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Characterization and identification of extracellular vesicles-coupled miRNA profiles in seminal plasma of fertile and subfertile rabbit bucks

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ABSTRACT

Seminal plasma (SP) provides essential nutrients, transport, and protection to the spermatozoa during their journey through the male and female reproductive tracts. Extracellular vesicles (EVs) are one of the main components of the SP with several biomolecular cargoes, including miRNAs, that can influence spermatozoa functions and interact with the cells of the female reproductive tract. This study aimed to isolate, characterize, and identify the miRNA expression profiles in the SP-EVs isolated from fertile (F) and subfertile (S) rabbit bucks that could serve as fertility biomarkers. In this study, the methods to isolate and identify EVs including exosomes, from SP of 3 F and S bucks have been developed. Ultracentrifugation and size exclusion chromatography analysis were using to isolate EVs from SP of F and S males that were qualitative and quantitively characterised using transmission electron microscopy, nanoparticle tracking analysis and western blotting. In addition, total RNA, including miRNA, was isolated, sequenced and identified from SP-EVs samples. Different SP-EVs concentrations ($8.53 \times 10^{11} \pm 1.04 \times 10^{11}$ and $1.84 \times 10^{12} \pm 1.75 \times 10^{11}$ particles/mL of SP; $P = 0.008$), with a similar average size (143.9 ± 11.9 and 115.5 ± 2.4 nm; $P = 0.7422$) in F and S males, respectively was observed. Particle size was not significantly correlated with any kinetic parameter. The concentration of SP-EVs was positively correlated with the percentage of abnormal forms ($r = 0.94$; $P < 0.05$) and with the percentage of immotile spermatozoa ($r = 0.88$; $P < 0.05$). Small-RNA-seq analysis identified a total of 267 and 244 expressed miRNAs in the F and S groups, respectively. Two miRNAs (let-7b-5p and let-7a-5p) were the top most abundant miRNAs in both groups. Differential expression analysis revealed that 9 miRNAs including miR-190b-5p, miR-193b-5p, let-7b-3p, and miR-378−3p, and another 9 miRNAs including miR-7a-5p, miR-33a-5p, miR-449a-5p, and miR-146a-5p were significantly up- and downregulated in the F compared to the S group, respectively. The SP from F and S rabbit males contains EVs with different miRNA cargo correlated with spermatogenesis, homeostasis, and infertility, which could be used as biomarkers for male fertility and potential therapies for assisted reproductive technologies.

1. Introduction

In many (perhaps all) biological fluids, there are two main categories of extracellular vesicles (EVs) defined by their size: small EVs (sEVs <200 nm) and large EVs (lEVs >200 nm) [1]. However, EVs can be generally classified according to their biogenesis as follows: exosomes (30−100 nm), microvesicles (100−1.000 nm), apoptotic bodies (50−4.000 nm), uterosomes and prostasomes (40−500 nm) [2−4]. EVs studies in different fluids have now clearly

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achieved widespread interest, however not all conditions concerning collection, isolation, and characterization have been thoroughly investigated. The International Society for Extracellular Vesicles [1] assumes the existence of specific issues when working with these particles due to their size and amount, often making them difficult to obtain as relatively pure preparations and to characterize correctly. EVs separation and concentration, as well as characterization, are needed to attribute them to a function or a biomarker.

Recent reviews highlight the role of EVs in reproduction [5–8]. In particular, the ejaculate is a mixture of spermatozoa (SPZ, approximately 5%) and a non-cellular liquid named seminal plasma (SP, 95%) coming from the testicles, epididymis, and accessory glands. Seminal plasma (SP) contains sugars, oligosaccharides, glucans, lipids, inorganic ions (calcium, magnesium, potassium, sodium, and zinc), proteins and RNA molecules that may be encapsulated in EVs [9]. In the case of ejaculates from rabbit, Davis [10] already described the existence of EVs that have a relevant role in the SPZ activity. These rabbit SP-EVs are limited by membranes rich in enzymes that hydrolyse ATP and ADP [11], contain abundant cholesterol (or demosterol) that can be transferred to head membrane of SPZ reversing or blocking capacitation and acrosome reaction [12], phospholipids, which makes the SPZ membrane more stable [13] and Vitamin E and carotenes related to oxidative stress [14,15]. In addition, it has described that SP-EVs contain functional molecules including microRNAs (miRNAs), mRNAs, DNA, lipids, and proteins that are a novel source of biomarkers to detect infertility problems and potential therapies for assisted reproductive technologies [9,16]. Among species of zootechnical interest, bull SP has more than 82 bovine miRNAs that share strong homology with 1645 miRNA sequences from 74 different other species and were identified to play a major role in fertility [17]. Recently, differences in the abundance of four miRNAs in ejaculated and epididymal spermatozoa have been associated with boar fertility [18]. Also, some miRNAs contained in semen exosomes from men have been considered non-invasive biomarkers for prostate cancer diagnosis [19], erectile dysfunction, varicocele, and testicular injury [20]. Nonetheless, as far as we know, possible miRNA biomarkers in rabbit SP-EVs have not been analysed.

In European rabbit farms, artificial insemination (AI) is routinary applied using heterospermic pools (ejaculates from several males), and it is assumed that the male contribution to fertility is negligible [21]. Nonetheless, for breeding selection programs, it is mandatory using the ejaculate of a single male to inseminate groups of 25–35 female rabbits (homospermic insemination). In this case, the relationship between seminal quality and semen fertilising ability is generally found to be weak. In this sense, identification of specific miRNAs present in the SP-EVs could help determine if those are modulating the productive results of a specific buck [11,22], and in the case of rabbits, it would be a very novel study.

Thus, the purpose of this research was to isolate, characterize, and identify the miRNA expression profiles in the SP-EVs isolated from fertile and subfertile rabbits, contributing to the progress of clinical and livestock biomarkers of male fertility.

2. Material and methods

2.1. Ethics statement

All experiments were accomplished in accordance with the European Directive 2010/63/EU EEC for animal experiments and accepted by the Technical University of Madrid's Research Ethics Commission (Research code: PROEX 302/15).

2.2. Animals and seminal parameters

Initially, in order to have SP from males with high and low proven seminal quality, 14 sexually mature male California x New Zealand white rabbits (Oryctolagus cuniculus) of 10–12 months of age were used and were housed in individual cages under standard environmental conditions (16 h/8h light/dark cycle, 22–24 °C and 55–60% relative humidity) (PROEX 302/15). An ejaculate was collected from these animals twice a week with an artificial vagina for 1 month (8 ejaculates from each animal). The macroscopic quality of each ejaculate was assessed after removing the gel fraction by color, discarding the ejaculates with abnormal colors (yellow or red) and by volume, discarding the ejaculated with low volumes (<0.2 mL). Sperm viability was also evaluated with the eosin staining test and the percentage of abnormal forms (10 μL of semen + 10 μL of eosine, count of 200 cells), concentration (with Bürker chamber) and kinetic parameters (CASA program, Microptic S.L., Barcelona, Spain) were assessed, observing a minimum of 200 cells in each of the 3 replicates that were analysed from each ejaculate. The setting parameters were: 15 and 150 pixels^2 of minimal and maximal SPZ area, respectively; 30 frames for minimal track length; 25 μm/s for minimal curvilinear velocity (VCL) of motile SPZ and 15 μm/s for maximal VCL of slow SPZ; 10, 25 and 50 μm/s for minimal average path velocity for slow, medium and fast SPZ, respectively; 70% straightness index (STR) was defined for progressive motility and a frame rate of 100 frame/s. From these analyses, 3 males of high (HSQ) and 3 of low (LSQ) seminal quality were chosen, whose individual and average results are shown in Table 1.

Furthermore, to confirm that the seminal parameters of these males were associated with low fertility and prolificacy, 153 does (nulliparous and multiparous) were inseminated with homospermic doses from each of the 6 males selected in the study (3 HSQ and 3 LSQ males), diluted in a commercial diluent (Inserbo S.L., Lérida, Spain). Around 25 females/male were inseminated with a concentration of 5×10^6 SPZ/mL in a volume of 0.5 mL. All rabbit does were treated with 20 μg gonadorelin i.m. (Gestavet, Hypra, Spain) to induce ovulation. Fertility ([number of pregnant rabbits/number of rabbits inseminated] × 100) and prolificacy (born alive and stillbirths) were calculated and are shown in Table 2. Because HSQ males' reproductive parameters were better than those of LSQ males ($P < 0.001$), we categorized them as Fertile (F) and Subfertile (S), respectively.

