Title: **Heterologous in vitro fertilization is a good procedure to assess the fertility of thawed ram spermatozoa**

Short Title: **Fertility prediction by heterologous IVF**

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Abstract

A heterologous in vitro fertilization (IVF) test using calf oocytes with zona-pellucida was employed to assess the fertility of thawed ram sperm samples. Six males with significant differences in fertility (*P*= 0.003) were used. The males were classified as having high fertility (≥ 42%) and low fertility (≤ 41%). Male fertility was not influenced by number of inseminated ewes (*P*=0.584), insemination technician (*P*=0.156), insemination date (*P*=0.323) or farm (*P*=0.207). Thawed sperm samples were employed to assess several sperm parameters for each male: motility, acrosomal integrity, viability, membrane stability, membrane phospholipid disorder, mitochondrial membrane potential and chromatin stability. These samples were used to carry out a heterologous in vitro fertilization. In vitro-matured calf oocytes (n=716) were inseminated with thawed ram semen and in vitro cultured for 40 hours. Overall, at thawing, variability among males respect to sperm quality was high. Despite this variability, there were not differences (*P*<0.05) between fertility groups. Yield of hybrid embryos ranged from 31 to 59% between males. There were not differences between males (*P*=0.340). However, there were differences between fertility groups (high fertility: 55%; low fertility: 39%; *P*=0.020). Multiple regression analysis showed that the heterologous in vitro fertility was the only predictive parameter for in vivo male fertility. Correlation between both parameters was fair (r^2=0.760; *P*=0.025). These results indicate that heterologous in vitro fertilization tests can be useful to predict the fertility of ram spermatozoa using calf oocytes with intact-zona pellucida.

Keywords: heterologous in vitro fertilization, ram, fertility, spermatozoa, intact-zona oocytes

1. Introduction

The assessment of semen quality is very important prior to performing artificial insemination or in vitro fertilization to assure a good fertility results. So far, many studies have focused in the relationship between sperm parameters and in vivo fertility, with very different outcomes [1-4].

Most methods for in vitro semen evaluation measure general characteristics of the spermatozoa (motility, membrane integrity, organelle integrity, etc…), all essential to fertility. Other methods attempt to mimic in vitro the process of fertilization in vivo. Membrane integrity and functionality are requirements for the viability of the spermatozoa, and they are usually assessed with membrane- impermeant dyes [5]. Early stages of sperm capacitation and increased membrane permeability indicative of spermatozoa damaged can be measured using different fluorophores [6-8]. Acrosome intactness, a prerequisite for fertilization, can be examined in vitro using phase-contrast microscopy or using different fluorescent conjugated lectins, which can be combined with viability stains [5]. Other determinant of fertilization ability is the mitochondrial status, since mitochondrial function might be useful as a measure of sperm quality [9]. Mitochondrial membrane potential can be determined employing specific fluorophores, such as the Mitotracker dyes [10,11]. Finally, the degree of DNA integrity is important because early embryo development depends on the presence of normal DNA [3]. One of the methods to determine DNA damage is the SCSA (sperm chromatin structure assay) used to assess the degree of susceptibility of the DNA to acid-induced denaturation.

However, the relationship between sperm quality, assessing all different sperm characteristics, and fertility vary greatly among studies [6,11-16], being necessary to find methods more accurate to evaluate the fertility of sperm sample.

In vitro fertilization is the most adequate method to assess the fertility, since this procedure evaluates the spermatozoa-oocyte interactions occurring during in vivo fertilization, allowing measurement of different endpoints in the early stages of the embryo development. Some authors have used homologous IVF assays as a predictor of fertility using zona-intact oocytes [17-20]. However, it is often difficult to obtain oocytes of the same species, especially when dealing with wild or endangered ones. An alternative is employing oocytes of laboratory animals or domestic species, which can be easily obtained. Thus, some authors have used oocytes of laboratory animals, as the hamster, since its oocytes can be penetrated by spermatozoa of other species [21-24] or oocytes of domestic species obtained at slaughterhouses [25]. However, these studies were carried out using oocytes free of zona pellucida. The zona pellucida is the first barrier in the spermatozoa-oocyte interaction. On its surface there are receptors for the attachment and binding of capacitated spermatozoa and it is involved in the subsequent induction of the acrosome reaction [26]. Moreover, modifications to the zona pellucida following fertilization prevent polyspermy [27]. Thus, the IVF assays using zona-free oocytes might be considered incomplete for assessing fertility, since sperm fertility can be described as the ability of the spermatozoon to bind and cross the zona pellucida, to perform the fusion of its membrane with oocyte's oolema, to achive the formation of the male pronucleus and to conduct to the zygote cleavage. Others authors, have used zona-intact oocytes of domestic animals to evaluate spermatozoa functionality by heterologous IVF tests [28-31]. However, so far nobody has studied the relationship with in vivo fertility using heterologous in vitro fertilization systems employing oocytes with intact zona pellucida.

