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Effect of donkey seminal plasma on sperm movement and sperm–polymorphonuclear neutrophils attachment *in vitro*

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ABSTRACT

To evaluate the effect of seminal plasma in endometrial inflammation in donkeys, samples from fresh pure, fresh diluted and frozen-thawed semen of three different jackasses were co-incubated in water bath at 37 °C with uterine Jennie's secretions collected 6 h after artificial insemination with frozen-thawed donkey semen. Individual sperm movement parameters using the computerised sperm analysis system (CASA) and sperm–polymorphonuclear neutrophils (sperm–PMN) attachment observed in Diff-Quick stained smears were evaluated at 0, 1, 2, 3 and 4 h of co-incubation. Controls consisted of incubating diluted or frozen-thawed sperm in the absence of uterine secretions. For data analyses, a repeated measures ANOVA was performed with incubation time as intra-subject factor and with treatment and donkey as inter-subject factor, followed by a post-hoc Bonferroni's test. Greater values ($P < 0.05$) of sperm–PMN percentages and a loss of progressive motility were observed in frozen-thawed semen compared with pure and diluted fresh semen samples throughout the incubation time. In addition, the presence of seminal plasma in fresh and diluted semen samples reduced the inflammatory response of polymorphonuclear neutrophils produced after insemination by suppressing the sperm–PMN attachment *in vitro*. Motility sperm parameters analysed by CASA were also less affected than those in frozen-thawed semen samples. In conclusion, seminal plasma in jennies appears to have a modulation on the endometrial response after artificial insemination with frozen-thawed donkey semen. As a result, spermatozoa with the greater motility characteristics are selected.

1. Introduction

Insemination is considered the start of communication between spermatozoa and the female organism, involving a number of species specific immune mechanisms to achieve pregnancy (Schuberth et al., 2008). Post-breeding acute endometritis, characterised by a migration of PMN into the uterus as a necessary physiological response for successful outcome, has been observed in some mammals as gilts and mares (Palm et al., 2006; Rodríguez-Martínez et al., 2010; Rozeboom et al., 2001; Troedsson et al., 2001), but not in others such as cows (Schuberth et al., 2008). This inflammatory response triggered by sperm and other components of semen, normally starts within 1 h of breeding, peaks at 6–12 h and finally decreases after 24–36 h in mares (Katila, 1996; Kotilainen et al., 1994; Troedsson, 2006; Troedsson et al., 2001). An irritating persistent post-mating

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endometritis (PMIE) caused by repeated inseminations has been regarded as a cause of infertility in mares and sows (Palm et al., 2006; Troedsson, 2006; Troedsson et al., 2001; Rodríguez-Martínez et al., 2010; Rozeboom et al., 2001) with a higher inflammatory reaction when frozen-thawed semen is used in both reproductive healthy and ill mares (Barbacini et al., 2003; Nikolakopoulos and Watson, 1997; Sieme et al., 2004; Troedsson, 2006; Troedsson et al., 2001, 2005).

In some mammalian species, removing seminal plasma is a necessary process for sperm preservation in artificial conditions. Specifically in donkeys, an improvement of sperm quality after elimination of seminal plasma in refrigerated semen has previously been reported (Miró et al., 2009). In horses and pigs, however, seminal plasma is involved in the immunological response of endometrium through inhibiting complement activation, phagocytosis and PMN chemotaxis induced by spermatozoa (Alghamdi et al., 2004; O'Leary et al., 2006; Rodríguez-Martínez et al., 2008). *In vitro* incubation sperm from highly fertile bulls with seminal plasma from lowly fertile bulls decreases the oocyte penetration ability (Henault and Killian, 1996). Furthermore, differences in composition of seminal plasma between species and among males within the same species (Alghamdi et al., 2009; Robertson, 2005; Schuberth et al., 2008) result in variable fertility indexes (Maxwell et al., 2007; Moura et al., 2006; Robertson, 2005), as well as different uterine immune responses (Katila, 2001; Kotilainen et al., 1994; Matthijs et al., 2003; Pitnick et al., 2009) and freezability (Jobim et al., 2011; Zahn et al., 2005).

Preliminary studies show that, after artificial insemination (AI) using frozen-thawed Catalan donkey semen, there is an exacerbating inflammatory response in jennies that has been suggested to lead to very few pregnancy rates (Vidament et al., 2009). This fact has limited so far reproduction by artificial insemination of this endangered species (Miró et al., 2011).

Therefore, considering the relevance of seminal plasma in reproductive physiology and taking into account that each species has many different male and female physiologic mechanisms that are involved in the regulation of post-breeding endometritis, the aim of the present study was to evaluate the *in vitro* effects of seminal plasma in the uterine inflammation and sperm function in donkeys. With this purpose, the sperm–PMN binding and its influence over sperm movement patterns was evaluated by using different semen samples containing (fresh pure sperm) or not (diluted and frozen-thawed sperm) seminal plasma, co-incubated with uterine secretions collected after artificial insemination. Negative controls consisted of semen samples incubated in the absence of such uterine secretions.

2. Materials and methods

Six healthy Catalonian jennies with ages from 5 to 14 years, and three jackasses of known fertility, aged 5–8 years old, were included in the study. Uterine cultures, endometrial cytology and a biopsy while in estrus were performed before starting the experiment to confirm the reproductive health status and results from these findings were used to select the females used in the study.

2.1. Uterine secretions

To determine the most desirable time for AI in estrus, all females were exhaustively examined by clinical observation, transrectal palpation and ultrasonography (Esaote® MyLab™30 VET; Genova, Italy) using a linear transducer of 5 MHz. After detection of a follicle diameter of greater than 40 mm, softening of this follicle and absence of corpus luteum, jennies were inseminated using eight frozen-thawed semen French straws previously thawed with a water bath at 37 °C. Final sperm concentration was of 200×10^6 spermatozoa mL^{-1} and frozen-thawed semen was deposited in jennies' uterine body using an insemination equine catheter (Minitüb Ibérica SL; Tarragona, Spain).