2.3. Isolation of seminal Plasma-EVs

For the isolation of EVs from the same F and S rabbits classified in the previous test, another 6 ejaculates were obtained from each animal (n = 6 animals: 3 F and 3 S) for 3 weeks (2 per week). Each sample was subjected to 3 series of centrifugation at 4 °C (800×*g* 20 min, 2000×*g* 20 min, and 16000×*g* 60 min) and the resulting SP from each sample was frozen at −80 °C. Subsequently, the method of Cañón - Beltrán et al. [23], based on PURE-EV (HansaBioMed Life Sciences) size exclusion chromatography (SEC), followed by centrifugation was used. Briefly, columns were first washed with 30 mL PBS and then SP from each male (2.5 mL) was loaded on the top of a SEC column. When the sample was completely inside the column, 11 mL of PBS were loaded. Afterwards, the first 3 mL were discarded, and the following 2.5 mL of EV-rich fractions were collected. These samples were then subjected to centrifugation for 30 min at 2000×*g* to concentrate the SP-EVs through an Amicon Ultra-15 filter (Merck-Millipore Ltd., Ireland) in a final volume of 100 μL. Supernatants were then discarded, and each pellet was suspended in 200 μL cold PBS. The rest of the analysis was performed from this final volume.

Table 1
Sperm parameters for 6 selected rabbit bucks with high (HSQ = 3) and low (LSQ = 3) seminal quality. For each animal, 8 ejaculates were considered and analysed over a month. Individual values from cada male and LSMEAN ± SE from each group are presented below.

	HSQ males			LSQ males			HSQ	LSQ	P VALUE
	1	2	3	1	2	3	LSMEAN ± SE	LSMEAN ± SE	
Volume (mL)	1.35 ± 0.94	1.23 ± 0.11	1.29 ± 0.08	0.98 ± 0.140	2.07 ± 0.29	0.99 ± 0.1	1.21 ± 0.13	1.36 ± 0.12	0.3992
Viability (%)	90.75 ± 1.01	91.58 ± 0.61	91.63 ± 0.91	46.11 ± 1.42	35.56 ± 1.22	31 ± 1.17	90.69 ± 1.25	37.81 ± 1.20	0.0001
Abnormality (%)	4.75 ± 0.70	2.83 ± 0.53	4.96 ± 27.96	33.22 ± 2.86	41.28 ± 3.14	59.81 ± 0.81	5.09 ± 2.00	44.19 ± 1.92	0.0001
Concentration (× 10^6/mL)	455.0 ± 54.05	515.17 ± 82.67	468.75 ± 63.23	296.33 ± 37.03	214.78 ± 37.34	147.88 ± 38.88	411.2 ± 30.06	222.4 ± 28.88	0.0001
Non-progressives (%)	29.62 ± 4.62	30.67 ± 3.01	29.78 ± 2.81	13.56 ± 2.22	10.33 ± 1.38	9.38 ± 1.43	29.83 ± 1.53	11.00 ± 1.47	0.0001
Progressives (%)	53.75 ± 2.21	46.17 ± 7.25	61.63 ± 27.58	13.56 ± 2.42	13.56 ± 3.10	2.88 ± 0.79	55.88 ± 1.70	10.00 ± 1.64	0.0001
Motility (%)	82.75 ± 2.82	75.5 ± 8.44	91.38 ± 1.73	26 ± 4.55	24.11 ± 4.14	12.5 ± 2.13	85.46 ± 2.12	21.19 ± 2.04	0.0001
Fast (%)	65.38 ± 1.70	51.83 ± 9.43	68.25 ± 4.26	13.56 ± 3.99	13.0 ± 3.81	4.25 ± 2.08	59.63 ± 3.02	10.50 ± 2.90	0.0001
Medium (%)	8.00 ± 0.78	12.0 ± 2.34	13.5 ± 1.59	7.0 ± 1.53	3.78 ± 0.68	2.75 ± 1.52	13.42 ± 1.18	4.58 ± 1.14	0.0001
Slow (%)	9.38 ± 1.35	12.83 ± 2.12	9.75 ± 1.44	9.11 ± 1.61	7.33 ± 0.88	8.75 ± 0.01	12.20 ± 1.33	8.38 ± 1.28	0.0434
VCL (μm/s)	120.03 ± 3.56	88.83 ± 12.56	100.61 ± 6.03	61.28 ± 6.76	63.27 ± 8.22	35.60 ± 6.56	97.57 ± 5.23	54.06 ± 5.02	0.0001
VSL (μm/s)	80.44 ± 5.03	57.42 ± 9.58	65.31 ± 3.35	38.08 ± 3.50	44.97 ± 6.46	18.05 ± 3.56	64.75 ± 3.74	34.30 ± 3.60	0.0001
VAP (μm/s)	92.96 ± 4.73	68.65 ± 10.96	77.03 ± 4.45	43.43 ± 3.43	50.71 ± 7.13	21.31 ± 4.27	75.55 ± 4.19	39.15 ± 4.03	0.0001
LIN (%)	67.23 ± 4.30	62.97 ± 3.32	64.46 ± 2.21	63.97 ± 4.64	71.09 ± 4.71	52.62 ± 7.02	66.34 ± 2.79	62.96 ± 2.68	0.3878
STR (%)	86.55 ± 3.36	83.03 ± 1.52	82.11 ± 1.36	87.19 ± 71.9	88.35 ± 2.18	91.93 ± 10.33	84.75 ± 2.66	89.05 ± 2.55	0.2484
WOB (%)	77.43 ± 3.22	75.68 ± 2.96	77.30 ± 2.10	72.88 ± 3.53	79.89 ± 59.8	66.06 ± 7.35	77.67 ± 2.41	73.20 ± 2.31	0.1869
ALH (μm)	3.11 ± 0.22	2.78 ± 14.47	2.56 ± 0.12	2.32 ± 0.18	1.91 ± 0.24	1.48 ± 0.29	2.59 ± 0.14	1.92 ± 0.13	0.0012
BCF (Hz)	9.15 ± 0.23	8.08 ± 1.09	9.13 ± 0.20	7.80 ± 0.60	8.42 ± 0.52	2.53 ± 0.96	9.23 ± 0.49	6.39 ± 0.47	0.0001

LSMEAN: Least Square Mean, SE: stsndard error, Curvilinear velocity [VCL], rectilinear velocity [VSL], mean [VAP], linearity [LIN], straightness [STR], wobble coefficient [WOB], head trajectory width [ALH], frequency of trajectory intersections rectilinear and curvilinear [BCF].

2.4. Qualitative and quantitative characterization of SP-EVs

2.4.1. Transmission electron microscopy

Five microliters from three replicates (males) of each experimental group (n = 3 males F and 3 S) of SP-EVs were diluted (1:10) with PBS to perform the negative staining of EVs. An ionized carbon and collodion-coated copper electron microscopy grid was floated on a diluted sample drop, washed, and stained with 2% uranyl acetate for 20 s and visualized in a transmission electron microscope JEM-1010 (JEOL, Tokyo, Japan) equipped with a Megaview II CCD camera integrated into the iTEM Olympus Soft Image Solutions software (Olympus, Tokyo, Japan).

2.4.2. Nanoparticle tracking analysis

Similarly, 5 μL from three replicates (males) of each experimental group (n = 3 males F and 3 S) of SP-EVs were diluted (1:20) with PBS and analysed by nanoparticle tracking analysis (NTA, Nanosight NS500 Ltd., Minton, Park, UK) using the software NTA 3.1. to determine the size and concentration of the nanoparticles in each replicate. Three videos of 60 s each were recorded by sample with camera level set at 13, and temperature of 22 °C. For the analysis settings, the detection threshold was established at levels 2 to 3.

2.5. Western blotting

To have enough protein amount from EVs biomarkers to be detectable by western blot, a pool of SP-EVs of all the animals (90 μL per animal) of each experimental group was performed. Extracellular vesicle proteins were lysed by 1 × RIPA buffer (Cell Signalling Technology, 9806S), supplemented with 1 × protease phosphatase Inhibitor Cocktail (Roche, Basel, Switzerland). Protein concentrations in the EV samples were determined by the Bicinchoninic acid assay (BCA) (Micro BCA Protein Assay Kit; 23325). A total of 35 μg of protein per sample was suspended in Laemmli loading buffer, and then separated in a 4%–12% gradient SDS-PAGE polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences Whatman™). The membranes were washed in distilled water and blocked with PBS containing Tween 20 (0.1% (w/v)) (PBS-T) and supplemented with bovine serum albumin (BSA) (3% w/v) for 30 min at room temperature.

