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Short communication. *In vitro* oocyte maturation and fertilization rates in the Spanish Lidia bovine breed

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Abstract

The Lidia bovine breed is the most successful cattle breed on the Iberian Peninsula, also considered a hallmark of Spanish tradition and image around the world. The aims of the study were to characterize the oocyte recovery rates and to evaluate the effect of two standard in vitro maturation protocols on oocyte maturation (cumulus expansion and nuclear maturation) and fertilization rates after *in vitro* fertilization in this breed. For this purpose, 261 ovaries from Lidia cows were processed obtaining 1,125 viable cumulus oocyte complexes (COCs). The oocyte recovery rate obtained (4.31 viable COCs per ovary) was lower than those described previously in other studied breeds. Maturation rates were evaluated in two different oocyte maturation media with (M1) and without (M2) hormonal supplementation. The percentage of COCs with expanded cumulus cells was significantly lower in M1 (74.35%) compared with M2 (82.25%). Metaphase II (MII) rates (67.75% in M1 and 73.18% in M2) were similar to previous studies in different cattle populations. M2 significantly improved the percentage of COCs with their cumulus cells expanded ($p < 0.01$) and nuclear maturation rates ($p < 0.05$), but it did not affect the fertilization percentages obtained in this experiment. In conclusion, our study suggests that oocytes of the Lidia cattle breed can be obtained, matured and fertilized following standard protocols previously used in other cattle breeds.

Additional key words: breed effect; bullfight; *in vitro* fertilization; Lidia cattle breed.

The Lidia bovine breed, also known as fighting the bull, is the most successful cattle bred on the Iberian peninsula (Cañón *et al.*, 2008). It is also considered a hallmark of Spanish tradition and image around the world (Jiménez *et al.*, 2007). This animal population is reared following traditional procedures, characterized by a long history of isolation from the rest of Spanish cattle breeds. For hundreds of years, this breed has only been selected for their temperament and aggressiveness without considering other phenotypic characteristics. This fact has caused a loss in genetic variability and an increase in inbreeding depression due to this phenotypic selection performed over the years (Cañón *et al.*, 2008). The same authors have determined that this mainly occurs on isolated lineages, where only a few superior animals are the highest contributors to the gene pool (Cortés *et al.*, 2011). Bulls of this breed are isolated from the females to increase aggressiveness and are normally killed during a bullfight before being able to produce any offspring (Jiménez *et al.*, 2007). Only a very limited number of bulls are pardoned and subsequently used in for reproduction/breeding according to their performance during the Lidia. For this reason, the use of assisted reproductive techniques (ARTs) could be considered as an important tool to obtain more offspring from certain maternal lineages or from a particular bull killed during the Lidia (Katska-Ksiazkiewicz *et al.*, 2006). As part of the *in vitro* procedures, artificial oocyte maturation has a significant role in *in vitro* technologies

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Abbreviations used: ART (assisted reproductive techniques); COC (cumullus oophorus complexes); FSH (follicle stimulating hormone); IVF (*in vitro* fertilization); LH (luteinizing hormone); M1 (medium 1); M2 (medium 2); TALP (Tyrode's Albumin Lactate Piruvate medium).

in cattle (Russell *et al.*, 2006). Previous reports suggested that animal breed (Kafi *et al.*, 2002; Nicodemo *et al.*, 2010; Pauciullo *et al.*, 2012), maturation media (Choi *et al.*, 2001) and protocols (Hawk & Wall, 1994) had an influence in the developmental capacity of the oocytes matured *in vitro*. Furthermore, it has been demonstrated that oocyte maturation affects the *in vitro* fertilization (IVF) outcomes in several species and breeds (Palma & Sinowatz, 2004). To our knowledge, no previous studies have been performed to evaluate the developmental capacity of *in vitro* matured oocytes derived from Lidia cows. This is mainly due to the difficulty in obtaining a high number of ovaries from this breed to carry out these studies. Therefore, the aims of the study were to: 1) characterize the oocyte recovery rates from Lidia cow ovaries; and 2) evaluate the effect of two standard oocyte maturation protocols on *in vitro* maturation and fertilization rates of oocytes derived from Lidia cows.