Uterine secretions were collected 6 h after AI using small volume uterine lavage, introducing 50 mL of warmed (37 °C) lactate ringer in the uterus through an insemination equine catheter (Minitüb Ibérica SL), and massaging the uterus *via* the rectum and recovering at least 10% of sample in a syringe. The recovered volume was registered and PMN concentration was determined by flow cytometry and cytochemistry. Ovulation and drainage of uterine fluid was evaluated by ultrasonography after 24 h of insemination.

2.2. Fresh and diluted semen samples

At the time of uterine secretion sampling, semen was collected by artificial vagina Hannover model (Minitüb Ibérica SL) equipped with an in-line filter (Minitüb Ibérica SL) to get a gel-free semen sample. Semen volume was recorded and halved in two aliquots; one of this aliquot contained raw semen (fresh pure semen), while the other was diluted 1:3 with a skim-milk based semen extender (Kenney et al., 1975) for obtaining the diluted sperm sample.

2.3. Frozen-thawed semen samples

Semen of the same donkey was frozen by means IceCube 14S, Computer Controlled Rate Freezer (Minitüb Ibérica SL), using Gent extender (Minitüb Ibérica SL) and stored at −196 °C in liquid nitrogen. At the time of experiment, frozen straws were quickly thawed in water bath at 37 °C, analysed and used immediately.

For all semen samples, a routine quality sperm analysis was performed before each experiment (concentration, sperm viability, morphology and motility) and then was standardised to get approximately the same proportion of sperm per millilitre. Only those sperm samples with >50% of viability, <25% of sperm abnormalities and >60% progressive motility were included in this study.

2.4. Treatments

Treatments (T1, T2 and T3) consisted of *in vitro* co-incubation, in water bath at 37 °C for a 4 h period, of uterine secretions with sperm samples that came from each male at a 1:1 ratio. These co-incubation experiments utilised pure

fresh (T1), diluted fresh (T2), or frozen-thawed (T3) semen samples, all of them coming from the three individuals involved in this study. In addition, two more treatments were performed as controls: diluted fresh semen, C1; and frozen-thawed semen, C2, both being incubated separately without any uterine secretion. Treatment variables (sperm–PMN attachment and sperm motility) were evaluated at 0, 1, 2, 3 and 4 h of incubation.

2.5. Sperm concentration

As stated, sperm concentration was evaluated before starting the experiment, to adjust concentration in all treatments (*i.e.* fresh pure, diluted, frozen-thawed semen) and thus ensure that it was seminal plasma rather than sperm number which affected sperm–PMN binding and sperm motility. The sperm concentration of each semen sample was determined using a haemocytometer (Newbauer chamber).

2.6. Sperm morphology and viability

Sperm viability and morphological abnormalities were assessed using eosin-nigrosin stained smears. Following this stain, 200 sperm cells were assessed per slide (Miró et al., 2005). For each sample two replicates were made.

2.7. Sperm–PMN binding

Sperm–PMN attachment in T1, T2 and T3 was determined as described (Palm et al., 2006; Alghamdi et al., 2004). A sample of 10 μl was deposited in a slide and a Diff-Quick staining smear was performed. A minimum of 200 sperm cells were counted by light optic microscopy (Carl Zeiss, Germany) at 1000× magnification and expressed as the percentage of PMN bound to at least one spermatozoon.

2.8. Sperm motility

Sperm motion characteristics were evaluated after seminal extraction and in T1, T2, T3, C1 and C2 during a 4 h period through a computer assisted sperm analysis CASA (Integrated Semen Analysis System, ISAS® Ver. 1.0.15; Projects and Services R+D S.L., ProiSer, Valencia, Spain) equipped with a negative phase contrast microscope Olympus BH-2 (Microptic, Spain) with yellow light filter, warm-up plate and a digital video camera (Basler, Germany) connected to the software. A sample of 5 μl was deposited in a 37 °C pre-warmed slide and a 24 mm^2 coverslip was carefully set. Digital images at 200× magnifications were recorded and strictly analysed to suppress erroneous routes or artefacts and acquire at least 200 cells per sample. Nine descriptive motion parameters were generated: total motility (TMOT, %); progressive motility (PMOT, %); curvilinear velocity (VCL, $\mu m\,s^{-1}$); straight-line velocity (VSL, $\mu m\,s^{-1}$); average path velocity (VAP, $\mu m\,s^{-1}$); linearity (LIN, %) (Ratio between VSL and VCL); average lateral head displacement (ALH, μm); wobble (WOB) and beat-cross frequency (BCF, Hz; Mortimer, 2000). The program settings were: frames acquired 25; connectivity, 12; particles size, 4–75 μm^2; straightness threshold, 75%; low VAP cut off, 10 $\mu m\,s^{-1}$; medium VAP cut off, 45 $\mu m\,s^{-1}$; high VAP cut off, 90 $\mu m\,s^{-1}$; minimum of images to calculate ALH, 10.

Table 1
Ejaculate variables immediately after extraction (fresh semen) as mean ± S.E.M.

Parameter	Mean ± S.E.M.
Filtered volume (mL)	52.31 ± 18.20
Sperm concentration ($\times 10^6\ mL^{-1}$)	273.26 ± 11.54
pH	7.8 ± 0.29
Sperm viability (%)	77.19 ± 12.53
Sperm immature tail (%)	10.03 ± 3.95
Sperm coiled-tail (%)	1.36 ± 1.15
Sperm head abnormality (%)	2.36 ± 2.07
Tailess spermatozoon (%)	2.26 ± 1.96
Immature sperm with proximal cytoplasmic droplet (%)	1.41 ± 1.32
Immature sperm with distal cytoplasmic droplet (%)	0.48 ± 0.5
Total morphological abnormalities	17.89 ± 8.54

2.9. Statistical analysis

All data were analysed using IBM SPSS 19.0 for Windows (SPSS Inc., Chicago, IL). Sperm–PMN attachment is expressed in percentages and results of motility parameters are presented as mean ± standard error of the mean (S.E.M.).

