Production of Embryos by Interspecies Somatic Cell Nuclear Transfer (iSCNT) Between Bovine (*Bos taurus*) and Ovine (*Ovis aries*) Using Handmade Cloning (HMC)

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ABSTRACT

Objective: To determine the ability of the sheep oocyte (*Ovis aries*) to generate cloned bovine embryos (*Bos taurus*), through interspecies Somatic Cell Nuclear Transfer (iSCNT) by Handmade Cloning (HMC).

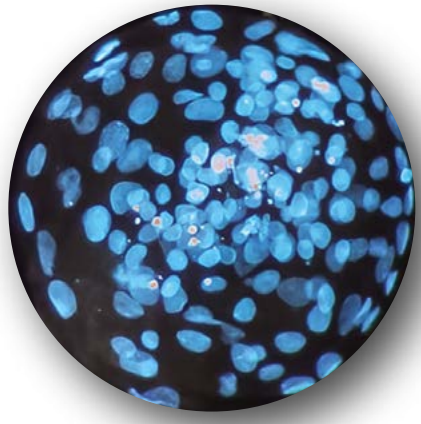
Design/methodology/approach: For iSCNT, fifth-passage bovine skin fibroblasts were used as karyoplasts, and *in vitro*-matured, manually enucleated ovine oocytes were used as cytoplasts. Cytoplast-karyoplast-cytoplast triplets were formed, which were fused by electrical pulses and activated for the development of cloned bovine embryos, which were cultured in Cleavage medium. Additionally, some ovine oocytes were activated as parthenogenetic embryos to serve as a control group. The *in vitro* development rate (IVD) of the iSCNT group (BOV-OV) *vs*. parthenogenetic ovine embryos (G-OV) was evaluated using a two-way Student t-test for paired data.

Results: It was observed that in the BOV-OV group, bovine cloned embryos produced by iSNCT using HMC only reached the morula stage (20 blastomeres), while in the G-OV blastocysts were observed with significant differences ($p \leq 0.05$).

Limitations on study/implications: Limited sample size of slaughterhouse ovaries.

Findings/conclusions: Although ovine oocytes were able to initiate the IVD of cloned bovine embryos produced by iSCNT using HMC, species divergence proved to be a critical limiting factor for reaching the blastocyst stage.

Keywords: bovine, ovine, handmade cloning, interspecies.



INTRODUCTION

Reproductive biotechnologies used in animals enable the growth of populations of species with high genetic value, especially when their numbers are

critically low. Moreover, these technologies support the conservation, preservation, and dissemination of genetic material from individuals with superior traits compared to others of their kind (Loi *et al.*, 2011; Lagutina *et al.*, 2013; Yelisetti *et al.*, 2016; Galli *et al.*, 2021; Mrowiec *et al.*, 2021).

The genetic rescue of selected individuals has led to numerous studies in domestic species, aiming to extrapolate findings to other animal populations or specific individuals. One such approach is Somatic Cell Nuclear Transfer (SCNT), or cloning, which is used to generate embryos with genetic material identical to that of an existing specimen. In 1938, Hans Spemann first reported a technique based on this principle, applying it to amphibians (as cited by Wilmut *et al.*, 1997). Later, in 1997, Wilmut and colleagues achieved the birth of the sheep *Dolly*. This milestone demonstrated that the genome of an adult somatic cell retains all its genetic information, despite being a specialized cell type. In other words, although the genome undergoes restrictions during development and changes during cellular differentiation, these are reversible, as they result from epigenetic modifications rather than permanent alterations to the genome (Verma *et al.*, 2015; Gouveia *et al.*, 2020; Galli *et al.*, 2021).

