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Effect of storage temperature during transport of ovaries on *in vitro* embryo production in Iberian red deer (*Cervus elaphus hispanicus*)

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Abstract

The aim of this work was to study the effect of storage temperature during the transport of ovaries on cleavage and blastocyst rates in Iberian red deer, because wild populations of this subspecies are usually far from laboratories. A total of 472 ovaries from 236 Iberian hinds were recovered and maintained in saline solution at 5–8 °C or 20–25 °C for 12 h. After storage, aspirated oocytes were matured with FSH/LH or EGF and the developed embryos were cultured with oviduct epithelial cells monolayer (OCM). A higher (P = 0.009) cleavage rate was obtained when the ovaries were stored at 5–8 °C. However, there were no differences between both storage temperatures in relation to the percentage of blastocysts obtained. Considering the management and production systems of Iberian red deer, this study provides important information about the ovary storage temperature during transport with the purpose of assuring an optimal *in vitro* embryo production.

Keywords: Iberian red deer; Ovary storage; *In vitro* fertilization

1. Introduction

The use of assisted reproduction technologies (ART) ensures the continuity and the genetic variability of the breeds, populations and species [1–3]. The collection of sperm samples, oocytes and embryos is an aspect of great importance in the development of these technologies. However, for wild species there are many drawbacks in relation to sample collection.

The Iberian red deer (*Cervus elaphus hispanicus*) is a wild subspecies of red deer that inhabit Spain and Portugal. The main appeal of this subspecies is its trophy, so the embryo production focused on achieving individuals with higher quality of trophy from hunted

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animals is an important goal. Nowadays, there is a remarkable interest in the use of ART for the management of Iberian red deer populations. Most of the technologies carried out in this specie have been related with the collection, storage and subsequent application of spermatozoa by means of artificial insemination [4–16]. However, *in vitro* embryos have not yet been produced so far. That could be due to the difficulty in obtaining the Iberian hind oocytes. For the *in vivo* oocyte collection, specific installations in farms are needed and in Spain there are no many private properties with these characteristics since the production of this cervid subspecies is mainly extensive. In such cases, oocyte collection from ovaries of hunted hinds offers an opportunity to obtain valuable genotypes. It is important to mention that many variables may adversely affect oocyte quality and subsequent embryo development such as temperature and time of ovary storage.

Under field conditions it is not always possible to collect and immediately transport the ovaries from Iberian hinds to the laboratory due to a lack of technicians and equipment. In addition, most Iberian red deer wild populations are far from laboratories. For these reasons, we have decided to study in depth some of the aspects that could affect the oocyte quality during the transportation, such as the ovary storage temperature.

It is known that the metabolic activities of cells are slowed down or completely arrested at low temperatures, whereas cellular autolysis could occur in ovaries during the long period of transportation at high temperatures [3]. Many authors have reported, in several species, that ovary storage at 38 °C for several hours decreased the rate of blastocyst formation [17,18]. Ovary storage at low temperatures in bovine and feline species did not show any effect on oocyte maturation or on the potential of oocytes to develop into blasctocysts [19,20]. However, oocytes from equine, porcine and canine were more sensitive at low temperatures [21–23].

With this background, the objective of this study was to test two ovary storage temperatures (5–8 °C *vs.* 20–25 °C) easily to obtain and maintain for 12 h in order to identify the field conditions that better preserve oocyte development competence and subsequent embryo development.

2. Materials and methods

All chemicals and media were from Sigma (Madrid, Spain). Biladyl® and Triladyl® were from Minitüb (Germany). Estrus sheep serum (ESS) was collected from sheep in oestrus and heat inactivated at 56 °C for 45 min.

2.1. Oocyte collection and maturation

A total of 472 ovaries were collected from 236 Iberian hinds selectively hunted and eviscerated in an authorized place in their natural habitat (game reserve) at the beginning of the hunting season (September–October). Collection of ovaries was carried out in two successive days. Ovaries were placed into glass containers with saline solution and transported to the laboratory at different temperatures according to the experimental design within 12 h after being removed. From each hind, one ovary was stored at 5–8 °C and the other one at room temperature (20–25 °C). Containers were transported into expanded polystyrene boxes with cold accumulators for the 5–8 °C treatment. In both treatments, immature oocytes were aspirated from 2 to 6 mm follicles using a 19 gauge needle connected to a 5 mL syringe containing 0.5 mL of TCM 199 supplemented with Hepes (2.38 mg/mL), heparin and gentamycin (40 μg/mL). Aspirated cumulus oocytes complexes (COCs) were washed in TCM 199-gentamycin, and those with dark homogeneous cytoplasm and surrounded by tightly packed cumulus cells were selected and randomly placed in four-well plates containing 500 μL of TCM 199 supplemented with cysteamine (100 μM), 10% fetal calf serum (FCS) and FSH/LH (10 μg/mL) or EGF (10 ng/mL), and matured at 38.5 °C under a humidified atmosphere of 5% CO_2 in air for 24 h.

