Theriogenology Wild 4 (2024) 100075

Contents lists available at ScienceDirect

Theriogenology Wild

journal homepage: www.journals.elsevier.com/theriogenology-wild

Optimized heterologous in vitro fertilization with Iberian ibex sperm and domestic goat oocytes

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ARTICLE INFO

Keywords:
Heterologous IVF
Ibex
Goat
Embryo quality
Lineages

ABSTRACT

Assisted reproductive technologies are key to maintain genetic stocks of endangered wild species, such as the Iberian ibex (*Capra pyrenaica*). Due to the low availability of ibex ovaries, heterologous in vitro fertilization (IVF) with domestic goat (*Capra hircus*) oocytes constitutes an excellent alternative to determine the fertilization capacity of ibex sperm doses. The aim of this study was to optimize heterologous ibex-goat IVF procedures by testing two different IVF media (TALP and SOF) and to determine whether estrous sheep serum (ESS) is required for fertilization. We found that TALP medium provides optimal conditions to conduct heterologous ibex-goat IVF, yielding blastocyst rates above 50%, and that supplementation with ESS is not required. No differences were found in embryo quality between embryos fertilized in TALP, SOF alone, or SOF supplemented with 2 or 20% ESS, based on the analysis of cell lineages development at day (D) 8 and D10 of development. Optimized heterologous ibex-goat IVF, together with embryo quality assessment through lineages development analysis, constitute an excellent system to assess fertilization capacity of ibex sperm doses, and opens the possibility of performing homologous in vitro embryo production in this species.

1. Introduction

Assisted reproductive technologies (ARTs) are very valuable tools to preserve genetic diversity and to protect wild species. The Iberian ibex (*Capra pyrenaica*) is a wild caprine species comprising four subspecies, from which two are already extinct and two are included in the IUCN red list of threatened species [1]. Although sperm collection can be complex in this species, viable epididymal sperm has been recovered *post mortem* and cryopreserved [2–6], making Iberian ibex an excellent model to establish ARTs that can be applied to other wild ungulate species. *In vitro* fertilization constitutes an optimal test to check the fertilization ability of cryopreserved epididymal sperm, but access to ibex oocytes is very limited [7].

Heterologous IVF performed on oocytes obtained from a phylogenetically close domestic species can be used to circumvent the limited access to oocytes from wild animals. Heterologous IVF has been successfully performed employing endangered felids sperm and cat oocytes [8–10]; antelope [11] or oryx [12] sperm and bovine oocytes; Iberian red deer and sheep oocytes [13]; or Iberian ibex sperm and goat oocytes [2,3]. In the case of the studies focused on Spanish Ibex [2,3], heterologous IVF was conducted in SOF medium supplemented with estrous sheep serum (ESS) at an optimal concentration of 2%. Although ESS constitutes an important source of variability and potential media contamination, it is required for sheep IVF due to its sperm capacitating effect [14,15] and is widely used to supplement SOF medium in homologous domestic goat IVF [16–18]. However, other studies have reported high fertilization rates following homologous goat IVF conducted in TALP, a medium initially developed for bovine IVF [19], without ESS supplementation and thus in defined conditions [20–22].

The aim of this study was to compare two different IVF media, and to evaluate the effect of ESS supplementation during ibex-goat heterologous IVF in terms of fertilization rates, ability to develop to the blastocyst and post-hatching stages and embryo quality assessed by lineage development analysis.

2. Materials and methods

All reagents were purchased from Sigma-Aldrich unless otherwise stated.

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https://doi.org/10.1016/j.therwi.2024.100075
Received 30 November 2023; Received in revised form 23 January 2024; Accepted 23 January 2024
Available online 26 January 2024

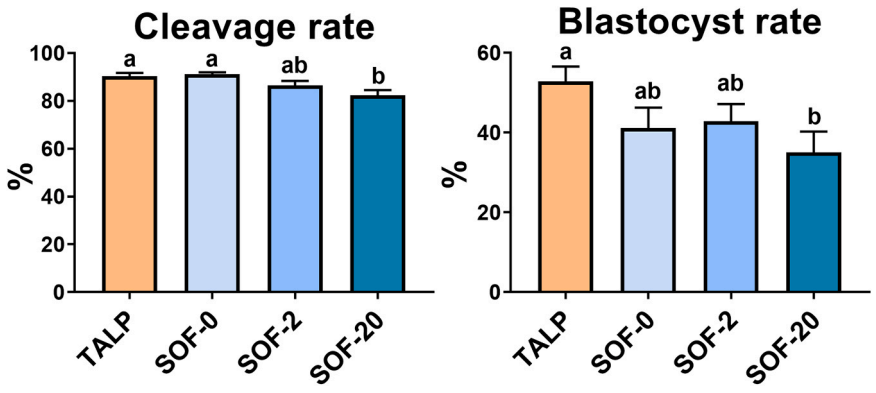

Fig. 1. Heterologous in vitro embryo production rates with Iberian ibex sperm and domestic goat oocytes. Cleavage rates at 48 h post-fertilization and blastocyst rates at day 8 after fertilization in FERT medium or SOF medium alone (SOF-0) or supplemented with 2% (SOF-2) or 20% (SOF-20) estrous sheep serum. Mean ± s.e.m. Different letters indicate statistically significant differences (One-way ANOVA; $p < 0.05$).

