

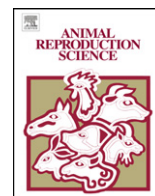


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# Fertility after post-cervical artificial insemination with cryopreserved sperm from boar ejaculates of good and poor freezability

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### ABSTRACT

This study compared the field fertility outcomes in frozen–thawed (FT) sperm from boar ejaculates with different freezability (good, GFE/poor, PFE) while testing the reliability of the post-cervical artificial insemination (post-CAI) in FT sperm. The assay was conducted over eight months with 86 weaned sows being inseminated by post-CAI. Every ejaculate in a total of 26 from 15 Piétrain boars was divided into a refrigerated semen portion (FS; control treatment) and a cryopreserved portion (FT sperm), and the ejaculates were in turn classified as GFE or PFE in function of the sperm progressive motility and viability at 240 min post-thaw. As result, one of four possible treatments was randomly given to each sow: FS-GFE, FS-PFE, FT-GFE and FT-PFE. The number of pregnant and farrowing sows in FT-GFE did not significantly differ from those of FS control treatments. Contrarily, the probabilities of pregnancy were two times lower after inseminations with FT-PFE ( $P < 0.05$ ) compared to FT-GFE, which indicates that ejaculates with high post-thaw sperm progressive motility and viability are more likely to result in pregnancies than those with poor in vitro sperm function. There were no differences in litter size or the risk of backflow among treatments. Further trials are required to determine the optimal volume and concentration of FT sperm in post-CAI to obtain a more reliable method for farmers interested in cryopreserved sperm.

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## 1. Introduction

The use of frozen–thawed (FT) sperm in artificial insemination (AI) technologies represents around 1% of total inseminations (Roca et al., 2006a), mainly due to the poor reproductive output obtained with FT sperm compared to refrigerated semen (FS) (Watson, 2000). The success of fertilization when FT sperm doses are used depends not only on their quality or a proper timing between insemination and ovulation (Bolarín et al., 2006; Großfeld et al., 2008; Martínez et al., 2002), but also on the site of sperm deposition. Both volume and survival limitations of FT sperm

restrict the use of cervical AI to FS (Martínez et al., 2001) as a high concentration of FT sperm is necessary ( $5\text{--}6 \times 10^9$  spermatozoa per dose) to match the results of FS obtained with only  $3 \times 10^9$  spermatozoa per dose (Roca et al., 2006a). However, when sperm is deposited in the uterus lower concentration is required for fertilization (Bathgate, 2008; Rozeboom et al., 2004; Vázquez et al., 2005). As sperm is placed nearer the oocyte (Sumransap et al., 2007) the limited survival of FT sperm is no longer a problem. Two methods of intrauterine AI are currently available: the deep intrauterine insemination (DUI), developed by Krueger and Rath (2000) and Martínez et al. (2001, 2002, 2006), and the post-cervical insemination (post-CAI), developed by Gil et al. (2000, 2004) and Gil (2006). Both post-CAI and DUI are suitable to improve fertility rates when FT sperm is used (Roca et al., 2006b). In DUI, the sperm is left at

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the proximal segment of one uterine horn (Martínez et al., 2006), whereas in post-CAI the sperm is placed in the uterine body (Roca et al., 2006a). DUI is successfully performed with FT sperm and only demands  $0.600 \times 10^9$  spermatozoa per dose (Roca et al., 2006b), but the implementation of post-CAI to FT sperm has not yet occurred. It will emerge from the adaptation of the technical procedure currently used in FS, which demands  $1 \times 10^9$  spermatozoa per dose (Gil, 2006). The trials done by Gil et al. (2000) and Fraser et al. (2007) represent, to our knowledge, the only published records of post-CAI with cryopreserved sperm.

On the other side, it is demonstrated that FT sperm from good freezability ejaculates (GFE) has higher rates of in vitro oocyte penetration than FT sperm from poor freezability ejaculates (PFE) (Gil et al., 2005); however, there is still no work for in vivo fertility rates in FT sperm from GFE and PFE. Post-thaw conventional sperm parameters are currently the most used to measure the boar ejaculate freezability (Casas et al., 2008a; Gil et al., 2005, 2008; Hernández et al., 2007; Holt et al., 1997). In the present study, ejaculates were statistically classified into good and poor freezability (GFE and PFE, respectively) by assessing the sperm progressive motility and the viability at 240 min post-thaw. This time point was set to assure the survival of FT sperm within the insemination-to-ovulation interval recommended for cryopreserved doses (Vázquez et al., 2005; Waberski et al., 1994).