The data obtained from the analysis of all sperm parameters were first tested for normality and homoscedasticity using the Kolmogorov–Smirnov and Levene's tests. When necessary, data were transformed using arcsine square root (arcsin $\sqrt{x}$) before repeated measures ANOVA was run. Post-hoc *t*-test with Bonferroni adjustment was also used for pair-wise comparisons. In these models, sperm motility parameters and sperm–PMN binding were the dependent variables, the intra-subject factor was the co-incubation time between sperm and PMN (*i.e.* 0, 1, 2, 3 or 4 h) and the inter-subject factors were the treatment (*i.e.* fresh pure, diluted or frozen-thawed semen incubated in the presence/absence of uterine secretions; T1, T2, T3, C1 and C2) and the donkey.

In all the statistical analyses, the level of significance was set at $P < 0.05$.

3. Results

Table 1 contains mean ± S.E.M. data for several ejaculate variables obtained immediately after extraction, such as filtered volume and pH, sperm concentration, sperm viability and morphology categories (*i.e.* abnormalities in heads and tails, sperm with cytoplasmic droplets (Tables 2–4).

3.1. Sperm–PMN attachment

Multivariate contrasts when assessed using a repeated measures model showed there was an effect of time ($P < 0.001$) and treatment ($P < 0.001$), and an interaction between time and treatment ($P < 0.001$). In contrast, there was neither a male effect ($P > 0.05$) or interaction between time, treatment and male ($P > 0.05$).

Table 2
Total (TMOT) and progressive sperm motility (PMOT) (mean ± S.E.M.) obtained at the start (0 h) and after 1, 2, 3 and 4 h after starting the experiment. Different letters (a–d) indicate differences ($P < 0.05$) between treatments within a given time point (columns), whereas different numbers (1–4) indicate differences ($P < 0.05$) between time points within the same treatment (rows). Treatments were: pure fresh (T1), diluted fresh (T2), and frozen-thawed sperm (T3) co-incubated with uterine secretions. Controls were diluted fresh and (C1) and frozen-thawed sperm (C2) incubated in the absence of uterine secretions.

Treatment	Co-incubation time					Treatment means
	0 h	1 h	2 h	3 h	4 h	
TMOT (%)						
T1	$75.9 \pm 5.6^{a,1}$	$73.6 \pm 5.9^{a,1}$	$59.7 \pm 2.1^{a,2}$	$40.9 \pm 2.7^{a,3}$	$30.4 \pm 4.9^{a,4}$	56.1 ± 1.9^{a}
T2	$70.7 \pm 1.4^{a,b,1}$	$68.1 \pm 4.3^{b,1,2}$	$65.6 \pm 2.2^{b,2}$	$60.2 \pm 1.6^{c,3}$	$30.1 \pm 3.5^{a,4}$	$58.9 \pm 1.2^{a,b}$
T3	$66.9 \pm 4.0^{b,1}$	$57.8 \pm 7.5^{c,2}$	$53.0 \pm 0.6^{c,2}$	$44.9 \pm 1.3^{b,3}$	$28.9 \pm 5.7^{a,4}$	50.3 ± 1.7^{c}
C1	$69.1 \pm 2.9^{a,b,1}$	$67.1 \pm 3.3^{b,1}$	$70.1 \pm 3.4^{d,1}$	$65.9 \pm 2.8^{d,1}$	$49.9 \pm 4.2^{b,2}$	64.4 ± 1.5^{d}
C2	$65.2 \pm 5.2^{b,1,2}$	$65.8 \pm 5.8^{b,1,2}$	$68.1 \pm 1.8^{b,d,1}$	$60.1 \pm 2.1^{c,2}$	$43.4 \pm 3.2^{b,3}$	60.5 ± 1.6^{b}
Time means	69.6 ± 1.7^{1}	$66.5 \pm 2.4^{1,2}$	63.3 ± 0.9^{2}	54.4 ± 0.9^{3}	36.5 ± 1.9^{4}	
PMOT (%)						
T1	$68.2 \pm 4.2^{a,1}$	$63.6 \pm 1.9^{a,1}$	$58.8 \pm 2.2^{a,2}$	$55.1 \pm 2.1^{a,2}$	$50.6 \pm 2.7^{a,3}$	59.3 ± 1.2^{a}
T2	$74.9 \pm 0.5^{b,1}$	$65.3 \pm 3.5^{a,2}$	$62.9 \pm 0.8^{b,2}$	$55.1 \pm 3.4^{a,3}$	$49.8 \pm 1.5^{a,b,4}$	61.6 ± 0.9^{a}
T3	$71.8 \pm 3.7^{a,b,1}$	$58.9 \pm 0.5^{b,2}$	$60.3 \pm 1.3^{a,b,2}$	$40.4 \pm 2.9^{b,3}$	$45.2 \pm 3.7^{b,3}$	55.3 ± 1.1^{b}
C1	$74.4 \pm 3.1^{b,1}$	$76.9 \pm 4.8^{c,1}$	$86.7 \pm 5.3^{c,2}$	$74.9 \pm 5.2^{c,1}$	$60.2 \pm 4.5^{c,3}$	74.6 ± 2.0^{c}
C2	$75.1 \pm 2.8^{b,1,2}$	$73.8 \pm 0.5^{c,1}$	$77.0 \pm 1.8^{d,2}$	$69.9 \pm 4.5^{d,3}$	$55.3 \pm 3.0^{a,c,4}$	70.2 ± 1.1^{d}
Time means	72.9 ± 1.3^{1}	67.7 ± 1.0^{2}	69.1 ± 1.0^{2}	59.1 ± 1.6^{3}	52.2 ± 1.4^{4}	