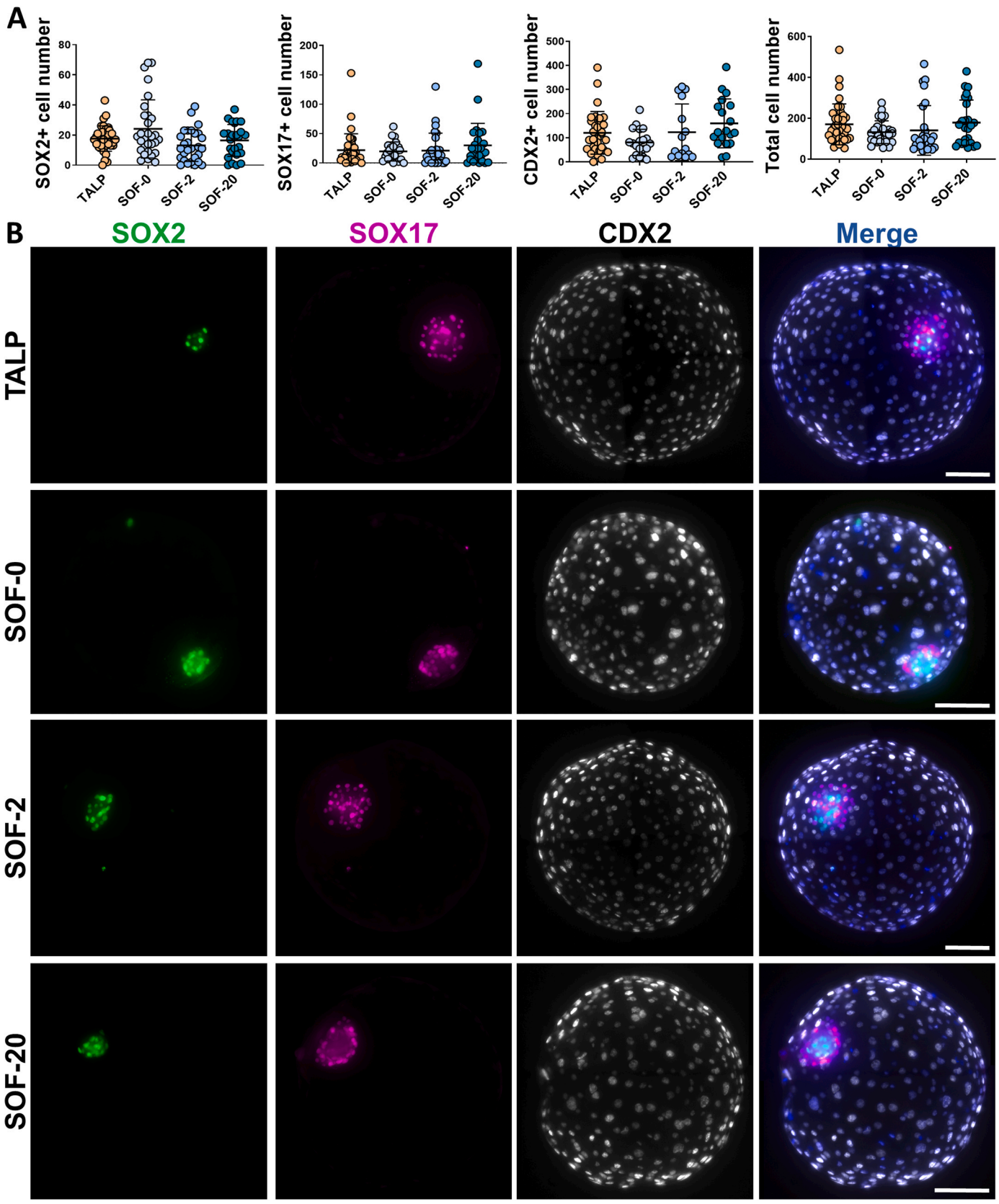

Fig. 2. Lineages development in heterologous ibex-goat blastocysts. A) Scatter plots indicating SOX2 + , SOX17 + , CDX2 + and total cell numbers in D8 blastocysts after IVF in the conditions indicated above. Graphs represent mean ± s.e.m. No statistically significant differences were detected by One-way ANOVA; Kruskal-Wallis test; $p > 0.05$. B) Representative immunofluorescence images of D8 blastocysts after IVF in FERT, SOF-0, SOF-2 or SOF-20 medium, stained for epiblast (SOX2, green), hypoblast (SOX17, magenta), trophectoderm (CDX2, white) and DAPI (blue). Scale bars: 100 μm.

Table 1
Post-hatching embryo and epiblast survival of heterologous ibex-goat embryos at D10, 12 and 14 of development.

Day	Embryo survival (%)	Epiblast survival (%)
10	227/243 (93.42)	174/204 (85.29)[a]
12	59/60 (98.33)	19/59 (32.2)[b]
14	33/33 (100)	6/33 (18.18)[b]

Values with different superscripts in the same column differ significantly (Chi-square test; $p < 0.05$).

2.1. Oocytes collection and in vitro maturation

Ovaries from adult domestic goats (*Capra hircus*) were obtained from local slaughterhouses and transported to the laboratory in 0.9% saline solution supplemented with penicillin and streptomycin at room temperature (RT). After washing the ovaries, cumulus oocyte complexes (COCs) were aspirated from 2 to 8 mm diameter follicles, using a 23 g needle connected to an aspiration pump (VMAR 5100, Cook) adjusted to − 25 mmHg. COCs were collected in a 50 ml tube with Euroflush (IVM Technologies). COCs with a compact cumulus and homogeneous cytoplasm were selected and matured for 24 h in TCM-199 supplemented with 40 μg/ml gentamicin sulphate, 10% (v/v) fetal bovine serum (FBS) and 10 ng/ml epidermal growth factor (EGF) at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity.

