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Zona pellucida removal modifies the expression and release of specific microRNAs in domestic cat blastocysts

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Research Article

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Summary

The *in vitro* culture of domestic cat embryos without the zona pellucida affects their implantation capacity. MicroRNAs (miRNAs) have an important role in embryo–maternal communication and implantation. The objective of this study was to evaluate the expression of specific miRNAs in domestic cat blastocysts cultured without the zona pellucida. Two experimental groups were done: (1) domestic cat embryos cultured with the zona pellucida (zona intact control group, ZI); and (2) cultured without the zona pellucida (zona free group, ZF). The cleavage, morula and blastocyst rates were evaluated. The blastocysts and their spent medium were used for miRNA expression analysis using RT-qPCR (*miR-21, miR-24, mi25, miR-29, miR-96, miR-98, miR-103, miR-191, miR-196, miR-199, miR-130, miR-155* and *miR-302*). The pre-mature microRNAs (pre-miRNAs) and miRNAs were evaluated in the blastocysts and only miRNAs were evaluated in the spent medium. No differences were observed in the cleavage, morula and blastocyst rates between the ZF and ZI groups ($P > 0.05$). For miRNAs analysis, *miR-103* and *miR-191* had the most stable expression and were selected as internal controls. ZF blastocysts had a higher expression of *miR-21*, *miR-25*, *miR-29* and *miR-199* and a lower expression of *miR-96* than their ZI counterparts ($P < 0.05$). Furthermore, higher levels of *miR-21*, *miR-25* and *miR-98* were detected in the spent medium of ZF blastocysts ($P < 0.05$). In conclusion, *in vitro* culture of domestic cat embryos without the zona pellucida modifies the expression of *miR-21*, *miR-25*, *miR-29*, *miR-199* and *miR-96* at the blastocyst stage and the release of *miR-21*, *miR-25* and *miR-98*.

Introduction

Significant progress has been made in the *in vitro* embryo production of domestic cats and wild felids in the last 40 years (Pope, 2019a, 2019b). However, the developmental capacity after embryo transfer (ET) remained low compared with other species as bovine and murine (Veraguas *et al*., 2017a; Veraguas *et al*., Veraguas *et al.,* 2020a). *In vivo* developmental capacity decreased even more when felid embryos were generated using SCNT or interspecific SCNT (iSCNT) (Gómez *et al*., 2009). Removal of the zona pellucida has been used to enhance the production of cloned embryos and live offspring by handmade cloning and embryo aggregation in different species (Rodríguez-Alvarez *et al*., 2010; Gambini *et al*., 2012; Buemo *et al*., 2016). Embryo aggregation enhances the developmental capacity of domestic cat cloned embryos and embryos from cheetah, tiger and kodkod generated using iSCNT (Moro *et al.,* 2015a, 2015b; Veraguas *et al.,* 2020b). However, *in vitro* culture of domestic cat embryos without the zona pellucida affects the gene expression pattern at the blastocyst stage (Veraguas-Davila *et al.,* 2021). Domestic cat blastocysts cultured without the zona pellucida had reduced expression of *SOX2* and *NANOG* and increased expression of *BAX* (Veraguas-Davila *et al.,* 2021). This did not affect the *in vitro* development of ZF domestic cat embryos, but no implantations were achieved after ET (Veraguas-Davila *et al.,* 2021). Furthermore, ZF domestic cat blastocysts had an increased expression of trophectoderm genes *YAP1* and *EOMES* (Veraguas-Dávila *et al*., 2022). Similarly, in ZF mouse blastocysts ICM and TE had altered expression of differentiation genes and the embryo implantation rate was lower compared with ZI embryos (Fan *et al*., 2022). This indicates that the removal of the zona pellucida affects the expression of pluripotency and differentiation genes, along with the implantation capacity of *in vitro*-produced embryos in some species.

Mechanisms involved in maternal recognition and implantation of domestic cat embryos are barely understood. The molecular signals that allow maternal recognition during the

development of felid embryos have been scarcely studied. Furthermore, different studies have hypothesized that an intact zona pellucida must be present in domestic cat embryos to ensure implantation after ET (Kanda *et al.*, 1995; Denker, 2000; Pope, 2014; Veraguas-Davila *et al.*, 2021). The surface of the zona pellucida in domestic cat oocytes contains numerous spherical and elliptical pores of different sizes (Lunn and Wright, 2009). The porous nature of this matrix allows the penetration of specific molecules; this does not depend on the relative size of the molecules but on other biochemical and physicochemical properties (Prasad *et al.*, 2000). Possibly, the zona pellucida of domestic cat embryos has a crucial role in the regulation of molecular signals during maternal recognition and implantation.

Most miRNAs inhibit protein synthesis by repressing translation, promoting mRNA deadenylation or inducing mRNA scission (Krol *et al.*, 2010). For this reason, miRNAs have a crucial role in cell-to-cell communication (Gross *et al.*, 2017a). Extracellular miRNAs can be detected in all the biological fluids participating in several physiological and pathological processes (Turchinovich *et al.*, 2013). Furthermore, all types of *in vitro*-cultured cells release miRNAs into the medium by binding to apolipoproteins, inside extracellular vesicles (EVs) or during apoptosis (Hawke *et al.*, 2021b; Gong *et al.*, 2017; Zhang *et al.*, 2016). Different miRNAs have been involved in embryo–maternal communication, some of them are released by endometrial cells and others by preimplantation embryos (Abu-Halima *et al.*, 2017; Gross *et al.*, 2017b; Capalbo *et al.*, 2016). The miRNAs expressed by preimplantation embryos are involved in trophectoderm formation, invasion, and implantation (Doridot *et al.*, 2013; Paul *et al.*, 2019). Significant differences in the expression profile of miRNAs have been detected in implanted blastocysts compared with non-implanted ones, and between receptive and non-receptive endometrium (Liu *et al.*, 2016; Paul *et al.*, 2019). Furthermore, several miRNAs described in the spent culture medium of *in vitro*-produced embryos have been proposed as biomarkers of embryo quality or predictors of *in vivo* development (Kropp *et al.*, 2014; Kropp and Khatib, 2015). Different miRNAs have been related to *in vitro* fertilization (IVF) failure and embryo degeneration (*miR-25*), morula to blastocyst transition (*miR-130*), blastocyst formation (*miR-24*, *miR-199a-5p*, *miR-196*, *miR-302*), trophoblast differentiation, invasion, and migration (*miR-21*, *miR-96*, *miR-29*, *miR-155*), and implantation and immune response (*Let7/miR-98*) (Liang *et al.*, 2017; Reza *et al.*, 2019). Despite that, the evaluation of miRNAs expressed by domestic cat preimplantation embryos has not been reported.

Embryonic miRNAs can traverse the zona pellucida carried by EVs or binding to apolipoproteins (Kim *et al.*, 2019; Hawke *et al.*, 2021a). The zona pellucida, along with other extra-embryonic coats, works as a mailbox between embryos and the maternal environment (Denker, 2000; Herrler and Beier, 2000). We hypothesize that *in vitro* culture of domestic cat embryos without the zona pellucida might affect the expression of miRNAs. The abnormal expression of specific miRNAs might be one of the factors that affect the implantation capacity of these embryos. For these reasons, the objective of this study was to compare the expression of specific miRNAs in domestic cat blastocysts cultured with or without the zona pellucida and in the spent culture medium of both groups.