For this purpose, 261 ovaries from 3 to 8 years-old Lidia cows belonging to three different lineages were collected in two replicates at the local slaughterhouse (Cooperativa del Valle de Los Pedroches, Pozoblanco, Spain) and transported to the laboratory within two hours in a 0.9% NaCl aqueous solution at 30-37°C. Thereafter, the ovaries were washed in a warm physiological saline solution supplemented with kanamycin (25 mg mL^{-1}) until all remaining traces of blood were removed. Cumulus oocyte complexes (COCs) were aspirated from follicles between 4 and 8 mm in diameter with a 18G needle. After sedimentation, oocytes were poured into Petri dishes and selected under a stereomicroscope with a warm plate. Recovered oocytes were classified according to their morphology (Hazeleger *et al.*, 1995). Only those with an homogeneous cytoplasm and at least three layers of cumulus cells were used in this study. A series of standard Tyrode's albumin lactate pyruvate (TALP) media were used throughout the entire experiment. COCs were washed twice in warm (38.5°C) TALP media supplemented with Hepes salts (H-TALP, according to Parrish *et al.* [1988]) and matured in groups of 100 on four well Nunclon™ dishes for 24 h, at 38.5°C in a 5% CO_2 humid atmosphere. Oocyte recovery rates were recorded for further analysis. To evaluate the effect of the hormonal supplementation on maturation and fertilization rates, oocytes were cultured in two different maturation media: Medium 1 (M1) was TCM199 modified bicarbonate-buffered (Sigma Aldrich Spain), supplemented with 10% of fetal calf serum, 0.4 mmol L^{-1} glutamine; 0.2 mmol L^{-1} sodium pyruvate and 50 mg mL^{-1} gentamicin without any hormonal supplementation; and Medium 2 (M2) was the same medium M1 supplemented with 25 µg mL^{-1} FSH, 6.25 µg mL^{-1} LH and 2 µg mL^{-1} estradiol. After maturation, percentages of oocytes with expanded cumulus cells were determined in each group following our laboratory criteria (Ocana Quero *et al.*, 1994). A total of 727 cultured oocytes (369 in M1 and 358 in M2) were denuded by vortexing, fixed and stained with standard Hoechst 33342 protocol (Flaherty *et al.*, 1995) to evaluate their nuclear maturation status.

The remaining oocytes were fertilized with a pool of frozen semen from three different Retinta bulls of proven fertility. For this purpose, thawed spermatozoa were previously selected through a discontinuous Percoll gradient (45 and 90% (v/v); Pharmacia) according to Parrish *et al.* (1995) and adjusted to a final concentration of $1 \cdot 10^6$ sperm mL^{-1} in equilibrated IVF-TALP medium (Parrish *et al.*, 1988) supplemented with 6 mg mL^{-1} BSA and 20 mg mL^{-1} heparin. Oocytes were washed twice and co-incubated in groups of 60 with sperm at 38.5°C in 5% CO_2 on equilibrated IVF-TALP medium. After 20 h presumptive zygotes were denuded by vortexing and stained as previously described by Flaherty *et al.* (1995), to determinate pronuclei formation rates.

Statistical analysis used was a Z-score test (z) with two tails (Demyda Peyrás *et al.*, 2012). The intragroup differences for total viable oocytes, maturation rates and fertilization rates between replicates were achieved using Fisher exact test, using Minitab software Version 15.1 (Minitab, Inc, College State, Pennsylvania).

Oocyte recovery rates observed in our study are shown in Table 1. A total of 1,356 oocytes of different categories were recovered from 261 ovaries; with an average of 5.20 oocytes per ovary punctured. After morphological selection, 1,125 oocytes were used throughout this experiment, resulting in 4.31 viable oocytes suitable for maturation per ovary punctured.

Table 1. Oocyte collection rates in Lidia cattle breed

Total animals slaughtered	153
Total ovaries collected	261
Total oocytes collected	1,356
Total oocytes collected per ovary[1]	5.19
Total viable oocytes	1,125
Total viable oocytes per ovary[1]	4.31

[1] Statistical differences between the two replicates were assessed using Fisher exact test. No differences were observed between replicates ($p > 0.05$).

These results were lower than previous reports by several authors in other cattle breeds: 4.60 in Podolian and 5.83 in Maremmana (Pauciullo *et al.*, 2012); 5.33 in Czech Simmental and 6.50 in dairy Holstein (Machatkova *et al.*, 2008); 9.5 in Belgium Holstein and 11.1 in Belgium Blue (Van Soom *et al.*, 1993). We only found a lower rate reported previously in Czech beef breeds (Machatkova *et al.*, 2008) and in zebu Nelore cows (Dode *et al.*, 2001). It is noteworthy that the low number of replicates could influence the results obtained. This is due to the great difficulty in obtaining enough ovaries derived from Lidia cows to perform an experiment. However, there were not statistical differences between replicates (Fisher exact test, $p > 0.05$). In this sense, the low number of oocytes obtained per ovary is consistent with the moderate fertility previously observed in this breed (Jiménez *et al.*, 2007). Recently, Evans *et al.* (2010) suggested that a lower number of follicles are reflective of the environment during fetal development. It was observed in beef heifers restricted to 0.6 of their maintenance energy requirement, from shortly before conception to the end of the first trimester of pregnancy. Conversely, Lidia breed cows are reared in extensive production systems in the Spanish "*dehesas*" with no nutritional imbalances throughout the whole year (Jiménez *et al.*, 2007). Several authors identify an antagonistic association between high milk production and *in vivo* (Olsen *et al.*, 2011) and *in vitro* (Khatib *et al.*, 2010) fertility traits. More recently, other authors (Peñagaricano & Khatib, 2012) were more specific, suggesting the same association between milk protein yield and *in vitro* fertility. But Lidia breed is characterized by a medium milk production, with no genetic selection performed in this sense. One possible explanation is that oocyte recovery rates can be influenced by their selection process for the last five centuries, focused mainly on their aggressiveness (Silva *et al.*, 2006). In this sense, a previous study showed that hostile animals have lower reproductive performance (Phocas *et al.*, 2006). Moreover, it has been suggested that the main cause of this lower reproductive performance is the greater basal concentrations of glucocorticoids and catecholamines shown in more temperamental cattle, leading to a "stress like" situation (Burdick *et al.*, 2011). Likewise, high genetic selection pressure only for a few specific production traits might have the same deleterious effect on reproductive traits, as it has been demonstrated in high-producing dairy cows (Walsh *et al.*, 2011). Oppositely, effectiveness of selection for reproductive traits has been widely demonstrated (Álvarez *et al.*, 2005; Cushman *et al.*, 2005). Moreover, recent work has established that the number of COC's obtained from individual cows in an IVP program can be increased by genetic selection (Merton *et al.*, 2009). Finally, another possibility is that the low number of COCs obtained could be derived directly from the animals breed. Recent work reports that the outcome of IVP bovine embryos depends on the breed of the donor ovary (Abraham *et al.*, 2012). The same differences have also been observed in a native Hungarian pig breed (Egerszegi *et al.*, 2001).