In Fig. 1, mean ± SEM data are depicted for binding percentages between sperm and PMN immediately after starting the experiment (0 h) and after 1, 2, 3 and 4 of sperm–PMN co-incubation. No significant differences among treatments (*i.e.* fresh, diluted and frozen-thawed semen) were observed at the beginning of the experiment (*i.e.* 0 h). After 1, 3 and 4 h of co-incubation, however, sperm–PMN binding was greater ($P < 0.05$) in frozen-thawed than in diluted and pure fresh semen, and in the former greater than in the latter. At 2 h, frozen-thawed semen had a greater sperm–PMN binding than in diluted and fresh semen, but there were no significant differences between the diluted and fresh semen. The maximum peak of sperm–PMN binding was found after 3 h of co-incubation in frozen-thawed semen, where a tendency to form large cell aggregates was detected (Fig. 2). Thus, there was

Table 3
Sperm velocity variables (curvilinear (VCL), straight linear (VSL) and average pathway (VAP) velocities), as mean ± S.E.M., obtained at the start (0 h) and after 1, 2, 3 and 4 h from initiation of the experiment. Different letters (a–d) indicate differences ($P < 0.05$) between treatments within a given time point (columns), whereas different numbers (1–4) indicate differences ($P < 0.05$) between time points within the same treatment (rows). Treatments were: pure fresh (T1), diluted fresh (T2), and frozen-thawed sperm (T3) co-incubated with uterine secretions. Controls were diluted fresh and (C1) and frozen-thawed sperm (C2) incubated in the absence of uterine secretions.

Treatment	Co-incubation time					Treatment means
	0 h	1 h	2 h	3 h	4 h	
VCL ($\mu m\,s^{-1}$)						
T1	$108.6 \pm 3.2^{a,1}$	$99.8 \pm 1.5^{a,2}$	$80.8 \pm 3.9^{a,3}$	$101.1 \pm 8.6^{a,d,2}$	$86.6 \pm 10.2^{a,d,3}$	95.3 ± 2.4^{a}
T2	$102.0 \pm 0.3^{b,1}$	$99.1 \pm 1.6^{a,1}$	$109.1 \pm 1.4^{b,2}$	$92.6 \pm 7.6^{b,3}$	$80.0 \pm 9.0^{a,4}$	96.6 ± 1.8^{a}
T3	$105.4 \pm 2.2^{a,b,1}$	$105.8 \pm 1.2^{b,1}$	$83.6 \pm 0.6^{a,2}$	$84.6 \pm 5.9^{c,2}$	$62.1 \pm 7.6^{b,3}$	88.3 ± 1.6^{b}
C1	$103.0 \pm 0.9^{b,1}$	$106.5 \pm 3.5^{b,1}$	$118.6 \pm 3.9^{c,2}$	$106.9 \pm 8.0^{d,1}$	$107.2 \pm 7.3^{c,1}$	108.4 ± 2.1^{c}
C2	$102.5 \pm 1.7^{b,1}$	$100.8 \pm 6.5^{a,1}$	$90.0 \pm 4.5^{d,2}$	$98.5 \pm 6.4^{a,b,1}$	$90.0 \pm 5.3^{d,2}$	96.4 ± 2.2^{a}
Time means	104.2 ± 0.7^{1}	102.4 ± 1.3^{1}	96.4 ± 1.3^{2}	96.7 ± 3.3^{2}	85.2 ± 3.5^{3}	
VSL ($\mu m\,s^{-1}$)						
T1	$44.5 \pm 1.4^{a,1}$	$54.2 \pm 2.6^{a,2}$	$40.1 \pm 2.6^{a,3}$	$45.8 \pm 3.4^{a,1}$	$40.0 \pm 2.2^{a,3}$	44.9 ± 1.1^{a}
T2	$54.9 \pm 4.5^{b,2}$	$53.4 \pm 1.8^{a,1}$	$57.9 \pm 3.1^{b,2}$	$43.7 \pm 1.9^{a,3}$	$41.5 \pm 5.7^{a,3}$	50.3 ± 1.5^{b}
T3	$47.3 \pm 2.5^{a,c,1}$	$43.7 \pm 4.4^{b,2}$	$42.8 \pm 1.7^{a,2}$	$38.3 \pm 5.6^{b,3}$	$32.3 \pm 5.6^{b,4}$	40.9 ± 1.8^{c}
C1	$50.1 \pm 5.3^{b,c,1}$	$69.0 \pm 2.9^{c,2}$	$66.5 \pm 4.0^{c,2}$	$55.6 \pm 2.9^{c,3}$	$67.0 \pm 5.3^{c,2}$	61.6 ± 1.8^{d}
C2	$45.2 \pm 3.5^{a,1}$	$43.7 \pm 5.1^{b,1}$	$49.4 \pm 2.5^{d,2}$	$46.6 \pm 3.5^{a,1,2}$	$43.0 \pm 4.6^{a,1}$	45.6 ± 1.7^{a}
Time means	48.4 ± 1.5^{1}	52.8 ± 1.5^{2}	51.3 ± 1.2^{2}	$46.0 \pm 1.5^{1,3}$	44.8 ± 2.1^{3}	
VAP ($\mu m\,s^{-1}$)						
T1	$66.7 \pm 8.7^{a,1}$	$73.4 \pm 4.8^{a,2}$	$52.8 \pm 1.2^{a,3}$	$66.3 \pm 6.7^{a,d,1}$	$52.2 \pm 7.8^{a,3}$	62.3 ± 2.6^{a}
T2	$71.5 \pm 0.8^{b,1}$	$74.2 \pm 3.6^{a,1}$	$80.1 \pm 3.4^{b,2}$	$60.4 \pm 4.9^{a,b,3}$	$60.4 \pm 7.6^{b,3}$	69.3 ± 1.8^{b}
T3	$66.2 \pm 5.8^{a,1}$	$64.9 \pm 2.4^{b,1}$	$58.5 \pm 2.1^{c,2}$	$55.2 \pm 5.7^{b,2}$	$36.8 \pm 5.9^{c,3}$	56.3 ± 2.0^{c}
C1	$73.7 \pm 4.1^{b,1}$	$100.9 \pm 3.6^{c,2}$	$95.7 \pm 2.9^{d,2,3}$	$91.6 \pm 3.7^{c,3}$	$96.3 \pm 6.6^{d,2}$	91.6 ± 1.9^{d}
C2	$66.0 \pm 2.1^{a,1,4}$	$75.3 \pm 3.0^{a,2}$	$59.5 \pm 3.3^{c,3}$	$68.8 \pm 4.5^{d,1}$	$62.3 \pm 4.9^{b,3,4}$	66.4 ± 1.6^{b}
Time means	68.8 ± 1.9^{1}	77.7 ± 1.6^{2}	69.3 ± 1.1^{1}	68.5 ± 2.3^{1}	61.6 ± 2.9^{3}	

