BOAR SPERMATOZOA AND PROSTAGLANDIN $F_{2\alpha}$

QUALITY OF BOAR SPERM AFTER THE ADDITION OF PROSTAGLANDIN $F_{2\alpha}$ TO THE SHORT-TERM EXTENDER OVER COOLING TIME

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ABSTRACT

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been used to improve reproductive performance in swine. The goal of the present work was to determine how the addition of $PGF_{2\alpha}$ affects boar sperm quality. Eleven different treatments were evaluated: eight with only $PGF_{2\alpha}$ (0.625, 1.25, 2.50, 5, 10, 12.50, 25 and 50 mg $PGF_{2\alpha}$/100 ml) and three binary treatments (0.625 mg $PGF_{2\alpha}$/100 ml + 200 µg/ml hyaluronic acid (HA), 1.25 mg $PGF_{2\alpha}$/100 ml + 200 µg/ml HA, 0.625 mg $PGF_{2\alpha}$/100 ml + 7.5 µM caffeine (Caf)). All these substances were added to sixteen ejaculates from sixteen healthy and sexually mature boars (n=16), and each ejaculate was considered as a replicate. Our study also assessed the effects of these eleven treatments over different periods of preservation.

Sperm quality was tested immediately after the addition of treatments (time 0), and after 1 day, 3 days, 6 days and 10 days of cooling at 15 ºC. To evaluate sperm quality, five parameters were analysed: (1) sperm viability, acrosome and mitochondrial sheath integrity (using a multiple fluorochrome-staining test), (2) sperm motility, (3) sperm morphology and (4) agglutination (using a computer assisted system) and (5) osmotic resistance (using the ORT).

Parametric (analysis of variance for repeated measures) and non-parametric tests (Friedman test) were used as statistical analyses. Treatments with $PGF_{2\alpha}$ concentrations higher than 12.5 mg/100 ml were cytotoxic while the others did not damage boar spermatozoa. Thus, the other treatments may be used to produce profitable effects without adverse effects. Moreover, the addition of $PGF_{2\alpha}$ at 5 mg/100 ml to sperm diluted in BTS may maintain sperm viability and motility better after 6 days of cooling, because significant differences were observed ($P<0.05$) compared with control at the same time.

KEYWORDS: boar, sperm quality, prostaglandin $F_{2\alpha}$, hyaluronic acid, caffeine

1. INTRODUCTION

Currently, the use of Artificial Insemination (AI) is a routine practice in the swine industry. Several studies have been carried out in order to improve fertility with AI use. Numerous pharmacological agents have been shown to enhance male reproductive performance in many species (Traas et al., 2004).

Prostaglandins (PGs) are extensively distributed in vertebrate tissues and are important in a wide array of physiological processes (Kingsley et al., 2005). PGs are present in seminal fluid (Templeton et al., 1978) and in cervical mucus (Charbonnel et al., 1982). Furthermore, Roy and Ratnam (1992) showed that human spermatozoa are able to synthesize PGs. The role of PGs and other arachidonic acid metabolites in the events leading to the acrosome reaction and fertilization have been investigated (Joyce et al., 1987). On one hand, several studies of prostaglandins (PGE_1 and PGE_2) and their effects on sperm function have been carried out (Aitken and Kelly, 1985; Gottlieb et al., 1988; Shimizu et al., 1998). The effects of different types and amounts of prostaglandins (19-OH-PGE, 19-OH-PGF, PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$) on human sperm function have also been reported (Bendvold et al., 1984). While the addition of $PGF_{1\alpha}$ reduced sperm motility, 19-OH-PGE stimulated sperm motility and penetration capacity positively and 19-OH-PGF diminished ATP concentration in sperm. The other assayed prostaglandins (PGE_1, PGE_2, and $PGF_{2\alpha}$) had no effect on sperm function (Gottlieb et al., 1988). Conversely, Herrero et al. (1997) found that PGE_1 is a capacitating factor in vitro for mouse spermatozoa.

On the other hand, several authors have demonstrated that $PGF_{2\alpha}$ improves fertility (Gustaffson et al., 1975; Gamcik et al., 1980). The physiological role of $PGF_{2\alpha}$ and other seminal components in a sow's reproductive tract contributes to the timing of ovulation in response to mating (Claus, 1990). Cheng et al. (2001) added $PGF_{2\alpha}$ to boar

76 sperm doses 72 hours before AI without observing the inactivation of this hormone. Kos
77 and Bilkei (2004) also reported that $PGF_{2\alpha}$ significantly improves reproductive
78 performance whenever extended sperm is supplemented with it.
79 In the present study, treatments assessed were not only done using several
80 concentrations of $PGF_{2\alpha}$ but also a binary combination of $PGF_{2\alpha}$ and either HA or
81 caffeine. HA improves the velocity and the retention of motility in both fresh ejaculated
82 (Huszar et al., 1990) and cryopreserved/thawed human spermatozoa (Sbracia et al.,
83 1997) and in boar spermatozoa (Peña et al., 2004). Moreover, HA also induces the
84 acrosome reaction of spermatozoa in humans (Slotte et al., 1993; Sabeur et al., 1998),
85 monkeys (VandVoort et al., 1998), hamsters (Stanley and Turner, 1986) and boars
86 (Peña et al., 2004), and it has been successfully used to decrease polyspermy in
87 conventional porcine in vitro fertilization (IVF) (Suzuki et al., 2000). Caffeine is a
88 phosphodiesterase inhibitor that stimulates sperm motility and increases intracellular
89 cAMP levels, thus stimulating the capacitation of spermatozoa in humans (Rees et al.,
90 1990), boars (Funahashi and Nagai, 2001) and bulls (Coscioni et al., 2001). Therefore,
91 caffeine is usually used to induce sperm capacitation in several IVF systems (Funahashi
92 and Day, 1993; Funahashi and Nagai, 2001).
93 In light of this background, our hypothesis was set. This assumed that the hormone
94 $PGF_{2\alpha}$ might be added to seminal doses in order to improve reproductive performance
95 without damaging sperm quality.
96 To test this hypothesis, we assessed eleven treatments chosen after carrying out
97 preliminary trials. All these treatments contained different concentrations of $PGF_{2\alpha}$, and
98 three were also combined with HA and caffeine. The effects of these treatments in
99 commercial seminal doses in a short-term extender (Beltsville thawing solution, BTS)