Materials and methods

All chemical reagents were purchased from Sigma-Aldrich Chemicals Company (St. Louis, MO, USA), except for those otherwise indicated.

Ethics statement

All procedures that involved animal manipulation were approved by the Ethics Committee (Comité de Bioética de la Facultad de Ciencias Veterinarias de la Universidad de Concepción). Certificate of approval no. CBE-08-2020.

Animals

Healthy female and male domestic cats aged between 6 months and 5 years were selected as oocyte and sperm donors for *in vitro* embryo production.

Experimental design

Two experimental groups were done. (1) Domestic cat embryos generated by IVF and cultured *in vitro* (Zona intact; ZI group). (2) Domestic cat embryos generated by IVF and cultured *in vitro* without the zona pellucida (Zona free; ZF group). The ovaries from domestic cats were collected by ovariohysterectomy. Immature cumulus–oocyte complexes (COCs) were collected from these ovaries by slicing and then subjected to *in vitro* maturation (IVM). Subsequently, the *in vitro* matured COCs were subjected to IVF using epididymal sperm. Only in the ZF group, the zona pellucida of presumptive zygotes was removed. Then ZF embryos were cultured *in vitro* using the well-of-the-well system (WOW). In addition, ZI embryos were cultured normally in four-well dishes. Embryos were cultured *in vitro* for 7 days. The cleavage, morula and blastocyst rates were evaluated. The total cell number of blastocysts was estimated. Finally, the expression of pre-miRNAs and mature miRNAs (*miR-21, miR-24, miR-25, miR-29, miR-98, miR-103, miR-130, miR-155, miR-191, miR-196, miR-199, miR-302* and *miR-96*) was evaluated in blastocysts by RT-qPCR. In addition, only mature miRNAs were evaluated in spent medium. The geometric mean of *miR-103* and *miR-191* was used as an internal control.

Ovariohysterectomy and COCs collection

The ovaries of female domestic cats were collected by ovariohysterectomy. Anaesthesia was induced using an (i.m.) dose of 0.5 mg/kg xylazine (Xilazina 2%, Virbac Chile) and 5 mg/kg ketamine (Ketamina 100; Chemie Chile) and maintained by administering xylazine/ketamine (i.v.) at the same dose. Atipamezole (Antisedan, Zoetis) was used to eliminate the effects of xylazine once the surgical procedure ended, using a unique dose of 0.02 to 0.05 ml ovaries were transported in a sterile solution containing 0.9% NaCl and 0.1% gentamycin (Gentamicina 10%, Veterquimica, Chile) at 38.5°C. Immature COCs were recovered from the ovaries by slicing in a 100 mm Petri dish containing 10 ml of medium-199 with Earle's salts supplemented with 0.18 mM HEPES, 5% fetal bovine serum (FBS) and 50 µg/ml gentamycin (He199) at 38.5°C. Only grade I and grade II immature COCs were subjected to IVM.

In vitro *maturation*

In vitro maturation of immature COCs was performed in four-well dishes containing 500 µl of medium-199 with Earle's salts supplemented with 0.3% fraction-V BSA, 0.1 IU/ml FSH-LH (Pluset, Serono, Italy), 1 µg/ml 17β-estradiol, 0.36 mM sodium pyruvate, 2 mM glutamine, 2.2 mM calcium lactate, 20 ng/ml epidermal growth factor (EGF), 10 µl/ml insulin, transferrin, selenium (ITS) and 50 µg/ml gentamycin (IVM-199) in an humidified gas atmosphere with 5.0% CO_2, at 38.5°C for 24–26 h.

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Sperm collection

Only male domestic cats older than 10 months of age were used as sperm donors. The anaesthesia procedure was the same as that described for ovariohysterectomy. Additionally, lidocaine (Lidocalm, 2%, Dragpharma, Chile) was administered in the genital area as local anaesthesia. Testes were transported in sterile 0.9% NaCl solution with 0.1% gentamycin at room temperature. The caudal portions of the epididymis were cut into small pieces of ~1 mm in a 100 mm Petri dish containing 10 ml of He199 supplemented with 0.3% Fraction-V BSA instead of FBS (He199-BSA) at 38.5°C. The medium with the sperm was passed through a sterile nylon filter (40 µm) into a 50 ml sterile tube. The filtrate was centrifuged at 1500 rpm for 5 min, and the pellet was resuspended in 5 ml of 20% Andromed medium (diluted in sterile distilled water according to the manufacturer's instructions; Minitube, Tiefenbach, Germany) and refrigerated at 4°C for 24 h (Veraguas *et al.*, 2020a).

In vitro *fertilization*

Once IVM was finished, COCs were washed in TALP medium supplemented with 6 mg/ml BSA, 0.36 mM sodium pyruvate, 1 mM glutamine, 2.2 mM calcium lactate, 1% MEM nonessential amino acids (NEAA), 0.5% MEM essential amino acids (EAA), 0.01 mg/ml heparin sodium salt and 50 µg/ml gentamycin (TALP-IVF). Refrigerated sperm were allowed to swim up for 30 min in He199-BSA at 38.5°C (Veraguas *et al.*, 2020a). The supernatant was collected and then centrifuged at 1500 rpm for 5 min. The pellet was collected and resuspended in TALP-IVF. For IVF, 20–30 COCs were placed in four-well dishes containing 500 µl of TALP-IVF and co-incubated with 1.5 to 2.5 × 10^6 spermatozoa/ml, in a humidified atmosphere of 5% CO_2 in air, at 38.5°C, for 24 h. Subsequently, cumulus cells were removed from the presumptive zygotes using a 0.5 mg/ml hyaluronidase solution and vortexed for 6 min.

In vitro *embryo culture*

In the ZI group, presumptive zygotes were cultured in supplement SOF medium (Veraguas *et al.*, 2018). However, FBS was replaced by ITS (10 µl/ml) to avoid the presence of contaminating miRNAs carried by serum-EVs. The culture was done in four-well dishes containing 500 µl of SOF medium supplemented with 0.37 mM trisodium citrate, 2.77 mM myo-inositol, essential and nonessential amino acids (final concentration 1×), 50 µg/ml gentamycin, 10 µl/ml ITS, 20 ng/ml EGF and 3 mg/ml essentially fatty acid-free BSA (SOF-B); 10–20 embryos were placed into each well.

In the ZF group, the zona pellucida from presumptive zygotes was removed by incubation in 2 mg/ml of pronase for 4 min, at 37°C. Subsequently, presumptive zygotes were washed three times in He199 supplemented with 30% FBS (He199-30) to remove pronase, and then washed three times in SOF-B to eliminate FBS. The ZF embryos were cultured in four-well dishes using the WOW system (Vajta *et al.*, 2008). In the WOW system, several microwells were made in the culture dish, and each ZF embryo was individually cultured in one microwell to prevent the disaggregation of blastomeres (Vajta *et al.*, 2008). In this study, the microwells were created using an aggregation needle (DN-09N, BLS Ltd). Eighty microwells were made in a four-well dish, and up to 20 ZF embryos were cultured per well.