Table 4
Percentages of linearity (%LIN) and Wobbless (%WOB), and values of amplitude of head lateral displacement (ALH) and beat cross frequency, as mean ± S.E.M., obtained at the start (0 h) and after 1, 2, 3 and 4 h from initiation the experiment. Different letters (a–d) indicate differences ($P < 0.05$) between treatments within a given time point (columns), whereas different numbers (1–4) indicate differences ($P < 0.05$) between time points within the same treatment (rows). Treatments were: pure fresh (T1), diluted fresh (T2), and frozen-thawed sperm (T3) co-incubated with uterine secretions. Controls were diluted fresh and (C1) and frozen-thawed sperm (C2) incubated in the absence of uterine secretions.

Treatment	Co-incubation time					Time means
	0 h	1 h	2 h	3 h	4 h	
LIN (%)						
T1	$49.5 \pm 2.8^{a,1}$	$58.6 \pm 5.2^{a,c,2}$	$57.1 \pm 2.8^{a,b,2}$	$48.1 \pm 3.2^{a,b,1,3}$	$42.9 \pm 5.6^{a,3}$	51.2 ± 1.7^{a}
T2	$65.1 \pm 2.8^{b,c,1}$	$58.5 \pm 1.8^{a,2}$	$55.7 \pm 1.6^{a,2,3}$	$52.4 \pm 5.5^{a,c,3}$	$41.4 \pm 2.5^{a,4}$	54.6 ± 1.3
T3	$67.1 \pm 5.1^{b,1}$	$44.4 \pm 4.4^{b,2}$	$55.9 \pm 8.8^{a,b,3}$	$44.9 \pm 2.8^{b,2}$	$30.5 \pm 3.8^{b,4}$	48.6 ± 2.2
C1	$63.7 \pm 2.5^{b,c,1}$	$59.7 \pm 5.2^{a,1,2}$	$59.2 \pm 5.4^{b,1,2}$	$55.8 \pm 5.5^{c,2,3}$	$50.4 \pm 2.5^{c,3}$	57.8 ± 1.9
C2	$61.3 \pm 3.5^{c,1}$	$52.8 \pm 6.1^{c,2}$	$58.5 \pm 6.5^{a,b,1,3}$	$54.7 \pm 4.5^{c,2,3}$	$45.4 \pm 4.0^{a,4}$	54.5 ± 2.2
Time means	61.3 ± 1.5^{1}	$54.8 \pm 2.0^{2,3}$	57.3 ± 2.2^{2}	51.2 ± 1.9^{3}	42.1 ± 1.6^{4}	
WOB (%)						
T1	$61.8 \pm 9.7^{a,1}$	$75.7 \pm 1.4^{a,2}$	$70.3 \pm 2.3^{a,3}$	$67.5 \pm 7.2^{a,b,3,4}$	$64.5 \pm 7.2^{a,1,4}$	68.0 ± 2.5^{a}
T2	$81.9 \pm 2.3^{b,1}$	$77.1 \pm 1.2^{a,c,1,2}$	$74.5 \pm 8.7^{a,2}$	$69.9 \pm 7.9^{a,3}$	$65.1 \pm 7.8^{a,3}$	73.7 ± 2.5^{b}
T3	$74.5 \pm 9.2^{c,d,1}$	$62.9 \pm 5.7^{b,2}$	$71.5 \pm 8.0^{a,1}$	$63.7 \pm 5.9^{b,2}$	$55.1 \pm 5.2^{b,3}$	65.5 ± 3.0^{a}
C1	$78.5 \pm 5.9^{b,c,1}$	$80.5 \pm 2.8^{c,1}$	$80.7 \pm 5.8^{b,1}$	$78.8 \pm 5.0^{c,1}$	$78.4 \pm 6.7^{c,1}$	79.4 ± 2.3^{c}
C2	$70.5 \pm 7.5^{d,1,2}$	$68.4 \pm 4.2^{b,1}$	$73.9 \pm 7.5^{a,2,3}$	$75.9 \pm 4.8^{c,3}$	$73.6 \pm 4.6^{c,2,3}$	72.5 ± 2.6^{b}
Time means	73.4 ± 3.1^{1}	72.9 ± 1.4^{1}	74.2 ± 2.9^{1}	$71.2 \pm 2.7^{1,2}$	67.3 ± 2.8^{2}	
ALH (μm)						
T1	$4.1 \pm 0.6^{a,1}$	$3.2 \pm 1.1^{a,2,3}$	$2.9 \pm 0.5^{a,3}$	$3.8 \pm 0.1^{a,1,2}$	$3.4 \pm 0.4^{a,2}$	3.5 ± 0.3^{a}
T2	$2.8 \pm 0.9^{b,1}$	$3.1 \pm 1.0^{a,1,2}$	$3.5 \pm 0.3^{b,2}$	$3.2 \pm 0.7^{a,1,2}$	$3.4 \pm 0.3^{a,2}$	3.2 ± 0.3^{a}
T3	$3.1 \pm 0.2^{b,1}$	$4.0 \pm 0.3^{b,2}$	$2.9 \pm 0.7^{a,1}$	$3.2 \pm 1.3^{a,1}$	$2.1 \pm 0.3^{b,3}$	3.1 ± 0.2^{a}
C1	$3.0 \pm 0.7^{b,1}$	$4.6 \pm 1.2^{b,2}$	$4.6 \pm 0.4^{c,2}$	$4.7 \pm 0.8^{b,2}$	$4.6 \pm 0.3^{c,2}$	4.3 ± 0.3^{b}
C2	$3.1 \pm 0.5^{b,1}$	$2.5 \pm 0.5^{c,2}$	$3.7 \pm 0.5^{b,3}$	$3.8 \pm 0.6^{a,3}$	$3.1 \pm 0.5^{a,1}$	3.2 ± 0.3^{a}
Time means	3.2 ± 0.3^{1}	3.5 ± 0.4^{1}	3.5 ± 0.2^{1}	3.7 ± 0.3^{1}	3.3 ± 0.2^{1}	
BCF (Hz)						
T1	$8.2 \pm 0.1^{a,1}$	$8.4 \pm 0.4^{a,1}$	$8.0 \pm 0.5^{a,1}$	$8.1 \pm 0.4^{a,1}$	$9.2 \pm 0.8^{a,2}$	8.4 ± 0.3^{a}
T2	$7.8 \pm 0.3^{a,1}$	$8.4 \pm 1.0^{a,1,2}$	$8.4 \pm 0.9^{a,1,2}$	$7.9 \pm 0.5^{a,1}$	$8.8 \pm 0.8^{a,2}$	8.3 ± 0.4^{a}
T3	$7.9 \pm 0.6^{a,1}$	$11.2 \pm 0.8^{b,2}$	$10.3 \pm 0.4^{b,3}$	$11.5 \pm 0.6^{b,2}$	$9.9 \pm 0.7^{a,b,3}$	10.2 ± 0.4^{b}
C1	$7.2 \pm 1.1^{b,1}$	$10.5 \pm 1.2^{b,2}$	$10.9 \pm 0.6^{b,2}$	$10.6 \pm 0.6^{c,2}$	$10.7 \pm 0.7^{b,2}$	10.0 ± 0.5^{b}
C2	$7.7 \pm 0.3^{a,b,1}$	$8.2 \pm 0.7^{a,1,2}$	$8.8 \pm 0.1^{a,2}$	$8.3 \pm 0.6^{a,1,2}$	$7.6 \pm 0.7^{c,1}$	8.1 ± 0.3^{a}
Time means	7.8 ± 0.2^{1}	9.3 ± 0.4^{2}	9.3 ± 0.2^{2}	9.3 ± 0.2^{2}	9.2 ± 0.7^{2}	

