Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

AETE CONTRIBUTIONS – PROCEEDINGS 2025

Presidents Letter

Dear AETE members,

We would like to welcome you all in Cork, Ireland for the upcoming 41st AETE event! While the summer is rapidly approaching and we are experiencing sunny days in most parts of Europe, the preparations for our next AETE meeting are at full speed. This year you will again have the opportunity to extend your attendance with two extra AETE days when you book the pre- and post-conference day! Therefore the 41st AETE conference promises to become again a very nice and vibrant meeting for scientific and social exchanges that will be held at the Clayton Hotel Cork City from September 3rd to 6th.

Our invited speakers will share the latest information on the role of a (healthy) maternal environment on fertility successes via sperm selection, embryo quality, but also with attention for embryo recipients, and we scheduled two parallel ART workshops for Thursday afternoon, to exchange and interact on the latest insights on equine and ruminant ART. We are also very happy to announce our Pioneer Award winner Urban Besenfelder for this year, who had major contributions to our field by his work on early embryonic development. He will share his collected insights of the last 30 years.

On top of this, we need to thank you all, for the very large number of worldwide high quality abstract contributions for this years AETE meeting! The abstracts will be presented during the conference, via posters or short orals and five of them will be selected for the student competition. I would also like to take the opportunity to thank everyone for the support in the reviewing of these abstracts, being a large job that we could not have managed without your help.

The LOC has put together an excellent program for the social events with a welcome reception in the city hall of Cork, a gala dinner in Cork City Gaol and a farewell party in a pub setting in Cork. Furthermore, the LOC organized two preconferences, one on the Wednesday for OPU, ICSI and IVF in horses, and another one on ruminants where pedigree Holstein farms in the area of Cork will be visited and latest practices in dairy will be shared. In addition, on the Saturday there will be two options for a post-conference tour to Fota Wildlife Park, Barryscourt Castle and finally a Wiskey tasting or to Coolmore racing stables, the equine veterinary hospital and back via the scenic route of the Golden Valley. Please check the latest information on our website for more details and be sure to be in time if you would like to join, as there is a limited number of places.

Last, but certainly not least, we feel very privileged by the large interest and support of our sponsors, which makes it possible to organize the AETE meeting in the atmosphere that we like where we try to keep registration prices low while including all the social events. Many thanks for your continuing support and trust in our AETE society and a warm welcome to our new sponsors!

We are looking forward to meet you all in Cork, and first would like to wish you a nice summer!

On behalf of the entire AETE board and the LOC,

Hilde Aardema
President of the AETE

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THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

STUDENT COMPETITION

Endometrial organoids medium does not support epiblast development during ovine post-hatching development

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Embryo mortality during conceptus elongation is a major contributor to early pregnancy loss in livestock. Despite its relevance, the molecular mechanisms governing this developmental period remain poorly understood. We recently established an *in vitro* post-hatching culture system based on N2B27 medium that supports key events of ovine post-hatching development up to early gastrulation (Ramos-Ibeas et al., 2022). However, conceptus elongation still appears to require additional cues, presumably derived from uterine secretions. Endometrial organoids (EOs) retain both the structural and functional characteristics of the in vivo endometrium when cultured in endometrial organoids medium (EOM) (Turco et al., 2017). Incorporating EOs derived from pregnant ewes during conceptus elongation into the post-hatching culture system offers a promising model to study the effects of uterine secretions on embryo development. However, optimal conditions for such co-culture remain to be defined. In this study, we evaluated whether EOM, which contains the growth factors, hormones, and other molecules required for EOs derivation and culture, can support ovine post-hatching development. Day (D) 6 and 7 blastocysts were randomly cultured in either EOM (n=117) or N2B27 (n=113) until D12. Embryo survival was assessed as the percentage of embryos maintaining blastocoel integrity, and embryo area was measured from images captured prior to fixation. Embryos were then processed for immunofluorescence analysis of lineages development using SOX2 (epiblast), SOX17 (hypoblast), and CDX2 (trophectoderm) markers. No significant differences were detected in embryo survival (77.6±4.3 vs. 83.4±4.9%; n=3 replicates), embryo area (504.7±30.8 vs. 606.5±61.9 µm2), or in the % of hypoblast migration along the inner embryo surface (44.9±2.9 vs. 51.5±3.2%) between embryos cultured in EOM or N2B27 medium, respectively (mean±s.e.m; Mann-Whitney test, P>0.05). However, a significant reduction in both the proportion of embryos with surviving SOX2+ epiblast cells (33/91 [36.3%] vs. 54/77 [70.1%]; Chi-square test, P<0.05) and the number of SOX2+ epiblast cells per embryo (9.3±2 vs. 42.1±8.8; Mann-Whitney test, P<0.05) was observed in embryos cultured in EOM compared to N2B27 medium. These results demonstrate that EOM fails to support epiblast development during ovine post-hatching *in vitro* culture.

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Keywords: post-hatching *in vitro* culture, ovine, endometrial organoids

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

STUDENT COMPETITION

Changes in bull epididymal extracellular vesicles miRNA cargo in response to natural heat stress

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Maturation in the epididymis is critical for sperm to acquire fertilising ability. During this process, sperm incorporate epididymis extracellular vesicles (EVs), which contain a variety of molecules, including small RNAs (miRNAs). These miRNAs could potentially be delivered to the oocyte during fertilisation and influence embryo development. Importantly, environmental factors have been shown to modulate the cargo of seminal plasma EVs. Heat stress negatively affects bull fertility by decreasing sperm motility and conception rate. However, whether heat stress can affect the epididymal EV-miRNA cargo is unknown. Thus, this study aimed to characterise the miRNA content of EVs secreted by the epididymis of heat stressed bulls. For this, we flushed the cauda epididymis of bulls collected at a local slaughterhouse in Madrid (Spain) in November-April (control; n=4), and in July-August (heat stress-HS; n=5). Heat stress was determined based on the average Temperature and Humidity Index (THI) on the week prior to sample collection (63.4 ± 3.2 and 78.0 ± 0.3 for control and HS, respectively). EVs were isolated from the epididymal fluid using size exclusion chromatography, followed by ultrafiltration for EV concentration, and characterized using flow cytometry (CytoFLEX S, Beckman Coulter) by the detection of CFSE, HSP70, CD63, CD81 and CD9 markers. After total RNA isolation and miRNA sequencing, the processed reads were mapped to the bovine reference genome (ARS-UCD1.3), annotated and quantified with miRDeep2 based on bovine precursor and mature miRNAs available from the mirBase database. Normalization of miRNA read counts and differential expression analysis between groups was done using DEseq2. Interestingly, EV concentration was lower in the HS group compared to the control (5.6E+04 ± 7.4E+03 EV/µl vs. 1.8E+05 ± 5.8E+04 EV/µl, respectively; P=0.022). A total of 1207 miRNAs were identified through RNAseq analysis, but none showed significant differential abundance between groups. However, one miRNA was only detected in samples of the HS group (bta-miR-2350), and four miRNAs (bta-miR-2483-5p, bta-miR-2419-5p, bta-miR-2376, and bta-miR-2419-3p) showed a trend for higher abundance and one a trend for lower abundance (bta-miR-502a) in the HS group. Exploratory enrichment analysis (miRWalk - GSEA results) based on the exclusive and higher abundant miRNAs suggested involvement of MAPK, Ras, FoxO, AMPK, and Wnt signalling pathways, which are associated with stress response, cell survival, and metabolic adaptation. The lower abundant miRNA showed an involvement in the p53 signalling pathway, a crucial regulator of cell cycle, apoptosis, and DNA repair. Our findings suggest that heat stress induces changes in EV secretion and miRNA cargo in the epididymis. However, whether this affects sperm function and embryo development remains to be studied.

This study was funded by grant PID2022-140855OA-I00 (funded by MICIU/AEI/ 10.13039/501100011033 and by ERDF/EU) and grant DN101120104 (BullNet; funded by Marie Skłodowska-Curie Actions).

Keywords: epididymis, cattle, RNAseq

Animal Reproduction

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STUDENT COMPETITION

Comparison of the mitochondrial hydrogen peroxide production in bovine oocytes and zygotes

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This study aimed to compare mitochondrial hydrogen peroxide (mt-H_2O_2) dynamics between bovine oocytes and zygotes to begin addressing whether redox regulation differs across developmental stages. Understanding such differences is crucial, as low mt-H_2O_2 levels support redox signaling, whereas excess can lead to oxidative stress, requiring tight control to maintain cellular fitness (Murphy MP, Biochem J, 417, 1–13, 2009). mt-H_2O_2 dynamics were monitored using HyPer7-MLS, a mt-targeted, H_2O_2-specific ratiometric sensor (Pak VV, Cell Metabolism, 31, 642–653.e6, 2020), which we validated in preliminary experiments in bovine oocytes and zygotes. cRNA encoding HyPer7-MLS was synthesized for microinjection. Cumulus-oocyte complexes (COCs) were collected from abattoir-derived ovaries. A pre-*in vitro* maturation phase with IBMX delayed meiotic resumption and synchronized nuclear maturation. For oocyte experiments, germinal vesicle (GV) oocytes were denuded, injected with cRNA, and co-cultured with intact COCs during *in vitro* maturation (IVM). For zygote experiments, intact COCs underwent IVM, followed by denudation and microinjection at the MII stage, preceding *in vitro* fertilization (IVF) to generate presumptive zygotes. After IVM or IVF, samples were imaged under control (IVM/IVC medium) or pro-oxidant conditions (100 mM tert-butyl hydroperoxide (t-BOOH)) every 30 seconds for up to 60 minutes using a spinning disk confocal microscope (NOLIMITS Unitech facility) with 405 and 488 nm excitation for ratiometric F488/F405 analysis. Imaging settings were constant across groups, and F488/F405 ratios were extracted from the image data using NIS-Elements (Nikon) software. Data were compared using the two-tailed Mann–Whitney test in Prism v10; differences with p-values <0.05 were considered statistically significant. To further confirm mt- targeting, co-localization was tested by incubating injected oocytes with the mitochondrial dye PK-Mito for 1 hour, followed by washing and imaging. Co-localization confirmed mitochondrial targeting of HyPer7-MLS (52 oocytes). A total of 93 *in vitro* matured oocytes (54 control, 39 pro-oxidant) and 121 presumptive zygotes (56 control, 43 pro-oxidant) were analyzed. Although phototoxicity limited extended imaging, the initial 15-minute window was suitable to assess mt-H_2O_2 levels and compare the mitochondrial responsiveness of oocytes and zygotes to oxidative stress. Under control conditions, no significant differences were observed between groups. However, under pro-oxidant challenge, oocytes showed significantly higher F488/F405 ratios than presumptive zygotes. This study establishes the first use of HyPer7 to compare mt-H_2O_2 dynamics between oocytes and zygotes in mammals, revealing a differential response that may reflect enhanced mitochondrial antioxidant capacity in zygotes. Ongoing efforts aim to optimize imaging protocols for full time-lapse monitoring of mt-H_2O_2 levels throughout oocyte maturation, while minimizing phototoxicity to ensure reliable data acquisition.

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Keywords: oocyte, zygote, ROS

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

STUDENT COMPETITION

TEAD3 is not required for bovine blastocyst formation

Aitana Salvo-Jiménez[1], David Morato[1], Carlos Irala Gil[2], Leopoldo González-Brusi[1], Inés Flores Borobia[1], Priscila Ramos-Ibeas[1], Pablo Bermejo-Álvarez[1]

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Blastocyst formation requires the differentiation of the initially totipotent blastomeres into the inner cell mass (ICM) and the trophectoderm (TE). While the molecular regulation of such cell differentiation event is well studied in mice, recent loss-of-function studies have highlighted significant differences between mice and other mammals. In mouse embryos, TE/ICM differentiation is driven by Hippo signaling pathway, with TEAD4 acting as the major and indispensable regulator. In striking contrast, TEAD4 ablation does not prevent TE differentiation in cattle, rabbits and humans (Pérez-Gómez et al. Development 2024). In the absence of a critical role for TEAD4 in TE differentiation, other TEAD family members may substitute TEAD4, and between them, TEAD3 is expressed by the bovine blastocyst. The objective of this study has been to elucidate the role of TEAD3 in first linage differentiation in cattle by testing the developmental ability of TEAD3 KO embryos generated by cytosine base editor (CBE) technology. *In vitro* matured bovine oocytes (n=158, 2 replicates) were divided in two groups: one was microinjected with CBE-encoding mRNA and a sgRNA against TEAD3 (n=90, targeted group, containing KO embryos), and the other was microinjected with CBE encoding mRNA alone, serving as microinjection control (n=68, control group, formed by wild-type WT embryos). Microinjected oocytes were fertilized *in vitro* and developed to Day (D) 8 in SOF medium. D8 embryos were fixed and subjected to immunohistochemistry to detect TE (CDX2+) and ICM (SOX2+). Following image acquisition, embryos from C+G group were genotyped by Sanger to determine which ones contain the stop codon introduced by CBE. No significant differences were observed in the ability to reach the blastocyst stage between both microinjection groups (Blastocyst rate: 25±0.8 vs. 26.6±7.9%, for control vs. targeted, mean±s.e.m., t-test P>0.05). Of the 17 D8 embryos genotyped in the targeted group, 15 (88%) were KO. TEAD3 KO blastocysts displayed similar total, TE and ICM cell numbers than WT (Total: 95.9±10.4 vs. 93.7±5.6; TE: 89.2±10 vs. 82.3±6; ICM: 19.5±2.4 vs. 17.9±3.5, for WT (26) vs. KO (15), respectively, mean±s.e.m, t-test P>0.05). In conclusion, TEAD3 is not essential for TE specification and blastocyst formation in cattle.

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Keywords: Trophectoderm, TEAD3, CRISPR

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STUDENT COMPETITION

Sperm metabolomic profiles reflect bull fertility status

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Early and accurate assessment of bull fertility is critical for the success of artificial insemination (AI) programs in cattle production. However, current selection tools — including genomic predictions and standard semen evaluations — offer limited reliability in forecasting field fertility outcomes. To address this limitation, we explored the sperm metabolome as a potential source of novel fertility-associated biomarkers. Twenty-four Holstein-Friesian bulls were selected from a base population of 840 bulls throughout AI centres in Ireland which each had at least 500 insemination records. Fertility was defined based on an adjusted sire fertility index (average fertility score was 0), with high fertility (n=12 bulls) having a mean adjusted fertility score (± SEM) of +3.7% (± 0.15%) and low fertility (n=12 bulls) having a mean adjusted score of -8.4% (± 0.95%). High-throughput untargeted metabolomics (Metabolon Inc., Durham, NC, USA) of the frozen-thawed sperm, which had passed standard microscopy-based quality control checks, identified raw peak intensities for 615 metabolites. After normalizing the metabolites to the total protein concentration of each sample and subsequent data filtration, 547 were retained for downstream analyses. Initial qualitative analysis of the pre-processed dataset revealed the metabolites clustered in 8 biochemical super-pathways [lipids (55.1%), amino acids (17.4%), xenobiotics (6.7%), peptides (5.7%), nucleotides (5.0%), carbohydrates (4.2%), cofactors and vitamins (4.1%), and energy substrates (1.5%)]. Unsupervised clustering and principal component analyses showed considerable overlap between the high and low fertility groups, indicating largely conserved metabolic profiles likely due to the fact that the low fertility bulls are suboptimal compared to the reference population rather than infertile. Nonetheless, subsequent unpaired t-tests combined with fold-change thresholding identified 18 differentially abundant metabolites between fertility groups ($P<0.1$, absolute fold change > 1.5), with significant enrichment in pathways relating to lipid and energy metabolism. Further interrogation of the differentially abundant metabolites revealed metabolic differences associated with calcium channel inhibition and reactive oxygen species production in the low fertility bulls. To evaluate the predictive capacity of the dataset, a Random Forest-based receiver operating characteristic analysis was employed. Using a subset of 5 metabolites — 3-phosphoglycerate, phenylalanine, ceramide, citrate, and citrulline — this machine learning approach was capable of distinguishing fertility status with high predictive accuracy (AUROC=0.877; $P=0.02$). Overall, these data support metabolomics as a promising omics-based approach to enhance bull fertility evaluation and improve selection strategies in AI programs.

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Keywords: sperm, bull fertility, metabolome

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AI / TAI / FTET

Identification of predictive biomarkers of bovine endometrial receptivity on day 7 in peripheral white blood cells

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Embryo transfer (ET) efficiency in cattle remains low, with live calf rates ranging from only 30% to 40%. A receptive endometrium is essential for embryo implantation and successful gestation, but there are currently no predictive biomarkers to that can distinguish a receptive from a non-receptive endometrium. During the estrous cycle, the endometrium undergoes molecular, functional and structural modifications under hormonal control to support pregnancy. We hypothesized that these changes may influence gene expression at the peripheral level on Day 7 post-estrous (D7), a critical time point increasingly explored for transcriptomic biomarkers of endometrial receptivity. We aimed to identify a transcriptomic signature in peripheral white blood cells (PWBC) predictive of gestation success on D7. Blood samples were collected on D7 from 200 Holstein heifers across 18 farms, just before ET using IETS-Q1/Q2 *in vitro* produced embryos. Pregnancy status was determined on Day 45 via ultrasound and only heifers that produced a live calf were classified as pregnant (PG). A subset of 100 samples (40 PG, 60 non-PG on D45) was analyzed by RNA-seq. Gene Set Enrichment Analysis (GSEA) of the 25,798 expressed transcripts revealed functional enrichment for immune tolerance, ion and metal transport pathways in PG heifers. Differential expression analysis (DESeq2), however, revealed no transcripts differentially expressed (FDR ≤ 0.10) between PG and non-PG heifers. To further explore predictive signatures, BORUTA, a machine learning approach with a method relying on Random Forest classifiers, identified transcripts in PWBC whose expression could discriminate between PG and non-PG heifers. The BORUTA algorithm was first applied to the complete set of expressed transcripts to rank them by importance, and then to the top 1,000 most important transcripts. This analysis identified 47 informative transcripts, of which 24 had Ensembl 113 annotations. These transcripts were involved in transcriptional regulation, amino acid, lipid metabolism, enzymatic redox control, cell adhesion, extracellular matrix interactions, signal transduction, ion transport, cellular communication and immune signaling. To evaluate the predictive accuracy of the biomarkers identified by the algorithm, multiple BORUTA runs were performed using 80% of samples for training and 20% for testing. The model achieved between 90 and 96% accuracy on training sets and 60 -75% on test sets. Although none of the transcripts reached classical statistical significance in differential expression (FDR ≤0.10), the combined expression profiles classified heifers as PG or non-PG at the time of ET. These results suggest that the PWBC transcriptome reflects systemic physiological adjustments associated with endometrial receptivity. Future studies could improve results by increasing the number of animals in the experimental design or by including other omics data in the prediction model. This study was funded by APISGENE (PRATEG and CINEMORE research programs) and by the French National Association for Research and Technology (ANRT) through a CIFRE doctoral fellowship.

Keywords: endometrium, receptivity, cattle, biomarkers

Animal Reproduction

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AI / TAI / FTET

Endometrial cytology and fertility in dairy cows

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This study is part of a research project aimed at reducing unnecessary hormone use in dairy herds and identifying biomarkers of bovine infertility. The research was conducted at the dairy cattle farm of the University of Bologna, involving 64 multiparous cows (21 with 2 parities [P2], and 43 with ≥3 parities [P3+]) assigned to either natural (N) or synchronization (S) protocols. The N protocol relied on heat detection via automatic monitoring and reproductive assessment after a voluntary waiting period of 70 days, while the S protocol used Double Ovsynch, with the first artificial insemination (AI) performed around 80 days in milk (DIM). Pregnancy diagnoses were performed at 30±2 days after AI. Cows pregnant after the first AI were classified as fertile (F1). The others underwent a second AI using the same assigned protocol (automated estrus detection or re-synch) and were then classified as fertile (F2) if pregnant or sub-fertile (SF) if not. This study focused on evaluating endometrial cytology to identify cows with reduced fertility. Although only cytology results are presented here, blood and urine samples were also collected for further analyses, such as hormonal and biochemical profiling, metabolomics, lipidomics, and oxidative stress. Cytological samples were collected at +10, +35, +45 DIM, and at first and second AI, using a cytobrush. Smears were air-dried and stained with Wright's stain. Polymorphonuclear cell (PMN) percentages were determined by counting at least 200 cells per smear. Data are expressed as mean ± standard deviation. PMN rates were tested for normality using the Shapiro-Wilk test and compared using the Mann–Whitney test or Friedman test followed by the Wilcoxon test. Fertility results were analysed using the Chi-square test for contingency tables (IBM SPSS Statistics 29). Significance was set at $P<0.05$. No differences in fertility were observed between protocols, as well as in PMN rates at any time point. In both groups, the PMN rate at +10 was higher ($P<0.05$) than at the other time points. Therefore, subsequent analyses were performed on pooled data from both protocols. Regarding parity, at 45 DIM, P2 cows had significantly lower PMN rates than P3+ (1.40±2.51% vs 3.05±4.60%, $P<0.05$), while at second AI, an opposite trend was observed (P2: 6.39±14.50% vs P3+: 1.38±4.26%, $P<0.05$). PMN rates at +10, +35, +45 DIM and first AI were similar between F1 and non-pregnant cows. However, at second AI, PMN were significantly higher ($P<0.05$) in non-pregnant cows (SF: 3.93±10.51%) than in F2 cows (0.42±0.69%). These findings align with previous research showing that parity influences endometrial inflammation dynamics, with different PMN thresholds for diagnosing cytological endometritis postpartum. This highlights the need for parity-specific diagnostic criteria. This study provides new insights into PMN dynamics under different AI protocols; however, practical applications for improving fertility prediction and reducing hormone use require further investigation, particularly through the integration of complementary biomarkers and a larger population.

Funding: PRIN 2022, project 2022CBNFEX.

Keywords: endometrial cytology, fertility, cattle

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

AI / TAI / FTET

Reproductive outcomes in high-producing lactating dairy cows following timed artificial insemination or timed embryo transfer with cryopreserved *in vitro*-produced embryos

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Timed embryo transfer (TET) could improve efficiency in dairy cattle by enhancing fertility during warmer seasons and increasing the value of surplus calves. Previously, we evaluated the efficiency of TET programs in high-producing lactating dairy cows and found that the rejection rate for TET (7.2%) was not affected by season, synchronization protocol (SP), days in milk (DIM), body condition score (BCS), or parity (Cuevas-Gómez, Anim Reprod, 21, 6, 2024). In this follow-up study, we compared the results of pregnancy per service (P/S) following timed artificial insemination (TAI) or TET with cryopreserved abattoir-derived *in vitro*-produced (IVP) beef embryos. A total of 587 Holstein-Friesian cows from three farms in southern Spain were blocked by parity, DIM, and SP (Double Ovsynch and G6G, using D-cloprostenol and buserelin [Veteglan and Veterelin, respectively, Calier, Spain] and randomly assigned to receive either TAI (n=313) or TET (n=274; performed by a single veterinarian). Based on CL ultrasound assessment, non-rejected cows (n=230) assigned to TET were further randomized to receive either commercial slow-frozen (ET-SF, n=182) or vitrified (ET-VIT, n=48) IVP embryos. Pregnancy was diagnosed by transrectal ultrasound on d 28 after synchronized ovulation and confirmed on d 63. In a subset of 115 cows, an additional confirmation was performed on d 41. Statistical analyses were conducted using generalized linear mixed models (GLIMMIX, SAS). Separate models were used to compare type of service: (i) TAI vs. TET; and (ii) TAI vs. ET-SF vs. ET-VIT. Each model included type of service, season (cold, warm), BCS (<2.75, 2.75-3, >3), parity (1st, 2nd, >2nd), service number (1st, and >1st), and SP as fixed effects. Farm was included as a random effect. All variables and two-way interactions were tested and retained in the models when P≤0.25. Overall, P/S on d 28 was lower following TET (21.4%) compared with TAI (36.1%; P<0.01). Although the interaction between season and type of service was not significant (P=0.12), post-hoc analyses revealed differences among factor levels. Notably, no differences in P/S were found between TET (17.6%) and TAI (22.2%) during the warm season, whereas during the cold season, P/S was lower for TET (26%) compared with TAI (49.1%; P<0.01). Unlike previous reports, TET did not improve P/S during the warm season in high-producing cows. In addition, P/S on d 28 was lower for ET-SF (18.8%) than for TAI (36.1%, P<0.01), while no differences were observed between ET-VIT (31.2%) and TAI or ET-SF. Pregnancy loss (PL) between d 28 and 63 was greater for TET (57.1%) compared with TAI (25.9%, P<0.001), resulting in lower P/S on d 63 (9.1% vs. 26.8%, respectively; P<0.01). No differences in PL were observed between ET-SF (58.8%) and ET-VIT (53.3%). Outcomes were not affected by SP. Most losses occurred between d 28 and 41 compared to d 41 to 63 in all groups: TAI (16.7% vs. 5.7%), ET-SF (37.5% vs. 20%), and ET-VIT (46.7% vs. 12.5%). In conclusion, TET using cryopreserved IVP embryos resulted in poorer reproductive outcomes compared to TAI in high-producing dairy cows.

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Keywords: embryo transfer, fertility, cattle

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

AI / TAI / FTET

Effect of cumulative heat–humidity index on pregnancy outcomes in dairy cattle: a preliminary summer study

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Heat stress is known to impair dairy-cow fertility, yet the magnitude of this effect under temperate European summers, and its interaction with embryo traits, remains unclear. To determine whether cumulative temperature–humidity index (THI) measured before breeding alters pregnancy establishment after artificial insemination (AI) or embryo transfer (ET), and to assess the influence of embryo origin and cryopreservation. Between 28 June and 5 September 2024, 174 breedings were recorded in a commercial Holstein herd in central Poland: 104 AI and 70 ET. Cows were balanced for lactation number (1 to 7), and body-condition score (3.0±1.0). ET recipients received fresh in-vitro embryos (n = 35), slow freeze in-vivo embryos (n = 30) or slow freez in-vitro embryos (n = 5); all embryos were Grade 1 and 2 (IETS). Ambient temperature and humidity were logged every 3h by a Netatmo® Weather Station. A THI was calculated for each record as (1.8×T+32) − (055−0.0055×RH) (1.8×T−26). Two complementary THI metrics were created: (i) 31 adjacent 2-day windows (day 0→-1 … -30→-31) and (ii) 31 cumulative windows (day 0→-1 … 0→-31). Daily THI was averaged within each window. Pregnancy was diagnosed ultrasonographically on day 30. Separate logistic-regression models were built for AI and ET; discrimination was assessed by the area under the ROC curve (AUC). Breeding-method effects were compared with a global χ^2 test followed by pairwise Fisher's exact tests (Holm corrected) (Python 3.11; statsmodels 0.14 and scikitlearn 1.4). Daily mean THI ranged 65–77 (70.7 ± 3.0) and never exceeded the moderate-stress threshold (78). Pregnancy rates were 36% for AI, 11% for fresh in-vitro ET, 10% for slow freez in-vivo ET and 0% for slow freez in-vitro ET (χ^2, P = 0.002). For AI, odds ratios across the 31 adjacent windows were 0.93–1.08 and across the 31 cumulative windows 0.94–1.07 (all P > 0.25; AUC 0.46–0.57). THI effects on ET were likewise non-significant. Under moderate summer conditions, neither short (2-day) nor cumulative (up to 31-day) THI indices impaired fertility, whereas embryo category strongly influenced ET outcome. The winter phase (2025/26) will determine whether colder environments modify these relationships.

Keywords: embryo, heat stress, cattle

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

AI / TAI / FTET

Geographical variation in antral follicle count in mares is not solely explained by breed or management factors

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Antral follicle count (AFC) is a determining factor of success in the *in vitro* production of equine embryos (Cuervo-Arango, Reproduction Fertility and Development, 31, 1894-1903, 2019). Differences in AFC exist between individual horses and are reported across geographic regions, but these are poorly understood. This study aimed to evaluate whether AFC is influenced by geographic location and identify management practices associated with higher AFC. An observational study was conducted using data collected during 316 Ovum pick-up (OPU) sessions in four clinics located in the UK (n=33), Netherlands (n=188), Ireland (n=11) and Brazil (n=84). OPU was performed between December and April (winter in Europe, summer in Brazil) during 2 seasons (2024-2025). Experienced veterinarians counted all visible follicles at the time of OPU (number aspirated and number not aspirated). In Europe, mares were checked for suitability prior to OPU whereas in Brazil they were not. Mares were presented up to 3 times per season, with ≥ 3 weeks between sessions. Only data from Warmblood mares was included. Climate (weekly temperature ranges and solar radiation) was assessed using public records from the 4 areas. Husbandry factors were explored using questionnaires completed by owners on mare signalment, nutrition, housing (closed questions) and exercise (scale from 1 to 5). Data distribution was assessed by Shapiro-Wilk test, in case of normal distribution one-way ANOVA followed by Tukey's test was used (≥ 3 groups) while in non-normal distribution, Kruskal-Wallis with Dunn's test (≥ 3 groups) or Mann-Whitney U test (2 groups) were applied. AFC was lower in Brazil (10.25 ± 0.56) compared to the UK (20.12 ± 1.15) and The Netherlands (19.10 ± 0.52) ($P<0.01$). Climate factors varied markedly between Brazil and Europe (Temperature: $P<0.001$; Solar radiation: $P<0.001$), but not within European sites. Brazil was the only clinic that had mares with a diverse profile, therefore husbandry factors were explored for them only (n=71). In these mares, adults had a greater AFC than both young (≤3 years: $P=0.02$) and old mares (≥16 years: $P<0.01$). Higher AFC was found in competition mares (n=38; in training/high athletic performance level, housed in a stable and receiving concentrate in addition to forage) compared to broodmares (n=29; those not in work, living in a field and receiving forage only) ($P=0.03$). Each of the three factors (exercise, housing and feeding) was significant individually (in training vs. not in work, stable vs. field, concentrate and forage vs. forage only) but as they were strongly interrelated it could not be determined which one was the root cause of the higher AFC. Interestingly, AFC was higher at the clinic in the UK, despite a lower proportion of competition mares compared to the Brazilian cohort (z-test, $P<0.001$). In conclusion, AFC varied significantly by geographic region. Husbandry practices influenced AFC within Brazilian mares, but do not fully explain regional disparities. Larger, prospective experimental studies are needed to clarify which individual factors influence AFC so as to develop evidence-based recommendations for optimal management of mares in OPU programmes.