The aim of the present work was to compare the field fertility outcomes in FT sperm between two groups of boar ejaculates with different freezability (GFE/PFE) while testing the reliability of the post-CAI in FT sperm.

## 2. Material and methods

### 2.1. Animals and sample collection

Fifteen mature healthy Piétrain boars aged 20 months and 86 multiparous (2–4 parity) sows from Duroc and hybrid (Landrace  $\times$  Duroc) breeds were stalled in two commercial herds (Selecció Batallé SA, Girona, Spain, and Semen Cardona SL, Barcelona, Spain). Boars were fed under standard protocols and provided with water *ad libitum* while being submitted to a collection rhythm of twice per week during 13 weeks. The sperm-rich fractions of the ejaculates (50–200 ml) were obtained by the gloved-hand technique and filtered to remove the gel. From all the ejaculates, 64 were randomly selected and the others were destined to commercial purposes. They were pre-diluted 2:1 (v/v) in a long-term extender (Vitasem LD, Magapor SL, Spain) at 37 °C inside a collecting recipient, and transported to the AI centre (BioGirona SL, Girona, Spain). One portion of each ejaculate (FS portion; refrigerated portion) was stored at 17 °C for two days before inseminations, after being rediluted in the long-term extender to obtain 30 ml doses at  $1 \times 10^9$  spermatozoa dose<sup>-1</sup>. A minimum of two doses was packed per ejaculate, like commercial seminal doses, to be used as controls in the fertility assay. The remaining portion of each ejaculate (FT portion; frozen–thawed portion) was rediluted to 1:5 (v/v) and sent to our laboratories in sealed plastic bags at 17 °C. Quality parameters were analyzed the

following day to cryopreserve the FT portions of those ejaculates which fulfilled a minimal sperm quality (see Section 2.4).

### 2.2. Experimental design

This study comprised 13 weeks randomly selected within eight different months. Each week (one assay) a group of sows was weaned at 5pm on Day 0 and hormonally treated at 5pm on Day 1 with PMSG/hcCG (Gestavet®Hipra, Girona, Spain) to synchronize oestrus. Sows with a weaning-to-oestrus interval (WEI) of four days were selected for the assay after verification, in the presence of a boar, of the standing reflex and the swelling of the vulva. Sows were tested for signs of oestrus twice daily through snout-to-snout contact between each sow and a boar, until double post-CAIs were performed (Day 6). Each week, at least three of the 64 ejaculates were collected from different boars the very same day oestrus was detected (Day 4). Each ejaculate was divided into two portions: the FS (control) and the FT portion. This last portion was obtained on Day 5 after quality assessment of the FS portion. From the 64 initial ejaculates, only the FS portions of 26 ejaculates in all the 15 boars fulfilled the standards of quality for cryopreservation and were maintained in the study. Due to the complexity of the design, the number of ejaculates that were cryopreserved and the number of sows in oestrus were different among weeks, but it was always verified that at least one sow was inseminated with FT sperm and one with FS from a same ejaculate. While inseminations were performed on-farm, four straws per ejaculate were thawed in our laboratories to assess the progressive motility and the viability of the FT portion at 240 min post-thawing. As result, four treatments were randomly given to the sows: FS-GFE and FS-PFE (control treatments), FT-GFE and FT-PFE. Each sow received a single treatment. The fixed effects of assay, month, boar, ejaculate and treatment were included in the analysis of the fertility parameters.

The experimental protocol was designed in accordance with the guidelines established by the Animal Welfare Directive of the Autonomous Government of Catalonia (Spain).

### 2.3. Sperm quality assay

Samples from all the ejaculates were taken from the FS portion (17 °C) and the FT portion (240 min post-thawing) for the quality assessment of sperm motility, viability and morphology. Morphological evaluation of sperm was only carried out at 17 °C as a quality control, since morphological alterations after freezing are mainly related to the sperm volume due to osmotic challenge (García-Herreros et al., 2008; Petrunkina et al., 2005).