In Spain slaughtered sheep are generally old, and the oocytes quality is not good, being the oocytes of other species, slaughtered younger and sexually matured, an alternative. The objective of this study was to evaluate the performance of a heterologous in vitro fertilization test that employed zona-intact calf oocytes, for assessing the fertility of thawed ram semen. The sperm samples used in this study were selected for heterogeneity in in vivo fertility after intra uterine laparoscopic insemination.

2. Material and methods

2.1. Material

Fluorescence probes were purchased from Invitrogen (Barcelona, Spain). Chromatographically purified acridine orange was purchased from Polysciences Inc. (Warrington, PA, USA). Other chemicals were of reagent grade and were purchased from Sigma (Madrid).

2.2. Semen collection

All animal procedures were performed in accordance with the Spanish Animal Protection Regulation RD223/1988, which conforms to European Union Regulation 86/609. Adult males were maintained and managed at Centro Regional de Selección y Reproducción Animal of Valdepeñas (CERSYRA). A total of 6 males of Manchega sheep breed (age > 3 years) were used. Semen collection was performed using artificial vagina. Volume, concentration, wave motion (0: no movement to 5: strong wave movement) and sperm motility were assessed shortly after collection. Only, the ejaculates with values of wave motion and sperm motility higher of 4 and 80%, respectively, were frozen.

2.3. Semen cryopreservation

After initial semen evaluation, each ejaculated was diluted with the freezing extender. The diluent used was prepared as previously described [32]. The ejaculates were diluted to a final concentration of 200 x 10^6 spermatozoa/mL. Diluent 1 contained 3.25% (w/v) TRIS, 0.935% (w/v) D-fructose, 1.702% (w/v) citric acid, 2% glycerol, 25% egg yolk and 50000 IU penicillin G. Composition of diluent 2 was: 3.953% (w/v) dextran B, 0.688% (w/v) sodium citrate, 0.158% (w/v) TEST, 0.363% (w/v) glycine 10.188% (w/v) lactose, 1.186% (w/v) raffinosse, 0.506% fructose (w/v), 50000 IU penicillin and 12% glycerol. Diluent 1 was added 3:2 to semen and slowly cooled from 30 to 5ºC in 2 h. Then, the samples were further diluted (3:1) with the diluent 2 at this temperature and held for equilibration at 5ºC for 2 h (total refrigeration time at 5ºC was thus 4 h). At the end of the cooling and equilibration period, the extended semen was loaded into 0.25-mL plastic straws and frozen. The straws were frozen in a programmable biofreezer (Planner) at 20ºC/min to -100ºC, and at 10ºC/min from -100ºC to -140ºC and then plunged into liquid nitrogen.

2.4. Semen evaluation

The straws were thawed for 20 sec at 37ºC and aliquots were used to assess sperm quality. Percentage of individual sperm motility evaluated subjectively was recorder. Also, the acrosome integrity and viability were noted. Acrosome integrity was evaluated after a 1:10 dilution in 2% glutaraldehyde in 0.165 M cacodylate/HCl buffer (pH 7.3). The percentage of spermatozoa with intact acrosomes , i.e., those showing a normal apical ridge (% NAR), was assessed by phase-contrast microscopy. The viability was assessed by nigrosin-eosin stain as previously described [33]. An aliquot of thawed semen was mixed with the stain (1:2) for 20 sec at 37ºC and a smear was carried out after incubation. The percentage of live spermatozoa, i.e., those remaining unstained (% Viability), was assessed by bright field microscopy.