Keywords: antral follicle count, ovum pick up, mare

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

AI / TAI / FTET

Bacteriological and cytological findings in uterine swabs from broodmares in Ireland

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Endometritis is a leading cause of reduced fertility in mares and remains a major challenge in equine reproductive practice. It is primarily associated with bacterial infection and is typically diagnosed using endometrial cytology, microbial culture, or both. Uterine swabbing is a widely used, practical, and cost-effective method for preliminary reproductive tract assessment, allowing microbiological and cytological evaluation, especially under field conditions. Although equine endometritis has been studied extensively elsewhere, no data on its prevalence in Ireland have been published. This retrospective study aimed to present bacteriological and cytological findings from 4,489 uterine swab samples collected from Irish broodmares and submitted to the Irish Equine Centre (Naas, Co. Kildare) between 2019 and 2024 for screening and diagnostic purposes before breeding. Due to confidentiality agreements with the owners, information regarding the clinical status of the mares and the sampling context was not available. All samples underwent aerobic bacterial culture, and 1,724 were also assessed cytologically. Cytological endometritis was defined as the presence of ≥2 polymorphonuclear neutrophils per field at 400× magnification on Diff-Quik–stained slides. Bacteriological endometritis was defined by the presence of one or more bacterial isolates from the uterine swab samples and bacteriological cultures were classified as negative if no growth was observed at 24 and 48 hours. Cytological endometritis was diagnosed in 4.8% (n = 83) of samples that were cytologically evaluated, including 0.5% (n=9) with positive cytology but no bacterial growth. Additionally, 4.3% (n = 74) of cytologically evaluated samples were positive in both cytological and bacteriological tests. Among all cultured samples, bacterial growth (≥1 isolate) was detected in 60.6% (n = 2,718) of samples. Single isolates were found in 50.2% (n=1,365) of samples with positive bacterial growth, two isolates in 33.5% (n=911), and three or more isolates in 16.3% (n=442). The most frequently isolated bacteria (including samples with multiple isolates) were β-haemolytic Streptococcus spp. (56.6%, n=1,538), Escherichia coli (46.7%, n=1,268), α-haemolytic Streptococcus spp. (18.4%, n = 501), Staphylococcus spp. (9.4%, n = 256), Neisseria spp. (8.2%, n=224), Staphylococcus aureus (4.9%, n=134), and Klebsiella aerogenes (4.1%, n=111). This dataset offers valuable insights into the prevalence and bacterial profile of endometritis in Irish broodmares, highlighting β-haemolytic Streptococcus spp., Escherichia coli, and α-haemolytic Streptococcus spp. as the most commonly isolated bacteria. The bacterial profile is broadly consistent with findings from other countries, supporting the relevance of international research to Ireland. Furthermore, these results emphasize the importance of combined microbiological and cytological diagnostics for targeted reproductive management.

Keywords: endometritis, prevalence, Ireland

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

AI / TAI / FTET

Associations between the number of uterine bacterial isolates and cytological endometritis in mares

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The combined use of cytology and bacteriological culture is the most common method for diagnosing endometritis in mares. Uterine samples are primarily collected via uterine swabs (US) or uterine lavage (UL). US are commonly taken for both screening and diagnostic purposes, whereas UL are performed mainly as a diagnostic test. There is currently no published data on the use of these diagnostic methods in Ireland nor the link between the number of isolates with the diagnosis of cytological endometritis (CE). The latter is important in mixed growth of bacteria (≥3 isolates) regarding appropriate use of antimicrobials. Thus, we retrospectively evaluated the effect of single or mixed bacterial growths on the prevalence of CE for these two methods. A dataset including 3,956 endometrial samples (US: n = 1,724; UL: n = 2,232), collected between 2019 and 2024 from mares in Ireland and processed at the Irish Equine Centre (Naas, Co. Kildare), was analysed. Information on endometrial cytological evaluation and aerobic bacterial culture (blood and McConkey agar plates) was obtained. Cytology results were considered positive for CE when ≥2 polymorphonuclear neutrophils per high-power field were observed. Bacteriological cultures were classified as negative when no growth was observed at 24 and 48 hours after culture, and positive otherwise. The number of isolates (0, 1, 2 or ≥3) was also recorded, and bacterial identification was carried out using the VITEK2 system (Biomerieux) and phenotypic tests when appropriate. Generalized mixed effects models (RStudio v4.4.3) were used to assess the effects of bacterial growth on the prevalence of CE. The year nested within the farm of origin was included as a random effect. For US samples, the prevalence of CE was 4.8% (n = 83), while 46.8% (n = 807) were positive on bacterial culture, of which 9.2% (n = 74) were CE positive. Among positive cultures, 56.8% (n = 458) had 1 isolate, 32.8% (n = 265) had 2, and 10.4% (n = 84) had ≥3. The predicted probability of CE did not differ between groups (P > 0.05), with CE rates of 8.3 ± 1.3%, 8.7 ± 1.7%, and 15.5 ± 4.0% for samples with 1, 2, and ≥3 bacterial isolates, respectively. As for UL samples, the prevalence of CE was 36.6% (n = 816), while 81.3% (n = 1,814) were positive on bacterial culture, of which 42.3% (n = 768) were CE positive. Among positive cultures, 44.3% (n = 804), 35.7% (n = 648), and 20.0% (n = 362) had 1, 2, or ≥3 isolates, respectively. Samples with a single bacterial isolate showed greater predicted probability of CE compared to those with ≥3 isolates (45.4 ± 2.0% vs. 33.9 ± 2.7%, respectively, P = 0.002). For all other comparisons, no differences were found (P > 0.05). In conclusion, prevalence of CE was low in US positive cultures, suggesting further investigation may be indicated before antimicrobial treatment. For UL, the prevalence of CE was higher, and the probability increased in cases with a single isolate compared to those with ≥3 isolates. This suggests that when interpreting UL results, single isolates may better represent true uterine infection, while mixed cultures could reflect external contamination or non-pathogenic flora, considering the quality of the sample as a limiting factor.

Keywords: uterine pathology, culture, equine

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

AI / TAI / FTET

Associations between vaginal microbiota and pregnancy outcome in mares

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The composition and dynamics of the vaginal microbiome are increasingly recognized as critical factors influencing reproductive health across animal species. However, little is known about the vaginal microbiota in mares. Factors such as breeding, reproductive tract anatomy, injuries, and antibiotic exposure can alter the microbial environment, potentially influencing fertility. In a continuation of studies characterizing microbial shifts during the estrous cycle and after artificial insemination (AI), this study investigated associations between vaginal bacterial species and pregnancy outcomes in mares. Vaginal swabs were collected from 24 clinically healthy mares, aged from 5 to 20 years, immediately before AI and again three days later. Four mares were maiden, while the others had previously foaled. All mares were inseminated with cooled semen. None showed signs of exaggerated post-breeding-induced endometritis. Pregnancy was confirmed by rectal ultrasonography 15 days after ovulation. Isolates from 1007 cultures were identified by MALDI-TOF. Bacterial species presence was correlated with pregnancy outcome in the same cycle. Among 24 mares, 13 (54%) were diagnosed pregnant. A total of 33 bacterial species were identified in isolates. Escherichia coli was the most prevalent, detected in 13 mares (59.1%) that became pregnant. Bacteria more frequently found in mares that became pregnant included Enterococcus casseliflavus (80%), Streptococcus zooepidermicus (75%), and Staphylococcus capitis (66.7%). In contrast, Enterococcus faecalis was more commonly isolated from mares that did not become pregnant (75%). Several species were exclusively found in one group. Acinetobacter sp, Paenibacillus amylolyticu, Rahnella aquatilis, Streptococcus equinus, and Streptococcus gallolyticus were isolated only in some mares that became pregnant, while Staphylococcus schleiferi and Staphylococcus vitulinus were only found in some mares that did not become pregnant. However, since these and some other species were detected at low rates, it may indicate that they are not consistently associated with the vaginal microbiome. Although semen is deposited in the uterus in mares, human studies suggest vaginal bacteria may reflect the uterine environment and reproductive health. This study assessed specific vaginal bacterial profiles as potential indicators of pregnancy outcome in mares. The study focused solely on bacteria. No screening for fungi was performed, as clinical signs of fungal infections were absent and such infections are rare in mares. However, altered bacterial microbiota may increase susceptibility to fungi, so undetected fungal presence could have indirectly contributed to bacterial shifts observed in this study. Though limited by sample size, the study provides valuable insight into an under-researched area of equine reproduction and supports the need for further investigations using larger cohorts and advanced models. These efforts may ultimately contribute to the development of microbiota-based diagnostic or therapeutic strategies in mares.

Keywords: vaginal microbiota, pregnancy, mare

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

AI / TAI / FTET

Duroc boar semen prepared by single layer centrifugation and stored at 4 degrees without antibiotics: an artificial insemination trial

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Antimicrobial resistance is an increasing problem in the swine production industry. Cold storage of boar semen at 4°C instead of conventional storage at 17°C could reduce antibiotic use at the semen station and during inseminations. However, bacteria can survive at 4°C. Colloid centrifugation can effectively remove bacteria from semen (Morrell et al., Theriogenology; 126:272-278, 2019; Ngo et al.,Theriogenology; 226:194-201, 2024) but it is not known if boar spermatozoa would then survive cold storage. This study investigated the use of Single Layer Centrifugation (SLC) through Porcicoll followed by cold storage at 4°C with and without antibiotics on pregnancy rates and litter sizes. Semen was collected at a commercial boar station (Norsvin, Hamar). Ejaculates from three Duroc boars were pooled and extended 1:1 in Androstar Premium with or without antibiotics. In addition, control semen doses with similar concentration (1.8x109 spermatozoa in 90mL) were prepared in AndroStar Premium with antibiotics from the same semen pool at the semen station. The semen was transported to the laboratory at approximately 20 °C. Here the control doses were stored at 17 °C while the rest of the semen was prepared by Single Layer Centrifugation (SLC) through Porcicoll. Extended semen (80 mL) was layered on top of 60 ml Porcicoll and centrifuged for 20 min at 300g. The sperm pellets were then resuspended in AndroStar Premium, with or without antibiotics and cooled gradually over 4 hours to 4°C. The semen doses were transported to the NMBU farm at their respective temperatures where they were stored for insemination 24 and 48h after semen collection. The control doses were stored conventionally while the SLC doses were stored at 4 °C. Three rounds of inseminations were performed with hybrid sows (parity 2-4); the sows were divided into three groups, matching parity between the groups as far as possible. One group was inseminated with the control semen dose (n = 16), the second group with cold stored semen at 4°C with antibiotics (n = 18) and the third group with cold stored semen at 4°C without antibiotics (n = 18). One-way analysis of variance (ANOVA) was used to compare results of the three groups. Pregnancy rates were 88% (14/16), 89% (16/18) and 89% (16/18), respectively. Number of live born piglets per litter were 16.4 ± 3.2, 17.5 ± 3.3 and 17.0 ± 3.2, respectively. No differences were observed in pregnancy rates (P = 0.66) or number of live born piglets per litter (P = 0.59) between the three groups. These results show that boar spermatozoa survive and retain their fertility after SLC and cold storage. There was no difference in fertility between SLC and cold storage at 4°C with and without antibiotics. The method could thus be an effective way to replace antibiotics in semen samples to inhibit bacterial growth, but a larger study is warranted.

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Keywords: Cold storage, boar, semen

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Comparative OPU-IVP efficiency across Bos taurus breeds under commercial and genetic selection programs

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OPU-IVP is essential for bovine genetic improvement, but embryo yield remains variable despite standardized protocols. Limited studies have addressed these variations, and none have compared multiple Bos taurus breeds under a commercial and genetic selection program. This retrospective study (2019-2025) aimed to compare OPU-IVP performance among five Bos taurus breeds under the same laboratory conditions. 663 OPU-IVP sessions (maximum 5 per donor) were analyzed from Aubrac, Charolaise, Holstein, Blonde d'Aquitaine, and INRA 95 donors. Given the unbalanced dataset, the effects of breed, parity, and semen type on OPU-IVP outcomes were assessed using a general linear model across relevant subgroups. In Holstein heifers (n=213), sexed semen significantly decreased segmentation rate compared to conventional semen (74.8 ± 19.4% vs 89.0 ± 12.3%, P<0.001) without affecting the number of segmented embryos (8.00 ± 6.6 vs 9.8 ± 6.0). It also tended to decrease the G1 (Grade 1, IETS) blastocyst rate (36.0 ± 16.0% vs 45.2 ± 20.8%, P=0.07) without changing the number of G1 embryos (3.7 ± 3.5 vs 4.8 ± 3.6). Parity effects were evaluated on two subgroups. In Holstein (n=163), non-lactating and non- pregnant cows (11.5 inseminated oocytes per session) produced more embryos than heifers (10.2 inseminated oocytes per session), with higher segmentation rate (87.5 ± 12.1% vs. 74.9 ± 19.4%, P<0.001), G1 embryos (5.4 ± 3.4 vs. 3.7 ± 3.5, P<0.05), and G1 blastocyst rate (50.7 ± 23.1% vs. 36.0 ± 16.0%, P=0.01). In contrast, no parity effect was observed in beef breeds (Aubrac, Charolaise, Blonde d'Aquitaine, 16.9 and 15.01 inseminated oocytes per session respectively for 34 heifers and 164 non-pregnant cows) for segmentation rate (88–89%), G1 embryo production (8.3-8.6), or G1 blastocyst rate (50.1-54.9%). To test the breed effects, 500 sessions using conventional semen were analyzed: Aubrac (n=49, 9.6 ± 6.1) and Charolaise (n=32, 9.8 ± 6.2) produced significantly more G1 embryos than Holstein (n=195, 4.7 ± 3.6) and INRA 95 (n=107, 3.2 ± 2.0; P<0.001). This difference was explained by: (1) a higher number of inseminated oocytes per session in Aubrac (16.8 ± 8.6) and Charolaise (17.3 ± 10.5) vs. Holstein (11.1 ± 6.7) and INRA 95 (7.8 ± 5.2; P<0.001); (2) a tendency for a higher segmentation rate in Aubrac (92.7 ± 9.9) compared to other breeds (86.7–89.4%; P=0.074); and (3) higher G1 blastocyst rates in Aubrac (57.6%) and Charolaise (54.9%) vs. Holstein (45.2%) and INRA 95 (45.9%; P<0.001). Blonde d'Aquitaine (n=117) showed intermediate values for all parameters. Despite an unbalanced dataset, this field study demonstrates that sexed semen reduces segmentation rate and tends to reduce G1 blastocyst rate in Holstein heifer donors. Parity influenced OPU-IVP outcomes in Holstein, with cows outperforming heifers. This effect was not observed in beef breeds. Breed significantly impacted OPU-IVP performance, with Aubrac and Charolaise outperforming Holstein and INRA 95 in embryo yield and quality. These breed-specific differences in G1 embryo production and G1 blastocyst rates could reflect variations in underlying ovarian reserve and oocyte competence.

Keywords: OPU-IVP

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Donor age as a limiting factor in the conservation of endangered cattle breeds through assisted reproductive technologies

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Assisted reproductive technologies (ARTs) and the storage of cryopreserved viable gametes, embryos, and ovarian tissues (cryobanking) hold considerable promise for preserving genetic biodiversity in small and threatened animal populations. Their application extends beyond wildlife conservation, as it is equally critical for safeguarding local livestock populations and preserving their unique genetic traits. In this context, ARTs and cryobanking were applied to the genetic conservation of Varzese-Ottonese-Tortonese (VOT) cattle, an FAO-listed endangered Italian breed with a total population of 847 individuals. The project involved the preservation of germplasm from 27 female VOT donors: 15 donors underwent on-farm techniques, specifically Multiple Ovulation and Artificial Insemination (MOAI, n=2) or Ovum Pick-Up followed by IVP (OPU, n=13); 12 donors, culled for meat production, age-related issues, and infertility, were subjected to genetic rescue (GR) by IVP. For IVP, VOT bull semen was used according to the breeder association's mating plans. Additionally, from GR, oocytes derived from early antral follicles were retrieved for long-term *in vitro* culture and subsequent IVM to cryopreserve MII (Barros, Reproduction,165, 221, 2023). Preantral follicles were preserved as ovarian cortex fragments (Alkali, Reproduction, 166, 299, 2023). Extra pieces were histologically processed for morphometric analysis in heifers (20-21 months) and cows (32-153 months). Overall, 142 embryos (MOAI, n=2; OPU, n=43; GR, n=97), 35 MII oocytes, and 120 ovarian cortex fragments were retrieved. As proof of principle that VOT cryopreserved embryos can sustain cross-bred implantation, 22 grade 1 embryos (OPU, n=19; GR, n=3) were randomly assigned for transfer into synchronized Holstein surrogate dams, successfully resulting in the birth of 4 calves (calving rate: 18%). Notably, full-term pregnancies occurred only with embryos produced by oocyte donors younger than 113 months (4 out of 7 ET, 57%), while no pregnancies resulted from older donors (114-204 months, 0 out of 15 ET). Embryo origin (7/7 vs 12/15 OPU-derived), freezing/thawing procedures, and recipient parity (6/7 vs 12/15 nulliparous for the young and old groups, respectively) were not different, suggesting that advanced maternal age in donor cows negatively impacts embryo quality and implantation efficiency. The demographic study highlighted critical age distribution, showing that 25.11% of the VOT female population is over 7 years old. Furthermore, morphometric analysis indicated a significant decrease in preantral follicle density with age (heifers: 290.4±144.5; cows: 32.71±6.9 follicles/mm3, P<0.005), complicating the genetic preservation scenario for this breed. Our results indicate the need to prioritize younger females in conservation programs, as advanced age compromises reproductive performance, limits the success of ARTs, and reduces the availability of gametes for cryobanking to exploit the full ovarian reserve.

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Keywords: biodiversity, aging, fertility

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Cysteamine mitigates seasonal variation in bovine oocyte nuclear maturation during *in vitro* maturation

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Heat-stressed cows produce poor-quality oocytes for *in vitro* fertilization (IVF) (Al-Katanan et.al, J Dairy Sci. 85(2):390-6, 2002.). Supplementing the *in vitro* maturation (IVM) medium with cysteamine has been shown to enhance the outcome of the *in vitro* production (IVP) systems (Merton et.al., Theriogenology. 1;80(4):365-71, 2013). However, limited research has quantified the magnitude of heat stress effects or the capacity of cysteamine to counteract these impairments. This pilot study aimed to assess seasonal variation in nuclear maturation of bovine oocytes and evaluate the potential of cysteamine to counteract heat stress-induced impairments during IVM. High-quality cumulus–oocyte complexes (COCs), characterized by multiple cumulus cell layers and homogeneous cytoplasm, were selected. COCs were washed five times in pre-warmed washing medium and transferred to 400 µL of maturation medium (BO-IVM, IVF Bioscience, Falmouth, UK), with or without 100 µM cysteamine, then incubated for 22–24 hours. Nuclear maturation was evaluated via Hoechst 33342 staining to determine metaphase II (MII) progression. The experiment was repeated in three different times in winter (n=388; 120,132,136) and in three different times in summer (n=312; 98,110,104). Data were analyzed using mixed-effects logistic regression; the model included two categorical predictor variables (season and treatment) in the fixed-effects part, interaction term (season × treatment), and replicate nested within season in the random-effects part. Oocytes collected in winter had significantly higher MII rates, and cysteamine significantly enhanced maturation in both seasons ($P<0.01$). In winter, MII rates increased from 72.1 ± 1.5% without cysteamine to 82.1 ± 0.6% with cysteamine. In summer, rates rose from 50 ± 1.5% to 70.5 ± 2.4%. The mean effect of cysteamine supplementation was larger in summer (21 ± 2%) than in winter (10 ± 2%), but the interaction between season and supplementation was not significant. Heat-stressed cows experience elevated reactive oxygen species (ROS), which damage DNA, lipids, and proteins (Ayemele, Antioxidants (Basel). 29;10(12):1918, 2021). Cysteamine facilitates cysteine uptake, boosting intracellular glutathione (GSH) levels and reducing ROS (Wilmer et.al, Biochim Biophys Acta.1812(6):643-51, 2011). In conclusion, oocyte quality is inherently better in winter. Although cysteamine significantly improves nuclear maturation in both seasons, it does not completely overcome the detrimental effects of heat stress. Addition of cysteamine can, however, partially mitigate the impact of elevated oxidative stress on oocyte maturation during the summer.

Keywords: bovine oocytes, cysteamine, nuclear maturation

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

In vitro embryo development following *in vitro* fertilization with conventional or sex-sorted semen from the same bull

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The aim of this study was to compare embryo development rates for *in vitro* produced (IVP) embryos produced using either conventional (CV) or sex-sorted (SS) semen from the same team of bulls. Oocytes were collected once per week for up to four successive weeks from Holstein-Friesian (DAIRY, n=51) and Angus (BEEF, n=37) donors using transvaginal ovum pickup (OPU). Oocytes were matured *in vitro* for 24 hours and oocytes from a single donor were fertilized using either CV or SS semen from a panel of proven fertility bulls (Dairy=8 and Beef=5). If a donor yielded ≥6 oocytes at a given OPU session, half were fertilized with CV semen and the other half with SS semen from the same bull. Blastocysts were cultured *in vitro* until Day 7. Blastocyst yield was calculated based on the number of presumptive zygotes placed into culture. Embryo development data were available from a total of 101 OPU sessions and 204 *in vitro* fertilization (IVF) events. The effect of semen type (CV or SS) on blastocyst yield and the number of blastocysts produced per IVF were determined using generalized linear mixed models in SAS. The first analysis included breed, parity and semen type as fixed effects. For the second analysis, two breed-specific datasets (i.e., DAIRY and BEEF) were created to analyse the effect of sire and semen type on embryo development outcomes. Fixed effects in the breed-specific models included semen type, sire and the two-way interaction between semen type and sire. In the first analysis, cleavage rate was not different between BEEF and DAIRY (58.8% vs 51.7%, P=0.16) but was less for CV compared with SS semen (48.7% vs 61.9%, P<0.0001). Blastocyst yield and the number of blastocysts produced were greater for BEEF than DAIRY (24.0% vs 12.1%, P=0.002 and 2.3 vs 0.5, P=0.001, respectively). Both blastocyst yield and the number of blastocysts produced per IVF were less for CV compared with SS semen (14.5% vs 21.5%, P=0.005 and 1.2 vs 1.6, P=0.009, respectively). In the second analysis on the BEEF dataset, cleavage rate was not different between CV and SS (59.8% vs 63.6%, P=0.35). There was no effect of semen type (CV vs. SS) on blastocyst yield (25.0% vs 28.2%, P=0.41) or the number of blastocysts produced per IVF (2.3 vs. 2.7, P=0.11). The sire used for IVF affected blastocyst yield for BEEF (P=0.013) but there was no sire by semen type interaction. From the analysis of the DAIRY dataset, cleavage rate was lesser for CV compared to SS semen (46.2% vs 62.4%, P=0.0027) there was a tendency for blastocyst yield to be less for CV than SS (11.2% vs 18.8%, P=0.07), but the number of blastocysts produced per IVF was not different (0.7 and 0.9, P=0.26). The dairy sire used for IVF had an effect on the number of blastocysts produced per IVF (P=0.0025) and a sire by semen type interaction was detected (P=0.03) due to the fact that 7/8 bulls produced fewer blastocysts with CV than SS semen but 1/8 bulls produced fewer blastocysts with SS than CV semen. In conclusion, embryo development rates were greater for BEEF than DAIRY and were greater for SS than CV semen.

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Keywords: sex-sorted semen, *in vitro* fertilization

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Assessment of *in vitro* and in vivo embryo production outcomes in female cattle donors infected with epizootic hemorrhagic disease

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Epizootic hemorrhagic disease (EHD) is a vector-borne illness caused by an Orbivirus. The little-known serotype 8 (EHDV-8) first emerged in Europe in 2022. Recently, we reported pregnancy losses in cattle not previously associated with this serotype. However, the impact of EHDV-8 on the oocyte and early embryo remains unknown. Thus, this study aims to evaluate the impact of EHDV-8 infection on the quality of oocytes and embryos in cattle. As part of a conservation program (May 2022–November 2024), 17 Cárdena Andaluza native breed donors aged 4.0–12.6 years and housed at the CENSYRA Reproduction Center (Badajoz, Spain) underwent periodic *in vitro* embryo production (IVP) and in vivo embryo collection (IVD). In addition, monthly blood sampling for disease monitoring was performed in each donor, and all animals tested positive for EHDV-8 after asymptomatic natural infection by PCR from September 2023 to March 2024. We compared the outcomes of IVP and IVD procedures pre- (May 2022–July 2023) and post- (October 2023–November 2024) EHDV-8 infection. A total of 94 ovum pick-up (OPU) and IVP sessions (61 pre- and 33 post-EHDV-8), and 66 multiple ovulation and IVD sessions (41 pre- and 25 post-EHDV-8) were analyzed. Notably, 12 out of 17 donors underwent at least one IVP and one IVD session both before and after infection. Generalized linear mixed models were used to compare reproductive outcomes between periods, with each session considered as the individual unit and donor included as a random effect. Infection status (pre- or post-EHDV-8) and season (warm [June–September] vs. cold) were included as fixed effects. Regarding IVP outcomes per OPU session, no differences in the number of cumulus-oocyte complexes (COCs) recovered pre- and post-EHDV-8 infection (8.5 ± 0.85 vs. 8.9 ± 1.03) were observed. The number of grade 1 COCs decreased (2.2 ± 0.37 vs. 1.1 ± 0.28; P=0.013), while the number of grade 4 COCs increased (0.6 ± 0.13 vs. 1.5 ± 0.35; P=0.003) after infection. However, cleavage rates (56.7 ± 3.41% vs. 62.4 ± 4.75%), number of viable embryos (1.1 ± 0.24 vs. 1.4 ± 0.38), and percentage of viable embryos from total COCs recovered (25.6 ± 2.76% vs. 26.8 ± 3.74%) were similar between periods. Regarding IVD outcomes per session, a similar number of corpora lutea detected by ultrasonography on the day of embryo recovery (4.9 ± 0.54 vs. 5.7 ± 0.70) and number of total structures recovered (2.1 ± 0.44 vs. 3.1 ± 0.73) were observed pre- and post-EHDV-8 infection. Although the number of transferable embryos (1.3 ± 0.28 vs. 2.0 ± 0.51) was similar between periods, we observed a higher percentage of transferable embryos among the total structures recovered (42.8 ± 4.83% vs. 58.3 ± 5.42%; P=0.047) after infection. In conclusion, EHDV-8 infection in female cattle donors was associated with altered oocyte quality but did not impair overall embryo production outcomes, either *in vitro* or in vivo.

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Keywords: disease, oocyte/embryo quality, cattle.

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Does intrafollicular oocyte transfer near ovulation improve the results of in vivo embryo production using this technique?

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Intra-follicular oocyte transfer (IFOT) is an alternative to improve embryonic quality, decrease the number of procedures necessary for in vivo production, and avoid the limitations of the *in vitro* process. The IFOT technique consists of injecting immature oocytes into a pre-ovulatory follicle, inseminating the recipient-cow with a single dose of semen, and flushing the uterus seven days later. However, reported IFOT embryo yields are still very low (12.9% and 17.3%). The objective was to evaluate the timing of IFOT in relation to ovulation in the in vivo embryo production in Bos indicus cows. In the study, the cumulus oocyte complexes used (COCs; quality 1 and 2) were recovered from slaughterhouse ovaries. The cows were submitted to the ovulation synchronization protocol based on progesterone/estradiol (P4/E2) for the formation of the dominant follicle at the time of IFOT. On D0, all cows (n=14) received 2mg of estradiol benzoate and an intravaginal device containing 1g of P4. After seven days (D7), 500µg of Cloprostenol (PGF2α) was administered. On D8, the P4 device was removed and 1mg of estradiol cypionate, 500µg of PGF2α and 300IU of eCG were administered and 40 hours later, 20µg of Buserelin (GnRH) was administered. The IFOT was performed 22 hours after GnRH administration. Ultrasonographic exams were performed every 3 hours after GnRH administration to determine the time of ovulation. Artificial insemination (AI) was performed 10 hours after IFOT (D11). After seven days of AI, embryos were collected in vivo by washing the uterine horn. Furthermore, *in vitro* fertilization was performed in a subgroup of oocytes (n=159) to verify the quality of the oocytes used in the study. After defining ovulation in relation to IFOT, the females were divided into two experimental groups: Near Group (n=9) - IFOT performed within 7 hours before ovulation and Later Group (n=5) - IFOT performed more than 11 hours before ovulation. Statistical analysis was performed by SAS. The mean interval between IFOT and ovulation was 5.0 ± 0.7 h for the Near Group and 15.8 ± 1.8 h for the Later Group. The follicular diameter at IFOT (Near: 12.7 ± 0.6 mm and Later: 12.0 ± 0.9 mm; P=0.49), the number of oocytes injected (Near: 29.2 ± 0.7 and Later: 28.6 ± 1.2; P=0.63) and the total number of structures recovered (Near: 3.3 ± 1.8 and Later: 3.4 ± 2.7; P=0.49) were similar between the experimental groups. However, the total number of embryos recovered was greater in cows submitted to IFOT near ovulation (Near: 1.2 ± 0.6 and Later: 0.0 ± 0.0; P=0.001). In addition, the recovery rate was 11.9% and 11.4% in the Near and Later groups, respectively. However, only the group that received IFOT near to ovulation obtained embryo production (4.2%). The oocytes submitted to *in vitro* fertilization obtained a 62.3% cleavage rate and a 25.8% blastocyst rate. It is concluded that despite increasing the number of embryos produced in vivo, IFOT performed up to 7 hours before ovulation does not present satisfactory results in the in vivo embryo production in Bos indicus cows. However, additional studies with larger samples are needed to better evaluate the potential of this technique.