#### 2.3.1. Sperm motility

Sperm cells from the FS portion were pre-heated at 37 °C for 20 min before the sample was analyzed. A Makler chamber (Sefi-medical Instruments, Haifa, Israel) was used in all samples instead of slides. Motility was examined at 100 $\times$  magnification under a negative phase-contrast objective with an Olympus BX41 microscope (Olympus

Europe GmbH, Hamburg, Germany) and the software CASA (Computer Assisted Sperm Analysis; SCA production 2002, Microptic SL, Barcelona). A minimum of three replicates per ejaculate with 1500 spermatozoa in each replicate was analyzed. Each spermatozoon was characterized as “progressive” if it had more than 45% straightness (STR).

### 2.3.2. Sperm viability

Samples were diluted to  $10 \times 10^6$  spermatozoa  $\text{ml}^{-1}$  in Beltsville Thawing Solution (BTS; Pursel et al., 1973) before viability assessment using the fluorescent LIVE/DEAD® Sperm viability kit (Molecular probes, Eugene, OR, USA). Two replicates per ejaculate were evaluated for the percentage of viable sperm, one replicate under a fluorescence microscope (Zeiss Axio Imager.Z1, Carl Zeiss, Germany) and one by flow cytometry (Cell Lab Quanta SC®, Beckman Coulter, Fullerton, CA, USA), depicting a single mean per ejaculate. Two hundred spermatozoa and 20,000 events were counted under the microscope and by flow cytometry, respectively, and one positive control for each fluorescent probe was assayed.

### 2.3.3. Sperm morphology

The percentage of sperm without anomalies (absence of abnormal head shape, tail folding, tail coiling or proximal droplets) and the percentage of sperm with proximal droplets were detected at  $200\times$  magnification under a positive phase-contrast objective with an Olympus BX41 microscope and the software CASA. A minimum of 300 spermatozoa was examined for each ejaculate sample before cryopreservation as a quality control.

## 2.4. Sperm cryopreservation

Twenty-six ejaculates from 15 different boars, which had over 60% sperm progressive motility, 70% morphologically normal sperm and 80% sperm viability, were cryopreserved, after holding at  $17^\circ\text{C}$  overnight, using the Westendorf method for porcine (Westendorf et al., 1975; modified from Carvajal et al., 2004). All the ejaculates were centrifuged at  $17^\circ\text{C}$  and  $640 \times g$  for 3 min. Pellets were recovered with 3–4 ml of supernatant and diluted at  $1.5 \times 10^9$  spermatozoa  $\text{ml}^{-1}$  into a freezing media containing lactose-egg yolk (LEY). After cooling to  $5^\circ\text{C}$  during 150 min, sperm was diluted at  $1 \times 10^9$  spermatozoa  $\text{ml}^{-1}$  in a second media containing LEY with 6% glycerol and 1.5% Orvus ES Paste (Equex STM, Nova Chemical Sales Inc., Scituate, MA, USA). Sperm was packed in 0.5 ml plastic straws (Minitub Ibérica SL, Tarragona, Spain) and transferred to a programmable freezer (Icecube14S-B, Minitub Ibérica SL, Tarragona, Spain). The freezing programme (SY-LAB software, Minitub Ibérica SL, Tarragona, Spain) consisted of 5 min 13 s of cooling at the following rates:  $-6^\circ\text{C min}^{-1}$  from 5 to  $-5^\circ\text{C}$  (1 min 40 s),  $-39.82^\circ\text{C min}^{-1}$  from  $-5$  to  $-80^\circ\text{C}$  (1 min 53 s), held for 30 s at  $-80^\circ\text{C}$  and  $-60^\circ\text{C min}^{-1}$  from  $-80$  to  $-150^\circ\text{C}$  (1 min 10 s). The straws were then plunged into liquid  $\text{N}_2$  ( $-196^\circ\text{C}$ ) for further storage.