Aliquots of thawed semen were used to carry out flow cytometry analysis. We assess the membrane stability with YO-PRO-1, the membrane phospholipid disorder with Merocyanine 540, the mitochondrial membrane potentital with Mitotracker Deep Red, the acrosome integrity with PNA-FICT and the viability with propidium ioide (PI). We prepared two staining solutions using flow cytometer sheath fluid (BD FACSFlow™). One of them was prepared by adding 3 nM Hoechst 33342 (stock:9 μM in milli-Q water), 50 nM YO-PRO-1 (stock: 100 μM in DMSO), 1 μM Merocyanine 540, 15 μM propidium ioide (stock: 7.5 mM in milli-Q water) and 100 nM of Mitotracker Deep Red (stock: 1 mM in DMSO). The other was prepared by adding the same concentration of Hoechst 33342 and PI, and 10 μg/mL of PNA-FITC (stock of 0.2 mg/mL). We diluted 20 μL of sample in 0.5 mL of each staining solution in polypropylene tubes for flow cytometry. The tubes were allowed to rest for 15 min in the dark and then analyzed using a LSR-I flow cytometer (BD Biosciences, San José, CA, USA). We used the three lasers of the cytometer to excite the different fluorochromes. A 325 nm Helium-Cadmium UV laser for exciting the Hoechst 33342, a 488 nm Argon-Ion laser for exciting YO-PRO-1, Merocyanine 540, PNA-FITC and PI, and a 633 nm Helium-Neon laser for exciting Mitotracker Deep Red. We acquired the FSC (forward-scatter light) and SSC (side-scatter light) signals plus the fluorescence light of each fluorochrome using four photodetectors. FL1 was used for YO-PRO-1 and PNA-FITC (530/28BP filter), FL2 for Merocyanine 540 (575/26BP filter), FL3 for propidium ioide (670LP filter), FL5 for Hoechst 33342 (424/44BP filter) and FL6 for Mitotracker Deep Red (670/40BP filter). The acquisition was controlled using the Cell Quest Pro 3.1 software. All the parameters were read using logarithmic amplification. We set up an acquisition template in the software which allowed us first to discriminate spermatozoa from debris within the events acquired. FSC/SSC and FL6/FL3 (Hoechst

33342 vs. PI) dot plots were used to discard debris. The filtered events were displayed in dot plots showing either FL1/FL3 (YO-PRO-1 vs. PI), FL6/FL3 (Mitotracker Deep Red vs. PI) and FL2/FL3 (Merocyanine 540 vs. PI). We acquired 10000 spermatozoa from each sample, saving the data in FCS v. 2 files.

Chromatin stability was assessed using the metachromatic staining Acridin Orange (AO), which fluoresces green when combined with double stranded DNA, and red when combined with single stranded DNA (denatured). Spermatozoa were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM EDTA; pH=7.4) to $2x10^6$ cells/mL. Samples were flash frozen in LN2 and stored at -80ºC until analysis. For the analysis, the samples were thawed on crushed ice and 200 µl were put on a cytometry tube. Then, we added 400 µl of an acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH=1.2). Exactly 30 s after adding the acid-detergent solution, we added 1.2 mL of staining solution (6 µg/mL of acridine orange in a buffer containing 37 mM citric acid, 126 mM Na_2HPO_4, 1.1 mM disodium EDTA and 150 mM NaCl; pH=6). We left the sample staining for 3 min, and then we run it through a Becton Dikinson LSR-1 flow cytometer. Acridine orange was exciting with an Ar-ion las providing 488 nm light. The red fluorescence was detected using a long pass (670LP) filter (FL-3) and the green one using a band pass (530/28BP) filter (FL-1). Sample acquisition was carried out with a CellQuest v. 3 software. Flow Cytometry data (FCS files) were processed and saved as tabbed text using WinMDI v. 2.8 (The Scripss Research Institute, La Jolla, California). We calculated the DNA Fragmentation Index (DFI) for each spermatozoon as the ratio of red fluorescence respect to total fluorescence (red+green). High values of DFI, indicates chromatin abnormalities. We also calculated DFI%, as the percentage of spermatozoa with DFI >25, and High DNA

Stainability (HDS) as the percentage of the spermatozoa with green fluorescence higher than channel 600 (of 1024 channels).