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Keywords: IFOT, oocytes, embryo, cows

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Influence of CL and its size on the production of sheep embryos *in vitro*

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The corpus luteum (CL) is a transient gland that can directly influence follicular dynamics, oocyte quality, and embryo development. The objective of this study was to evaluate the influence of the absence or presence of a Small, Medium, or Large CL in slaughterhouse ovaries on *in vitro* embryo production. A total of 332 ovaries were used in this experiment. The number of ovaries for each group was Without CL=129, Small=90, Medium=62, and Large CL=51. Cumulus-oocyte complexes (COCs) were collected from each group of ovaries (Without CL=339, Small=274, Medium=197, and Large CL=185) and matured independently in TCM-199 medium, supplemented with 10% (v/v) FBS (Biowest; Mayimex, Mexico), 5 µg mL−1 FSH (Folltropin-V; Bioniche, Canada), 5 IU mL−1 hCG (Chorulon; Merck Shar, USA), and 1 µg mL−1 17-β estradiol (Sigma Aldrich, Mexico), plus 200 µL mineral oil (Sigma Aldrich, Mexico). Fertilization was performed with fresh semen from a Katahdin ram of known fertility. Semen collection was conducted using an artificial vagina. Semen was kept at 22°C for 90 min, washed in a commercial fertilization medium (Modified Tyrode; In Vitro S.A, Mexico), and centrifuged at 225×g for 3 min. Sperm capacitation and selection were performed using the Swim-up technique. After maturation, the COCs were washed and placed in wells with fertilization medium plus mineral oil. COCs were fertilized with 1×106 sperm mL−1 and cultured for 22 h. The putative zygotes were washed and placed in independent wells according to the experimental group: from ovaries Without CL, with a Small CL (≤3 mm), Medium CL (4-8 mm), or Large CL (>8 mm in diameter). Embryo development was carried out in commercial sequential media (Cleavage and Blastocyst, Cook, USA) for 72 and 96 h, determining the morulae rate, blastocyst rate (rates calculated from the COCs), and blastocyst diameter. Statistical analysis was performed using GLIMMIX, GLM, and ESTIMATE procedures from SAS, according to the data distribution of the response variables ($P<0.05$). A difference ($P<0.05$) was only evident in the morulae rate between the Without CL group (n=264, 78.59 ± 3.13%) vs. the Large CL group (n=162, 88.01 ± 3.70%). In the case of blastocyst rate, the Medium CL group showed greater efficiency (n=102, 52.63 ± 5.48%) compared to the Without CL group (n=132, 39.48 ± 3.64%). However, in the case of blastocyst diameter, no difference was evident. In conclusion, *in vitro* morulae and blastocysts production rate was better in the Medium and Large CL ovaries groups compared to the Without CL ovaries group, under the conditions of this study.

Keywords: oocyte, embryos, corpus luteum

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

The use of OPU-ICSI does not influence foal health during the first 30 days post partum. A survey with 181 ICSI foals

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Increasing international emphasis on equine welfare and breeding techniques necessitates data regarding the health of ICSI offspring. The aim of this study was to retrospectively evaluate whether pregnancies obtained by OPU-ICSI could influence the foal sex ratio, risk of dystocia and retained placenta, and risk of neonatal mortality and morbidity during the first 30 days post partum. The study was performed as a survey for breeders of ICSI foals from VetEmbryo's clinic during the years 2019-2024 with ICSI embryos vitrified at day 7, 8, 9 and 10 after ICSI. Not all breeders responded to every question, resulting in varying totals for each category of responses. The average gestation length was 343.1 days (n=181), with no difference between the group of fillies and colts. The foal sex ratio was 55% colts and 45% fillies. The gestation length and sex ratio were similar to previously reported numbers for AI pregnancies (Lanci, Theriogenology, 215, 125–131, 2024 & McCue, EVJ, 44, 22-25, 2012). The sex ratio shifted towards more fillies with embryos showing a slower speed of development. For embryos reaching the blastocyst stage at days 7, 8, 9 and 10 after ICSI, the proportion of fillies were 38%, 45%, 48%, 58%, respectively. From 167 responders, 164 (98.2%) reported an uncomplicated foaling without intervention. There was one case (0.6%) of red bag delivery handled without complications, one case (0.6%) where both mare and foal died due to severe dystocia, and one case (0.6%) of premature delivery (1 month too early) resulting in a non-viable foal. These incidences are numerically lower than previously reported incidences for AI pregnancies (McCue, EVJ, 44, 22-25, 2012). From 163 responders, there were two (1.2%) cases of retained placenta, which is also lower than previous data from thoroughbreds (ISHII, J Equine Sci., 24(2), 26-29, 2013). From 165 responders, 155 foals (94%) were viable and healthy at birth without any assistance needed. Five foals (3%) could stand but were initially unable to nurse, two foals (1.2%) could not stand or nurse and died, one foal (0.6%) was euthanized due to microphtalmia, and one foal (0.6%) was found dead presumably due to asphyxiation, as the fetal membranes were intact covering the foal's nose. Furthermore, no critical illnesses were observed in the first 30 days post partum for 157 foals (97.5%). One foal (0.62%) developed a fever, was hospitalized and recovered, one foal (0.62%) experienced constipation and recovered, one foal (0.62%) died on day 2 after difficulties with nursing, and one foal (0.62%) was killed by an aggressive recipient mare. These incidences are comparable to previously reported numbers for AI pregnancies (McCue, EVJ, 44, 22-25, 2012). The results demonstrate that ICSI pregnancies are not associated with a higher incidence of dystocia, retained placenta, neonatal mortality or morbidity during the first 30 days post partum, compared to AI pregnancies. Additional research is needed to investigate the health status of ICSI offspring beyond 30 days of age.

Keywords: ICSI, foals, health

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

High live foal rates after ICSI: The role of culture media, warming protocol, and recipient mare selection

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The goal of equine ICSI is a live foal, but there is a need for more data on how to improve the live foal rate. Especially in the context of current international discussions on equine welfare, raising concerns about the increased risk of pregnancy losses during ICSI pregnancies (Lewis, Reproduction, Fertility and Development, 35, 338–351, 2023 & Claes, Theriogenology, 187, 215-218, 2022). The aim of this study was to determine how the embryo culture medium, vitrification day, warming method, and recipient mare type affected the ongoing day 50 pregnancy rate and live foaling rate. Data from VetEmbryo's commercial OPU-ICSI program was analyzed retrospectively and the study included 413 equine OPU-ICSI embryos. Inseminated oocytes were cultured following three different medium protocols with different glucose concentrations: (1) 0.89 mM glucose, on day 5 shift to 17.5 mM glucose, (2) 0.89 mM glucose, on day 5 shift to 11 mM glucose, and (3) 11 mM glucose, on day 5 shift to fresh medium of the same composition. Blastocysts were grouped based on vitrification day 7, 8, 9 and10 after ICSI and were vitrified with a commercial kit (VitriFreezeTM, Fertipro), on a VitriFit carrier (Cooper Surgical) and warmed by one of two protocols: (1) 3-step warming with a commercial kit (VitriThawTM, Fertipro) or (2) 1-step direct warming in G-MOPSTM PLUS or Gx-MOPSTM PLUS (Vitrolife). All embryos were transferred to recipient mares without or with a foal at foot (between 30 and 120 days of age) four days after ovulation from 2021-2024. To investigate the effect of the mentioned factors, we performed multivariable logistic regression analyses with all 413 embryos and day 50 pregnancy as outcome (yes/no). All analyzed variables had a significant effect on the day 50 pregnancy rate with the following odds ratios [95% CI]: Culture medium (1,2,3) 1.463 [1.157 to 1.863], warming method (3-step or 1-step) 1.717 [1.158 to 2.556], embryo vitrification day (7,8,9,10) 0.6231 [0.4947 to 0.7795], and foal at foot (yes/no) 0.3963 [0.2336 to 0.6639]. The ongoing day 50 pregnancy rate in recipient mares without a foal at foot was 63.6% vs. 40.3% in recipient mares with a foal at foot (Fisher's exact test, P<0.001). The day 50 pregnancy rate with embryos warmed by the 1-step method was 65.2% vs. 52.2% with the 3-step method (P<0.01). Continuing the analysis with embryos transferred only to recipient mares without a foal at foot and embryos warmed by the 1-step method (n=176), there was a significant effect of culture media on the day 50 pregnancy rate (p=0.0361), but there was no longer an effect of embryo vitrification day (P=0.269). Looking into the effect of culture media within this group of transfers, ICSI embryos produced in media 3 had a live foaling rate of 75.3% compared to 59.8% in media1 and 2 (P=0.036) independently of embryo vitrification day. In conclusion, very high live foal rates can be achieved with equine ICSI, through optimization of the culture media protocol, by using a 1-step embryo warming method, and by selecting recipient mares without a foal at foot.

Keywords: ICSI, pregnancy, foal.

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Effective identification of ovarian status in gilts via transabdominal ultrasonography

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Fixed-time artificial insemination (FTAI) is a highly demanded reproductive strategy in swine production, as it enhances reproductive efficiency by maximizing the use of genetically superior boars and reducing the costs associated with multiple inseminations. Successful application of FTAI depends, however, on knowledge of the timing of ovulation relative to the onset of estrus. The aim of the present study was to evaluate the effectiveness of transabdominal ultrasonography (T-US) for detecting ovulation timing in gilts. Estrus detection involved snout-to-snout contact with vasectomized boars and back pressure by a skilled technician. Gilts exhibiting a standing reflex in the presence of the boar were classified as in estrus, with the day of onset designated as day 0. T-US examinations were performed using a high-resolution MyLab™ X1 equipped with a 5–8 MHz microconvex transducer (Esaote, Genoa, Italy). The transducer was horizontally placed on the right and left ventrolateral abdominal walls, just dorsal to the last pair of teats. Fisher's exact test and Student's t-test were applied as appropriate for categorical and continuous variables, respectively. In Experiment 1,126 gilts (Topigs TN70, Topigs Norsvin, Madrid, Spain), aged 9 months with at least two previous estrous cycles, were included. The efficiency of the procedure was assessed based on the successful visualization of preovulatory follicles at day 0 and the duration of the examination. An overall efficiency rate of 97.6% was achieved, with ovaries successfully visualized in 123 gilts. T-US lasted 5.9±2.1 min in successful examinations, and 10.2±2.6 min when ovaries were not detected. In Experiment 2, a subset of 48 gilts were studied by T-US for ovulation timing and mean follicular size starting at 0 h of the onset of estrus and continuing at 24-hour intervals until ovulation. Ovulation was defined as either the complete disappearance of preovulatory follicles or a number reduction of at least 50% compared to the previous T-US. Mean follicular size was expressed as mean diameter of preovulatory follicles. In all gilts, the duration of the estrus was 48 h. A greater proportion of gilts ovulated within the first 24 h of estrus onset (60.4%) than at 48 h (39.6%) ($P<0.05$). Mean follicular diameter at 0 h of the onset of estrus was similar between gilts ovulating within 24 h and those ovulating at 48 h (~ 0.7±0.07 cm). In gilts that had not ovulated by 24 hours after onset of estrus, follicular diameter remained unchanged; however, these gilts ovulated within the following 24 h. In conclusion, ovarian T-US is effective as procedure to monitor ovarian status in gilts, thus being a valuable tool to optimize the scheduling of single-dose FTAI.

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Keywords: pig, ovulation time, artificial insemination

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

High recovery of partially in vivo matured oocytes by ovum pick-up in sows and promising blastocyst rates following *in vitro* fertilization

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Transvaginal ultrasound guided Ovum Pick-Up (OPU) is challenging in sows. Recent advancements in equipment and protocols for sedation and epidural anaesthesia have significantly improved oocyte collection efficiency and animal welfare (Oltedal et al., Theriogenology, 226, 68-75, 2024). Partial in vivo maturation (IVM), where oocytes undergo initial maturation within the follicle prior to OPU, may enhance developmental competence and embryo quality. The objective of this study was to assess the *in vitro* embryo production (IVP) efficiency when using partially in vivo matured oocytes. OPU was performed 4 days (OPU-D4) and 5 days (OPU-D5, expected day of ovulation) post-weaning in a commercial sow herd. Sows (parity 5-7) were administered 0.4 mg/kg IM meloxicam (Dopharma Raamsdonksveer, Holland), restrained in a claw-trimming chute, sedated using an IM combination of butorphanol (CP-Pharma, Burgdorf, Germany) and detomidine (Orion Pharma, Espoo, Finland) and lifted. Epidural anesthesia using procaine hydrochloride (VetViva Richter, Wels, Austria) was applied to reduce straining and discomfort. Follicles were aspirated with an 18G 70 mm needle and 60 mmHg vacuum using a customized OPU system for sows. The setup included a SonoSite M-turbo ultrasound with C11x transducer for imaging and needle guidance connected to tubing and an aspiration pump (Minitube, Germany). Media used for IVP was purchased from EmbryoCloud (Murcia, Spain). Cumulus-oocyte complexes (COCs) were aspirated, washed and transported in TL-HEPES-PVA to the laboratory at 37°C in a portable incubator (Minitube, Germany). COCs from the OPU-D4 group were matured for 25 h in IVM-2 medium, while OPU-D5 COCs underwent 3 h of IVM prior fertilization. Fresh extended semen (Norsvin, Hamar, Norway) was centrifuged with a 80/40% BoviPure gradient (Nidacon, Mölndal, Sweden) for 25 min at 300g and COCs were co-incubated for 4 h at a 1000:1 sperm/oocyte ratio. Presumptive zygotes were denuded by pipetting and cultured at 38.9°C in a humified atmosphere containing 6% CO2 and 7% O2. Differences between the OPU-D4 and OPU-D5 groups were analyzed by T-test for the number of oocytes collected and by Chi-Squared test for the percentage of expanded COCs recovered, recovery and blastocyst rates (number of blastocysts divided by the total number of oocytes). On average, 21.2 ± 9.5 oocytes were collected per sow for OPU-D4 (n=19 sows) and 17.9 ± 10.0 oocytes for OPU-D5 (n=12 sows, P=0.39). The oocyte recovery rate was higher (P<0.05) in the OPU-D5 group (78.2%, 215 oocytes/275 follicles) compared to OPU-D4 (62.6%, 402 oocytes/642 follicles). In addition, more expanded oocytes (P<0.05) were collected on day 5 (86.0%), compared to day 4 (74.7%). However, similar blastocyst rates (P=0.9) were obtained for OPU-D4 (18/59, 30.5%) and for OPU-D5 (18/61, 29.5%). In conclusion, a consistently high number of partially in vivo matured oocytes and high blastocyst rates were obtained after OPU on both days, underlining the potential of this technology in pigs. However, a substantial number of sows already had ovulated on day 5, indicating day 4 as the optimal day of OPU. Ongoing studies aim to further optimize OPU-IVP efficiency and assess the effect of partial in vivo maturation on embryo quality.

Keywords: Transvaginal OPU, porcine, IVP

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Mortality of calves derived from *in vitro* produced embryos

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Calves derived from *in vitro* produced (IVP) embryos have greater risk of developmental abnormalities, including large offspring syndrome (LOS) and perinatal mortality compared with calves derived from artificial insemination (AI). The objective of this study was to characterize the main causes of death in calves derived from IVP that had been submitted for postmortem examination (PME). The calves were generated over four years (2021-2025) from studies designed to evaluate pregnancy per embryo transfer (P/ET) of fresh and frozen IVP embryos from dairy and beef breeds, P/ET for IVP embryos produced using conventional or sex-sorted semen from dairy and beef breeds, and P/ET of embryos produced from 2 to 3 month old dairy calves (juvenile *in vitro* fertilization and embryo transfer, "JIVET"). A total of 1592 embryos were transferred (Dairy Fresh=328, Dairy Frozen=328, Beef Fresh=576, Beef Frozen=273 and Dairy JIVET Fresh=87), resulting in 746 pregnancies that reached full-term. A total of 50 calves were stillborn or died postpartum, of which 44 calves were submitted for PME (Dairy Fresh=11, Dairy Frozen=6, Beef Fresh=19, Beef Frozen=6 and Dairy JIVET Fresh=2). Cause of death (COD) was identified based on the findings of the initial PME and from samples sent for additional diagnostic testing. Descriptive statistical procedures were carried out in SAS 9.4. Cause of death was categorized as dystocia (n=15), congenital defect (n=11), infection (n=5), multiple causes (n=4), other (n=5; abomasal bloat, umbilical haemorrhage, premature placental separation, intestinal torsion and umbilical cord wrap) and diagnosis not reached (n=4). The time of death was categorized as 1-5 (1=stillborn or died ≤ 30 minutes after birth; 2= >30 mins and ≤ 24 hours after birth; 3= > 24 hours to ≤ 7 days after birth; 4=8 to 21 days after birth; and 5= > 21 days after birth). Congenital defects identified during PME (as COD or as incidental finding) in 28 calves (63.6%) and were multiple defects (13), LOS (6), intestinal atresia (4), cardiac defects (2) and other (3). Mean ± SD gestation length for all calves was 277.7 ± 7.9 days and mean birthweight was 44.5 ± 14.5 kg. The % (and n) of calf death events for each of the 5 time categories were as follows: 61.4% (27/44), 13.6% (6/44), 9.1% (4/44), 6.8% (3/44), and 9.1% (4/44) for categories 1, 2, 3, 4, and 5, respectively. Of the 44 calves that were submitted for PME, congenital defect was found to be a contributor (primary or otherwise) to COD for 47.7% (n=21 calves). The number of calf deaths when conventional semen or sex-sorted semen had been used for IVF were similar (54.5% vs 45.5%, respectively). Of the 11 diagnoses of congenital defect as a primary COD, the organ most commonly affected was the intestines (5), followed by the liver (2) and heart (1). In conclusion, dystocia and congenital defect were the two primary COD identified in calves derived from IVP embryos

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Keywords: Congenital defects, IVP embryos

Animal Reproduction

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SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Asynchronous embryo transfer in cattle alters the transcriptome of maternal blood and major organs in male foetuses

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High progesterone (P4) concentrations have been shown to accelerate uterine receptivity, leading to improved pregnancy rates, longer conceptuses and enhanced foetal development in cattle. The objective of this study was to understand the impact of transferring a bovine embryo into a uterus primed with high P4 concentrations on the foetal organs (heart, liver and gonads) and maternal blood transcriptome. Grade 1 blastocysts were produced by *in vitro* fertilisation with semen from a single proven sire. Heifers were randomly assigned to one of two groups; synchronous (ET_D7, n=5), which received a single fresh day 7 blastocyst on day 7 of the cycle, or asynchronous (ET_D9, n=5) which received a single fresh day 7 blastocyst on day 9 of the cycle. Blood samples taken 2 days before embryo transfer confirmed higher P4 concentrations in the asynchronous vs. synchronous group (6.16 ± 1.13 ng/ml vs. 4.24 ± 0.35, P=0.01), suggesting advanced endometrial priming. On day 42, maternal blood and foetal organ samples (n=5 per group) were collected post slaughter for RNA-sequencing. Foetal sex was confirmed to be male by PCR of the Amelogenin gene from the fetal tail. Foetal weight from the corresponding male foetuses ranged from 1.25 to 1.69 grams (mean 1.44 ± 0.15 grams). Foetuses in the asynchronous group were heavier than those in the synchronous group (1.61 ± 0.08 grams vs 1.34 ± 0.05 grams, P=0.002). Principal component analysis showed clear separation between groups in the heart and liver; the separation of the gonads was less distinct. Differential gene expression was conducted using the edgeR package (FDR < 0.05, logFC > 1). Differentially expressed genes (DEG) between asynchronous and synchronous groups were detected in the foetal heart (n=393; 308 upregulated, 85 downregulated), liver (n=717; 121 upregulated and 596 downregulated) and gonads (n=172; 110 upregulated and 62 downregulated). In the maternal blood 135 DEG (55 upregulated and 80 downregulated) were identified using unadjusted p-values due to the low number of genes that passed FDR correction. Key findings included the upregulation of oxidative phosphorylation in the heart of foetuses derived from the ET_D9 group, with strong enrichment of representative genes of interest including UQCRFS1, COX7A2L and ATP5F1B, suggesting enhanced energy metabolism. Upregulated genes in the maternal blood enriched biological processes mainly related to the immune system, with key genes including PRG3, and ISG15, suggesting ongoing maternal adaptation to the foetus. These results indicates that early differences in P4 concentrations, amongst other factors, achieved through asynchronous transfer can lead to lasting impacts on foetal development and maternal immune function later in gestation.

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Keywords: uterine receptivity, foetal development, embryo transfer

Animal Reproduction

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SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Long-term haematological analysis in pigs derived from assisted reproductive technologies

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Rising livestock demand has increased use of assisted reproductive technologies (ART). Evidence indicates long-term effects of ART on molecular physiology and metabolism. This study explores ART's impact on haematological parameters in a colony of pigs from 1 to 5 years of age. Animals were born after artificial insemination (AI) and transfer of embryos *in vitro* produced with (RF-IVP) or without (C-IVP) oviductal and uterine fluids during *in vitro* fertilization and embryo culture media (París-Oller et al., J Anim Sci and Biotechnol, 2021). Pigs were maintained under identical conditions and sampled every six months. Number of animals in each group decreased throughout life: 33 to 9 (AI); 22 to 10 (C-IVP); and 14 to 4 (RF-IVP). The number of blood samples was the same as the number of animals and were collected via jugular venipuncture in lithium heparin tubes and immediately transported to laboratory. A haematology analyzer (Siemens ADVIA® 120, USA) assessed red blood cell (RBC) count, haemoglobin concentration (HB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), cell haemoglobin concentration mean (CHCM), red blood cell distribution width (RDW), haemoglobin concentration distribution width (HDW), white blood cell (WBC) count; and the differential count of neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Reticulocyte percentage, mean corpuscular volume of reticulocytes (MCVr), haemoglobin content in reticulocytes (CHr) and platelet count were analyzed. Data were analyzed by mixed-effects model and likelihood-ratio test to determine effects of age, group and sex on the variables. Post-hoc test for multiple comparisons Tukey's method were used. $P<0.05$ was considered significant. Age affected all variables. MCH, CH, CHCM, WBC, and platelet indices (MPV, MPC, MPM, PMDW) increased through life. Biphasic trajectories occurred in MCV, HTC, RBC, neutrophil counts, monocyte counts, PLT counts, and lymphocytes (second-year decline followed by increase). Progressive decline with age was observed in monocyte percentage and reticulocyte parameters. Irregular oscillatory patterns dominated HB, RDW, MCHC, CHDW, HDW, eosinophil and basophil parameters, and advanced platelet indices (large PLT, PCT, PDW, MCVr, PCDW). HCT, RBC, and HB reached lowest values in AI group, highest in RF-IVP. HCM, MCHC, and lymphocyte percentage showed lowest means in AI, highest in C-IVP. RDW, WBC, neutrophil parameters, and lymphocyte count were lowest in C-IVP, highest in AI. Monocyte count exhibited lowest values in C-IVP, highest in RF-IVP group. CH and PCDW were higher in females than males, whereas HDW, PDW, MPC were higher in males. Long-term results confirm slight persisting haematological differences in naturally and artificially conceived pigs, although the clinical relevance is unnoticeable.

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Keywords: porcine, metabolism, ART

Animal Reproduction

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SUPEROVULATION, OPU-IVP, AND EMBRYO TRANSFER

Long-term blood plasma biochemical analysis in pigs derived from assisted reproductive technologies.

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Rising livestock demand has increased use of assisted reproductive technologies (ART). Evidence indicates long-term effects of ART on molecular physiology and metabolism. This study explores ART's impact on biochemical parameters in a colony of pigs from 1 to 5 years of age. Animals were born after artificial insemination (AI) and transfer of embryos *in vitro* produced with (RF-IVP) or without (C-IVP) oviductal and uterine fluids during *in vitro* fertilization and embryo culture media (París-Oller et al., J Anim Sci and Biotechnol, 2021). Pigs were maintained under identical conditions and sampled every six months. Number of animals in each group decreased throughout life: 33 to 9 (AI); 22 to 10 (C-IVP); and 14 to 4 (RF-IVP). The number of blood samples was the same as the number of animals and were collected via jugular venipuncture in lithium heparin tubes and immediately transported to laboratory. Plasma was obtained through centrifugation (1200 g, 20 min, 4ºC, Eppendorf 5810 R) of blood collected via direct jugular venipuncture in lithium heparin tubes. Plasma was assessed using a chemistry analyzer (Olympus AU400, Japan) for creatinine (CREA), urea, amylase, creatine kinase (CK), cholesterol, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lipase, total protein (TP), albumin (ALB), globulin (GLOB), triglycerides (TRIGL), and total bilirubin (TBIL). Data were analyzed by mixed-effects model and likelihood-ratio test to determine effects of age, group and sex on the variables. Post-hoc test for multiple comparisons Tukey's method was used. Statistical significance was set at $P < 0.05$. Age significantly influenced all variables. Glucose, cholesterol, lipase, CK, ALP, AST, ALT were not affected by any other variable. TP, GLOB, UREA and TRIGL showed progressive elevation with age, with peak values between 4-5 years. ALB, cholesterol, CK, ALP, AST, GGT exhibited characteristic juvenile decline, with ALP and AST showing reduction during early development (155 to 52 U/L and 121 to 37 U/L respectively) followed by stabilization, while ALT displayed oscillatory changes. Glucose and TBIL showed initial elevation peaking at 2 years, before decreasing. CREA and amylase fluctuated irregularly. Critical developmental transitions occurred at 1.5-2 years and 4-5 years, representing key physiological maturation milestones. ALBU showed the highest mean concentration in the RF-IVP group and lowest in the AI group, while GGT showed the opposite pattern. ALBU, GLOB and urea were influenced by sex, with females higher ALBU and urea levels, while males showed elevated GLOB values. Long-term results confirm slight persisting biochemical differences in naturally and artificially conceived pigs, although the clinical relevance is unnoticeable.

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Keywords: porcine, metabolism, ART

Animal Reproduction

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FOLLICULOGENESIS AND OOGENESIS

Characterization of protein expression and lipid droplet distribution pattern in granulated cattle oocytes

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For *in vitro* embryo production in cattle, cumulus-oocyte-complexes (COCs) that present several layers of tightly packed cumulus cells and a homogeneous, darkish ooplasm are widely accepted as indicative of good quality and are therefore preferentially selected. Nevertheless, COCs obtained from antral follicles display a wide range of morphological features, among which granulation of the ooplasm is frequently observed. A rather recent publication demonstrates that granulation does not negatively affect developmental competence of these oocytes and post-transfer pregnancy outcome (Rosa et al., 2024, Zygote v. 32, pp. 28-37). Therefore, the present study aimed to evaluate the protein expression pattern of immature cattle oocytes exhibiting a granulated ooplasm (n=5) compared to those that display a homogeneous ooplasm (n=4). COCs were obtained by aspirating 2 - 5 mm follicles of slaughterhouse ovaries. For both oocyte groups four to five biological replicates (eight to eleven oocytes each) were analyzed via LC-MS/MS. Differentially abundant proteins between both oocyte groups were determined using a Student's t-test with permutation-based FDR correction (FDR=0.05). A total of 2,130 proteins could be identified and quantified, five of which were significantly differentially abundant between granulated and homogeneous oocytes. Ten candidate proteins (Student's t-test; P<0.01 and log2FC≥|0.6|) were subjected to functional enrichment analysis using the online tool STRING (v12.0; https://string-db.org/). Pathways associated with fatty acid metabolism were particularly enriched. To supplement this result, lipid droplet distribution within the ooplasm was assessed on a large-scale for both oocyte groups, utilizing Nile Red staining and confocal microscopy. While homogeneous oocytes (n=42) displayed mainly small sized lipid droplets that were evenly distributed throughout the ooplasm, lipid droplets in granulated oocytes (n = 40) presented higher heterogeneity in size and distinct formation of clusters. Mean fluorescence intensity values from maximum projections of the oocytes were compared to quantify lipid accumulation, revealing a significantly higher lipid content in granulated oocytes compared to homogeneous oocytes (60.9±33.4 vs. 40.3±29.5; Wilcoxon rank-sum test, P=0.0019). In conclusion, the proteomes of immature cattle oocytes presenting a granulated or a homogeneous ooplasm are remarkably similar with only minor variations. Proteins related to lipid metabolism are mainly affected, likely accounting for the distinct differences in lipid droplet accumulation between both groups. Given that lipids serve as the primary energy source for cattle oocytes during maturation and subsequent developmental events (de Andrade Melo-Sterza et al., 2021, Int. J. Mol. Sci. v. 22), any alterations in their metabolism could negatively influence not only oocyte developmental competence but also the quality of developing blastocysts, impacting aspects like cryotolerance. Therefore, future lipidomic investigations promise to yield a more profound understanding of the differing lipid landscapes of granulated and homogeneous oocytes.