100 were also evaluated after different periods of preservation time (immediately, 1, 3, 6 and
101 10 days of cooling).
102
103 2. MATERIAL AND METHODS
104 2.1. Sperm samples
105 Sixteen ejaculates from sixteen sexually mature and healthy Pietrain boars were used to
106 analyse the effects of $PGF_{2\alpha}$ on boar sperm quality. The boars were housed in climate
107 controlled buildings (relative humidity and temperature) and fed with an adjusted diet.
108 These boars were subjected to an extraction rhythm of twice a week by means of the
109 gloved-hand technique (Hancock and Howell, 1959). The total volume of the sperm-
110 rich fraction was filtered through gauze and, after 1:5 v/v dilution in BTS (Tecnovit,
111 Valencia, Spain), samples were cooled at 15ºC and transported to the laboratory.
112 This study was carried out over eight weeks, in winter 2005 (from mid-January to mid-
113 March). In the first, third, fifth and seventh weeks, four ejaculates from four different
114 boars were received, and the analyses were performed corresponding to immediately
115 afterwards, the first and the third days. In the second, fourth, sixth and eighth weeks, the
116 assessments corresponding to the sixth and tenth days of cooling were carried out. Each
117 ejaculate was split up into 60 aliquots (11 treatments + control, and 5 different
118 preservation times; that is, 12x5).
119 Although the level of antibiotics in short-term extenders may be insufficient,
120 contamination was never observed using phase contrast microscopy at 20x
121 magnification.
122 2.2. $PGF_{2\alpha}$ treatments and different times assayed
123 Eleven treatments consisted of different concentrations of $PGF_{2\alpha}$ (Dinolytic®,
124 Pharmacia, 5 mg $PGF_{2\alpha}$/ml) and three of them also combined $PGF_{2\alpha}$ with hyaluronic

125 acid (HA, Sigma-Aldrich®) or caffeine (Caf, Panreac®). The treatments were: (1) 0.625
126 mg $PGF_{2\alpha}$/100 ml, (2) 1.25 mg $PGF_{2\alpha}$/100 ml, (3) 2.50 mg $PGF_{2\alpha}$/100 ml, (4) 5 mg
127 $PGF_{2\alpha}$/100 ml, (5) 10 mg $PGF_{2\alpha}$/100 ml, (6) 12.50 mg $PGF_{2\alpha}$/100 ml, (7) 25 mg
128 $PGF_{2\alpha}$/100 ml, (8) 50 mg $PGF_{2\alpha}$/100 ml, (9) 0.625 mg $PGF_{2\alpha}$/100 ml + 200 μg/ml HA,
129 (10) 1.25 mg $PGF_{2\alpha}$/100 ml + 200 μg/ml HA, and (11) 0.625 mg $PGF_{2\alpha}$/100 ml + 7.5
130 μM Caffeine.
131 On the other hand, sperm samples were subjected to different preservation times at 15ºC
132 after the addition of $PGF_{2\alpha}$, HA or caffeine, according to each case. Measurements of
133 sperm quality parameters (viability and integrity, motility, morphology, agglutination
134 and osmotic resistance) were performed at five time points: immediately after addition
135 of $PGF_{2\alpha}$, and after 1 day, 3 days, 6 days and 10 days. Therefore, the effects of $PGF_{2\alpha}$
136 on boar sperm quality were tested according to the amount of added substance, binary
137 combinations with two other compounds and the preservation time.
138 Sperm quality was evaluated before and after applying the different treatments.
139 2.3. Evaluation of sperm viability, acrosome and mitochondrial sheath integrity
140 Sperm viability was assessed using a multiple fluorochrome-staining test, and
141 examination under an epifluorescence microscope (Leica DMLR-XA) according to
142 Bussalleu et al. (2005). A Leica 40X 1.32 HCX PL APO objective was used. Sperm
143 samples were stained using four different fluorochromes, the first two of which were
144 nuclear fluorochromes: (a) bisbenzimide (Hoescht 33258; specific for viable cells;
145 Sigma, St. Louis, MO, USA), (b) propidium iodide (specific for nonviable cells; Sigma,
146 St. Louis, MO, USA), (c) the Mitotracker® Green FM fluorochrome (specific for
147 functional mitochondria; Molecular Probes®, Eugene, OR, USA) and (d) the lectin
148 *Trypsin inhibitor* from Soybean (SBTI) conjugated with the fluorochrome Alexa Fluor®
149 488 (specific for proacrosin; Molecular Probes®, Eugene, OR, USA). Stained samples

were then observed. For each sample, 300 spermatozoa (3 drops, 100 spermatozoa per drop) were counted. A spermatozoon was considered viable (VB) when it showed an intact nucleus, intact acrosome and intact mitochondrial sheath. When a spermatozoon presented damage to at least one of these three cell components, it was considered non-viable. Moreover, the percentages of spermatozoa with acrosome reacted and with mitochondrial sheath damaged were also analyzed.

2.4. Evaluation of sperm motility

To evaluate sperm motility, a computer assisted sperm analyser (CASA) system was used consisting of a phase contrast microscope (Olympus BX41) with a heat plate (at 37ºC) equipped with Sperm Class Analyser software (SCA® motility module, Microptic, Barcelona).

Fifteen µl of sperm in a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) were observed using an Olympus 10x 0.30 PLAN objective (negative phase contrast field). Each analysis captured several fields and at least 1000 spermatozoa were counted.

After acquiring fields, the software provided different sperm motility parameters: sperm progressive motility (PMOT, %); curvilinear velocity (VCL, µm/s), which is the average velocity measured over the actual point-to-point track followed by the cell; average path velocity (VAP, µm/s), which corresponds to the average velocity of the smoothed cell's pathway; straight line velocity (VSL, µm/s), which represents the average velocity measured in a straight line from the beginning to the end of a track; amplitude of lateral head displacement (ALH, µm); beat cross frequency (BCF, Hz), which is the frequency at which the sperm cell's head crosses the sperm cell's average pathway; linearity (LIN, %), which is provided by the quotient of VSL/VCL; and straightness (STR, %), which results from dividing VSL/VAP and motility parameter

175 wobble (WOB, %). The parameters used were taken from Verstegen et al. (2002) and
176 Maes et al. (2003). Before every sperm motility analysis, samples were incubated at 37
177 ºC for 20 min and three measurements were taken for each one.
178 2.5. Evaluation of sperm morphology and sperm agglutination
179 Sperm morphology was assessed using a computer assisted sperm analyser (CASA), a
180 phase contrast microscope system (Olympus BX41) equipped with a Sperm Class
181 Analyser provided by a production module (SCA® 2002, Microptic, Barcelona). For
182 each analysis, 5 µl of sperm sample was placed on a slide and mounted with a cover
183 slip. Slides were then incubated for 30 min in 100% of humidity at 25 ºC to immobilize
184 the spermatozoa. Spermatozoa were then subjectively analysed at 200x magnification
185 (Olympus 20 x 0.40 PLAN objective, positive phase contrast field) and differentiations
186 were made among mature, immature with proximal cytoplasm droplets and aberrant
187 spermatozoa (coiled tails, tails folded at the connection piece, at the intermediate piece
188 or at the Jensen's ring) (Verstegen et al., 2002; WHO, 2000). Three hundred
189 spermatozoa were analysed in each case.
190 Sperm agglutination was also assessed by counting two hundred spermatozoa at 200x
191 magnification using the same objective for sperm morphology (Olympus 20 x 0.40
192 PLAN objective, positive phase contrast field). We distinguished between agglutinated
193 and non-agglutinated spermatozoa, considering that the spermatozoa sticking to each
194 other were agglutinated.
195 2.5. Osmotic resistance test (ORT)
196 Spermatozoa osmotic resistance was tested using a Schilling (1986) modified test as
197 described by Rodríguez-Gil and Rigau (1995). Aliquots were incubated at 37ºC in 900
198 µl of a hypo-osmotic solution containing 1% (w/v) sodium citrate, and adjusted to pH
199 7.4 (osmotic pressure 102 ± 5 $mOsm \cdot Kg^{-1}$). At the same time, aliquots were incubated