The membranes were incubated with primary antibodies diluted in PBS-T containing BSA (5% w/v) overnight at 4 °C with gentle shaking. Four primary antibodies were used as EVs biomarkers: anti-heat shock protein 70 (anti-HSP70 mAb, C92F3A-5 Enzo Life Sciences, NY, USA); anti-tetraspanin cell surface protein CD9 (anti-CD9 mAb, 13403, Cell Signalling Technology, D3H4P, Danvers, MA, USA); and anti-ALIX protein (anti-ALIX mAb, sc-53540, Santa Cruz Biotechnology, CA, USA). As a negative control, anti-calnexin (anti-CANX mAb, sc-23954, Santa Cruz Biotechnology, CA, USA) was used to indicate the absence of cell contamination in EV samples. After primary antibodies incubation, the membranes were washed with PBS-T and incubated for 2 h under agitation with secondary antibodies. The following horseradish peroxidase-conjugated secondary antibodies were used: goat anti-rabbit or horse anti-mouse IgG-HRP (sc7074, sc7076, Cell Signaling Technology, Danvers, MA, USA). The membranes were newly

Table 2
Reproductive parameters from rabbit females inseminated with semen from rabbit bucks with high (HSQ = 3) and low (LSQ = 3) seminal quality.

HSQ males				LS Mean ± sem	LSQ males			LS Mean ± sem	P > f
	1	2	3		1	2	3		
n	24	33	25		24	24	23		
Fertility[1] (%)	75	72.7	68	71.95 ± 5.22	37.5	45.83	39.13	40.85 ± 5.61	***
Born alive	10.78 ± 0.40	11.67 ± 0.39	12.24 ± 0.25	11.56 ± 0.31	8.89 ± 0.36	9.00 ± 0.95	6.22 ± 1.23	8.10 ± 0.44	***
Stillbirths	0.11 ± 0.08	0.08 ± 0.06	0.06 ± 0.06	0.08 ± 0.07	1.00 ± 0.37	0.27 ± 0.19	1.11 ± 0.26	0.76 ± 0.11	***

n: No. inseminated rabbit females. [1] (Number of pregnant does/Number of inseminated does) x100; ***: P < 0.001. LS mean: Least Square mean.

washed three times in PBS-T for 5 min and incubated for 1 min in the Immobilon Forte Western HRP substrate (#WBLUF0100, Millipore, Burlington, MA) and revealed by chemiluminescence with an Image Quant LAS500 biomolecular imager (GE Healthcare Life Sciences, USA, 29005063). The mouse ureter was used as a control for the CANX protein.

2.6. Total RNA extraction, library preparation, and sequencing

The miRNeasy Micro Kit (Qiagen, Hilden, Germany) was used to isolate total RNA, including miRNA, from SP-EVs samples, following the manufacturer's instructions. The RNA concentration and size distribution were evaluated using an Agilent RNA 6000 Pico kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Next-generation sequencing (NGS) small-RNA libraries were created with a QIAseq miRNA Library Kit (Qiagen), following the manufacturer's instructions. Quality and quantity assessments of the libraries were done using the Agilent DNA High Sensitivity kit on an Agilent 2100 Bioanalyzer (Agilent Technologies) and a Qubit DNA HS Assay Kit on a Qubit 4 Fluorometer (Thermo Fisher Scientific), respectively. Subsequently, the libraries were combined in equimolar ratios and then sequenced as single-end reads on a NovaSeq6000 sequencing instrument (Illumina, Inc., San Diego, CA, USA).

2.7. Sequencing data analysis

Using the bcl2fastq software (Illumina Inc., San Diego, CA), FASTQ files were generated for each sample, and their quality was verified using the FastQC tool version 0.11.9. The CLC Genomics Workbench software, version 21, was used to analyze the data. Raw sequencing reads were trimmed based on specific parameters, such as a quality score greater than 30 (Q-score >30), a maximum of two ambiguous nucleotides, and a read length of at least 15 nucleotides, and adapter sequences were removed. Rabbit (Oryctolagus cuniculus) reference genome (OryCun2.0) was used to map the reads, and the mirBase database (release 22) was used to annotate against rabbit precursor and mature miRNAs. The CLC Genomics Workbench RNA-Seq Analysis and Quantify miRNA tools were used for this purpose, and default software parameters were applied.

The trimmed mean of M-values normalization method (TMM normalization) [24] was used to normalize the raw expression data, which was presented as TMM-adjusted Counts Per Million (CPM). To compare the expression analysis of the two groups, the Differential Expression tool of the CLC Genomics Workbench was used. MiRNAs were considered differentially expressed (DE) if they met certain criteria, such as a fold change (FC) of at least 2, P-adjusted value (FDR [25], < 0.05, and an average of CPM greater than 5. The raw FASTQ files and processed CSV files have been deposited in the NCBI's Gene Expression Omnibus (GEO) with the accession number GSE209607.

2.8. Target gene prediction and ontological classification

The human miRNA homologous for the DE rabbit miRNAs were identified from the miRBase database and used for target gene prediction using the miRWalk database [26]. Within the miRWalk, validated target genes from miRTarBase (version 7.0) and commonly target genes predicted by miRDB (release 5.0) and TargetScan (version 7.1) were selected for ontological classification analysis using the DAVID bioinformatics web tool (https://david.abcc.ncifc rf.gov/). Pathways and biological processes (BP) were determined from the KEGG database [27], and GOTERM_BP_DIRECT annotation set, respectively. Irrelative and low gene count terms were filtered out from the pathways and BP lists.

2.9. Statistical analysis

Several analysis of variance considering the type of buck as the main effect were done to 1) compare seminal parameters of 8 ejaculated obtained during a month from selected HSQ ($n = 3$) and LSQ ($n = 3$) males, 2) to analyze whether the seminal quality of these males affected fertility and prolificacy results of females inseminated, and 3) to study the differences in the concentration and size of particles from each male. Also, a Pearson correlation analysis to check if the seminal quality could be related to the size and concentration of EVs-SP particles was assessed [28].

3. Results

3.1. Size distribution, concentration and identification of EVs

The biophysical and molecular characterization of the SP-EVs from the F and S rabbits are shown in Fig. 1. Electron microscope observation (Fig. 1A) confirmed the presence of EVs in both groups, with sizes ranging from 30 to less than 200 nm. Additionally, NTA results supported TEM observation, showing an EV populations diameter of 143.9 ± 11.9 nm for F and 115.5 ± 2.4 nm for S rabbits ($P = 0.7422$) (Fig. 1B). Regarding particle concentration, the NTA analysis recorded a lower average ($P = 0.008$) in F (1.0×10^{12} EV/mL) than in S males (1.8×10^{12} EV/mL) (Fig. 1B).

Western blot analysis validated the expression of some classical exosome markers in the EVs of both groups of males: HSP70, CD9, and ALIX proteins, as well as CANX (Fig. 1C), an endoplasmic reticulum protein that serves as a negative control for cell contamination and is therefore not usually present in EVs. These data confirm that the previously described seminal exosomes are indeed exosomes and microvesicles and that they exist at high concentrations in rabbit semen.

No significant correlation was observed between the kinetic parameters of the SPZ and EVs size. The lower concentration in F compared to S males was positively correlated with the percentage of abnormal forms ($r = 0.94$; $P < 0.05$) and immobile SPZ ($r = 0.88$; $P < 0.05$).