In both experimental groups, on day 2 of *in vitro* culture (IVC), cleavage embryos were selected and the rest were discarded. At day 5 of IVC, morulae were selected, and the remaining embryos were discarded. The morulae were cultured in medium-199 with Earle's salts supplemented with 0.37 mM trisodium citrate, 2.77 mM myo-inositol, essential and nonessential amino acids (final concentration 1×), 50 µg/ml gentamycin, 3 mg/ml essentially fatty acid-free BSA, 10 µl/ml ITS and 20 ng/ml EGF (M199-IVC). The culture was carried out in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C, for 7 days. The cleavage, morula and blastocyst rates were estimated on days 2, 5 and 7, respectively. For sample collection, only embryos at the blastocyst stage on day-7 of culture were selected.

Morphological evaluation of the blastocysts

Blastocysts were fixed in a 3% glutaraldehyde solution for 72 h at 4°C. Fixed blastocysts were stained with 5 µg/ml Hoechst 33342 for 20 min. Stained embryos were placed on a slide with a drop of glycerin and then covered with a coverslip. Visualization was achieved using the EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific).

Gene expression analysis

RNA extraction and gDNA digestion in blastocyst samples

For the analysis of pre-miRNAs and miRNAs, ZI and ZF day-7 blastocysts were pooled within their respective groups. Ten pools were made in both groups; pools had five day-7 blastocysts. The samples were stored at −80°C.

Total RNA extraction of blastocysts was performed using the Ambion® Cells-to-cDNA™ II Kit (Thermo Fisher Scientific, Austin, TX, USA) as previously described (Veraguas *et al.*, 2018). Blastocyst samples were washed three times in cold PBS to eliminate the remaining culture medium. For this, 100 µl of PBS was added and then samples were centrifuged at 11,000 rpm for 5 min. Next, 50 µl of lysis buffer was added and the samples were incubated at 75°C for 10 min. For gDNA digestion, the samples were treated with DNase I (0.04 U/µl) and incubated at 37°C for 15 min and at 75°C for 5 min.

cDNA synthesis of pre-mature miRNAs in the blastocyst samples

The cDNA synthesis for the pre-miRNAs analysis was done using the SuperScript™ IV First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific). Next, 10 µl of total RNA was used in a 20-µl final reaction containing: 50 ng/µl random primers hexamers and 10 mM of each dNTP. The mixture was incubated at 65°C for 5 min and kept at 4°C for at least 1 min. Then, the reaction was completed by adding 4 µl 5× SS IV buffer, 1 µl 100 mM DTT, 1 µl ribonuclease inhibitor and 1 µl SuperScript™ IV reverse transcriptase (200 U/ µl). The mixture was centrifuged and then incubated at 23°C for 10 min, 55°C for 10 min and 80°C for 10 min. The cDNA samples were kept at −20°C until qPCR was performed.

miRNA extraction in medium samples

For this, 400–500 µl of culture medium was collected only in the wells in which the morulae reached the blastocyst stage on day 7 of IVC. miRNA extraction of medium samples was carried out using the mirPremier® microRNA Isolation Kit (SNC10). This kit uses a purification column system, which allows to concentrate medium samples in a small volume (30–40 µl). The samples were first

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Table 1. Primer sequences and RT-qPCR conditions used for the expression analysis of pre-microRNAs

Pre-miRNAs	Primer sequences (5′→3′)	Annealing T (°C)	Product length (bp)	*Accession numbers (NCBI)
Let-7/mir98	F: CCAGGGAAAGTAGTAAGTTGTATAGT; R: CCGGGGTGAGGTAGTAAGTT	58	89	NC_018741.3; NR_033116.1
mir21	F: TGTCGGGTAGCTTATCAGACTG; R: TGTCAGACAGCCCATCGA	58	72	NC_000017.11
mir24	F: CGGTGCCTACTGAGCTGAT; R: CGACTCCTGTTCCTGCTGAA	58	68	NC_000079.7; CM001390.3
mir25	F: GGCCGGCACTGTCAGACCGAGA; R: GGCCAGCGTTGAGAGGCGGAGA	58	83	CM001393.3; NR_030941.1
mir29	F: CTCCCCACCAAACGATGA; R: TGAAGAATTGATCCCCAGTCA	58	101	NC_007115.7
mir130	F: TGCGGGCCGGAGCTCTTTT; R: TACACGGCCAATGCCCTTTTAAC	58	83	CM001387.3
mir155	F: CATCCTCCGAGTGCTGG; R: CCTATCACGATTAGCATTAACAGC	58	61	NC_037328.1
mir191	F: TGGCAGGAGAGCAGGGAA; R: GGCTGGACAGCGGGCAA	58	91	CM001379.3
mir196	F: CTGAGTGAATTAGGTAGTTTCATGTTG; R: CGTGAGTCGGGTGGTTTAAT	58	75	CM001391.3; NR_030828.1
mir199	F: CGCTTACCCGAGCCCAGCCTAAC; R: GGACAGCCGGCCCCGCC	58	101	NC_018724.3
mir302	F: CCACCACTTAAACGTGGATGTA; R: CAGCGTAAGTATACCAGACAGG	58	150	CM001381.3; NR_049443.1
mir96	F: TTTCCCATATTGGCACTGCAC; R: TGGCCGATTTTGGCACTA	58	78	NC_018724.3
mir103	F: TTGTGCTTTCAGCTTCTTTACA; R: CGGTTCTTTCATAGCCCTGT	58	78	CM001380.3; NR_049450.1
mir103	F: GCAGAGCAGCATTGTACAG; R: GGTCCAGTTTTTTTTTTTTTTTCATAG	58	23	CM001380.3; NR_049450.1:48–70
mir103	F: GCAGAGCAGCATTGTACAG; R: GGTCCAGTTTTTTTTTTTTTTTCATAG	58	23	CM001380.3; NR_049450.1:48–70

*Two accession numbers indicate that sequences alignment was done, a common sequence between domestic cat (*Felis silvestris catus*) and different species was uses for primer design: dog (*Canis lupus familiaris*); human (*Homo sapiens*); zebrafish (*Danio rerio*); horse (*Equus caballus)*; bovine (*Bos taurus*); mouse (*Mus musculus*).

centrifuged at 10,000 *g* for 5 min to remove cellular debris; 300–400 µl of supernatant were transferred and used in the next steps. The lysis mix supplement with 2-mercaptoethanol (1 µl/ml) was added to the medium samples in 1:1 (vol/vol) and then samples were incubated at 55°C for 5 min. Samples were centrifuged at 15,000 *g* for 5 min to remove gDNA and large RNA, the supernatant was transferred, and the pellet was discarded. Then, a 1.1 volume of 100% ethanol was added to the samples and mixed by vortexing. The binding, wash, and dry column steps were carried out according to the manufacturer's instructions. Once the dry column step was carried out, 30–40 µl of dilution buffer were added to the columns and then these were centrifuged. Finally, 30–40 µl miRNAs samples were obtained and these were stored at −80°C.

cDNA synthesis of miRNAs in medium and blastocyst samples

To convert miRNAs into cDNA, the MystiCq™ microRNA cDNA Synthesis Mix was used (MIRRT) according to the manufacturer's instructions. For this we used: 7 µl of total RNA from blastocyst samples or 7 µl of purified miRNA from medium samples. A final reaction of 20-µl volume was made. First, the poly(A) tailing reaction was done with 2 µl of poly(A) tailing buffer (5×) and 1 µl of poly(A) polymerase; the mix was centrifuged and then incubated at 37°C for 60 min, and 70°C for 5 min. Finally, the first-strand cDNA synthesis reaction was done by adding: 9 µl of the MystiCq microRNA cDNA reaction mix, and 1 µl of ReadyScript reverse transcriptase. The mixture was centrifuged and then incubated at 42°C for 20 min, and 85°C for 5 min. The cDNA samples were stored at −20°C.