200 at 37°C in 900 µl of an adjusted (pH 7.4) iso-osmotic solution of 3.2% (w/v) sodium
201 citrate (osmotic pressure 305 ± 7 mOsm·Kg^{-1}).
202 After 1 h of incubation, aliquots were stained by the modified double-staining method,
203 and the viability and percentage of altered acrosomes were counted. The percentage of
204 osmotic resistance was calculated by applying the formula described by Sánchez (1991).
205 2.6. Statistical analyses
206 Data from each ejaculate from each boar (every ejaculate was considered as a case;
207 n=16) were managed using Microsoft Excel® (Microsoft Office 2003, Microsoft
208 Corporation, USA) and SPSS for Windows (SPSS Inc.; Version 13.0). Eighteen
209 variables were subjected to statistical analysis: viable spermatozoa, acrosome reacted
210 spermatozoa, mitochondrial sheath damaged spermatozoa, PMOT, MOT, VCL, VAP,
211 VSL, ALH, BCF, LIN, STR, WOB, mature, immature, aberrant, agglutinated
212 spermatozoa and ORT. These variables were assessed in controls and compared to each
213 other using either a one-way ANOVA or a Kruskal-Wallis test when, even transformed,
214 the distribution of the variable was non-normal. In both tests, the boar was the factor.
215 No significant differences (P>0.05) were observed among boars.
216 Since the treatments were assessed within the aliquants from the same sample (paired
217 data), ratios were calculated per variable and treatment with respect to control as
218 follows: ratio (variable x treatment) = treatment value/control value to remove the
219 individual effect of boars. Although the results are presented in the tables as raw, the
220 statistical analyses were performed using ratio data.
221 Ratio variables were tested for normality using the Kolmogorov–Smirnov test and for
222 homoscedasticity with the Levene's test (setting the significance level at 5%). To
223 achieve normal distribution and accomplish the assumptions of analysis of variance
224 when data (x) were non-normal, three transformations were performed (square root (x),

$\log_{10}$ (x) and arcsine square root (x)), and the best one of them was chosen. Parametric statistics were used for transformed variables that reached normality (ALH, BCF, %LIN, %STR, %WOB, frequencies of mature, immature and aberrant spermatozoa, frequency of agglutinated spermatozoa and % of osmotic resistance). These tests consisted of one-way analysis of variance (ANOVA) for repeated measures (where the cooling time was the intrasubject factor, the treatment was the intersubject factor and the each ratio parameter was the dependent variable) with post hoc Dunnett's test. Moreover, each treatment was compared with control for each period of time using a t-test with Bonferroni correction.

When no transformation remedied the normality (viable spermatozoa, acrosome reacted spermatozoa, mitochondrial sheath damaged spermatozoa, PMOT, MOT, VCL, VAP, VSL), non-parametric procedures were used with raw ratio data. Friedman's test was performed as a non-parametric alternative to repeated measures ANOVA and the Wilcoxon matched pairs test was used to evaluate differences between control and treatments as well as the effects of preservation time.

In all statistical analyses, the significant level was set at 5%. Results are expressed as means ± standard deviation (SD).

3. RESULTS

Results of sperm quality parameters, expressed as mean ± SD of sixteen replicates per cell, are shown in Tables 1 (sperm viability), 2 and 3 (sperm motility). In these tables, there are two different superscripts (*a, b*). Whereas *a* means statistical (P<0.05) differences in preservation times (within rows, between days 1, 3, 6 or 10 and time 0), *b* means statistical differences (P<0.05) between $PGF_{2\alpha}$ treatments and control (within columns).

250 3.1. Analysis of sperm viability, acrosome and mitochondrial sheath integrity

251 The Fisher test showed significant differences among treatments and cooling times
252 (P<0.001; DF=59; Chi-square=929.442). Compared with control, there was a
253 significant increase in 2.5 mg $PGF_{2\alpha}$/100 ml treatment and significant decreases were
254 observed in the other treatments. The frequency of viable spermatozoa diminished
255 significantly (P<0.001) in 25 and 50 mg of $PGF_{2\alpha}$/100 ml treatments. These decreases
256 were maintained during different preservation times (1, 3, 6 and 10 days).

257 After one day of cooling, no treatment significantly increased the sperm viability
258 compared with control and, most significantly, reduced sperm viability. After three days
259 of preservation, significant decreases (P<0.01) were observed in all treatments, except
260 in 5 mg $PGF_{2\alpha}$/100 ml (73.33 ± 4.06), compared with control on the third day (73.96 ±
261 4.79). Moreover, significant increases (P<0.01) were also observed in 5 mg $PGF_{2\alpha}$/100
262 ml at days 6 and 10 compared with the control at the same time (70.04 ± 3.63 vs 59.79 ±
263 4.23; 56.33 ± 3.39 vs 51.96 ± 3.46). After six days of preservation, the frequency of
264 viable spermatozoa was significantly higher in binary treatments than in their respective
265 control.

266 Regarding differences among preservation times, significant decreases were observed
267 since the first day in almost all $PGF_{2\alpha}$ treatments. However, a large decrease was
268 observed at days 6 and 10 rather than at days 1 and 3.

269 Comparing the frequencies of acrosome reacted spermatozoa, significant impairments
270 were observed in treatments of 25 and 50 mg $PGF_{2\alpha}$/100 ml either immediately after
271 treatment application (38.72 ± 2.02; 52.35 ± 2.74) or after 1 day of preservation (45.66
272 ± 2.13; 59.83 ± 2.56). Moreover, acrosome integrity was more affected than
273 mitochondrial sheath integrity through preservation time in all treatments (16.10 ± 1.12
274 vs 5.90 ± 0.40, respectively, in control after 3 days of cooling).

275 3.2. Analysis of sperm motility

276 Table 2 shows the values of the following kinetic parameters: VCL, VAP, VSL and
277 BCF. The analysis of VCL using the Fisher test showed significant differences
278 (P<0.001; DF=59; Chi-square=909.523). Significant differences (P<0.001) were
279 observed in VCL between control and most treatments and a large reduction was
280 observed in 25 and 50 mg $PGF_{2\alpha}$/100 ml treatments at day 0. Preservation time
281 significantly reduced this kinetic parameter from the first day. After 6 as well as 10 days
282 of cooling, VCL was significantly higher in 2.5 and 5 mg $PGF_{2\alpha}$/100 treatments than in
283 control at the same time.