3.2. MiRNA expression profiles

Small-RNA-seq analysis revealed an average number of 51 million raw reads per library with an average of 32 million reads being retained after quality control assessment. An average of 90% of reads were mapped to the rabbit genome with an average of 3% of mapped reads annotated to miRNAs from the mirBase database (Supplementary Table S1). Heatmap clustering and principal component analysis (PCA) showed a clear clustering of the biological replicates of the F group with less variability compared to the biological replicates of the S group (Fig. 2). While one replicate from the S group shows greater variation than the other two replicates, it still closely clusters with the S group, as opposed to the F group, and both groups are separated from each other based on the miRNA expression profiles as demonstrated in the heatmap (Fig. 2B). A miRNA with at least 1 read count in each of the three replicates was considered to be expressed. A total of 267 and 244 miRNAs were expressed in the F and S groups, respectively, with 233 miRNAs being expressed in both groups (Fig. 3A). A complete list of all expressed miRNAs is presented in Supplementary Table S2, and the top 20 highly expressed miRNAs are presented in Table 3. Interestingly, let-7b-5p and let-7a-5p were the top most abundant miRNAs in both groups accounting for approximately 20% of the total miRNA sequence reads in both groups (Table 3).

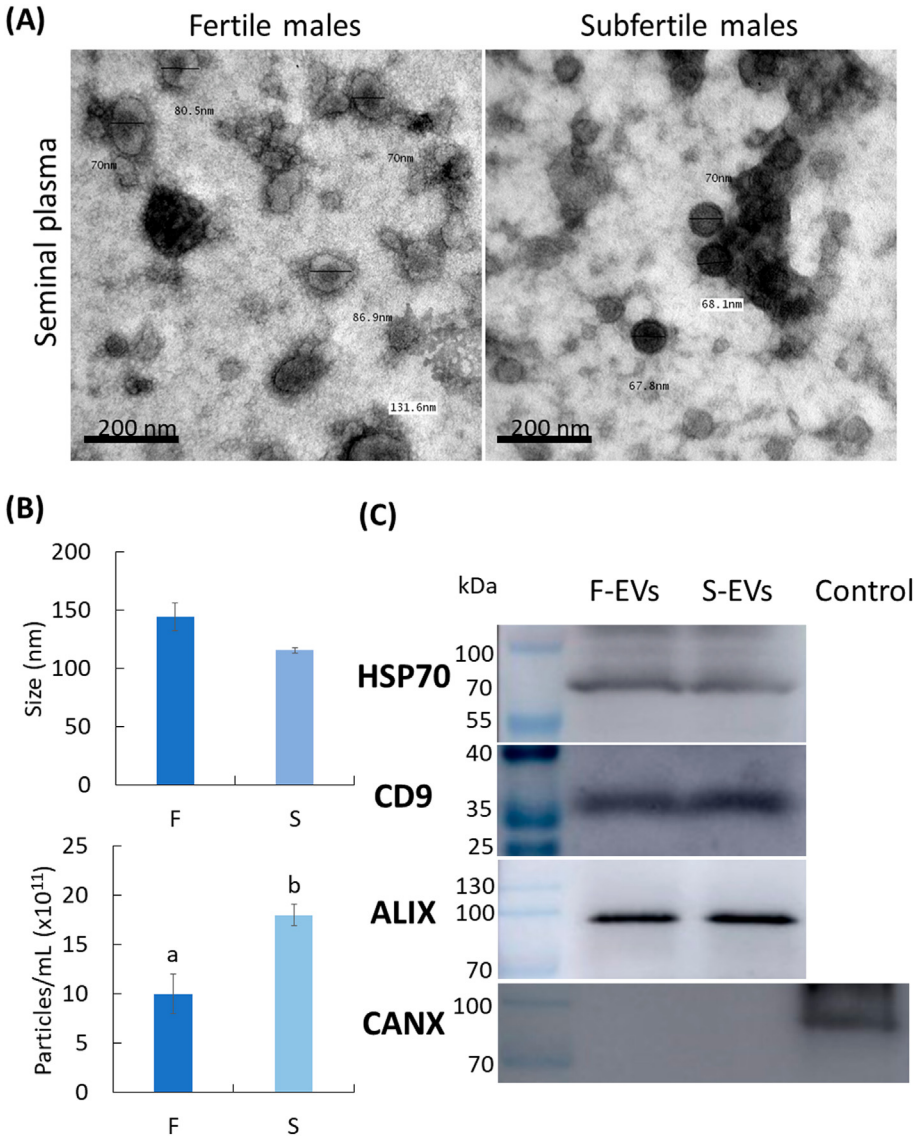

Fig. 1. Fertile and Subfertile rabbit EVs characterization: A) Representative images of EVs (30–200 nm size) in the seminal plasma of Fertile (F) and subfertile (S) males by transmission electron microscopy (TEM). B) Rabbit F-EVs and S-EVs average size and concentration measured by Nanosight (NTA) from 6 samples (three animals/group). C) Western blot of exosome protein markers (HSP70, CD9, and ALIX) in fertile (F-EVs) and subfertile (S-EVs) rabbits. Additionally, we verified the presence of CANX, an endoplasmic reticulum protein, which serves as a negative control for cell contamination. Cropped western blot membrane images are shown here, while full-length blots are presented in Supplementary Fig. S1.

3.3. Differentially expressed miRNAs and ontological classification

Differential expression analysis of miRNAs revealed that 9 miRNAs (including miR-190b-5p, miR-193b-5p, let-7b-3p, and miR-378–3p) were significantly in higher abundance, and 9 miRNAs (including miR-7a-5p, miR-33a-5p, miR-449a-5p, and miR-146a-5p) were in lower abundance (FC $\geq$ 2, FDR <0.05, and CPM >5 in the enriched group) in the F compared to the S group (Fig. 3B, Table 4).

Target gene prediction revealed a total of 483 and 500 genes are targeted by the up- and downregulated miRNAs, respectively, with 56 genes targeted by both. Signaling pathways including regulating pluripotency of stem cells, AMPK, PI3K-Akt, and FoxO were the top pathways targeted by the elevated miRNAs in the F group (Fig. 4A, Supplementary Table S3). On the other hand, MAPK signaling, Cellular senescence, Wnt signaling, and EGFR tyrosine kinase inhibitor resistance pathways were the top pathways targeted by the elevated miRNAs in the S group (Fig. 5A, Supplementary Table S3). Regulation of protein catabolic, transcription, and gene expression were the top BP targeted by the elevated miRNAs in the F group (Fig. 4B, Supplementary Table S4). Elevated miRNAs in the S group were predicted to target genes involved in transcription regulation, protein dephosphorylation, and signal transduction (Fig. 5B, Supplementary Table S4).

4. Discussion

Reproductive performance prediction based on semen quality values of males is very important from a zootechnical perspective. Regardless of the species, predicting male fertility for genetic improvement or clinical reasons depends on fertility and prolificacy results obtained after homospermic inseminations that are time-consuming. As a result, new techniques that can complement the routine seminal analysis carried out in insemination centers could

