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

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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 h. 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 fertility potential is very important prior to performing artificial insemination or in vitro fertilization (IVF) to assure good 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, DNA integrity etc) all essential to fertility [5–11]. Other methods attempt to mimic in vitro the process of fertilization in vivo.

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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 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. Other authors, have used zona-intact oocytes of domestic animals to evaluate spermatozoa functionality by heterologous IVF tests [26–29]. However, so far nobody has studied the relationship with in vivo fertility using heterologous in vitro fertilization systems employing oocytes with intact zona pellucida. The use of this kind of test to assess the fertility can be an alternative when it is difficult to obtain oocytes from the same species.

In Spain, sexually matured sheep is generally slaughtered at old age. Slaughtering of matured – generally old – sheep is usually focalized in several slaughterhouses. Variability between breeds, regarding corporal and reproductive status, is very high for the slaughtered sheep, and this it could reflect on increased variability of oocytes quality. Oocytes from other species, routinely slaughtered younger and sexually matured is an alternative, since the variability between animals is lower. The objective of this study was to evaluate the performance of a heterologous in vitro fertilization test, which employed zona-intact calf oocytes for assessing the fertility of thawed ram semen. The sperm samples used in this study were selected for heterogeneity regarding its in vivo fertility, after intrauterine laparoscopic insemination.

## 2. Material and methods

### 2.1. Materials

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 six 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 described by Fiser et al. [30]. The ejaculates were diluted to a final concentration of $200 \times 10^6$ spermatozoa/mL. Diluent 1 was added 3:2 to semen and slowly cooled from 30 to 5 °C in 2 h. Then, the samples were further diluted (3:1) with the diluent 2 at this temperature and held for equilibration at 5 °C for 2 h (total refrigeration time at 5 °C was 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 20 °C/min to −100 °C, and at 10 °C/min from −100 °C to −140 °C and then plunged into liquid nitrogen.

### 2.4. Semen evaluation

The straws were thawed for 20 s at 37 °C and aliquots were used to assess sperm quality. Percentage