284 Average path velocity (VAP) showed statistical differences as well (P<0.001; DF=59;
285 Chi-square=742.045). Preservation time significantly reduced this parameter, especially
286 from the third day. Statistical differences were observed immediately in many
287 treatments but significant decreases were only observed in 2.5, 25 and 50 mg
288 $PGF_{2\alpha}$/100 ml treatments. In the 50 $PGF_{2\alpha}$/100 ml treatment, the value of VAP was fast
289 reduced at time 0. A significant (P<0.05) increase of this parameter was observed in 2.5
290 and 5 mg $PGF_{2\alpha}$/100 ml treatments at days 6 and 10.

291 The results of the straight line velocity (VSL) were similar to VAP and VCL (P<0.001;
292 DF=59; Chi-square=695.773). When VSL was measured immediately after the addition
293 of 50 mg $PGF_{2\alpha}$/100 ml, significant differences were observed. As in VCL and VAP, a
294 significant increase in VSL was immediately observed in 0.625 and 1.25 mg $PGF_{2\alpha}$/100
295 ml. After 1 day at 15ºC, VSL was reduced significantly in the following treatments:
296 12.5 mg $PGF_{2\alpha}$/100 ml, 25 mg $PGF_{2\alpha}$/100 ml and 50 mg $PGF_{2\alpha}$/100 ml. After 10 days,
297 VSL was reduced in control and in all $PGF_{2\alpha}$ treatments, except for 2.5 $PGF_{2\alpha}$/100 ml
298 and 5 $PGF_{2\alpha}$/100 ml concentrations where it was significantly higher than in control.

299 Beat cross frequency (BCF) results showed significant differences in overall time points
300 in 25 and 50 mg $PGF_{2\alpha}$/100 ml treatments. In both 12.5 mg $PGF_{2\alpha}$/100 and in binary
301 treatments significant decreases were observed from the third day. Conversely, BCF
302 was better preserved in 2.5 and 5 mg $PGF_{2\alpha}$/100 treatments, revealing significant
303 increases with control after 6 and 10 days of cooling.
304 With regard to ALH, significant differences were observed when comparing several
305 treatments with control. Two treatments: 0.625 and 1.25 mg $PGF_{2\alpha}$/100 ml showed a
306 significant increase compared to control at time 0 (3.27 ± 0.55 and 3.55 ± 0.37,
307 respectively, vs 2.80 ± 0.53 in control). Conversely, the 50 mg $PGF_{2\alpha}$/100 ml treatment
308 showed a fast decrease after the experiment was started (0.71 ± 0.19). The preservation
309 time significantly reduced the ALH after 10 days of cooling in all treatments including
310 the control, except in 5 mg $PGF_{2\alpha}$/100 ml (2.45 ± 0.25, after 10 days, vs 2.47 ± 0.22,
311 immediately) and 12.5 mg $PGF_{2\alpha}$/100 ml (2.70 ± 0.72 vs 2.73 ± 0.58) where no
312 significant differences were found, and 2.5 mg $PGF_{2\alpha}$/100 ml (2.67 ± 0.72 vs 2.23 ±
313 0.47) where a significant increase was observed.
314 Linearity (LIN) significantly increased after the addition of 2.5 mg $PGF_{2\alpha}$/100 at 0, 1, 6
315 and 10 days of preservation (53.37 ± 4.71 in treatment vs 39.84 ± 3.12 in control; 52.75
316 ± 4.50 vs 45.76 ± 4.15; 47.77 ± 4.83 vs 42.69 ± 4.72 ; 48.7 ± 4.77 vs 42.01 ± 4.85) . On
317 the other hand, this parameter was significantly reduced when 50 mg $PGF_{2\alpha}$/100 was
318 added (23.55 ± 2.79 vs 39.84 ± 3.12).
319 Straightness (STR) results showed significant increases in 2.5 (70.77 ± 5.75), 5 (64.77 ±
320 3.47), 10 (64.80 ± 2.60) and 12.5 (69.20 ± 3.72) mg $PGF_{2\alpha}$/100 ml and 0.625 $PGF_{2\alpha}$
321 mg/100 ml + Caf 7.5 µM (63.93 ± 5.24) at time 0. Significant differences were also
322 observed between control (65.52 ± 4.06) and 2.5 and 5 mg/100 ml (71.67 ± 2.49; 69.75
323 ± 2.82) treatments at day 1 and between control and the 5 mg $PGF_{2\alpha}$ /100 ml treatment

at days 6 and 10 (64.25 ± 4.20 vs 67.82 ± 4.69; 66.57 ± 4.36 vs 70.37 ± 3.92). After 10 days of cooling, significant differences were found between control (66.57 ± 4.36) and the following two treatments: 1.25 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml (69.36 ± 5.19) and 0.625 mg $PGF_{2\alpha}$/100 ml + Caf 7.5 µM (71.93 ± 5.45).

The motility parameter wobble (WOB) presented similar results to LIN and STR. Therefore, at day 0, the addition of 25 mg $PGF_{2\alpha}$/100 ml (57.33 ± 3.26) or 50 mg $PGF_{2\alpha}$/100 ml (39.99 ± 3.01) significantly decreased the WOB. On the other hand, significant increases were observed between the control (66.81 ± 4.06) and the three following treatments: 2.5 (75.12 ± 4.47), 5 (75.67 ± 2.58) and 10 mg $PGF_{2\alpha}$/100 ml (74.40 ± 3.04). The increases concerning these three treatments were also found after 6 (71.77 ± 4.14, 73.47 ± 4.68, 74.02 ± 4.45 vs 65.11 ± 4.86) and after 10 days of cooling (73.07 ± 3.81, 71.32 ± 4.58, 71.18 ± 4.51 vs 62.71 ± 4.10).

On the other hand, significant differences (P<0.05) were observed in the 0.625 mg $PGF_{2\alpha}$/100 ml treatment at day 10 (50.27 ± 2.25) compared to the same treatment at time 0 (64.07 ± 5.01). Moreover, significant differences were observed at 10 days between the control (62.71 ± 4.10) and the 0.625 mg $PGF_{2\alpha}$/100 ml concentration.

Sperm PMOT is shown in Table 3. Both total motility and progressive motility present similar data. Significant decreases of the percentages of progressive motility compared with control (55.19 ± 4.37) were immediately observed after the addition of 50 mg $PGF_{2\alpha}$/100 ml (0.99 ± 0.05) and 25 mg $PGF_{2\alpha}$/100 ml (48.31 ± 2.64). By contrast, significant increases were observed in the other treatments, except in 0.625 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml and 0.625 mg $PGF_{2\alpha}$/100 ml + Caf 7.5 µM treatments.