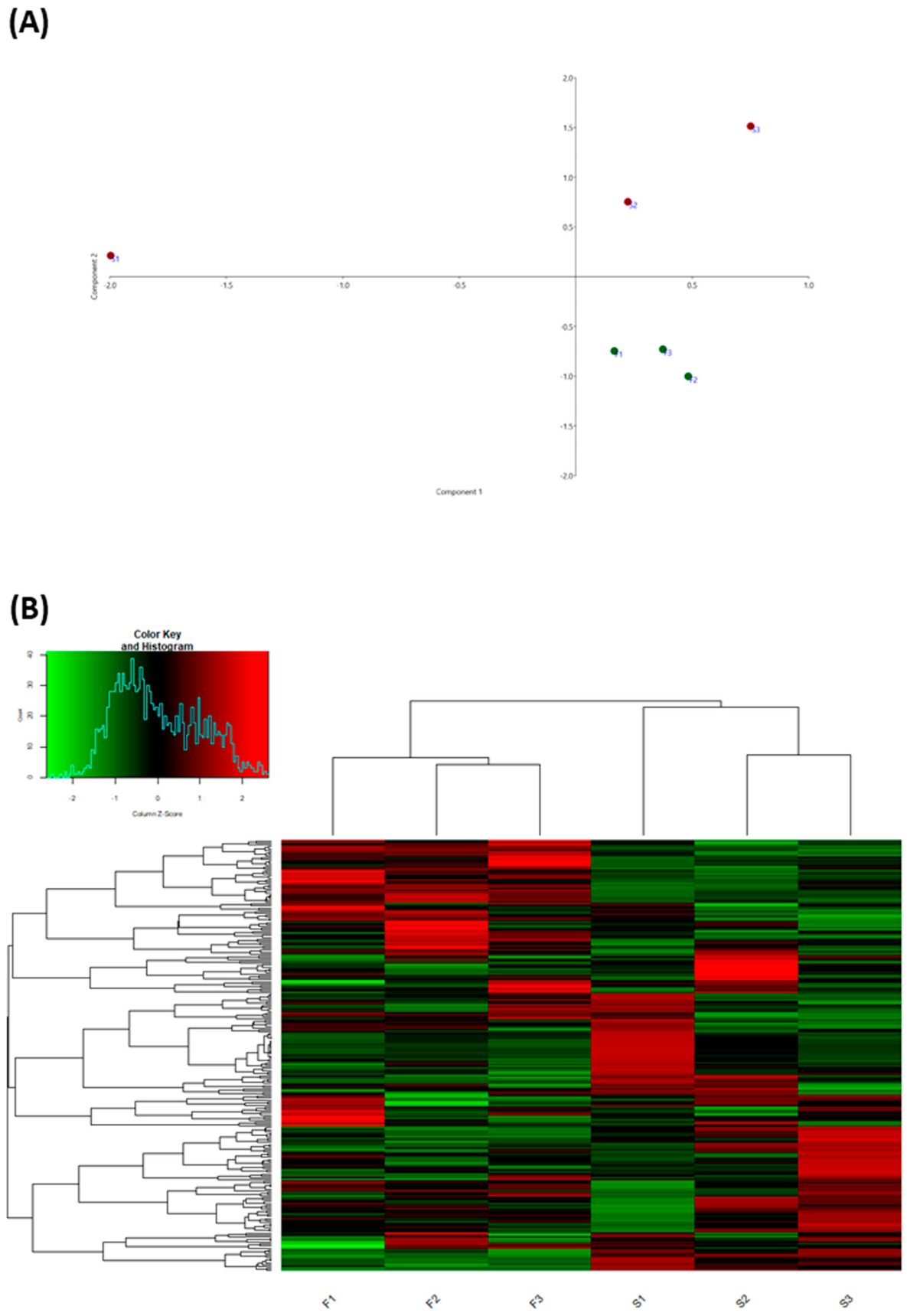

Fig. 2. Samples clustering. A) Principal Component Analysis. B) Heatmap and hierarchical clustering of expressed miRNAs. Red and green colors represent high and low expressed miRNAs, respectively. F1–F3: Fertile group replicates; S1–S3: Subfertile group replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

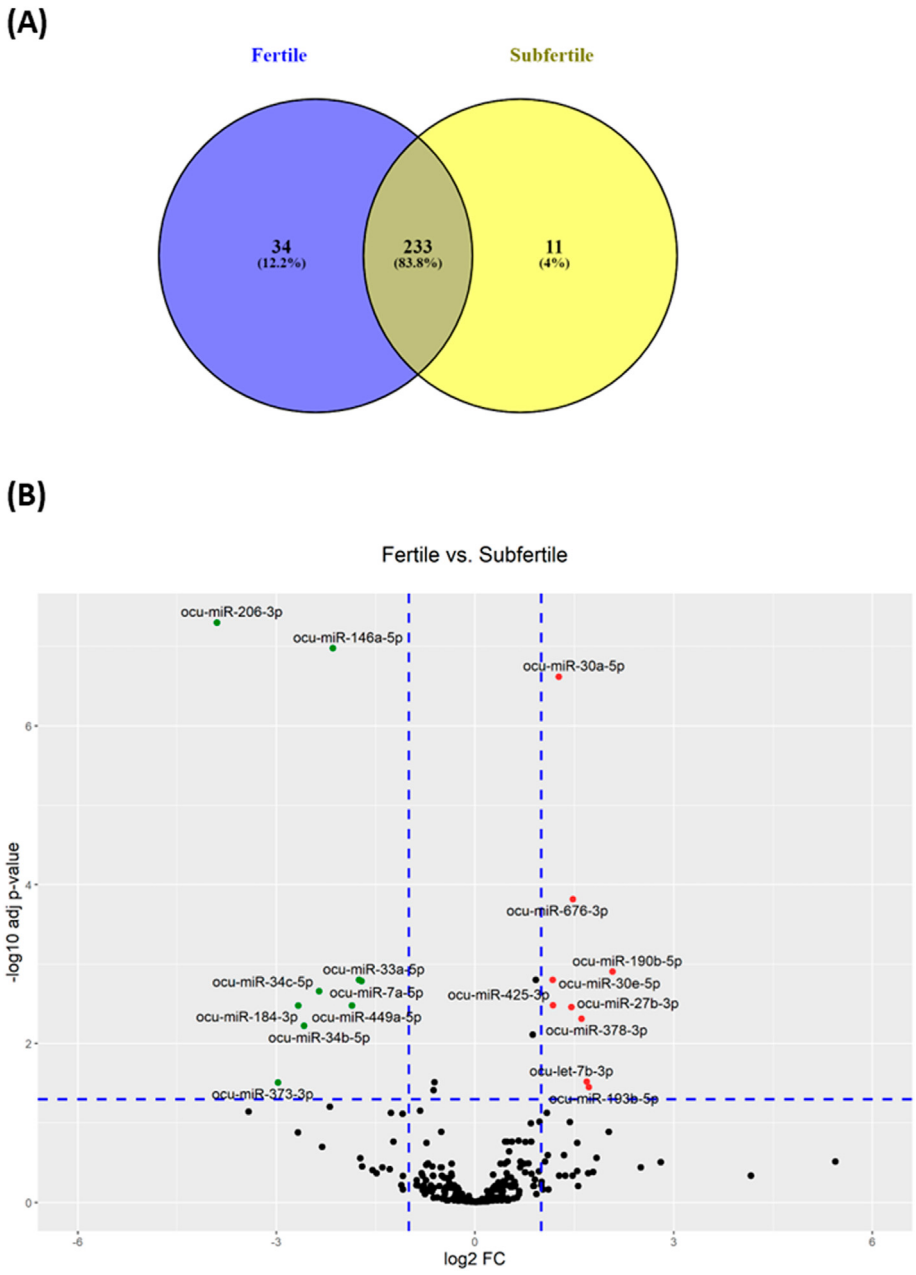

Fig. 3. Differential expression analysis. A) Venn diagram for commonly and exclusively expressed miRNAs in Fertile and Subfertile groups. B) Volcano plot of expressed miRNAs. Up- and downregulated miRNAs in the Fertile compared to the Subfertile SP-EV groups are labelled with red and green points, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

contribute to a better understanding of male fertility more quickly and efficiently.

As we expected, fertility and prolificacy obtained in the current study after the homospermic artificial insemination of females were strongly affected by the seminal quality of the different groups of males. According to Table 1, LSQ males with only 37% sperm viability, 21% motility (10% progressive) and 44% sperm abnormalities achieved only 40% pregnancy, whereas HSQ males achieved better fertility (71.95%) with 91% sperm viability, 85% motility (>55% progressive) and only 5% sperm abnormality. We consider this assumption necessary to properly classify the two groups of males in this study. In accordance with our results, Lavara et al. [29], found significant correlations between fertility and the total percentage of motile cells ($r = 0.31$), the LIN index ($r = -0.32$), and the percentage of abnormal SPZ ($r = 0.32$) of the male used in heterospermic inseminations using low sperm concentrations (6×10^6 SPZ/mL) as those applied in the current study. On the other hand, in homospermic inseminations and high dilutions (1:19), Theau-Clèment et al. [30] stated that selected ejaculates by high mass motility can increase productivity in 1 rabbit born alive by insemination. Therefore, in this study, F and S males were appropriately grouped based on their seminal quality, and SP-EVs isolated from their ejaculates were analysed for differences.

As far as we know, the EVs isolation technique applied in the current study had not been performed previously on the rabbit SP. Castellini et al. [12], after laying raw rabbit semen onto a two-layer Percoll gradient, centrifuging at $500 \times g$ for 10 min, and ultracentrifuging at $105{,}000 \times g$ for 2 h obtained small granules with a

Table 3
List of top 20 most abundant miRNAs in the extracellular vesicles obtained from the seminal plasma of Fertile (F) and Subfertile (S) groups.

