed by PMN, most remained attached on the PMN surface or into a surrounding halo. Spermatozoa not attached to PMN were viable, and most of those bound to PMN were also viable and showed high tail beating. Finally, only sperm concentrations higher than 500×10^6 spermatozoa/mL showed free sperm cells after 3 h of incubation, and percentages of spermatozoa not attached to PMN were higher at 3 h than at 1 h, exhibiting high motility. We can thus conclude that semen activates PMN in the donkey, and that the percentage of spermatozoa phagocytosed by PMN is low. Furthermore, because percentages of spermatozoa not attached to PMN were higher after 3 h than after 1 h of incubation, we suggest that PMN-sperm interaction plays an instrumental role in the reproductive strategy of the donkey.

Keywords: seminal plasma; sperm; polymorphonuclear neutrophils; donkey

1. Introduction

Most European donkey breeds are currently in danger of extinction. However, there has been, in the recent years, an increased global demand for donkey products. As such, donkey skin, which is

used in China to make a traditional donkey-hide gelatin (Ejiao); meat; and milk, which represents an alternative to its bovine counterpart for infants with some allergic and inflammatory conditions, are also attracting the interest of the cosmetic industry. Despite this, our current knowledge of the donkey reproductive physiology is still scarce and, despite horse reproduction technologies being used, many differences between these two species exist.

Jackasses show high spermatogenic efficiency [1], and their sperm concentration, total cell number, sperm kinematic parameters and percentages of progressively motile and morphologically normal spermatozoa are higher than in stallions [2,3]. In addition, while frozen-thawed donkey spermatozoa have good post-thaw viability and motility [4–6], are able to penetrate zona pellucida free bovine oocytes in vitro [7] and yield good conception rates (50%) following artificial insemination (AI) of mares [1], their pregnancy rates are poor (0–28%) when used to inseminate jennies [8].

Oliveira et al. investigated the most suitable jackass sperm concentration for inseminating jennies and mares and concluded that increasing insemination doses up to 10^9 spermatozoa/mL augmented fertility rates in jennies (four inseminations per cycle), achieving figures similar to those obtained in mares [9]. In addition, these authors demonstrated that in order for optimal fertility of frozen-thawed donkey sperm to be reached, deep-horn intrauterine insemination should be used together with four inseminations per cycle (concentration: 10^9 spermatozoa/mL).

Natural breeding and AI induce a physiological endometrial inflammatory response, entailing neutrophils influx into the uterus. Kotilainen et al. observed that infusing spermatozoa rather than bacteria into the mare uterus provoked the influx of Polymorphonuclear neutrophils (PMN) [10]. They also reported that the highest PMN infiltration occurred when mares were inseminated with frozen-thawed or concentrated fresh semen, and that the intensity of the PMN reaction relied on sperm concentration and/or insemination volume. Therefore, the deposited semen is responsible for the uterine inflammatory response, triggering strong chemotaxis of PMN along with cytokine expression soon after insemination [11–15]. Related with this, it is worth mentioning that when inseminated with frozen-thawed semen, jennies show an exacerbated inflammatory response, higher than in mares [16], which was suggested to lead to reduced pregnancy rates [17].

On the other hand, not only does removing seminal plasma (SP) result in an improvement of quality and survival of cooled-stored donkey semen [18], but is also a required step prior to sperm cryopreservation [4]. However, SP has positive effects on sperm motility and survival into the uterus, since it helps avoid oxidative damage [19] and decreases the binding of sperm to PMN and other phagocytic cells [5,20–22]. Despite this, the mechanism through which SP modulates endometrial inflammation remains to be elucidated. Moreover, and as explained before, sperm concentration and velocity are higher in the donkey than in the horse, and their relationship with the interaction between PMN and spermatozoa is also unknown. In fact, our current knowledge comes from in vitro studies focused on sperm:PMN binding in the donkey, the rich PMN samples being obtained through uterine washings at 6 h post-AI [22,23]. However, performing too many uterine washings may produce significant injuries on the endometrium and reduce the reproductive performance, which is especially critical in the case of endangered breeds.

Against this background, the objectives of the present study were: (1) to set a method aimed at obtaining a PMN- rich fraction from jenny peripheral blood; and (2) to determine whether the whole semen, with the presence of SP, and different sperm concentrations affect the percentages of spermatozoa attached to PMN.

2. Results

2.1. Experiment 1: Recovering Donkey PMN from Peripheral Blood Samples

We tested seven protocols, as described in Materials and Methods. Protocols 1 to 4 yielded concentrations lower than 100×10^3 PMN/μL. Similarly, protocols 5 and 6 also harvested concentrations lower than 100×10^3 PMN/μL, although they were slightly higher in some biological replicates.

In contrast, Protocol 7 showed significantly ($p < 0.05$) higher PMN concentrations than the others, with values greater than 100×10^3 cells/μL in all cases (Table 1).

Table 1. PMN concentration ($\times 10^3$ cells/μL) obtained after conducting different PMN isolation protocols (Experiment 1).

Protocol	[PMN] ($\times 10^3$ cells/μL)
1	8.23 ± 0.87^{a}
2	$6.20 \pm 1.23^{a,b}$
3	3.37 ± 0.95^{b}
4	56.16 ± 6.31^{c}
5	93.70 ± 7.07^{d}
6	126.07 ± 10.01^{d}
7	307.07 ± 19.77^{e}

Different superscript letters (a, b, c, d, e) mean significant ($p < 0.05$) differences between protocols. Data are shown as mean ± SEM for seven separate experiments.

2.2. Experiment 2: Evaluation of PMN-sperm Binding (FMLP and DMSO)

2.2.1. Sperm Motility

Sperm motility was evaluated through Computer-Assisted Sperm Analysis (CASA). However, we found difficulties while using this device, since CASA systems track the head to determine sperm movement, and a significant number of sperm cells were attached to PMN and showed tail beating. Therefore, it was required for us to distinguish between attached and non-attached (free) sperm populations.

Evaluation of sperm motility in the free population, which was evaluated by CASA, evidenced that after 3 h and 4 h of incubation, most spermatozoa of this population were immotile. However, a high percentage of spermatozoa attached to PMN showed high tail beating and, occasionally, some were released and exhibited high velocity and progressiveness (average path velocity, VAP ≥ 95 μm/s; percentage of straightness, STR ≥ 73%; percentage of linearity, LIN ≥ 65%; amplitude of lateral head displacement, ALH ≤ 2.7 μm; and frequency of head displacement, BCF ≥ 13.5 Hz). Interestingly, only treatments with sperm concentrations higher than 500×10^6 sperm/mL showed free motile spermatozoa after 3 h of incubation (Supplementary File 1). For this reason, the relationship between PMN and spermatozoa was investigated in treatments containing 500×10^6 sperm/mL (Table 2; Figures 1–5).

Table 2. Sperm viability in different treatments and after incubation at 37 °C for 4 h (Experiment 2). Sperm concentration was set at 500×10^6 sperm/mL in all cases.

Treatment	0 h	1 h	2 h	3 h	4 h
PMN + SP	$77.9 \pm 4.7^{1,a}$	$65.2 \pm 7.0^{1,2,a}$	$55.8 \pm 6.0^{2,3,a}$	$43.2 \pm 4.3^{3,a}$	$30.5 \pm 3.3^{4,a}$
PMN + SP + FMLP	$73.9 \pm 6.8^{1,a}$	$62.5 \pm 7.1^{1,2,a}$	$53.6 \pm 7.1^{2,3,a}$	$38.7 \pm 4.5^{3,4,a}$	$28.9 \pm 3.4^{4,a}$
PMN + SP + DMSO	$74.9 \pm 7.4^{1,a}$	$62.3 \pm 8.1^{1,2,a}$	$54.7 \pm 6.8^{2,3,a}$	$43.0 \pm 8.3^{3,4,a}$	$30.7 \pm 7.3^{4,a}$
SP + DMSO	$74.5 \pm 6.1^{1,a}$	$64.2 \pm 7.0^{1,2,a}$	$55.0 \pm 6.8^{2,3,a}$	$43.3 \pm 4.8^{3,a}$	$31.2 \pm 4.9^{4,a}$
SP	$77.7 \pm 6.3^{1,a}$	$68.1 \pm 5.3^{1,2,a}$	$55.5 \pm 5.8^{2,3,a}$	$45.1 \pm 6.5^{3,a}$	$28.3 \pm 4.8^{4,a}$

Different superscripts (a, b) mean significant ($p < 0.05$) differences between treatments within a given time point, and different numbers (1, 2) mean significant ($p < 0.05$) differences between incubation times within a given treatment. Data are shown as mean ± SEM for seven experiments. PMN: polymorphonuclear neutrophils; SP: seminal plasma; DMSO: dimethyl sulfoxide; FMLP: formyl-methionyl-leucyl-phenylalanine.

2.2.2. Sperm Viability

Sperm viability decreased significantly ($p < 0.05$) after 3 h of incubation, irrespective of the treatment (Table 2). In addition, no significant differences ($p > 0.05$) in the viability of free spermatozoa, were observed between treatments. On the other hand, dimethyl sulfoxide (DMSO) alone or in the presence of PMN showed no detrimental effects on sperm survival.

At the beginning of the experiment (0 h), percentages of viable spermatozoa attached to PMN in the presence of FMLP were significantly ($p < 0.05$) higher than in the other treatments. In addition, although percentages of viable spermatozoa bound to PMN in the treatment containing DMSO were significantly ($p < 0.05$) lower at 1 h and higher at 3 h than in the other ones, no significant differences between treatments were observed at 2 h and 4 h (Figure 1). In the case of the treatment containing SP, neither variations along the incubation time, nor significant differences with other treatments after 2 h and 4 h of incubation were observed ($p > 0.05$; Figure 1).

2.2.3. Sperm-PMN Binding

Percentages of spermatozoa attached to PMN increased along the incubation time, reaching significantly ($p < 0.05$) higher values with regard to the beginning of the experiment after 4 h of incubation. However, the presence of FMLP or DMSO did not differ from the treatment that did not contain these two agents (i.e., PMN+SP; Figure 2). Moreover, the number of spermatozoa attached to PMN (sperm:PMN ratio) showed a progressive decrease along the incubation period, and was significantly ($p < 0.05$) higher in the PMN + SP treatment than in the ones containing FMLP or DMSO after 0 h, 2 h and 3 h of incubation (Figure 3).

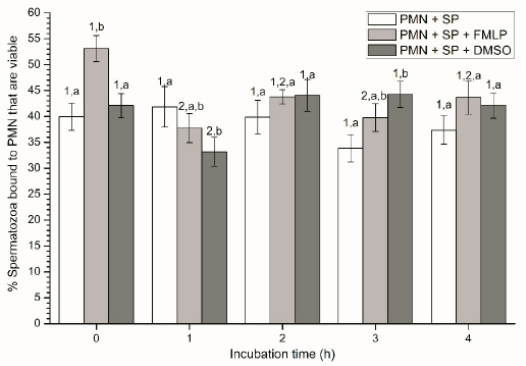

Figure 1. Percentages of viable spermatozoa bound to PMN in the three treatments (PMN + SP, PMN +SP + FMLP and PMN + SP + DMSO) throughout the incubation time (4 h; Experiment 2). Different superscripts (a, b) mean significant ($p < 0.05$) differences between treatments within a given time point, and different numbers (1, 2) mean significant ($p < 0.05$) differences between incubation times within a given treatment. Data are shown as mean ± SEM for seven experiments. Sperm concentration: 500×10^6 sperm/mL. PMN concentration: 100×10^6 PMN/mL. PMN: polymorphonuclear neutrophils; SP: seminal plasma; DMSO: dimethyl sulfoxide; FMLP: formyl-methionyl-leucyl-phenylalanine.

2.2.4. Sperm Attachment to PMN via Their Head or Tail

Percentages of spermatozoa attached to PMN via sperm head varied between 37% and 52% on average (Figure 4). At 0 h, percentages of spermatozoa attached to PMN via sperm head were significantly ($p < 0.05$) higher in the treatment containing SP than in that containing SP and DMSO. In contrast, at 1 h, no significant differences ($p > 0.05$) in the percentages of spermatozoa attached to PMN via sperm head were observed between treatments. Whereas those percentages were significantly ($p < 0.05$) higher in the treatment with SP than in that containing SP and FMLP after 2 h of incubation, these differences were not observed at 3 h, when percentages of spermatozoa attached to PMN via sperm head were significantly ($p < 0.05$) higher in the treatment containing SP and DMSO than in those containing either SP, or SP, and FMLP. Finally, at the end of the experiment, data resembled to those of 0 h, since percentages of spermatozoa attached to PMN via sperm head were significantly ($p < 0.05$) higher in the treatment containing SP than in that containing SP and DMSO (Figure 4).

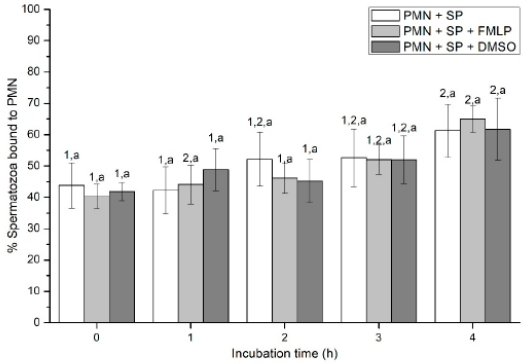

Figure 2. Percentages of spermatozoa bound to PMN in the three treatments (PMN + SP, PMN + SP + FMLP and PMN + SP + DMSO) throughout the incubation time (4 h; Experiment 2). Different superscripts (a, b) mean significant ($p < 0.05$) differences between treatments within a given time point, and different numbers (1, 2) mean significant ($p < 0.05$) differences between incubation times within a given treatment. Data are shown as mean ± SEM for seven experiments. Sperm concentration: 500×10^6 sperm/mL. PMN concentration: 100×10^6 PMN/mL. PMN: polymorphonuclear neutrophils; SP: seminal plasma; DMSO: dimethyl sulfoxide; FMLP: formyl-methionyl-leucyl-phenylalanine.