less ($P < 0.05$) sperm–PMN binding throughout all experimental periods when fresh semen (T1) was compared with diluted fresh and frozen-thawed semen (T2 and T3; Fig. 1).

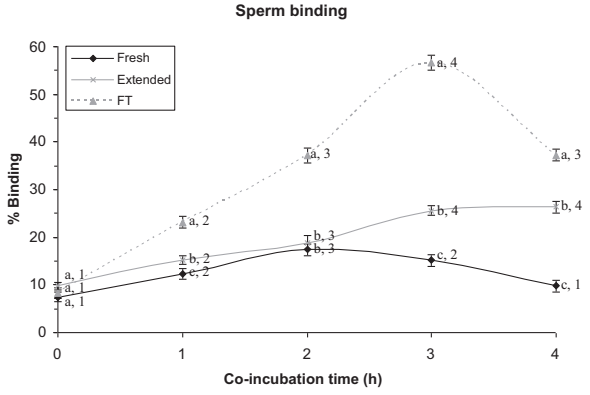

Fig. 1. Sperm binding to leukocytes 0, 1, 2, 3 or 4 h after co-incubation of PMN with fresh, extended or frozen-thawed (FT) spermatozoa. Different letters (a–c) indicate differences ($P < 0.05$) between treatments within a given time point (columns), whereas different numbers (1–4) indicate differences ($P < 0.05$) between time points within the same treatment (rows).

3.2. Sperm motility

For sperm motility variables (except ALH), there was an effect of time ($P < 0.01$) and treatment ($P < 0.005$), and an interaction between time and treatment ($P < 0.01$). In contrast, there was not a male effect ($P > 0.05$) or interaction between time, treatment and male ($P > 0.05$).

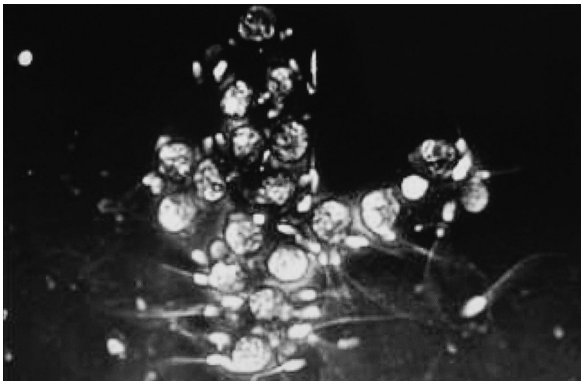

Fig. 2. Sperm–PMN attachment of frozen semen with a tendency to form large aggregates at 3 h of incubation.