Among the limitations of this technique is the restricted availability of oocytes from wild animals. As an alternative, iSCNT involves using a somatic cell from the species to be cloned as the nuclear donor (karyoplast) and inserting it into an enucleated oocyte (cytoplast) obtained from a species belonging to a completely different order or taxon. This approach offers the possibility of generating viable and transferable cloned embryos (Beyhan *et al.*, 2007; González-Grajales *et al.*, 2016; Yu *et al.*, 2016; Saini *et al.*, 2018; Ammari *et al.*, 2022). Therefore, iSCNT is employed to conserve wildlife species from which oocytes cannot be collected, species with challenging reproductive management, or those with limited oocyte availability (Saikhun *et al.*, 2002; Pan *et al.*, 2014). Using this method, the gaur (*Bos gaurus*) (Lanza *et al.*, 2000; Srirattana *et al.*, 2012) and the gray wolf (*Canis lupus*) (Oh *et al.*, 2008) have been successfully cloned.

Interspecies Somatic Cell Nuclear Transfer is more efficient when the donor karyoplast and the recipient cytoplast come from species with similar reproductive physiology, pregnancy length, and placental type (Melo *et al.*, 2022).

In species that can naturally hybridize or share a closer phylogenetic relationship, the likelihood of embryos developing and producing live offspring is higher (Lagutina *et al.*, 2013; Mrowiec *et al.*, 2021). However, Dominko *et al.*, (1999) fused ovine somatic cells with enucleated bovine oocytes to study nucleus-cytoplasm interactions in embryos generated via iSCNT, achieving a blastocyst rate of 13.9%.

Even when enucleated, the oocyte retains the ability to reprogram the nucleus of a somatic cell, returning it to an undifferentiated and totipotent state capable of producing a viable embryo (Jiang *et al.*, 2011; Seaby *et al.*, 2013; Saini *et al.*, 2018; Galli *et al.*, 2021), without altering the transplanted genome (Dominko *et al.*, 1999; Gurdon & Wilmut, 2011). In a study published by Hamilton (2004), ovine oocytes were used as recipients for bovine somatic cell nuclei to clone bovine embryos via iSCNT; however, the results were inconclusive, with only four cloned embryos reaching at least the 8-cell stage. The

present study aimed to determine the ability of ovine oocytes (*Ovis aries*) to generate bovine cloned embryos (*Bos taurus*) through iSCNT by Handmade Cloning (HMC).

The hypothesis was that, since both species belong to the same family and mammalian oocytes contain all the necessary information to produce an embryo, using ovine oocytes as cytoplasts will enable the *in vitro* development (IVD) of cloned bovine embryos produced via iSCNT from adult bovine fibroblasts. This approach could serve as an alternative for the reproduction and conservation of individuals with valuable, productive traits.

MATERIALS AND METHODS

This study was carried out at the Assisted Animal Reproduction Laboratory W-210 of the Universidad Autónoma Metropolitana, Iztapalapa Campus, in Mexico City, Mexico. All reagents were purchased from Sigma-Aldrich unless otherwise specified.

Collection of Auricular Skin and Derivation of Bovine Fibroblasts

Following the protocols described by Tovar *et al.*, (2008) and Navarro-Maldonado *et al.*, (2015), skin biopsies were taken from the dorsal side of the ear of an adult female Simbrah bovine, housed at the Agricultural Production Research Center of the Universidad Autónoma de Nuevo León (CIPA-UANL). A 1 cm^2 area on the back of the ear was shaved, washed with a 2% chlorhexidine solution (Laboratorio Aranda, Mexico), and disinfected with 70% alcohol. Three skin samples, each 0.3 cm^2 in diameter, were collected using a biopsy punch (Pickett, 2011) and transported in tubes containing 10 mL of Dulbecco's Modified Eagle Medium (DMEM, *In Vitro*, S.A., Mexico), supplemented with 10% Fetal Bovine Serum (FBS, Biowest, Nuaillé, France), 2% antibiotic-antimycotic (Antibac-Antifun, *In Vitro*, S.A., Mexico), and kept at 4-8 °C.