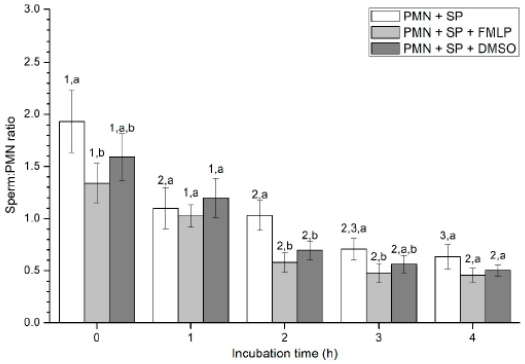

Figure 3. Number of spermatozoa bound to PMN (sperm:PMN ratio) in the three treatments (PMN+SP, PMN+SP+FMLP and PMN+SP+DMSO) throughout the incubation time (4 h; Experiment 2). Different superscripts (a, b) mean significant ($p < 0.05$) differences between treatments within a given time point, and different numbers (1, 2) mean significant ($p < 0.05$) differences between incubation times within a given treatment. Data are shown as mean ± SEM for seven experiments. Sperm concentration: 500×10^6 sperm/mL. PMN concentration: 100×10^6 PMN/mL. PMN: polymorphonuclear neutrophils; SP: seminal plasma; DMSO: dimethyl sulfoxide; FMLP: formyl-methionyl-leucyl-phenylalanine.

Percentages of spermatozoa attached to PMN via sperm tail ranged between 46% and 69% on average, and significant ($p < 0.05$) differences between incubation times and between treatments within the same incubation time were found (Figure 5). In effect, percentages of spermatozoa bound to PMN via the sperm tail at 0 h were significantly ($p < 0.05$) higher in the treatment with SP (PMN + SP) than in that containing SP and DMSO (PMN + SP + DMSO). In addition, whereas percentages of spermatozoa bound to PMN via the tail were significantly ($p < 0.05$) higher after 1 h and 3 h than at 0 h, no significant different differences ($p > 0.05$) between 0 h, 2 h and 4 h were observed. After 4 h of incubation, the treatment containing DMSO showed significantly ($p < 0.05$) higher percentages of spermatozoa bound to PMN via the tail than the other two treatments (Figure 5).

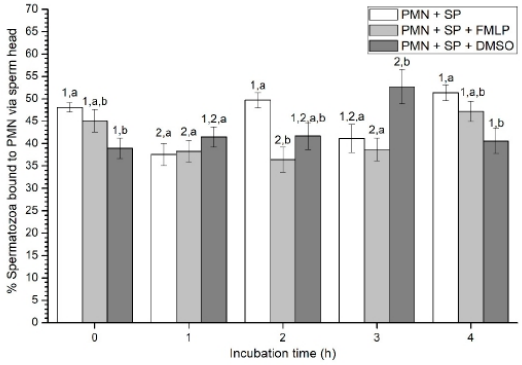

Figure 4. Percentages of viable spermatozoa bound to PMN via sperm head in the three treatments (PMN + SP, PMN + SP + FMLP and PMN + SP + DMSO) throughout the incubation time (4 h; Experiment 2). Different superscripts (a, b) mean significant ($p < 0.05$) differences between treatments within a given time point, and different numbers (1, 2) mean significant ($p < 0.05$) differences between incubation times within a given treatment. Data are shown as mean ± SEM for seven experiments. Sperm concentration: 500×10^6 sperm/mL. PMN concentration: 100×10^6 PMN/mL. PMN: polymorphonuclear neutrophils; SP: seminal plasma; DMSO: dimethyl sulfoxide; FMLP: formyl-methionyl-leucyl-phenylalanine.

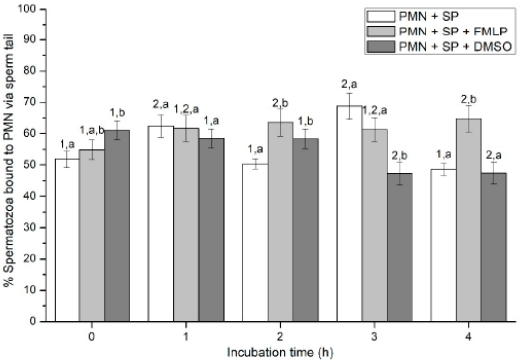

Figure 5. Percentages of viable spermatozoa bound to PMN via sperm tail in the three treatments (PMN + SP, PMN + SP + FMLP and PMN + SP + DMSO) throughout the incubation time (4 h; Experiment 2). Different superscripts (a, b) mean significant ($p < 0.05$) differences between treatments within a given time point, and different numbers (1, 2) mean significant ($p < 0.05$) differences between incubation times within a given treatment. Data are shown as mean ± SEM for seven experiments. Sperm concentration: 500×10^6 sperm/mL. PMN concentration: 100×10^6 PMN/mL. PMN: polymorphonuclear neutrophils; SP: seminal plasma; DMSO: dimethyl sulfoxide; FMLP: formyl-methionyl-leucyl-phenylalanine.

2.3. Experiment 3: Evaluation of PMN-sperm Binding (Sperm Concentration)

Sperm-PMN Binding

Since results from Experiment 2 showed no significant effects from the presence of FMLP or DMSO, the evaluation of how different sperm concentrations (100×10^6, 250×10^6, 500×10^6, and 1000×10^6 spermatozoa/mL) affected sperm-PMN binding only took into consideration the treatments that did not contain either FMLP or DMSO.

Percentages of spermatozoa attached to PMN were significantly ($p < 0.05$) higher in the treatment containing 1000×10^6 spermatozoa/mL than in those containing lower sperm concentrations, at 0 h

and 1 h. However, these differences between sperm concentrations were not observed after 2 h of incubation. Regardless of sperm concentration, sperm:PMN ratios decreased along the incubation time, reaching the lowest levels after 3 h of incubation (Figure 6).

With regard to sperm viability, percentages of viable spermatozoa ranged from 30% to 50% on average, without much variation throughout the incubation time (Figure 7). While samples with the highest sperm concentration (1000×10^6 spermatozoa/mL) showed the highest percentage of viable spermatozoa attached to PMN at 0 h and 1 h, no significant differences with the other concentrations were observed after 2 h of incubation.

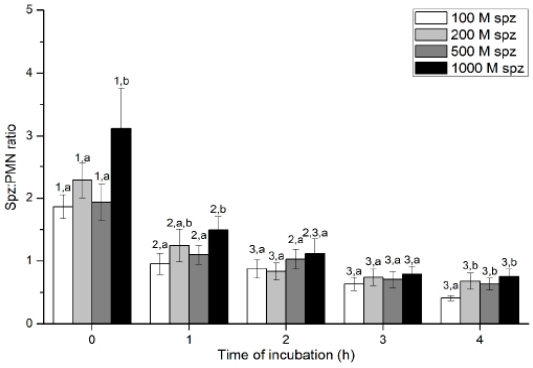

Figure 6. Number of spermatozoa bound to PMN (sperm:PMN ratio) after incubation with four different sperm concentrations (100×10^6, 250×10^6, 500×10^6, and 1000×10^6 spermatozoa/mL) for 4 h (Experiment 3). Different superscripts (a, b) mean significant ($p < 0.05$) differences between treatments within a given time point, and different numbers (1, 2) mean significant ($p < 0.05$) differences between incubation times within a given treatment. Data are shown as mean ± SEM for seven experiments. PMN concentration: 100×10^6 PMN/mL.

3. Discussion

Endometrial inflammation in the jenny after insemination was previously investigated in vitro using uterine flushing collected at 6 h post-AI [22]. Although an enriched PMN fraction from that fluid was obtained (100×10^3 cells/mL on average) in the aforementioned study, continued uterine washings can detrimentally impact the female reproductive tract and, in turn, cause infertility. Since the Catalan donkey is an endangered breed and in order to avoid the aforementioned undesirable side effect, the first aim of the current work was to set an in vitro model by isolating PMN from the peripheral blood. From the seven protocols tested, one (which was designated as Protocol 7) clearly showed the highest recovery PMN rate, yielding $\geq 100 \times 10^3$ cells/mL. As distinct from our results, other protocols tested in other species [24,25], including the equine [26,27], were proven to be unable to isolate enough PMN from the peripheral blood.

In vitro experiments evaluating the activity of PMN in the horse previously used different activating agents, such as formyl-methionyl-leucyl-phenylalanine (FMLP) or recombinant equine interleukin 8 (reqIL-8) [26]. In the current study, while the whole semen (including spermatozoa and SP) was found to be able to stimulate PMN, the presence of FMLP did not alter the percentages of spermatozoa bound to PMN, nor was the viability of free and bound-to-PMN sperm populations over the incubation time affected. These data are in line with previous reports conducted in donkeys, in which SP was found to modulate sperm-PMN binding and sperm motility [22]. On the other hand, the decrease in sperm survival throughout the incubation time was independent from the presence of PMN. While the viability of the sperm population attached to PMN at 0 h was higher using FMLP, perhaps as a result of a fast PMN stimulation, no differences between treatments and over incubation

were observed. This suggests that the viability of the sperm population attached to PMN is higher than that of the unattached (free) sperm population.

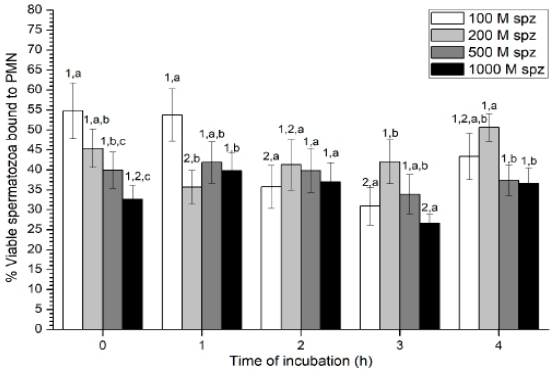

Figure 7. Percentages of viable spermatozoa bound to PMN after incubation with four different sperm concentrations (100×10^6, 250×10^6, 500×10^6, and 1000×10^6 spermatozoa/mL) for 4 h (Experiment 3). Different superscripts (a, b) mean significant ($p < 0.05$) differences between treatments within a given time point, and different numbers (1, 2) mean significant ($p < 0.05$) differences between incubation times within a given treatment. Data are shown as mean ± SEM for seven experiments. PMN concentration: 100×10^6 PMN/mL.

Remarkably, we observed that few spermatozoa appeared to be phagocytosed by PMN, since while many attached the PMN surface, only some sperm cells were found to be within a stained halo surrounding less colored PMN. While one could not completely exclude that there was non-observed phagocytosis, which would need further specific studies including time-lapse microscopy, it was very apparent that an important proportion of spermatozoa remained surrounding PMN. Polymorphonuclear neutrophils are the first barrier in front of pathogens, via phagocytosis or through releasing their own DNA, histones, and enzymes, including cathepsin (CAT), elastase (ELA) and myeloperoxidase (MPO). In the latter case, this creates complexes known as neutrophil extracellular traps (NETs; NETosis), which prevent the dissemination of pathogens [28–32]. According to Branzk et al. [33], whether PMN trigger phagocytosis or NETosis depends on the size of the pathogen; in effect, while the contact of bacteria with PMN induces phagocytosis, the presence of larger pathogenic cells (such as the yeast ones) activates NETosis. The sperm:PMN binding observed herein warrants further research on two different realms. On the one hand, studies on the potential ligand/receptor mechanism through which PMN triggers phagocytosis upon sperm:PMN binding are required to confirm our reduced percentages of sperm phagocytosis by PMN. On the other hand, and because the formation of NETs was reported on in humans and other species [20,34–37], further research on whether and how NETs are formed when PMN and sperm are incubated in the donkey is much warranted. NETs are known to trap mammalian spermatozoa in the vicinity of PMNs (i.e., without direct contact), which results in a significant loss of sperm motility [34]. For this reason, we suggest that in a similar fashion to that investigated in other species [31,32,38], additional studies focused upon staining of DNA and PMN enzymes should be conducted. Remarkably, addressing this issue is of high relevance in this context, since the dynamics of sperm:PMN binding observed in our study suggests that sperm phagocytosis could be inhibited, which, as indicated by Branzk et al. [33], would lead to the activation of NETosis. Indeed, deficiencies in the activity of phagocytic receptors were suggested to disrupt the size-dependent selectivity of NET-release due to inefficient phagocytosis. Moreover, blocking of Dectin-1, a phagocytic receptor present in human PMN, with a specific antibody is known to lead to lower rates of phagocytosis and higher NETosis response. Based on the low percentages of phagocytosis, we suggest that seminal plasma factors could inhibit PMN

receptors involved in the induction of phagocytosis, which would lead to the activation of NETosis in a similar fashion to that reported by Branzk et al. [33] when Dectin-1 is inhibited.

In the present study, we observed that a significant number of spermatozoa attached to PMN were viable and showed tail beating. In addition, not only were the percentages of viable spermatozoa higher in the bound-to-PMN than in the free sperm population, but sperm cells that were apparently released from PMN after 3 h of incubation also showed good motility. Moreover, we also noticed that during the first hours of incubation, spermatozoa tended to attach PMN through the tail rather than through the head. Since we do not know whether this observation has any relationship with the sperm ability to release from PMN, future studies should also contemplate, through time-lapse microscopy, the binding dynamics between spermatozoa and PMN and the relevance of each sperm compartment. All these data suggest that the attachment of spermatozoa to PMN does not always result in the clearance of the formers, but this apparent transient binding could play an important role in the donkey reproduction strategy, since it is quite likely that sperm cells released from PMN are able to fertilize.