Keywords: granulated ooplasm, proteomics, lipid content

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS AND OOGENESIS

Comparative analysis of morphology, gene expression, and luteinization in bovine granulosa cells among various culture systems

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The *in vitro* culture of ovarian granulosa cells (GCs) is essential for studying their role in regulating ovarian follicle development, steroidogenesis, and oocyte maturation. Over the last few decades, 2D cell culture has been a common method for the culture of GCs *in vitro*. However, this approach has been shown to significantly alter cell behaviors compared to their in vivo counterparts. To address these limitations, the objectives of this study are (i) to identify the optimal cell culture system for preserving GCs morphology and functions, and (ii) to find an ideal culture for successful *in vitro* luteinization. Bovine GCs were collected from healthy, vascular small ovarian follicles (2-6 mm), and subjected to various cell culture systems (2D, suspension, or 3D). In 2D culture, the surface of the culture plate was subjected to specific physical treatments to facilitate cell adhesion. Conversely, the surface of the suspension culture plate was left untreated and exhibits a predominantly hydrophobic nature. The 3D culture employed a U-bottom culture plate whose surface was treated to acquire a cell-repellent characteristic. The study consisted of two phases: (i) assessing the impact of the culture system on the quality of GCs and (ii) evaluating luteinization. GCs were cultured in 2D or suspension conditions for 4 days, followed by a 1-day induction of luteinization. The *in vitro* luteinization GCs was induced by treating the cells with insulin and forskolin. In the second experiment, GCs were cultured in 2D and 3D environments for 4 days, followed by a 1-day induction of luteinization. At the end of each phase, cells and culture supernatant were collected to assess the functions and luteinization potential of the GCs in various culture systems, more than six independent replicates were performed in the present study. Following the designated culture periods, both cells and culture supernatants were collected to assess their physiological and molecular characteristics. In the 2D and suspension cultures, GCs displayed a stretched and flat morphology, forming a monolayer in the case of 2D culture. In contrast, under suspension culture, GCs exhibited growth in both single and multiple layers. Successful spheroid formation occurred in 3D culture, where GCs presented a round shape on the outer edge and an oval/circular phenotype internally, closely resembling in vivo morphology. The relative mRNA expression of marker genes, including FSHR, CYP19A1, STAR, ESR2, LHCGR, and FOXL2, was notably downregulated in GCs cultured in 2D, suspension, or 3D conditions ($P<0.001$) in comparison to freshly isolated GCs. However, suspension better preserved FSHR and STAR mRNA expression compared to 2D cell culture ($P<0.05$). while 3D cell cultures maintained STAR mRNA expression compared to 2D culture systems. Furthermore, the mRNA expression of PCNA, CYP11A1, and HSD3B exhibited significant ($P<0.001$) upregulation in the suspension cell and 3D culture system in contrast to fresh and 2D cell culture. The secretion levels of E2 and P4, along with the expression of marker genes (CYP11A1, STAR, and HSD3B), exhibited a significant increase in both suspension and 3D cultures compared to the 2D culture system ($P<0.05$). Following the induction of in-vitro luteinization, a significant increase ($P<0.05$) in P4 production and the expression of CYP11A1, STAR, and HSD3B in luteinized GCs was observed within the 2D and suspension culture systems. However, the concentration of E2 remained similar between 2D and suspension cultures. Conversely, the 3D culture system led to a decline in the expression of CYP11A1, STAR, and HSD3B mRNA, accompanied by a reduction in P4 production. E2 secretion decreased; however, it remained significantly higher in the 3D culture compared to the 2D culture. In conclusion, considering all findings, we propose that behavior of bovine GCs is influenced by *in vitro* culture. More precisely, both suspension and 3D cultures partially maintain the function of GCs, demonstrating advantages over 2D culture. Moreover, suspension culture emerges as the optimal method for inducing *in vitro* luteinization.

Keywords: Granulosa Cells, Various Culture Systems, Morphology, Gene Expression, and Luteinization

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS AND OOGENESIS

A transcriptomic approach to investigate early folliculogenesis in the bovine

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The vast potential of the ovarian reserve is still largely unexploited. It mainly consists of primordial (PMF), primary (PF), and secondary (SF) follicles, a reservoir of oocytes inaccessible to assisted reproductive technologies due to challenges in replicating complex developmental processes *in vitro* (Telfer, Physiol Rev, 103(4), 2623, 2023). The mechanisms by which oocytes from the dormant PMF pool are selected and proceed through differentiation remain largely unknown. Advances have been made in understanding folliculogenesis and establishing culture systems for PMF, but clinical applications for fertility preservation at this stage remain ineffective (Dey, J Assist Reprod Genet, 41(12), 3287, 2024). This study aims to deepen the understanding of folliculogenesis by dissecting the mechanisms that drive early folliculogenesis in the bovine model. PMF, PF, and SF were mechanically isolated from ovarian cortex fragments obtained from ovaries of 12- to 24-month-old heifers (N=33) (Monferini & Dey, Reproduction, 167(6), e240060, 2024). Homogeneous pools of isolated PMF, PF, and SF were collected in triplicate; total RNA was extracted, and 50bp paired-end bulk RNA sequenced reads were generated on Illumina NextSeq2000. Sequenced data were trimmed using Trimgalore, mapped to the bovine reference transcriptome ARS UCD 1.3, and quantified by Salmon. Differential gene expression (DGE) analyses were conducted in R using DESeq2 for two pairwise comparisons: PF vs. PMF and SF vs. PF. Functional enrichment was carried out with WebGestalt to identify overrepresented pathways. A Likelihood Ratio Test (DESeq2) was applied to compare all three stages simultaneously and investigate the preantral follicle gene expression dynamics. Enrichment analyses were then performed on the resultant gene clusters to determine their function. As a result, 689 and 3206 differentially expressed genes (DEGs, FDR<0.05) were identified between the two pairwise comparisons, PF vs. PMF and SF vs. PF, respectively, revealing that signaling pathways, such as PI3K-Akt, Wnt, mTOR, and extracellular matrix (ECM) interaction guide the transition between stages. The preantral follicle gene expression dynamics revealed 4 clusters of genes, each with shared expression patterns. Three clusters contain significantly enriched pathways (FDR<0.05) related to cell cycle and ECM interactions. These pathways provide meaningful insights into gene expression across the three follicular stages, complementing the previous pairwise comparisons. To strengthen our findings, studies have been conducted to experimentally validate the main outcomes and assess the role of the crosstalk between follicle components and the surrounding stroma. Overall, our study contributes to dissecting the potential mechanisms underlying PMF to SF folliculogenesis for the first time in cattle, laying a foundation for developing *in vitro* approaches for fertility preservation efforts applicable in clinical settings and genomic rescue in threatened species and livestock.

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Keywords: folliculogenesis, RNA-Seq, bovine

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS AND OOGENESIS

MicroRNA expression profiling in bovine ovulation and early corpus luteum formation: insights from two independent studies

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MicroRNAs (miRNAs) play key regulatory roles in ovarian function, yet their role in ovulation and corpus luteum (CL) formation is not well understood. This study integrates two independent datasets to profile miRNA expression in bovine theca, granulosa, and CL tissues across six timepoints spanning the pre-ovulatory to early luteal phase. Study 1 (UCD) analysed samples from synchronised beef heifers (n=24; 2 ± 0.5 years; 587.6 ± 98 kg) collected at 0 (T1), 22 (T2), 48 (T3) or 96 h (T4) post-GnRH administration, while Study 2 (PSU) analysed samples from non-lactating Holstein cows (n=8; 4.5 ± 1.5 years; ~720 kg) sampled at 96 h (T5), and 144 h (T6) post-GnRH administration. MicroRNA expression profiling of all samples was performed using the NanoString nCounter platform. The resulting UCD and PSU datasets were collated and analysed in R using the limma package with empirical Bayes moderation and FDR correction; batch effects, if present, were adjusted using ComBat. Functional pathway enrichment was performed with DIANA-mirPath for annotated miRNAs, miRPathDB for KEGG visualisation and miRBase for ortholog discovery. A total of 237 miRNAs were detected, of which let-7a-5p, let-7b-5p, miR-125b-5p, and miR-199a-3p were the most abundantly expressed in all tissues. No significantly differentially expressed miRNAs were identified in theca tissue. In granulosa tissue, 44 miRNAs were differentially expressed during ovulation (T1 vs. T2). Pathway analysis of these miRNAs revealed significant enrichment in oocyte meiosis, steroid biosynthesis, GnRH signaling, MAPK signalling, and Wnt signaling pathways. In CL tissue, 127 miRNAs were differentially expressed between T5 and T6 and enriched FoxO signaling, TGF-beta signaling, apoptosis, cell cycle regulation and metabolic pathways. Co-expression analysis via weighted gene co-expression network analysis and manual clustering were applied to identify trends between tissue types and timepoints. RT-qPCR validation was performed on 14 selected miRNAs, based on differential expression and functional relevance. Four miRNAs – miR-132-3p, miR-214-3p, and miR-382-5p – were retained for further ongoing functional studies based on consistent validation and biological roles. This dual-study approach identifies conserved miRNAs as potential biomarkers for fertility management and enhances our understanding of miRNA-mediated regulation of bovine ovarian function, offering valuable insights for improving reproductive health and fertility management in cattle.

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Keywords: miRNAs, ovulation, cattle

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS AND OOGENESIS

Effects of nanoplastics on bovine oocyte competence and mitochondrial function

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The presence of nanoplastics (NPs; < 1 µm) in the environment has gained increasing concerns about their potential effects on health and reproduction. Studies have shown that NPs can pass biological barriers, including the reproductive tract in rodents, and enter the ovary (Liu, Z. J Hazard Mater. 2022;424(Pt C):127629.) To explore the effects of NPs on oocyte developmental competence, we exposed bovine maturing cumulus-oocyte complexes (COCs) to NPs. This research is also of interest for human reproduction, as bovine early development until implantation is largely comparable to human (Menezo, YJ. Reprod Biomed Online. 2002;4(2):170-175.). We previously reported that 50 nm polystyrene (PS) NPs can enter the cumulus cells and oocytes. Exposure during *in vitro* maturation (IVM), at a concentration of 3 µg/mL, resulted in impaired oocyte nuclear maturation rate and temporally delayed embryo development (Yang, J. Reprod Fertil Dev. 2025;37(1)). To investigate the underlying mechanisms, RNA sequencing was performed on RNA isolated from bovine COCs after the 23h IVM in medium (NaHCO3-buffered M199 supplemented with 100 IU/mL Penicillin-streptomycin, 0.05 IU/mL FSH, 0.1 µM cysteamine, and 10 ng/mL EGF) with or without 50 nm PS-NPs (Polysciences Europe GmbH, Hirschberg an der Bergstrasse, Germany) at 3 µg/mL at 39°C and 5% CO2 in air. RNA samples were from 6 independent replicates with groups of 50 COCs. Libraries were sequenced to a depth of 20 million reads per sample, and data were analyzed using Gene Set Enrichment Analysis (GSEA) with Kyoto Encyclopedia of Genes and Genomes pathway and gene ontology databases. In addition, mitochondrial function of COCs was assessed using Alamar blue assay (Sigma-Aldrich, Darmstadt, Germany; 210 COCs per group in 6 independent replicates) and CellTiter-Glo Luminescent Cell Viability Assay (Promega, WI, United States; 72 COCs per group in 3 independent replicates). One-way ANOVA followed by Dunnett's multiple comparisons was used for statistical analysis. A p-value < 0.05 was considered statistically significant. Differentially expressed genes were defined as those with an FDR < 0.05. There were no differentially expressed genes detected between NP-exposed and control COCs. The GSEA suggested downregulation of oxidative phosphorylation in the NP-exposure group. However, neither the mitochondrial enzyme activity nor the ATP content differed in response to 50 nm NPs. In conclusion, while 50 nm PS-NPs can enter maturing oocytes and impair oocyte nuclear maturation and developmental competence, their effects may not be driven by detectable changes in transcriptomic level or mitochondrial function. Further research is required to clarify the mechanisms by which nanoplastics affect oocyte developmental competence.

Keywords: nanoplastics, reproductive toxicity, oocyte maturation, bovine

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS AND OOGENESIS

In vitro culture of cumulus-oocyte complexes from sheep early antral follicles: achievements, challenges, and future directions

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The potential use of early antral follicles (EAFs) provides a valuable source for assisted reproduction and fertility preservation. However, these oocytes are incompetent and require a growth phase to acquire meiotic competence. To unlock the potential of this genetic resource, we conducted a comprehensive study that systematically evaluated key factors influencing the *in vitro* culture of EAF-derived cumulus-oocyte complexes (COCs). These factors included culture strategy (single vs. group), seasonal variation (breeding vs. non-breeding season), donor age (prepubertal vs. adult), and culture duration (5 vs. 7 days), to optimize a reproducible culture system. Here, we recapitulate the major achievements, ongoing challenges, and future perspectives derived from this work. COCs isolated from EAFs (350–450 µm) were cultured in TCM199 medium supplemented with 0.15 µg/mL zinc sulfate, 10^{-4} IU/mL FSH, 10 ng/mL estradiol, 50 ng/mL testosterone, 50 ng/mL progesterone, and 5 µM cilostamide. Following long *in vitro* culture and maturation, assessments were performed on morphology, viability, oocyte diameter, global transcriptional activity, gap junction communication, chromatin configuration, meiotic resumption, and developmental competence, along with analyses of reactive oxygen species levels, mitochondrial activity, and ultrastructural features. Key findings demonstrated that COCs collected during the breeding season (n=227) exhibited greater cumulus cell cohesiveness and compactness (P<0.000), viability, and development to metaphase II compared to the non-breeding season (n=294). In the age-related comparison, COCs derived from adult ewes (n=226) outperformed those from prepubertal donors (n=196), showing greater oocyte diameters (116.3±0.37 µm vs. 113.4± 0.34 µm µm, P<0.01), higher meiotic competence following maturation (59.4% vs. 21.0%), and more robust gap junction communication. Regarding culture strategy, group culture (n=181), although not differing from single culture (n=227) in terms of morphology or viability, significantly enhanced meiotic resumption (P<0.01). Moreover, extending the culture period from 5 to 7 days resulted in improved oocyte growth (118.1 vs. 116.3 µm, P<0.05) and a notable increase in blastocyst formation rates (0% vs. 11.1%, P<0.05). Overall, these findings highlight the impact of seasonality on oocyte quality, the limited developmental potential of prepubertal COCs, and the benefits of extended culture and group culture in enhancing oocyte competence. However, despite the promising outcomes, the lower maturation and blastocyst rates compared to standard *in vitro* embryo production systems highlight the necessity for further optimization. This includes standardizing culture conditions, exploring innovative 3D culture systems to better preserve COC integrity, refining the culture medium, and addressing the challenges related to follicle isolation. Tackling these aspects will bring us closer to establishing an efficient and reliable culture system, thereby enhancing fertility preservation strategies.

Keywords: early antral follicles, cumulus-oocyte complexes, *in vitro* culture

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS AND OOGENESIS

Isolation and characterization of mesenchymal stromal cells from equine follicular aspirates

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Mesenchymal stromal cells (MSCs) are multipotent cells known to be able to differentiate into different cell types. These MSCs have an immunomodulatory capacity and have been isolated from different tissue sources. In horses, transvaginal oocyte aspiration or ovum pick up (OPU) is routinely used to collect oocytes for experimental or clinical purposes. Similar to humans, discarded follicular aspirates in horses contain a diverse population of cells. Hence, the aim of the present work was to isolate and characterize MSCs from ovarian follicular aspirates of mares subjected to OPU. Cells were isolated from a total of six mares included in an experimental OPU program. The cells were seeded in culture flasks containing Dulbecco´s Modified Eagle Medium (DMEM; Gibco, Paisley, UK), supplemented with 10% Fetal Bovine Serum (FBS; Sigma Aldrich, Louis, USA), 1% penicillin and streptomycin (Gibco, Grand Island, NY, USA) and 0.5% amphotericin B (Gibco, Paisley, UK). Cells were incubated at 37ºC in a humidified atmosphere with 5% CO2 and expanded to reach 80% confluence (9 to 14 days). At this point, cells were detached using 0.05% Trypsin-EDTA (Gibco, Grand Island, NY, USA) and seeded at 5,000 cells/cm² in 75 or 175 cm2 culture flasks for expansion. After three passages, cells were cryopreserved at a concentration of 1 x 10^6 cells per ml in a freezing medium composed of 10% dimethyl sulfoxide (DMSO; Sigma Aldrich, Missouri, USA) and 90% FBS, and stored at -80°C. For flow cytometry analysis, one ml of MSCs from each mare was thawed at 37 °C in culture medium, centrifugated at 430xg for 5 min to remove the cryoprotectants and placed in a 75 cm² culture flask to allow re-expansion. For flow cytometry analysis, once the cells reached 80% confluence, they were trypsinized and counted. Subsequently, 1 x 10^5 cells were resuspended in 150 µl of phosphate-buffered saline (PBS, pH 7.2; Gibco, Paisley, UK) and incubated for 30 min at 4°C with fluorochrome-conjugated monoclonal antibodies. Following the minimal criteria for reporting animal MSCs (Guest, D.J., Frontiers in Veterinary Science, 9, 817041, 2022), two positive surface markers (CD90 and CD29), which are commonly detected in MSCs, and three negative surface markers (CD45, CD19 and MHC-II), along with their respective isotype controls were used. Cells were washed once with PBS and 10,000 events were acquired using an ACEA NovoCyte™ flow cytometer. Cells obtained from all mares were respectively positive for the MSCs markers CD90 and CD29, and negative for CD45, CD19 and MHC-II in 100% of the selected cell population. Differentiation assays (adipogenic, chondrogenic and osteogenic lines) are being carried out, however these initial findings demonstrate the presence of MSCs in equine follicular aspirates, and opens the possibility of investigating a new field in equine reproduction.

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Keywords: equine, follicular fluid, MSC

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS AND OOGENESIS

Impact of ethylparaben on porcine oocyte maturation

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Parabens are a group of chemicals widely used as artificial preservatives in cosmetics and personal care products. Despite being considered safe, growing evidence links their environmental exposure to endocrine disruption due to interference with endogenous hormone activity. Porcine oocytes, due to their morphological and meiotic similarities to human oocytes, can be used as a relevant model for studying the reproductive impact of endocrine disruptors. This study assessed the effects of ethylparaben on *in vitro* oocyte maturation by evaluating cumulus cell expansion, nuclear maturation stage and oocyte diameter. A total of 15 to 25 prepubertal gilts were utilized to obtain the 250 COCs employed for each of the four experimental replicates. Ovaries were collected at the slaughterhouse, and cumulus-oocyte complexes (COCs) were recovered. COCs were placed in four-well Nunc® dishes (≈50 per well) and incubated for 20–22 hours within 500 µL of NCSU-37 medium containing varying concentrations of ethylparaben (Control: 0 µM; T1: 250 µM; T2: 500 µM; T3: 750 µM; T4: 1000 µM). Subsequently, COCs were then transferred to NCSU-37 medium without hCG, eCG, and dibutyryl cAMP and cultured under the same conditions for 20–22 hours. Following incubation, the COCs were photographed with a digital camera attached to a stereomicroscope (Nikon SMZ800, Japan), and cumulus cell expansion was measured with ImageJ® software. Once decumulated, oocytes were photographed with a digital camera (Nikon® D40, Japan) connected to an inverted microscope (Nikon® Diaphot 300, Japan). Oocyte diameter was then measured also using ImageJ® software. For nuclear maturation analysis, oocytes were fixed in glutaraldehyde, washed in PBS/PVA, and stained with Hoechst. Oocytes were assessed under a fluorescence microscope (Leica® DM4000 Led, Wetzlar, Germany, 460/490 nm) and classified as follow: (a) mature oocytes if they had a metaphase plate and the first polar body (metaphase II) and (b) immature (germinal vesicle (GV) or metaphase I stage) or degenerated oocytes. The results showed that the expansion of the COCs was lower in the treatment groups compared to the control. In particular, a dose-dependent decrease in the expansion of the COCs was observed until 750 µM (T3) (C: 2453.31 ± 841.70 µm, T1: 1234.81 ± 526.88 µm, T2: 852.08 ± 410.05 µm, T3: 690.19 ± 323.78 µm, T4: 651.05 ± 356.03 µm; $P<0.001$). Moreover, ethylparaben treatment decreased the proportion of mature oocytes and increased the number of immature and degenerated oocytes in a dose-dependent manner. Thus, the percentage of mature oocytes after IVM was significantly lower in the treatment groups (T1-T4) (0-24.44%) compared to the control (59.74%) ($P<0.05$). Moreover, the results showed a dose-dependent decrease in oocyte diameter, being significantly smaller in the T3 (143.41 ± 7.33 µm) and T4 (141.63 ± 6.51 µm) groups compared to the control (146.95 ± 4.77 µm) ($p < 0.05$). The present study demonstrates that ethylparaben impairs oocyte maturation in a dose-dependent manner, suggesting its potential role in fertility reduction, as evidenced using the swine model.

Keywords: paraben, endocrine disruptor, oocyte maturation

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS AND OOGENESIS

The vulnerability of primordial follicles to diet-induced mitochondrial damage and its persistence until ovulation despite preconception care

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Obesity and consumption of high-fat/high-sugar (HF/HS) diets compromise female fertility, in part through impaired oocyte quality driven by mitochondrial dysfunction. Yet, it remains unclear at which stage of folliculogenesis oocytes become vulnerable, and whether diet normalization prior to conception can fully reverse this damage. In this integrated study, we examined mitochondrial ultrastructure in oocytes across follicular stages using transmission electron microscopy (TEM), following exposure to an obesogenic diet. Female Swiss mice were fed a HF/HS diet for ≥7 weeks and analyzed either directly or after diet normalization as a preconception dietary intervention. Mitochondrial ultrastructure was assessed in primordial follicle oocytes (in ovarian sections, n = 10-27 follicles in 6 ovaries from 6 mice per treatment group), as well as in ovulated metaphase II (MII) oocytes (n=3-5 COCs from 3-5 mice per treatment group). A chi-square analysis was used to compare proportions of abnormal mitochondria across conditions. Mature MII oocytes from HF/HS-fed mice exhibited a high proportion (46.44%) of mitochondria with ultrastructural abnormalities, including vacuolization, elongation, and disrupted inner membrane. This is compared with 3.02% in the controls ($P<0.05$). Following 4 weeks of diet normalization, the proportion of mitochondrial ultrastructural defects dropped significantly ($P<0.05$), yet a residual 9.5–9.9% of mitochondrial abnormalities persisted—suggesting incomplete recovery. To identify the origin of this persistence, we assessed early follicular stages. Oocytes from primordial follicles of HF/HS-fed mice showed on average a 10% increase in mitochondrial abnormalities compared to controls ($P<0.05$). These findings indicate that oocytes already acquire mitochondrial damage at the earliest dormant follicular stage in response to direct dietary exposure. In conclusion, our data demonstrate that HF/HS diet impairs mitochondrial integrity of the oocytes arrested in the primordial follicle pool, but the majority of the damage seems to be acquired during folliculogenesis. Diet normalization partially improves oocyte quality upon ovulation, and the persistent mitochondrial defects may reflect irreversible damage originating from affected primordial follicles. These findings highlight the need for early and sustained metabolic health optimization in women of reproductive age, well before conception is planned. Moreover, the results are highly relevant to livestock species, where metabolic challenges due to diet composition, negative energy balance or heat stress may similarly compromise oocyte quality and long-term fertility potential.

Keywords: Obesogenic diet, subfertility, primordial follicles, Britt hypothesis

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS AND OOGENESIS

ZP1 and ZP3 proteins are essential for zona pellucida formation and fertility in rabbits

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The zona pellucida (ZP) is an extracellular matrix that surrounds the oocytes and preimplantational embryos and plays relevant roles in folliculogenesis, gamete interaction, block to polyspermy and embryo protection. In eutherian mammals the ZP is composed by three to four glycoproteins (ZP1, ZP2, ZP3 and ZP4). Murine knockout (KO) models have provided relevant information about the functional role of the different ZP proteins. However, mouse ZP is composed of three proteins (ZP1, ZP2 and ZP3), while both human and rabbit ZP have four proteins. The aim of this study was to determine the role of ZP1 and ZP3 proteins on ZP formation and fertility in rabbits. ZP1 or ZP3 KO rabbits were generated using CRISPR-Cas9 technology. Founder rabbits carrying frame-disrupting KO alleles composed of a 26-nucleotide deletion in ZP1 and 1-nucleotide insertion in ZP3 were selected to generate the KO lines. Homozygous animals for such mutations were obtained following a conventional crossbreeding programme. Litter size was roughly similar between heterozygous (+/-) and wild-type (WT) animals (6.9±0.6 vs. 9.3±0.5 vs. 8.3±0.5 pups in 14, 6 and 8 crosses for ZP1+/-, ZP3+/- and WT, respectively), but KO animals fail to produce any litter (10 crosses for ZP1 KO, 3 crosses for ZP3 KO). In both KO models, no oocytes were collected following superovulation by a subcutaneous dose of 3 µg of corifollitropin alfa (Elonva®) and 7,5 IU hCG (Coriogan®) followed 72 hours later by ovulation induction with an intramuscular dose of 2 µg buserelin acetate (Suprefact®). The analysis of oocytes collected from heterozygous animals revealed that the ZP of ZP1+/– oocytes was morphologically normal with a similar thickness to WT oocytes (18.50±0.43 (n=52) vs 18.97±0.38 (n=42) for ZP1+/- and WT, respectively), whereas the ZP of ZP3+/– oocytes was thinner than that of WT oocytes (9.66±0.33 (n=51) vs 18.97±0.38 (n=42) for ZP3+/- and WT, respectively). To analyse the effect of the ablations on folliculogenesis, ovaries were fixed in 4% formaldehyde and embedded in paraffin, and sections of 5 µm thickness were prepared and stained with hematoxylin and eosin to be scanned using the Digital Slide Scanner Pannoramic MIDI II (3DHistech). The ablation of ZP1 or ZP3 prevented the formation of the ZP. Finally, to determine if the ablation of ZP1 or ZP3 altered the expression of other ZP genes, PCRs were performed on cDNA obtained from ovarian tissue. ZP2, ZP3 and ZP4 transcripts were detected in animals lacking ZP1, and ZP1, ZP2 and ZP4 transcripts were identified in animals lacking ZP3, demonstrating that ZP1 or ZP3 ablation did not impede the expression of the other ZP proteins. In conclusion, ZP1 or ZP3 ablation results in female infertility by preventing ZP formation without altering the transcription of all other ZP proteins.

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Keywords: zona pellucida, oocyte

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Effect of ectoine on the *in vitro* quality of liquid-stored bovine semen during extended storage

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Ectoine, a compatible solute, serves as a protective shield for cells by protecting macromolecules and cell structures from extreme environmental stress. The objective of this study was to assess the ability of ectoine to enhance sperm quality parameters in liquid-stored bovine semen during extended storage. Semen was collected from bulls (n=7) in a commercial stud and diluted in INRA96 containing either 0 (control), 1, 5, 25 and 50 mM of ectoine. The semen was packaged into straws (0.25 ml at 20 x 106 sperm per ml) and held at 18oC over 7 days. Motility (computer assisted sperm analysis) as well as the viability, acrosomal status, mitochondrial membrane potential (MMP), esterase activity, calcium influx and superoxide production (flow cytometry) were assessed on Day 1, 3, 5 and 7 post collection. Statistical analysis was performed using IBM SPSS (Statistical Package for Social Sciences), and data were analysed using repeated-measures ANOVA. Results are presented as mean ± sem. There was a significant interaction between treatment and time ($P<0.01$) for both total and progressive motility, indicating that the rate of motility decline varied depending on ectoine concentration. The control group exhibited a continuous decline in motility across the storage period ($P<0.001$), whereas 25 mM ectoine significantly preserved motility. On Day 7, total motility (48.5 ± 5.64%) and progressive motility (31.1 ± 6.00%) in the 25 mM group were notably higher compared to the control (20.6 ± 5.56% and 9.6 ± 2.94%, respectively). Similarly, storage with 25 mM ectoine maintained sperm viability over time compared to control samples ($P<0.05$). A treatment-by-time interaction was also evident for the proportion of viable sperm exhibiting high MMP ($P<0.05$). Although there were no differences among groups on Day 1, after 7 days of storage, the control group experienced an 88% decrease in viable sperm with high MMP ($P<0.001$). In contrast, the 25 and 50 mM ectoine treatments significantly preserved MMP over time ($P<0.05$). Additionally, ectoine effectively preserved membrane stability and functional capacity during extended storage, as reflected by a significantly higher percentage of viable sperm with low membrane fluidity and intact acrosomes in the 25 mM (95.3 ± 1.01%) and 50 mM (95.5 ± 0.49%) treatments compared to the control (89.8 ± 1.26%) on Day 7 ($P<0.05$). Ectoine had no effect on calcium influx at any time point ($P>0.05$), suggesting that its protective effects are not mediated through alterations in intracellular calcium signaling. Similarly, no effect of ectoine was observed on superoxide production ($P>0.05$). Overall, this study demonstrates the potential application of ectoine to extend semen shelf-life and mitigate the decline in semen quality over time.