Real-time quantitative polymerase chain reaction (qPCR)

Gene expression analysis was performed by real-time qPCR using the standard curve method as previously described (Veraguas *et al*., 2017b). Standard curves were generated using 2 µl of PCR products for each specific miRNA. These PCR products were purified previously using the E.Z.N.A gel extraction kit (Omega, BioTek, Santiago, Chile) and quantified using a spectrophotometer (Epoch, BioTek Instruments, Inc., Winooski, VT, USA). The standard curves were generated using eight 10-fold dilutions from the PCR products. For qPCR analysis, each sample was loaded in duplicate (technical replicates). PCR was performed using 2 µl of cDNA from samples, 1 µl of primers (10 pmol each, forward and reverse), 5 µl of the KiCqStart SYBR Green ReadyMix, Low ROX (Sigma-Aldrich) and 2 µl of betaine solution (5 M; B0300) in a final volume of 10 µl. The reaction was run on an MX3000P Real-Time PCR device (Agilent, Santa Clara,

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Table 2. Primer sequences and RT-qPCR conditions used for the expression analysis of microRNAs

miRNAs	Primer sequences (5′→3′)	Annealing temperature (°C)	Product length (bp)	*Accession number (NCBI)
mir98	F: GCGCAGTGAGGTAGTAAG; R: CAGGTCCAGTTTTTTTTTTTTTTTAAC	58	22	NC_018741.3; NR_033116.1:22–43
mir21	F: GCAGTAGCTTATCAGACTGATG; R: GGTCCAGTTTTTTTTTTTTTTTCAAC	58	22	NR_029493.1:8–29
mir24	F: GGTGCCTACTGAGCTGA; R: GGTCCAGTTTTTTTTTTTTTTTTACTGA	58	23	CM001390.3; NR_029575.1:6–28
mir25	F: CATTGCACTTGTCTCGGT; R: GGTCCAGTTTTTTTTTTTTTTTCAG	58	22	CM001393.3; NR_030941.1:52–73
mir29	F: CAGTAGCACCATTTGAAATCG; R: GGTCCAGTTTTTTTTTTTTTTTAACC	58	22	NR_030039.1:81–102
mir130	F: GGCTCTTTTCACATTGTGC; R: AGGTCCAGTTTTTTTTTTTTTTTTAGTAG	58	22	CM001387.3; NR_029544.1:3–24
mir191	F: CAACGGAATCCCAAAAGCA; R: TCCAGTTTTTTTTTTTTTTTCAGCT	58	23	CM001379.3; NR_029690.1:16–38
mir196	F: GCAGTAGGTAGTTTCATGTTGT; R: GTCCAGTTTTTTTTTTTTTTTTCCCA	58	22	CM001391.3; NR_030828.1:24–45
mir199	F: CAGACAGTAGTCTGCACATTG; R: GGTCCAGTTTTTTTTTTTTTTTTAACC	58	22	AY194865.1
mir96	F: CAGTTTGGCACTAGCACA; R: GGTCCAGTTTTTTTTTTTTTTTTAGCA	58	23	NR_049449.1:14–36
mir103	F: GCAGAGCAGCATTGTACAG; R: GGTCCAGTTTTTTTTTTTTTTTTCATAG	58	23	CM001380.3; NR_049450.1:48–70

*Two accession numbers indicate that sequences alignment was done, a common sequence between domestic cat (*Felis silvestris catus*) and different species was uses for primer design: dog (*Canis lupus familiaris*); human (*Homo sapiens*); zebrafish (*Danio rerio*); horse (*Equus caballus*); bovine (*Bos taurus*); mouse (*Mus musculus*).

CA, USA). Only experiments with an efficiency within the range 90–110% and a correlation coefficient of at least 0.9 were used for analysis. The software NormFinder was used to select the most stable miRNAs for their use as internal controls (Andersen *et al.*, 2004).

The primers used for the analysis of pre-miRNAs were designed using the RNAcentral database (https://rnacentral.org). These sequences were also verified in the NCBI database for the *Felis catus* species (https://www.ncbi.nlm.nih.gov/genome/?term=felis+catus) and compared against other species. The primers and PCR conditions are shown in Table 1.

To identify the sequences of miRNAs, sequence alignment was carried out between the sequences of pre-miRNAs from *Felis catus* and the sequences of miRNAs from different species using the NCBI database (Table 2). To design miRNAs primers, the software miRprimer was used (Busk, 2014). This software designed miRNA-specific forward and reverse primers, which allowed the use of SYBR green for quantification by RT-qPCR (Forero *et al.*, 2019; Balcells *et al.*, 2011). The primers for miRNAs and their PCR conditions are shown in Table 2.

Statistical analysis

The Wilcoxon nonparametric test was used to evaluate *in vitro* development and gene expression analysis between the ZI and ZF groups. The *t*-test was used to evaluate the total cell number of blastocysts between groups. The statistical software InfoStat was used for these analyses (2020 InfoStat/L Version; University of Cordoba, Argentina).

Results

In vitro *development of domestic cat embryos cultured with or without the zona pellucida*

In the ZF group, presumptive zygotes were cultured in microwells to prevent disaggregation of blastomeres. No statistical differences were found in cleavage, morula and blastocyst rates between the embryos cultured in the ZI and ZF groups ($P > 0.05$) (Table 3). This indicates that the culture system used in this study ensures the *in vitro* development of ZF domestic cat embryos (Figure 1).

Morphological evaluation of domestic cat blastocysts cultured with and without the zona pellucida

Day-7 blastocysts from the ZI and ZF groups were fixed and stained for total cell counting. Ten blastocysts were evaluated in both experimental groups. No statistical differences were observed between the total cell number of blastocysts (mean ± standard deviation, SD) from the ZI (279.3 ± 129.9) and ZF groups (319.3 ± 126.0) ($P > 0.05$; Figure 2).

Expression analysis of pre-miRNAs and miRNAs in domestic cat blastocysts cultured with and without the zona pellucida

The expression of all the pre-miRNAs and miRNAs selected in this study was analyzed using the software NormFinder. According to this, *miR-103* and *miR-191* had the most stable expression among samples (Figure 3). For this reason, the geometric mean of *pre-miR-103* and *pre-miR-191,* along with *miR-103* and *miR-191* were

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Table 3. *In vitro* development of domestic cat embryos cultured with (ZI) and without (ZF) zona pellucida for 7 days.

Groups	N*	Total oocytes	No. cleavage (% mean ± standard deviation, SD)	No. morulae/cleavage (% mean ± SD)	No. blastocysts/cleavage (% mean ± SD)	No. hatching blastocysts/ cleavage (% mean ± SD)
ZI	12	455	201 (44.2 ± 18.1)	95 (47.3 ± 14.1)	56 (27.9 ± 9.2)	9 (4.5 ± 9.8)
ZF	12	585	240 (41.0 ± 19.9)	122 (50.8 ± 26.5)	86 (35.9 ± 19.7)	

*N: number of replicates. No significant differences were found between groups ($P > 0.05$).