2.2. In vitro fertilization

After 24 h, COCs were washed in order to remove cumulus cells by gentle pipeting. Then the oocytes were transferred into four-well plates containing 450 μL of synthetic oviduct fluid as described by Berg and Asher [24] for red deer (IVF-SOF) with 20% ESS.

For IVF, epididymal spermatozoa from one male frozen with Triladyl® as described by Soler et al [4] were used. Thawed epididymal spermatozoa were selected by centrifugation on a Percoll® gradient (45/90%). The sperm pellet was diluted to 10×10^6 spermatozoa/mL in IVF-SOF with 20% ESS and incubated at 38.5 °C in 5% CO_2 for 10 min. Spermatozoa were co-incubated with the oocytes at a final concentration of 10^6 spermatozoa/mL for 18 h at 38.5 °C in 5% CO_2, 5% O_2 and 90% N_2 humidified atmosphere.

2.3. In vitro culture

Presumptive zygotes were washed with SOF-BSA [25] and cultured with SOF-BSA supplemented with 10% FCS in the presence of cow oviduct epithelial cells

monolayer (OCM) at 38.5 °C under a humidified atmosphere of 5% CO_2 in air for 8 days. Half of the culture medium (250μL) was renewed (fresh medium) every 48 h during *in vitro* development.

Cleavage rate was assessed at 24 and 48 hours post insemination (hpi) and the embryo rate reaching blastocyst stage was assessed at 8 days post insemination (dpi) by phase-contrast optic. At this time, blastocysts were stained with Hoechst 33342 (Fig. 1).

2.4. Preparation of oviduct epithelial cells monolayer

Ipsilateral cow oviduct to the ovary bearing a *corpus haemorragicum* was collected at local slaughterhouse one week before ovary collection and dissected free from surrounding tissues. After several washing of the oviduct, the mucosa was expelled by gentle squeezing using a sterile glass slide along the outside of the oviduct, from the uterotubal junction to the infundibulum on a Petri dish. Resulting epithelium fragments were washed three times by consecutive suspensions and centrifugations (500 × g for 5 min) in TCM-199 medium and cultured in four-well plates containing 500 μL of TCM-199 supplemented with 10% FCS and 40 μg/mL gentamycin. Cells were cultured at 38.5 °C under a humidified atmosphere of 5% CO_2 in air. Fragments spontaneously formed vesicles after