Once in the laboratory, the samples were washed in DPBS (Dulbecco's Phosphate-Buffered Saline without calcium or magnesium, *In Vitro*, S.A., Mexico), supplemented with 2% of a 100X antibiotic-antimycotic solution Antibac-Antifun at pH 7 (10,000 IU/mL Sodium Penicillin G, 10,000 μg/mL Streptomycin Sulfate, and 25 μg/mL solubilized Amphotericin B, *In Vitro*, S.A., Mexico) for 3 min, with the wash repeated twice (Santos *et al.*, 2021). The skin samples were then cultured as explants in 35 mm Petri dishes with 3 mL of supplemented DMEM (88% DMEM, 10% FBS, 2% Antibac-Antifun), enriched with 1 ng/mL Epidermal Growth Factor (EGF), and incubated at 38.5 °C with 5% CO_2 in 95% air and saturated humidity for at least 7 days or until a confluent fibroblast layer was obtained (Navarro-Maldonado *et al.*, 2015). Fibroblast confluence (>90%) was assessed, and cells were detached from the extracellular matrix using an enzymatic solution of 0.05/0.05% Trypsin-Versene (*In Vitro*, S.A., Mexico). The fibroblasts were collected, with half of the population subcultured for cell passages and the remainder cryopreserved in a DMSO-based medium (Sigma-Aldrich, USA). These steps were repeated until five cell passages were completed, as required for iSCNT. Cell concentration was determined at each passage using a Neubauer chamber, following Freshney (2010).

Collection and *In Vitro* Maturation (IVM) of Ovine (*Ovis aries*) Cumulus-Oocyte Complexes

Cumulus-oocyte complexes (COC) were obtained from ovine ovaries collected at a local slaughterhouse. The ovaries were transported in physiological saline solution (PSS) supplemented with 2% antibiotics at 30 °C for 1 to 2 h (Martínez-Ibarra *et al.*, 2019). Upon arrival at the laboratory, the ovaries were washed three times with isothermal PSS. The COC were then collected by puncture/aspiration of ovarian follicles (2 to 5 mm in diameter) using a 10 mL syringe with an 18-gauge hypodermic needle in TCM-199 medium with HEPES (*In Vitro*, S.A., Mexico), supplemented with 100 IU/mL of heparin at 30 °C. The COC were evaluated and selected based on quality grades I and II for *in vitro* maturation (IVM) (Yadav *et al.*, 1997).

In vitro maturation was performed in TCM-199 without HEPES, supplemented with 10% FBS, 10 ng/mL Epidermal Growth Factor (EGF), recombinant Follicle-Stimulating Hormone (FSH; 0.1 IU/mL; Gonal-F, Merck), 5 IU/mL equine Chorionic Gonadotropin (eCG; Gonaforte, Parfarm S.A., Mexico), and 0.6% antibiotics. Groups of 30 COC were then cultured in four-well dishes (Nunc, USA), each containing 500 μL of IVM medium covered with mineral oil for 20 h (Martínez-Ibarra *et al.*, 2019).

Cloning of Bovine-Ovine (BO-OV) Embryos via iSCNT

Preparation of Bovine Karyoplasts

Bovine fibroblasts from the fifth passage were used as nuclear donors (karyoplasts) for iSCNT (Beltrán & González, 2016; Cortez *et al.*, 2017). Before iSCNT, cells were detached from their extracellular matrix by adding a 0.05/0.05% Trypsin-Versene enzymatic solution (*In Vitro*, S.A., Mexico) for 8-10 min. They were then placed under incubation conditions, followed by enzyme inactivation using DMEM supplemented with 1% FBS.

Preparation of Ovine Cytoplasts

After 20 h of IVM, cumulus cell expansion in the COC was evaluated. The COC were removed from the IVM medium and incubated in a 1.5 mL tube with 500 μL of hyaluronidase (0.5 mg/mL in TCM-199 without HEPES) for 5 min under the same conditions. The suspension was gently pipetted to disperse the cumulus cells. The contents were transferred to a 35 mm Petri dish, and denuded oocytes were recovered and washed in 500 μL of TCM-199 supplemented with 2% FBS (T2). Oocytes showing a first polar body (PB), indicating arrest at Metaphase II (MII), were selected. To induce protrusion of the metaphase plate, MII oocytes were incubated in the same IVM medium supplemented with Demecolcine (0.5 μg/mL) for 1 h. To remove the zona pellucida (ZP) and enucleate the oocytes, a Petri dish was prepared with 30 μL microdrops of: T2, Pronase (2 mg/mL in TCM-199 supplemented with 10% FBS or T10), TCM-199 supplemented with 20% FBS (T20), T10 (supplemented with 0.05% Cytochalasin B), and T20.