Four straws were thawed per ejaculate after at least 12 h in liquid nitrogen to assess the post-thaw sperm quality. Each straw was shaken for 20 s under water at  $37^\circ\text{C}$  and then diluted to 1:3 (v/v) in BTS at the same temperature

(Casas et al., 2008b). Sperm progressive motility and viability were examined after sperm was incubated for 240 min in the water bath ( $37^\circ\text{C}$ ).

## 2.5. Fertility assay

Multiparous (2–4 parity) Duroc and hybrid sows (Landrace  $\times$  Duroc) were stalled in a Selecció Batallé SA herd (Girona, Spain). They were aged  $24.75 \pm 7.38$  months, fed under a standard protocol and provided with water *ad libitum*. Post-CAIs were performed on 86 sows by the same veterinarian after positive back pressure test was verified. Both the FS and the FT treatments were applied using the Soft-quick® catheter (Import-vet SA, Barcelona, Spain), which consists of inserting a 72 cm (28.3 in.) flexible fiberoptic into a conventional cannula. Post-CAI inseminations with FT sperm were carried out with the same volume recommended in the literature for FS (Echegaray et al., 2003; Fraser et al., 2007; Roberts and Bilkei, 2005; Salvador et al., 2005; Williams, 2002). Inseminations with the FS portions (control groups) were completed in 41 sows with one dose (30 ml at  $1 \times 10^9$  spermatozoa) per sow, while 45 sows were inseminated with the FT portions, each with 15 straws of 0.5 ml that were thawed and diluted in 22.5 ml of BTS (1:3, v/v) to yield a final concentration of  $7.5 \times 10^9$  spermatozoa in 30 ml (one dose). After inseminations, approximately 3 ml of tempered BTS was flushed through the cannula to introduce residual sperm.

As a safeguard for fertility, the insemination practices were repeated twice in each sow according to Gil (2006) and Watson and Behan (2002). Waberski et al. (1994) state that ovulation occurs within 24–48 h from the onset of oestrus, and report optimal fertilization rates for FT sperm when inseminations are performed within 4–6 h prior to ovulation. This insemination-to-ovulation interval for FT sperm corresponds to the shorter lifespan compared to FS, this last with a fertility confidence threshold of 24 h. As the first sows in oestrus were detected at 4 pm, ovulation was predicted for the same hour two days later. The first dose was applied at 12 am and the second at the moment of predicted ovulation (4 pm), following the same schedule in controls of FS.

Pregnancy was detected by ultrasounds (Echoscan T-100, Import-vet SA, Barcelona, Spain) 28 days after inseminations. Data concerning rates of pregnancy, farrowing and the litter size (live born and total born) were recorded by the veterinarian, as well as observations about the insemination performance (resistance to the catheter, bleeding or backflow).

## 2.6. Statistical analyses

Data were introduced in the statistical package SPSS v15.0 (SPSS Inc., Chicago, IL, USA). Significance was fixed at  $P \leq 0.05$ . Percentages for progressive motility and viability in the sperm quality assay and values for the litter size are given as mean  $\pm$  standard error of the mean (SEM), whereas data on pregnancy, farrowing and backflow are set as binomial variables with dummy codification. The classification of the 26 ejaculates for freezability (GFE/PFE) was made through a non-hierarchical k-means cluster anal-

**Table 1**

Percentages of sperm progressive motility, sperm viability and sperm morphology for each treatment (mean ± SEM). It is stated the number of different boars and ejaculates that contributed each treatment ( $n$  = total number in the whole experiment). Notice that three boars were collected ejaculates with different freezability in different assays. Each ejaculate was obtained one FS portion and one FT portion so that the number of boars and ejaculates should coincide between portions in GFE and in PFE. Sperm morphology was only assessed on FS as a quality control and progressive motility and viability were assessed on FT sperm at 240 min post-thawing, as the basis for the classification into GFE and PFE. FS, refrigerated semen; FT, frozen–thawed sperm; GFE, good freezability ejaculates; PFE, poor freezability ejaculates; N, spermatozoa without anomalies (absence of abnormal head shape, proximal droplets, tail folding or tail coiling); PD, spermatozoa with proximal droplets.