2.5. Artificial insemination trials

Thawed sperm samples of all males were used to inseminate a total of 551 ewes in eight farms. Sperm samples from each male were used to inseminate between 11 and 262 females. The ewes were synchronized using progestagen pessaries (30 mg fluorogestone acetate, FGA; Chronogest, Intervet, The Netherlands) for 13 days followed by 500 IU equine chorionic gonadotrophin (eCG) at pessary removal. Ewes were inseminated intra uterine by laparoscopy at 55-58 h after pessary removal. Two technicians carried out all intra uterine inseminations in different dates.

We considered that a male scored a successful fertilization when the female lambed. Fertility rate for each male was calculated as follows: number of lambed ewes/number of ewes inseminated x 100. This rate was called Male fertility.

The males were classified according to fertility in two groups: high fertility, those with fertility above mean (male fertility $\geq 42\%$) and, low fertility, those with fertility below mean (male fertility $\leq 41\%$).

2.6. Heterologous in vitro fertilization (IVF)

Heterologous IVF was carried out four times for each male and a minimum of twenty oocytes were used each time (minimum 20; maximum 40). Calf ovaries about 1 year old were collected at an abattoir and transported to our laboratory in saline (30ºC) between 1 and 2 h after removal. Immature oocytes were collected from ovaries, using 19-gauge needle, in TCM-199 supplemented with HEPES (2.39 mg/mL), heparin (2 µl/mL) and gentamycin (40 µg/mL). Aspirated cumulus oocyte complexes (COC) were washed in with the same medium, and those with dark homogeneous cytoplasm and surrounded by tightly packed cumulus cells were selected and placed in four-well plates containing

500 µl of TCM-199 supplemented with cysteamine (100 µM) and epidermal growth factor (EGF) (10 ng/mL) and matured at 38.5ºC in 5% CO_2.. After 24 h, COC were washed in synthetic oviduct fluid supplemented with essential and non essential amino acids [34] and cumulus cells were removed by gentle pipeting. Oocytes were transferred into four-well plates with 400 µl of fertilization medium (SOF supplemented with 10% of estrous sheep serum, ss, and 40 µg/mL gentamycin) under mineral oil.

Thawed spermatozoa were selected on a Percoll® discontinuous density gradient (45/90) and were capacited in the fertilization medium for 10 min. Sperm was co-incubated with oocytes at a final concentration of 10^6 mL^{-1} at 38.5ºC in 5% CO_2..

Oocytes were evaluated visually with an inverted microscope 40 h later for cleavage (two to eight cells). Then, the oocytes were fixed and stained with Hoechst 33342 to assure fertilization by the presence of 2 or more nuclei. The percentage of cleaved oocytes was called Heterologous in vitro fertility.

2.7. Statistical analysis

Statistical analyses were performed using SPSS for Windows version 15.0 (SPSS Inc, Chicago, III). All variables were transformed using arc sin (percentage) or decimal logarithm. Dates were considered statically significant when $p < 0.05$.

First, an ANCOVA was carried out to know that variables could be indicative of male fertility including five independent variables: insemination technician, insemination date, farm, male as factors and number of inseminated ewes as covariate. Also, all sperm parameters and heterologous in vitro fertility were compared between fertility groups using a GLM-ANOVA. Comparisons were made by Bonferroni. Multiple regression analyses were used to calculate regression equations and to predict the male fertility on the basis of the analyses made in vitro.

3. Results

Male fertility rates ranged from 22 to 62% with a mean value of ~ 42% (Table 1). Differences in fertility rates among males were significant (*P* = 0.003) (Table 2). Fertility only depended on the male. Thus, the insemination technician, the insemination date, the farm or the number of inseminated ewes per male did not influenced the significantly fertility rates (Table 2).