2.2. Evaluation of epididymal sperm

Epididymal spermatozoa from the same male, recovered within 8 h after death, was used for all experimental replicates to avoid a possible confounding male effect on embryo development. The following parameters were assessed after sperm thawing, before IVF: motility, quality of motility, viability, plasma membrane integrity, acrosome integrity and morphological abnormalities. The percentage of motile spermatozoa was 80% and the quality of motility (scored on a scale from 0 [lowest] to 5 [highest]) was 3. Both parameters were subjectively evaluated using a phase-contrast microscope (Zeiss, Germany). Sperm viability was assessed by nigrosine-eosin staining, which labels non-viable spermatozoa [23], and 65% spermatozoa were viable. Plasma membrane integrity was evaluated with the hypo-osmotic swelling test (HOST) [24]. Spermatozoa showing sperm tail swelling were classified as having an intact plasma membrane and represented 78% of sperm. The percentage of spermatozoa with an intact acrosome was assessed in sperm samples fixed with buffered 2% glutaraldehyde solution at 37 °C under a phase-contrast microscope [25]. Spermatozoa showing a crescent-shaped apical ridge were classified as having an intact acrosome (72%). However, spermatozoa with an irregulary shaped apical ridge, no apical ridge, or loose and vesiculated acrosomal cap were classified as not showing acrosome integrity (28%) [26]. Morphological abnormalities were evaluated in glutaraldehyde-fixed spermatozoa. Those spermatozoa with cytoplasmic droplets were considered morphologically normal, since this morphology is physiological in epididymal sperm cells. Only 1% of spermatozoa showed abnormal shapes. Moreover, this male showed a 40% fertility rate by artificial insemination with frozen sperm (2 pregnant out of 5 females).

2.3. Heterologous in vitro fertilization

Matured COCs were partially denuded by gentle pipetting and fertilized with frozen-thawed Bovi-Pure® (Nidacon) - separated ibex epididymal spermatozoa at a final concentration of 2×10^6. Gametes were co-incubated in 50 μl droplets of four different media: TALP medium (Supplementary Table 1) [19] supplemented with 50 IU/ml heparin (TALP) or synthetic oviductal fluid (SOF) (Supplementary Table 1) [27] supplemented with 50 IU/ml heparin alone (SOF-0) or combined with 2% (SOF-2) or 20% (SOF-20) (v/v) heat-inactivated estrous sheep serum (ESS), covered by mineral oil, at 38.5 °C in an atmosphere of 5% CO2 with maximum humidity.

2.4. In vitro embryo culture up to the blastocyst stage

At 18 h post-insemination, presumptive zygotes were denuded by pipetting and cultured in 50 μl droplets of SOF supplemented with 0.3% bovine serum albumin (BSA) under mineral oil at 38.5 °C under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 with maximum humidity. Cleavage was assessed at 48 h, and at day (D) 4 after fertilization, embryos were transferred to SOF supplemented with foetal bovine serum (FBS). Blastocyst rates were assessed at D6, 7 and 8.

2.5. Post-hatching embryo culture

At D 6 and 7 after fertilization, blastocysts were transferred to Nunclon Sphera low attachment dishes (Thermo Scientific, Roskilde, Denmark) in groups of 10–15. Blastocysts were cultured in N2B27 medium [1:1 Neurobasal and DMEM/F12 medium supplemented with penicillin/streptomycin, 2 mM L-glutamine, N2 and B27 supplements (Thermo Fisher Scientific)] supplemented with 20 ng/ml activin A and 10 μM ROCK inhibitor Y27632 (StemCell Technologies) [28] at 38.5 °C in a water saturated atmosphere of 5% CO_2, 5% O_2, and 90% N_2, and half of the culture medium was replaced every other day.

2.6. Immunofluorescence to analyze lineages development

Zona pellucida was removed by brief incubation in PBS pH 2 at room temperature (RT) when present. Zona-free embryos were fixed in 4% paraformaldehyde (PFA) for 15 min at RT and washed in phosphate-buffered saline (PBS) - 1% bovine serum albumin (BSA). If immunofluorescence was not performed right after fixation, embryos were kept in PBS – 1% BSA at 4 °C, and incubated in 125 mM glycine in PBS-1% BSA for 2 h at 4 °C right before immunostaining. Next, embryos were permeabilized in 1% Triton X-100 in PBS for 15 min at RT, and blocked in 10% Donkey Serum - 0.02% Tween 20 in PBS for 1 h at RT. Then, embryos were incubated overnight at 4 °C with primary antibodies to detect epiblast (SOX2; Invitrogen, 14–9811-80; 1:100 dilution), hypoblast (SOX17; R&D, AF1924; 1:100 dilution) and trophectoderm (CDX2; Biogenex, MU392A; 1:100 dilution). After 4 washes in PBS-1% BSA, embryos were incubated in Donkey anti-Rat IgG Alexa 488, Donkey anti-Goat IgG Alexa 555 and Donkey anti-Rabbit IgG Alexa 647 antibodies (Life Technologies) and counterstained with 10 μg/ml DAPI for 1 h at RT. Finally, embryos were washed 4 times in PBS-1% BSA. Then, embryos were mounted in PBS drops in CoverWell™ incubation chambers (Invitrogen) and imaged at a structured illumination equipment composed by a Zeiss Axio Observer microscope coupled to ApoTome.2.

2.7. Experimental design and end-point analyses

To compare the performance of different media for heterologous ibex-goat in vitro fertilization, domestic goat oocytes were co-incubated with ibex sperm in TALP [19] (n = 295), SOF [27] alone (SOF-0; n = 300) or supplemented with 2% (SOF-2; n = 301) or 20% (SOF-20; n = 310) estrous sheep serum. Cleavage rates were evaluated at 48 h post-fertilization and blastocyst rates at D8 (6 experimental replicates). In a first experiment, epiblast, hypoblast, trophectoderm and total cell number were analyzed in D8 blastocysts (3 experimental replicates) to assess embryo quality. In a second experiment, to further characterize embryo quality, D6/7 blastocysts were cultured in a recently established post-blastocyst culture system that allows ovine embryos to develop up to peri-gastrulating stages [28]. Embryos were cultured up to D14 (1 experimental replicate), D12 (1 experimental replicate) or D10 (3 experimental replicates). One experimental replicate provided enough embryos to analyze both D8 and D14 embryos fertilized in the four different experimental conditions, and another one was used to analyze