Treatment	FS portion (control)		FT portion	
	GFE	PFE	GFE	PFE
Boars ( $n = 15$ )	9	9	9	9
Ejaculates ( $n = 26$ )	13	13	13	13
Sperm progressive motility (%)	67.67 ± 0.19 <sup>1</sup>	64.77 ± 0.15 <sup>1</sup>	51.55 ± 0.25 <sup>2</sup>	4.94 ± 0.22 <sup>3</sup>
Sperm viability (%)	90.59 ± 0.15 <sup>1</sup>	88.01 ± 0.15 <sup>1</sup>	46.18 ± 0.27 <sup>2</sup>	21.66 ± 0.26 <sup>3</sup>
Sperm morphology (%)	N 85.84 ± 0.21	N 90.50 ± 0.19	–	–
	PD 7.38 ± 0.21	PD 3.07 ± 0.13	–	–

Values in the same row with different superscripts are significantly different (<sup>1,2,3</sup> $P < 0.01$ ).

ysis for dissimilarities, using sperm progressive motility and viability values at 240 min post-thaw. The sow ( $N = 86$ ) was the experimental unit, nested within five categorical independent variables with the following hierarchical order: assay, month, boar, ejaculate and treatment (four treatments of freezability). To determine which of them had to be included in the analyses regression was tested at each dependent variable. Data on pregnancy, farrowing and backflow were analyzed with binary logistic regression analyses (Wald's algorithm and Cox and Snell pseudo- $R$ -square). Each variable was grouped into the independent variables that stayed; the degrees of freedom (df) of significant tests are specified in the results. When regression analyses resulted non-significant, Spearman or Pearson correlations were calculated depending on data compared.

The three binomial dependent variables were run a generalized linear model (Type III) with sequential Bonferroni post hoc tests; the odds ratios (OR, Exp(B)) provided information about the direction and strength of the association. Probabilities or “relative risks”, for a positive outcome at each category of the independent variables were calculated from the ORs in relation to a reference category (Kåreholt, 2003).

The arcsin transformed percentages from the sperm quality assay and the two dependent variables related to the litter size were run a multivariate general linear model (GLM; Type III and IV, respectively) after verifying normal distribution of values and residuals (K–S test). The effects size of independent variables (the variation accounted) was estimated by the partial eta-square values, to a maximum of 1. Significant differences in association with an independent variable (Roy's largest root) were followed by Bonferroni post hoc tests for comparison among categories.

### 3. Results

Thirteen ejaculates with sperm progressive motility and viability over 30% were classified as GFE, and the other 13 ejaculates were labelled as PFE. Each group of ejaculates was collected from nine different boars, from which three contributed ejaculates to more than one treatment (GFE and PFE) due to differences in freezability among collections.

#### 3.1. Sperm quality assay

The sperm progressive motility and the sperm viability were statistically associated with treatment (Roy's largest root  $P < 0.01$ ;  $df = 3$ ; partial eta-square = 0.94). Percentages of sperm progressive motility, sperm viability and sperm morphology (sperm without anomalies and sperm with proximal droplets) of the 26 ejaculates are shown in Table 1 for each treatment.

Both the GFE and the PFE had over 85% of FS without anomalies and fewer than 10% of proximal droplets, which fulfilled the quality standards we determined before cryopreservation. No significant differences were found in the sperm progressive motility or viability between control treatments (FS). Among FS and FT portions differences were found in the two parameters ( $P < 0.01$ ). Compared to FS of the same freezability, the sperm progressive motility and viability at 240 min after freezing decreased 16.12% and 44.41%, respectively, in GFE, and 59.83% and 66.35%, respectively, in PFE. Differences were also found between FT-GFE and FT-PFE treatments ( $P < 0.01$ ). Compared to FT-GFE, the percentage of sperm progressive motility in FT-PFE was the 46.61% lower and the sperm viability the 24.52% lower.

#### 3.2. Fertility assay

Irrespective of the assay, month, boar or ejaculate, and despite the logistic regression coefficients being low (pregnancy pseudo- $R$ -square = 0.194; farrowing pseudo- $R$ -square = 0.165), the treatment given to sows conditioned pregnancy and farrowing outcomes after post-CAI inseminations ( $P < 0.01$ ;  $df = 3$ ;  $n = 86$ ). As a result, data on pregnancy and farrowing were split for the four treatments as shown in Table 2. The farrowing rates were calculated from two miscarriages in treatment FS-PFE and one miscarriage and a sow slaughtered in treatment FT-GFE.