Name	Human homologous	F group CPM	Name	Human homologous	S group CPM
ocu-let-7b-5p	hsa-let-7b-5p	137134.4	ocu-let-7b-5p	hsa-let-7b-5p	120607.5
ocu-let-7a-5p	hsa-let-7a-5p	79541.8	ocu-let-7a-5p	hsa-let-7a-5p	81015.05
ocu-miR-30d-5p	hsa-miR-30d-5p	70130.65	ocu-let-7f-5p	hsa-let-7f-5p	68212.33
ocu-let-7f-5p	hsa-let-7f-5p	53367.64	ocu-miR-200c-3p	hsa-miR-200c-3p	47498.3
ocu-miR-10b-5p	hsa-miR-10b-5p	53318.43	ocu-miR-141–3p	hsa-miR-141–3p	46485.18
ocu-miR-10a-5p	hsa-miR-10a-5p	46805.01	ocu-miR-10a-5p	hsa-miR-10a-5p	45828.85
ocu-miR-200c-3p	hsa-miR-200c-3p	44931.5	ocu-miR-10b-5p	hsa-miR-10b-5p	44655.45
ocu-miR-141–3p	hsa-miR-141–3p	41944.39	ocu-miR-30d-5p	hsa-miR-30d-5p	37197.53
ocu-miR-30a-5p	hsa-miR-30a-5p	41467.98	ocu-miR-1911–5p	hsa-miR-1911–5p	37040.78
ocu-miR-1911–5p	hsa-miR-1911–5p	38239.87	ocu-miR-29a-3p	hsa-miR-29a-3p	35785.61
ocu-miR-29a-3p	hsa-miR-29a-3p	35070.39	ocu-miR-26a-5p	hsa-miR-26a-5p	35781.25
ocu-miR-191–5p	hsa-miR-191–5p	33576.06	ocu-miR-29c-3p	hsa-miR-29c-3p	30625.09
ocu-miR-29c-3p	hsa-miR-29c-3p	29435.21	ocu-miR-21–5p	hsa-miR-21–5p	28887.09
ocu-miR-21–5p	hsa-miR-21–5p	27656.35	ocu-miR-191–5p	hsa-miR-191–5p	24551.93
ocu-miR-26a-5p	hsa-miR-26a-5p	25087.18	ocu-miR-148a-3p	hsa-miR-148a-3p	18683.14
ocu-miR-148a-3p	hsa-miR-148a-3p	17639.57	ocu-let-7c-5p	hsa-let-7c-5p	17958.9
ocu-let-7c-5p	hsa-let-7c-5p	16946.59	ocu-miR-30a-5p	hsa-miR-30a-5p	17245.07
ocu-miR-509b-3p	hsa-miR-514a-3p	15268.44	ocu-miR-1298–5p	hsa-miR-1298–5p	12526.79
ocu-miR-125b-5p	hsa-miR-125b-5p	14476.14	ocu-miR-509b-3p	hsa-miR-514a-3p	12393.36
ocu-miR-30e-5p	hsa-miR-30e-5p	13263.53	ocu-miR-205–5p	hsa-miR-205–5p	11934.07

CPM: average Counts Per Million mapped reads.

prevalently round shape and a diameter of 0.5 μm, far to the size that it is considered appropriate for EVs according to the minimal requirements of MISEV2018 publication. After adding another chromatography step on a Sephadex G-200 column and another ultracentrifugation, Minelli et al. [11] obtained round vesicles with a diameter of 70 nm, but vesicles with a diameter >160 nm were also observed. Abumaghaid et al. [31] isolated EVs from the primary culture of rabbit testis, prostate and epididymis using isolation kits,

Table 4
Differentially expressed (DE) miRNAs in the extracellular vesicles obtained from the seminal plasma of Fertile (F) compared to Subfertile (S) groups.

Name	Human homologous	Sequence	FC	FDR
ocu-miR-190b-5p	hsa-miR-190b-5p	UGAUAUGUUUGAUAUUGGGUUG	4.2	0.00124
ocu-miR-193b-5p	hsa-miR-193b-5p	CGGGGUUUUGAGGGCGAGAUGA	3.3	0.035167
ocu-let-7b-3p	hsa-let-7b-3p	CUAUACAGCCUACUGCCUUCCC	3.2	0.030057
ocu-miR-378–3p	hsa-miR-378a-3p	ACUGGACUUGGAGUCAGAAGGC	3.0	0.004843
ocu-miR-676–3p	hsa-miR-676–3p	CCGUCCUAAGGUUGUUGAGUU	2.8	0.000152
ocu-miR-27b-3p	hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC	2.7	0.003455
ocu-miR-30a-5p	hsa-miR-30a-5p	UGUAAACAUCCUCGACUGGAAGCU	2.4	2.4E-07
ocu-miR-425–3p	hsa-miR-425–3p	CAUCGGGAAUGUCGUGUCCGCCC	2.3	0.003272
ocu-miR-30e-5p	hsa-miR-30e-5p	UGUAAACAUCCUUGACUGGAAGCU	2.3	0.001571
ocu-miR-7a-5p	hsa-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGUU	−3.3	0.001634
ocu-miR-33a-5p	hsa-miR-33a-5p	GUGCAUUGUAGUUGCAUUGCA	−3.4	0.001571
ocu-miR-449a-5p	hsa-miR-449a	UGGCAGUGUAUUGUUAGCUGGU	−3.6	0.003305
ocu-miR-146a-5p	hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUUG	−4.4	1.05E-07
ocu-miR-34c-5p	hsa-miR-34c-5p	AGGCAGUGUAGUUAGCUGAUUGC	−5.1	0.002193
ocu-miR-34b-5p	hsa-miR-34b-5p	UAGGCAGUGUAAUUAGCUGAUUGU	−6.0	0.005982
ocu-miR-184–3p	hsa-miR-184	UGGACGGAGAACUGAUAAGGGU	−6.4	0.003305
ocu-miR-373–3p	NA	AAAGUGCUUCCCUUUUGUGUGU	−7.9	0.03087
ocu-miR-206–3p	hsa-miR-206	UGGAAUGUAAGGAAGUGUGUGG	−14.9	5.01E-08
	Human homologous	Sequence	FC	FDR
ocu-miR-190b-5p	hsa-miR-190b-5p	UGAUAUGUUUGAUAUUGGGUUG	4.2	0.00124
ocu-miR-193b-5p	hsa-miR-193b-5p	CGGGGUUUUGAGGGCGAGAUGA	3.3	0.035167
ocu-let-7b-3p	hsa-let-7b-3p	CUAUACAGCCUACUGCCUUCCC	3.2	0.030057
ocu-miR-378–3p	hsa-miR-378a-3p	ACUGGACUUGGAGUCAGAAGGC	3.0	0.004843
ocu-miR-676–3p	hsa-miR-676–3p	CCGUCCUAAGGUUGUUGAGUU	2.8	0.000152
ocu-miR-27b-3p	hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC	2.7	0.003455
ocu-miR-30a-5p	hsa-miR-30a-5p	UGUAAACAUCCUCGACUGGAAGCU	2.4	2.4E-07
ocu-miR-425–3p	hsa-miR-425–3p	CAUCGGGAAUGUCGUGUCCGCCC	2.3	0.003272
ocu-miR-30e-5p	hsa-miR-30e-5p	UGUAAACAUCCUUGACUGGAAGCU	2.3	0.001571
ocu-miR-7a-5p	hsa-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGUU	−3.3	0.001634
ocu-miR-33a-5p	hsa-miR-33a-5p	GUGCAUUGUAGUUGCAUUGCA	−3.4	0.001571
ocu-miR-449a-5p	hsa-miR-449a	UGGCAGUGUAUUGUUAGCUGGU	−3.6	0.003305
ocu-miR-146a-5p	hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUUG	−4.4	1.05E-07
ocu-miR-34c-5p	hsa-miR-34c-5p	AGGCAGUGUAGUUAGCUGAUUGC	−5.1	0.002193
ocu-miR-34b-5p	hsa-miR-34b-5p	UAGGCAGUGUAAUUAGCUGAUUGU	−6.0	0.005982
ocu-miR-184–3p	hsa-miR-184	UGGACGGAGAACUGAUAAGGGU	−6.4	0.003305
ocu-miR-373–3p	NA	AAAGUGCUUCCCUUUUGUGUGU	−7.9	0.03087
ocu-miR-206–3p	hsa-miR-206	UGGAAUGUAAGGAAGUGUGUGG	−14.9	5.01E-08

FC: Fold Change, FDR: False Discovery Rate, NA: Not applicable.