Oocytes were passed through each microdrop and manually enucleated for HMC in T10 with Cytochalasin B using a microblade (Bioniche, USA), as described by Vajta *et al.* (2001). This procedure removed the metaphase plate along with the polar body (PB)

(Cortez *et al*., 2017; Vazquez-Avendaño *et al*., 2024). Once enucleated, the cytoplasts were placed in T20 microdrops before fusion with the karyoplasts.

Formation and Fusion of Cytoplast-Karyoplast-Cytoplast Triplets

In a 35 mm Petri dish, 15 μL microdrops were prepared containing: T20, phytohemagglutinin (5 mg/mL in TCM-199 with HEPES), T2, fusion medium (0.3 M D-mannitol, 1 mg/mL polyvinyl alcohol), and T20. Ovine cytoplasts were placed individually into the T20 microdrops. Groups of five cytoplasts were then briefly immersed (3 to 4 s) in the phytohemagglutinin microdrop to facilitate the formation of triplets, composed of ovine cytoplast, bovine karyoplast, and ovine cytoplast. These cellular triplets were equilibrated in the fusion medium and aligned on the positive electrode of the fusion chamber (BTX, 0.5 mm gap, model 450, Holliston, MA, USA). Fusion was induced using a single direct current (DC) pulse of 1.0 kV/cm for 9 μs. The fused structures were then transferred to T20 microdrops for reconstitution into a single cell (reconstructed cloned embryo). This process was repeated with the remaining cytoplasts (Vajta *et al*., 2001).

***In Vitro* Development (IVD) of Cloned Bovine (BO-OV) Embryos via iSCNT**

The reconstructed cloned bovine (BO-OV) embryos generated through iSCNT via HMC were chemically activated by incubating them in 1 mL of T2 supplemented with 8 μg/mL of calcium ionophore (A23187) for 5 min, followed by three washes in 1 mL of T20. They were then transferred to 200 μL of Cleavage medium (Cook Medical, IN, USA) containing 2 mM 6-DMAP (6-dimethylaminopurine), covered with mineral oil, to initiate nuclear reprogramming (Vazquez-Avendaño *et al*., 2024). Each embryo was individually placed in 2 μL microdrops of the same supplemented Cleavage medium and incubated for 4 h under previously described conditions.

For embryo culture, the Well of the Well (WOW) system was used. Microwells were created inside a four-well dish (Nunc, USA) using a punch tool, corresponding to the number of viable reconstructed cloned embryos (Navarro-Maldonado *et al*., 2015). Each cloned embryo was placed individually into a microwell containing 100 μL of Cleavage medium covered with mineral oil and incubated for 7 days under the same conditions to assess the IVD rate.

Development of Ovine Parthenogenetic Embryos (G-OV)

A group of ovine oocytes at the MII stage were chemically activated following the same protocol used for cloned embryo activation, in order to produce parthenogenetic embryos as a control for the culture system (G-OV).

Determination of Embryo Quality Based on Number of Nuclei Present

To assess embryo quality based on the number of nuclei, DAPI staining, and epifluorescence microscopy were used. This method allows visualization of blastomere nuclei and analysis of cellular fragmentation.

At the end of IVD, embryos were washed in DPBS without antibiotics and fixed in 4% paraformaldehyde for 15 min. They were then incubated in a DAPI (4',6-diamidino-2-

phenylindole) solution at 1 μg/mL for 10 min, covering the culture dish. Excess stain was removed by washing with DPBS. For evaluation under epifluorescence microscopy, UV-2A filters with a wavelength range of 330-380 nm were used. The presence of nuclei in the blastomeres was observed (Tarnowski *et al.*, 1991). To compare the IVD rate between the BO-OV and G-OV groups, a two-tailed paired Student's t-test was applied using GraphPad Prism version 9.5 (MA, USA), with statistical significance set at $p<0.05$.