Multiple peer comparisons showed that the numbers of pregnant and farrowing sows were significantly higher after inseminations with FS control treatments than with the FT-PFE treatment ( $P < 0.01$ ;  $df = 1$ ), with no differences in FS treatments and the FT-GFE treatment. Differences between FT-GFE and FT-PFE were detected for pregnancy ( $P < 0.05$ ;  $df = 1$ ) but not for farrowing, which showed that

**Table 2**

Fertility assay after double post-CAI (mean  $\pm$  SEM). It is stated the number of different assays (weeks), months (from February = 2 to December = 12 except July, August and September), boars and ejaculates that contributed each treatment ( $n$  = total number in the whole experiment). Notice that three boars were collected ejaculates with different freezability in different assays. Each ejaculate was obtained one FS portion and one FT portion so that the four factors listed above should coincide between portions in GFE and in PFE. FS, refrigerated semen; FT, frozen–thawed sperm; GFE, good freezability ejaculates; PFE, poor freezability ejaculates.

Treatment	FS portion (control)		FT portion	
	GFE	PFE	GFE	PFE
Assays ( $n$ = 13)	9	7	9	7
Months ( $n$ = 8)	2, 3, 4, 5, 6, 12	3, 4, 10, 11, 12	2, 3, 4, 5, 6, 12	3, 4, 10, 11, 12
Boars ( $n$ = 15)	9	9	9	9
Ejaculates ( $n$ = 26)	13	13	13	13
Sows inseminated ( $n$ )	21	20	26	19
Total volume inseminated per sow (ml)	30 (2 $\times$ )	30 (2 $\times$ )	30 (2 $\times$ )	30 (2 $\times$ )
Sperm number per insemination ( $\times 10^9$ spz)	1	1	7.5	7.5
Sows having backflow ( $n$ )	4	2	8	4
Pregnant sows at 28 days ( $n$ )	17 <sup>1</sup>	17 <sup>1</sup>	16 <sup>A</sup>	5 <sup>B,2</sup>
Pregnancy rate (%)	80.95	85	61.54	26.32
Farrowed sows ( $n$ )	17 <sup>1</sup>	15 <sup>1</sup>	14	5 <sup>2</sup>
Farrowing rate (%)	80.95	75	53.85	26.32
Live born litter size ( $n$ )	10.06 $\pm$ 0.89	11.27 $\pm$ 0.61	8.64 $\pm$ 0.94	9.40 $\pm$ 0.93
Total born litter size ( $n$ )	10.65 $\pm$ 0.74	12.13 $\pm$ 0.57	9.36 $\pm$ 1.15	10.60 $\pm$ 1.21

Values in the same row with different superscripts are significantly different (<sup>A,B</sup> $P < 0.05$ ; <sup>1,2</sup> $P < 0.01$ ).

those ejaculates with high post-thaw progressive motility and viability were more likely to successfully fertilize sows ( $r = 0.328$  and  $0.410$ , respectively;  $P < 0.05$  and  $0.01$ ). The number of farrowed sows neither improved nor worsened after inseminations with FT-GFE compared to the other three treatments. Even so, a positive correlation was found between farrowing and progressive motility and viability in FT sperm ( $r = 0.295$  and  $0.357$ , respectively;  $P < 0.05$ ) and between farrowing and pregnancy in FS ( $r = 0.856$ ;  $P < 0.01$ ) and in FT sperm ( $r = 0.914$ ;  $P < 0.01$ ).

Data showed that the odds of becoming pregnant after insemination with FS-GFE, FS-PFE and FT-GFE were respectively 11.90, 15.87 and 4.48 times the odds of becoming pregnant after inseminations with FT-PFE (taken as the reference category) ( $P < 0.01$  or  $0.05$ ;  $df = 1$ ). That is, the probability of pregnancy when FS (either GFE or PFE) or FT-GFE is used for insemination increases three and two times respectively compared to inseminations with FT-PFE.