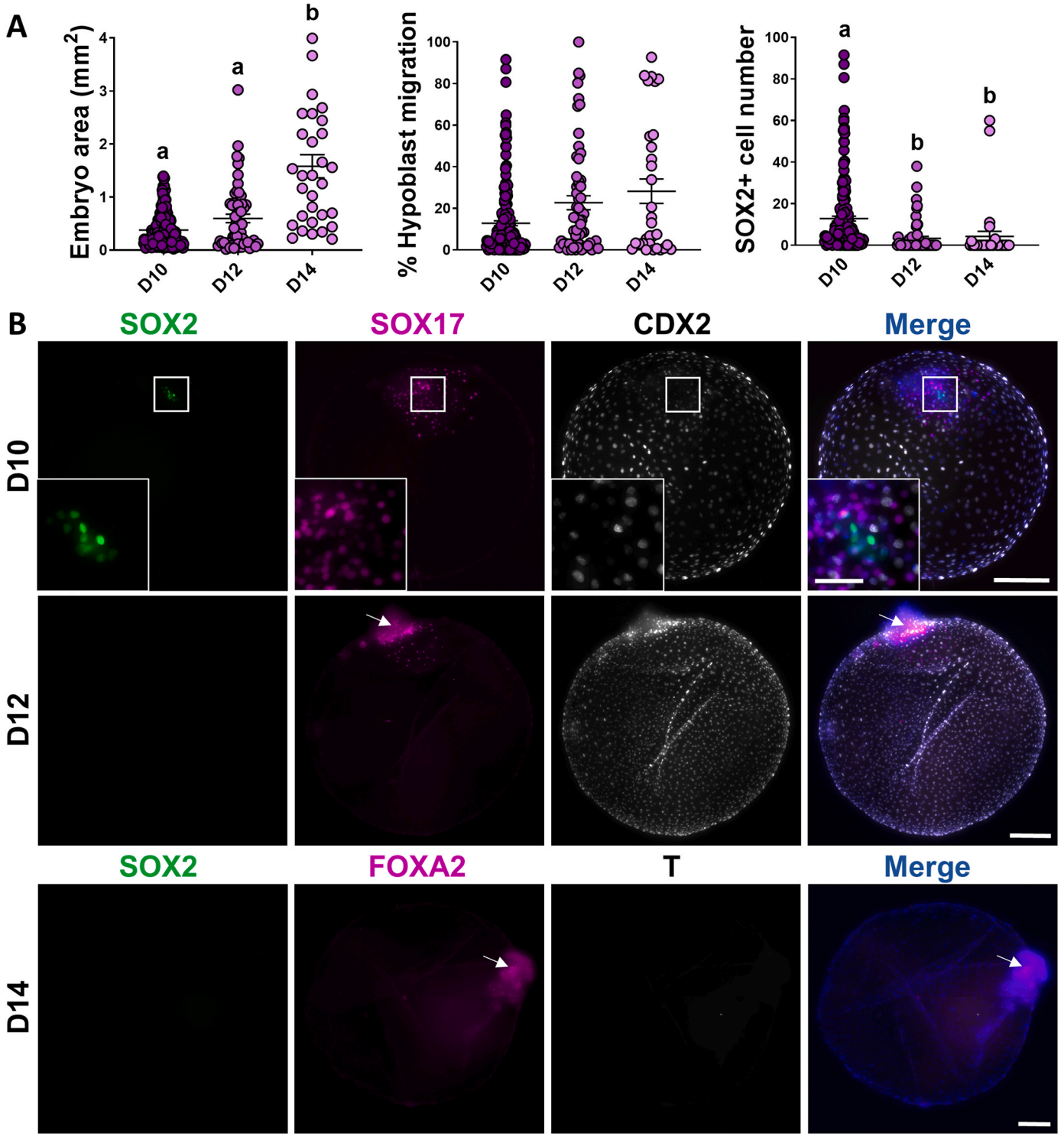

Fig. 3. Post-hatching development in heterologous ibex-goat embryos. A) Scatter plots indicating embryo area, percentage of hypoblast migration and SOX2 + cell number in D10, 12 and 14 heterologous ibex-goat embryos. Graphs represent mean ± s.e.m. Different letters indicate statistically significant differences detected by One-way ANOVA; Kruskal-Wallis test; $p < 0.05$. B) Representative immunofluorescence images of D10, 12 and 14 embryos. D10 and D12 embryos were stained for epiblast (SOX2, green), hypoblast (SOX17, magenta), trophectoderm (CDX2, white) and DAPI (blue), and D14 embryos were stained for epiblast (SOX2, green), hypoblast (FOXA2, magenta), mesoderm (T, white) and DAPI (blue). Scale bars: 200 μm for embryos; 50 μm for epiblast magnifications. White arrows indicate degenerating hypoblast cells.

Table 2
Embryo and epiblast survival of D10 heterologous embryos after IVF in TALP, SOF-0, SOF-2 or SOF-20 medium.

IVF medium	Embryo Survival (%)	Epiblast survival (%)
TALP	79/81 (97.53)[a]	55/69 (79.71)
SOF-0	46/57 (80.7)[b]	37/42 (88.09)
SOF-2	59/60 (98.33)[a]	50/55 (90.91)
SOF-20	43/45 (95.55)[a]	32/38 (84.21)

Values with different superscripts in the same column differ significantly (Chi-square test; $p < 0.05$).

both D8 and D12 embryos. At the end of the culture period, embryo survival was analyzed (alive embryos were able to maintain the blastocoel, whereas dead embryos collapsed), and surviving embryos were collected for lineages development analysis by immunofluorescence. Embryo area was measured using the Contour plugin in ZEN 3.2 (Carl Zeiss Microscopy GmbH, Germany). To quantify hypoblast migration, each spherical embryo was divided into two halves in the Z axis and orthogonal projections were performed. Then, total area and the area covered by SOX17 + hypoblast cells were quantified in each projection, and the percentage of hypoblast migration was calculated as the area covered by hypoblast divided by the total area. The percentage of hypoblast migration was calculated for each embryo as the mean of both projections. Epiblast survival was scored as the presence of SOX2 + cells at the end of the culture period. SOX2 + cell number was counted manually using the multi-point counter plugin in ZEN 3.2.