At the beginning of the experiment, percentages of spermatozoa bound to PMN were significantly higher in the treatment containing the highest sperm concentration (1000×10^6 spermatozoa/mL). This could be explained by the higher likelihood of a given PMN to interact with a given spermatozoon, as sperm concentrations increase. However, it is worth noting that these differences in sperm:PMN binding observed between sperm concentrations were not found after 3 h of incubation. In addition, whereas both sperm:PMN ratios and percentages of viable spermatozoa decreased throughout incubation, percentages of spermatozoa attached to PMN increased over successive incubation times. These data indicate that PMN could be progressively activated throughout incubation, thereby attaching a higher number of spermatozoa. Nevertheless, percentages of viable spermatozoa attached to PMN (approximately 50%) showed no variations between sperm concentrations and incubation times. This suggests that the higher the sperm concentration, the higher the possibility of observing free spermatozoa after 3 h and 4 h of incubation. This hypothesis would be in agreement with our CASA observations, as only concentrations higher than 500×10^6 sperm/mL showed free motile spermatozoa at the end of the experiment.

Oliveira et al. observed an increase in fertility rates after inseminating jennies with high concentrated sperm doses [9]. Since jackasses usually show good semen quality with higher sperm concentration and motility than in stallions [1,3], one could suggest that this better sperm quality could play an important physiologic role for the post-breeding endometrial reaction in the jenny. Indeed, as explained before, high sperm concentrations with good motility appear to be required in order for spermatozoa to be released from PMN after binding. However, an important question herein is whether spermatozoa that ultimately fertilize the oocyte belong to the sperm population that are released from PMN. Therefore, future studies should also focus on the relationship between PMN and spermatozoa at post-breeding in the donkey.

4. Materials and Methods

4.1. Animals

Three Catalonian jackasses, aged 5–11 years, and three Catalonian jennies, aged 5–8 years, were used in this study. All animals were in good health conditions and of proven fertility. While conducting experiments, they were housed at the experimental farm, Faculty of Veterinary Science, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès; Spain). Jackasses were maintained in individual paddocks, whereas jennies were grouped in a big paddock. Animals were fed grain forage, straw and hay, and water was provided ad libitum. The study was approved by the Ethics Committee, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain; Code: CEEAH 1424, 23. 04.2018).

4.2. Experiment 1. Isolation of Donkey PMN from Peripheral Blood

Peripheral blood was collected from three different jennies via jugular venipuncture with 10 mL BD Vacutainer® tubes containing 18.0 mg of Ethylenediaminetetraaceticacid (EDTA; BD-Plymouth, UK). Eight tubes were collected per jenny and protocol, and seven protocols were tested. Samples were protected from the light, and immediately transported to the laboratory at room temperature within the following 10 min. To isolate PMN and adjust the concentration to 100×10^3 PMN/mL, different protocols used in other species were evaluated. Each protocol was replicated seven times.

4.2.1. Protocol 1

This protocol was based on the method set by Baumber et al. [26] to isolate equine neutrophils. Briefly, whole blood samples were centrifuged at 200× *g* and room temperature for 15 min. Supernatants (blood plasma) were discarded and pellets containing leukocytes and erythrocytes (2.5 mL) were layered onto four tubes containing 2.5 mL of 59% and 2.5 mL of 75% Percoll solutions (Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany). Samples were centrifuged at 400× *g* and room temperature for 20 min. Neutrophils were collected from the interface of the 59/75% Percoll gradient, resuspended in 5 mL PBS and centrifuged at 200× *g* and room temperature for 10 min. Pellets were resuspended in 1 mL PBS and samples were analyzed through a hematological cytometer (ADVIA 120 Siemens Medical Solutions; Fernwald, Germany) to determine the concentration of PMN per mL.

4.2.2. Protocol 2

This protocol was based on the method set by Siemsen et al. [25] designed to isolate non-human neutrophils. Blood samples were collected in tubes containing EDTA and then incubated at 37 °C in a water bath for 30 min, which was the time required for red blood cells to stack. The leukocyte-rich plasma fraction was aspirated and layered onto four tubes containing 2.5 mL of 70% and 2.5 mL of 85% Percoll solutions. Samples were centrifuged at 400× *g* and room temperature for 20 min. Neutrophils were collected from the interface of the 70/85% Percoll gradient, resuspended in 2 mL PBS and washed twice at 200× *g* for 10 min with PBS. The resulting pellets were resuspended in 1 mL PBS and then analyzed through a hematological cytometer (ADVIA 120 Haemathology System, Siemens Healthcare GmbH, Erlangen, Germany) to determine the concentration of PMN per mL.

4.2.3. Protocol 3

The third protocol was based on that of Roth and Kaeberle, which was set to isolate bovine PMN [24]. In brief, blood samples collected with vacutainer tubes were gathered in 15-mL conical tubes and centrifuged at 400× *g* for 15 min. The leukocyte layer was aspirated with some plasma and placed onto 4 mL of Ficoll (Hystopaque 1077®, Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany); samples were centrifuged at 300× *g* and room temperature for 30 min. Supernatants were discarded and the white layer just below the Ficoll one was aspirated and transferred into a clean tube. Samples were added with two volumes of distilled water, mixed and incubated at room temperature for 5–7 s to lyse the residual erythrocytes. Following this, samples were immediately mixed in an equal volume of 1.7% NaCl solution to restore isotonicity, and subsequently centrifuged at 250× *g* for 10 min. Supernatants were discarded and pellets were resuspended in 1 mL of Lactate Ringer (B Braun, Rubí, Barcelona, Spain). Resuspended pellets were analyzed using a hematological cytometer (ADVIA 120 Siemens Medical Solutions) to determine the concentration of PMN per mL.

4.2.4. Protocol 4

This protocol was based on that from Loftus et al. [27], which aims at isolating equine PMN. Blood samples were cooled down and maintained at 4 °C until visibly sedimented, which lasted for approximately 20 min. The leukocyte rich plasma layer was transferred into a new tube and centrifuged at 250× *g* and 4 ºC for 10 min. Thereafter, the pellet was resuspended in 40 mL of ice-cold 0.9% NaCl,

and the resulting cell suspension was layered onto 10 mL Hystopaque 1077® (Sigma- Aldrich®) and centrifuged at 300× *g* and room temperature for 40 min. The liquid layer was discarded and the pellet was resuspended in 4 mL ice-cold PBS. Following this, 1 mL of the resulting cell suspension was layered onto 9 mL of 90% Percoll and centrifuged at 650× *g* and room temperature for 40 min. The neutrophil fraction was collected, mixed with 4 mL PBS, and centrifuged at 300× *g* for 8 min. Pellets were subsequently resuspended in 1 mL PBS, and then analyzed with a hematological cytometer (ADVIA 120 Siemens Medical Solutions) to determine the concentration of PMN per mL.

4.2.5. Protocol 5

The fifth protocol was a modification from Protocol 1 which as previously mentioned (see Section 4.2.1), was based on Loftus et al. [27]. In brief, blood samples were collected in tubes containing EDTA. Samples were incubated in a water bath at 37 °C for 30 min to allow red blood cells to stack, forming rouleaux. The leukocyte-rich plasma layer was subsequently aspirated, transferred into 15-mL conical tubes, and centrifuged at 402× *g* and 4 °C for 5 min. Cells were resuspended with 4 mL ice-cold PBS and then centrifuged at 250× *g* and 4 °C for 5 min. Resulting pellets were resuspended in 500 µL ice-cold PBS containing 2% paraformaldehyde, and maintained on ice for 1 h. Thereafter, cells were centrifuged and resuspended in 500 µL PBS, and the resulting sample was analyzed with a hematological cytometer (ADVIA 120 Siemens Medical Solutions) to determine the concentration of PMN per mL, prior to adjusting the concentration to 100×10^6 PMN/mL in ice-cold PBS.

4.2.6. Protocol 6

This protocol consisted of a modification of Protocol 2, which was based on that of Loftus et al. [27] for isolating equine PMN (see Section 4.2.2). With this purpose, blood samples were collected in tubes containing EDTA and then incubated in water bath at 37 °C for 30 min to allow red blood cells to stack. The leukocyte-rich plasma layer was aspirated, transferred into 15-mL conical tubes and centrifuged at 402× *g* and 4 °C for 5 min. Samples were resuspended in 4 mL ice-cold PBS and then centrifuged at 250× *g* and 4 °C for 5 min. Thereafter, cells were resuspended in 500 µL ice-cold PBS, and then analyzed with a hematological cytometer (ADVIA 120 Siemens Medical Solutions) to determine the concentration of PMN per mL. Cell concentration was adjusted to 100×10^6 PMN/mL in ice-cold PBS.

4.2.7. Protocol 7

Blood samples were collected in tubes containing EDTA and incubated in a water bath at 37 °C for 30 min, to stack red blood cells. The leukocyte-rich plasma layer was aspirated, transferred into 15-mL conical tubes, and subsequently centrifuged at 402× *g* and 4 °C for 5 min (modified from Loftus et al. [27]). The supernatant was aspirated and discarded; the pellet was resuspended in 4 mL ice-cold PBS, and centrifuged at 402× *g* and 4 °C for 5 min. Next, the supernatant was aspirated and discarded, and the upper white layer of the pellet, which contained PMN, was collected and transferred into a 1.5-mL tube. Samples were analyzed with a hematological cytometer (ADVIA 120 Siemens Medical Solutions) to determine the concentration of PMN per mL. Cell concentration was adjusted to 100×10^6 PMN/mL in ice-cold PBS.

4.3. Experiments 2 and 3: Effects of SP and Sperm Concentration on PMN-sperm Binding

4.3.1. Semen Collection and Process

Semen was collected with an artificial vagina (Hannover model) equipped with an in-line filter (Minitüb Ibérica SL; Reus, Spain) to get a gel-free semen sample. Semen volume was recorded and diluted 1:1 (*v*:*v*) with a skim-milk-based semen extender [39]. Sperm concentration was evaluated using a Neubauer chamber (Paul Marienfeld GmbH & Co. KG; Lauda-Königshofen, Germany), and four treatments containing different sperm concentrations (100×10^6, 200×10^6, 500×10^6, and 1000×10^6 sperm/mL) were prepared.

4.3.2. Treatments

Eleven treatments containing different sperm concentrations, PMN, FMLP and DMSO were prepared, as follows. In the case of Experiment 2, which tested the role of FMLP activation on PMN [26], these treatments were: (a) PMN + 100 × 10^6 sperm/mL + SP + FMLP 0.1 mM; (b) PMN+ 200 × 10^6 sperm/mL + SP + FMLP 0.1 mM; (c) PMN + 500 × 10^6 sperm/mL + SP+ FMLP 0.1 mM; (d) PMN + 1000 × 10^6 sperm/mL + SP + FMLP 0.1 mM; (e) PMN + 500 × 10^6 sperm/mL + SP + DMSO; (f) 500 × 10^6 sperm/mL + SP + DMSO; and (g) control (500 × 10^6 sperm/mL + SP sample). Treatments g and f were the controls for DMSO, which was the vehicle for FMLP dilution. Treatment g was the control for sperm viability and motility throughout incubation. In the case of Experiment 3, which aimed at assessing the effects of different sperm concentration on sperm:PMN binding, we tested the following treatments: (a) PMN + 100 × 10^6 sperm/mL + SP; (b) PMN + 200 × 10^6 sperm/mL + SP; (c) PMN + 500 × 10^6 sperm/mL + SP; and (d) PMN + 1000 × 10^6 sperm/mL + SP.

All semen samples were incubated in a water bath at 37 °C, and their viability, sperm-PMN binding and motility evaluated after 0 h, 1 h, 2 h, 3 h and 4 h of incubation.

4.3.3. Evaluation of Sperm Viability

Sperm viability was determined through eosin-nigrosin staining [40]. A minimum of 200 spermatozoa/sample were examined under a bright-field, optical microscope (Olympus Europe, Hamburg, Germany) at 1000 × magnification. We recorded percentages of viable spermatozoa (eosin-negative), and those of viable spermatozoa attached to PMN.

4.3.4. Sperm–PMN Binding

Sperm–PMN attachment was determined as previously described [20,41]. In brief, 10 µL of each sample was placed onto a slide prior to mixing with Diff-Quick stain (QCA, Amposta, Spain) and smearing [22]. A minimum of 200 sperm cells were counted under a bright-field, optical microscope at 1000 × magnification. The following three parameters were recorded: (a) percentages of spermatozoa attached to PMN; (b) sperm:PMN ratio (number of spermatozoa attached per PMN); and (c) percentages of spermatozoa attached to PMN either via their head or their tail.

4.3.5. Sperm Motility

Sperm motion characteristics in the free (unattached) sperm population were evaluated by means of a computer assisted sperm analysis (CASA) system (Integrated Semen Analysis System, ISAS® Ver.1.0.15; Projects and Services R+D SL, Proiser; Valencia, Spain). This system consisted of a negative phase-contrast microscope (Olympus BH-2) with a yellow light filter and a warm-up plate, and a digital video camera (Basler, Ahrensburg Germany) connected to a computer containing the ISAS software. Samples were placed into a pre-warmed Neubauer chamber (37 °C) and examined at 1000 × magnification, with at least 200 spermatozoa being counted per analysis.

4.4. Statistical Analyses

A statistical package (IBM SPSS Statistics 25.0; Armonk, New York, NY, USA) was used to analyze the results obtained in this work. Shapiro-Wilk and Levene tests were conducted to assess data distribution and homogeneity of variances, respectively. When required, data were transformed through arcsine $\sqrt{x}$. Following this, one-way ANOVA (experiment 1) or a linear mixed model (experiments 2 and 3) followed by post-hoc Sidak test for pair-wise comparisons were run. The treatment was considered to be the fixed-effects factor, the donkey was the random-effects factor and the incubation time was the intra-subjects factor. When, even after linear transformation, data did not show normal distribution and/or variances were not homogenous, non-parametric alternatives (Kruskal-Wallis and Mann-Whitney tests for Experiment 1; and Friedman and Wilcoxon tests for Experiments 2 and 3) were

used. The level of significance was set at $p \leq 0.05$, and data are shown as mean ± standard error of the mean (SEM).