The odds of sows farrowing after insemination with FS-GFE and FS-PFE were 11.90 and 8.40 times respectively the odds of sows farrowing after inseminations with FT-PFE ( $P < 0.01$ ;  $df = 1$ ). That is, the probability of sows farrowing when they had been inseminated with FS (either GFE or PFE) increases three times with respect to inseminations with FT-PFE.

Numbers of live born and total born piglets were not associated with any independent variable. Descriptive data on the litter size are given in Table 2 for each treatment. The highest partial eta-square in the analysis was weak (0.156), which shows that independent variables in the model, including treatment, had negligible effects on the litter size. No correlation appeared to be significant between the litter size and treatment, progressive motility or viability, either in FS or in FT sperm.

Observations were also made on the post-CAI performance during the 172 insertions of the assay (86 sows inseminated twice). Sperm was flushed easily and resistance from the cervix to the entrance of the catheter was only reported for five insertions (2.91%) in four sows

(two sows from FS-GFE and FS-PFE control treatments and two sows from the FT-GFE treatment), and all except one resulted in negative pregnancy. Bleeding was detected once (0.58%) in the FT-GFE treatment, with positive pregnancy, and backflow was reported in 21 insertions (12.21%) in 18 sows (Table 2). None of the independent variables in the assay, including treatment, was correlated with the risk of backflow with either FS or FT sperm.

#### 4. Discussion

The main challenge in developing suitable AI techniques for FT boar sperm is to overcome the lower fertility percentage in comparison to FS, both in terms of pregnancy and farrowing and also with respect to the litter size (Bathgate, 2008; Bathgate et al., 2008; Fraser et al., 2007). The reduced lifespan of FT sperm and the increase in dose cost that results from the freezing process make intrauterine AI adequate in such context (Roca et al., 2003, 2006a). This AI technique shortens the way of sperm to the utero-tubular junction (UTJ), where fertilization occurs, and demands lower sperm concentration than cervical AI. Post-thaw selection for the GFE followed by intrauterine AI can substantially improve fertility, as it is demonstrated in the present trial that especially focuses on the post-CAI. We showed that chances for positive pregnancy in AI with FT boar sperm are doubled when there is a selection for GFE and use of post-CAI, maintaining similar outcomes in pregnancy, farrowing and prolificity as in FS.

Selection of GFE has been usually based on multiparametric analysis (Gil et al., 2005) but we have simplified the procedure by dealing only with the sperm progressive motility and the viability at 240 min post-thaw. Both parameters are able to cluster ejaculates into GFE and PFE with the same confidence as when including 11 more variables, as we tested in anterior studies (data not shown), since they summarize the integrity of sperm after freezing that is the key for selection inside the oviducts (Fazeli et al., 1999; Pursel et al., 1978; Taylor et al., 2008). At this respect,

cryopreservation triggers a capacitation-like state in sperm characterized by decreased viability and high hyperactivated motility (Watson, 1995), this last being manifested in CASA parameters by a reduction in the sperm straightness and hence in the sperm progressivity (Schmidt and Kamp, 2004).

In this study we have also observed no effects of the freezability selection of ejaculates on the viability, progressivity and fertility results in the FS portion. Recent assays in our laboratories (data not shown) have demonstrated that description of the ejaculates freezability through conventional sperm quality parameters is not feasible before freezing (i.e., in the FS portion) but it could be after achieving the 5 °C, when it is known the cold shock reaches its maximum (Andrabi, 2007; Watson, 2000). Roca et al. (2006c) also observed a poor significance for the sperm cryosurvival of the FS outcomes, which respond to different characteristics among boars that are still to be described.

Nevertheless, the description of a general pattern in the relationship of the boar sperm quality parameters with the fertility outcomes, either in FS or in FT sperm, is still controversial. Some studies indicate it largely depends on boars (Popwell and Flowers, 2004), so that a sperm quality parameter that is a good predictor of the in vivo or the in vitro fertility for one boar may not be applicable for others. Similarly, the threshold between good and poor freezability, statistically established at 30% in the present study, should be removed regarding interests on selective or productive farming. High-value genetic boars do not necessarily deal with good reproductive performance, so a PFE with a given genetic value would be also chosen for sperm banking, as also discussed by Holt et al. (2005). Contrarily, interests on the reproductive performance of boars should benefit of an increase in the freezability threshold, allowing more ejaculates to be discarded as GFE in order to achieve higher confidence in the fertility outcomes.