RESULTS AND DISCUSSION

Bovine Fibroblast Cultures

After five cell passages, a total fibroblast concentration of 7,892,900 cells was obtained, consistent with the surface area provided for cell growth. These results align with findings reported by Takashima (1998) and Seluanov *et al.*, (2010), who concluded that by the fifth passage, cells reach genetically stable, suitable conditions for use as nuclear donors. Similarly, Baranyi *et al.*, (2019) and Cortez *et al.*, (2021) agree that genetic stability and appropriate phenotypic characteristics are maintained between the fifth and tenth passages. In contrast, exceeding this range may lead to genetic drift, changes in growth rate, and morphological alterations.

In Vitro Maturation of Ovine COC

Five experimental replicates were performed using 364 ovine ovaries, from which a total of 614 oocytes were collected (100% of the initial group), averaging 1.6 oocytes per ovary. Of these, 352 oocytes (57%) were classified as grades A and B according to ASEBIR criteria (2015). The next selection focused on oocytes that successfully reached IVM (MII stage); among the 352 selected, 216 oocytes (61.36%) achieved MII. However, only 200 were used as cytoplasts following enucleation.

Cloning of Bovine-Ovine (BO-OV) Embryos via iSCNT

From the 200 enucleated ovine oocytes, a total of 100 cytoplast-karyoplast-cytoplast triplets were successfully formed.

Table 1 and Figure 1 show the *in vitro* development (IVD) results of bovine-ovine cloned embryos (BO-OV) (n=100) versus ovine parthenogenetic embryos (G-OV) (n=82), after 5 days of *in vitro* culture, respectively.

Of the 100 reconstructed cloned bovine embryos produced via iSCNT, 49 (49%) underwent cleavage at various stages of IVD, while 51 (51%) exhibited fragmentation. The highest developmental stage reached was the morula, with 15 embryos containing 15 to 20 blastomeres (30.61%). These results surpass those reported by Hamilton (2004), who achieved only 4 cloned embryos (19%) beyond the 8-cell stage using iSCNT. However, they fall short of the outcomes reported by Tecirlioglu *et al.*, (2006), who obtained a blastocyst (4.8%). Notably, those authors used fetal bovine fibroblasts and traditional cloning techniques involving micromanipulators for enucleating ovine oocytes and fusing cytoplast-karyoplast pairs. They also cultured cloned bovine embryos using the WOW system. In contrast, the present study employed adult female bovine fibroblasts and manually

Table 1. *In Vitro* Development (IVD) Rate of Bovine-Ovine Cloned Embryos (BO-OV) (n=100) and Ovine Parthenogenetic Embryos (G-OV) (n=82) at 5 Days of Culture.

Embryo Type	Total (N)	Fragmented (%)	Cleaved (%)	Embryonic Cleavage Stages Based on the Number of Nucleated Cells				
				<6 cells	7 - 10 cells	11 - 14 cells	Morulae 15 - 20 cells	Blastocysts N (%)
				N (%)	N (%)	N (%)	N (%)	
BO-OV	100	51 (51)	49 (49)	11 (22.44)	11 (22.44)	12 (24.48)	15 (30.61)	0 (0)[a]
G-OV	82	27 (32.92)	55 (67.07)	6 (10.90)	7 (12.72)	14 (25.45)	8 (14.54)	20 (36.36)[b]

Different superscript letters (a, b) indicate statistically significant differences (P=0.05).