2.8. Statistical analysis

Data were analyzed using the GraphPad Prism (GraphPad Software, San Diego, CA, USA) package and a value of $P < 0.05$ was considered significant. Differences in cleavage and blastocyst rates, cell counts, embryo area and hypoblast migration were analyzed by One-way ANOVA when data distribution was normal. When normality test failed, statistical differences were analyzed by non-parametric One-way ANOVA (Kruskal-Wallis test). Differences in embryo survival and epiblast survival were analyzed by Chi-square test.

3. Results

3.1. Heterologous in vitro embryo production with Iberian ibex sperm and domestic goat oocytes

Similar cleavage and blastocyst rates were found when IVF was performed in TALP, SOF alone (SOF-0) or SOF supplemented with 2% (SOF-2) estrus sheep serum (ESS). However, cleavage rate was significantly lower in SOF medium supplemented with 20% ESS (SOF-20) than in TALP medium or SOF-0. Blastocyst rate at D8 was significantly higher in TALP medium than in SOF-20 (Fig. 1). When lineages development was analyzed in D8 blastocysts, epiblast (SOX2 +), hypoblast (SOX17 +), trophectoderm (CDX2 +) and total cell number was similar between blastocysts fertilized in TALP, SOF alone or supplemented with 2% or 20% ESS (Fig. 2).

3.2. Post-hatching development of heterologous embryos

Ibex-goat hybrid embryos showed high survival rates during post-hatching culture (Table 1) growing extensively from D10 to D14 (Fig. 3**A**). However, in contrast to cattle or ovine embryos [28,29], hypoblast migration was incomplete and many embryos showed a compact mass of degenerating hypoblast cells at D12 and 14 (Fig. 3**B**). A similar trend over post-hatching culture was noted for epiblast, as both the percentage of embryos with surviving epiblast cells (Table 1) and the number of SOX2 + epiblast cells were significantly lower at D12 and 14 than at D10 (Fig. 3**A**). Epiblast did not develop into an embryonic disc, and initiation of gastrulation was not observed (Fig. 3**B**), in contrast to sheep embryos [28].

Given that no further development was observed beyond D10, the effects of the IVF media on subsequent embryo development were analyzed at D10. Survival was significantly lower in embryos fertilized in SOF-0 (Table 2). However, no significant differences were detected in embryo area, hypoblast migration, the percentage of embryos with surviving epiblast and the number of SOX2 + cells (Table 2, Fig. 4).

4. Discussion

The development of assisted reproductive technologies is crucial to preserve endangered wild species. Access to Iberian ibex female gametes is very limited, but cryopreserved ibex epididymal sperm are available [4], and heterologous IVF using domestic goat oocytes is an excellent system to test the fertilization capacity of stored ibex sperm doses. Here, we have optimized heterologous ibex-goat IVF, achieving over 50% blastocyst rates when IVF was performed in TALP medium, in the absence of estrous sheep serum (ESS).

Heterologous ibex-goat IVF has been previously performed employing SOF medium alone or supplemented with 2 or 20% ESS, yielding 1%, 7% and 0% blastocyst rates, respectively [3]. A posterior study reported a 4.5% blastocyst rate (per oocyte) using SOF with 2% ESS, the best IVF medium according to the first study [2]. The higher blastocyst rates obtained herein (35–53%) could be associated to the use of adult instead of prepubertal goat oocytes, as prepubertal oocytes also show a reduced developmental ability compared to adult ones during domestic goat IVF. For instance, Leoni *et al.* reported 6.7 vs. 18.44% blastocyst rate from prepubertal vs. adult oocytes, respectively employing SOF medium supplemented with 10% ESS [18]; and Romaguera *et al.* reported 10.9 vs. 20.8% using TALP medium [20].

Based on previous reports on heterologous ibex-goat IVF, we tested SOF medium alone or supplemented with 2 or 20% ESS, and TALP medium, which is regularly used for bovine IVF [19] and has been also employed in domestic goat IVF [20]. ESS is essential for sheep IVF due to its sperm capacitating effect [14,15] and blastocyst rates ranging from 32 to 46% have been obtained in domestic goat IVF with adult oocytes and SOF medium supplemented with 10% ESS [16,17], but ESS composition is variable and unknown. In addition, its use can increase the risk of media contamination [17], and serum supplementation has been associated to large offspring syndrome in ovine [30] and bovine [31]. Our data evidences that ESS supplementation is not required for heterologous ibex-goat IVF and that it can be detrimental for fertilization and blastocyst rates at 20%, encouraging the use of a defined fertilization medium, such as TALP, which yielded a very high blastocyst rate in this study (52.9 ± 3.67%; mean ± s.e.m.).

Heterologous artificial insemination has been previously used to evaluate the fertilization capacity of cryopreserved ibex sperm in vivo [32]. However, apparent fertility of sperm doses is profoundly influenced by the variable fertility of the females, which entails that a great number of females need to be inseminated per sperm sample to obtain significant conclusions. Furthermore, a number of technical limitations should be taken into account, such as the individual response to oestrus synchronization and ovulation, the influence of environmental conditions (e.g. reproductive seasonality), the technical limitations for laparoscopic insemination, etc. Conversely, heterologous IVF with phylogenetically related domestic species may be a more suitable method to evaluate the fertilizing capacity of sperm samples in rare or wild species, as fertilization takes place under in vitro controlled conditions and it does not require the use of valuable homologous oocytes.