Therefore, morphological quality of the oocytes collected in this study can be also influenced by genetic and breed factors (Domínguez, 1995). However, the percentage of viable oocytes obtained in our study was similar to those observed in other breeds (Fischer *et al.*, 2000; Ribeiro *et al.*, 2011).

Oocyte *in vitro* maturation rates achieved in our study using two different maturation media are shown in Table 2.

Table 2. Maturation and fertilization rates of oocytes derived from Lidia breed cows after *in vitro* maturation in two different maturation media.

	Medium 1		Medium 2	
	n	%	n	%
Total oocytes	573	100	552	100
Oocytes with expanded cumulus[1]	426	74.35%^{A}	454	82.25%^{B}
Oocytes with nuclear maturation[1]	250/369	67.75%^{a}	262/358	73.18%^{b}
Fertilization rates	98/204	48.03%	105/194	54.12%

[1] On each row, values followed by different letters (^{a,b}) differ statistically ($p < 0.05$), and values followed by different capital letters (^{A,B}) highly differ statistically ($p < 0.01$) (two tails Z test for proportions). Statistical differences between the two replicates were assessed using Fisher exact test. No differences were observed between replicates in cumulus expansion, nuclear maturation or fertilization rates ($p > 0.05$).

Highly significant statistical differences ($p < 0.01$) were observed in the percentage of COCs with expanded cumulus after maturation between M1 (74.35%) and M2 (82.25%). Cumulus cell expansion was higher when the maturation medium was supplemented with FSH, LH and estradiol, as previously demonstrated (Younis *et al.*, 1989; Rose & Bavister, 1992). Similar results were obtained in nuclear maturation rates: 67.75% in M1 and 73.18% in M2; however, the amplitude differences were statistically lower than those observed previously in cumulus cell expansion rates ($p < 0.05$). It has been shown that oocyte donor breed affects its developmental competence in other species (Ptak *et al.*, 2003; Rátky *et al.*, 2005). However, our results were within the average rates previously reported in other cattle breeds (Camargo *et al.*, 1997; Kafi *et al.*, 2002; Wang *et al.*, 2007; McLaughlin & Telfer, 2010; Nicodemo *et al.*, 2010). It may suggest that donor breed may not produce an important influence in the *in vitro* oocyte nuclear maturation in some bovine populations.

Finally, fertilization rates obtained are in agreement with previous observations by our group (Ocana Quero *et al.*, 1995) and with those of other authors (Sumantri *et al.*, 1997). However, higher pronucleus formation rates were obtained in previous experiments (Kafi *et al.*, 2002). It is noteworthy that fertilization rates were similar in both maturation media, supplemented with hormones or not. This finding is in accordance with previous results obtained by other authors (Sartori *et al.*, 2010). Until now fertilization failures have not been related with oocyte sources in other species or breeds (Squires, 2005; England & Russo, 2006; Burns *et al.*, 2010). However, some recent studies have suggested that the existence of specific genes activated during oocyte maturation play a major role in the fertilization process (Zheng & Dean, 2007; Meczekalski, 2009). On the other hand, male influence has also been suggested as the primary cause of failed fertilization in livestock (Bar-Anan *et al.*, 1980), due to a lack of ability of sperm to penetrate the oocyte (Sartori *et al.*, 2010). Despite the controversy found in literature, the acceptable fertilization rates observed in our study do not appear to be an important issue during the *in vitro* fertilization process of Lidia breed oocytes.

In conclusion, our study suggests that oocytes belonging to Lidia cattle breed can be obtained, matured and fertilized following standard protocols previously described in other cattle populations. However, the total number of COCs and viable oocytes obtained from ovaries derived from Lidia cows are lower than those obtained in other breeds previously studied. Finally, the use of appropriate hormone supplementation in the maturation media enhances maturation rates, without affecting the fertilization process of these oocytes. Further studies are necessary to optimize the overall success of IVF protocols in this particular breed.

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