Heterologous in vitro fertility rates ranged from 31 to 59% with a mean value of ~ 47% (Table 1, Fig. 1). There were not significant differences between males for heterologous in vitro fertility (*P* = 0.340). However, the males classified as of high fertility (55.11±3.06%) had significantly higher (*P* = 0.020) in vitro fertility than those of low fertility (38.90.±3.06%).

The values for the sperm parameters after thawing are showed in Table 1. There was a great variability between males with respect to the values of sperm quality. However, the two fertility groups did not show significant differences ($P \geq 0.05$) for the different sperm parameters (Table 3).

To calculate expected fertility, all tested parameters were included in a predictive equation. Stepwise multiple regression analysis was used to select the independent variables that best predicted fertility values. Only the heterologous in vitro fertility showed a relationship with in vivo male fertility ($r^2 = 0.76$; *P* = 0.025). Thus, the males with higher heterologous in vitro fertility were those with higher in vivo fertility (Fig. 1).

4. Discussion

In the present study we assessed the relationship between different sperm parameters and heterologous in vitro fertility with the male fertility. Heterologous in vitro fertilization tests were useful to assess the fertility of thawed ram sperm since a high relationship was showed between in vitro fertility and in vivo male fertility.

Hybrid embryos may occur almost exclusively between closely related species [35]. Slavík et al. [36] produced hybrid zygotes to the 8 cell-stage by in vitro fertilization of in vitro matured bovine oocytes with ram semen and others authors [28-30] obtained hybrid embryos to the same stage using bovine oocytes and spermatozoa of different antelope species.

So far, heterologous IVF tests using zona-free oocytes and ovum have been used to assess the sperm functionality [21-25,37]. Nevertheless, some authors have used zona-free hamster tests to study the relationship with in vivo fertility [38]. However, the zona-free hamster assay evaluates only a part of the process of fertilization, since it does not measure the ability of spermatozoa to bind and penetrate the zona pellucida and later on the cleavage rate [38]. Others authors have used these assays using zona-intact oocytes [28-31]. However, in these works have been not studied the relationships with in vivo fertility.

Alternatively, homologous IVF tests if have been used to assess the fertility of thawed spermatozoa. Thus, Papadopoulos et al. and O’Meara et al. [19,20] studied the relationship between in vitro and in vivo fertility for thawed ram semen using cervical artificial insemination. In these works, the in vitro fertility was related with non-return rate, but not with the pregnancy rate [19]. However, when these authors inseminated oocytes with low sperm concentration (0.0625 x 10^6 spermatozoa/mL), the cleavage rate at 48 hours in an IVF system showed a relationship with the pregnancy rate [20]. Our results do not agree with that study since a relationship between the in vitro fertility and male fertility was showed, despite using a normal sperm concentration (10^6 spermatozoa/mL) to inseminate calf oocytes. These differences could be due to the different insemination technique. Thus, we inseminated intrauterinely by laparoscopy, whereas they carried out cervical inseminations [19,20]. The conditions of spermatozoa after intrauterine insemination may be more similar to those of in vitro fertilization. Cryopreservation damages severely to spermatozoa and when this type of semen is used in cervical insemination the fertility is clearly reduced, partly due to the high structural complexity of the ewe cervix which prevents deep artificial insemination [39-42]. Since the routine IVF procedures are designed to maximize blastocyst yields, in order, to find relationships between in vivo and in vitro fertility using cervical insemination, we would need to subject the spermatozoa used in the vitro fertilization systems to limiting conditions with the purpose of mimicking the conditions of the sperm subpopulation after cervical insemination. Thus, O’Meara et al. [20] using an extreme sperm concentration in an IVF procedure were able to predict the fertility of ram thawed sperm. However, our results agree with those obtained by Smith et al. [43] who found significant correlations ($r = 0.521$; $P < 0.003$) between ram fertility after laparoscopic artificial insemination and cleavage rate obtained in a homologous IVF assay using a high sperm concentration (2×10^6 spermatozoa/mL) to inseminate the oocytes.

Although, the number of spermatozoa used in our intrauterine inseminations trials was high (50×10^6 spermatozoa/per straw), we could clearly find relationships between in vivo and in vitro fertility. It could be due to that the number of spermatozoa decreased after the uterotubal junction, as it has been showed by Suarez et al. [44]. Thus, the number of spermatozoa reaching the oocyte would be much lower, resembling the situation of the in vitro fertilization test.