5. Conclusions

In conclusion, the whole semen, including seminal plasma, activates PMN and induces sperm-PMN binding in jennies. However, we observed low percentages of phagocytosis upon sperm:PMN binding, and not only were the percentages of viable spermatozoa higher in the bound-to-PMN than in the free sperm population, but sperm cells that were apparently released from PMN after 3 h of incubation also showed good motility. Therefore, the fact that sperm previously attached to PMN could be later released, especially when concentration was at least 500×10^6 spermatozoa/mL, suggests that this binding does not necessarily lead to the induction of phagocytosis. For this reason, and together with the fact that NETosis has been extensively reported on in other mammalian species, further research should be aimed at elucidating: (a) through which ligand/receptor spermatozoa and PMN bind; (b) whether the contact between spermatozoa and PMN in the presence of seminal plasma always triggers phagocytosis or rather some sperm cells remain fertile upon releasing from PMN, and (c) if, in the donkey, clearance of spermatozoa through PMN also occurs via NETosis rather than phagocytosis. In the latter case, while one would expect that this could also be the case, the previously reported variations between other species along with the differences related to the semen deposition site support the need for specific experiments in the donkey.

Supplementary Materials: The following are available online at http://www.mdpi.com/1422-0067/21/10/3478/s1, **Supplementary File S1**. Movie showing sperm: PMN interaction (sperm concentration: 500×10^6 spermatozoa/mL; PMN concentration: 100×10^6 PMN/mL). It is noticeable that most sperm bound to the PMN surface exhibited high tail activity.

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Abbreviations

AI	Artificial insemination
ALH	Amplitude of lateral head displacement
BCF	Frequency of head displacement
CASA	Computer-Assisted Sperm Analysis
CAT	Cathepsin
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELA	Elastase
FMLP	Formyl-methionyl-leucyl-phenylalanine
LIN	Linearity

MPO	Myeloperoxidase
NETs	Neutrophil Extracellular Traps
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PMN	Polymorphonuclear Neutrophils
SEM	Standard error of the mean
SP	Seminal plasma
STR	Straightness
VAP	Average path velocity

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3.3. Artículo 3

Donkey jackass seminal plasma fractions attending the proteins molecular weight and their role in the control of jenny post-AI endometrial reaction.

Abstract

Artificial Insemination (AI) with frozen-thawed semen in donkeys results in low fertility rates. However, the addition of seminal plasma, removed during the freezing process, improves partially the obtained fertility. Jenny express a high inflammatory response after AI with frozen semen. High amount of polymorphonuclear neutrophils (PMN) have been observed into the jenny uterus six hours after AI. PMN are the main filter reducing and, possibly, selecting the spermatozoa that will reach the oviduct. PMN consider the spermatozoa like bacteria. Part of spermatozoa are phagocytosed, but most remain attached on the PMN surface or in the surrounding halo. Another mechanism of PMN antimicrobial action is the activation and extrusion of their DNA and associated proteins creating neutrophil extracellular traps (NETs) that block but don't kill the bacteria. Most of donkey spermatozoa attached to PMN surface are viable showing high tail beating. Seminal plasma is a complex fluid and proteins are an important part of its compounds. Part of this proteins are involved in the control of the jenny post-AI inflammatory response or related phenomena. However, there is a large amount of proteins in jackasses seminal plasma, significantly higher than in horse one, and the study of all of these proteins could be complex and very expensive. In our study six seminal plasma fractions attending the proteins molecular weight (<3, 3-10, 10-30, 30-50, 50-100 and >100 kDa) were obtained and incubated during 0,1,2,3 and 4h in an in vitro model with PMN and spermatozoa. Then free sperm motility was analysed by a CASA system and sperm motile subpopulations were statistically defined. Sperm viability was evaluated by an eosin-nigrosin stain and PMN-spermatozoa binding by a Diff-Quik staining smear. SP fractions including proteins between 30-50 kDa and 50-

100kDa showed the best maintenance of donkey sperm motile subpopulations. On the other hand, both fractions are involved in the control of the jenny post-breeding inflammatory response, showing significantly higher percentage of viable unbound spermatozoa after 3h of incubation than other treatments. Several proteins, described in horse or in donkey SP and involved in the post-AI endometritis control, in the sperm motility maintenance and in the oxidative stress control, have a molecular weight between 30 to 100kDa. Further studies are needed in other to evaluate a possible in vivo effect of these fractions and to define the proteins included and their specific role in the donkey reproduction strategy.

1. Introduction

Donkey jack shows a high spermatogenic efficiency and a relatively short length of spermatogenesis (Neves *et al.,* 2002). In general, donkey ejaculates show excellent semen quality characteristics, reduced sperm abnormalities, high percentage of live spermatozoa and high sperm motility, with higher velocity and linearity than horse sperm. Four sperm motile subpopulations were detected by a computerised system of sperm motility analysis (CASA). The characteristics of the motility patterns of these subpopulations provides a good overall indicator of semen quality (Miró *et al.,* 2005).

Donkey frozen-thawed semen shows good sperm survival and motility (Flores *et al.,* 2008; Rota *et al.,* 2008; Sabatini *et al.,* 2014). Cryopreserved jackass semen is able to penetrate zona pellucida-free bovine oocytes matured *in vitro,* to decondense and form the male pronucleus (Taberner *et al.,* 2010). Surprisingly, *in vivo,* cryopreserved donkey jack semen is able to fertilize mares to produce mules, but inseminating jennies the pregnacy rates are poor, significantly lower than in mares (Vidament *et al.,* 2009; Oliveira *et al.,* 2012; Canisso *et al.,* 2011; Glatzel *et al.,* 1982).

In mammals natural breeding or AI induce a physiological endometritis. This endometrial inflammatory response is characterised by an important

polimorphonuclear neutrofils (PMN) influx into the uterus provoked by spermatozoa rather than bacteria entrance (Kotilainen *et al.,* 1994). The amount of post-AI endometritis depends on the semen deposition site by natural breeding. Species with vaginal semen deposition, like ruminants, show a reduced inflammatory response. Thowsands milions of spermatozoa are deposited in the cranial part of the vagina, the cervix is the main filter and only thowsands arrive into the uterus, hundreds reach the oviduct and one fertilise one oocyte (Larson & Larson, 1985; López-Gatius *et al.*, 1993). But in the species with intrauterine deposition, like pig, horse or donkey, thousands of millions of spermatozoa are deposited into the uterus and PMN are the main filter, only thousands cross the uterus and also hundreds rise to the oviduct. Frozen-thawed semen induces in mares higher post-AI endometritis than fresh semen and this reaction seems to be related also with sperm concentration and inseminating volume. (Kotilainen *et al.,* 1994). Artificial Insemination involving the placing of frozen-thawed semen into the jenny uterine body is associated with an exacerbate endometrial response, higher than in mares. Large numbers of PMN and some eosinophils have been observed in the uterine lumen 6 hours after insemination of jennies with frozen-thawed semen (Rota *et al.,* 2008; Miró *et al.,* 2013)

The deposited semen is responsible for the uterine inflammatory response inducing the strong chemotaxis of PMNs as well as cytokine expression just after insemination (Troedson *et al.,* 2001; Rozeboom *et al.,* 2001; Matthijs *et al.,* 2003; Gorgens *et al.,* 2005). The jenny endometrial post-AI inflammatory pattern, analyzed by uterine biopsies, is characterised by a diffuse infiltration of PMN in the luminal epithelium and stratum compactum, along with eosinophils in the stratum compactum and the stratum spongiform surroundind the endometrial glands. Furthermore, an intense cycloxygenase-2 (COX-2) immunohistochemical labelling was detected in the luminal epithelium and glandular cells of the stratum compactum (Vilés *et al.,* 2013a).

A recent study, analysing *in vitro* the sperm-PMN interaction in donkey, showed that a reduced percentage of spermatozoa are phagocytosed by PMN, most

remain attached on the PMN surface or into a surrounding halo (Miró *et al.,* 2020).

PMN are the first barrier in front of pathogens, via killing bacteria by phagocytosis or through releasing lytic enzymes. However, it is worth mentioning that PMN have been found to be able to release their own DNA, histones, and enzymes, including cathepsin, elastase (ELA) and myeloperoxidase (MPO), creating complexes known as neutrophil extracellular traps (NETs; NETosis), which prevent bacteria dissemination (Brinkman *et al.,* 2004, Fuchs *et al .,* 2007, Kazzaz *et al.,* 2016, Nakazawa *et al.,* 2017, Rebordão *et al.,* 2018). Interestingly, most bacteria attached to NETs are still viable when released after DNase treatment (Menegazzi *et al.,* 2012).

NETs are stimulated by bull, stallion and human sperm, leading to their entrapment (Hahn *et al.,* 2012). Recently, Mateo-Otero *et al.,* (2020) showed the ability of jackass semen to induce jenny NETosis. The incubation time and the sperm concentration are positively correlated with the percentage of PMNs that realese NETs (Mateo-Otero *et al.,* 2020) and with the existence of free motile spermatozoa (Miró *et al.,* 2020).

Donkey spermatozoa has a midpiece larger than horse one (Miró *et al.,* 2017), with a high mitochondrial activity evaluated by JC-1 probe and flow citometry (Papas *et al.,* 2020). As a result of this donkey spermatozoon runs faster than horse one (Miró *et al.,* 2005). But the high metabolic activity of donkey spz produces large amounts of lactic acid (Miró *et al.,* 2005) and reactive oxigen species (ROS) (Papas *et al.,* 2020). Additionally, the post-AI PMN activity will produce much more ROS, until 100 times higher (Aitken *et al.,* 1990; Ford *et al.,* 1999).

ROS is important in the sperm capacitation process (Shi-Kai & Whan-Xi, 2017). However, high levels of ROS will induce a severe cellular damage and decreased fertility as a result of the high levels of polyunsaturated fatty acids in the sperm membrane (Muiño-Blanco *et al.,* 2008; Guthrie & Welch, 2012).

Seminal plasma is produced by the epididymis and accessory sex glands, including ductus deferens ampullae in donkey (Gacem *et al.,* 2020). In mammals, seminal plasma plays an important role for the sperm function, both in the male and female reproductive tract (Rodríguez-Martínez *et al.,* 2011). However, there is a controversy with semen preservation. The elimination of seminal plasma (SP) increases the survival spermatozoa and the maintenance of motility patterns in cooled donkey semen (Miró *et al.,* 2009). And most of seminal plasma is eliminated during the donkey sperm cryopreservation process (Flores *et al.,* 2008) as in other species.

Post-thaw addition of seminal plasma positively affects the post-thaw quality of semen from poor freezing stallions (Sichtar *et al.,* 2019). Rota *et al.,* (2012) reported an increase in jenny pregnancy rates (8/13, 61.5%) by post-thaw semen resuspension in seminal plasma. *In vitro* using a PMN rich uterine lavage post-AI, seminal plasma showed a significant decrease in the PMN/spz attachment and a maintenance of sperm motility patterns (Miró *et al.,* 2013). SP decrease the COX-2 immunohistochemical labeling both in the epithelium and surrounding the endometrial glands in jennies postAI, but no effect was observed in PMN or Eosiniophils counts in the endometrial biopsies (Vilés *et al.,* 2013) or in the uterine lavage (Vilés *et al.,* 2013, Rota *et al.,* 2012).

The raw semen, including seminal plasma, is able to induce PMN-spz interaction, and the sperm concentration seems to be important in this interaction and to ensure some sperm survival (Miró *et al.,* 2020). On the other hand, seminal plasma seems to stimulate that PMN realese NETs (Mateu-Orero, 2020). Then seminal plasma role is complex, inducing but controlling the inflammatory response.

In mammals, seminal plasma is a complex fluid, containing ions, sugars, lipids, hormones, amino acids or growth factors (Alghamdi *et al.,* 2009). Proteins represent an important part. They include spermadhesins, proteins containing fibronectin tipe II domains, and proteins exhibiting enzymatic, inhibitory, and immunomodulation activities (Jonakova *et al.,* 2010). However, seminal plasma

composition differs between species (Jonakova *et al.,* 2010), and also between individuals (Baumber & Ball, 2005).

Seminal plasma levels for glucose, proteins, lipids, cholesterol, calcium or phosphorus are significantly higher in donkey semen than in horse one (Talluri *et al.,* 2017). Donkey seminal plasma protein values are between 4 to 10 times higher than in stallion seminal plasma (Talluri *et al.,* 2017; Papas *et al.,* 2019). Enzymatic protein like LDH (Lactate deshidrogenase), with values three times higher than for stallion (Talluri *et al.,* 2017), could be related with the large amount of lactic acid released by donkey spermatozoa (Miró *et al.,* 2005). Furthermore, Talluri *et al.,* (2017) observed a positive correlation between the jack seminal volume or the sperm concentration and GOT (Glutamic oxalacetic transaminase), GPT (Glutamic piruvic transaminase) or ACP (Acid phosphatase).

On the other hand, SP is an important source of antioxidants, containing non-enzymatic and enzymatic components to control the ROS balance (Muiño-Blanco *et al.,* 2008). The antioxidant enzimatic system involved catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GSR) and glutathione peroxidase (GPX), all of them with significantly higher values in donkey SP than in horse one (Papas *et al.,* 2019). Furthermore, SOD has a positive correlation with donkey sperm cryotolerance (Papas *et al.*, 2020).

Cisteine-Rich secretory protein-3 (CRISP-3) isolated from horse seminal plasma and seminal-DNase have been described to protect spermatozoa from binding to PMN (Dotty *et al.,* 2011), and free-spermatozoa from NETs (Alghamdi & Foster, 2005).

The existence of this kind of proteins and/or other proteins with similar activity or with a role in the control of post-AI endometritis and ROS production in donkey remains unknown. Donkey SP has a large amount and undefined types of proteins. To study all SP proteins and their effect on spz:PMN interaction and ROS production is like impossible. However, fractioning SP attending proteins

molecular weight could be a good method to identify the groups of proteins involved in the jenny post-AI endometrial reaction control.

2. Material and methods

a. Animals.