After 6 and 10 days of cooling, five treatments significantly increased the percentage of PMOT sperm compared with control at the same time (0.625 from 10 mg $PGF_{2\alpha}$/100 ml). Preservation time significantly reduced the PMOT not only in control but also in all

349 treatments. Indeed, after three days of cooling, all treatments significantly reduced the
350 values obtained for PMOT at time 0.
351 3.3. Analysis of sperm morphology and sperm agglutination
352 With regard to sperm morphology, no significant differences were observed (P>0.05) in
353 the frequency of mature spermatozoa comparing control (92.92 ± 2.89) and treatments
354 at time 0. Furthermore, no significant differences (P>0.05) were observed when a
355 comparison among preservation times was performed in all treatments (for controls,
356 results were: 93.06 ± 3.47 at day 1, 90.85 ± 2.84 at day 3, 89.37 ± 4.45 at day 6 and
357 89.79 ± 324 at day 10). Moreover, neither frequencies of immature spermatozoa with
358 distal cytoplasmic droplet nor aberrant spermatozoa with coiled tails were affected by
359 treatment and time preservation (P>0.05).
360 In contrast, sperm agglutination was significantly (P<0.001) increased after both six
361 (19.36 ± 1.38) and 10 days of preservation (35.14 ± 2.32) when compared with day 0
362 (2.25 ± 0.11).
363 At the sixth day, the frequency of agglutinated spermatozoa was significantly different
364 between control and the following treatments: 12.50 mg $PGF_{2\alpha}$/100 ml (28.57 ± 1.74),
365 25 mg $PGF_{2\alpha}$/100 ml (27.81 ± 1.71), 50 mg $PGF_{2\alpha}$/100 ml (29.69 ± 1.58), and 0.625 mg
366 $PGF_{2\alpha}$/100 ml + 7.5 μM Caffeine (29.14 ± 1.85). Significant differences were also
367 observed in comparisons between day 10 and day 0 in all treatments.
368
369 3.4. Osmotic resistance test
370 At day 0 and compared with control (83.64 ± 2.51), ORT results showed a significant
371 decrease (P<0.05) when $PGF_{2\alpha}$ concentration was higher than 25 mg/100 ml compared
372 with controls immediately (79.48 ± 2.19 in 25 mg $PGF_{2\alpha}$/100 ml; 72.75 ± 1.93 in 50 mg
373 $PGF_{2\alpha}$/100 ml). These differences were maintained throughout the ten days. No

374 significant differences were observed when comparisons between preservation times
375 were performed during the first 3 days of cooling. Conversely, osmotic resistance ORT
376 significantly (P<0.05) decreased after 6 days of cooling (79.24 ± 2.34) compared to day
377 0 in all treatments except 2.5 mg $PGF_{2\alpha}$/100 ml (82.84 ± 2.67) and 5 mg $PGF_{2\alpha}$/100 ml
378 (83.19 ± 2.03). In contrast, significant differences were found in all treatments after ten
379 days of cooling (72.14 ± 2.10 in control).
380
381 4. DISCUSSION
382 The goal of this study was to know the effects of different treatments of $PGF_{2\alpha}$ on boar
383 sperm quality. Differential effects of those treatments with different cooling times were
384 assessed as well. To evaluate sperm quality, various analyses were carried out: whereas
385 sperm motility and morphology were counted by using a CASA system, sperm viability
386 was assessed by staining with a multiple fluorochrome-staining test (Bussalleu et al.,
387 2005). As CASA systems have been validated in different mammal species (human, dog
388 and boar) (Maes et al., 2003), both sperm motility and morphology were analysed in an
389 objective way. On the other hand, multiple fluorochrome-staining (Bussalleu et al.,
390 2005) provides another way to evaluate sperm quality. Although previous experiments
391 studied how different concentrations of $PGF_{2\alpha}$ affected the motility of diluted boar
392 sperm (Maes et al., 2003), we used different $PGF_{2\alpha}$ treatments and different
393 preservation time points as previously reported. Furthermore, sperm quality has not only
394 been assessed using sperm motility; sperm morphology, agglutination and sperm
395 viability and mitochondrial sheath and acrosome integrity were also used.
396 In sperm viability analysis, $PGF_{2\alpha}$ toxicity was observed when the concentration was
397 higher than 12.5 mg/100 ml. Thee data agree with sperm motility (because a significant
398 reduction was also observed in VCL, VSL, VAP, LIN, STR, WOB and PMOT) and in

399 osmotic resistance test results where this same effect was also observed. After 3 days of
400 preservation, in all treatments except 5 mg/100 ml, a significant reduction was observed
401 compared with control at the same time. After six days of preservation, sperm viability
402 decreased significantly in most treatments but significant increases were obtained in 5
403 mg $PGF_{2\alpha}$/100 ml and in the three binary treatments. These results suggest that the
404 addition of these treatments to extenders may maintain sperm viability after six days of
405 cooling. However, as some sperm motility parameters are only slightly related to these
406 viability data and successful research has been carried out in long-term semen extenders
407 (Dubé et al., 2004; Haugan et al., 2007), further research should be done.
408 Acrosome integrity was damaged more than mitochondrial sheath integrity when the
409 concentration of $PGF_{2\alpha}$ was higher than 12.5 mg/ml. The acrosome is a
410 Golgi/endoplasmic reticulum derived acidic secretory organelle (Silva and Gadella,
411 2006) that triggers the acrosome reaction after spermatozoa bind to the zona pellucida.
412 However, early acrosome reactions can occur and then spermatozoa become infertile.
413 Spermatozoa can lose their acrosomes when they die (the false or degenerative
414 acrosome reaction) (Cross and Meizel, 1989).
415 A significant increase in sperm progressive motility was observed in 1.25 mg
416 $PGF_{2\alpha}$/100 ml + HA 200 μg/ml but not in the other binary treatments. Hyaluronic acid
417 has been used as an improver of sperm motility and velocity in post-thawed sperm
418 (Peña et al., 2004) and caffeine stimulates sperm motility and capacitation (Funahashi
419 and Nagai, 2001). Therefore, additional research should be done using other
420 concentrations of both HA and caffeine.
421 There was no significant effect on sperm morphology. The frequency of mature
422 spermatozoa is not modified with the treatments applied in this study. Thus, the
423 frequency of sperm morphology aberrations such as coiled tails or big head frequencies

were not affected by $PGF_{2\alpha}$. However, sperm agglutination increased through cooling time. Thus, sperm agglutination increased significantly at day 6. As previous papers have shown (Sánchez, 1991), this effect is not rare.

Apart from concentrations higher than 12.5 mg/100 ml, the impairments observed on sperm viability, motility, agglutination and osmotic resistance are not caused by treatments but by preservation time. No bacterial growth was observed during the cooling period. This finding agrees with a previous study comparing short (BTS) and long-term (Mulberry III, Androhep™ and Acromax) commercial extenders (Vyt et al., 2004). This report showed that the bacterial contamination, assessed as a determination of colony forming units (CFU), remained more or less stable during a 7-day cooling period.