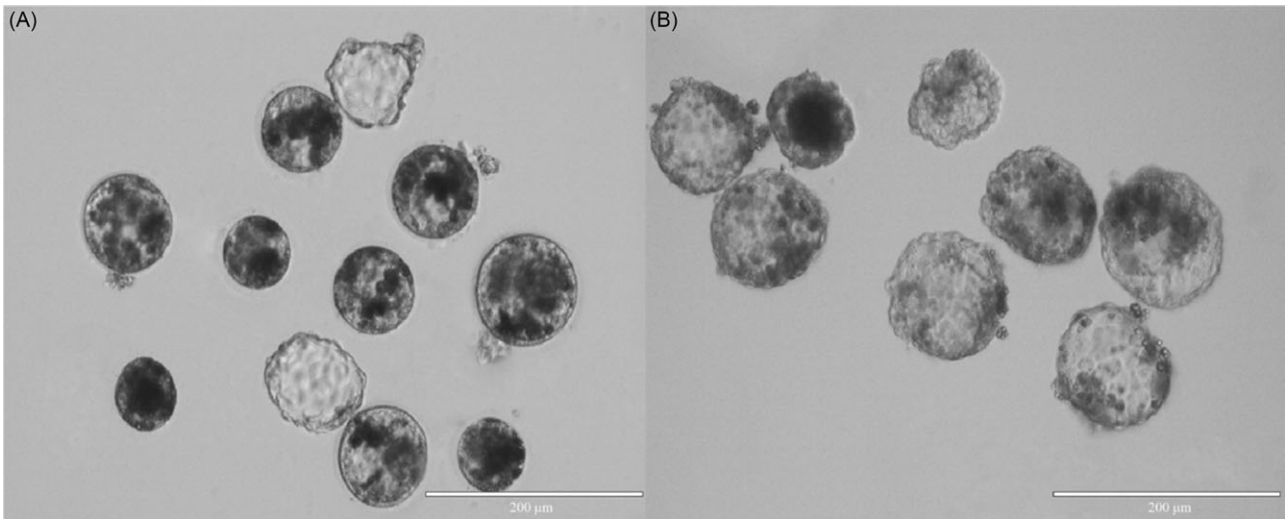

Figure 1. *In vitro* development of domestic cat embryos. (A) Domestic cat blastocysts generated by IVF and *in vitro* cultured for 7 days (20×). (B) Domestic cat blastocysts generated by IVF and *in vitro* cultured without the zona pellucida for 7 days (20×).

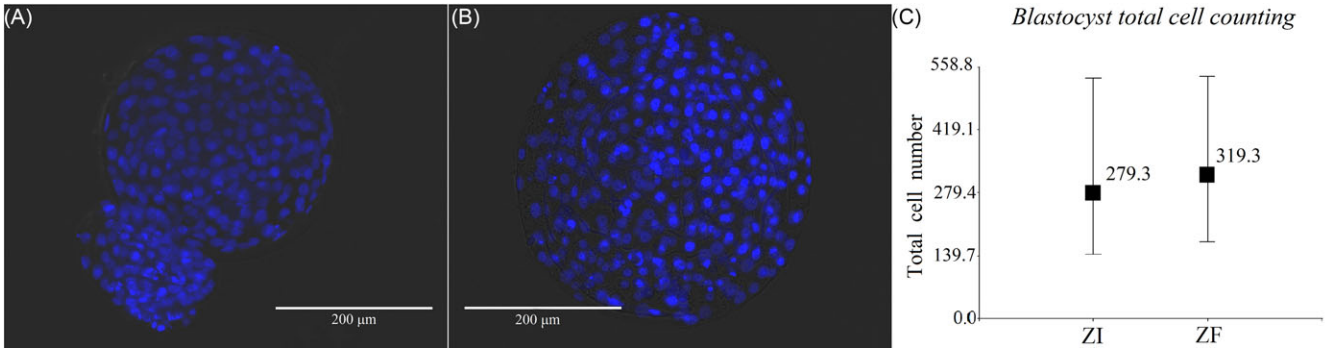

Figure 2. Total cell counting of blastocysts. (A) Hatching domestic cat blastocyst fixed and stained with Hoechst (20×). (B) ZF domestic cat blastocysts fixed and stained with Hoechst (20×). C) Total cell number of blastocysts (mean; min/max) from the ZI (279.3; 143/535) and ZF groups (319.3; 172/539).

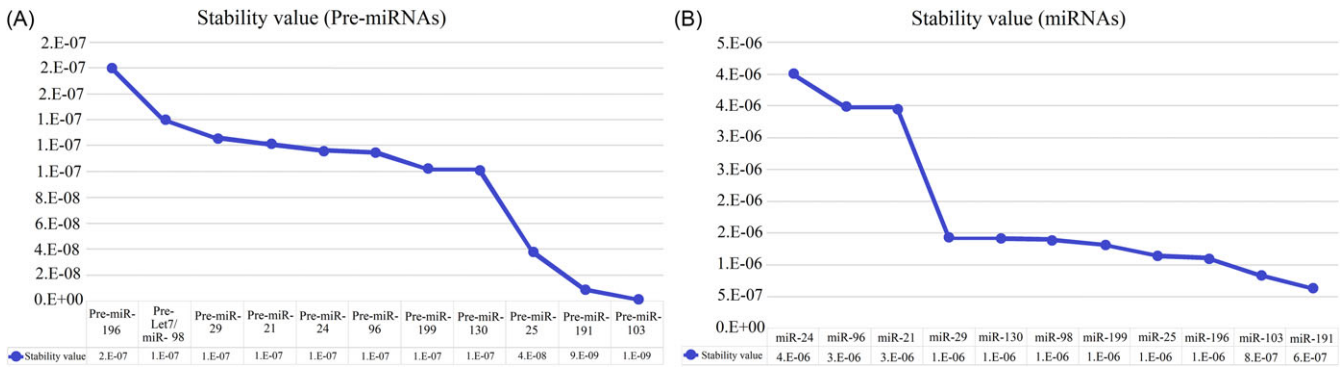

Figure 3. Internal control analysis. Stability value (mean) of pre-miRNAs (A) and miRNAs (B) evaluated in ZI and ZF blastocysts by RT-qPCR. The lower values indicate a higher stability.