Five Catalonian jackass aged 3-11 years old, and three jennies, aged 5–8 years old, were included in this study, all of them were in good health conditions, good body condition and of proven fertility. The animals were housed at the Experimental Farm, Veterinary Faculty, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès; Spain). Jackasses were maintained in individual paddocks and jennies were into a group of ten jennies in a big paddock. Animals were feed grain forage, and straw, hay and water were provided *ad libitum*. The study was approved by the Ethics Committee, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain; Code: CEEAH 1424.

b. Seminal plasma obtention

Semen was collected with an artificial vagina (Hannover model) equipped with an in-line filter (MinitübIbérica SL; Reus, Spain). Different aliquots of raw semen were distributed in 50 mL conical tubes. Semen samples were centrifuged at 3000 x g at 4ºC for 10 minutes (JP Seleca S.A., Barcelona, Spain). The supernatant was recovered into another 50 mL conical tube and was centrifuged again (this action was repeated 4 times). Then seminal plasma was examined under a phase-contrast microscope (Olympus Europe, Hamburg, Germany) to ensure that there were no spermatozoa. Centrifugations, by the same conditions, were repeated as needed until samples were sperm-free. The number of centrifugation cycles ranged between five and seven, depending on the ejaculate Free spermatozoa seminal plasma samples were stored in 15 mL Corning tubes at -80ºC.

c. Fractions of seminal plasma

SP from 5 jackass was thawed at laboratory temperature and pooled. Different SP fractions were obtained by ultracentrifugation with filters for protein purification and concentration (Amicon® filters, Merck KGaA, Darmstadt, Germany) attending the protein molecular weight. Each fraction was centrifuged at 4000xg 10 minutes and the supernatant was used to obtain the next fraction. After the entire process the following fractions were obtained. <3kDa/ 3-10kDa/ 10-30kDa/ 30-50kDa/ 50-100kDa/ >100kDa.

d. Donkey PMN isolation and concentration

Peripheral blood was collected from three different jennies via jugular venipuncture with 10mL BD Vacutainer R tubes containing 18,0 mg of Ethylenediaminetetraacetic acid (EDTA; BD-Plymouth, UK). Eight tubes were collected from each jenny and PMN were isolated following a recently described protocol (Miró *et al.,* 2020). Briefly, blood samples were incubated in EDTA tubes for 30 minutes in water bath at 37ºC, to stack red blood cells. The leukocyte-rich plasma layer was aspirated, placed in 15mL conical tubes and centrifuged at 402 x g at 4ºC for 5 min (modified from Loftus *et al.,* 2010). The supernatant was aspirated and discarded, and the pellet was resuspended in 2 ml of ice-cold PBS and centrifuged at 402 x g at 4ºC for 5 minutes. Then, the supernatant was aspirated and discarded, and the upper white layer of the pellet (this layer contains neutrophils) was collected and placed in a 1,5mL tube. Sample were analyzed by means an haematological cytometer (ADVIA 120 Siemens Medical Solutions, Fernwald, Germany) for the amount of PMN/ml. PMN concentration was adjusted to $100x10^6$ PMN/ml adding ice-cold PBS.

e. Semen collection and preparation

During the neutrophil isolation and concentration process, semen was collected with an artificial vagina (Hannover model) equipped with an in-line filter (MinitübIbérica SL; Reus, Spain) to get a gel-free semen sample. Semen volume

was recorded, and sperm concentration was evaluated using a Neubauer chamber (Paul Marienfeld GmbH & Co. KG; Lauda-Köngshofen, Germany). The obtained ejaculate was extended with a skim-milk-based extender (Kenney *et al.,* 1975) and divided in two fractions. One fraction was adjusted to 500x10^6 spz/ml concentration. The second one was centrifuged through a single layer of a silane-coated silica-based colloid (Equicoll*, Sweden) using the protocol for small tubes described by Morrell *et al.,* (2011). Briefly, 1,5 mL of extended semen was carefuly pipetted on top of 4mL of Equicoll* in a 15mL conical tube. Four tubes under the same conditions were centrifuged at 300xg, at 20ºC for 20 min. The supernatant and the colloid were discarded. The sperm pellet was diluted with the same skim-milk-based extender and was adjusted to a final concentration of the 500x10^6 spz/mL.

f. Treatments

Seven treatments were prepared. All of them using the same PMN and sperm concentration (100x10^6 PMN/ml+ 100 µl 500x10^{6}spz/ml) (A, B, C, D, E, F, G). Six treatments adding different obtained SP fractions (A, B, C, D, E, F) and a CONTROL treatment, PMN+spz (G).
Detail of different treatments:

A. 100 µl 100x10^6 PMN/ml+ 100 µl 500x10^{6}spz/ml+ 100 µl <3KD fraction
B. 100 µl 100x10^6 PMN/ml+ 100 µl 500x10^{6}spz/ml+ 100 µl 3-10KD fraction
C. 100 µl 100x10^6 PMN/ml+ 100 µl 500x10^{6}spz/ml+ 100 µl 10-30KD fraction
D. 100 µl 100x10^6 PMN/ml+ 100 µl 500x10^{6}spz/ml+ 100 µl 30-50KD fraction
E. 100 µl 100x10^6 PMN/ml+ 100 µl 500x10^{6}spz/ml+ 100 µl 50-100KD fraction
F. 100 µl 100x10^6 PMN/ml+ 100 µl 500x10^{6}spz/ml+ 100 µl >100KD fraction
G. 100 µl 100x10^6 PMN/ml+ 100 µl 500x10^{6}spz/ml (CONTROL)

All semen samples were incubated in a water bath at 37ºC, and their viability, sperm:PMN binding, motility and ROS production evaluated after 0, 1, 2, 3 and 4 h of incubation.

g. Sperm abnormalities and viability

Eosin-nigrosin stained smears (Bamba, 1988) were used to evaluate the sperm viability and abnormalities. A minimum of 200 spermatozoa per sample were analyzed under a bright-field, optical microscope (Olympus Europe, Hamburg, Germany) at 1000 x magnification. Viability and abnormalities of free spermatozoa and spermatozoa attached to PMN were evaluated.

h. Sperm:PMN binding

Sperm:PMN attachment in treatments A to I was determined as previously described (*Palm et al.,* 2006; *Alghamdi et al.*, 2004). 10 µl of each sample was deposited onto a slide and a Diff-Quick (QCA, Amposta, Spain) staining smear was performed. A minimum of 200 spermatozoa per sample were analyzed under a bright-field, optical microscope at 1000 x magnification. The following parameters were recorded: (a) number of spermatozoa attached to PMN, (b) presence of PMN agregates and number of PMN agregates (c) percentages of spermatozoa attached to PMN via their head or their tail.

i. Sperm motility

Sperm motion characteristics in the free spermatozoa were evaluated by a computer assisted sperm analysis system (CASA) (Integrated Semen Analysis System, ISAS® Ver.1.0.15; Projects and Services R+D SL, ProiSer, Valencia, Spain). The system is equipped with a negative phase contrast micro-scope Olympus BH-2, with a yellow light filter, warm-up plate and a digital video camera (Basler, Germany) connected to the computer with the ISAS software. A sample of 5 µL was deposited onto a 37◦C pre-warmed slide and a 24 mm^2 coverslip was carefully set. Digital images at 1000× magnification, were recorded and strictly analysed to suppress erroneous routes or artefacts and acquire at least 200 cells per sample.

Motility descriptors obtained after CASA analysis are:
Curvilinear velocity (VCL): The mean path velocity of the sperm head along its actual trajectory (units: μm/s).
Linear velocity (VSL): The mean path velocity of the sperm head along a straight line from the first to its least position (units: μm/s).
Mean velocity (VAP): The mean velocity of the sperm head along its average trajectory (units: μm/s).
Linearity coefficient (LIN): (VSL/VCL) x 100 (units: %).
Straightness coefficient (STR): (VSL/VAP) x 100 (units: %).
Wobble coefficient (WOB): (VAP/VCL) x 100 (units: %).
Mean amplitude of lateral head displacement (ALH): The mean value of the extreme side-to-side movement of the sperm head in each beat cycle (units: μm).
Frequency of head displacement (BCF): The frequency with which the actual sperm trajectory crosses the average path trajectory (units: Hz).
Dance: (VCL/ALH) (units: $\mu m^2/s$).
Absolute angular mean displacement (MADabs): The absolute value of the advancing angle of the sperm trajectory (units: angular degrees).
Algebraic angular mean displacement (MADalg): The algebraic value of the advancing angle of the sperm trajectory, provided that negative values indicate a clockwise displacement (units: angular degree).

j. Statistical analyses

Data were analyzed using a statistical package (IBM® SPSS® 25.0 for Windows; IBM corp., Armonk, NY, USA) and plotted with GraphPad Prism 8.0 for Windows (GraphPad Software Inc.; San Diego, CA, USA). Data were tested for normal distribution with Shapiro-Wilk test, and homoscedasticity and Levene test. The effects of centrifuging seminal plasma with different filters to harvest < 3 kDa, 3-10 kDa, 10-30 kDa, 30-50 kDa, 50-100 kDa, and > 100 kDa fractions on ROS generation, sperm viability (unbound, and bound population to PMN), and sperm binding to PMN (sperm:PMN ratio, % sperm bound to PMN) was tested through a linear mixed model followed by Sidak's test for pair-wise comparisons. The

fraction was the fixed-effects factor, the animal was the random-effects factor and the incubation time (4 h at 38 ºC) was the intra-subjects factor.

Sperm motile subpopulations were set according to the procedure described in Catalán *et al.,* (2020). In brief, the individual kinematic parameters recorded with CASA analysis (VSL, VCL, VAP, LIN, STR, WOB, ALH, BCF and DANCE) were used to run a Principal Component Analysis (PCA). As a result of the PCA, which used Varimax procedure and Kaiser normalization, provided two PCA components which explained 88,44% of variability. In addition, each spermatozoon was assigned with regression scores for each of these two PCA components. Based on these regression scores, spermatozoa were classified into motile subpopulations with a two-step cluster analysis, using the log-likelihood distance and the Schwarz's Bayesian Criterion. After identifying automatically the number of different subpopulations, which resulted to be four, percentages of spermatozoa belonging to each of these subpopulations for each SP-fraction and incubation time were calculated. These percentages were subsequently used to evaluate the effects of SP-faction and incubation time with a linear mixed model, as described before.

In all analyses, the level of s for statistical significance were set at $p \leq 0.05$. Data are shown as mean ± standard deviation (SD).

3. Results

Four sperm subpopulations with specific motility characteristics were described (Table1). The spermatozoa of Subpopulation 1 (SP1) showed high velocity (VCL and VAP), very high ALH with high BCF, but low progressiveness (low LIN and STR). Subpopulation 2 (SP2) contained spermatozoa with an excel. lent movement, high velocity (VCL, VSL and VAP), linear trajectories (high LIN and STR) and low head displacement (ALH) with high frequency (BCF). Subpopulation 3 (SP3) included spermatozoa with low velocity but good linear coefficients (LIN and STR) and low head displacement with good frequency. And subpopulation 4 (SP4) was characterized by spermatozoa with anarchy in their movements, in general, low velocity and low progressive movements.

Table 1. Sperm motile subpopulations and motility descriptors.

	SP1 (N=6616)		SP2 (N=8484)		SP3 (N=6306)		SP4 (N=9049)	
	Mean ± SD	Range (min, max)	Mean ± SD	Range (min, max)	Mean ± SD	Range (min, max)	Mean ± SD	Range (min, max)
VCL	152.05 ± 29.05	88.95, 304.00	123.42 ± 21.94	68.06, 218.59	54.00 ± 19.74	10.00, 105.92	68.57 ± 21.12	10.14, 132.50
VSL	35.91 ± 20.03	0.00, 135.91	77.62 ± 22.13	27.62, 169.34	31.87 ± 13.45	3.76, 71.49	16.65 ± 9.32	0.00, 46.51
VAP	90.60 ± 22.46	19.47, 230.51	97.40 ± 21.52	51.17, 199.72	38.84 ± 15.30	3.91, 75.03	35.10 ± 13.83	4.20, 81.88
LIN	23.49 ± 11.43	0.00, 63.97	63.25 ± 15.54	28.45, 98.51	59.64 ±14.75	31.97, 99.21	24.09 ± 10.29	0.00, 44.57
STR	39.83 ± 19.30	0.00, 92.68	79.46 ± 12.37	30.37, 99.72	82.11 ± 10.60	32.47, 100.00	47.76 ± 19.35	0.00, 93.80
WOB	59.90 ± 11.49	11.84, 94.02	79.09 ± 11.69	44.38, 100.00	72.32 ± 12.78	36.74, 100.00	51.03 ± 11.92	9.44, 96.70
ALH	6.08 ± 1.37	2.35, 14.40	3.92 ± 1.21	0.66, 9.70	2.19 ± 0.89	0.23, 5.91	3.24 ± 1.01	0.55, 7.00
BCF	8.24 ± 3.62	0.00, 22.00	8.88 ± 3.10	0.00, 20.00	7.63 ± 3.30	0.00, 20.00	6.40 ± 2.74	0.00, 19.00
DANCE	951.78 ± 389.09	257.76, 4103.72	497.53 ± 215.35	61.09, 1816.66	131.87 ± 85.21	3.37, 479.16	240.97 ± 137.77	5.93, 769.68
MDAabs	111.24 ± 30.92	0.00, 240.19	83.11 ± 44.51	0.00, 266.35	100.79 ± 40.76	0.00, 286.16	122.78 ± 26.64	0.00, 226.72
MDAalg	0.16 ± 9.63	-39.55, 42.46	0.00 ± 9.88	-43.82, 43.71	-0.16 ± 7.77	-41.68, 34.40	-0.25 ± 8.24	-41.01, 43.59

Motility descriptors are described in section 2.