Our results showed that post-thawing sperm quality varied between males. However, these differences did not discriminated among fertility groups, which showed no differences for the sperm parameters assessed. Moreover, any of the sperm parameters obtained by flow cytometry was of predictive value for fertility, in the stepwise multiple regression analysis. These outcomes agree with those obtained by O'Meara et al. [16] who did not found any relationship between sperm quality after thawing, employing sperm functional tests similar to ours, and comparing with ram fertility after cervical insemination. Furthermore, Hallap et al. [11] did not found any correlation between the mitochondrial status and in vivo fertility, although they showed that the percentage of spermatozoa with unstable membrane, using the triple fluorochrome combination [Merocyanine 540/YO-PRO-1/H33342] was related to the non-return rates [6]. Likewise, others authors have showed relationships between different sperm characteristics and in vivo fertility. Thus, Januskasuskas et al. [13] found a relationship between viability assessed by flow cytometry and non-return rates ($r = 0.68$; $P < 0.01$). Furthermore, García-Macías et al. [15] showed a correlation between DNA integrity evaluated by Sperm-Bos-Halomax and flow cytometry and the fertility. In addition, in the previous reports the fertility was related to routine sperm parameters, such as subjectively assessed motility or the morphology.

We were not able to find correlations between the sperm quality and fertility. Some sperm attributes assessed in this study (i.e: motility, intact acrosomes, membrane stability and DNA stability), might not have a critical role when intrauterine insemination is performed, since the semen is deposited close to the insemination site. Moreover, it is possible that some techniques used to assess the sperm quality were not sufficiently powerful to predict differences in the fertility or that the sperm population after intrauterine insemination were different from the population evaluated in the different tests, which is more heterogeneous. Thus, the methods used in the laboratory to separate spermatozoa (swim-up, Percoll®…) can be useful to obtain a sperm population with attributes of importance to fertilize the oocytes, as it has been suggested by Rodríguez-Martínez [2,3] and for this reason we have noted a relation between in vitro fertility of spermatozoa subject to a separation procedure using Percoll® and in vivo fertility. Nevertheless, we must keep in mind that the number of males used in this study was low. For studying relationship between sperm quality and in vivo fertility, it would be necessary to have a larger male population, with a higher heterogeneity with regard to the fertility.

In conclusion, the results of this study indicate that heterologous in vitro fertilization assays using zona-intact calf oocytes are good procedures to predict the fertility of thawed ram semen after laparoscopic intrauterine insemination, whereas, other tests used to evaluate the sperm quality were not related to fertility.

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Figure 1. Relationship between in vivo male fertility (AI) and heterologous in vitro fertility (IVF)

Table 1. Descriptive statistics (mean, standard deviation (SD), and range) for male fertility, heterologous in vitro fertility and sperm parameters for thawed ram spermatozoa.

Parameters	**Mean (%)**	**SD (%)**	**Range: min-max (%)**
Male fertility	42.33	6.84	22-62
Heterologous in vitro fertility	47.00	4.11	31-59
Motility	50.00	5.32	30-70
NAR	46.83	10.84	13-74
Viability	45.16	7.88	20-66
YO-PRO-1-/PI-	80.82	3.91	65-91
M540-/PI-	65.80	4.74	50-85
Mitotracker+/PI-	67.08	7.86	35-90
PNA+/PI-	97.39	0.95	93-99
DFI	1.03	0.12	0.7-1.6
HDS	6.70	0.54	5-8

Sperm parameters: NAR: spermatozoa with normal acrosomal apical rigde; YO-PRO-1-/PI-: live spermatozoa with stable membrane; M540-/PI-: live spermatozoa with low membrane phospholipid disorder; Mitotracker+/PI-: live spermatozoa with high mitochondrial membrane potential; PNA+/PI-: live spermatozoa with intact acrosome; DFI: % spermatozoa with DFI (DNA fragmentation index) higher that 25%; HDS: % spermatozoa with high DNA stainability (green fluorescence higher than channel 600)

Table 2. GLM of male fertility on number of inseminated ewes, insemination technician, insemination date, farm and male (Model: r^2=0.83; *P* = 0.002).