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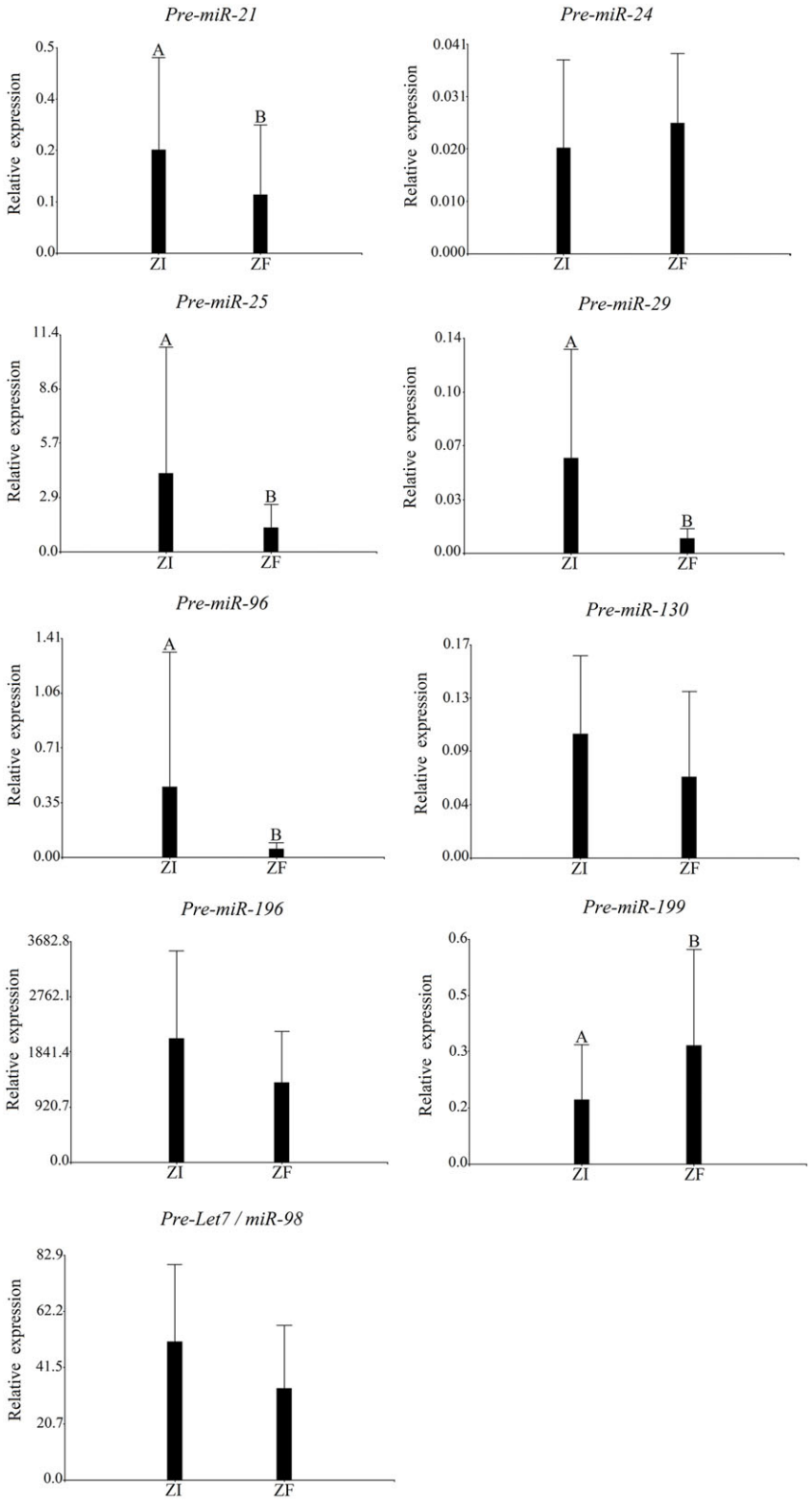

Figure 4. Relative expression analysis (mean ± SD) of pre-miRNAs. *pre-miR21, pre-miR24, pre-miR25, pre-miR29, pre-miR96, pre-miR130, pre-miR196, pre-miR199* and *pre-let7/miR98* were evaluated in day-7 blastocysts from the ZI and ZF groups. The geometric mean of *pre-miR103* and *pre-miR191* was used as an internal control. (A, B) Different superscripts indicate significant differences between groups ($P < 0.05$).

selected as internal controls for the analysis of pre-miRNAs and miRNAs, respectively.

The relative expression of pre-miRNAs was analyzed first. No expression of *pre-miR-155* and *pre-miR-302* was detected in blastocysts from the ZI and ZF groups. No statistical differences were observed in the relative expression of *pre-miR-24*, *pre-miR-130*, *pre-miR-196* and *pre-let7/miR-98* between blastocysts from the ZI and ZF groups ($P > 0.05$). The relative expression of *pre-miR-21*, *pre-miR-25*, *pre-miR-29* and *pre-miR-96* was higher in blastocysts from the ZI group than in those from the ZF group ($P < 0.05$). However, *pre-miR-199* was highly expressed in blastocysts from the ZF group compared with their ZI counterparts ($P < 0.05$; Figure 4).

Regarding the relative expression of miRNAs, no statistical differences were observed in the relative expression of *miR-24*,

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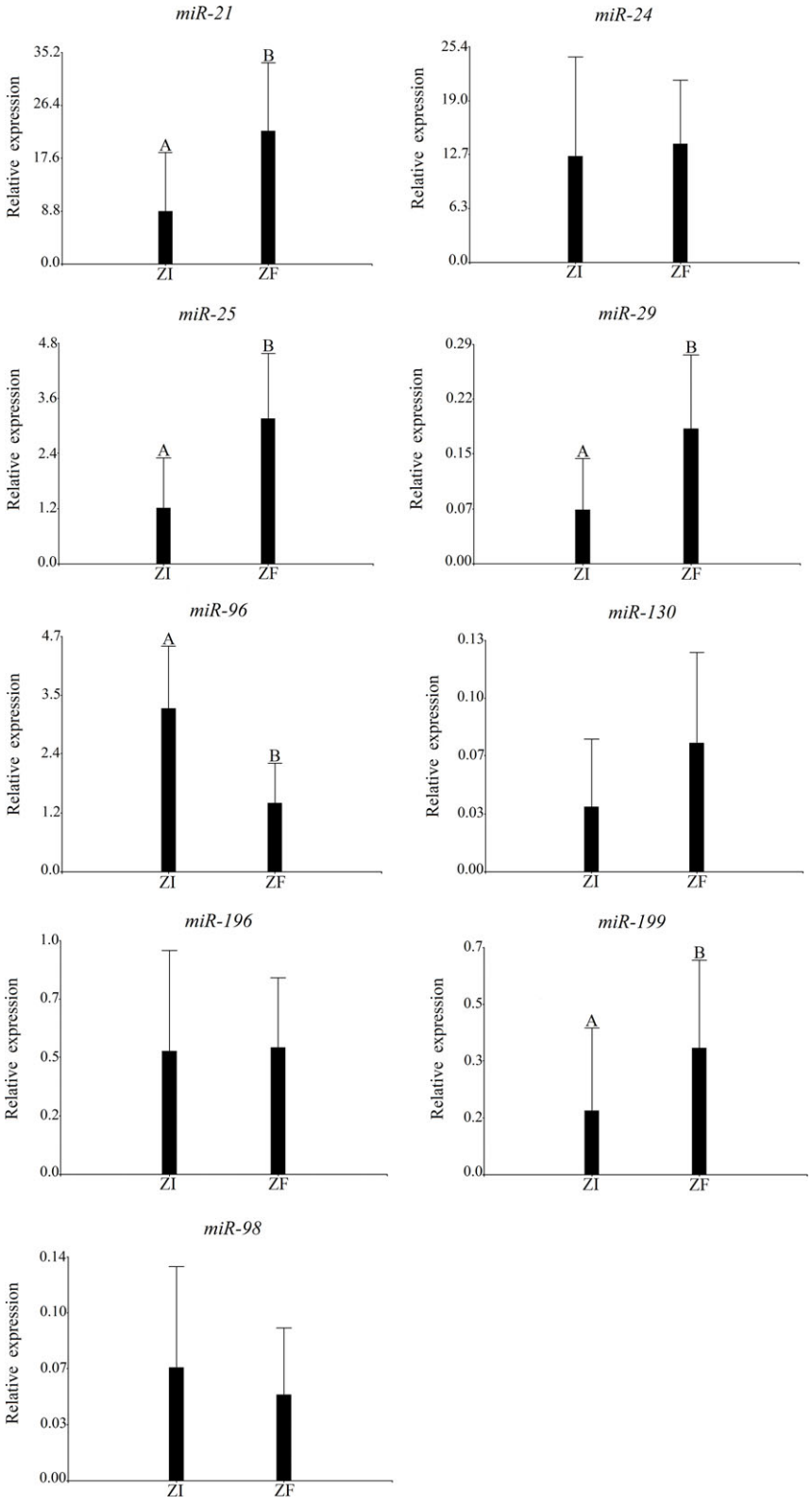