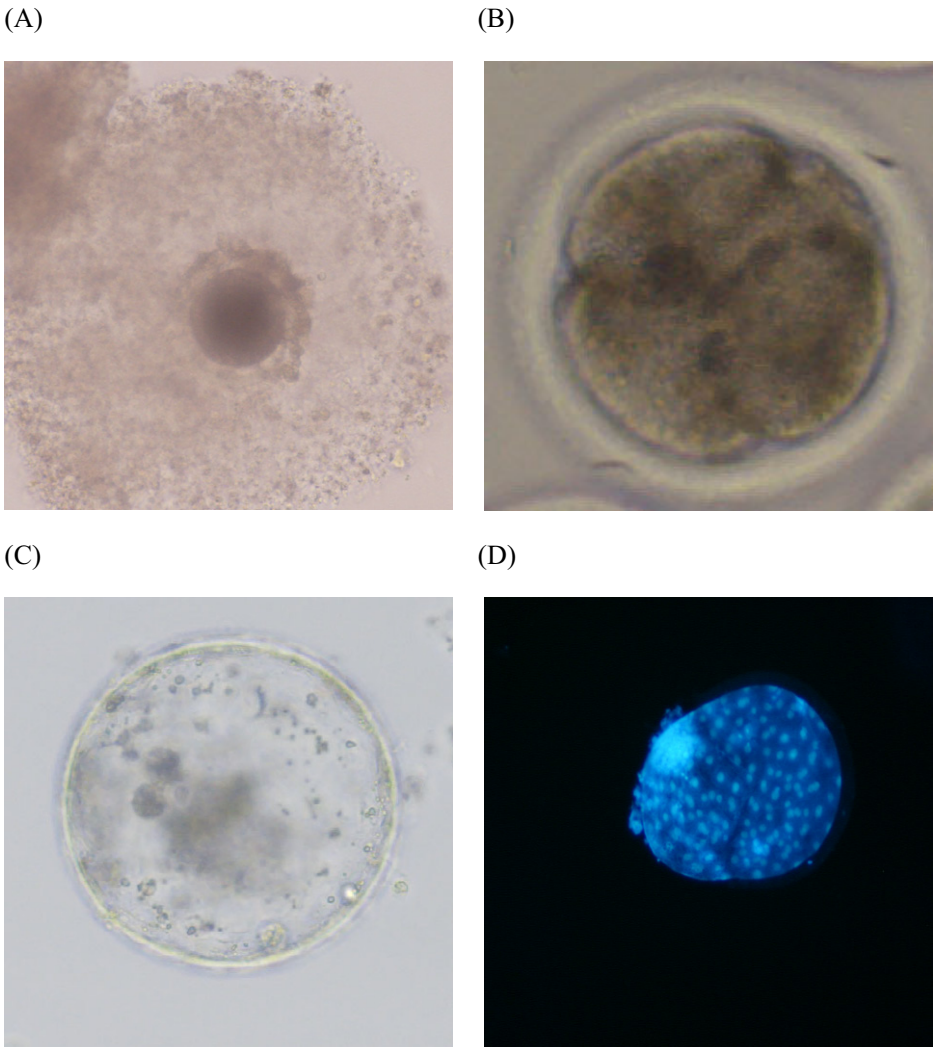

Fig. 1. Oocytes aspirated from post-mortem ovaries and embryos obtained after *in vitro* maturation, fertilization and culture in Iberian red deer. (A) Oocytes with cumulus cells expanded after *in vitro* maturation; (B and C) 4-cell embryo and blastocyst after *in vitro* fertilization and culture examined by phase-contrast optics after 48 hpi and 8 dpi, respectively; (D) Blastocyst stage embryo after staining with Hoechst 33342 and examined under fluorescence microscope after 8 dpi.

2–3 days of culture. These vesicles formed monolayers within 7 days after initiation of the culture. Medium was renewed every 48 h and replaced 24 h before culture of zygotes by SOF-BSA supplemented with 10% FCS.

2.5. In vitro fertilization in ovine

Due to the practical limitations cited above in relation to the location of deer game reserves, ovary storage at 30–35 °C for a short time was not a possible alternative for study. For this reason, an additional trial in a domestic species (ovine), in which it is accepted that the optimal temperature of ovary storage is 30–35 °C [26–28], was carried out as a way to provide extra information about how oocyte competence and embryo development is affected by storage methods. Thus, we decided to include an additional experiment in which the two storage conditions considered in the deer's work (5–8 °C and 20–25 °C for 12 h) were compared with a storage treatment at 30–35 °C for 4 h (Control).This temperature could be maintained with a portable incubator.

From each ewe, one ovary was fixed to Control treatment and the other one simultaneously to 20–25 °C or 5–8 °C treatments. Ovaries of all treatments (Control, 22–25 °C and 5–8 °C) were sliced using a micro-blade and the follicle content released in medium TCM-199 supplemented with Hepes (2.38 mg/mL), heparin and gentamycin (40 μg/mL). Aspirated cumulus oocytes complexes (COCs) were handled in the same way as the deer assay and matured in 500 μL of TCM 199 supplemented with cysteamine (100 μM), 10% fetal calf serum (FCS) and FSH/LH (10 μg/mL) at 38.5 °C under a humidified atmosphere of 5% CO_2 in air for 24 h. After 24 h, COCs were washed in order to remove cumulus cells by gentle pipeting and transferred into four-well plates containing 450 μL of synthetic oviduct fluid (SOF) as described by Cognie et al [29] with 10% ESS.

Ram sperm samples used in the *in vitro* fertilization were frozen with Biladyl® as described by García-Álvarez et al [30] and selected as it was above described. Spermatozoa were co-incubated with the oocytes at a final concentration of 10^6 spermatozoa/mL for 18 h at 38.5 °C in 5% CO_2, 5% O_2 and 90% N_2 humidified atmosphere.