In general, the incubation time induces a decrease in the sperm movement and in the motility quality, the percentage of spermatozoa included in SP1 and SP2 showed a tendency to decrease and those included in SP3 and SP4 increased progressively (Figs 1,2,3,4). The treatments including seminal plasma protein between 30-50kDa and 50-100kDa showed the best maintenance of rapid sperm subpopulations. SP1 was maintained during 3h and SP2, the best one, was maintained, at least for 4h. In addition, both treatments showed low increase of SP3 and the maintenance of SP4.

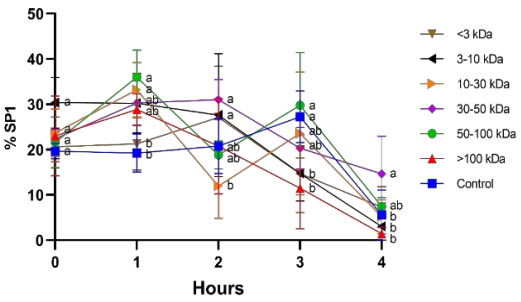

Fig 1

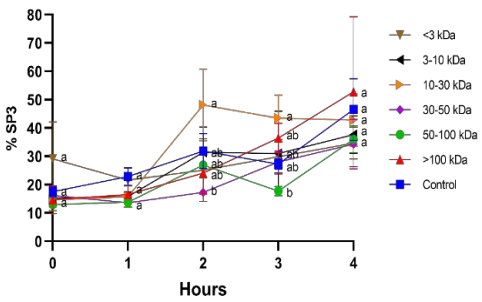

Fig 2

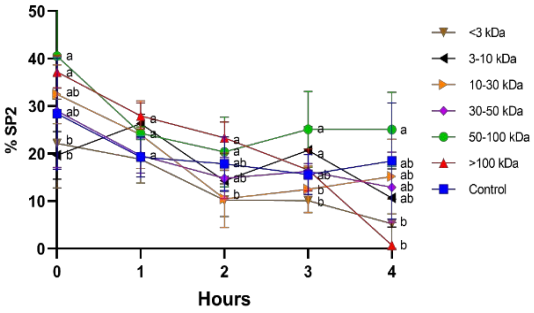

Fig 3

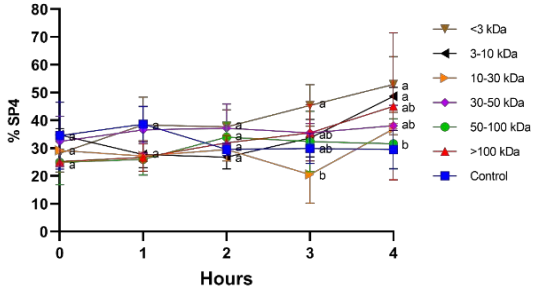

Fig 4

Figs1,2,3,4. Evolution of sperm motile subpopulations (SP1, SP2, SP3 and SP4) for different treatments and along the incubation time.

Treatments including seminal plasma proteins higher than 10kDa induced a rapid sperm/PMN interaction, with significantly higher percentage of spermatozoa bound to PMN at time 0. Then the differences between treatments were reduced, however all the treatments showed a progressive increase, while the 30-50kDa treatment maintained similar values along the incubation time and 10-30kDa treatment showed a significant decrease at 4h (Fig 5).

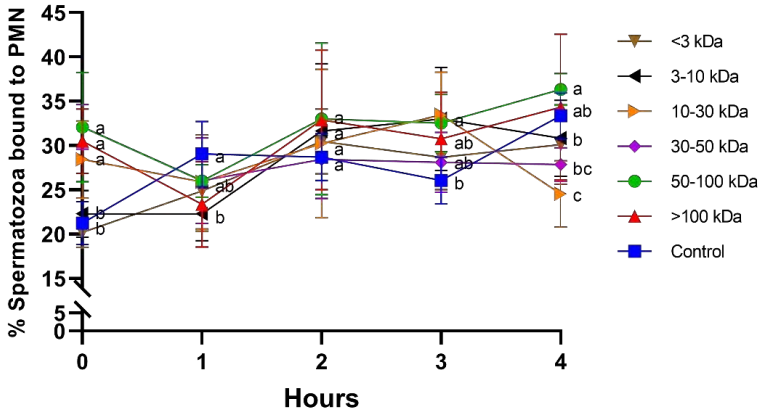

Fig 5. Percentage of spermatozoa bound to PMN for different treatments and different times of incubation.

The analysis of PMN with attached spermatozoa showed showed that the number of spermatozoa bound was significantly higher for the treatments including seminal plasma proteins higher than 10kDa. However, after 1 hour of incubation no differences between treatments were observed (Fig6).

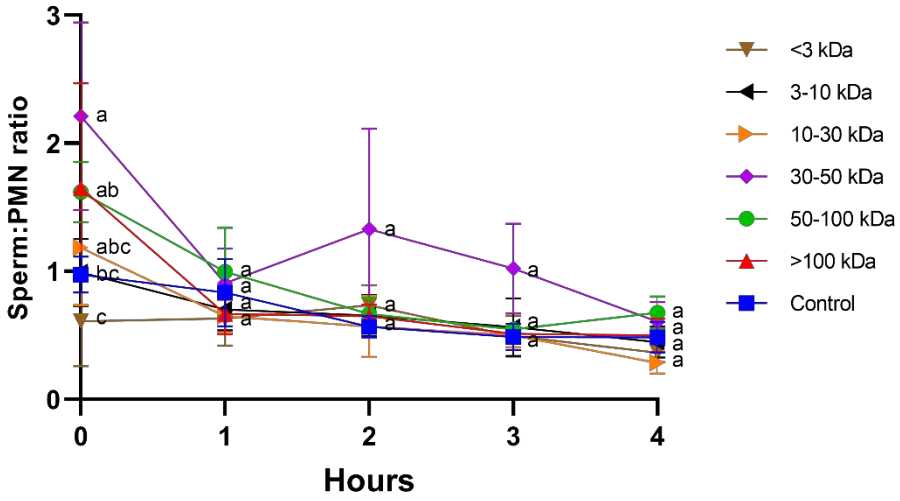

Fig 6. Sperm:PMN ratio (number of spermatozoa attached to one PMN) for different treatments and times of incubation.

There was no differences between treatments for the percentage of viable spermatozoa bound to PMN at 0 and 1 h of incubation. The treatment 30/50 Treatments showed the highest values for each time of incubation. And the treatment 50/100 the lowest with values significantly different between both treatments at 2 and 3h of incubation. Then at 4h the differences between treatments disappear (Fig 7).

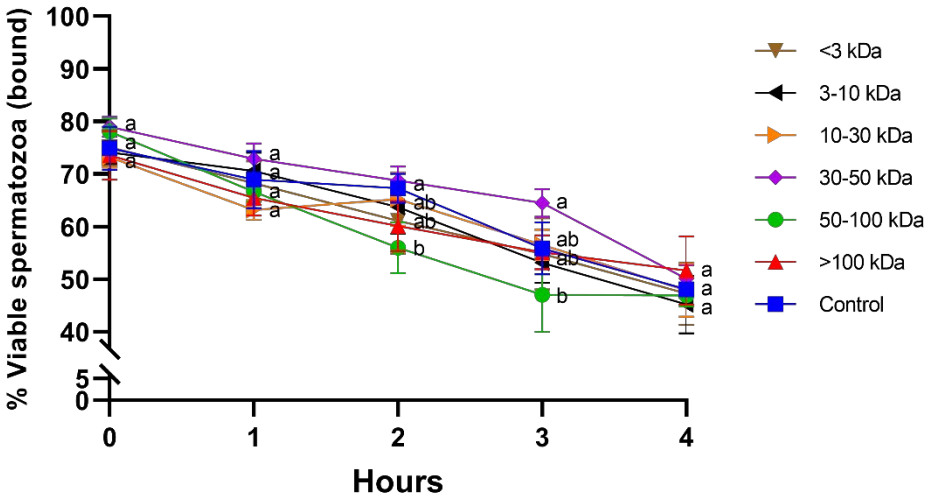

Fig 7. Percentage of viable spermatozoa bound to PMN for different treatments and times.

The percentage of unbound viable spermatozoa had no differences between treatments at 0 and 1h of incubation. Nevertheless, after two hours of incubation the differences increased progressively, and treatments 30/50 and 50/100 showed significantly more free viable spermatozoa than other treatments without differences between them (Fig 8).

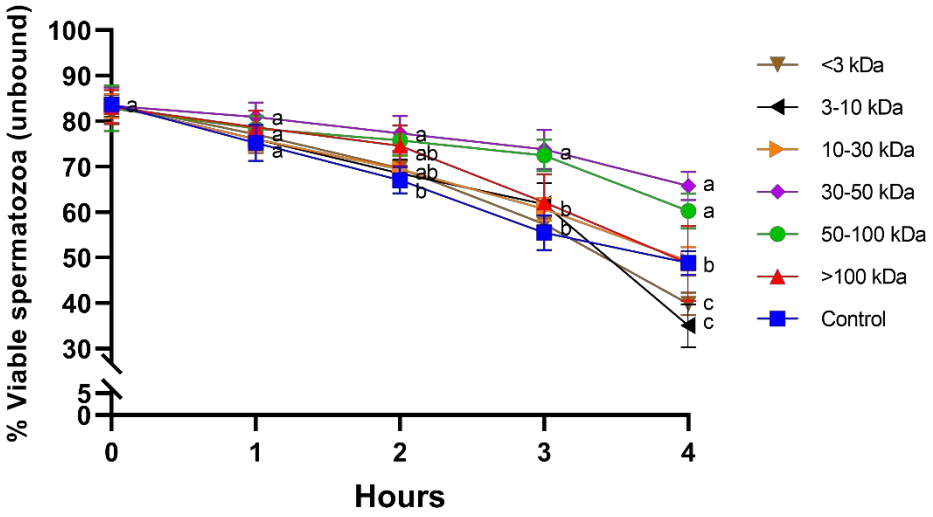

Fig 8. Percentage of unbound viable spermatozoa for different treatments and time of incubation.

4. Discussion

Sperm motile subpopulations with specific motility patterns have been described previously in donkey (Miró *et al.,* 2005; Miró *et al.,* 2009) and in other species such common marmoset, gazelle, pig, dog, horse or red deer (Abaigar *et al.,* 1999, Abaigar *et al.,* 2001, Rigau *et al.,* 2001, Thurston *et al.,* 2001, Quintero-Moreno *et al.,* 2003, Quintero-Moreno *et al.,* 2004, Martinez-Pastor *et al.,* 2005). Mammals of very different phylogenetic origin show this structure suggesting the existence of a relationship between the maintenance of the subpopulations distribution of an ejaculate an its fertilizing ability (Quintero-Moreno *et al.,* 2003, Quintero-Moreno *et al.,* 2004, Flores *et al.,* 2008). In our study the treatments with SP fractions including proteins between 30-50 kDa and 50-100kDa showed the best maintenance of sperm motile subpopulations. Both treatments resulted in high values for the rapid sperm motile subpopulations, SP1 and SP2, without significant differences between them. It is noteworthy the maintenance of SP2 including the spermatozoa with better movements. On the other hand, 30-50 and 50-100 treatments showed a low increase of SP3 with slow but progressive spermatozoa and a maintenance of SP4 the worst, with strange movements.

The information about the donkey seminal plasma proteins is reduced. A low number of proteins are described and the function of most of them remain unknown.

Horse Lactate dehydrogenase (LDH) has a molecular weight of 35 kDa showing important levels in horse seminal plasma (Pesch *et al.,* 2006) and significantly higher levels in jackass one (Talluri *et al.,* 2017). In stallion seminal plasma LDH is positively correlated with sperm concentration, sperm motility and progressive motility or living sperm, and negatively correlated with pathomorphology of sperm and semen volume. These findings could be a sign that extracellular LDH ensures metabolism of spermatozoa, perhaps even in anaerobic conditions (Pesch *et al.,* 2006). LDH catalyzes the oxidation of pyruvate to lactate during anaerobic glycolisis, but this is a reversible process.

Donkey spermatozoa produce a large amount of lactate with a significant correlation with sperm motility and velocity, being a good predictor of donkey semen quality (Miró *et al.*, 2005), then LDH could be very important in the control of lactate levels.

The antioxidant enzyme system involving SOD, CAT, GSR and GPX has been described in several species (Alvarez *et al.,* 1987, Beconi *et al.,* 1993, Martí *et al.,* 2007, Papas *et al.,* 2019). A recent study evidenced that the activity for all of these enzymes are significantly higher in jackass than in stallion seminal plasma. The activity of these four enzymes has no correlation with semen quality in stallion but SOD and CAT activities are possitively correlated with the percentage of motile spermatozoa and the sperm motility patterns in donkey male (Papas *et al.,* 2019). On the other hand, SOD activity in seminal plasma is possitively corelated witht cryotolerance of donkey spermatozoa (Papas *et al.,* 2020). And GPx was correlated with age and fertility in Arabian stallions (Waheed *et al.,* 2013). The molecular weight for the antioxidant seminal plasma enzymes is 68 kDa for Catalase (CAT) (Summer & Gralen, 1938), 80 kDa for Glutathione Reductase (GSR) (García-Alonso *et al.,* 1993), Glutathione Peroxidase (GPx-1) consists of a tetramer of identical subunits of 22-23 kDa (Lusbos *et al.,* 2011) or SOD is protein of 32.5 kDa (Nozik-Grayck *et al.,* 2005).

Donkey semen, including seminal plasma, induce a post-AI inflammatory response in the jenny uterus, with a high PMN infiltration and a PMN-spermatozoa interaction (Miró *et al.,* 2020). However, *in vivo* post-thaw addition of seminal plasma increases the fertility rates (Rota *et al.,* 2012) or reduces the cicloxigenase-2 (COX-2) expression in the endometrium post-AI (Vilés *et al.,* 2013). *In vitro* donkey seminal plasma reduces significantly the PMN-spermatozoa binding (Miró *et al.,* 2013). Seminal plasma proteins have been shown to modulate the interaction PMN-spermatozoa. In horse, seminal plasma protein CRISP3 suppresses PMN-sperm binding (Doty *et al.,* 2011). CRISP3, with a molecular weight of 25 kDa, is the major seminal plasma protein in stallions (Schambony *et al.,* 1998). In the horse great amounts of

CRISP3 are synthesised in the accessory glands, ampulla and seminal vesicle (Schambony *et al.,* 1998). Donkey's ampulla are significantly larger than horse one but the presence of CRISP3 in the seminal plasma and its molecular weight and origin remains unknown.