There are two main conclusions from the results of this study: (A) sperm function can be maintained in cooling conditions for up to 3 days and (B) the addition of prostaglandin $F_{2\alpha}$ at concentrations of 2.5, 5 and 10 mg/ml does not damage the spermatozoa. As several authors have previously reported, this hormone is used to contract the uterus myometrium in order to improve AI procedures (Friel et al., 2005; Baldi et al., 1991; Gil et al., 1998). Contraction of myometrium plays a fundamental role in fertilization because these contractions transport spermatozoa to the site of fertilization (Kos and Bilkei, 2004). In order to improve reproductive performance, stimulation of uterine contractility is carried out by adding hormones to boar semen doses. Different authors showed the enhancing effects of oxytocin (Odenhal et al., 1990), prostaglandin (Gil et al., 1998) and estradiol (Kirwood and Thacker, 1991) on AI procedures.

Thanks to this work, we conclude that prostaglandin $F_{2\alpha}$ may be used in the above mentioned concentrations without damaging sperm quality. Binary treatments, with

caffeine and HA, may also be used at the reported concentrations without damaging spermatozoa. Therefore, these substances used at these concentrations may be added to sperm doses diluted in BTS to produce profitable effects on AI.

Relevant data are also provided by sperm viability and motility results on $PGF_{2\alpha}$ 5 mg/100 ml treatment after 6 days of cooling. Results for sperm parameters are correlated and significant increases compared with control at the sixth and tenth days were found. Thus, this treatment may be used to improve the BTS extender, but since BTS is a short-term extender, further research might assess the effects of $PGF_{2\alpha}$ 5 mg/100 ml on sperm quality using a long-term extender.

ACKNOWLEDGEMENTS

This work was supported by the project PTR1995.0761.OP entitled “Design of a prototype and technical protocol to improve sperm quality of boar seminal doses for AI” and by a pre-doctoral fellowship granted to M. Yeste (BES-2003-2298) from the Spanish Ministry of Education and Science (MEC).

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587
588 FIGURE CAPTIONS
589 **Table 1** Frequencies of viable spermatozoa (with both intact mitochondrial sheath and
590 acrosome). Results are expressed as means ± SD. (Superscript a) Statistical differences
591 (P<0.05) in preservation times between days 1, 3, 6 or 10 and time 0 (within rows).
592 (Superscript b) Statistical differences (P<0.05) between $PGF_{2\alpha}$ treatments and control
593 (within columns)
594 **Table 2** Values of curvilinear velocity (VCL, μm/s); average path velocity (VAP,
595 μm/s); straight line velocity (VSL, μm/s) and beat cross frequency (BCF, Hz). Results

596 are expressed as means ± SD. (Superscript a) Statistical differences (P<0.05) in
597 preservation times between days 1, 3, 6 or 10 and time 0 (within rows). (Superscript b)
598 Statistical differences (P<0.05) between $PGF_{2\alpha}$ treatments and control (within columns)
599 **Table 3** Frequencies of progressive motility. Results are expressed as means ± SD.
600 (Superscript a) Statistical differences (P<0.05) in preservation times between days 1, 3,
601 6 or 10 and time 0 (within rows). (Superscript b) Statistical differences (P<0.05)
602 between $PGF_{2\alpha}$ treatments and control

Treatment\time	Immediately	1 day	3 days	6 days	10 days
Sperm viability and mitochondrial sheath and acrosome integrity					
Control	80.79 ± 6.04	76.37 ± 6.06 [a]	73.96 ± 4.79 [a]	59.79 ± 4.23[a]	51.96 ± 3.46[a]
0.625 mg $PGF_{2\alpha}$/100 ml	77.44 ± 6.40[b]	76.01 ± 4.16	70.71 ± 4.11[a, b]	58.88 ± 3.36[a]	50.78 ± 3.54[a]
1.25 mg $PGF_{2\alpha}$/100 ml	77.33 ± 4.02[b]	74.11 ± 4.22[a, b]	70.31 ± 3.60[a, b]	58.55 ± 3.64[a]	51.43 ± 3.19[a]
2.5 mg $PGF_{2\alpha}$/100 ml	82.42 ± 6.20[b]	75.50 ± 4.15[a]	68.41 ± 2.65[a, b]	60.33 ± 3.09[a]	50.59 ± 3.37[a, b]
5 mg $PGF_{2\alpha}$/100 ml	78.83 ± 5.21[b]	74.25 ± 4.63[a, b]	73.33 ± 4.06[a]	70.04 ± 3.63[a, b]	56.33 ± 3.39[a, b]
10 mg $PGF_{2\alpha}$/100 ml	75.25 ± 5.58[b]	70.80 ± 5.13[a, b]	67.50 ± 4.85[a, b]	57.58 ± 4.23[a, b]	45.58 ± 3.24[a, b]
12.5 mg $PGF_{2\alpha}$/100 ml	72.67 ± 3.13[b]	68.12 ± 3.41[a, b]	63.17 ± 3.39[a, b]	47.10 ± 3.77[a, b]	55.41 ± 3.26[a, b]
25 mg $PGF_{2\alpha}$/100 ml	43.71 ± 2.82[b]	32.30 ± 2.70[a, b]	20.88 ± 1.73[a, b]	13.23 ± 1.20[a, b]	8.14 ± 0.95[a, b]
50 mg $PGF_{2\alpha}$/100 ml	23.14 ± 1.52[b]	8.33 ± 1.20[a, b]	4.64 ± 0.62[a, b]	3.66 ± 0.47[a, b]	3.24 ± 0.55[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + HA 200 μg/ml	72.11 ± 6.67[b]	68.44 ± 5.19[a, b]	63.77 ± 4.85[a, b]	66.89 ± 4.77[a, b]	50.13 ± 3.82[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml + HA 200 μg/ml	73.04 ± 5.49[b]	67.06 ± 5.09[a, b]	65.41 ± 4.98[a, b]	63.67 ± 3.76[a, b]	41.87 ± 3.35[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + Caf 7.5 μM	70.89 ± 5.54[b]	65.22 ± 4.05[a, b]	64.78 ± 4.81[a, b]	65.56 ± 3.03[a, b]	40.11 ± 3.88[a, b]

Table 1 Frequencies of viable spermatozoa (with both intact mitochondrial sheath and acrosome). Results are expressed as means ± SD. (Superscript a) Statistical differences (P<0.05) in preservation times between days 1, 3, 6 or 10 and time 0 (within rows). (Superscript b) Statistical differences (P<0.05) between $PGF_{2\alpha}$ treatments and control (within columns)