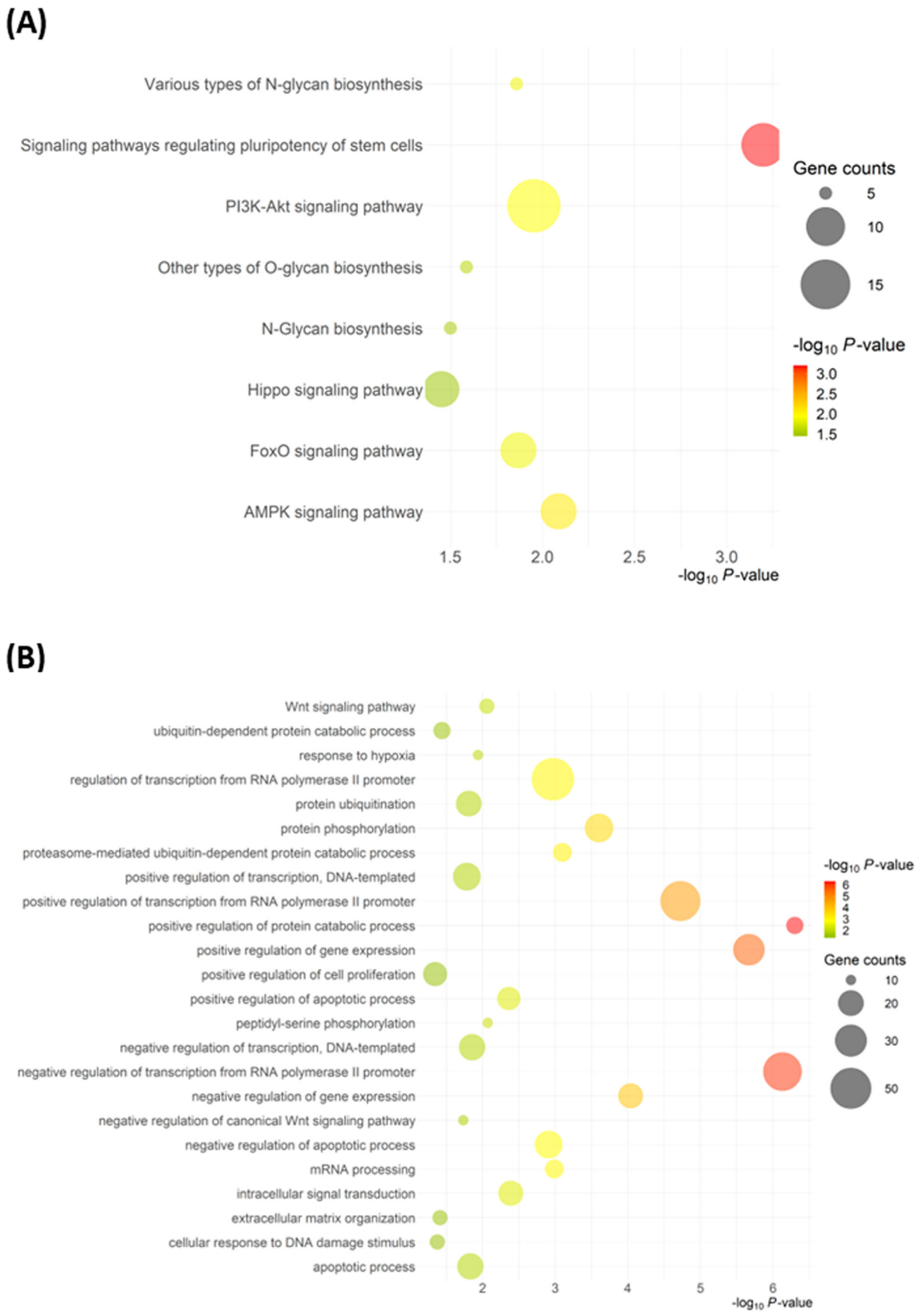

Fig. 4. Ontological classification of differentially expressed miRNA target genes. Bubble plots for the pathways (A) and biological processes (B) targeted by the elevated miRNAs in the Fertile group.

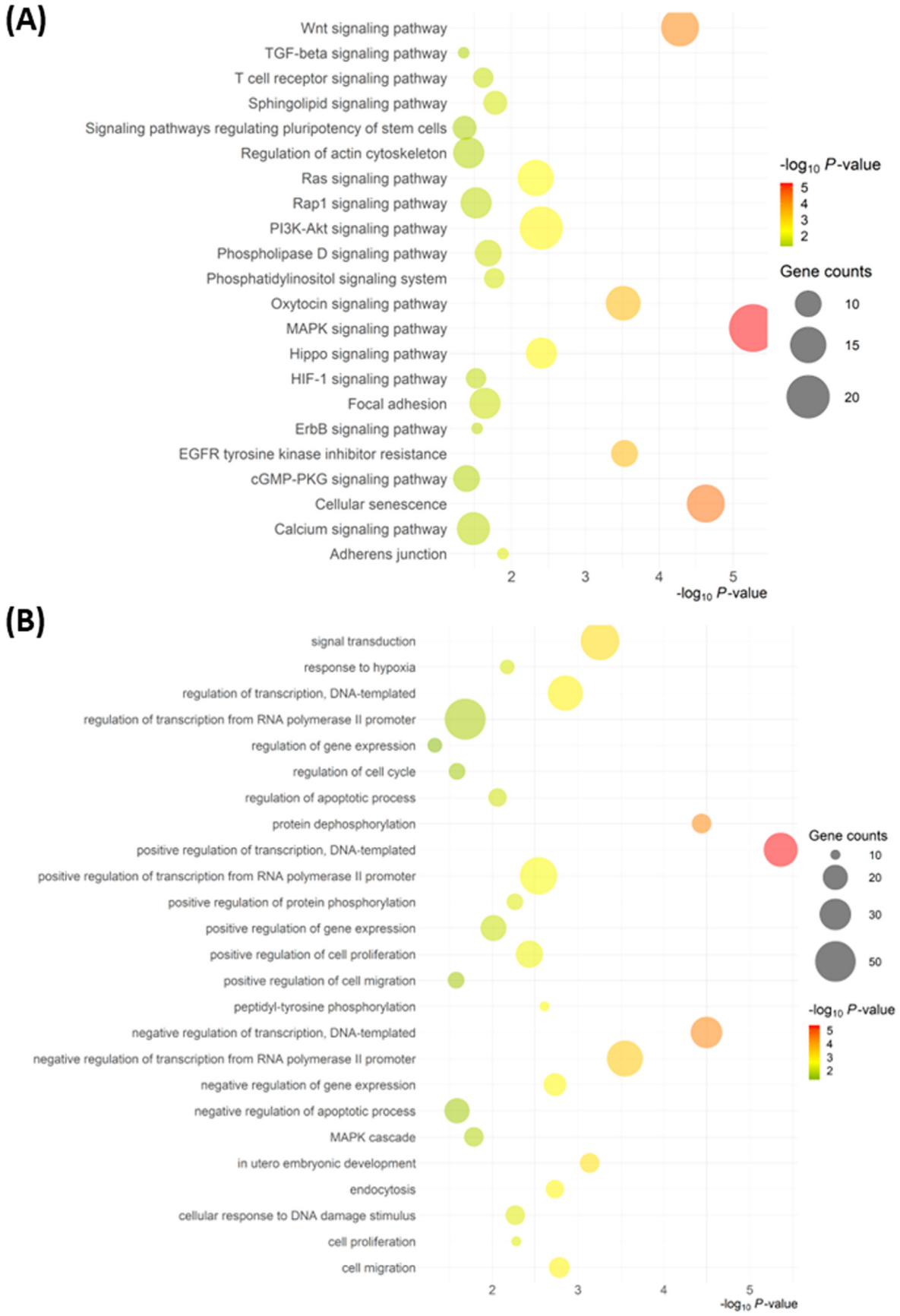

Fig. 5. Ontological classification of differentially expressed miRNA target genes. Bubble plots for the pathways (A) and biological processes (B) targeted by the elevated miRNAs in the Subfertile group.

visualized them by TEM and finally, with NTA, observed sizes ranging from 106.4 to 111.0 nm. Du et al. [22] performed ultracentrifugation (12,000×*g* for 30 min and 120,000×*g* for 1 h at 4 °C) and filtration (0.2 μm filters) methods to isolate exosomes from boar SP. These techniques allowed them to achieve a concentration of 2×10^{10} particles/mL calculated by the Nanosight NS300 system with a size of 50–100 nm observed by electron microscopy. In the current study, size exclusion chromatography methods used with the two groups of seminal samples have allowed the isolation of high concentrations of SP-EVs as calculated by the Nanosight. TEM demonstrated that most of the spherical membranous structures in the rabbit samples have a very similar diameter to those of typical exosomes, which is considered between 40 and 120 nm by Raposo and Stoorvogel [2]. Our results are slightly higher than those defined by these authors, but others such as Candenas and Chianese [9], also argue that exosomes could reach a diameter of 150 nm due to the similar morphology and overlapping sizes of SP particles. For example, in humans, Tamessar et al. [32] have defined the different sizes of EVs isolated from testes (30–200 nm), epididymis (50–250 nm), vas deferens (unknown), seminal vesicles (unknown) and prostate (30–500 nm). Recently, the MISEV2018 publication recommends using the term "small EVs" when the particles range is < 200 nm [1].