Keywords: sperm, bull fertility, fresh semen, flow cytometry

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Testing sustainably-obtained natural grape marc extracts for bull semen cryopreservation

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Plant extracts contain bioactive compounds, such as polyphenols, known for their antioxidant and antimicrobial effects. The EU-funded NeoGiANT project aims to apply sustainably-obtained natural extracts from wine industry by-products to reduce the use of antibiotics in animal production. Using these natural extracts for semen cryopreservation is an opportunity in cattle breeding, with an extensive use of artificial insemination. Therefore, this study evaluates two extender formulations containing grape marc extracts (E1 and E2, confidential composition, LIDSA, Spain) on bull semen criopreservation (11 bulls, Friesian, Rubia Gallega, and Belgian Blue, 1 ejaculate/bull; Xenética Fontao, Spain). Ejaculates were split and extended with the same volume of BoviFree CSS (Minitüb) as CTL (control, neither antibiotic nor extract), CTLAB (antibiotic as per CSS), and extract supplementation as formulations E1 and E2. Upon arrival to the laboratory (<24 h at 5°C), sperm concentration was adjusted to 92×106 mL-1 using the same extenders, packed in 0.25-ml straws, frozen (IceCube biofreezer), and stored in LN_2. Samples were evaluated before (pre-freezing, PF) and after (post-thawing, PT) cryopreservation (thawing 37°C, 30 s) for sperm motility (OpenCASA v2) and microbial contamination (colony counting after plate culture with serial dilution and MALDI-TOF identification). Data were analyzed by linear mixed-effects models (R statistical environment), with treatment as fixed effect and the bull as random effect (results as %, mean±SEM). Microbiological testing after refrigerated storage showed no contamination or very low contamination in CTLAB, $P<0.05$, but it was significantly higher in treatments without antibiotics, both PF and PT. Total and progressive motility were higher in CTLAB compared to E2 especially post-thawing (PF: 76.5±2.8 vs. 71.4±4.0, $P<0.05$, and 20.9±3.6 vs. 19.0±3.3, $P>0.05$; PT: 51.1±7.1 vs. 32.4±3.0, $P<0.01$, and 27.9±4.7 vs. 11.4±2.8, $P<0.001$); however, E1 performed similarly to CTLAB (PF: 77.1±3.3 and 42.0±3.4; PT: 21.4±3.9 and 16.9±3.6, respectively). Sperm velocity was not affected by the extracts, but PT linearity (LIN) and straightness (STR) were lower for E2 ($P<0.05$ vs. CTLAB), but not for E1. E2 effects could be due to specific polyphenols depressing sperm motility, maybe having other applications. In conclusion, the E1 formulation might have potential for the development of more sustainable extenders, despite of no evidence for antimicrobial effects. The improved methodology developed in NeoGiANT enables for environmentally-friendly, polyphenol-rich natural extracts. Further studies will focus on the antioxidant effects of these formulations.

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Keywords: grape marc extract, bull sperm, cryopreservation

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

A machine learning model for frozen-thawed bull semen quality prediction

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The reproductive performance achieved in cattle farming is dependent on both female and male factors, including semen quality. In recent times, cattle production is increasingly making use of artificial insemination (AI) which requires frozen-thawed bull semen (FTBS). FTBS is variable in quality at collection and vulnerable to depreciation in quality due to cryopreservation and subsequent transport. Also, FTBS quality assessment techniques in the field are often subjective and consequently potentially sub-optimal for predicting fertility performance. This study aims to improve quality assessment of FTBS. It is important for farmers to know the quality of semen before AI to have confidence in the projected fertility performance of the semen. To achieve this goal, a machine learning (ML) model is proposed for predicting semen quality. The model proposed applies a rigorous approach by using multiple objective parameters (motility and morphology) to improve the prediction of conception risk (CR) values. The semen parameters employed in the model include straight-line velocity (VSL), total motility (TM), progressive motility (PM), motile dose (MD), TM and PM after incubation for twenty minutes, normal morphology, acrosomal membrane integrity (AMI) and mitochondria membrane activity (MMA). The parameters were applied in predicting semen fertility potential (SFP) which was then correlated to CR values obtained from veterinary ultrasound pregnancy diagnosis following AI. The proposed model is a regression model and makes use of random forest, a supervised ML algorithm. This study makes use of 201 semen samples (SS) from 34 bulls, of which 80% (160 SS) was used for model development and the remaining 20% (41 SS) for testing and validation. The mean actual CR value (MACR) is 46.94% pregnancies while the mean predicted CR (MPCR) or SFP is 46.95% pregnancies. The model accuracy is given by the percentage ratio of the difference between MPCR and the root mean square error (RMSE) to the MPCR. The performance of the developed model in predicting SFP values is at approximately 64% but increases to 85% accuracy when the prediction model is optimised. This is an increment of between 21-45% when compared to the values obtained in similar research by Sellem et al. (2015) with accuracy of between 24-40%. Both assessment techniques are more objective and less prone to error when compared to the subjective state-of-the-art method which makes use of light microscopes in determining semen quality. All the highlighted methods do not consider confounding factors such as cow reproductive physiology or farm level management. Notwithstanding, the improvement offered in the proposed method will potentially give cattle farmers a higher level of confidence in the quality of semen being applied for AI and help save on cost and time.

Keywords: bull, frozen-thawed semen, fertility prediction

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Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Interpreting sire *in vitro* fertility using machine learning

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Artificial insemination (AI) and *in vitro* fertilization (IVF) are widely used in cattle breeding. Yet, fertilization capacity often varies between these two techniques, despite using semen from the same sire. This study evaluated differences between sires classified as high-performance (HP) and low-performance (LP) based on *in vitro* embryo production (IVP) capacity. Through analysis of semen quality parameters from fresh and post-thaw (pt) semen samples, we developed a Python-based analytical pipeline implementing machine learning algorithms to process computer-assisted sperm analysis (CASA) data to predict sire IVF potential. Our findings provide novel insights into sire selection for IVF success rates and improve embryo development outcomes. Semen samples were collected from 40 Estonian Holstein bulls across two separate collection events. All samples were analyzed with CASA. IVF procedures were performed in triplicate for each ejaculate (utilizing 300 oocytes per replicate), totaling 240 IVF rounds conducted with Stroebech Media under standardized protocols. A control bull with previously determined stable IVF results was included throughout the IVF experiments. Embryo development was assessed on days 7 and 8 post-fertilization, including evaluation of blastocyst rates, morphological grading, and kinetic scoring. Blastocyst rate was calculated as the number of blastocysts over the total number of fertilized oocytes. Morphology score was defined by scoring blastocyst quality as poor, good, or excellent based on the IETS guidelines. The kinetic score was determined by classifying blastocyst developmental stages as early/non-expanded, expanded, and hatching/hatched. Based on these criteria, bulls were ranked and classified as HP or LP. Using CASA data from both fresh and post-thaw ejaculates, an analytical tool was developed in Python implementing Linear Discriminant Analysis (LDA) to predict fertility potential. The dataset consisted of 38 ejaculates, of which 34 were used for training, and 4 (3 LP and 1 HP) for testing. On the training set, the model achieved an accuracy of 94%, with a precision and F1-score of 0.96 for LP bulls, and 0.91 for HP bulls. On the test set, it reached an accuracy of 75%, with a precision of 1.00 and F1-score of 0.80 for LP bulls, and a precision of 0.50 and F1-score of 0.67 for HP bulls. The model showed good performance on the training data, but the small size of the test set limits the generalizability of the results. Straight line velocity (VSL), curvilinear velocity post thaw (VCL_pt), and distance average path post thaw (DAP_pt) are some of the most significant parameters for HP sires. In contrast, lateral head displacement post thaw (ALH_pt) and average path velocity before and post thaw (VAP, VAP_pt) are strongly associated with LP sires. Our preliminary results indicate that there is a difference in CASA parameters that determine bull fertility *in vitro*. Further experiments are needed to train the model with more ejaculates to improve its predictability.

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Keywords: *in vitro* fertilization, machine learning, bovine

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Age affects antimüllerian hormone levels and sperm motility but not testosterone levels in bulls from Asturiana de la Montaña and Asturiana de los Valles breeds

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Antimüllerian hormone (AMH) levels are related to the ovarian reserve in females; it has gained attention as a marker of male fertility in human medicine. AMH is produced by Sertoli cells and reflects testicular function. Since there is limited information about AMH levels in bulls and their relationship with sperm quality, the objective of this study was to evaluate AMH levels in bulls of different ages to establish general levels and assess the influence of age. As a secondary objective, we investigated possible relationships with testosterone levels and sperm quality. Bulls (n=56) were Asturiana de la Montaña and Asturiana de los Valles breeds (autochthonous breeds in Asturias, Northern Spain), ranging from 12 to 26 months of age. Semen was obtained by electroejaculation, extended with the same BioxCell (IMV) volume, and analysed within 6 hours. Blood plasma was obtained by venipuncture and centrifugation and analyzed for testosterone (Immulite 2000, Siemens) and AMH (ELISA, Abyntek ABK1-E6659 for Bos taurus). Sperm quality was evaluated using CASA for motility and flow cytometry for viability, apoptosis, mitochondrial and acrosomal status, and reactive oxygen species (ROS). The association among variables was studied using Pearson correlations, and the effects of age on the variables were analyzed by linear regression and linear models after categorizing the bulls into three groups: yearlings (<15 months of age), young (<20 months), and adults. Data were analyzed in the R statistical environment (results presented as medians and first and third quartiles). The AMH levels (ng/ml) decreased with age (yearlings 2.14 [1.25, 2.89]; young 1.74 [1.13, 2.15]; adult 0.86 [0.61, 0.96], with a regression coefficient (R2) of 0.25 (P<0.001); however, testosterone was not influenced by age and showed a high dispersion among bulls (9.63 [3.08, 14.35] ng/ml]). Most sperm quality variables were not significantly affected by age, with sperm motility generally improving with age. Younger bulls exhibited higher values for mitochondrial ROS but lower values for cytoplasmic ROS. Correlations were not significant in any case. In summary, we report AMH levels in bulls of different ages, demonstrating that they decrease with age, are lower in the older group, and show no relationship with testosterone levels or sperm quality variables. However, the bulls showed good sperm quality, and they may have good testicular function, concealing a potential role as a biomarker. Since AMH levels in young bulls are associated with testicular development, they may predict lifelong reproductive performance. In fact, despite the lack of significant correlations, differences in sperm quality among younger bulls, including ROS levels, may indicate incomplete testicular maturity. Future studies should investigate associations within age groups, follow bull cohorts over time and include fertility data.

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Keywords: bull, antimüllerian hormone, sperm quality

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Functional role of β-N-acetyl-glucosaminidase in sperm reservoir dynamics in bovine oviduct

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Successful fertilization requires precise sperm-oviduct interactions, including storage and timely release, potentially regulated by epithelial glycans and oviductal glycosidases. This study investigated the role of β-N-acetyl-glucosaminidase (BNAG) and its substrate, β-N-acetyl-glucosamine (NAG), in modulating bovine sperm binding and release from bovine oviductal epithelial cells (BOECs). Reproductive tracts from adult cows were collected post-mortem and transported on ice. Oviducts were classified as peri-ovulatory (PO, n=5) or non-periovulatory (NOPO, n=5) based on ovarian morphology. Oviductal fluid was flushed from the uterotubal junction to the infundibulum, centrifuged (13,000 g, 10 min), and stored at −20°C. BOECs for primary cultures were obtained from PO oviducts (n=4) by mechanical extrusion (Lamy et al., Biol Reprod 94:18, 2016). BNAG activity in oviductal fluid was measured by spectrofluorometry using 4-methylumbelliferone substrates (Sigma, St. Louis, MO, USA) at pH 7.0 and normalized by protein concentration (Bradford assay). BNAG activity was significantly higher in PO vs. NOPO samples (P<0.05, t-test). BOECs were cultured in TCM199 (Thermo Fisher Scientific, NY, USA) with 10% FBS (Serendipia, Buenos Aires, Argentina) at 38.5°C in 5% CO□. Epithelial identity was confirmed by immunofluorescence (anti-cytokeratin Abcam ab9377, Cambridge, UK). To assess NAG's role in sperm adhesion, co-culture assays were performed using cryopreserved bovine sperm incubated in non-capacitating TALP medium (NCM) with increasing NAG concentrations (5, 10, 50 mM). Treatments included: (1) simultaneous incubation, (2) sperm preincubation, or (3) BOEC preincubation (20 min at 38.5°C, 5% CO_2). In groups 2 and 3, cells and sperm were washed before co-incubation (20 min at 38.5°C). BOECs preincubation with NAG significantly increased sperm binding in a dose-dependent manner, while sperm preincubation reduced it (P<0.005, ANOVA). To test BNAG's involvement in sperm release, BOECs were pre-treated with the BNAG inhibitor VP115 (7 nM; Pingitore et al., Bioorg Chem 120:105650, 2022) for 18 h. After sperm co-culture and removal of unbound sperm, BOECs were incubated for 4 h in capacitating medium (CM) with or without 17β-estradiol (E2, 100 pg/mL), progesterone (P4, 100 ng/mL), or both. Released sperm were removed and cells washed before fixation. BNAG inhibition significantly reduced sperm release in CM + P4 and CM + P4 + E2 groups compared to controls (n=4; P<0.05, ANOVA). In all cases, sperm bound to BOECs were quantified under fluorescence microscopy following Hoechst staining. These findings support that BNAG activity increases during the periovulatory period and may regulate sperm-oviduct interaction dynamics. Targeting BNAG could improve assisted reproductive technologies, such as *in vitro* fertilization and sperm storage in cattle.

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Keywords: β-N-acetyl-glucosaminidase, sperm-oviduct interaction, β-N-acetyl-glucosamine

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

CASA-based analysis of frozen-thawed homospermic and heterospermic bovine semen from split ejaculates shows no difference in post-thaw motility

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Heterospermic (HS) semen is a product which contains semen from two or more sires in the same semen straw. It has been marketed as a product that increases pregnancy rates above conventional homospermic semen and it has been hypothesized that it can potentially widen the window in which cows can be inseminated, due to the mixing of sperm with differential rates of capacitation (Beatty et al. 1969, Journal of Reproduction and Fertility, 19, 491-502; Stewart et al. 1974, Journal of Reproduction and Fertility, 36, 107-116). The objective of this study was to compare the post-thaw motility of homospermic and HS semen, obtained by Computer Assisted Sperm Analysis (CASA), and assess if there were any differences when semen from three bulls was mixed in the same semen straw. Across two bull semen collection centers, a total of 15 bulls (12 Angus and 3 Hereford) were split into 5 packs, each containing 3 bulls from the same breed. For each pack, two to three ejaculates were collected per bull and each ejaculate split into a homospermic treatment, containing only semen from each individual bull, and an HS treatment, containing a mix of semen from the three bulls. Semen from each treatment was then placed into straws (0.25 mL with 20 million sperm), cooled to 5ºC and cryopreserved as per routine procedures (slow rate freezing in programmable freezer followed by submersion in liquid nitrogen at -196ºC). Motility based quality controls were performed on individual ejaculates prior to mixing and cryopreservation. Two ejaculates per bull and HS mixes were analyzed, with two straws per ejaculate being pooled and two technical replicates performed. Total and progressive motility, as well as Average Path Velocity (VAP), Curvilinear Velocity (VCL), Straight Line Velocity (VSL), Linearity (LIN), Straightness (STR), Amplitude of Lateral Head Displacement (ALH), Beat Cross Frequency (BCF) and Wobble (WOB) were assessed by CASA at 0, 3 and 6 h post-incubation with a total of 8 fields of view and a minimum of 1000 sperm analyzed. Statistical analysis was conducted with IBM SPSS Statistics software and data were analyzed using repeated measures ANOVA. Results are presented as mean ± sem. There was no difference in total or progressive motility between homospermic (44.5 ± 1.40% and 39.2 ± 1.32%, respectively) and HS (44.5 ± 1.40% and 39.9 ± 2.28%, respectively) treatments (P>0.05). There was also no difference between the two treatments for any other kinematic parameter (P>0.05). Total motility decreased from 0 to 6 h post-incubation in both the homospermic (47.8 ± 1.42% and 40.6 ± 1.56%, respectively) and the HS (50.8 ± 2.45% and 39.3 ± 2.69%, respectively) treatment (P<0.01). Similarly, progressive motility and ALH also decreased over time in both treatments (P<0.01). There was no treatment by incubation time interaction for any of the parameters evaluated (P>0.05). Overall, this study demonstrates that there is no increase in post-thaw motility, when semen from different bulls is mixed in the same semen straw. There is a need to establish if other parameters, including field fertility, differ between the two treatments.

Keywords: bovine, sperm, mixed semen

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Embryo development following IVF with heterospermic or homospermic semen in cattle

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Heterospermic semen (HS) involves the mixing of semen from multiple bulls in a single semen straw. Limited evidence suggests that mixing semen leads to improved post-thaw semen quality, widens the window in which cows can be inseminated and lessens the potential negative impact of a sub-fertile bull. To date, there is limited research investigating the influence of HS on *in vitro* embryo production. Therefore, the aim of this study was to assess embryo development following IVF with HS or homospermic semen (HO). Semen was collected from 12 Angus bulls at two commercial artificial insemination centres. Each ejaculate (2-3 per bull) was split to generate HO and HS straws which were subsequently frozen. Quality control checks were done on individual ejaculates prior to mixing. Four independent semen packs (P) were generated, each consisting of straws from three individual bulls (HO1-3) and HS straws containing an equal number of sperm from each of the same three bulls in that pack. All straws contained approximately 20 million sperm/straw. All media were obtained from Stroebech Media® (Hundested, Denmark). Ovaries were collected from heifers and cows at a commercial abattoir. Following follicular aspiration, cumulus-oocyte-complexes (COCs) were matured for 24 h at 38.8℃ in 5% CO2 in air. Semen straws were thawed at 37℃ and sperm were washed twice by centrifugation. Matured COCs were randomly allocated to one of four groups for IVF and inseminated with approximately 1 million sperm/ml of either HS or each of the three HO bulls. Three replicates were carried out per pack with an average of 135 COCs per treatment per replicate (6463 COCs in total). Gametes were co-incubated for 22 h at 38.8℃ in 5% CO2 in air. Presumptive zygotes were denuded by vortexing and cultured in 50 mL droplets of medium under oil at 38.8℃ in 5% CO2, 5% O2, 90% N2. Embryo development was assessed by recording cleavage rate at 72 h post-insemination and blastocyst yield at Day 8 (Day 0 = day of IVF). Data were analysed with PROC GLIMMIX of SAS; treatment and semen pack were included in the model as fixed effects, while replicate was included as a random effect. Overall, treatment affected cleavage rate (HS: 80.7 v. HO: 77.0%; P=0.006), which was driven by differences observed in two of the four individual packs (P1: 84.0 v. 76.7%, P=0.001; P2: 77.3 v. 79.5%, P=0.39; P3: 80.3 v. 75.0%, P=0.0558; P4: 81.2 v. 77.1%, P=0.12). Similarly, blastocyst yield was affected by treatment (HS: 34.5 v. HO: 30.8%, P=0.0151), with a difference observed in one of the four packs when analysed individually (P1: 35.3 v. 25.5%, P = 0.0143; P2: 22.8 v. 22.5%, P = 0.9; P3: 39.5 v. 36.8%, P = 0.37; P4: 42.7 v. 40.5%, P = 0.48). In conclusion, use of HS for IVF resulted in improved cleavage and blastocyst rates compared to HO, albeit with variation between packs. In some situations, HS could compensate for bulls that exhibit reduced performance. Subsequent research will genotype blastocysts derived from HS to determine paternity bias.

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Keywords: mixed semen, bovine, embryo development

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Characterization of bovine sperm subpopulations using computer assisted sperm analysis

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Recent work by our group has shown that sperm from low fertility bulls used in artificial insemination have an impaired ability to hyperactivate. Other studies have shown that hyperactive sperm penetrate into the uterine glands, illicit an immune response and prepare the female reproductive tract for an impending pregnancy. Although this is not fully understood, it does demonstrate that sperm subpopulations could play a critical role in the establishment of pregnancy. This study aimed to characterize the sperm subpopulations present in bull semen. Semen straws from six bulls of proven field fertility were thawed and analyzed using Computer Assisted Sperm Analysis (CASA; IVOS II, Hamilton Thorne, USA), with two ejaculates per bull used and each analyzed in duplicate. Statistical analyses were performed using SPSS Statistics version 29.0.2.0 (IBM Corp., USA), where dendrograms were first generated to identify the optimal number of sperm clusters. Secondly, a K-means cluster analysis was performed using the straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and linearity (LIN) kinematics. The resulting clusters were then characterized based on their mean ± s.e.m values for these kinematics, and four different subpopulations were identified: Subpopulation 1 consisted of 19.5 ± 0.79% of the sperm population, with non-progressive yet highly active sperm that could be regarded as exhibiting a “hyperactivated-like” movement, characterized by elevated VCL and ALH, alongside low LIN (VSL = 56.5 ± 2.95µm/s; VCL = 140.7 ± 2.66µm/s; ALH = 7.9 ± 0.23µm; LIN = 41.02 ± 1.81%). Subpopulation 2 contained 34.8 ± 1.04% of the sperm and represented those which moved rapidly and progressively, as shown by their high VSL and LIN (VSL = 103.4 ± 5.61µm/s; VCL = 167,8 ± 11.97µm/s; ALH = 6.6 ± 0.45µm; LIN = 62.5 ± 1.97%). Subpopulation 3 consisted of 30.3 ± 1.77% of the sperm and displayed the fastest movement; however, the progressive motility was more limited, as indicated by higher VCL and ALH and lower LIN (VSL = 110.3 ± 7.97µm/s; VCL = 194.0 ± 9.46µm/s; ALH = 7.9 ± 0.29µm; LIN = 57.01 ± 2.81%). Subpopulation 4 represented 15.4 ± 1.67% of the sperm, which were the non-progressive and the least motile, with low values across all the kinematic parameters (VSL = 28.5 ± 1.66µm/s; VCL = 67.2 ± 3.27µm/s; ALH = 4.0 ± 0.21µm; LIN = 45.2 ± 2.16%). It is worth noting that these analyses were conducted solely with motile sperm, which accounted for 45.1 ± 0.81% of the total sperm across all bulls. There was no difference between bulls on the percentages of subpopulations 1, 2, and 3 ($P>0.05$). In contrast, there was an effect of bull on subpopulation 4, which was driven by one bull with a higher proportion of this subpopulation ($p < 0.01$). This study confirms the presence of 4 sperm subpopulations within the motile population of sperm. Further studies are required to understand the role of these sperm subpopulations, if any, within the female reproductive tract.

Keywords: bull, semen subpopulations, CASA

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Microfluidic chip-based sperm selection improves motility and functional quality of sex-sorted bull semen compared to swim-up and washing techniques

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The selection of high-quality spermatozoa is critical in assisted reproductive technologies (ART), particularly when working with sex-sorted semen, which is often associated with reduced viability. This study aimed to compare three sperm selection techniques—Swim-Up, Sperm Washing, and a Microfluidic Chip (Yeni Biotechnology Inc, Izmir, Turkey)—in terms of their ability to enhance sperm motility and function in sex-sorted bull semen. All samples originated from a single ejaculate batch of one bull and were processed using three thawed sex-sorted straws (Anadolu Stockbreeding, Turkey). Each technique was repeated ten times (n=10/group). CASA evaluations were conducted using the SCA system (Microptics, Barcelona, Spain), while flow cytometric analyses were performed using the CytoFLEX (Beckman Coulter, Brea, CA, USA) to assess mitochondrial membrane potential (HMMP), membrane integrity (SYBR/PI), acrosome integrity (FITC/PI), capacitation status (CAP) and lipid peroxidation (MLPO). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test (P<0.05). The Chip group demonstrated superior performance, with a mean total motility of 83.1%, progressive motility of 73.9%, and velocity values of VCL: 101.7 µm/s, VSL: 70.6 µm/s, and VAP: 86.4 µm/s. These results were significantly higher than those of the Swim-Up group (69.4%, 46.7%, 74.9, 48.7, and 62.7 µm/s) and the Sperm Washing group (34.5%, 23.5%, 67.4, 44.5, and 53.5 µm/s, respectively). Flow cytometry further confirmed enhanced mitochondrial activity, better acrosomal and membrane integrity, lower oxidative stress, and reduced premature capacitation in the Chip-selected spermatozoa (P<0.05). Despite improved quality, sperm concentration was significantly reduced after chip-based selection compared to the other techniques (P<0.05). In conclusion, microfluidic chip-based sperm selection significantly improves the motility and functional integrity of sexed bull sperm compared to Swim-Up and Sperm Washing methods, offering a robust strategy for optimizing ART outcomes. Further analyses will evaluate the impact of these selection techniques on *in vitro* embryo production quality and will evaluate semen from more bulls.

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Keywords: Sperm Swim-Up, Sperm Washing, Sperm Microfluidic Chip

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Optimization of *in vitro* Fertilization Outcomes with Bovine X-Sorted Semen

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The increasing economic pressure in dairy farming has elevated the demand for female calves due to their superior utility in milk production. Assisted reproductive technologies, especially *in vitro* fertilization (IVF) combined with X-sorted semen, offer a pathway to selectively produce female offspring. However, X-sorted semen has reduced fertilization potential compared to unsorted semen, primarily due to the mechanical and chemical stresses introduced during the sorting and cryopreservation processes. These stresses result in lower spermatozoa motility, compromised membrane integrity, and premature capacitation, contributing to suboptimal blastocyst rates. This study aimed to optimize bovine IVF outcomes using commercially available X-sorted semen by refining processing protocols and co-incubation conditions to improve spermatozoa motility, fertilization rates, and embryo development. Bovine cumulus-oocyte complexes (COCs) were aspirated from ovaries of Holstein-Friesian cows collected at the abattoir, matured *in vitro*, and fertilized *in vitro* using commercially available X-sorted semen from a single Holstein-Friesian bull, processed through modified Percoll® (Merck Life Science S.r.l., Milano, Italy) gradients (G1: 90%-45%, 4 mL; G2: 90%-45%, 0.8 mL; and G3: 78.7%-67.5%, 0.8 mL). Spermatozoa concentration and motility were analyzed. Post-IVF embryo development was assessed after co-incubation of gametes for either 10 or 18 hours. Presumptive zygotes (n=33-35/group) were fixed for pronuclei (PN) assessment or cultured *in vitro* for 8 days to evaluate blastocyst formation and morphology. Data were analyzed using Fisher's exact test or T-test, with $P<0.05$ considered statistically significant. G2 was excluded due to poor performance, while G3 yielded significantly higher sperm motility ($P=0.0462$) and improved fertilization rates (27/31, 87% 2PN) compared to G1 (9/34, 26% 2PN; $P<0.0001$). Despite high PN formation with G3, blastocyst rates were low. However, shortening the gamete co-incubation time to 10 hours significantly enhanced blastocyst yield (G3: 56/225, 25% vs G1: 19/124, 15%). In summary, we observed that increasing gradient density and reducing the co-incubation time of gametes positively impacted X-sorted IVF, enhancing both sperm motility and embryo production rates. While studies indicate that different bulls may require specific conditions for optimal fertilization and embryo yield, small adjustments to semen processing, tailored to the characteristics of sorted sperm, can significantly improve IVP efficiency.

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Keywords: IVF, spermatozoa, blastocyst

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Flow cytometry determination of optimal EVs concentration and the time necessary for sperm interaction in ovine (Ovis orientalis aries)

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Extracellular vesicles (EVs) play a crucial role in fertilization, as nanoparticles composed of bioactive proteins, microRNAs, and other molecules involved in cellular communication within reproductive fluids. While EVs have been isolated and characterized in small ruminants, their specific role in sperm functionality remains to be elucidated. In other species, EVs derived from oviductal fluid have been shown to be internalized by sperm, facilitating guidance toward the fertilization site and promoting capacitation-associated signaling pathways. The aim of this study was to isolate EVs from estrous sheep serum (ESS)—a non-reproductive fluid commonly incorporated into *in vitro* fertilization capacitation media—as well as from follicular (FF), oviductal (OF), and uterine (UF) reproductive fluids, to evaluate their interaction with ram sperm over incubation periods of 15, 30, 60, and 120 min. Fluids were obtained from six ewes, pooled in groups of two, and processed for EV isolation using size exclusion chromatography. Sperm samples were collected from three rams via artificial vagina, pooled and adjusted to a final concentration of 10x106 sperm/mL and then incubated at 38°C with EVs at three concentrations (109, 108, and 107 EV/mL). EV concentration and size distribution were quantified by nanoparticle tracking analysis. To assess EV uptake, EVs were labeled with CFSE (5,6-carboxy-succinimidyl-fluorescein ester), and sperm percentage (%) incorporating EVs was determined via flow cytometry. Commercial exosomes expressing green fluorescent protein served as gating control. Data analysis employed one-way ANOVA, with Bonferroni post-hoc tests for multiple comparisons. Results indicated that at 109 EV/mL, CFSE expression % was significantly higher ($P<0.05$) than 108 and 107 EV/mL concentrations (27.46 ± 1.50 vs 3.82 ± 1.50 and 0.09 ± 1.50 EV/mL respectively). Specifically, at 109 EV/mL there were differences in two fluids between times. In ESS, CFSE expression % was higher ($P<0.05$) than in control at 15 min (47.9 ± 9.02 vs 12.09 ± 9.02 EV/mL respectively) as well as at 30 min (35.73 ± 6.23 vs 10.83 ± 6.23 EV/mL respectively). In FF, CFSE expression % was higher ($P<0.05$) than in control at 30 min (47.92 ± 5.16 vs 10.83 ± 5.16 EV/mL respectively) as well as at 60 min (39.48 ± 3.93 vs 8.01 ± 3.93 EV/mL respectively). No differences were found for OF and UF ($P>0.05$) in any incubation times. Our findings confirm that EVs of ESS and FF bind to sperm after 30 min of incubation at the optimal concentration of 109 EV/mL, while for OF and UF the incubation time is not determinant for binding to occur. This allows to determine the optimal concentration necessary next to the time to achieve the EVs-sperm interaction in ovine to follow research into how EVs from female reproductive fluids influence sperm physiology, with potential improvements in reproductive protocols in ovine species.