IVF efficiency is usually evaluated by fertilization rates and developmental rates up to the blastocyst stage. However, embryo quality is another relevant parameter to analyze, as sperm cryodamage, DNA fragmentation and epigenetic alterations affecting chromatin condensation can affect subsequent embryo development [33–35]. Beyond the conventional morphological grading performed at the blastocyst stage,

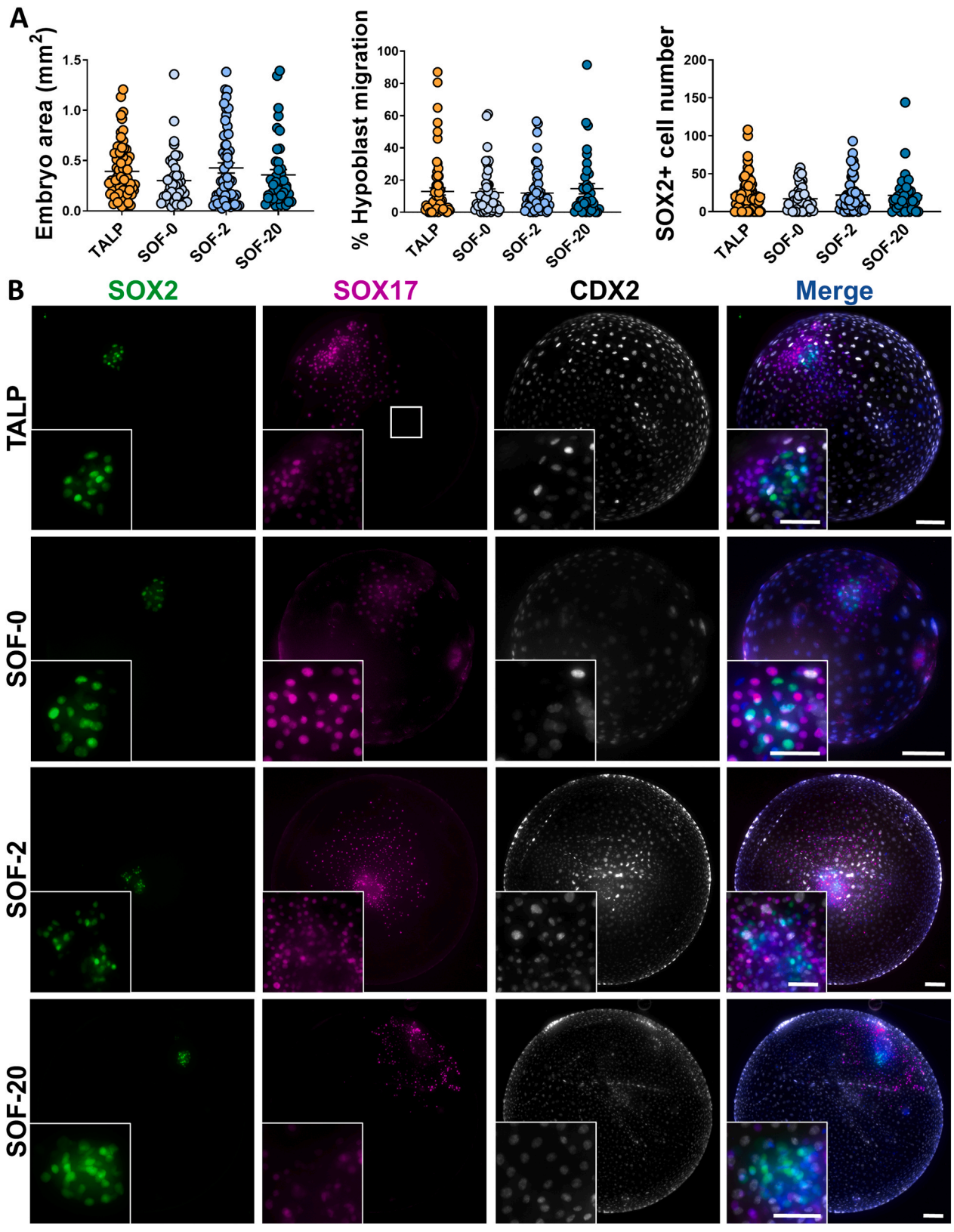

Fig. 4. Post-hatching development in heterologous ibex-goat D10 embryos. A) Scatter plots indicating embryo area, percentage of hypoblast migration and SOX2 + cell number in D10 embryos after IVF in the conditions indicated above. Graphs represent mean ± s.e.m. No statistically significant differences were detected by One-way ANOVA; Kruskal-Wallis test; $p > 0.05$. B) Representative immunofluorescence images of D10 embryos after IVF in FERT, SOF-0, SOF-2 or SOF-20 medium, stained for epiblast (SOX2, green), hypoblast (SOX17, magenta), trophectoderm (CDX2, white) and DAPI (blue). Scale bars: 100 μm for embryos; 50 μm for epiblast magnifications.

the analysis of cell lineages development using specific epiblast, hypoblast and trophectoderm markers provides a deeper insight of embryo quality [36], and post-hatching in vitro culture system allows to objectively evaluate real developmental ability beyond the blastocyst stage [28,29]. Although ibex-goat hybrid embryos showed limited development in vitro from D12 onwards, epiblast, hypoblast and trophectoderm development could be successfully quantified at D8 and D10, allowing the assessment of post-hatching developmental ability. D8 blastocysts and D10 post-hatching embryos showed proper epiblast, hypoblast and trophectoderm development in all IVF conditions, reflecting proper embryo viability beyond blastocyst hatching and adding an additional test to infer the suitability of stored ibex sperm doses to produce offspring through artificial insemination for conservation purposes [6].