Nonetheless, further characterization of isolated EVs has been made by western blotting, and the expression of 3 of the classic exosome markers HSP70, CD9 and ALIX in the EVs isolated, demonstrated their presence in both types of males [1,33–36].

In SP-EVs of F and S males, the mean particle size was similar, and this feature and kinetic parameters were not correlated. Similar results have been observed in SP-isolated exosomes collected from normozoospermic, severe asthenozoospermic and post-vasectomy azoospermic men, which displayed similar features in terms of shape, size and expression of canonic exosome markers [37], suggesting that the size of exosomes is independent from the presence or the status of spermatozoa in seminal fluid.

However, there was a higher concentration of particles found in the SPs of the three S rabbits, which were more likely to have abnormal forms and no motility, than in the SPs of the three F rabbits. The statistical correlation we found may make sense since all samples were isolated and concentrated in the same way. However, other different parameters that are not related to the concentration of EVs seem to modulate sperm motility. Thus, exosomes obtained from normozoospermic or vasectomized men added to SPZ cultures increased their motility, while exosomes from asthenozoospermic men decreased it [37]. In pigs, it has been shown that the ATP produced by the exosomes of the SP can affect the motility of the SPZ since it is transferred to their intermediate piece, where the mitochondrial metabolism of these cells is located [38], demonstrating that sperm stored in seminal plasma rather than diluted or removed show improvement in survival and motility.

SP-EVs contain a unique and specific amount of highly stable miRNAs which represent the physiological status of semen, have regulatory functions, and could be useful as biomarkers for the diagnosis of male infertility [39,40]. In the current study, the SP-EVs miRNA expression profiles of both groups were clearly separated, indicating the differences between F and S males in their SP-EV miRNA cargos. The miRNA expression profiles revealed that let-7b-5p and let-7a-5p were the top most abundant miRNAs accounting for approximately 20% of the total miRNA sequence reads in both rabbit male groups. In addition, Let-7c and Let-7f were among the top 20 most abundant miRNAs in both groups. In agreement with our findings, the Let-7 family was highly abundant in boar [41] and human [40] SP-EVs. In the human study, Let-7b was the most abundant miRNA in all donors accounting for 19.5% of the total miRNA reads. Moreover, the Let-7 family members were reported as the most abundant miRNAs in the testes and the sperms of juvenile and adult mice [42]. This miRNA family is known to target several genes (including *IL-10* and *IL-13*) associated with inflammatory responses [43] and is also known as a regulator of male germ cell differentiation [44]. Previously, let-7b has been proven to target the estrogen receptor (ERα) gene [45] which is also expressed in testicular germ cells and mature sperm [46]. As a mediator of the physiological effects of estradiol, the expression of ERα was highly correlated with male infertility in terms of sperm quality [47]. This may indicate the role of Let-7 family members in regulating the expression of ERα in testicular cells and sperms. However, further studies are needed to explore the roles of the Let-7 family in spermatogenesis. In addition to the let-7 family, five miRNAs (miR-10a, miR-10b, miR-200c, miR-21, and miR-148a) were among the top 20 most abundant miRNAs in both groups. The same five miRNAs have been reported as the most abundant in boar SP-EVs in two different studies [41,48]. The high abundance of this group in the SP-EVs of different species may indicate their indispensable role in spermatogenesis and homeostasis regardless of the fertility status.

Differential expression analysis indicated 18 miRNAs as significantly differentially expressed between the two male groups. Among the DE-miRNAs, miR-34b-5p, miR-34c-5p, and miR-449a-5p were downregulated in the F compared to the S group. These three miRNAs have the same sequence of the seed region and thus, they may target the same group of genes. Previous studies confirmed that these miRNAs represent the paternal miRNAs as they are present in sperms but absent in oocytes [49,50]. Yuan et al. [51] found that miR-34b/c and miR-449a/b/c are essential for normal spermatogenesis and male fertility, but their presence in sperm is dispensable for fertilization and preimplantation development. In bovine, expression of miR-34b was lower in the sperm and seminal plasma of high-fertile compared to low-fertile bulls and it has been predicted to target genes related to spermatogenesis, embryo implantation, and fetal development [52]. Other studies reported that the lower expression levels of miR-34b/c-5p in testicular tissue and spermatozoa are highly correlated with the non-obstructive azoospermia, the most severe form of male infertility which is identified by no sperm in the ejaculate due to failure of spermatogenesis [16,53,54]. However, miR-34c was significantly increased in the seminal plasma of patients with asthenozoospermia, a medical term for reduced sperm motility [55]. These results together with our findings may indicate the importance of retaining these miRNAs in the testicular tissue and sperm cells instead of releasing them into the SP-EVs as they play an important role in spermatogenesis. In contrast, Eikmans et al. [56] stated that the lower expression of both miR-34b and c in the SP of men was correlated with the low concentration of spermatozoa. In the current study, we also found miR-184–3p as one of the downregulated miRNAs in F compared to the S group. In boars, the expression level of miR-184 was lower in SP-EVs of normal semen compared to samples containing spermatozoa with cytoplasmic droplets [57], which is correlated with low semen quality, low piglets number, and low litter size [58]. Moreover, miR-184 has been highly expressed in the SP-EVs of asthenozoospermia male patients [59] and, thus could be a potential biomarker of semen quality and male fertility. Among the DE-miRNAs, miR-27b was highly expressed in the SP-EVs of the F compared to the S group. The same miRNA has been reported among the low-expressed miRNAs in the SP-EVs of subfertile compared to fertile men [60]. Previously, miR-27b has been proven to target Cysteine-rich secretory protein 2 mRNA (*CRISP2*) and regulate its protein level in the sperms of asthenozoospermic patients [61] suggesting its potential therapeutic target for treating male infertility.

Ontological classifications of miRNA-predicted target genes exhibited several signaling pathways and regulatory biological processes that could be affected by the SP DE-miRNAs. Interestingly, one of the biological processes which is targeted by the highly abundant miRNAs in the S group was in-utero embryonic development. The presence of miRNAs in sperms and seminal plasma was correlated not only with the semen quality but also with fertilization and preimplantation embryonic development [62]. It is well known that seminal plasma contains several active components that interact with the endometrial epithelium and regulate embryonic development and implantation [63]. SP-EVs miRNAs could be one of these factors that may regulate early embryonic development and thus the pregnancy outcome.

In conclusion, the SP-EVs of rabbits contain different miRNAs cargoes that target genes involved in transcription regulation, protein dephosphorylation, and signal transduction pathways linked to spermatogenesis, homeostasis, and sperm quality. These miRNAs can be used as biomarkers for male fertility and potential therapies for assisted reproductive technologies. However, further studies are required to explore the exact role of SP-EVs miRNAs in male and female fertility interactions.

CRediT authorship contribution statement

Osama G. Sakr: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, supervised the project and designed the experiments. All the authors reviewed the manuscript. **Ahmed Gad:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, supervised the project and designed the experiments. All the authors reviewed the manuscript. **Karina Cañón-Beltrán:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, supervised the project and designed the experiments. All the authors reviewed the manuscript. **Yulia N. Cajas:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, supervised the project and designed the experiments. All the authors reviewed the manuscript. **Radek Prochazka:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, supervised the project and designed the experiments. All the authors reviewed the manuscript. **Dimitrios Rizos:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Pilar G. Rebollar:** and.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.06.020.

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