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Keywords: extracellular vesicles, reproductive fluids, flow cytometry

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Pilot insights into the impact of freeze-thaw cycles on the integrity of ovine oviductal extracellular vesicles and their fusion potential with sperm

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In mammals, sperm capacitation occurs within the oviduct, where extracellular vesicles (EVs) are thought to mediate molecular exchanges priming gametes for fertilization. Although EVs are applied *in vitro* to mimic this environment (Franchi A et al., J.Cell.Biotechnol; 121(4):2877-2888, 2020), the impact of freeze-thaw cycles on their biological activity remains poorly understood. This study aimed to (i) assess whether repeated freeze-thawing compromises the integrity of oviductal EVs in sheep, and (ii) determine whether it affects their fusion ability with sperm. Oviductal EVs were isolated by size-exclusion chromatography from oviductal fluid collected from pre-ovulatory ewes at a local abattoir. Pooled oviductal EVs (n=2/rep) were subjected to either one freeze-thaw cycle (oEVs) or up to three cycles (oEVs-rethawed). Then, they were stained with CFSE (5 µM; 30 min; 37°C) that fluoresces only in the presence of active esterases, thus excluding non-functional EVs and labeling only those with enzymatic activity. Finally, they were co-incubated with fresh sperm (107 spz/mL; 10^8 EVs/mL) for 120 min. CFSE mean fluorescence intensity (MFI; in arbitrary units (AU)) and the percentage of CFSE-positive sperm were quantified by flow cytometry as a proxy for the transfer of EVs cargo. Controls-including unstained EVs and buffer-only samples confirmed selective CFSE binding and debris exclusion. MFI values for EVs and sperm were normalized to their respective CFSE-negative controls to omit non-specific staining. Comparisons between oEVs and oEVs-rethawed were assessed using a t-test, and staining dynamics over time were analyzed via one-way ANOVA with Bonferroni post hoc test (n=3). Our results showed that following staining, oEVs-rethawed displayed significantly lower CFSE labeling compared to oEVs (1.07 ± 0.09 vs 1.75 ± 0.83 AU; P=0.03), though this difference was no longer significant after 3h (1.03 ± 0.09 vs 2.01 ± 1.13 AU; P= 0.07). Functionally, oEVs induced a higher proportion of CFSE-positive sperm at 15 and 60 min than oEVs-rethawed (25.94 ± 14.70% vs 1.42 ± 0.96%, P=0.02; 25.72 ± 15.03% vs 4.04 ± 3.34%, P=0.04), with no significant differences at 30 or 120 min (19.05 ± 5.57% vs 3.03 ± 1.95%, P=0.23; 22.16 ± 11.54% vs 4.78 ± 3.94%, P=0.19). Notably, at 60min, sperm incubated with oEVs-rethawed exhibited greater CFSE intensity than those exposed to oEVs (0.96 ± 0.01 vs 0.89 ± 0.11 AU; P=0.04). This suggests that compromised yet persistent oEVs-rethawed may enter a state that favors the diffusion of their contents into target cells. Finally, no significant differences in any of the parameters evaluated were observed between the studied time points. Our findings align with prior evidence indicating that freeze-thaw cycles disrupt EVs via membrane destabilization (Gelibter S, Marostica M et al., JEV; 11(2):e12162, 2022), and reveal for the first time in sheep oviductal EVs that this compromise significantly alter their ability to mediate essential molecular exchanges with sperm. Further studies are needed to determine whether this phenomenon also applies to the physiological delivery of EVs cargo.

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Keywords: extracellular vesicles, freeze-thaw cycles, sperm

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Influence of protected choline supplementation to Creole sheep rams on reproductive and seminal characteristics

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In addition to other factors, nutrition plays an important role in influencing ram reproductive traits and sperm quality. The objective of this study was to evaluate the supplementation of protected choline in the diet of Creole rams to improve reproductive and semen characteristics. Eight Creole males of known fertility, approximately 1.5 years old, with an average weight of 42 kg and a body condition score of 3.5 (on a scale of 1 to 5), were used for this experiment. The basal diet was designed to consume 1.70 kg of DM, with 3.6 Mcal d-1 of metabolizable energy. Animals underwent a 12-d adaptation period and were subsequently assigned to one of two treatments. T1 (control) = basal diet without choline, and T2 = basal diet + 3 g of protected choline (ReaShure®) for 60 d. Animals were weighed weekly, and scrotal circumference was determined from the start of the experiment until 60 days later. Semen was collected from each animal twice weekly using an artificial vagina to assess reaction time and kinetic parameters (mCASA, Isperm). The experimental design was completely randomized. Statistical analysis was performed using MIXED of the SAS program, considering a repeated measures model with a $P<0.05$. Scrotal circumference was similar between T1 and T2 throughout the experiment, with no difference in the last measurement (32.10 ± 1.21 vs. 33.25 ± 1.21 cm). Reaction time was lower ($P<0.05$) in T2 vs. T1 (11.24 ± 1.34 vs. 16.19 ± 1.23 s) in the last week of measurement. However, no differences were found in sperm concentration or kinetic parameters such as total motility, progressive motility, and velocity of spermatozoa. Therefore, it is recommended to increase the number of repetitions per treatment. In conclusion, the consumption of protected choline did not improve the kinetic parameters of Creole ram semen, but it influenced the reaction time of males under the conditions of this study.

Keywords: Creole ram, choline, reproductive capacity

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Sperm viability in refrigerated semen samples from Creole rams

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Sperm viability depends on several animal-related factors, such as age, nutrition, health status, and breed. Furthermore, it has been observed that there are individual differences in fertility between rams of the same breed with similar physical and physiological characteristics. The present study aimed to evaluate sperm viability in refrigerated samples from eight Creole rams, to identify differences between them. Rams of known fertility, approximately 2.5 years old, with an average weight of 52 kg, a body condition score of 3.5 (on a scale of 1 to 5), and a scrotal circumference of 32-34 cm were used for this experiment. The animals consumed 1.80 kg of DM, from a basal diet with 3.6 Mcal d-1 of metabolizable energy. Semen samples were collected using an artificial vagina from eight Creole rams, identified with consecutive numbers (1-8), during five weeks (five replicas) within the reproductive season. At the time of semen collection, pure semen samples were placed in a water bath (37°C) and a diluent composed of 60% distilled water, 20% Triladyl (Triladyl®, Minitube, Germany), and 20% egg yolk was added, adjusting the sample concentration to 30-40 × 106 mL^{-1} of spermatozoa. Eight tubes with 2 mL of diluted semen were prepared, corresponding to each Creole ram. Samples from rams were refrigerated at 4°C for five days. For the determination of motility, a 10 µL sample of semen was placed on a coverslip slide to count the number of motile and progressively motile sperm at 40×. A total of 100 sperm were counted per measurement. Total motility (TM), progressive motility (PM), live sperm count (LSC) using eosin-nigrosin staining, and membrane integrity (MI), using the Hypoosmotic Swelling Test, were determined every 24 h for five days. This experiment was repeated five times (in five consecutive weeks). Rams was considered as a fixed effect in the statistical model, and data for each variable were analyzed using MIXED from SAS, considering a repeated measures model and the effect of experimental replication, with a $P<0.05$. Ram 7 showed consistency in lower ($P<0.05$) percentage of MI, during the five days of refrigeration of its seminal samples (67.7±1.75, 59.2±1.75, 49.9±1.75, 41.3±1.75, and 31.6±1.75) compared to ram 6 (75.5±1.75, 69.6±1.75, 61.1±1.75, 52.0±1.75, and 44.5±1.75). In MP, ram 7 was also lower than ram 6, but only on day 5 (25.6 vs. 36.2%). The other rams (1, 2, 3, 4, 5, and 8) showed no statistical differences in these variables. Likewise, no differences were seen between rams in the EV and TM variables. In conclusion, rams 6 and 7 showed significant differences in sperm viability of their semen samples refrigerated for five days, under the conditions of the present study.

Keywords: semen, creole, sperm viability

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Supplementation of INRA96 medium with penicillamine, hypotaurine, and epinephrine does not alter protein tyrosine phosphorylation of thawed stallion spermatozoa

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Conventional *in vitro* fertilisation (IVF) in horses requires a prolonged incubation (22 h at 38.5°C) in a capacitating medium (FERT-TALP) containing penicillamine, hypotaurine, and epinephrine (PHE), before co-incubation with oocytes for 3 h (Matheus R. Felix, Biol of Reprod 107(6):1551-64, 2022). However, this prolonged incubation is unfeasible when using cryopreserved spermatozoa due to its shortened lifespan. We previously reported that frozen-thawed stallion spermatozoa maintained motility and viability when incubated for 22 h with PHE in a commercial semen extender (INRA96) at 30°C (Luis-Calero M. Reprod Dom Anim 59(3):e14593, 2024). Therefore, we aimed to investigate protein tyrosine phosphorylation (PY) induction, a key marker of capacitation, in spermatozoa incubated in INRA96. Frozen-thawed spermatozoa from three stallions (seven replicates, n=7) were incubated for 22 h at 30°C, followed by an additional 3 h at 38.5°C under 5% CO2 in two different media (FERT-TALP or INRA96), with or without PHE, resulting in four experimental groups: FT-CTR, FT-PHE, INRA-CTR, and INRA-PHE. After incubation, spermatozoa were fixed, permeabilised, incubated with an anti-phosphotyrosine antibody (clone 4G10), and stained with a fluorescent secondary antibody (Alexa FluorTM Plus 488). Samples were mounted on slides using DAPI antifade and evaluated by fluorescence microscopy. At least 100 spermatozoa per slide were evaluated. The observed PY patterns were (1) equatorial region and midpiece; (2) equatorial region and tail; (3) apical, equatorial region and midpiece; and (4) apical, equatorial region and tail. These patterns were grouped into PYe (1 and 2) or PYa (3 and 4) according to the implication of the PY in the apical region, previously associated with fertilisation (Matheus R. Felix, Biol of Reprod 107(6):1551-64, 2022; Matheus R. Felix, Biol of Reprod 112(5): 867–879, 2025). The percentage of spermatozoa showing total PY (tPY), PYa, or PYe was compared between groups by one-way ANOVA after normality assessment using the Shapiro-Wilks test (statistical significance: $P<0.05$). The percentages of tPY were: 26.66 ± 3.20 vs 25.55 ± 3.63 vs 25.86 ± 4.10 vs 24.01 ± 4.07 for FT-CTR vs FT-PHE vs INRA-CTR vs INRA-PHE, respectively (% mean ± standard error of the mean). The percentages of PYa were: 21.63 ± 3.56 vs 23.56 ± 4.08 vs 23.62 ± 4.15 vs 20.81 ± 3.42, and for Pye were: 5.04 ± 1.93 vs 1.99 ± 1.17 vs 2.24 ± .67 vs 3.20 ± 1.21, respectively. No significant differences were found between groups for tPY, PYa or PYe ($P>0.05$). In conclusion, neither the incubation medium (FERT-TALP or INRA96) nor PHE supplementation affected the PY pattern of frozen-thawed stallion spermatozoa after 22 h at 30°C followed by 3 h at 38.5°C. Further investigation on the capacitation of frozen-thawed spermatozoa is necessary to establish commercial equine IVF protocols using cryopreserved sperm.

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Keywords: stallion frozen spermatozoa, protein tyrosine phosphorylation, *in vitro* fertilisation

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Impact of paternal age on the sex ratio of equine *in vitro* produced embryos obtained via ovum pick up and intracytoplasmic sperm injection

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The determination of sex in mammals is an intriguing question debated in different species. Paternal-related factors, including age, have been studied for their potential influence on offspring sex ratio (OSR) (Edwards et al. 2014, TREE, 29.3, 158-164). Studies in humans, horses, dogs, and laboratory animals have reported a decline in male births with increasing paternal age, supporting the hypothesis that male aging contributes to a decreased likelihood of male offspring (Stone et al. 2013, Fertil. Steril., 100.4, 952-958; Santos et al. 2015, Theriogenology, 84.7, 1238-1245; Martins et al. 2019, Theriogenology, 135, 169-173; Matsuo et al. 2009, J. Obstet. Gynaecol. Res., 35.1, 164-168). Santos et al. reported that older stallions tend to produce a higher proportion of female offspring within in vivo breeding program, so far, no studies have yet examined this aspect in equine *in vitro* ARTs. The *in vitro* model may offer a clearer perspective on the influence of paternal age by reducing the effects of the uterine environment. However, *in vitro* conditions may influence sperm characteristics, thereby introducing potential bias resulting from artificial influences. This pilot study aimed to investigate the effect of stallion age on the sex ratio of equine IVP embryos, generated through OPU-ICSI. Embryo production and sex determination followed the methods described by Lazzari et al. (2020, J. Equine Vet. Sci., 89, 103097). Prior to embryo freezing or ET, a spontaneously extruded trophectoderm cell biopsy was collected from the ICSI hole by gentle pipetting and stored at -20°C. A simultaneous multiplex PCR amplification was then performed, targeting the constitutive glycoprotein alpha-galactosyltransferase 1 gene and the equine sex-determining region of the Y chromosome. A retrospective analysis of the equine IVP embryo sex ratio (ESR) was conducted using 254 sex-determined embryos derived from 81 stallions of Warmblood, Arabian, and Quarter Horse breeds. Only frozen semen was employed, and stallion age at semen collection time was retrieved from sanitary documentation. Stallion ages were categorized into four groups: ≤10 (n=42 stallions), >10≤15 (n=55 stallions), >15≤20 (n=17 stallions) and >20 (n=7 stallions). The analysis of the stallion age-dependent ESR revealed the following results, reported as the number of females (F) on the number of males (M): [≤10= 0.62 (34 F/ 54 M)], [>10≤15= 0.89 (26 F/ 29 M)], [>15≤20= 0.65 (19 F/ 29 M)] and [>20= 1.17 (34 F/ 29 M)]. No significant differences between stallion age groups were observed (P<0.05) (Chi-square test, Social Science Statistics 2025). However, a trend was noted indicating a higher proportion of female embryos from the oldest stallion group (P=0.06). Further analyses with an expanded stallion cohort are needed. Additionally, monitoring the pregnancies of transferred sex-determined embryos in the future could help to identify any potential bias in pregnancy loss related to offspring sex and its impact on the ultimate OSR. Shedding light on factors influencing sex determination in horses may help to optimize breeding programs according to desired offspring gender.

Keywords: equine, OPU, ICSI, sex determination, stallion

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Co-incubation of boar sperm with seminal extracellular vesicles from high-fertility boars increases sperm parameters rather than those from low-fertility boars

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Boar sperm contains spermatozoa and a plethora of components in the seminal plasma (SP), acquired through transit in the male genital tract. Extracellular vesicles (EVs) and their cargo (proteins, small RNAs, etc.) have recently become an exponential field of research. However, artificial insemination (AI) doses produced by AI companies are highly diluted based solely on sperm concentration, with little consideration given to the role or functionality of remaining EVs and the fertility differences. The aim of this study was to evaluate the potential effects of EVs on semen quality of low fertility (LF) boars in a long-term incubation at 38°C with EVs derived from high- fertile (HF) and LF animals. For this study, 32 AI doses from boars with known fertility based on farrowing rates (>100 inseminations) were selected. 16 LF and 16 HF animals (4 pools LF/HF of 4 animals) were used. From each pool, 20 mL of a commercial AI dose (~600×10^6 spermatozoa) were centrifuged twice (1500×g, 5 min and 14,000×g, 10 min, 4 °C) to remove the sperm pellet, free membranes, and debris. SP was then concentrated using PES PierceTM 100K MWCO filters (ThermoFisher Scientific), EVs were isolated and purified by Size Exclusion Chromatography (SEC) columns (IZON), and finally re-concentrated to obtain 0.5 mL of purified EVs. Six treatments were performed: Control group of commercial extender (Duragen; Magapor) (CTL); 0.25 mL of isolated pure EVs (low-concentrated, lcEVs) and 0.5 mL (high-concentrated EVs, hcEVs) from HF (hcEVs_HF and lcEVs_HF) or LF (hcEVs_LF and lcEVs_LF) were co-incubated with sperm pellets (30×10^6 spermatozoa) for 0, 1, 3, 6, 12 and 24 hours at 38°C. Viability and acrosome integrity (Propidium iodide (PI)/PNA-FITC) and oxidative stress (YO-PRO-1/Dihydroethidium (DHE)) were analyzed. For statistical analysis, after normality and homoscedasticity confirmation, data were analyzed using two-way ANOVA, with incubation time and fertility group as fixed factors, including their interaction in the model, and Tukey's test for multiple comparisons (P<0.05; GraphPrism, USA). The results showed that after 1 h, the addition of EVs, regardless of their concentration or fertility, increased (P<0.05) the number of live sperm with intact acrosomes (PI-/PNA-), and the number of non-oxidized live sperm (YO-PRO-1-/DHE-). In contrast, after 3 h, hcEVs_HF displayed the highest quality in both parameters. However, by 6 hours, only lcEVs_HF provide an increased incubation protection. The positive effect of the EVs was reduced through time, potentially linked to a loss of EVs integrity or diminished interaction with sperm cells over time. In conclusion, EVs from HF induced protection. These findings underscore their potential as biomarkers and tools for advancing reproductive technologies and although the procedure can be complex, the implantation of the addition of seminal EVs should be considered.

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Keywords: sperm, porcine, extracellular vesicles, fertility

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Sperm thermoresistance test: an accurate tool to identify subfertile boars

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One of the major challenges in swine production is the presence of subfertile animals: 5–10% of boars with apparently normal sperm quality still show reduced fertility. Early detection of these subfertile boars could help minimize economic losses. Given the limited understanding of subfertility causes, the sector urgently needs rapid and reliable tools for early identification. In this context, a thermoresistance test may reveal sublethal sperm damage by mimicking the in vivo conditions under which sperm function. The aim of this study was to evaluate potential differences in semen quality between high- and low-fertility animals after 1-hour incubation at 38°C. For this study, 24 boars with a known fertility history—based on farrowing rates from at least 100 inseminations—were selected. Thus, 12 high fertility (HF) animals and 12 low-fertility (LF) animals were obtained. From each animal, 10 mL of a commercial artificial insemination (AI) dose (~300 × 10^6 spermatozoa) was centrifuged (1500×g, 5 min) to remove seminal plasma and eliminate its potential effects and resuspended in the same volume with defined Beltsville Thawing Solution (BTS) extender. Samples were incubated for 1 hour at 38°C and 5% CO2, and semen quality was assessed at two time points: immediately after centrifugation and after 60 minutes of incubation. Viability and acrosome integrity (Propidium iodide (PI)/PNA-FITC), mitochondrial activity (YO-PRO-1/Mitotracker Deep Red (MTDR)), and oxidative stress (YO-PRO-1/Dihydroethidium (DHE)); PI/CM-2DCFDA; YO-PRO-1/MitoSOX Red (MTsox, for mitochondrial superoxide) were analyzed. For statistical analysis, normality and homogeneity of variances were first confirmed. Then, data were analyzed using two-way ANOVA, with incubation time and fertility group as fixed factors, including their interaction in the model. Post hoccomparisons were performed using Tukey's multiple comparison test ($P< 0.05$; GraphPad Prism, USA). The results showed that the thermoresistance test increased the percentage of apoptotic and oxidized sperm (YO-PRO-1+/MTsox+) and active mitochondria and oxidized sperm (MTDR+/MTsox+) in LF relative to the HF. Interestingly, the percentage of live-non apoptotic and oxidized sperm YO-PRO-1-/DHE+ cells was affected by fertility, incubation time, and their interaction, showing significantly higher differences LF compared to HF animals. These results suggest that LF males present a higher proportion of non-apoptotic but oxidized spermatozoa, especially affected by superoxide anions (determined with MTsox and DHE). This could potentially indicate a reduced defensive response to reactive oxygen species in LF spermatozoa, and this could also be related to their lower fertility. In conclusion, a thermoresistance test could aid in the identification of animals with lower fertility by observing a greater proportion of non-apoptotic spermatozoa oxidized, particularly from superoxide anions.

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Keywords: porcine, thermoresistance test, fertility, sperm quality

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Detection and localization of Transient receptor potential channel 4 (TRPC4) in boar frozen-thawed spermatozoa

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Sperm cryopreservation, a relevant tool for the long-term preservation of genetic material, negatively affects spermatozoa, decreasing membrane integrity and motility, causing acrosomal damage, and increasing calcium intake mediated by sperm channels. Despite the well-studied CatSper channel, little is known about the role of transient receptor potential channels (TRPC). Among the TRPC, TRPC4 is a possible regulator of sperm motility, with caffeine and pH as key modulators. This study aimed to detect TRPC4 in post-thawed boar spermatozoa and whether its localization could be modified by changes triggered by 1) TRPC4-specific blocker (TRPC4-block), 2) caffeine, or 3) alkaline pH 8.2. Commercial boar artificial (AI) insemination doses (10 mL, ~300 ×106 spermatozoa; n= 12) were centrifuged (1500×g, 10 min) and cooled from 15°C to 5°C for 2 h diluted with LEY (1:1; 80% 310 mM lactose, 20% egg yolk (pH 6.15, 330±5 mOsm/kg)) and in LEYGO (1:1; 89.5% LEY, 1.5% Equex STM, and 9% glycerol (pH 6.15, 1700±5 mOsm/kg)). Then, 0.5 mL straws were filled and sealed for freezing 4.5 cm above liquid nitrogen (LN2) vapors for 20 min and stored in LN2. Thawing was conducted in a water bath at 37°C for 20 s, and extended in Beltsville Thawing Solution (1:1, pH 7.2, 328±5 mOsm/kg) at 37°C. After thawing, the samples were incubated for 2 h at 37°C in the presence of different concentrations of the TRPC4-block (0, 2.75, 5.5, 11 and 22 µM; ab269883, abcam) (Experiment 1); a combination of the TRPC4-block and caffeine (2 mM) (Experiment 2); and a combination of the TRPC4-block and alkaline pH (Experiment 3). For TRPC4 immunolocalization by epifluorescence, boar spermatozoa were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 15 min, and incubated for 1 h in blocking solution (PBS-1% BSA), and overnight at 4°C with the primary TRPC4-antibody (1:100; ab153810, abcam). After extensive washing, samples were incubated for 1 h at 37°C with a secondary antibody Alexa-488 (1:500; A32731, Invitrogen), and for 15 min at 4°C with the acrosomal integrity marker (PNA- Alexa 568) and the DNA marker (Hoechst 33342). Statistical analysis was performed after normal distribution and homoscedasticity assessment, using ANOVA and Tukey's test for multiple comparisons ($P<0.05$, GraphPad, USA). We detected the presence of TRPC4 in boar spermatozoa over the postacrosomal region and the intermediate piece. In contrast, in other studies, TRPC4 protein has not been detected in mouse spermatozoa, but a weak TRPC4 signal in the head and a strong signal in the midpiece and tail in human spermatozoa. In addition, the number of boar spermatozoa with TRPC4 was significantly higher than that of TRPC4-negative sperm ($P<0.05$). Finally, neither caffeine nor alkaline pH significantly affected the staining patterns. In conclusion, the presence and specific localization of TRPC4 on boar spermatozoa pave for further studies to characterize the potential function of this channel in boar sperm physiology.

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Keywords: sperm, porcine, TRPC, sperm channels, calcium

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Suitability of an environmentally-friendly grape marc extract as an extender supplement for boar semen refrigerated storage

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Antimicrobial resistance (AMR) is a major global challenge, largely driven by the excessive use of antibiotics, which are used on extenders for artificial insemination (AI). The pig industry uses refrigerated storage for several days, requiring antibiotics to prevent microbial growth; large-volume semen doses combined with a high post-AI backflow of the inseminated dose from the sow results in a notable release of antibiotic-containing extender to the environment. Natural plant extracts, with antimicrobial properties, are a promising approach for substituting antibiotics in animal production. In this study, we tested a polyphenol-rich grape marc extract with antimicrobial and antioxidant properties (K, proprietary composition), suitable for solid formulation of pig semen extenders. Contrasting to previous studies, this extract has as added value, being obtained through an environmentally-friendly and sustainable method (LIDSA, USC), revalorising agricultural waste (grape marc as source). Semen was collected from 8 boars, 2 ejaculates each (n=16), and extended with antibiotic-free Vitasem (Magapor) with no extract (Control, CTL) or including two levels of extract, K1 (0.005%) and K2 (0.02%). Semen doses were stored up to 7 days at 17°C and assessed by CASA (OpenCASA v. 2) and flow cytometry (BD Accury) at days 0, 3 and 7. Data were analyzed by linear mixed-effects models (results as %, mean±SEM). The K1 formulation yielded results similar to CTL after 3 days (P>0.05) for total motility (MOT, 81.9±1.6 vs. 83.2±1.8), progressivity (PROG, 51.0±2.1 vs. 50.8±1.7), acrosomal status (ACR, 18.4±1.0 vs. 15.8±1.5), mitochondrial status (MIT, 78.5±1.1 vs. 82.4±1.7), and viability (VIAB, 85.8±1.0 vs. 89.7±1.9). K1 performance at day 7 was slightly lower (P<0.05) for MOT (71.0±2.4 vs. 83.2±1.8) and PROG (44.4±2.5 vs. 52.8±2.2), being similar for ACR (19.5±1.2 vs. 18.0±2.1), MIT (77.0±1.1 vs. 82.2±1.4), and VIAB (83.9±0.8 vs. 88.0±1.9). K2 yielded significantly lower quality at days 3 (MOT 67.6±2.3, PROG 44.7±2.1, ACR 21.4±1.3, MIT 74.3±1.5, VIAB 81.4±1.6%) and 7 (MOT 59.1±3.0, PROG 36.2±2.6%, ACR, 24.2±1.9, MIT, 70.7±1.3, VIAB, 78.4±1.4). Some polyphenols could inhibit pig spermatozoa motility and ultimately affecting their physiology, which could be the case for K2. Indeed, our studies have showed a higher sensitivity to polyphenols comparing to bull spermatozoa. In conclusion, K1 could be suitable for storing boar semen within the typical timeframe in which sows are inseminated; however, adjustments are needed for improved quality in longer periods. Since these extracts revalorise agricultural waste and the extraction has a minimal environmental impact, they offer an opportunity for producing more sustainable semen extenders. However, it is necessary to carry out fertility trials and testing the antimicrobial potential of the new formulations.

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Keywords: boar, natural extracts, antibiotics

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Effect of oviductal fluid protein supplementation from different origins on bovine embryo development and quality *in vitro*

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The addition of low concentrations of oviductal fluid (OF) to serum-free culture media has been shown to enhance embryo development and quality. However, the composition of OF varies not only throughout the oestrous cycle but also under stress conditions such as high ambient temperature or high milk production. The aim of this study was to evaluate the effect of OF protein supplementation from different animal groups during *in vitro* culture on bovine embryo development and quality. In total 24 animals were assigned into six groups and synchronised. Oviductal fluid was collected on Day 1 of the oestrous cycle from Simmental heifers, low-yielding cows, and high-yielding cows during winter (Temperature-Humidity Index: THI <68) or summer (THI ≥72) seasons using transvaginal endoscopy (Papp SM, Theriogenology 132, 53-61, 2019). Samples of OF from four animals per group were pooled and concentrated using Amicon Ultra-2 centrifugal units. Protein concentrations were determined and adjusted to 5 µg/µl. Cumulus-oocyte complexes were recovered from slaughterhouse ovaries. After *in vitro* maturation and fertilization, presumptive zygotes (20 replicates; n=4903) were randomly assigned to one of seven groups: heifers winter (HEW), heifers summer (HES), low-yielding cows winter (LYW), low-yielding cows summer (LYS), high-yielding cows winter (HYW), high-yielding cows summer (HYS), and a control group (CON). The control group was cultured in synthetic oviductal fluid (SOF) supplemented with ITS (5 µg/mL insulin + 5 µg/mL transferrin + 5 ng/mL selenium) and 1 mg/ml PVP, while the culture medium of experimental groups was additionally supplemented with OF proteins at a final concentration of 0.5 mg/ml. Groups of 30–40 presumptive zygotes were cultured in 100 µl drops. Cleavage was assessed on Day 2, and blastocyst rates were recorded on Days 7 and 8. Blastocysts obtained on Day 7 were either cryopreserved by slow freezing or snap-frozen for gene expression analysis. Cryopreserved embryos were thawed and individually cultured for 72 hours and the re-expansion and hatching rates were recorded. Gene expression of PPP2R5B, LUM, HSPA1A, CPT1A, COX5A, and DNMT3B was evaluated by RT-qPCR (housekeeping gene GAPDH) and analysed with ANOVA. Embryo developmental data were analysed with a generalized linear model ANOVA and P-value of 0.05 for significance. Supplementation with OF proteins significantly enhanced blastocyst development compared to the control group (Day 7 24.8-31.5% vs. 14.4%; Day 8 42.4-49.1% vs. 30.9%), without affecting the cleavage rate. Moreover, the presence of OF proteins accelerated blastocyst formation by Day 7 (58.5-70.3% vs. 46.6%). Post-thaw re-expansion rates did not differ significantly between groups; however, the hatching rate was significantly higher in the control group compared to OF-treated groups (62.5% vs. 25-34%), but only marginally so for HYW (41.5%). Gene expression analysis revealed significant differences only in LUM expression between the control group (i) and high-yielding cows (ii) or heifers and low-yielding cows (iii). In conclusion, OF protein supplementation improved embryo development but reduced blastocyst cryotolerance. The influence of ambient temperature and milk production on OF revealed minor effects on embryo production *in vitro*.