Figure 5. Relative expression analysis (mean ± SD) of miRNAs in ZI and ZF blastocysts. *miR-21, miR-24, miR-25, miR-29, miR-96, miR-130, miR-196, miR-199* and *miR-98* were evaluated in day-7 blastocysts from the ZI and ZF groups. The geometric mean of *miR-103* and *miR-191* was used as an internal control. (A, B) different superscripts indicate significant differences between groups ($P < 0.05$).

miR-130, *miR-196*, and *miR-98* between blastocysts from the ZI and ZF groups ($P > 0.05$). However, the relative expression of *miR-21*, *miR-25*, *miR-29* and *miR-199* was higher in ZF blastocysts than in their ZI counterparts ($P < 0.05$). Furthermore, the relative expression of *miR-96* was lower in ZF blastocysts than in those from the ZI group (Figure 5).

Expression analysis of miRNAs in the spent medium of embryos cultured with and without the zona pellucida

The presence of *miR-103* was scarcely detected in the spent culture medium of ZI and ZF embryos. For this reason only *miR-191* was used as internal control in this type of sample.

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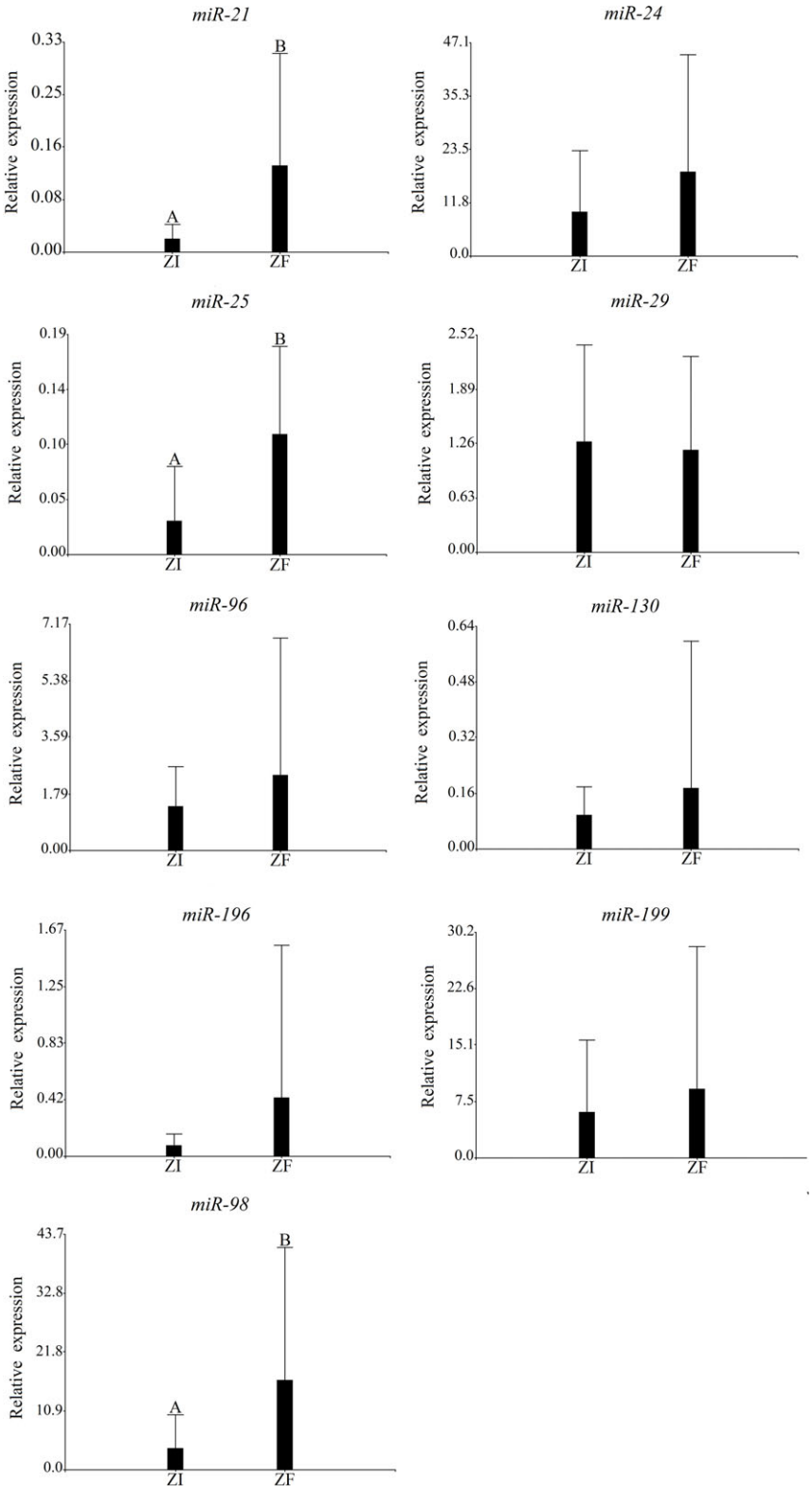

Figure 6. Relative expression analysis (mean ± SD) of miRNAs in spent culture medium. The levels of *miR-21, miR-24, miR-25, miR-29, miR-96, miR-130, miR-196, miR-199* and *miR-98* were evaluated in the spent culture medium of day-7 blastocysts from the ZI and ZF groups. The expression of *miR-191* was used as an internal control. (A, B) different superscripts indicate significant differences between groups ($P < 0.05$).

No statistical differences were found in the relative expression of *miR-24*, *miR-29*, *miR-96*, *miR-130*, *miR-196* and *miR-199* between the spent culture medium from ZI and ZF embryos ($P > 0.05$). However, *miR-21*, *miR-25* and *miR-98* were highly expressed in the spent culture medium from ZF embryos ($P < 0.05$; Figure 6).

Discussion

The results of our study demonstrated that *in vitro* culture of domestic cat embryos without the zona pellucida until the blastocyst stage resulted in the overexpression of *miR-21*, *miR-25*, *miR-29* and *miR-199,* and a reduced expression of *miR-96* compared with blastocysts cultured with an intact zona pellucida.

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This is in accordance with the significant differences observed in the relative expression of *pre-miR-21*, *pre-miR-25*, *pre-miR-29, pre-miR-96* and *pre-miR-199* between ZF and ZI blastocysts. Furthermore, the presence of different miRNAs was detected in spent culture medium, with higher levels of *miR-21*, *miR-25*, and *miR-98* in spent medium of ZF blastocysts. It was described that miRNAs are capable of passing through the zona pellucida, which indicates a possible regulatory role of the zona pellucida (Hawke *et al*., 2021a). In this study, the differences observed in the expression of miRNAs in the blastocysts and their spent medium, could be caused by the absence of the zona pellucida. In addition, the evaluation of these miRNAs might reveal important information about embryo–maternal crosstalk.