The analysis of donkey PMN-spermatozoa interaction showed that a reduced percentage of spermatozoa are phagocytosed by PMN, most remain attached on the PMN surface or into a surrounding halo (Miró *et al.,* 2020). Birnkmann *et al.,* (2004, 2007) demonstrated another mechanism of PMN antimicrobial action, the activation and extrusion of their DNA and associated proteins creating neutrophil extracellular traps (NETs) that block the bacteria. Most of donkey spermatozoa attached to PMN surface are viable showing high tail batting (Miró *et al.,* 2020) like bacteria attached to NETs that remain viable (Menegazi *et al.,* 2012). NETs production have been demostrated in horse PMN:spermatozoa interaction (Alghamdi and Foster 2005) and recently in donkey (Mateu-Orero *et al.,* 2020). A DNAse present in horse seminal plasma is able to digests the extruded DNA and frees entangled spermatozoa (Alghamdi and Foster 2005). The meaning of this mechanism remains unknown but seems important to increase the fertility rates. The equine DNAse molecular weight are 33 kDa (Alghamdi and Foster 2005) and its presence in donkey seminal plasma remains unknown.

Donkey seminal plasma has a large amount of proteins (Talluri *et al.,* 2017, Papas *et al.,* 2019). The detailed study of this proteins is a hard and expensive work, but fractioning the seminal plasma could be easier. The main part of positive seminal plasma effects are induced by the 30-50kDa an 50-100kDa fractions. Included in both fractions there are important proteins involved in the control of the jenny post-breeding inflammatory response or in the ROS scavenge described in donkey seminal plasma. However most of them remain unknown. Further studies are needed to analyse in vivo the effect of this fractions addition to frozen/thawed semen or to describe the different proteins and their role. These studies could contribute to increase the obtained fertility rates by frozen/thawed semen in donkeys.

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4. DISCUSIÓN

La utilización de Prostaglandinas, o combinaciones de estas con otras hormonas en la inducción de celos, es una práctica común en distintas especies de producción. En la yegua las prostaglandinas son, con diferencia, las hormonas más utilizadas. No obstante, existen aún importantes lagunas en la comprensión del proceso de lisis del cuerpo lúteo (CL) inducido por las prostaglandinas en las distintas especies. En nuestro caso, la inducción de celos en burras era un instrumento necesario para el estudio de la reacción inflamatoria post-inseminación, hecho que se aprovechó para el estudio de la luteólisis en la burra. El CL es una glándula productora de progesterona por lo que su funcionalidad se ha evaluado tradicionalmente mediante los niveles de progesterona en sangre (progesteronemia). La ecografía Doppler color nos permite evaluar la vascularización de los tejidos y puede ser también un instrumento importante para la evaluación rápida de la funcionalidad del CL. Así pues, en nuestro primer estudio se examinaron la progesterona plasmática, el flujo sanguíneo luteal y el área del cuerpo lúteo después de inducir la luteólisis. Así mismo, se monitorizaron le ecogenicidad del CL y el tono uterino.

Tras ecografiar a las distintas burras, todos los CL activos detectados mostraron una fuerte ecogeniciad y un área central hiperecogénica (máxima reflexión de ultrasonidos, imagen muy brillante) (Taberner *et al.,* 2008; Díez, 1992). En contraste, la yegua puede presentar dos tipos de CL activos, unos con un área central hipoecogénica (restos de hemorragia) y otros ecogénicos y uniformes (Ginther 1986; Díez, 1992). En todos los casos, la zona periférica corresponde al tejido luteal y en los de burra, o los ecogénicos de yegua, el centro es una zona de fibrosis, aunque con mayor ecogenicidad en la burra.

El tono uterino escaso en la palpación transrectal y la presencia de edema uterino en la ecografía se asocian en la yegua al celo y a bajos niveles de progesterona (Ginther, 2012). Las burras en celo muestran también poco tono uterino, pero, sin embargo, prácticamente no muestran edema uterino.

En nuestro estudio, partiendo de valores de progesterona plasmática mayores a 20 ng/ml, se ha observado que estos empezaban a reducirse 1h después de la administración de prostaglandinas, produciéndose un descenso muy significativo en las primeras 24h para llegar a valores basales de 2 ng/ml tras el tercer día, aunque sin diferencias importantes entre los días 1, 2 y 3 postratamiento. Estos resultados son similares a los reportados para burras Martina Franca (Carluccio *et al.,* 2008). En la yegua, sin embargo, se produce una rápida actividad luteolítica con una caída de la progesterona plasmática a menos de 1 ng/ml tras 10h del tratamiento con $PGF_{2\alpha}$ (Bergfelt *et al.*, 2006). También se han observado reducciones similares de progesterona plasmática en la vaca tras 30 minutos (Ginther *et al.,* 2007; Goff *et al.,* 1984). Por otro lado, en yeguas se ha descrito un aumento temporal de la progesterona plasmática junto con LH, FSH y cortisol a los 10 minutos del tratamiento con $PGF_{2\alpha}$ (Ginther *et al.,* 2009). Este comportamiento no ha sido observado en las burras de este estudio.

El aumento del flujo sanguíneo luteal en yeguas, tanto si se produce una luteólisis natural como inducida, es un hecho fisiológico común (Acosta *et al.,* 2002; Ginther, 2012; Miyamoto *et al.,* 2009; Shirasuna *et al.,* 2012; Ginther *et al.,* 2007a; Ginther *et al.,* 2007b). Este aumento transitorio del flujo sanguíneo luteal también lo hemos observado en las burras de este estudio entre 1 y 3h post inoculación de la prostaglandina. Por otro lado, en vacas se ha observado un aumento temprano de este flujo sanguíneo, entre las 0-0,5h después del incremento natural de prostaglandinas o su administración exógena, permaneciendo elevada hasta dos horas después, momento en el que la vascularización del CL empieza a disminuir (Acosta *et al.,* 2002; Carluccio *et al.,* 2008). Curiosamente, la falta de cambios en la vascularización intraluteal en respuesta al tratamiento con $PGF_{2\alpha}$ parece ser la responsable de la acción luteolítica refractaria observada en vacas antes del día 5 después de la ovulación (Ginther *et al.,* 2007).

La síntesis de prostaglandinas a partir del ácido araquidónico y la expresión de COX-2 en la fase lútea tardía se ha observado en vacas y ovejas (Tsai y

Wiltbank, 1997, 1998). En yeguas, se ha observado un aumento de cortisol después de la administración de un bolo simple de $PGF_{2\alpha}$ (Ginther *et al.,* 2009). Por lo tanto, aunque son necesarios más estudios, la luteólisis debe entenderse como un proceso fisiológico de inflamación que implica una liberación de prostaglandinas, otras hormonas y citoquinas, lo que resulta en la necrosis del tejido lúteo inducida por isquemia (Galvão *et al.,* 2013). Como se ha descrito en la vaca, un aumento en el flujo sanguíneo lúteo puede ser desencadenado por la prostaglandina vasodilatadora I2 o la prostaciclina y la liberación de óxido de nitrógeno de las arteriolas que rodean el CL; esto probablemente también causa vasodilatación en el CL activo de las burras después de la luteólisis inducida. La secreción de angiotensina II y la endotelina 1 se estimulan así a partir de vasos microcapilares en el CL, lo que provoca la vasoconstricción de las arteriolas y, finalmente, la luteólisis (Miyamoto y Shirasuna, 2009; Ginther *et al.,* 2007; Acosta *et al.,* 2002, Miyamoto *et al.,* 2009).

Recientemente, Korzewa *et al. (*2014) describieron efectos citotóxicos y proapoptóticos in vitro para la $PGF_{2\alpha}$ que causaron la muerte de las células lúteas en la vaca. Además, se vio que, el efecto del dinoprost (Dinolytic ^{R}) (también utilizado en este estudio) es más similar a la luteólisis natural que la inducida por las prostaglandinas sintéticas. En vacas, la progesterona plasmática disminuye a las 0,5 horas después de la administración de $PGF_{2\alpha}$, pero el flujo sanguíneo lúteo permanece sin cambios hasta 8 horas, coincidiendo con el inicio de la luteólisis estructural reflejada por una reducción significativa en el volumen del CL (Ginther *et al.,* 2007). En yeguas, el CL disminuye mientras que la ecogenicidad del tejido luteal aumenta después de la luteólisis inducida. En las burras de este estudio, el CL disminuyó lentamente durante las primeras 48 horas en comparación con el flujo sanguíneo lúteo y progesterona plasmática, lo que sugiere que la luteólisis funcional se manifiesta antes que la luteólisis estructural. Sin embargo, este hecho necesita ser estudiado más a fondo.

Niswender *et al.* (2000) observaron en la oveja una correlación positiva altamente significativa entre la progesterona plasmática y el flujo sanguíneo luteal. Así mismo, los niveles diarios de progesterona en plasma de las burras podrían predecirse por la evaluación del flujo sanguíneo luteal con el Doppler color, lo que lo convierte en una herramienta útil no invasiva para evaluar rápidamente la función del CL, como se describe en otras especies (Bollwein *et al.,* 2002, Hergoz y Bollwein, 2007).

La inflamación endometrial en la burra ha sido previamente investigada *in vitro* utilizando lavados uterinos recogidos a las seis horas post inseminación artificial (Miró *et al.,* 2013). En dicho estudio se obtuvo un lavado rico en PMN (>100 x 10^3 células/ml de promedio), no obstante, los lavados uterinos continuados pueden afectar negativamente al tracto reproductivo femenino y generar infertilidad. Para poder realizar el siguiente experimental y evitar el efecto secundario mencionado, el primer objetivo del segundo artículo ha sido establecer un modelo *in vitro* aislando PMN de sangre periférica para así poder utilizarlos en los diferentes experimentales sin tener que recurrir los lavados uterinos. De los siete protocolos probados, uno (que fue designado como Protocolo 7) mostró claramente la mayor tasa de recuperación produciendo >100 x 10^3 células/ml, mientras que utilizando protocolos probados en otras especies (Roth y Kaeberle, 1981), incluído el equino, (Baumber *at al.,* 2002; Loftus *et al.,* 2010) no se pudo aislar suficientes PMN de la sangre periférica.

En estudios previos *in vitro* en los que se ha evaluado la actividad de los PMN en el caballo se han utilizado diferentes agentes activadores como formil-metionil-leucil-fenilalanina (FMLP) o la interleuquina 8 equina recombinante (reqIL-8) (Baumber *at al.,* 2002). En el estudio actual, al poner el semen completo (incluidos los espermatozoides y SP) en contacto con PMN, se observó que era capaz de estimular a los PMN y activarlos de la misma manera que lo haría una sustancia específica como el FMLP. Se vio que la presencia de FMLP no alteró los porcentajes de espermatozoides que se unieron a PMN, ni tampoco la viabilidad de las poblaciones de spz libres y unidos a PMN durante el tiempo de incubación, obteniendo unos resultados muy similares a los

obtenidos en estudios previos realizados en burros en los que se encontró que el SP modula la unión spz:PMN y la motilidad de los spz (Miró *et al.,* 2013). También se observó la disminución en la supervivencia de los espermatozoides a lo largo del tiempo de incubación fue independiente a la presencia de PMN. Por otro lado, sí se observó que la viabilidad de la población de spz unida a PMN a las 0h fue mayor usando FMLP, tal vez como resultado de una estimulación rápida de PMN, aunque no se observaron diferencias entre tratamientos ni a lo largo de la incubación. Esto sugiere que la viabilidad de la población de spz unida a PMN es mayor que la de los spz libres. Sorprendentemente, pocos espermatozoides parecían ser fagocitados por PMN, muchos se pegaron a la superficie y algunos se encontraron dentro de un halo teñido que rodea a los PMN y que se observa menos coloreado. Con todo esto no podemos afirmar por completo que la fagocitosis no ha sido observada y para ello son necesarios más estudios específicos para poder probar este hecho. Lo que sí habría que destacar es que una proporción importante de espermatozoides permanecía alrededor de los PMN. Los neutrófilos polimorfonucleares son la primera barrera frente a los patógenos a través de la fagocitosis o mediante la liberación de su propio ADN, histonas, enzimas, incluida la catepsina, elastasa (ELA) y mieloperoxidasa (MPO). En el último caso, esto crea unos complejos conocidos como trampas extras celulares de neutrófilos (NETs), que evitan la diseminación de patógenos (Brinkmann *et al*., 2004; Fuchs *et al*., 2007; Kazzaz *et al*., 2016; Nakazawa *et al*., 2017; Rebordão *et al*., 2018). Según Branzk *et al*., (2014) que el PMN desencadene la fagocitosis o la NETosis depende del tamaño del patógeno. En este estudio, se ha querido investigar sobre dos vertientes, por un lado, se ha estudiado el mecanismo ligando/receptor a través del cual el PMN desencadenar la fagocitosis sobre los espermatozoides. Es necesario estudiar la unión spz-PMN para confirmar los porcentajes reducidos de fagocitosis de spz por parte de los PMN. Por otro lado, ya que se ha visto la formación de NETs tanto en humanos como en otras especies (Alghamdi *et al*., 2009; Alghamdi *et al*., 2005; Schjenken y Robertson, 2020), está muy justificado el estudio sobre sí se forman los NETs y de qué manera lo harían al incubar espermatozoides de burro con PMN. Se sabe que los NETs atrapan

espermatozoides de mamíferos cerca de PMN (es decir, sin contacto directo), lo que resulta en una pérdida significativa de la motilidad de los espermatozoides (Alghamdi *et al.*, 2005). Por este motivo, consideramos que se debería seguir investigando sobre este tema de manera similar a cómo se ha hecho en otras especies (Nakazawa *et al.*, 2017; Rebordão *et al.*, 2018; Menegazzi *et al.,* 2012) centrándose en la tinción de los enzimas de ADN y PMN.