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Keywords: oviduct, embryo, cattle

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Oviduct epithelial cells enhance bovine embryo quality through oviduct-specific mechanisms

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Co-culture with oviduct epithelial spheroids (OES) was recently reported to enhance cattle embryo development and quality under high (20%) oxygen conditions (Pranomphon et al., Theriogenology, 217, 113–126, 2024). This supportive effect of OES may be due to their oxygen consumption, the microflows generated by their ciliary beating, as well as secretions from oviduct epithelial cells. Common features of oviductal and bronchial epithelial cells are oxygen consumption and the presence of ciliated cells. To assess oviduct cell specificity, the effect of co-culture with oviduct and bronchial epithelial spheroids (BES) on embryo development and quality were compared. Isthmic and bronchial mucosa fragments from oviducts and lungs were collected from adult cyclic cows at the slaughterhouse and cultured for 4 d, as previously described for OES (Pranomphon et al., 2024). OES and BES were then selected based on size, morphology (100–200 µm in diameter, vesicular shape) and outward ciliary beating. In parallel, oocytes from slaughterhouse ovaries were *in vitro* matured and *in vitro* fertilized (day 0). On day 1, presumptive zygotes were cultured for 7 d in 25 µL synthetic oviductal fluid medium with 5% fetal calf serum alone (CONT) or with 25 OES (OES group) or 25 BES (BES group) at 38.5°C in a humidified 20% CO2 atmosphere. Cleavage, blastocyst and hatching rates were recorded on days 2 and 7–8, respectively (7 replicates). On day 8, expanded and hatched blastocysts were fixed for evaluation of total cell numbers and inner cell mass (ICM) % after SOX2 immunodetection and Hoechst staining (4 replicates; n=20–28 blastocysts per condition). Data (presented as mean ± s.e.m.) were compared among groups using ANOVA and post-hoc t-tests ($P \leq 0.05$). The cleavage rates did not differ among groups (77.5 ± 2.4–80.9 ± 3.2%, n=275–576 zygotes per condition). On days 7 and 8, the blastocyst rates were higher in OES and BES groups compared to CONT, with no difference regarding spheroid origin (27.2 ± 2.4 and 33.0 ± 2.7 vs 17.2 ± 2.3% on day 7, respectively; 33.3 ± 2.3 and 39.3 ± 3.6 vs 22.6 ± 1.4% on day 8, respectively; $P<0.01$). The hatching rate on day 8 did not differ among groups (29.5 ± 5.3, 25.0 ± 6.2 and 18.7 ± 6.4% in OES, BES and CONT, respectively). The number of cells per blastocyst was enhanced only with OES compared to CONT (159.0 ± 9.0, 130.7 ± 10.3 and 109.1 ± 8.0 cells in OES, BES and CONT groups, respectively; $P<0.01$), with no difference among groups regarding the ICM % (20.8 ± 2.0, 19.4 ± 2.1 and 24.9 ± 2.7%, respectively). In conclusion, co-culture with spheroids improved bovine blastocyst development under 20% oxygen regardless of the origin of epithelial cells, probably due to oxygen decrease in the culture medium and microflows generated around embryos by spheroid ciliary beating. Furthermore, OES displayed an oviduct-specific positive effect on blastocyst quality, likely due to specific OES secretions. Further studies will explore the oviduct-specific mechanisms that enhance embryo quality, the effect of OES and BES on embryo metabolism and gene expression, and the OES/BES-embryo dialog.

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Keywords: oviduct, embryo, spheroid

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY and DEVELOPMENTAL BIOLOGY

Equine mesenchymal stromal/stem cell secretome improve fertilization rates of bovine oocytes

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This study aimed to evaluate the effect of a conditioned medium derived from equine Wharton's jelly mesenchymal stromal/stem cells (eWJ-MSC-CM), intended for potential in vivo applications, on the cytoplasmic maturation of bovine oocyte, as assessed through early IVF events. eWJ tissue was collected from the umbilical cords of 3 healthy mares at parturition. eWJ-MSCs were isolated, fully characterized, and cryopreserved. For CM production, the samples were thawed, plated, expanded in DMEM-F12 until 90% confluence, then washed and cultured in Ringer's lactate solution for either 6 h (CM6) or 24 h (CM24). A pooled sample of CM was prepared for each time point. Cell viability after conditioning was evaluated. Bovine cumulus-oocyte complexes (COCs) were collected from 2-8 mm follicles of abattoir-derived ovaries. Grade I compact COCs were selected and randomly assigned to one of four IVM groups: i) control (CTR) (n=142): basal IVM medium (TCM199-based); ii) FBS (n=139): CTR medium + 10% FBS; iii) CM6 (n=141): CTR medium + 10% CM6; iv) CM24 (n=142): CTR medium + 10% CM24. After 22 h, COCs were fertilized with 1.5x106/ml frozen/thawed motile spermatozoa. Subsequently, oocytes were denuded, stained with 10 µg/mL bisbenzimide (Hoechst 33342), and examined under an epifluorescence microscope. The following IVF parameters were evaluated: i) penetration rate (fertilized oocytes/total inseminated); ii) monospermy rate (oocytes with a single sperm head or male pronucleus/total fertilized); iii) fertilization efficiency (monospermic oocytes/total inseminated); iv) pronucleus (PN) formation (oocytes with a male pronucleus/total inseminated). The experiment was conducted in 3 replicates. Data are presented as mean percentages ± SEM and were analysed using a binomial generalized linear model (GLM) with logit link and Wald pairwise tests (IBM SPSS Statistics 29), with significance set at $P<0.05$. In all samples, eWJ-MSC were non-viable after conditioning for both 6 and 24 h. Significant differences ($P<0.05$) were found for degeneration, penetration, PN formation and fertilization efficiency, but not monospermy. CM6 (6.3±0.4%) showed a lower ($P<0.05$) degeneration rate compared to FBS (14.1±1.6%) and CM24 (20.7±1.1%), but not CTR (7.9±0.6%), which was lower than CM24 but not FBS. Penetration rate and fertilization efficiency were lower ($P<0.05$) in CM24 (58.5±1.6% and 49.1±2.2%, respectively) compared to CTR (82.4±1.0% and 72.6±3.0%), FBS (78.5±1.2% and 66.4±1.8%), and CM6 (85.6±2.1% and 74.4±1.7). PN formation was significantly higher ($P<0.05$) in CTR (57.5±3.1%) and CM6 (60.9±1.8) compared to FBS (46.8±1.6%) and CM24 (35.8±3.4%), which tended to be higher than FBS ($P=0.063$). In conclusion, supplementation of IVM with CM6 did not alter fertilization rates of bovine oocytes, while CM24 exerted a negative effect. FBS also appeared to slightly impair early fertilization events. Further investigation is warranted to fully characterize the composition of eWJ-MSC-CM and to assess subsequent developmental competence to the blastocyst stage.

Keywords: oocyte, *in vitro* maturation, cattle

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Effects of uterine-derived small extracellular vesicles and their miRNA cargo on bovine embryo development and quality in an individual culture system

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In vitro production (IVP) of bovine embryos still struggles to match the quality of in vivo-derived embryos. Uterine small extracellular vesicles (U-sEVs) show potential for improving IVP outcomes but remain underexplored in individual culture systems. Although individual culture lacks the cooperative benefits of group culture, it offers greater experimental precision and reproducibility, making it ideal for studying the effects of U-sEVs. We assessed the impact of U-sEV supplementation on bovine embryo development and quality in individual cultures and analysed the U-sEV miRNA cargo. Histotroph samples were collected 4.5 days post-ovulation from the uterine horn ipsilateral to the corpus luteum in three nulliparous heifers. Extracellular vesicles were isolated via Optiprep™ density gradient ultracentrifugation, characterized, and pooled to balance donor contribution. *In vitro* maturation, fertilization, and culture in serum-free medium were performed individually in 20 µL droplets. On day 4.5, U-sEVs diluted in 5 µL equilibrated culture medium were added to the droplets at 6.5, 12.5, and 25 µg protein/mL. Positive controls received 5 µL of medium without EVs, while negative controls received no medium or EVs. Zygotes were imaged immediately after supplementation and classified by developmental stage: slow (1–8 blastomeres), intermediate (9–16 blastomeres), or fast (>16 blastomeres). Blastocyst development was assessed on day 8, and fixed for differential-apoptotic staining. Additionally, total RNA was extracted from U-sEVs of individual heifers, and miRNA sequencing was performed using the DNBSEQ platform. Blastocyst rates were fitted in logistic regression and quality parameters in linear regression models in RStudio. In 10 replicates (n=915 presumptive zygotes), no differences in blastocyst development (P>0.86) were observed within U-sEV (n=589) or control groups (n=326), so data were pooled for further analysis. The blastocyst rate was higher (P<0.001) in U-sEV-supplemented groups (slow: 6.4 ± 1.1%, intermediate: 76.1 ± 4.0%, fast: 93.7 ± 1.7%) compared to controls (slow: 1.4 ± 0.4%, intermediate: 40.3 ± 5.3%, fast: 75.1 ± 4.1%). No differences in embryo quality parameters were found among groups (P>0.32). Sequencing revealed a total of 302 miRNAs, of which 12 accounted for more than 2% of total miRNA reads. Among these 12 miRNAs, seven were commonly expressed across the three heifers, including bta-let-7a-5p and bta-miR-21-5p. Precise timing of U-sEV supplementation, mimicking physiological conditions, improved the blastocyst rate in individually cultured zygotes without affecting blastocyst quality. bta-let-7a-5p and bta-miR-21-5p, known miRNAs associated with oocyte maturation and embryo implantation in mouse models, were highly expressed in U-sEVs isolated from nulliparous heifers. These findings contribute to advancing strategies aimed at bridging the *in vitro*–in vivo gap in embryo culture, with potential applications in livestock breeding and human reproductive technologies.

Keywords: cattle, blastocyst, EVs

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Extracellular vesicles from oxidatively stressed granulosa cells improve embryo development and redox balance after vitrification of *in vitro* matured bovine oocytes

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Oxidative stress (OS) during oocyte vitrification contributes to cellular damage. Recent evidence suggest that extracellular vesicles (EVs) released by stressed granulosa cells (GCs) can enhance the response of recipient cells to stress. Herein, this study aimed to characterize the miRNA cargo of EVs derived from oxidatively stressed GCs and their effects on embryo development following vitrification/warming (VIT) of *in vitro* matured (IVM) bovine oocytes. Furthermore, redox and energetic status of the resulting blastocysts was assessed by mitochondrial activity, reactive oxygen species (ROS) and glutathione (GSH) levels. Bovine GCs from 3–6mm follicles were cultured and exposed to 5μM H_2O_2 for 40 min to induce OS. Untreated GCs served as controls. EVs were isolated by size exclusion chromatography and their miRNA cargo analyzed. A total of 1337 immature cumulus-oocyte complexes (COCs) were divided into 3 groups: CT (Control), CT-EV (non-stressed GC-EVs), and OS-EV (oxidative-stressed GC-EVs) at 1x10^{9}particles/mL (6 replicates) underwent 24 h of IVM. Half of the COCs were vitrified/warmed at 22h. Subsequently, oocytes were *in vitro* fertilization and *in vitro* culture. Embryo development and blastocyst fluorescence intensity (arbitrary units) for mitochondrial activity (MitoTracker Red), ROS (CellRox Green), and GSH (CellTracker Blue) were analyzed using confocal microscopy. MiRNAseq data were analyzed with MirDeep2 and R/Bioconductor. Blastocysts data were expressed as mean±SEM and analyzed by one-way ANOVA with Bonferroni post-hoc test ($P \leq 0.05$). A total of 10 miRNAs, including miR-134, miR-2285av, and miR-2411-5p, were upregulated in OS-EVs (LFC≥2, FDR<0.05), targeting signaling pathways including MAPK, PI3K-Akt, RAS, and Hippo, and processes related to insulin signaling, cell cycle regulation, migration, apoptosis, and cell growth. Regarding embryo development, VIT significantly reduced cleavage and D7 and D8 blastocyst rates in the CT VIT (69.7±5.5, 8.2±1.1 and 13.6±1.3, respectively) group compared to CT (89.2±3.1, 20.9±1.4 and 28.8±1.5, respectively). However, OS-EV supplementation mitigated this effect, with OS-EV VIT embryos showing comparable development (D7: 14.7±4.2; D8: 25.7±5.89) to CT at both D7 and D8. Redox and energy assessments revealed reduced mitochondrial activity in OS-EV VIT (1.9±0.9) embryos, along with significantly lower ROS levels (1.6±0.5) compared to the CT (3.3±2.8 and 3.2±1.9, respectively), along with increased GSH content in OS-EV (4.71±3.24) than in CT group embryos (3.02±3.16). No significant differences were observed in the other groups. These findings suggest, for the first time, that EVs from oxidative-stressed GCs enhance oocyte resilience to vitrification-induced OS, improving embryo development and modulating redox and energetic homeostasis of resulting blastocysts.

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Keywords: microRNAs, ROS, cryopreservation

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Extracellular vesicles in bovine oocyte maturation: a comparative study of group vs. individual IVM systems

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Objectives: Extracellular vesicles (EVs) have been identified as pivotal embryotropins within group embryo culture systems during *in vitro* development (Pavani, Int J Mol Sci. 2018). However, to date, compelling evidence is still absent regarding the presence or functional role of EVs secreted by cumulus-oocyte complexes (COCs) cultured in groups or individual during *in vitro* maturation (IVM). The aim of this study is to isolate, identify and characterise EVs released in a group and individual IVM system.

Materials and Methods: For the group IVM system, three replicates were performed with 60 COCs each (total n=180), cultured in 500 µL of IVM medium in 4-well Nunc™ IVF Multi Dishes (Thermo Fisher Scientific, USA), (Wydooghe et al., Reproduction, Fertility and Development 2014). For the individual IVM system, ten replicates were carried out, each involving 18 COCs (total n=180). Each COC was placed in a 20 µL droplet of IVM medium under paraffin oil, within a 60 mm × 15 mm Petri dish (Thermo Fisher Scientific, USA) (Azari-Dolatabad Animal Reproduction Science, 2023). The IVM medium was modified bicarbonate-buffered TCM-199 supplemented with 50 µg/mL gentamicin and 20 ng/mL EGF. Bovine COCs were matured at 38.5°C in 5% CO_2 for 22 hours. After 22 h, COCs were removed and media collected: 1.4 mL from the group system, and 3.4 mL from the individual system by droplet aspiration. The total conditioned medium from each system was used for EV isolation, with 500 µL aliquots processed per replicate using size-exclusion chromatography (SEC) and 70 nm Smart Columns (IZON™, ICS-70). Of the EV-rich fraction from each system, 150 µL were used for characterization. The presence of EVs was assessed by flow cytometry with the lipophilic dye Vesy-dye LMB-490 (Vesiculab), Nanoparticle Tracking Analysis (NTA) to determine size and concentration, transmission electron microscopy (TEM) for morphological evaluation, and Western blotting (WB) for the detection of EV markers CD9, CD63, and TSG101.

Results: Flow cytometry detected more positively labelled events (428,929 vs. 91,159) and a higher acquisition rate (3105 vs. 1198 events/sec) in group-derived EVs. The analysis with NTA showed that EVs from the group system had a larger mean diameter (198.8 ± 15.3 nm) and higher concentration (1.48 ± 0.21 × 10^9 particles/mL) compared to EVs from the individual system (169.7 ± 11 nm; 1.32 ± 0.06 × 10^9 particles/mL). Consistently, TEM analysis revealed the characteristic cup-shaped morphology of EVs, and ImageJ (v1.54k) measurements confirmed a larger diameter in group-derived EVs (201 ± 6.98 nm) compared to those from the individual system (61.67 ± 25.72 nm). Western blotting revealed the presence of EV-specific markers CD9, CD63, and TSG101 in both groups. Data are shown as descriptive values from a single experimental replicate.

Conclusions: The observed differences in EV size and concentration are noteworthy but based on limited replicates and should be interpreted with caution. Still, this study confirms that bovine COCs release EVs under both group and individual IVM conditions, with group-derived EVs showing greater abundance and size. Further studies with more replicates are needed to validate these findings.

Keywords: Extracellular vesicle, cumulus oocyte complex, bovine, Size exclusion chromatography, group culture, individual culture

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Do oviduct epithelial spheroids sense embryonic development? A transcriptomic study in cattle

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In cattle, there is evidence *in vitro* that the early embryo-oviduct dialogue is modulated by embryo quality (Dissanayake, J. Mol. Med, 99(5):685-697, 2021; Hamdi, BMC Genomics, 25(1):520, 2024). We previously showed that well-differentiated oviduct epithelial spheroids (OES) support embryo development and improve their quality after only 4 days of co-culture (Pranomphon, Biol Res, 57(1):73, 2024). Our aim here was to examine if OES can sense embryo developmental capacity by changing the OES morphology and gene expression. All cultures were performed at 38.5°C under 20% O2 and 5% CO2. Isthmic mucosa fragments from oviducts of adult cyclic cows collected at a slaughterhouse were cultured for 4 days in TCM199+10% fetal calf serum (FCS). OES were selected based on their size, spherical shape with a central cavity (vesicular shaped) and outward ciliary beating. In parallel, cumulus-oocyte complexes from slaughterhouse ovaries were *in vitro* matured for 22 h and either (i) fertilized (day 0) using 1.106/mL frozen-thawed Percoll-washed bull semen for 18 h (embryos with high developmental ability; 26% blastocysts on day 7 based on preliminary data) or (ii) activated by 7% ethanol for 5 min (parthenotes with low developmental ability; 3% blastocysts on day 7). On day 1, 25 OES were co-cultured (a) without (controls) or with (b) 25 embryos or (c) 25 parthenotes for 5 days in 25 µL droplets of synthetic oviduct fluid+5% FCS. The number of OES in suspension (with vigorous ciliary beating, the others adhering to the bottom of the well) and their morphology (vesicular shaped) were evaluated under a stereomicroscope. Vesicular-shaped OES were analyzed for gene expression by Illumina RNA-sequencing (5 replicates). The effect of the embryos on OES was analyzed by chi² and Kruskal-Wallis tests (7 replicates). Differentially expressed genes (DEG) were identified using EdgeR (FDR 5%) and functional enrichment analysis with Metascape. After 5 days of culture, the proportions of OES in suspension were higher with embryos (86.7%) and parthenotes (89.3%) than controls (80.8%; $P<0.05$). The proportion of vesicular-shaped OES was also greater with embryos (76.8%) and parthenotes (80.4%) compared to controls (56.1%; $P<0.05$). In vesicular-shaped OES, 1226 and 1668 DEG were identified after co-culture with embryos and parthenotes, respectively, than controls. Among those genes, 22% (265/1226) were specifically affected by embryos and 42% (707/1668) by parthenotes. Genes specifically affected by embryos were mostly involved in immune response and cell response to lipid, while those specifically affected by parthenotes were mostly involved in cytoskeleton organization and cytoplasmic translation. The comparison of OES incubated with embryos vs. parthenotes evidenced 14 upregulated DEG in OES in contact with embryos, including 11 previously identified as interferon-τ-induced genes. This work provides new insights into the physiological and transcriptomic response of the oviduct epithelium to embryo quality and points out potential markers for embryo developmental capacity.

Keywords: oviduct spheroid, embryo, transcriptome

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Pre-binding to isthmic epithelial spheroids enhances bull sperm ability to fertilize oocytes and obtain blastocysts *in vitro*

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Following mating, spermatozoa interact with the female reproductive tract primarily at the isthmus, the initial segment of the oviduct, where a sperm reservoir forms via sperm head binding to isthmic epithelial cells (IEC). Spermatozoa are then released toward ampulla where fertilization takes place. This study aimed to assess how pre-binding to IEC and exposure to their secretions affect bull sperm fertilizing capacity and subsequent embryo development. Isthmic mucosa fragments from healthy pre-ovulatory cyclic cows were cultured for 4 days. Epithelial spheroids with outward ciliary beating were selected according to their uniform shape and size (100-µm diameter, containing on average 25±2% ciliated IEC; Mahé C. et al., Scientific Reports, 13(1): 10311, 2023). Frozen-thawed Percoll-washed spermatozoa from a pool of 2 bulls were incubated with IES for 15 minutes to allow binding (sIES complexes). After washing, sIES were either analyzed for bound sperm density (≈5×10⁴/mL) or used for *in vitro* fertilization (IVF on day 0; 1 sIES per oocyte; bound group). Parallel IVF groups included: free sperm in IES-conditioned medium (conditioned group), and free sperm in standard IVF medium (control), all at the same sperm concentration. Presumptive zygotes were cultured for 8 days in mSOF at 38.5°C under 5% O2, 5% CO2. Fertilization outcomes were evaluated on day 1 by pronuclear staining (via Hoechst and H3K9me3 staining). Embryo development was assessed at day 2 (≥2 and ≥6 cell stages) and days 7–8 (blastocyst rate) and blastocyst quality on day 8 (total cell number and % in the inner cell mass via Hoechst and SOX2 staining). Groups were compared using ANOVA followed by Tukey's post-tests and t-tests (P≤0.05 ; n=5 replicates). In a 1st set of experiments using 22-hour IVF, the bound group showed significantly higher rates of ≥6-cell embryos (day 2) and blastocyst formation (day 7) compared to the conditioned group (46.2% vs. 18.2% and 20.0% vs. 11.2%, respectively, P-value<0.05; n=218-230 oocytes/group), suggesting accelerated early development. No significant differences in oocyte penetration (36-45%), polyspermy (0-17%) or blastocyst quality were observed. A 2nd set of experiments using shorten 8-hour IVF was undertaken with bound and control groups. The bound group had significantly higher cleaved embryos ≥ 2 and ≥ 6 cells on day 2 (22.3% vs. 37.6% and 49.7% vs. 67.7%, respectively; P-value<0.01) and blastocyst rates at days 7–8 versus control (10.2 vs. 22.7% and 10.5% vs. 24.2%, P-value<0.01; N=270-288 oocytes/group). No significant difference in blastocyst quality was observed. In conclusion, sperm pre-binding to IEC positively influenced fertilization efficiency and embryo development, possibly by selecting highly competent sperm for fertilization or promoting capacitation, or both. In contrast, exposure to IEC secretions impaired outcomes, highlighting the distinct biological effects of sperm physical binding versus soluble factors in the oviductal microenvironment.

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Keywords: oviduct, spermatozoa, embryo development

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Functional and structural profiling of region-specific bovine oviductal organoids

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Embryo–maternal communication is critical for establishing a successful pregnancy in cattle. Conventional *in vitro* models, such as two-dimensional (2D) cell cultures, do not accurately replicate the physiological environment of the oviduct. Nowadays, three-dimensional (3D) culture systems like organoids have been developed to better mimic the structural and functional properties of native tissues. The aim of this study was to develop and characterize bovine oviductal organoids derived from the infundibulum (IF), ampulla (AP), and isthmus (IT), and to compare them with native oviduct tissue. Epithelial cells from postovulatory bovine oviducts were first cultured in 2D and then embedded in Matrigel matrix (M: 50% or 80%; Corning, Tewksbury, MA), drops covered with organoid medium based on Lawson et al. (Biology of Reproduction, 108(6), 854–865, 2023). Transmission electron microscopy (TEM) was used to assess structural features, while ZO-1 immunostaining and binomial distribution analysis were employed to evaluate epithelial polarity (3 replicates), comparing organoids cultured in 50% (IT: n=9; AP: n=18; IF: n=14) or 80% M (IT: n=14; AP: n=38; IF: n=46) over a 21-day period. TEM analysis confirmed apical–basal polarity and revealed a transition from cuboidal (IT) to cylindrical (IF) morphology in organoids cultured in 80% M. Binomial analysis showed that epithelial polarity was predominantly oriented outward in organoids from the IT and AP regions at both M concentrations (89% and 86% for IT; 100% and 79% for AP, respectively; $P<0.05$). In contrast, IF-derived organoids did not exhibit a significant predominance of outward polarization under either condition. Organoids cultured in 80% M, those most closely resembling native tissue, were further analyzed for the presence of ciliated cells (tubulin antibody) and oviduct-specific glycoprotein (OVGP1 antibody). Organoids were cultured for 16 days (4 replicates; IT: n=35; AP: n=62; IF: n=51) or 21 days (2 replicates / analysis in progress; IT: n=22; AP: n=21; IF: n=36); diameter and OVGP1 expression were statistically evaluated (t-test, Mann–Whitney U). Both ciliated cells and OVGP1 were detected in native tissue and all organoid samples. In organoids, OVGP1 expression showed a weak correlation with organoid diameter. However, IF organoids cultured for 16 days exhibited significantly higher numbers of OVGP1-positive cells compared to those cultured for 21 days (8.7 ± 1.6 vs. 1.8 ± 0.7 cells/organoid; $P<0.001$). Additionally, the diameter of IF organoids cultured for 16 days (226.2 ± 11.6 µm) was significantly larger than that of AP (195.8 ± 9.7 µm) and IT (193.5 ± 10 µm) organoids ($P<0.05$). In conclusion, bovine oviductal organoids cultured in 80% Matrigel exhibit epithelial polarity and region-specific differences in morphology and marker expression. The distinct size and OVGP1 dynamics of IF organoids may provide a basis for studying regional oviduct function and embryo–maternal interactions.

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Keywords: bovine, oviduct, organoids

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Supplementation of IVM and IVC media with n6/n3 fatty acids affects lipid metabolism and quality of bovine blastocysts

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Essential PUFAs, such as omega-6 linoleic acid (LA) and omega-3 α-linolenic acid (ALA), affect oocyte and embryo quality. Their biological outcome is, however, determined by the n6/n3 ratio, with a lower ratio exerting a more favorable effect. Moreover, oocyte maturation (IVM) conditions influence the developmental potential of embryos, while *in vitro* culture (IVC) affects blastocyst quality. This study aimed to investigate how supplementation of IVM or IVC media with a blend of LA/ALA FAs affects the quality and lipid metabolism of bovine blastocysts.

Material: three groups of bovine expanded blastocysts (D 7-8) cultured with FAs during: (1) IVM only (IVM/FA), (2) IVC only (IVC/FA), and (3) control (no FA).

Methods: a set of commercial bovine media (Bioscience) with a moderate dose (100 μM) of FA blend (in DMSO; literature data), with an LA/ALA ratio of 3:1 (as in serum of experimental heifers); assays: 1) lipidomic profiling of single embryos by desorption electrospray ionization mass spectrometry (DESI-MS), and 2) lipid droplet (LD) and chromatin staining with BODIPY 493/503 + DAPI, followed by confocal microscopy; analysis on 3D images of embryos in a lateral position, with ICM visible and blastocoel excluded; data obtained: total cell number (TCN), number of ICM and TE cells, fluorescence intensity per blastomere (FI; ImageJ; arbitrary units); analysis of the entire embryo, ICM, and TE separately; statistical analysis (normality of data distribution - Kolmogorov-Smirnov test, comparing outcomes among groups - one-way ANOVA or Kruskal-Wallis tests).

Results: analysis included 132 embryos (27 DESI-MS; 105 BODIPY/DAPI). DESI-MS detected 14,588 ions per embryo. Analysis with MetaboAnalyst software (the threshold for fold change – 2.0) revealed that, compared to the IVC/FA group, the IVM/FA embryos exhibited elevated levels of 388 and reduced levels of 560 ions. As for cell counts, FA treatment significantly reduced the TCN (IVM/FA 150.7±62.8; IVC/FA 125.0±41.4) and the number of ICM cells (IVM/FA 47.8±24.3; IVC/FA 47.7±15.9) relative to the control (TCN 186.6±58.7; ICM 69.2±16.2). Additionally, the number of TE cells decreased in the IVM/FA group (90.6±37.3; control 127.1±37.5). When comparing the experimental groups, the blastomere numbers did not differ. Given the variation in blastomere numbers among embryos, FI was normalized per single blastomere. FA supplementation during IVC significantly increased FI per blastomere. Although the number of blastomeres did not differ between the IVM/FA and IVC/FA groups, a lower level of FI was detected per blastomere of IVM/FA blastocysts in the entire embryos and TE cells, with no difference for ICM.

Regardless of timing, FA treatment negatively affected blastocyst quality, as evidenced by reduced cell counts. Higher FI in IVC/FA embryos suggests enhanced lipid accumulation during IVC. An ongoing analysis of DESI-MS data aims to identify specific lipid species essential for embryo development and uncover lipid metabolic pathways influenced by LA/ALA treatment.

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Keywords: lipidomics, embryo

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Estimation of bovine blastocyst quality after early *in vitro* embryo culture with mono-unsaturated oleic acid or saturated stearic acid

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We previously demonstrated a dose-dependent negative effect of saturated stearic acid (SA) on maturing bovine cumulus–oocyte complexes (COCs) and oocyte developmental competence, while mono-unsaturated oleic acid (OA) was harmless. Accordingly, we next reported that early embryos exposed to 50 µM SA from day 1 to 5 of culture were hampered in their ability to develop into a blastocyst, while 25 µM OA resulted in significantly higher cryosurvival rates, comparable to in vivo blastocysts (Aardema et al., Front Cell Dev Biol. 10, 1-14, 2022). Therefore, we hypothesized that OA exposure improves the quality and potentially the capacity of the blastocyst to sustain a pregnancy after transfer. Testing this hypothesis through an in vivo trial is logistically challenging, as a large sample size is required to achieve statistical power. Conversely, the embryo competence index (ECI), which consists of a formula based on the expression of eight biomarker genes selected from large in vivo datasets, provides an objective estimate of embryo competence for survival (Rabaglino & Hansen, Biochem Biophys Rep. 39, 101772, 2024). The current study aimed to determine whether the presumed quality difference between OA and SA exposed embryos is reflected by the ECI measured in the blastocyst. COCs, collected from 2 to 8 mm follicles of bovine slaughterhouse ovaries, were *in vitro* matured for 23 hrs (n=50/group, 4 runs), and fertilized (day 0). Embryo culture conditions included (1) culture in synthetic-oviductal-fluid (SOF) without (control) or with 25 or 50 µM SA, and/or 25 or 50 µM OA, during days 1 to 5 of culture, followed by standard SOF from days 5 to 8. The blastocyst rate as a measure of developmental competence was scored on Day 8. The mRNA expressions of the eight biomarkers GSTO1, TPI1, and CHSY1 (upregulated in competent embryos), and CCNA2, CDK7, EIF4A3, LSM4, and YWHAG (downregulated in competent embryos) in the snap-frozen blastocysts (10/sample) were measured by RT-qPCR and used to calculate the ECI. ECI values between groups were compared based on LS-means differences, with p-values adjusted for multiple testing. Compared to the control group, exposure to 50 µM of SA resulted in a significantly reduced blastocyst rate (24.1% vs 8.7%; P=0.02) and did not meet the minimal number of blastocysts required for RT-qPCR. Low concentrations of 25 µM of SA as well as a combination of 25 µM SA and OA had no effect on the ECI of blastocysts. However, the ECI value of blastocysts cultured with 25 µM OA (2.43) tended to be higher than those cultured with 25 µM SA (-0.68, P=0.08) or a combination of 50 µM SA and 50 µM OA (-0.83, P=0.07), and was significantly higher than those cultured with a combination of 50 µM SA and 25 µM OA (-1.74, P=0.03), but did not differ from the control group (1.07, P=0.4). In conclusion, these results demonstrate the potential of the ECI to objectively evaluate the effect of different culture conditions on the potential survival capacity of *in vitro* produced bovine embryos.