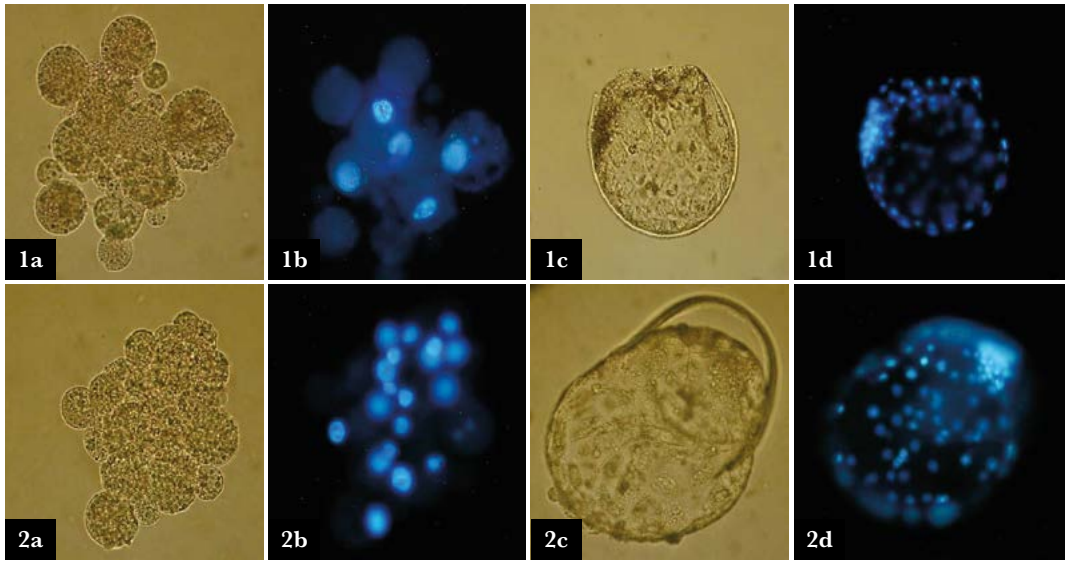


Figure 1. Bright-field and dark-field images of cloned bovine embryos and ovine parthenogenetic embryos produced via iSCNT and stained with DAPI. 1a) Fragmented cloned bovine embryo under bright-field microscopy. 1b) Fragmented cloned bovine embryo under dark-field microscopy, showing blastomeres lacking visible nuclei. 2a) Cloned bovine morula with 20 blastomeres under bright-field microscopy. 2b) Cloned bovine morula with 20 blastomeres under dark-field microscopy. 1c) Ovine parthenogenetic embryo at early blastocyst stage under bright-field microscopy. 1d) Ovine parthenogenetic embryo at the early blastocyst stage under dark-field microscopy. 2c) Ovine parthenogenetic embryo at hatched blastocyst stage under bright-field microscopy. 2d) Ovine parthenogenetic embryo at hatched blastocyst stage under dark-field microscopy. Images were captured using an epifluorescence microscope equipped with a UV-2A filter (wavelength 330-380 nm) at 400X magnification.

enucleated ovine oocytes using the HMC technique, and fused cellular triplets (Vajta *et al.*, 2001). Handmade cloning does not require micromanipulators for enucleation, and the oocytes are stripped of their ZP.

Development of Ovine Parthenogenetic Embryos (G-OV)

To rule out that the low rates of BO-OV clone embryos were due to culture failures, a group of 82 ovine oocytes was activated to produce parthenogenetic embryos (G-OV). After 7 days of IVD, 55 embryos (67.07%) underwent cleavage at various stages, while 27 (32.92%) showed fragmentation. The rate of parthenogenetic blastocyst formation was 20

embryos (36.36%), distributed as follows: 6 early blastocysts (30%), 4 late blastocysts (20%), 4 hatching blastocysts (20%), and 6 hatched blastocysts (30%).

DAPI staining confirmed the presence of blastomere nuclei in the ovine parthenogenetic embryos (G-OV) that reached the blastocyst stage (Figure 2).

It is known that in iSCNT, the degree of embryonic cleavage depends on the mitochondrial genome of one of the two species involved in the cloning process. Therefore, the fusion, activation, and development of cells from genetically distinct species often result in poor embryo development. One possible cause is reduced ATP production, coupled with the failure of interaction between subunits encoded by mitochondrial DNA (mtDNA) and the polypeptides involved in the electron transport chain (Jiang *et al.*, 2011; Selokar *et al.*, 2011).