Presumptive zygotes were cultured in SOF enriched with amino acids and BSA [31] for 8 days at 38.5 °C in 5% CO_2, 5% O_2 and 90% N_2 humidified atmosphere. Cleavage rate was assessed at 40–44 hpi and the embryo rate reaching blastocyst stage was assessed at 8 dpi by phase-contrast optic. This trial was replicated three times.

We also carried out another assay in ovine to assure that the resumption meiotic, as a result of loss of follicular regulation, had not been initiated. Ovaries were stored at 30 °C for 4 h (Control), or at 20–22 °C and 5–8 °C for 12 h. After that, oocytes were recovered in TCM-199 supplemented with Hepes (2.38 mg/mL), heparin and gentamycin (40 μg/mL) and washed in TCM-119-gentamycin. Finally, the oocytes were denuded of cumulus cells by pipetting in D-PBS containing 0.1% (w/v) hyaluronidase. Oocytes denuded (20–25 per treatment) were stained with Hoechst 33342 and the chromatin status in the germinal vesicle (GV) or germinal vesicle breakdown (GVBD) was recorded.

2.6. Statistical analysis

Statistical analyses were carried out using SPSS®, version 15 (SPSS Inc., Chicago, IL, USA). A mixed effects model including the storage temperature (5–8 °C *vs.* 20–25 °C) and maturation media (FSH/LH vs EGF) as fixed factors and the day of ovary collection as random effect was carried out to study the consequences of storage temperature on cleavage rate at 24 and 48 hpi and on the percentage of blastocysts obtained at 8 dpi over total and cleaved oocytes in Iberian red deer. Since no significant differences ($P < 0.05$) were found between both maturation media, results for this factor were not showed.

For the additional experiment using ovine data, a mixed effects model including the storage temperature (Control, 5–8 °C and 20–25 °C) as fixed factor and the day of ovary collection as random effect was also carried out to test the effects of storage temperature on cleavage rate at 40–44 hpi and on the percentage of blastocysts obtained at 8 dpi over total and cleaved oocytes.

Comparison of means was carried out using the Bonferroni test. Differences were considered statistically significant at $P < 0.05$. The data are expressed as means ± SEM.

3. Results

As shown in Table 1, the storage temperature only affected the cleavage rate at 48 hpi with significantly higher results when the hind ovaries were stored at 5–8 °C in relation to 20–25 °C (78.19% *vs.* 64.90%, $P = 0.009$). However, the percentage of blastocysts ob-

Table 1
Effect of ovary storage temperature on cleavage and blastocyst rate in Iberian red deer

Storage temperature	No. Cleaved embryo at 24 hpi (%)	No. Cleaved embryo at 48 hpi (%)	No. Blastocyst at 8 dpi (%)	
			Total	Cleaved
20–25 °C	23 (14.23 ± 2.60)	50 (64.90 ± 2.96)	7 (9.61 ± 2.36)	(13.88 ± 3.70)
5–8 °C	20 (20.80 ± 5.08)	38 (78.19 ± 1.85)	8 (17.71 ± 6.64)	(20.61 ± 8.01)
P value	0.294	0.009	0.295	0.475

Data are Mean ± SEM. Significant differences were considered within the same column when P value was < 0.05.

tained was not significantly different between both storage temperatures (Table 1).

In the experiment with ovine, the cleavage rate for the oocytes collected from ovaries stored at 5–8 °C was significantly lower (23%) in relation to Control and 20–25 °C treatments (47% and 43%, respectively; Table 2). However, there were no significant differences between treatments in relation to the percentage of blastocysts obtained (Table 2). On the other hand, chromatin of all the stained oocytes from Control and 20–25 °C treatments were in GV stage but, only a 91% was in this stage for the ovaries stored at 5–8 °C.

4. Discussion

The production of Iberian red deer under extensive conditions together with long distances to laboratories involves problems in relation to the biological sample collection (testis and ovaries) and transport. Studies aimed to show the viability of different cells depending on storage conditions, just as storage temperature, are needed in order to establish which conditions are optimal in this cervid subspecies. Several authors have carried out works about the influence of testis storage time at 5 °C on the sperm quality in Iberian red deer [32–34], but no studies on the effects of ovary storage conditions on *in vitro* embryo production were found on the literature. Thus, the evaluation of different temperatures during the ovary storage, easily to obtain under the particular conditions of the Iberian red deer production system, will allow us to identify which of them preserve better the quality of the biological samples (oocytes).