An adequate selection for freezability is not the only point to be considered in AI with FT sperm. It is also necessary to attain some important features when intra-uterine AI is used compared to cervical AI (Levis et al., 2002), i.e., less backflow, less time to infuse sperm, fewer sperm numbers per dose and smaller volume.

Backflow is the major mechanism of sperm elimination from the oviducts (Pursel et al., 1978; Rath et al., 2008; Satake et al., 2006). In cervical AI, the 70% of the total volume infused can flush out within the first hour after insemination (Matthijs et al., 2003; Steverink et al., 1998). Most of the extender is eliminated with up to 45% of the inseminated sperm, whereas sperm that has not been expelled or has not been able to bind to oviductal epithelial cells or neutrophils will be phagocytosed in the uterus by polymorphonuclear leukocytes (Matthijs et al., 2003). EDTA from the extender blocks phagocytosis and sperm capacitation until its elimination through backflow, but high volumes of liquid in the uterus could in turn initiate the recruitment of leukocytes (Fazeli, 2008). Reducing the inseminated volume has been demonstrated to be effective for relaxing both backflow and phagocytosis in cervical AI (Matthijs et al., 2003). Post-CAI is effective for reducing backflow to less than 20% in FS (Mezalira et al., 2005) and we verified it is as operative in FT sperm, as we barely observed

12% of backflow. The degree of phagocytosis in the uterus after post-CAI has not yet been quantified, but for cervical AI there are no differences between phagocytosis in FS and in FT sperm (Pursel et al., 1978).

Another feature that post-CAI accomplishes is the time-cost effectiveness, provided the catheter is easy to introduce and the genital tract of the sow puts up little resistance, without traces of bleeding. This simplicity of use has been reported in FS using a similar post-CAI device (Watson and Behan, 2002).

The development of new AI techniques should also guarantee good prolificity indexes. The possibility for bilateral fertilization (Martínez et al., 2006) and also the sperm volume and concentration of doses (Bathgate, 2008; Bathgate et al., 2008; Hofmo, 2006; Levis et al., 2002; Rozeboom et al., 2004) are positively correlated to the litter size, both in FS and in FT sperm. In relation to the concentration and volume of doses, it is recommended that a minimal population of  $0.005 \times 10^9$  spermatozoa in the UTJ of each horn was maintained (Rath et al., 1999). Paradoxically, as previously discussed, low volumes in the uterus benefit the recovery of sperm (Matthijs et al., 2003), taking apart it represents an important saving in sperm doses.

Post-CAI provides the possibility of bilateral fertilizations and maintains a good litter size in FS when  $0.5 \times 10^9$  spermatozoa ml<sup>-1</sup> and 15 ml doses are used (Gil et al., 2004). The optimization in spending doses compared to cervical AI and even the possibility to inseminate some nulliparous sows (Gil, 2006) have resulted in an increased number of FS inseminations with this technique. In this study we have attempted  $0.25 \times 10^9$  spermatozoa ml<sup>-1</sup> and 30 ml FT sperm doses, which represents the lowest volume and concentration tested to date in post-CAI with FT sperm. The mean litter size we obtained in GFE and PFE was non-statistically different to those attained in FS controls, which shows the good applicability of post-CAI to cryopreserved sperm and also the independence of prolificity over the ejaculate freezability. Future trials will be directed towards obtaining the sperm volume and concentration for the best performance of the technique.

In conclusion, good results in pregnancy and farrowing are attained with FT sperm if ejaculates with good post-thaw sperm progressive motility and viability (GFE) are previously selected. Post-CAI is a method that has been implemented on-farm in FS and provides successful results without specialized skills. The optimization of post-CAI features for FT sperm, as achieved for DUI with special reference to the sperm volume and concentration, will benefit the porcine livestock farmers interested in managing cryopreserved sperm.

#### Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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