Treatment\time	Immediately	1 day	3 days	6 days	10 days
VCL (µm/s)					
Control	70.49 ± 8.61	66.68 ± 8.84[a]	63.44 ± 9.07[a]	44.75 ± 8.36[a]	37.15 ± 7.89[a]
0.625 mg $PGF_{2\alpha}$/100 ml	73.70 ± 9.51[b]	75.50 ± 7.76[a, b]	64.51 ± 8.26[a]	43.47 ± 5.88[a]	40.03 ± 3.42[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml	73.60 ± 6.28[b]	65.30 ± 6.95[a]	56.43 ± 8.25[a, b]	49.16 ± 4.07[a, b]	38.30 ± 3.76[a]
2.5 mg $PGF_{2\alpha}$/100 ml	69.30 ± 8.63	50.85 ± 7.19[a, b]	49.21 ± 6.89[a, b]	48.25 ± 6.90[a, b]	38.62 ± 6.18[a, b]
5 mg $PGF_{2\alpha}$/100 ml	68.20 ± 5.16[b]	49.55 ± 7.85[a, b]	49.57 ± 7.69[a, b]	47.67 ± 6.18[a, b]	38.70 ± 7.02[a, b]
10 mg $PGF_{2\alpha}$/100 ml	64.75 ± 8.06[b]	47.22 ± 7.16[a, b]	51.40 ± 8.53[a, b]	34.92 ± 7.71[a, b]	31.70 ± 7.76[a, b]
12.5 mg $PGF_{2\alpha}$/100 ml	69.40 ± 6.95	42.10 ± 6.97[a, b]	42.10 ± 8.36[a, b]	21.50 ± 5.84[a, b]	20.30 ± 5.55[a, b]
25 mg $PGF_{2\alpha}$/100 ml	55.60 ± 7.44[b]	35.15 ± 6.74[a, b]	24.60 ± 6.59[a, b]	22.40 ± 5.97[a, b]	21.16 ± 4.82[a, b]
50 mg $PGF_{2\alpha}$/100 ml	14.81 ± 2.19[b]	17.60 ± 5.18[a, b]	17.40 ± 4.59[a, b]	18.20 ± 3.12[a, b]	16.21 ± 4.93[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml	62.80 ± 6.59[b]	68.20 ± 4.03[a, b]	45.60 ± 5.20[a, b]	34.57 ± 7.43[a, b]	19.70 ± 3.84[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml	66.87 ± 7.25[b]	64.47 ± 5.64[a, b]	40.03 ± 5.68[a, b]	37.23 ± 7.59[a, b]	22.83 ± 6.40[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + Caf 7.5 µM	63.50 ± 8.52[b]	62.63 ± 6.04[b]	38.13 ± 4.38[a, b]	23.42 ± 5.51[a, b]	22.07 ± 6.77[a, b]
VAP (µm/s)					
Control	40.70 ± 6.87	44.93 ± 6.07[a]	43.34 ± 6.67	29.72 ± 4.21[a]	24.36 ± 4.76[a]
0.625 mg $PGF_{2\alpha}$/100 ml	46.67 ± 4.76[b]	45.17 ± 5.16	38.06 ± 5.95[a, b]	14.23 ± 4.78[a, b]	11.13 ± 2.30[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml	44.20 ± 4.28[b]	43.93 ± 4.50	33.20 ± 5.19[a, b]	11.83 ± 1.67[a, b]	10.00 ± 1.56[a, b]
2.5 mg $PGF_{2\alpha}$/100 ml	35.30 ± 5.65[b]	35.07 ± 5.92[b]	38.47 ± 3.56[b]	35.50 ± 5.55[b]	35.87 ± 6.97[b]
5 mg $PGF_{2\alpha}$/100 ml	44.00 ± 3.39	38.87 ± 5.08[a, b]	39.52 ± 9.16	34.87 ± 5.86[a, b]	28.77 ± 7.49[a, b]
10 mg $PGF_{2\alpha}$/100 ml	40.82 ± 5.29	36.67 ± 5.23[b]	35.42 ± 5.97[a, b]	32.87 ± 6.41[a]	22.95 ± 7.69[a]
12.5 mg $PGF_{2\alpha}$/100 ml	43.10 ± 6.80	23.40 ± 5.11[a, b]	16.40 ± 3.56[a, b]	12.27 ± 4.32[a, b]	10.70 ± 4.87[a, b]
25 mg $PGF_{2\alpha}$/100 ml	31.90 ± 5.12[b]	18.10 ± 4.99[a, b]	13.40 ± 3.39[a, b]	12.32 ± 4.81[a, b]	11.80 ± 4.14[a, b]
50 mg $PGF_{2\alpha}$/100 ml	15.90 ± 2.84[b]	13.60 ± 3.83[b]	10.39 ± 3.24[a, b]	9.61 ± 3.28[a, b]	12.20 ± 5.22[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml	39.07 ± 5.45	41.80 ± 6.71[b]	26.00 ± 6.78[a, b]	21.70 ± 5.73[a, b]	10.80 ± 2.34[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml	44.83 ± 4.13[b]	39.80 ± 5.76[a, b]	22.97 ± 6.43[a, b]	22.00 ± 5.62[a, b]	14.67 ± 4.63[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + Caf 7.5 µM	43.20 ± 5.56	44.07 ± 6.87	21.20 ± 2.52[a, b]	13.73 ± 1.53[a, b]	13.70 ± 4.19[a, b]
VSL (µm/s)					
Control	24.09 ± 7.51	29.12 ± 5.12[a]	28.50 ± 7.19	19.91 ± 6.93[a]	16.60 ± 3.03[a]
0.625 mg $PGF_{2\alpha}$/100 ml	27.00 ± 1.77	26.87 ± 3.73	23.60 ± 5.89[a, b]	9.67 ± 2.54[a, b]	7.43 ± 1.79[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml	32.55 ± 1.06[b]	26.50 ± 5.16[a]	16.39 ± 3.30[a, b]	7.10 ± 1.05[a, b]	6.90 ± 1.49[a, b]
2.5 mg $PGF_{2\alpha}$/100 ml	24.45 ± 3.57	22.95 ± 5.65[b]	25.77 ± 3.14[a]	23.42 ± 3.28[a]	24.02 ± 5.41[b]
5 mg $PGF_{2\alpha}$/100 ml	28.47 ± 2.55[b]	27.02 ± 2.68	24.55 ± 4.31[a]	23.70 ± 4.90[a]	20.67 ± 5.91[a, b]
10 mg $PGF_{2\alpha}$/100 ml	26.60 ± 5.51	24.80 ± 5.09[b]	27.37 ± 4.28	21.77 ± 4.73[a]	16.07 ± 3.73[a]
12.5 mg $PGF_{2\alpha}$/100 ml	25.50 ± 4.89	13.52 ± 3.78[a, b]	10.90 ± 3.30[a, b]	8.58 ± 1.87[a, b]	6.58 ± 1.59[a, b]
25 mg $PGF_{2\alpha}$/100 ml	19.90 ± 3.51[b]	11.79 ± 3.01[a, b]	8.78 ± 3.62[a, b]	8.19 ± 2.89[a, b]	7.19 ± 2.86[a, b]
50 mg $PGF_{2\alpha}$/100 ml	8.50 ± 2.56[b]	8.11 ± 2.55[b]	6.04 ± 3.35[a, b]	5.90 ± 1.84[a, b]	7.40 ± 2.87[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml	23.07 ± 5.89	25.10 ± 6.11[a, b]	15.90 ± 3.74[a, b]	13.46 ± 3.22[a, b]	7.15 ± 1.65[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml	27.10 ± 4.47	22.40 ± 8.49[a, b]	13.67 ± 4.07[a, b]	12.93 ± 3.95[a, b]	10.83 ± 3.42[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + Caf 7.5 µM	27.16 ± 3.45	28.93 ± 8.05	13.00 ± 2.46[a, b]	8.83 ± 1.35[a, b]	10.03 ± 3.66[a, b]
BCF (Hz)					