Red deer embryos have been produced *in vitro* with variable results between different authors getting blastocyst rates around a 15% according to experimental design [24,35–39]. In those studies, ovaries collected postmortem were stored at temperatures between 30 °C and 35 °C and they were transported to the laboratory in 3 hours after slaughter, approximately. In this work, hind ovaries were stored at two temperatures easy to obtain (room temperature: 20–25 °C and a refrigeration temperature: 5–8 °C). We could not include an ovary storage treatment at 30–35 °C for short time since the distance from deer wild populations to laboratory was long (it takes around 12 h). In addition, a temperature around 30–35 °C could be difficult to maintain under field conditions.

In our study, it was possible to obtain embryos from stored ovary's oocytes for 12 h at 5–8 °C, with even higher results of cleavage rate at 48 h for this storage temperature in comparison with room temperature (20–25 °C), although blastocyst rate was similar in both treatments. According to our results, Matsusthita et al [19] reported that ovary storage temperature (10 °C *vs.* 30 °C) had no effect on oocyte developmental competence in bovine. Moreover, ovary transport at low temperatures had a positive effect on the oocyte maturation rates in feline [20] and canine [40] species. In opposite, for other species, ovary transport at high temperatures was more advantageous with higher matured oocyte and embryo rates, such as equine [21] and porcine [22] species.

Because we could not include an ovary storage treatment at 30–35 °C for a short time in red deer, we carried out an additional trial in a domestic species

Table 2
Effect of ovary storage temperature on cleavage and blastocyst rate in ovine

Storage temperature	No. Cleaved embryo at 40–44 hpi (%)	No. Blastocyst at 8 dpi (%)	
		Total	Cleaved
Control	67 (47.70 ± 0.72)[a]	23 (19.00 ± 7.21)[a]	(38.33 ± 13.96)[a]
20–25 °C	55 (43.50 ± 10.25)[a]	16 (16.00 ± 2.08)[a]	(33.67 ± 0.66)[a]
5–8 °C	31 (23.17 ± 3.49)[b]	8 (5.17 ± 3.03)[a]	(17.33 ± 10.10)[a]

Data are Mean ± SEM. Different letters within the same column are statistically different ($P < 0.05$).

(ovine). This trial was done with the purpose of providing additional information about how competency of oocytes is affected by the storage temperature. We found significant differences between treatments for the cleavage rate, which was lower when sheep ovaries were stored at low temperatures (5–8 °C), than for the storage at 20–25 °C and for the Control treatment. In relation to the blastocyst rate, no significant differences were observed among treatments. It is possible that the oocytes from ovine species are more sensitive against low temperatures than those from the cervid. Our data also showed that both cleavage and blastocyst rates were similar for the 20–25 °C and the Control treatment. It is possible that the same event happens in the Iberian red deer when the ovaries are stored at 30–35 °C for 4 h or 20–25 °C for 12 h.

It is known that when the oocytes are maintained in meiotic arrest at the GV stage for a time, they have the opportunity to acquire greater developmental competence *in vitro* [41]. As was pointed out by Blondin et al [42], one way to achieve greater developmental competence was to maintain the oocytes within the follicle for a time after slaughter and before fertilization. In our experiment with ewes, we found that when ovaries were stored at 30–35 °C for 4 h or at 20–22 °C for 12 h, the meiotic resumption was not initiated in the oocytes, due to the fact that the chromatin of all of them was at GV stage. However, for the cold storage treatment (5–8 °C for 12 h) only 91% of oocytes were in the GV stage. Moreover, for this treatment both cleavage and blastocyst rates were lower than for the other treatments, suggesting that in ovine species a prolonged exposure of ovaries to cold temperatures before oocyte collection could have a negative effect on the developmental competence of embryos. These results are in accordance with those by Moor and Crosby [43] who found that, in ovine, the exposure of oocytes to low temperatures induces chromosomal abnormalities and, as a consequence, a decrease in the development to expanded blastocyst.

In conclusion, our results showed that for *in vitro* embryo production in Iberian red deer, when the ovary storage time is long (about 12 h), no differences were found for the percentage of blastocyst obtained when the ovaries were transported at low temperatures (5–8 °C) or at room temperature (20–25 °C). Taking into account our results, we would choose 20–25 °C as an optimal ovary storage temperature during a long period of storage.

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