5. Conclusions

TALP medium provides optimal conditions to conduct heterologous IVF to test the fertilization ability of frozen-thawed Iberian ibex epididymal sperm doses in adult goat oocytes, being ESS supplementation not required. Embryo quality could be objectively evaluated through lineages development assessment up to D10 in vitro, but no differences were found between embryos fertilized in TALP or SOF alone or supplemented with ESS. The optimized heterologous ibex-goat IVF protocol, achieving blastocyst rates beyond 50%, combined with embryo quality assessment through lineages development, could be employed to predict the fertilization capacity of ibex sperm doses and opens the gate to homologous in vitro embryo production of ibex embryos.

Funding

This work has been funded by the projects StG-757886-ELONGAN from the European Research Council to PBA and PID2021-122153NA-I00 from the Spanish Ministry of Science and Innovation to PRI.

CRediT authorship contribution statement

Ramos Ibeas Priscila: Writing – original draft, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Bermejo-Álvarez Pablo:** Writing – review & editing, Funding acquisition, Conceptualization. **Santiago-Moreno Julián:** Writing – review & editing, Resources. **Peris-Frau Patricia:** Resources. **Marigorta Pilar:** Investigation, Formal analysis. **Flores-Borobia Inés:** Investigation, Formal analysis. **Carvajal-Serna Melissa:** Investigation, Formal analysis. **Martínez de los Reyes Nuria:** Writing – review & editing, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors want to acknowledge the slaughterhouses “Matadero Mondejano SL” and “Matadero Frigorífico de Valmojado SA” for gently providing goat ovaries to conduct the experiments.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2024.100075.

References

[1] J. Herrero, P. Acevedo, M. Arnal, D. Fernández de Luco, C. Fonseca, R. García-González, et al., *Capra pyrenaica* (amended version of 2020 assessment), IUCN Red. List Threat. Species (2021).
[2] J. López-Saucedo, M.T. Paramio, R. Fierro, D. Izquierdo, M.G. Catalá, M.A. Coloma, et al., Sperm characteristics and heterologous in vitro fertilisation capacity of Iberian ibex (Capra pyrenaica) epididymal sperm, frozen in the presence of the enzymatic antioxidant catalase, Cryobiology 68 (2014) 389–394.
[3] J. López-Saucedo, J. Santiago-Moreno, R. Fierro, D. Izquierdo, M.A. Coloma, M. G. Catalá, et al., Fertilization capacity of cryopreserved Iberian ibex epididymal sperm in a heterologous in vitro fertilization assay, Zygote 23 (2015) 136–144.
[4] J. Pradiee, M.C. Esteso, A. Lopez-Sebastián, A. Toledano-Díaz, C. Castaño, J. A. Carrizosa, et al., Successful ultrarapid cryopreservation of wild Iberian ibex (Capra pyrenaica) spermatozoa, Theriogenology 84 (2015) 1513–1522.
[5] J. Pradieé, M.J. Sánchez-Calabuig, C. Castaño, E. O'Brien, M.C. Esteso, P. Beltrán-Breña, et al., Fertilizing capacity of vitrified epididymal sperm from Iberian ibex (Capra pyrenaica), Theriogenology 108 (2018) 314–320.
[6] J. Santiago-Moreno, A. Toledano-Díaz, A. Pulido-Pastor, A. Gómez-Brunet, A. López-Sebastián, Birth of live Spanish ibex (Capra pyrenaica hispanica) derived from artificial insemination with epididymal spermatozoa retrieved after death, Theriogenology 66 (2006) 283–291.
[7] J. Santiago-Moreno, M.A. Coloma, A. Toledano-Díaz, C. Castaño, A. Gómez-Brunet, A. López-Sebastián, Assisted reproduction in Mediterranean wild ruminants: lessons from the Spanish ibex (Capra pyrenaica), Soc. Reprod. Fertil. Suppl. 67 (2010) 431–441.
[8] J.C. Andrews, J.G. Howard, B.D. Bavister, D.E. Wildt, Sperm capacitation in the domestic cat (Felis catus) and leopard cat (Felis bengalensis) as studied with a salt-stored zona pellucida penetration assay, Mol. Reprod. Dev. 31 (1992) 200–207.
[9] A.M. Donoghue, L.A. Johnston, U.S. Seal, D.L. Armstrong, L.G. Simmons, T. Gross, et al., Ability of thawed tiger (Panthera tigris) spermatozoa to fertilize conspecific eggs and bind and penetrate domestic cat eggs in vitro, J. Reprod. Fertil. 96 (1992) 555–564.
[10] N. Gañán, R. González, J.J. Garde, F. Martínez, A. Vargas, M. Gomendio, et al., Assessment of semen quality, sperm cryopreservation and heterologous IVF in the critically endangered Iberian lynx (Lynx pardinus), Reprod. Fertil. Dev. 21 (2009) 848–859.
[11] A.J. Kouba, M.W. Atkinson, A.R. Gandolf, T.L. Roth, Species-specific sperm-egg interaction affects the utility of a heterologous bovine in vitro fertilization system for evaluating antelope sperm, Biol. Reprod. 65 (2001) 1246–1251.
[12] T.L. Roth, L.M. Bush, D.E. Wildt, R.B. Weiss, Scimitar-horned oryx (Oryx dammah) spermatozoa are functionally competent in a heterologous bovine in vitro fertilization system after cryopreservation on dry ice, in a dry shipper, or over liquid nitrogen vapor, Biol. Reprod. 60 (1999) 493–498.
[13] A.J. Soler, N. Poulin, M.R. Fernández-Santos, Y. Cognie, M.C. Esteso, J.J. Garde, et al., Heterologous in vitro fertility evaluation of cryopreserved Iberian red deer epididymal spermatozoa with zona-intact sheep oocytes and its relationship with the characteristics of thawed spermatozoa, Reprod. Domest. Anim. 43 (2008) 293–298.
[14] D. Huneau, N. Crozet, M. Ahmed-Ali, Estrous sheep serum as a potent agent for ovine IVF: effect on cholesterol efflux from spermatozoa and the acrosome reaction, Theriogenology 42 (1994) 1017–1028.
[15] E. Del Olmo, O. García-Álvarez, A. Maroto-Morales, M. Ramón, P. Jiménez-Rabadán, M. Iniesta-Cuerda, et al., Estrous sheep serum enables in vitro capacitation of ram spermatozoa while preventing caspase activation, Theriogenology 85 (2016) 351–360.
[16] G.M. Bragança, A.S. Alcântara-Neto, R.I.T.P. Batista, F.Z. Brandão, V.J.F. Freitas, P. Mermillod, et al., Oviduct fluid during IVF moderately modulates polyspermy in in vitro-produced goat embryos during the non-breeding season, Theriogenology 168 (2021) 59–65.
[17] J.M. Souza-Fabjan, Y. Locatelli, N. Duffard, E. Corbin, J.L. Touzé, C. Perreau, et al., In vitro embryo production in goats: slaughterhouse and laparoscopic ovum pick up-derived oocytes have different kinetics and requirements regarding maturation media, Theriogenology 81 (2014) 1021–1031.
[18] G.G. Leoni, S. Succu, V. Satta, M. Paolo, L. Bogliolo, D. Bebbere, et al., In vitro production and cryotolerance of prepubertal and adult goat blastocysts obtained from oocytes collected by laparoscopic oocyte-pick-up (LOPU) after FSH treatment, Reprod. Fertil. Dev. 21 (2009) 901–908.
[19] J.J. Parrish, J.L. Susko-Parrish, M.L. Leibfried-Rutledge, E.S. Critser, W. H. Eyestone, N.L. First, Bovine in vitro fertilization with frozen-thawed semen, Theriogenology (1986) 591–600.
[20] R. Romaguera, X. Moll, R. Morató, M. Roura, M.J. Palomo, M.G. Catalá, et al., Prepubertal goat oocytes from large follicles result in similar blastocyst production and embryo ploidy than those from adult goats, Theriogenology 76 (2011) 1–11.
[21] M.J. Palomo, D. Izquierdo, T. Mogas, M.T. Paramio, Effect of semen preparation on IVF of prepubertal goat oocytes, Theriogenology 51 (1999) 927–940.
[22] A.I. Younis, K.A. Zuelke, K.M. Harper, M.A. Oliveira, B.G. Brackett, In vitro fertilization of goat oocytes, Biol. Reprod. 44 (1991) 1177–1182.
[23] E. Blom, A one-minute live-dead sperm stain by means of eosin-negrosin, Fertil. Steril. 1 (1950) 176–177.
[24] R.S. Jeyendran, H.H. Vanderven, M. Perezpelaez, B.G. Crabo, L.J.D. Zaneveld, Development of an assay to assess the functional integrity of the human-sperm membrane and its relationship to other semen characteristics, J. Reprod. Fertil. 70 (1984), 219-&.