Total sperm motility (TMOT) was less ($P < 0.05$) with all the treatments, but the reduction was greater in semen co-incubated with uterine secretions (T1–T3) than in semen incubated in the absence of these secretions (C1 and C2) after 3 and 4 h of co-incubation. Within treatments (T1–T3), for diluted semen (T2) there was a greater percentage of total motile spermatozoa than for pure fresh (T1) and frozen (T2) semen after 3 h of co-incubation with uterine secretions.

Similarly, to what occurred with total sperm motility, controls (C1 and C2) had greater sperm motility after 1 h of co-incubation and throughout the remaining experimental period, except after 4 h of co-incubation where there were no significant differences between T1 and C2. Again, T1 and T2 resulted in similar progressive motility data, and for frozen-thawed semen (T3) there was a lesser percentage of progressively motile spermatozoa after 1, 3 and 4 h of co-incubation with uterine secretions.

With regard to curvilinear velocity (VCL), there were no differences between treatments observed at the beginning of the experiment, but diluted spermatozoa incubated without uterine secretions (C1) had a greater VCL than the other treatments after 2 and 4 h of the experimental period. In contrast, a lesser VCL ($P < 0.05$) was observed in frozen-thawed semen (T3) co-incubated with uterine secretions at 4 h. In addition, the VCL was less ($P < 0.01$) at the end of the experiment (4 h) when compared to the beginning (0 h) in all treatments, except in C1.

Incubation of diluted semen in the absence of uterine secretions (C1) also resulted in a greater VSL at 1 h and throughout the remaining experimental period. It should be noted that the VSL was less at the end of experiment when compared to 0 h for all three treatments (T1, T2 and T3) but not in both controls, where VSL was greater (C1) or remained unaltered.

Average Pathway Velocity (VAP) was less from the beginning of the experiment and until the end of the co-incubation period in T1, T2, and T3. In contrast, spermatozoa from both controls (C1 and C2) did not reduce the VAP over the experimental period, and in the case of C1 increased the VAP. In addition, incubation of diluted sperm without uterine secretions (C1) resulted in a greater VAP than with the other treatments throughout all the experimental period.

Percentage of LIN significantly decreased in all the treatments from the beginning of the experiment, but the extent of this reduction was significantly higher in T3 than in the other treatments, especially at 4 h. In contrast, it was the C1 treatment which presented the lowest reduction in sperm LIN percentage.

With regard to WOB percentage, and despite no significant differences being observed at the start of experiment between diluted fresh semen co-incubated with uterine secretions (T2) and that incubated in the absence of such secretions (C1), a greater reduction in WOB percentages occurred with the former than the treatment (T2) from 1 h until the end of the experimental period. Again, frozen-thawed semen co-incubated with uterine secretions (T3) had a lesser WOB percentage after 1, 3 and 4 h of the incubation period.

Finally, ALH increased in C1 over the experimental period, while the values of BCF at 4 h were the greatest in C1 and least in C2.

4. Discussion

The uterus has features of a mucosa-associated lymphoid tissue that varies with the estrous phase in mares. A neutrophilic based population is observed just before ovulation, and its inflammatory activity is triggered by insemination to protect the uterine environment against sperm, bacteria and other substances acting as foreign bodies (Katila, 2001; Pitnick et al., 2009; Schuberth et al., 2008). However, the endometrial physiology and its response to semen vary with the composition, amount of sperm, seminal plasma components or added substances such as extenders (Gorgens et al., 2005; Palm et al., 2006). A greater inflammatory response triggered by frozen-thawed semen may be responsible for the lesser fertilisation and subsequent pregnancy obtained in donkeys (Miró et al., 2011; Vidament et al., 2009), possibly because of an exacerbating innate response to semen due to the absence of seminal plasma and/or presence of cryo-protectants that enhance irritation of the uterus as reported in horses (Gorgens et al., 2005; Nikolakopoulos and Watson, 1997; Watson et al., 2001).

During the incubation times, pure fresh semen followed by diluted fresh semen treatments resulted in less sperm–PMN attachment compared with frozen-thawed semen samples, and the difference increased over time with formation of large aggregates of sperm, PMN and endometrial cells. Similar results were observed in stallion fresh semen samples incubated with uterine mare secretions, where there was greater sperm–PMN attachment in the absence of seminal plasma compared with those diluted with extender or a mix of both. There was also a dose dependent effect of seminal plasma in reduction of sperm–PMN binding (Alghamdi et al., 2004; Rozeboom et al., 2001). BIn addition there is blocking of the effects of seminal plasma to interleukin-8-induced neutrophil chemotaxis (Rozeboom et al., 2001). The biological meaning of the large neutrophil aggregates is still incomplete but it appears as though the most relevant anti-chemotactic effect of seminal plasma is the inhibition of the strongly induced agglutination of neutrophils caused by sperm cells through a seminal plasma DNAase enzyme (Alghamdi et al., 2010).

The reduced sperm–PMN relation observed in fresh and diluted semen samples has been attributed to the presence of seminal plasma that suppresses the opsonisation, complement activation, chemiotaxis and, thus, blocks sperm phagocytosis (Alghamdi et al., 2004; Eisenbach, 2003; Katila, 1996; 2001; Troedsson et al., 2001, 2005; Schuberth et al., 2008). In contrast, seminal plasma promotes PMN binding and phagocytosis of non-viable spermatozoa (Troedsson et al., 2005). Transient binding of PMN to specific viable sperm cell subpopulations has been observed. It has been hypothesised that aged, dead or hyper-activated spermatozoa are targeted by neutrophilic granulocytes (Jaffe et al., 2006; Matthijs et al., 2003). The massive increase of PMN in the uterine lumen and

subsequent cell mass formations is also favoured by the presence of extenders, cryo-protectant substances and/or biochemical changes in the sperm membrane structure caused by the freezing-thawing process (Gorgens et al., 2005; Kotilainen et al., 1994; Matthijs et al., 2003). In this regard, membrane integrity is considered one of the key features for this selective reaction (Eisenbach, 2003; Gorgens et al., 2005; Mary, 2000; Taylor et al., 2008).