In the present study *miR-103* and *miR-191* were the most stable genes according to the NormFinder analysis and were selected as internal controls. Mahdipour *et al.* (2015) described similar results in bovine oocytes and early embryos in which *miR-103* and *miR-93* were the most stable genes. In addition, in porcine oocytes *miR-191* was one of the most stable genes according to the geNorm and BestKeeper software (Mahdipour *et al*., 2015). This allowed us to perform the specific miRNA analysis.

miR-21 is involved in oocyte maturation and embryo development (Dehghan *et al*., 2021). An overexpression of *miR-21* in COCs increases the *in vitro* fertilization, cleavage and blastocyst formation (Dehghan *et al*., 2021). In addition, the expression of *miR-21* significantly increases after implantation (Lv *et al*., 2018). Inhibition of *miR-21* in embryos suppresses *in vitro* and post-implantation development (Lv *et al*., 2018). In addition, over-expression of *miR-21* in preimplantation embryos has been related to an increase in blastocyst formation by regulation of the apoptosis process (Shen *et al*., 2009; Zhang *et al*., 2014). Overexpression of *miR-21* has been associated with the upregulation of *BCL2L1* and the downregulation of *CASP3* (Shen *et al*., 2009). Furthermore, the induction of *miR-21* in mesenchymal stem cells inhibits the expression of *SOX2*, regulating proliferation and differentiation (Trohatou *et al*., 2014). In the present study, *miR-21* was higher in ZF domestic cat blastocysts and in their spent culture medium. Despite that, we did not observe an increase in blastocyst formation rate. We previously described a lower expression of *SOX2* and an increased expression of *BAX* in ZF domestic cat blastocysts (Veraguas-Davila *et al.,* 2021). Despite the higher expression of *BAX*, no alteration was found in the expression of *CASP3* (Veraguas-Dávila *et al*., 2022). The overexpression of *miR-21* might be related to lower expression of *SOX2* and to the regulation of *CASP3* in ZF domestic cat blastocysts.

The presence of *miR-25* has been detected in spent culture medium of bovine blastocysts and degenerated embryos (Kropp *et al.,* 2014). Degenrated embryos and their spent medium had an overexpression of *miR-25* compared with embryos that reached the blastocyst stage (Kropp *et al*., 2014). Furthermore, overexpression of *miR-25* in circulating exosomes has been associated with embryonic mortality in cows (Pohler *et al*., 2017). In the present study, *miR-25* was highly expressed in ZF domestic cat blastocysts and in their spent medium without affecting the blastocyst formation rate. However, this might be related to the reduced implantation rate of the ZF domestic cat blastocysts (Veraguas-Davila *et al.,* 2021).

The *miR-29* family has an important role in the expression of pluripotency genes and cell reprogramming (Liang *et al*., 2018; Hysolli *et al*., 2016; Wang *et al*., 2016). An overexpression of *miR-29a* or *miR-29b* during somatic cell reprogramming increases the expression of pluripotency genes such as *OCT4* and *SOX2* (Liang *et al*., 2018; Hysolli *et al*., 2016). Furthermore, *miR-29a* is overexpressed in rat uterus during implantation and binds to the pro-apoptotic genes *BAK1* and *BMF* leading to inhibition of apoptosis in endometrial cells (Xia *et al*., 2014a). In domestic cat embryos generated by IVF an increased expression of *OCT4* is related to enhanced *in vitro* development (Veraguas *et al.,* 2017, 2020a). In accordance with this, no differences were observed in the relative expression of *OCT4* between ZF and ZI domestic cat blastocysts, and both groups had a similar blastocyst rate (Veraguas-Davila *et al.,* 2021). In this study, overexpression of *miR-29* observed in ZF domestic cat blastocysts was not related to an increase in blastocyst rate or an increase in the expression of pluripotency genes. However, it might be related to the regulation of apoptosis.

Regarding *miR-199*, it has been described that *in vitro*-produced embryos have lower expression of *miR-199a-5p* compared with *in vivo*-produced ones, leading to a reduced developmental capacity of blastocysts and to higher fetal mortality (Tan *et al*., 2016). In the present study, the higher expression of *miR-199* in ZF domestic cat blastocysts was not related to an increase in blastocyst formation rate.

miR-96 is involved in the implantation process by promoting luteal cell survival and progesterone synthesis by luteinized granulosa cells (Mohammed *et al*., 2017). Inhibition of *miR-96* increases apoptosis and decreases progesterone production (Mohammed *et al*., 2017). Furthermore, *miR-96* is highly expressed in stromal cells during pregnancy and is upregulated in implantation sites of mouse uterus (Yang *et al*., 2017). In the present study, the relative expression of *miR-96* was lower in ZF blastocysts than in their ZI counterparts. Correct expression of *miR-96* in domestic cat embryos might be important for luteal cell survival and implantation. For this reason, a reduced expression of *miR-96* could be related to the reduced implantation capacity of ZF domestic cat blastocysts.

miR-98 is a member of the *let-7* family, which is involved in embryo implantation (Liu *et al*., 2012). It has been reported that different members of the *let-7* were overexpressed in dormant blastocysts, which suggests a negative role for embryo implantation (Liu *et al*., 2012). Similarly, the upregulation of *miR-98* reduces ESC proliferation and increases apoptosis by inhibition of *BCL-XL* (Xia *et al*., 2014b). Furthermore, treatment of endometrial epithelial cells with *miR-98* downregulated immune system-related genes (Nakamura *et al*., 2019). This might indicate a possible role in the regulation of the maternal immune system during embryo–maternal crosstalk (Nakamura *et al*., 2019). Additionally, expression of *miR-98* has been detected in EVs released by *in vitro*-produced embryos (Andrade *et al*., 2019; Nakamura *et al*., 2019). *miR-98* has been associated with downregulation of tight junction mRNAs, which are crucial during embryo implantation (Andrade *et al*., 2019). For this reason, the expression of *miR-98* in EVs has been related to a delay in embryo implantation. In the present study, *miR-98* was highly expressed in spent culture medium of ZF blastocysts. The high presence of *miR-98* in spent medium might be a negative indicator of developmental capacity, and it could be related to the reduced implantation rate of ZF domestic cat blastocysts (Veraguas-Davila *et al.,* 2021). Additionally, *miR-21*, *miR-29c* and *let-7* have been involved in embryonic stem cell differentiation into trophectoderm cells (Liang *et al*., 2017; Viswanathan *et al*., 2009). The higher expression of *miR-21*, *miR-29* and *miR-98* observed in ZF blastocysts and in their spent medium might be related to the increased expression of *YAP1* and

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EOMES previously reported in these embryos (Veraguas-Dávila *et al.*, 2022).

In conclusion, *in vitro* culture of domestic cat embryos without the zona pellucida increases the relative expression of *miR-21*, *miR-25*, *miR-29* and *miR-199*, and reduces the expression of *miR-96* in the blastocyst stage. Additionally, *miR-21*, *miR-25* and *miR-98* were highly expressed in spent culture medium of ZF blastocysts. This demonstrates a possible regulatory role of the zona pellucida in the expression and release of embryonic miRNAs. In addition, these miRNAs might be related to altered expression of pluripotency and differentiation genes previously observed in ZF domestic cat blastocysts.

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Declaration of interest. The authors declare that there are no conflicts of interest to report.

Ethical standards. All the procedures were in accordance with the ethical standards of animal welfare and were approved by the Comité de Bioética de la Facultad de Ciencias Veterinarias de la Universidad de Concepción.

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