[25] B. Pintado Sanjuanbenito, B. Pérez Llano, Effect of glutaraldehyde concentration and fixative temperature on the number of spermatozoa with normal acrosomes in goat semen, Theriogenology 38 (1992) 527–533.
[26] V.G. Pursel, L.A. Johnson, Glutaraldehyde fixation of boar spermatozoa for acrosome evaluation, Theriogenology 1 (1974) 63–68.
[27] H.R. Tervit, D.G. Whittingham, L.E. Rowson, Successful culture in vitro of sheep and cattle ova, J. Reprod. Fertil. 30 (1972) 493–497.
[28] P. Ramos-Ibeas, L. González-Brusi, M.T. Used, M.J. Cocero, P. Marigorta, R. Alberio, et al., In vitro culture of ovine embryos up to early gastrulating stages, Development 149 (2022).
[29] P. Ramos-Ibeas, I. Lamas-Toranzo, Á. Martínez-Moro, C. de Frutos, A.C. Quiroga, E. Zurita, et al., Embryonic disc formation following post-hatching bovine embryo development in vitro, Reproduction (2020).
[30] J.G. Thompson, D.K. Gardner, P.A. Pugh, W.H. McMillan, H.R. Tervit, Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos, Biol. Reprod. 53 (1995) 1385–1391.
[31] Z. Chen, K.M. Robbins, K.D. Wells, R.M. Rivera, Large offspring syndrome: a bovine model for the human loss-of-imprinting overgrowth syndrome Beckwith-Wiedemann, Epigenetics 8 (2013) 591–601.
[32] J. Santiago-Moreno, M.A. Coloma, A. Toledano-Díaz, A. Gómez-Brunet, A. Pulido-Pastor, A. Zamora-Soria, et al., A comparison of the protective action of chicken and quail egg yolk in the cryopreservation of Spanish ibex epididymal spermatozoa, Cryobiology 57 (2008) 25–29.
[33] D.P. Braga, A.S. Setti, R.C. Figueira, A. Iaconelli Jr., E. Borges Jr., The negative influence of sperm cryopreservation on the quality and development of the embryo depends on the morphology of the oocyte, Andrology 3 (2015) 723–728.
[34] L. Simon, J. Castillo, R. Oliva, S.E. Lewis, Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes, Reprod. Biomed. Online 23 (2011) 724–734.
[35] L. Simon, K. Murphy, M.B. Shamsi, L. Liu, B. Emery, K.I. Aston, et al., Paternal influence of sperm DNA integrity on early embryonic development, Hum. Reprod. 29 (2014) 2402–2412.
[36] A. Perez-Gomez, L. Gonzalez-Brusi, P. Bermejo-Alvarez, P. Ramos-Ibeas, Lineage differentiation markers as a proxy for embryo viability in farm ungulates, Front. Vet. Sci. 8 (2021).