Despite some studies suggesting there are proteinaceus substances in seminal plasma involved in uterine immunomodulation in horses and swine (Alghamdi et al., 2004; Rodríguez-Martínez et al., 2010; Troedsson et al., 2005), other components in whole seminal plasma also seem to be implicated in selecting the viable and non-viable sperm and defending from the uterine inflammatory cells. However, the molecular mechanisms of these cell-to-cell interactions still remain unclear (Schuberth et al., 2008; Troedsson et al., 2005).

With regard to sperm movement, treatments and controls (always with greater values) were similar in all motility variables obtained by CASA at 0 h, and then decreased over time with a greater influence in treatments co-incubated with uterine secretions than in controls, changing from a rapid linear progressive movement to a slow non-linear non-progressive movement pattern. Total and progressive motilities followed by average velocity and linearity were the variables affected to the greatest extent (TMOT, PMOT, VAP and LIN). Related to this, it is worth noting that altered TMOT, PMOT and VAP due to the presence of PMN have previously been observed (Alghamdi et al., 2001; Reilas et al., 1997; Troedsson et al., 2001). Further, an initial rapid progressive motility is needed for the passage of sperm through specific sites in the female tract, and a force speed is required for efficient penetration of the zona pellucida (Olds-Clarke, 1996). Changes in velocity variables reduce the sperm ability to travel along the uterus and arrive at the fertilisation site (utero-tubal junction), so that less than 1% of inseminated spermatozoa are transported to the oviducts after insemination (Liu and Scott, 2000; Olds-Clarke, 1996). There was less variation for WOB, ALH and BCF variables throughout the co-incubation period and these were considered as the variables with the greatest impact on sperm binding Alghamdi et al., 2001) even though sperm head movements are less affected by uterine inflammatory cells. In addition and although spermatozoa could not move forward, some sperm cells were not impacted by inflammatory cells. This might represent a strategy for sperm selection as previously suggested (Alghamdi et al., 2001; Eisenbach, 2003; Jaffe et al., 2006). In fact, and considering sperm physiology, this fact appears to be directly related with spermatozoa energy and, eventually, this waste of energy could render sperm immobile and thus reduce viability (de Andrade et al., 2011; Kareskoski and Katila, 2008).

In the present study, there were few differences between treatments (T1–T3) in sperm movement after 4 h of co-incubation, confirming that sample types (fresh, diluted or frozen-thawed semen) do not impact the competency of inflammatory female cells collected after insemination from binding to sperm cells. In addition, the PMN influence on sperm movement also increased gradually over time as reported in horses (Alghamdi et al., 2001). However, sperm motility of frozen-thawed semen was affected to the greatest extent by treatment during the present experiment compared with pure and diluted fresh semen samples. This finding is consistent with those of Troedsson et al. (2005) where it was reported that removal or reduction of seminal plasma prior to cryopreservation affected sperm transport through the uterus. Furthermore, a stimulant effect of seminal plasma on sperm motility when in low concentrations and short-term exposures has also been reported (Jasko et al., 1992).

In horses, insemination with frozen-thawed semen has been successfully used with results over 60% (Katila, 2005; Samper and Morris, 1998; Sieme et al., 2004). Nevertheless, and despite frozen-thawed donkey semen having an apparently acceptable quality after thawing (with a 45–50% average viability), very poor pregnancy outcomes have been obtained after insemination in different donkey breeds (Canisso et al., 2008, 2011; Flores et al., 2008; Miró et al., 2009; Vidament et al., 2009). The intensive inflammatory response of the female endometrium along with the shorter longevity of frozen-thawed spermatozoa in absence of seminal plasma appear to limit the number of viable spermatozoa that are able to reach the oviduct and, subsequently, probabilities of fertilisation are clearly reduced as observed in horses (Katila, 2005). Interestingly, pregnancy rates have been reported to be greater when high quality spermatozoa suspended in seminal plasma is used to inseminate mares, with induced inflamed uteri compared with extended semen (77% compared with 5%; Alghamdi et al., 2004).

The role of seminal plasma has been gaining importance in reproductive physiology, because of the modulating mechanisms of the compounds that are in this plasma. Taken together results of previous and the present study could lead to development of procedures to enhance fertility rates using AI protocols with frozen-thawed semen in donkeys, as well as could allow for standardising and safe use of seminal plasma preparations within insemination doses as an *in vivo* anti-inflammatory treatment.

5. Conclusions

In conclusion, regardless of the sperm origin (fresh, diluted or frozen semen) insemination produces an inflammatory uterine response mainly characterised by the presence of active PMN in donkeys. Absence of seminal plasma in frozen-thawed sperm samples enhances the inflammatory activity of PMN *in vitro* as expressed by more sperm–PMN attachment with complex cell mass formations and more evident altered sperm motility variables (mainly velocity and progressiveness) than in fresh and diluted semen samples, in which seminal plasma is present. In addition, and although donkey's frozen-thawed semen has showed favourable viability characteristics and fertility *in vitro*, insemination protocols should be modified to achieve satisfactory results *in vivo*. Finally, our results warrant more research on the immuno-modulating mechanisms of seminal plasma in the reproductive breeding

process, as more knowledge in this area may enhance the understanding of inflammation and contribute to optimising reproductive technologies such as AI, not only in donkeys but also in other species susceptible to sperm-induced endometritis.

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