Nuestros resultados sugieren que la fagocitosis de espermatozoides podría inhibirse, lo cuál, según lo indicado por Branzk *et al.*, (2014), conduciría a la activación de la NETosis. De hecho, se ha sugerido que deficiencias en la actividad de los receptores fagocíticos puede provocar la interrupción de la selectividad dependiente del tamaño de la liberación de NETs debido a la ineficiencia de la fagocitosis. Además, se sabe que el bloqueo de la Dectina-1, un receptor fagocítico presente en PMN humanos, con un anticuerpo específico conduce a unas tasas más bajas de fagocitosis y una respuesta de NETosis más elevada. Basándonos en el bajo porcentaje de fagocitosis, tras este estudio sugerimos que algunos factores del plasma seminal podrían inhibir los receptores de los PMN implicados en la inducción de la fagocitosis, lo que conduciría a la activación de la NETosis de una manera similar a la reportada por Branzk *et al.*, (2014) cuando se inhibe la Dectina-1.

En este estudio se ha observado un número significativo de espermatozoides viables unidos a PMN y que mostraron un intenso movimiento de cola. Además, también se observó que el porcentaje de espermatozoides viables era mayor en la población unida a PMN que en los spz libres y que estos mostraban muy buena movilidad al ser liberados tras 3h de incubación. De la misma manera se vio que, durante las primeras horas de incubación, los spz tendían a unirse más por la cola que por la cabeza. Como no sabemos si esta observación tiene alguna relación con la capacidad de los espermatozoides para liberarse de PMN, los estudios futuros también deberían contemplar a través microscopía Time-lapse, la dinámica de unión entre espermatozoides y PMN, así como la relevancia de cada parte del spz. Esta unión transitoria

aparente podría desempeñar un papel importante en la estrategia de reproducción de burros, ya que es muy probable que los espermatozoides liberados por PMN puedan fertilizar.

Al inicio del experimento, los porcentajes de espermatozoides unidos a PMN fueron significativamente mayores en el tratamiento que contiene la mayor concentración de espermatozoides ($1000x10^6$ spz/ml). Sin embargo, vale la pena señalar que estas diferencias encontradas entre concentraciones en la unión spz:PMN, no se encontraron después de 3h de incubación. Adicionalmente, mientras que la relación spz:PMN y el porcentaje de espermatozoides viables disminuyeron con el paso del tiempo en la incubación, los porcentajes de spz unidos a PMN aumentaron en tiempos de incubación sucesivos. Estos datos indican que los PMN podrían activarse progresivamente a lo largo de la incubación, uniendo así un mayor número de espermatozoides. Sin embargo, no se mostraron variaciones en el porcentaje de spz unidos a PMN (aproximadamente 50%) entre las concentraciones y los tiempos de incubación. Esto sugiere que cuanto mayor es la concentración de espermatozoides, mayor es la posibilidad de observar spz libres después de 3h y 4h de incubación. Esta hipótesis encajaría con lo que se ha observado a través del programa CASA, ya que solo las concentraciones superiores a $500x10^6$ spz/ml mostraron espermatozoides móviles libres al final del experimento.

Oliveira *et al.* (2016), observaron un aumento en las tasas de fertilidad después de inseminar burras con dosis con altas concentraciones de espermatozoides. Dado que los burros generalmente muestran buena calidad de semen con una mayor concentración y movilidad de spz que los sementales (Canisso *et al.,* 2019; Miró *et al.,* 2005), se podría sugerir que esta mejor calidad espermática podría desempeñar un papel fisiológico importante para la reacción endometrial postmonta en la burra. En efecto, como se explicó anteriormente, parece que se requieren altas concentraciones de spz con buena movilidad para liberarse de los PMN después de la unión. Sin embargo, una pregunta importante aquí es si los espermatozoides que finalmente fertilizan el ovocito pertenecen a la población de espermatozoides que son liberados de PMN. Por

lo tanto, los estudios futuros también deberían centrarse en la relación entre PMN:spz en la postmonta/inseminación en el burro.

En el eyaculado de burro se ha descrito la existencia de subpoblaciones de espermatozoides mótiles según sus patrones específicos de motilidad (Miró *et al.,* 2005; Miró *et al.,* 2009) al igual que en otras especies como tití, gacela, cerdo, perro, caballo o ciervo común (Abaigar *et al*., 1999, Abaigar *et al.,* 2001, Rigau *et al.,* 2001, Quintero-Moreno *et al.,* 2003, Quintero-Moreno *et al.,* 2004, Martinez-Pastor *et al.,* 2005). Los mamíferos de origen filogenético muy diferente muestran esta estructura sugiriendo la existencia de una relación entre el mantenimiento de la distribución de subpoblaciones de un eyaculado y su capacidad de fertilización (Quintero-Moreno *et al.,* 2003, Quintero-Moreno *et al.,* 2004, Flores *et al.,* 2008). En nuestro tercer estudio, los tratamientos con fracciones de SP que incluyen proteínas entre 30-50 kDa y 50-100kDa mostraron el mejor mantenimiento de dichas subpoblaciones. Ambos tratamientos dieron como resultado valores altos para las subpoblaciones rápidas de espermatozoides móviles, SP1 y SP2, sin diferencias significativas entre ellos. Hay que destacar el mantenimiento de SP2 incluyendo los espermatozoides con mejores movimientos. Por otro lado, los tratamientos 30-50 y 50-100 kDa mostraron un bajo aumento de SP3 con espermatozoides lentos pero progresivos y SP4, con movimientos extraños.

La información sobre las proteínas plasmáticas seminales de burro es reducida. Se ha descrito un número escaso de proteínas y la función de la mayoría de ellas sigue siendo desconocida.

La deshidrogenasa de lactato de caballo (LDH) tiene un peso molecular de 35 kDa mostrando niveles importantes en plasma seminal del caballo (Pesch *et al.,* 2006) y niveles significativamente más altos en burro (Talluri *et al.,* 2017). En el semental, en el plasma seminal la LDH se correlaciona positivamente con la concentración de espermatozoides, la motilidad de los espermatozoides y la motilidad progresiva o spz vivos, y se correlaciona negativamente con la patomorfología del volumen de esperma y esperma. Estos hallazgos podrían

ser una señal de que la LDH extracelular asegura el metabolismo de los espermatozoides, tal vez incluso en condiciones anaeróbicas (*Pesch et al.,* 2006). La LDH cataliza la oxidación de piruvato a lactato durante la glucólisis anaeróbica, pero este es un proceso reversible. Los espermatozoides de burro producen una gran cantidad de lactato con una correlación significativa con la motilidad y velocidad de los espermatozoides, siendo un buen predictor de la calidad del semen de burro (Miró *et al.,* 2005), por lo que la LDH podría ser muy importante en el control de los niveles de lactato.

El sistema de enzimas antioxidantes que involucra SOD, CAT, GSR y GPX se ha descrito en varias especies (Alvarez *et al.,* 1987, Beconi *et al.,* 1993, Martí *et al.,* 2007, Papas *et al.,* 2019). Un estudio reciente evidenció que la actividad de todas estas enzimas es significativamente mayor en el plasma seminal del burro que en el del semental. La actividad de estas cuatro enzimas no tiene correlación con la calidad del semen en el semental, pero las actividades de SOD y CAT están posiblemente correlacionadas con el porcentaje de espermatozoides móviles y los patrones de motilidad espermática en el macho de burro (Papas *et al.,* 2019). Por otro lado, la actividad de SOD en el plasma seminal posiblemente se correlaciona con la criolerancia de los espermatozoides de burro (Papas *et al.,* 2020). La enzima GPX se correlacionó con la edad y la fertilidad en los sementales árabes (Waheed *et al.,* 2013). El peso molecular de las enzimas plasmáticas seminales antioxidantes es de 68 kDa para la Catalasa (CAT) (Summer & Gralen, 1938), 80 kDa para la Glutatión Reductasa (GSR) (García-Alonso *et al.,* 1993), la Glutatión Peroxidasa (GPX-1) consiste en un tetrámero de subunidades idénticas de 22-23 kDa (Lusbos *et al.,* 2011) y la SOD, que es una proteína de 32,5 kDa (Nozik-Grayck *et al.,* 2005).

El semen de burro, incluido el plasma seminal, induce una respuesta inflamatoria post-AI en el útero de las burras, con una alta infiltración de PMN y una interacción PMN-espermatozoides (Miró *et al.,* 2020). Sin embargo, la adición de plasma seminal *in vivo* después de descongelar la muestra de semen, aumenta las tasas de fertilidad (Rota *et al.,* 2012) o reduce la expresión

de cicloxigenasa-2 (COX-2) en el endometrio post-AI (Vilés *et al.,* 2013). El plasma seminal de burro *in vitro* reduce significativamente la unión de espermatozoides PMN (Miró *et al.,* 2013). Se ha demostrado que las proteínas plasmáticas seminales modulan la interacción PMN-espermatozoides. En los caballos, la proteína plasmática seminal CRISP3 suprime la unión de spz:PMN (Doty *et al.,* 2011). CRISP3, con un peso molecular de 25 kDa, es la principal proteína plasmática seminal en sementales (Schambony *et al.,* 1998). En el caballo, se sintetizan grandes cantidades de CRISP3 en las glándulas accesorias, la ampolla y la vesícula seminal (Schambony *et al.,* 1998). La ampolla del burro es significativamente más grande que la del caballo, pero la presencia de CRISP3 en el plasma seminal, su peso molecular y el origen aún se desconocen.

El análisis de la interacción entre PMN y espermatozoides de burro mostró que un porcentaje reducido de espermatozoides son fagocitados por PMN, la mayoría permanecen unidos en la superficie de PMN o en un halo circundante (Miró *et al.,* 2020). Birnkmann *et al.* (2004, 2007) demostraron otro mecanismo de acción antimicrobiana de PMN, la activación y extrusión de su ADN y proteínas asociadas creando trampas extracelulares de neutrófilos (NETs) que bloquean la bacteria. La mayoría de los espermatozoides de burro unidos a la superficie de PMN son viables y muestran un alto bateo de la cola (Miró *et al.,* 2020) como las bacterias unidas a los NETs que siguen siendo viables (Menegazi *et al.,* 2012). La producción de NETs ha sido demostrada en la interacción spz:PMN en caballo (Alghamdi y Foster 2005) y recientemente en burro (Mateo-Orero *et al.,* 2020). Una DNAsa presente en el plasma seminal de caballo es capaz de digerir el ADN extruido y liberar los espermatozoides enredados (Alghamdi y Foster 2005). El significado de este mecanismo sigue siendo desconocido, pero parece importante para aumentar las tasas de fertilidad. El peso molecular de la ADNsa equina es de 33 kDa (Alghamdi y Foster 2005) y su presencia en el plasma seminal de burro sigue siendo desconocida.

El plasma seminal de burro tiene una gran cantidad de proteínas (Talluri *et al.,* 2017; Papas *et al.,* 2019). El estudio detallado de estas proteínas es un trabajo duro y costoso, pero fraccionar el plasma seminal podría hacerlo más fácil. La mayor parte de los efectos positivos del plasma seminal está inducida por las fracciones de 30-50kDa y 50-100kDa. Incluidas en ambas fracciones encontramos proteínas importantes involucradas en el control de la respuesta inflamatoria posterior a la inseminación de la burra o en la eliminación de ROS descrita en plasma seminal de burro. Sin embargo, la mayoría de efectos siguen siendo desconocidos. Se necesitan más estudios para analizar *in vivo* el efecto de la adición de estas fracciones al semen congelado/descongelado o para describir las diferentes proteínas y su papel. Estos estudios podrían contribuir a aumentar las tasas de fertilidad obtenidas con semen congelado/descongelado en burros.

5. CONCLUSIONES FINALES

1. La administración exógena de prostaglandinas en burras en el día 10 postovulación induce un incremento de la vascularización del cuerpo lúteo entre 2 y 3 horas después, seguido de un descenso significativo entre las 3 y las 5 horas. Este descenso origina una isquemia del cuerpo lúteo y la pérdida de su funcionalidad con el consiguiente descenso de los niveles de progesterona.

2. A partir de neutrófilos polimorfonucleares (PMN) aislados de sangre puede obtenerse un buen modelo *in vitro* para el estudio de la respuesta inflamatoria postinseminación en la burra y de la relación PMN:espermatozoide.

3. El plasma seminal por sí solo es capaz de activar *in vitro* a los PMN sin necesidad de otro tipo de activador.

4. El estudio de la interacción PMN:espermatozoide muestra que pocos espermatozoides son fagocitados, la mayor parte permanecen unidos a la superficie del PMN o en un halo circundante. Así mismo, buena parte de estos espermatozoides se mantienen vivos. Esta observación posiblemente sea NETosis, aunque hacen falta tinciones específicas de DNA para confirmarlo.

5. Sólo concentraciones espermáticas superiores a $500x10^6$ spz/ml muestran espermatozoides vivos tras 3h de incubación junto a PMN. Se observa que algunos de estos espermatozoides con excelente motilidad se han liberado de su unión a la superficie de los PMN.

6. Las fracciones de plasma seminal que contienen proteínas entre 30 y 100 kDa son las que mejor mantienen la motilidad espermática y la estructura subpoblacional mótil de los espermatozoides de un eyaculado.

7. Estas fracciones entre 30 y 100 kDa son también las que mantienen más espermatozoides vivos libres tras 3h de incubación.

8. La mayor parte de proteínas descritas, en burro u otras especies, con un papel en el control de la respuesta inflamatoria del endometrio, el control del estrés oxidativo o la motilidad espermática, se encuentran en este intervalo de 30-100 kDa, aunque la mayor parte de proteínas y su función en la estrategia reproductiva del burro están aún por describir.

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