Control	5.90 ± 0.41	6.16 ± 0.71	6.32 ± 0.53[a]	5.55 ± 0.46[a]	4.45 ± 0.42[a]
0.625 mg $PGF_{2\alpha}$/100 ml	6.13 ± 0.72	5.97 ± 0.60	6.40 ± 0.95[a]	4.00 ± 0.62[a, b]	3.80 ± 0.50[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml	6.30 ± 0.14[b]	5.70 ± 0.26[a, b]	6.23 ± 0.40	3.77 ± 0.55[a, b]	3.48 ± 0.75[a, b]
2.5 mg $PGF_{2\alpha}$/100 ml	5.87 ± 0.36	5.75 ± 0.41[b]	6.05 ± 0.13	6.00 ± 0.36[b]	6.37 ± 0.54[a, b]
5 mg $PGF_{2\alpha}$/100 ml	5.90 ± 0.33	5.82 ± 0.15[b]	5.87 ± 0.71[b]	6.05 ± 0.57[b]	5.42 ± 0.97[a, b]
10 mg $PGF_{2\alpha}$/100 ml	6.05 ± 0.13	5.60 ± 0.65[a, b]	6.15 ± 0.83	6.17 ± 0.17[b]	4.50 ± 0.88[a]
12.5 mg $PGF_{2\alpha}$/100 ml	5.86 ± 0.31	6.00 ± 0.53	3.90 ± 0.33[a, b]	1.78 ± 0.37[a, b]	1.90 ± 0.69[a, b]
25 mg $PGF_{2\alpha}$/100 ml	5.31 ± 0.35[b]	5.65 ± 0.47[b]	1.37 ± 0.19[a, b]	0.72 ± 0.18[a, b]	1.17 ± 0.52[a, b]
50 mg $PGF_{2\alpha}$/100 ml	0.49 ± 0.05[b]	1.00 ± 0.31[a, b]	0.52 ± 0.08[b]	1.13 ± 0.34[a, b]	1.01 ± 0.37[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml	5.77 ± 0.29	5.97 ± 0.45	5.87 ± 0.61[b]	4.73 ± 0.86[a, b]	2.60 ± 0.83[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml	6.20 ± 0.60[b]	6.13 ± 0.40	5.63 ± 0.89[a, b]	5.23 ± 0.87[a]	0.87 ± 0.32[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + Caf 7.5 µM	5.93 ± 0.45	6.33 ± 0.47[a]	5.73 ± 0.87[b]	4.53 ± 0.74[a, b]	1.73 ± 0.85[a, b]

Table 2 Values of curvilinear velocity (VCL, µm/s); average path velocity (VAP, µm/s); straight line velocity (VSL, µm/s) and beat cross frequency (BCF, Hz). Results are expressed as means ± SD. (Superscript a) Statistical differences (P<0.05) in preservation times between days 1, 3, 6 or 10 and time 0 (within rows). (Superscript b) Statistical differences (P<0.05) between $PGF_{2\alpha}$ treatments and control (within columns)

Treatment\time	Immediately	1 day	3 days	6 days	10 days
Progressive motility (%)					
Control	55.19 ± 4.37	56.51± 4.47	51.25 ± 4.49[a]	34.71 ± 4.16[a, b]	25.07 ± 2.30[a]
0.625 mg $PGF_{2\alpha}$/100 ml	64.30 ± 1.23[b]	65.27 ± 4.92[b]	58.41 ± 4.27[a, b]	40.03 ± 0.32[a, b]	31.67 ± 0.56[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml	68.25 ± 2.05[b]	66.60 ± 4.39[b]	59.70 ± 4.83[a, b]	37.67 ± 2.05[a, b]	33.60 ± 1.22[a, b]
2.5 mg $PGF_{2\alpha}$/100 ml	65.67 ± 3.68[b]	63.32 ± 4.45[b]	54.71 ± 4.27[b]	46.40 ± 2.51[a, b]	36.52 ± 2.06[a, b]
5 mg $PGF_{2\alpha}$/100 ml	64.35 ± 4.51[b]	62.47 ± 3.16[a, b]	51.38 ± 4.17[a]	49.80 ± 4.24[a, b]	38.35 ± 3.25[a, b]
10 mg $PGF_{2\alpha}$/100 ml	66.32 ± 4.53[b]	65.97 ± 3.64[b]	58.50 ± 4.02[a, b]	39.65 ± 3.47[a, b]	35.17 ± 3.19[a, b]
12.5 mg $PGF_{2\alpha}$/100 ml	57.56 ± 2.31[b]	58.61 ± 1.14	4.32 ± 1.38[a, b]	1.32 ± 0.32[a, b]	0.70 ± 0.15[a, b]
25 mg $PGF_{2\alpha}$/100 ml	48.31 ± 2.64[b]	20.05 ± 0.83[a, b]	1.26 ± 0.53[a, b]	1.65 ± 0.11[a, b]	0.60 ± 0.20[a, b]
50 mg $PGF_{2\alpha}$/100 ml	0.99 ± 0.05[b]	0.72 ± 0.04[a, b]	0.73 ± 0.01[a, b]	0.49 ± 0.29[a, b]	0.70 ± 0.19[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml	55.03 ± 4.43	62.21 ± 4.51[a, b]	41.09 ± 3.72[a, b]	22.90 ± 3.53[a, b]	3.77 ± 4.53[a, b]
1.25 mg $PGF_{2\alpha}$/100 ml + HA 200 µg/ml	59.27 ± 4.53[b]	49.73 ± 4.17[a, b]	38.73 ± 3.89[a, b]	24.27 ± 3.76[a, b]	0.70 ± 0.43[a, b]
0.625 mg $PGF_{2\alpha}$/100 ml + Caf 7.5 µM	56.87 ± 4.09	57.17 ± 4.71[a]	42.36 ± 3.64[a, b]	8.10 ± 2.74[a, b]	0.93 ± 0.35[a, b]

Table 3 Frequencies of progressive motility. Results are expressed as means ± SD. (Superscript a) Statistical differences ($P<0.05$) in preservation times between days 1, 3, 6 or 10 and time 0 (within rows). (Superscript b) Statistical differences ($P<0.05$) between $PGF_{2\alpha}$ treatments and control