The condition of heteroplasmy observed in cloned animals —where different mtDNA genotypes coexist within the cloned embryo, originating from both the somatic donor cell and the recipient oocyte— is a factor that leads to metabolic disturbances. This is because the presence of mitochondria from different origins disrupts the respiratory chain and genetic stability, creating mitochondrial incompatibility that affects development and long-term health. Such incompatibility may influence physiological traits and predispose individuals to mitochondrial diseases, ultimately impacting reproductive efficiency (Mrowiec *et al.*, 2021).

On the other hand, although the present study aligns with findings by Jiang *et al.*, (2011), Seaby *et al.*, (2013), Saini *et al.*, (2018), and Galli *et al.*, (2021) who demonstrated that mammalian oocytes contain the necessary information to reprogram donor nuclei by dedifferentiating them to produce cloned embryos, even across unrelated species —this nuclear reprogramming remains incomplete. This is because the donor nucleus can induce abnormal epigenetic modifications that disrupt the expression of key genes involved in early embryonic development, such as: OCT4 (regulator of pluripotency), T-FAM (responsible for mitochondrial DNA stability, maintenance, and transcriptional control), BAX (pro-apoptotic), BCL-2 (anti-apoptotic), and GJA1 (major gap junction protein expressed in cumulus cells) (Ishino *et al.*, 2018; Mrowiec *et al.*, 2021; Adams *et al.*, 2022; Melo *et al.*, 2022).

Additionally, embryo cloning increases the incidence of developmental asynchrony, leading to a higher rate of developmental arrest compared to *in vivo* conditions. This explains the elevated fragmentation rate observed in early stages of *in vitro* development (Mrowiec

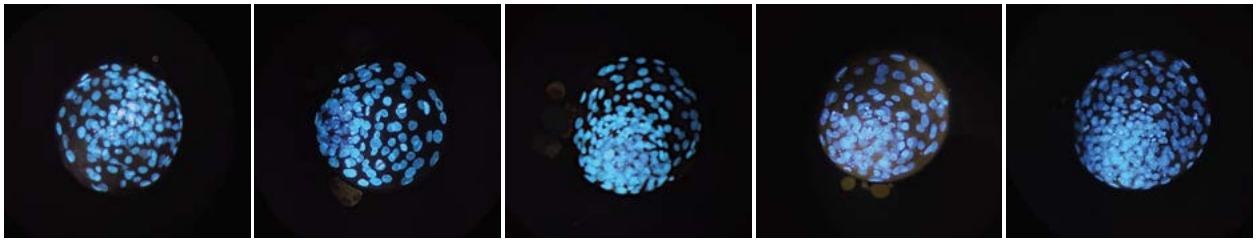


Figure 2. Ovine Parthenogenetic Blastocysts Stained with DAPI. Nuclei and the spatial arrangement of cells within the inner cell mass (ICM) and trophectoderm are clearly visible. Images were captured using an epifluorescence microscope equipped with a UV-2A filter (wavelength 330-380 nm) at 400X magnification.

et al., 2021; Adams *et al.*, 2022). Furthermore, when the taxonomic relationship between cytoplasts and karyoplasts is distant, embryonic development tends to be blocked at early stages. In contrast, development progresses more effectively when donor and recipient cells originate from species with close taxonomic proximity in terms of reproductive physiology, placental type, and gestation duration (Miyamoto, 2019; Malin *et al.*, 2022).

Interspecies Somatic Cell Nuclear Transfer could contribute to the generation of maternal-embryonic cell lines, which may be used for recloning (Loi *et al.*, 1998; Cibelli *et al.*, 2002). This would expand our understanding of embryonic development, as well as the enzymatic and genetic components involved, including incompatibility between mitochondrial DNA (mtDNA) and nuclear DNA, mitochondrial heteroplasmy, challenges in embryonic genome reactivation, and issues related to communication between mtDNA and nuclear genes (Mrowiec *et al.*, 2021).

CONCLUSIONS

Although ovine oocytes were capable of initiating the *in vitro* development of cloned bovine embryos using HMC, species divergence remains a critical factor in achieving the blastocyst stage. Nevertheless, the use of cytoplasts from species different from the nuclear donor opens up possibilities for generating embryos in species that share physiological and reproductive traits, offering a potential strategy for safeguarding valuable genetic material.

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