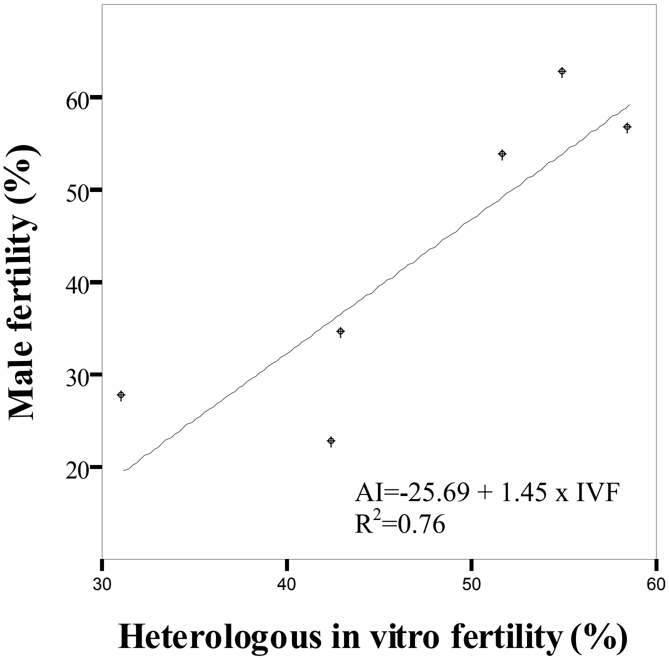
Dependent variable	**Independent variable**	***P***
Male fertility	Number of inseminated ewes	0.584
	Insemination technician	0.156
	Insemination date	0.323
	Farm	0.207
	Male	0.003

Table 3. Values (LSMean ± SEM) for the sperm parameters in the groups of high and low fertility

	Sperm Parameter (%)								
Fertility group	Motility	NAR	Viability	YO-PRO-1-/PI-	M540-/PI-	Mitotracker+/PI-	PNA+/PI-	DFI	HDS
High fertility	50.00±8.41^{a}	46.33±17.14^{a}	45.66±12.46^{a}	82.17±6.12^{a}	60.08±6.31^{a}	60.79±11.61^{a}	97.87±1.47^{a}	1.03±0.20^{a}	6.73±0.86^{a}
Low fertility	50.00±8.41^{a}	47.33±17.14^{a}	44.66±12.46^{a}	79.47±6.12^{a}	71.52±6.31^{a}	73.36±11.61^{a}	96.91±1.47^{a}	1.03±0.20^{a}	6.66±0.86^{a}

Different superscripts within a column differ significantly. Sperm parameters: NAR: spermatozoa with normal acrosomal apical ridge; YO-PRO-1-/PI-: live spermatozoa with stable membrane; M540-/PI-: live spermatozoa with low membrane phospholipid disorder; Mitotracker+/PI-: live spermatozoa with high mitochondrial membrane potential; PNA+/PI-: live spermatozoa with intact acrosome; DFI: % spermatozoa with DFI (DNA fragmentation index) higher that 25%; HDS: % spermatozoa with high DNA stainability (green fluorescence higher than channel 600)

Figure 1. García-Álvarez et al.



Dear Editor of Theriogenology,

Our manuscript “Heterologous in vitro fertilization is a good procedure to assess the fertility of thawed ram spermatozoa” is aimed at improving the current knowledge on methods to assess the fertility. In this study, we have assessed different sperm parameters, including the ability of spermatozoa to fertilize in vitro matured oocytes in a heterologous in vitro fertilization test, and the relationship of these sperm parameters with in vivo fertility. We have found that the heterologous in vitro fertility is correlated with in vivo fertility, but we have not found relationships between sperm quality and in vivo fertility. Our results may have a direct application for the insemination centers, since the fertility of thawed ram spermatozoa can be assessed by heterologous in vitro fertilization test.

All authors are agree to send this manuscript to Theriogenology, are in agreement with its content, and do not have any restriction in order to publish the obtained results.

We hope that this manuscript will be accepted for publication in Theriogenology.