Keywords: Blastocyst quality, Free fatty acids, Embryonic competence index (ECI)

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

The effect of catalpol supplementation during *in vitro* culture medium on bovine embryo development

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In vitro embryo production (IVEP) in cattle has become an increasingly prevalent biotechnological approach. Due to the numerous factors influencing its success, ongoing research aims to enhance both embryonic development and overall embryo quality. Catalpol has been proposed to modulate the cell cycle, thereby promoting cellular proliferation. This activity may support normal developmental processes by enhancing cell division and proliferation during the early stages of embryogenesis. This study aimed to evaluate the effects of catalpol, administered at varying concentrations in the culture medium, on embryonic development during *in vitro* embryo production in cattle. In this study, oocytes were collected from the ovaries of Holstein cattle over four sessions. The retrieved cumulus–oocyte complexes (COCs) were morphologically evaluated and subsequently transferred to a maturation medium. The COCs were incubated at 38.5°C in an atmosphere containing 5.5% CO_2 for 21–24 hours. Following *in vitro* maturation, the degree of cumulus expansion was assessed and classified into three grades: Grade I, Grade II, and Grade III. Following maturation, the oocytes were transferred to an *in vitro* fertilization (IVF) medium containing prepared sperm and incubated at 38.5°C in an atmosphere of 5.5% CO_2 for 22 hours. Prior to the *in vitro* culture stage, presumptive zygotes were randomly allocated into four experimental groups. The first group (Cat-5, n = 180) received 5 µmol/L catalpol, the second group (Cat-25, n=193) received 25 µmol/L catalpol, and the third group (Cat-50, n=173) received 50 µmol/L catalpol in the culture medium. The fourth group served as the control (n = 183) and received no catalpol supplementation. Embryos obtained following *in vitro* culture were evaluated based on the criteria established by the International Embryo Technology Society (IETS). The culture process was conducted in a commercial medium for 6–8 days. After assessing the developmental stages post-culture, only embryos that had reached the blastocyst stage were subjected to differential staining to determine total cell numbers. The TUNEL assay was employed to detect cell death and calculate the apoptotic index. Additionally, the levels of reactive oxygen species (ROS) and glutathione (GSH) within the embryos were measured using fluorescent staining techniques specific to ROS and GSH. In terms of statistical analysis, the Shapiro–Wilk test was used to assess the normality and homogeneity of variances. One-way ANOVA followed by Tukey's HSD post hoc test was applied to evaluate differences among groups. Relationships and proportions between categorical variables were analyzed using Chi-square and Fisher's exact tests. The blastocyst formation rates for the respective groups were 34.83%, 29.63%, 22.29%, and 27.17%. A statistically significant difference was observed among the groups ($P < 0.048$). Furthermore, it was found that the Cat-5 and Cat-25 groups exhibited lower ROS levels and apoptotic indices, along with higher GSH concentrations, indicating a dose-dependent effect. In conclusion, the addition of catalpol to the culture medium during *in vitro* embryo production in cattle was found to enhance blastocyst formation rates in the Cat-5 and Cat-25 groups. However, the blastocyst rate in the Cat-50 group was lower than that of both the control and the other treatment groups. These findings suggest that supplementation with catalpol at appropriate concentrations may improve embryonic development by increasing blastocyst yield, reducing intracellular ROS levels and apoptotic index, and elevating GSH levels in a dose-dependent manner.

Keywords: catalpol, bovine, *in vitro* culture, embryo

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

The effect of nonsteroidal anti-inflammatory drugs on embryo development and quality in bovine IVEP

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Prostaglandins (PG) are considered significant factors influencing embryo development and quality in *in vitro* embryo production. Elevated concentrations of PG during the early stages of *in vitro* culture (IVC) have been shown to negatively impact embryo development and quality, ultimately reducing the blastocyst formation rate. The objective of this study was to evaluate the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on embryo development and quality, aiming to mitigate the detrimental effects of PG during IVC of bovine oocytes. Commercial media (IVF Bioscience, United Kingdom) were used for all steps of IVEP system of oocytes aspirated from slaughterhouse ovaries. After IVM and IVF, presumptive zygotes were cultured during the first 3 days, in medium supplemented with NSAIDs: flunixin meglumine (FM, 5 ng/mL; n=265), meloxicam (Mel, 2 µg/mL; n=280), and carprofen (Car, 3 µg/mL; n=280) or non-supplemented with NSAIDs (control group, n=279). The media were changed on day 4 of the *in vitro* culture, after which all groups were incubated in the same medium. The embryos obtained were evaluated according to their developmental stage and quality on the 7th day of culture and subjected to differential staining (Hoechst+PI) and TUNEL assay for the assessment of their developmental competence, and quality, and apoptotic index, respectively. The relationships between categorical variables were analyzed using Fisher's exact test and the chi-square test, while the results of normally distributed variables were evaluated using one-way ANOVA post-hoc Tukey's HSD test. Following the supplementation of NSAIDs to the culture medium, the blastocyst formation rates were found to be higher in the FM (41.51%, 110/265), Car (34.09%, 90/264) and Mel (32.86%, 92/280) groups compared to the control group (21.50%, 60/279) ($P<0.05$) as well as the inner cell mass (46.46±4.17, 52.93±2.08, 47.93±3.78, and 42.56±1.82, respectively), trophectoderm (131.23±4.07, 134.23±3.57, 131.56±3.86, and 128.36±2.64, respectively), and total cell counts (177.70±7.9, 187.16±5.09, 179.5±7.11, and 170.92±3.39, respectively). In addition, the apoptotic index was found to be lower in the FM, Car and Mel groups compared to the control group (5.65, 3.63, 5.16, and 7.08%, respectively; $P<0.05$). The supplementation of flunixin meglumine, carprofen and meloxicam to the culture medium during bovine IVEP resulted in a higher rate of the blastocyst formation and an increase in the count of cells in the developing embryos. Along with this, NSAIDs have been demonstrated to reduce the number of apoptotic cells. Consequently, the supplementation of IVC media with NSAIDs has been demonstrated to enhance the development of blastocysts and improve their quality.

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Keywords: Blastocyst formation rate, cattle, *in vitro* culture.

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Effect of combined exposure to polystyrene nanoparticles and cadmium on *in vitro* maturation and embryo development of ovine oocytes

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A broad spectrum of environmental pollutants can affect the quality of the mammalian female gamete in both humans and animals, posing a serious threat to female fertility and reproductive health. Nanoplastics (NPs) are one of the main environmental pollutants today due to their fragmentation from plastic waste. These particles can easily enter our body raising concerns about their impact on our health. Cadmium (Cd) is one of the most toxic heavy metals which accumulates in organs and tissues over time, including the ovaries. This metal can leach from plastic waste, where it is sometimes used as an additive. Currently, no studies have been conducted to determine whether female fertility is impacted by exposure to a mixture of these two contaminants, which are frequently found together in the natural environment. Therefore, the aim of this study was to evaluate the combined effects of polystyrene nanoparticles (PSNPs) and Cd on IVM of ovine oocytes and subsequent preimplantation embryo development. Oocytes were recovered from the ovaries of slaughtered Sardinian lamb. PSNPs with a size of 50 nm (Bangs Laboratories, Inc.) and cadmium chloride (CdCl2; Sigma Aldrich) were added during IVM to evaluate the impact of exposure on nuclear maturation, apoptosis in cumulus cells (CCs) and embryonic development. Cumulus-oocyte complexes were subjected to IVM without (control group; CTR) or with PSNPs (50 µg/mL) and CdCl2 (100 nM; PSNPs+Cd group) for 24 h at 38.5°C and 5% CO2. After IVM apoptosis in the CCs was evaluated by TUNEL assay and partially denuded oocytes were fertilized with frozen ram semen. The presumptive zygotes were cultured *in vitro* to the blastocyst stage (day 8) at 38.5°C and 5% O2 and 5% CO2. All results consist of three replicates and were analyzed using the Chi-square test. Our results showed that nuclear maturation of the oocytes decreased ($P<0.05$) in PSNPs+Cd (50/84; 59.52%) compared to CTR group (76/91; 83.51%). Apoptotic index in CCs after IVM was increased ($P<0.05$) in PSNPs+Cd (12.20%) compared to CTR group (6.68%). No differences in CCs expansion rate were observed. Cleavage rate at 24 h was decreased in PSNPs+Cd (42/76; 55.26%) compared to CTR (57/77; 74.03%) while no difference was observed in blastocyst rate (11.84% vs. 16.88%, 9/76 and 13/77; respectively). In conclusion, the exposure to PS NPs+Cd altered nuclear maturation of oocytes and increased apoptosis in CCs. Even though PSNPs+Cd decreased cleavage rate, no effect on blastocyst rate was detected. Further analyzes are required to better understand the potential effect of PSNPs+Cd on embryo development.

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Keywords: nanoplastics, cadmium, oocytes

Animal Reproduction

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EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Prediction of cell stages and cleavage durations of IVP bovine embryos with a deep learning model

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Video microscopy has recently been introduced into the *in vitro* production of bovine embryos. Cleavage timing and the number of cells have been described to assess embryo quality (Angel-Velez et al. 2023, Reis et al. 2018) and viability (Reis et al. 2025, Sugimura et al. 2012). The follow-up of early cleavages creates new opportunities and is expected to improve field results. However, routine use is time-consuming and can be subject to variations between embryologists. Therefore, the automatization of the cleavage timing and cell number is of capital interest. The aim of this work was to develop a deep learning model, called CLEmbryo+CDT, to determine the cleavage durations and timings (CDT), and to count the number of cells in early embryonic stages (1 to 9+ cells). A batch of 1,225 embryos from one to sixteen cells was produced in semi-individual culture (Primovision®) with images captured every 15 minutes as described by Reis et al. (2018). Cleavage durations and cell numbers were annotated by a single embryologist according to the guidelines LeBrusq (2018). 137,419 images were used, of which 18,428 in cleavage stage and 35,249; 30,406; 4,468; 25,387; 679; 1,814; 75; 20,552 and 541 in 1, 2, 3, 4, 5, 6, 7, 8, 9+-cells stage respectively. 70% was devoted to learning, 5% to validation and 25% to testing. The learning process leverages a supervised contrastive deep learning framework (Khosla et al., NeurIPS, 2020) associated with a simple but effective space-time convolutional neural network and a basic post-processing step. Overall accuracy and per-stage F1-score were calculated for multiple trials. CLEmbryo+CDT achieved an overall accuracy of 85.15% + 0.92%. The F1-score for CDT and stages 1, 2, 4, and 8 cells was 70.10% + 3.45%, 96.74% + 0.56%, 90.08% + 1.29%, 78.36% + 2.50% and 91.98% + 2.45%, respectively. The model was very accurate to predict 1, 2, 4 and 8-cells with very low variability between trials as demonstrated by the standard deviation of the overall accuracy (less than 1%), and it was efficient to detect cleavages as well. Since the stages of 3, 5, 6 and 7 cells are biologically less common, they were very under-represented in the dataset (5.1% of the total number of frames). Such high imbalance explains that the F1-scores are lower for these stages. However, their impact on the overall performance of CLEmbryo+CDT is very low. CLembryo+CDT is the first model adapted to annotate the major parameters for early assessment of the bovine embryo quality: cleavage timing and duration and cell count. In addition, it overcomes the weakness of routine morphokinetics: it enables fast prediction of the most frequent cell stages and cleavage duration (3,03s per embryo with a GPU Nvidia RTX™ 5000). In the future, we will use CLEmbryo+CDT to assess quality and viability of bovine embryos.

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Keywords: embryo, deep learning, cattle

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Seasonal effects on *in vitro* oocyte maturation and embryo development in Holstein dairy cattle in northern Italy

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Dairy cattle are particularly vulnerable to heat stress, which negatively affects their reproductive performance, resulting in substantial economic losses. This study evaluated the influence of natural seasonal thermal variation, regardless of any potential mitigation strategies, on *in vitro* nuclear maturation and oocyte developmental competence in culled Holstein dairy cows in the Emilia-Romagna region (northern Italy, latitude 44.5°N). Ovaries were collected at a local slaughterhouse (also used to calculate the mean monthly THI) during two climatically distinct periods: winter (W; December to mid-March, with no heat stress, THI 55-60) and summer (S; June to mid-September, with moderate to severe heat stress, THI 75-82). Following collection, approximately 100 cumulus–oocyte complexes per replicate were matured *in vitro* for 22 h (8 replicates; W n=802, S n=830). Nuclear maturation was evaluated in a subset of oocytes using bisbenzimide (Hoechst 33342) staining. A second subset was fertilised *in vitro* using frozen semen from a bull of proven fertility, and the resulting zygotes were cultured until day 8. Cleavage and blastocyst rates (calculated as blastocysts per total cultured oocytes and per cleaved oocytes) were recorded on days 7 and 8. Data are expressed as mean±SD and were analysed using a binomial generalised linear Model (GLM) with a logit link function; statistical significance was set at $P<0.05$. Significant seasonal differences were observed across all evaluated parameters ($P<0.05$). Compared to S, W samples exhibited lower rates of degenerate and immature oocytes (20.1±12.7 vs 4.8±7.0 and 22.8±26.0 vs 15.6±10.8, respectively), and a higher proportion of oocytes reaching the metaphase II stage (57.1±22.9 vs 79.5±14.1). Similarly, cleavage rates were higher in W than in S (83.3±6.9 vs 68.6±14.9). Embryo developmental competence was also greater in W, as shown by higher total blastocyst yields on days 7 and 8 (8.9±6.7 vs 2.3±2.3 and 11.3±6.4 vs 5.5±4.2, respectively) and a greater percentage of embryos reaching the blastocyst stage (10.7±8.2 vs 3.6±3.8, on day 7; 13.5±8.0 vs 8.1±7.2 on day 8). These results confirm that summer-associated heat stress impairs reproductive potential at the oocyte level in Holstein dairy cattle in northern Italy. The animal category and physiological status likely contributed to the observed outcomes. Compared to similar studies in beef cattle, the impact of even moderate heat stress appears more pronounced on Holstein dairy cows, typically subject to high metabolic demands and potentially already exhibiting compromised reproductive function at the time of culling. Moreover, the observed delayed nuclear maturation during the early stages of IVF may reflect the reduced developmental competence, particularly in summer. Nonetheless, a possible overestimation of cleavage rates, due to the inclusion of abnormally cleaved embryos, cannot be excluded and warrants further investigation.

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Keywords: oocyte, heat stress, dairy cattle.

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

MiR-99a-5p and miR-92a regulate key signalling pathways involved in bovine embryonic development

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MicroRNAs (miRNAs) act as post-transcriptional regulators, controlling physiological pathways involved in early embryonic development (Cañon-Beltrán et al., 2024). Among them, miR-99a-5p and miR-92a are key miRNAs involved in embryogenesis-related signaling, such as the Rap1 and ErbB pathways. Data from our group (unpublished) showed higher expression levels of miR-99a-5p and miR-92a in in vivo-derived 4-, 8-, and 16-cell bovine embryos compared to those produced *in vitro*. This study aimed to evaluate the embryo uptake of miR-99a-5p and miR-92a via passive transfection (gymnosis) and assess the impact of their sequential supplementation on early development and quality of *in vitro* produced bovine embryos. Zygotes were cultured in IVC medium (Stroebech®) + 0.3% FCS depleted (Control) or supplemented with 1 µM of the following treatments (miRCURY LNA miRNA, Qiagen): miR-99a-5p from presumptive zygote stage to 54 hours post-insemination (hpi) (D2) (miR99a); miR-92a from 54 to 96 hpi (D2-D4) (miR92a); sequential culture with miR99a until 54 hpi, followed by miR92a until 96 hpi (miR99a+92a); or a control mimic (CM; negative control miRNA: miRCURY LNA miRNA mimic 5'FAM, Nº 339173, Qiagen). MicroRNA supplementation was performed directly in the culture medium, and gymnosis was confirmed by RT-qPCR. Developmental rates were evaluated on D2 (≥8-cell), D4 (≥16- cell), and D7 (blastocyst, BD7). BD7 were snap-frozen in LN2 (3 pools, n=10/group) for RT-qPCR analysis of genes related to embryo quality (CDK2, CDX2, DNMT3A, FOSL1, TEAD4), Rap1 (MAPK1, MAPK14), and ErbB (STAT3, JARID2, KAT6A) signaling pathways. H2AFZ and 18S were used as reference genes. Statistical analysis used one-way ANOVA and Tukey's test for all comparisons. No significant differences were found in cleavage rates on D2. The proportion of embryos reaching the ≥16-cell stage was lower (p<0.05) in miR92a and miR99a+92a groups. BD7 yield was higher ($p<0.05$) in the Control (29.6±0.7%) and CM (28.9±0.6%) groups compared to miR99a (23.2±1.3%), miR92a (23.6±0.5%), and miR99a+92a (24.6±1.3%). CDX2, CDK2, TEAD4, and MAPK1 expression levels were significantly increased in BD7 from the miR99a+92a group. MAPK14 was upregulated only in miR99a, while STAT3 was downregulated in miR92a and miR99a+92a. No significant changes were observed in DNMT3A, FOSL1, JARID2, or KAT6A. In conclusion, although miR-92a alone or combined with miR-99a-5p reduced early embryonic progression and blastocyst yield, sequential supplementation modulated key developmental gene expression in blastocysts. These findings highlight the potential of miR-99a-5p and miR-92a to influence critical pathways in bovine embryogenesis, providing a basis for improved strategies in *in vitro* embryo culture.

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Keywords: miRNAs, embryo, cattle, development, IVC

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Animal Reproduction

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EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Ovarian gene expression in juvenile calves exposed to nutrient restriction as fetuses

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Maternal nutrient restriction (NR) in early bovine gestation may have a detrimental influence on ovarian development in the progeny. This study aimed to investigate the impact of maternal NR from 10 days (d) before conception to 80 and 120 days of gestation (DG) on ovarian gene expression profile in juvenile offspring. Holstein-Friesian heifers (n=42; 14-17mo.; body weight 366±41kg) were randomly assigned to 3 experimental groups and, starting 10d before artificial insemination, were individually fed at: (i) 0.6 of their maintenance energy requirements (M) up to 80DG (NR80; n=16) or (ii) 120DG (NR120, n=16), and (iii) 1.8M until 120DG (Control, C; n=10). Estrous cycles were synchronized, and heifers were inseminated with sex-sorted semen from a single sire. After the end of the differential diet, heifers were group fed ad libitum until calving. Twenty-two single female calves were born (NR80, n=8; NR120, n=9; C, n=5) and were slaughtered at 4.5mo. of age (Frau A., Biology of Reproduction, DADE Special Issue, 1-16, 2024). Pairs of ovaries were collected and 1 cm3 ovarian tissue was harvested after middle antral follicle removal from a subset of samples (NR80, n=6 NR120, n=6; C, n=5) for RNA sequencing (BMR Genomics s.r.l. Padova, Italy). Total RNA was isolated from ovarian tissue using TRIzol™ Reagent (Invitrogen Corporation) and treated with DNase I according to manufacturer's protocols. The resulting RNA quantity and quality was checked with Agilent Tapestation (Agilent Technologies). One NR80 sample was excluded from the analysis due to low RNA quality. Raw reads were preprocessed with fastp v0.20.0 to remove adapter sequences and low-quality reads. High-quality reads (mean unique mapping rate: 86%) were aligned to the Bos taurus genome (Ensembl v110) using STAR v2.7.9a (sjdbOverhang set to read length). Transcript and gene abundances were estimated with RSEM v1.3.3. Genes with ≥10 counts in at least N samples (N=5 - smallest group size) were retained (11.053 genes). Differential expression analysis was performed using edgeR, considering genes with FDR ≤ 0.05 and |logFC| > 0.5 as significant. Differentially expressed genes (DEGs) were annotated via the bioMart R package (R 4.3.3) retrieving Gene Names and Entrez IDs. Principal component analysis (PCA) based on all expressed genes, indicated a clear separation between NR80 and NR120 sample groups, while NR80 and C showed a partial overlap, and no evident clustering was detected between NR120 and C. The number of DEGs was 11 between NR80 and NR120, 8 between NR120 and C, whereas no DEGs were observed between NR80 and C. Results do not support the hypothesis of an effect of NR in early pregnancy on ovarian transcriptome in juvenile progeny; this could be potentially due to a lack of impact or to the cellular heterogeneity of the whole ovarian homogenates. Indeed, the effect of NR on specific cellular types and the ovarian epigenetic status of calves exposed to nutrient restriction in utero is yet to be investigated.

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Keywords: programming, ovary, transcriptome

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Blastocyst cell number reduction by blastomeres separation impairs bovine epiblast development during post-hatching culture

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Early lineage differentiation events relies on cell-to-cell contacts which may be impaired in embryos with a reduced cell number. Blastomere separation at the 2-cell stage results in embryos that display a reduced cell number but reach the blastocyst stage at a similar pace than control non-split embryos, providing a good model to test the developmental outcomes of a reduced blastocyst cell number. The objective of this study has been to determine the developmental ability to post-hatching stages of bovine embryos resulting from blastomere separation at the two cell stages. *In vitro* matured oocytes (n=192) obtained from slaughterhouse ovaries were fertilized *in vitro*. Thirty hours after IVF, cleavage was assessed and embryos were randomly divided in two groups, one where each blastomere was separated (halved group), and other where intact embryos were cultured (control group). Blastomere separation was achieved by removing zona pellucida by brief incubation in 1.5 mg/ml pronase followed by gently manual separation of the blastomeres in DPBS media. To prevent blastomere aggregation in the halved group, both intact embryos and individualized blastomeres from control and halved groups were cultured in hand-made Well-of-the-Well (WOW) system until Day 7 (D7). D7 blastocysts were cultured in a post-hatching system based on N2B27 medium until D14. On D14, surviving embryos were fixed and subjected to immunofluorescence for lineage-specific markers: SOX2 (epiblast), SOX17 (hypoblast) and CDX2 (trophectoderm). The experiment was conducted in three independent replicates. Blastocyst rate (calculated per cleaved embryo for the control group, or per individualized blastomere in the halved group) was not significantly different between groups (49.2±7.8 vs. 25.2±8.4%, for control (C) and halved (H) groups, respectively, t test P>0.05). Embryo survival from D7 to D14 was not significantly different between groups (70±16.1 vs. 50±14.4%, for C and H groups, respectively, t test P>0.05), and embryo size did not differ significantly (706.5±89 vs. 590.2±86.6 µm, for C and H groups, respectively, t test P>0.05). Immunofluorescence analysis revealed no significant differences in hypoblast migration rate (13/21 (61.9%) vs. 9/20 (45%), for C and H groups, respectively, Chi square test p>0.05). However, the number of epiblast cells was significantly reduced in the halved group (27.9±7 vs. 9.7±3.1%, for C and H groups, respectively, t test P<0.05). These results suggest that proper epiblast specification (i.e., the lineage that will give rise to the proper embryo) requires a sufficient number of inner cell mass cells at the blastocyst stage.

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Keywords: Blastomere separation, epiblast, blastocyst.

Animal Reproduction

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EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Bovine embryonic disc characterisation in *in vitro*-produced (IVP) embryos at early developmental stages

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Pregnancy loss in cattle during the first trimester is primarily associated with impairments in blastocyst development and conceptus elongation and attachment. Additionally, evidence suggests that the method used to produce the embryo can alter endometrial responses by modifying both the endometrial transcriptome and embryonic gene expression. However, little is known about the phenotypic alterations that may arise from the origin of the embryo. Therefore, this study aims to phenotypically characterize bovine *in vitro* produced (IVP) embryos at early developmental stages, focusing on embryonic disc progression during the process of blastocyst expansion and hatching. Bovine blastocysts were produced from abattoir-derived ovaries from multiple collection days using commercial media for *in vitro* maturation, fertilisation and culture (Stroebech Media, ARTSMedia Denmark ApS). Mean blastocyst rate from starting cumulus-oocyte-complexes varied from 35 to 45%.The expression of SOX2 and SOX17, established biomarkers for inner cell mass (ICM)/epiblast and hypoblast cells, respectively, was evaluated in IVP blastocysts at days 7 (D7, blastocysts, n=7), 8 (D8, hatched blastocysts, n=4), and 9 (D9, hatched blastocysts, n=6), using wholemount immunofluorescent labelling. Isotype-matched IgG negative controls were included for each biomarker. Z-stacks were acquired using an LSM800 Airyscan confocal microscope and analysed with Fiji (Schindelin, et al., Nature Methods, 9(7), 676–682, 2012). Statistical analyses (one-way ANOVA followed by Tukey's HSD test) were performed in R. Significance was defined at $P<0.05$. The number of SOX2+ cells decreased significantly across time. From D7 (52.4%, 33/63) to D8 (28.3%, 56/198), there was a 24.1% reduction, followed by a further 16.8% decrease from D8 to D9 (11.5%, 24/209), resulting in a total reduction of 40.9% from D7 to D9. This reduction coincides with the developmental transition from the blastocyst stage to blastocyst expansion and hatching from the zona pellucida, during which the ICM begins to differentiate into epiblast and hypoblast lineages, with SOX2 becoming specific to epiblast cells. SOX17 expression was detected in D8 blastocysts and appeared to co-localise with SOX2. Additionally, by D9, 66.7% presented SOX17 labelling in cells directly underneath SOX2+ cells and in cells migrating towards the blastocoel, potentially indicating the initiation of hypoblast migration. Continued investigation is ongoing to fully characterize these developmental changes as the embryo progresses through ovoid, tubular and filamentous stages and to determine whether, and how, the origin of the embryo (in vivo derived vs. IVP) contributes to phenotypic variation during early development that may underlie embryonic loss.

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Keywords: bovine, embryonic disc, IVP.

Animal Reproduction

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EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

ROCK signalling pathway is required for blastocyst formation and cell proliferation in sheep

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The first lineage differentiation in mammalian embryos results in the formation of the inner cell mass (ICM) and the trophectoderm (TE) at the blastocyst stage. In mice, Rho-associated protein kinase (ROCK) signaling promotes the polarization of outer blastomeres at the morula stage and is essential for TE differentiation, since its inhibition from the 8-cell stage disrupts blastocyst formation. In contrast, studies in bovine embryos have reported enhanced TE proliferation following ROCK inhibition at later stages (from Day [D] 5 morulae or D8 blastocysts). This study aimed to determine whether ROCK signaling is required for TE differentiation and blastocyst formation in ovine by inhibiting this pathway at developmental stages preceding blastomere polarization. *In vitro*-produced D2 embryos containing at least 8 cells were randomly cultured in IVC medium (Stroebech media, Denmark), either alone (Control, n=102) or supplemented with 5 µM (n=68), 10 µM (n=62), 20 µM (n=107), 40 µM (n=115), or 100 µM (n=78) of the ROCK inhibitor Y-27632, until D8. Culture medium was refreshed at D5. Blastocyst development was assessed at D6, D7, and D8. At D8, blastocysts were fixed, and lineage development was analysed by immunofluorescence using ICM (SOX2) and TE (TFAP2C and GATA3) markers. Blastocyst formation was significantly reduced in a dose-dependent manner at D6 across all Y-27632 concentrations (68.2±5.9%; 36.8±6.1%, 24.5±4.1%, 21.3±1.9%, 10.4±2.8%, 0±0% for control, 5 µM, 10 µM, 20 µM, 40 µM, and 100 µM Y-27632; One-way ANOVA, $p < 0.05$; n=3 replicates). At D8, blastocyst formation was significantly impaired at 40 and 100 µM Y-27632, with no blastocysts developing at 100 µM (80.1±7.1%, 69.7±5.3%, 74.3±4.2%, 64.7±3.1%, 42.3±11.8%, 0±0%; mean±s.e.m. for control, 5 µM, 10 µM, 20 µM, 40 µM, and 100 µM Y-27632; One-way ANOVA, $p < 0.05$; n = 3 replicates). Blastocysts from the 10 µM, 20 µM and 40 µM groups showed significantly reduced total cell numbers (323.0±28.1, 272.0±29.2, 198.2±27.3, 152.3±12.6, 160.1±17.1; mean±s.e.m. for control, 5 µM, 10 µM, 20 µM, and 40 µM Y-27632; Kruskal-Wallis test, $P<0.05$). The number of TFAP2C+ TE cells was significantly decreased at 20 µM and 40 µM (211.9±27.3, 129.4±24.6, 94.7±21.4, 29.4±8.5, 68.4±14.9). GATA3+ TE cell numbers were also significantly reduced at 10 µM, 20 µM and 40 µM (215.3±22.6, 148.3±23.2, 105.8±19.0, 38.8±8.3, 63.6±13.6). Finally, the number of SOX2+ ICM cells was significantly reduced only at 20 µM (10.1±1.2, 8.5±1.1, 7.7±1.4, 4.8±0.8, 9.8±2.0; mean±s.e.m. for control, 5 µM, 10 µM, 20 µM, and 40 µM Y-27632; Kruskal-Wallis test, $P<0.05$). In conclusion, ROCK inhibition from stages preceding blastomere polarization impairs cell proliferation, particularly in TE cells, reducing blastocyst formation rates at high doses (100 µM Y-27632) and delaying blastocyst development even at low concentrations. Further experiments are needed to assess the specific role of ROCK signaling in blastomere polarization at the morula stage.

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Keywords: ROCK pathway, ovine, blastocyst

Animal Reproduction

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EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Preovulatory follicular fluid secretome supplementation during *in vitro* maturation affects the transcriptome of equine embryos recovered after uterine transfer

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In vitro embryo production is widely used in equine reproduction but can be improved, as current *in vitro* maturation (IVM) media do not fully meet oocyte' needs. We have previously attempted to add secretome from equine preovulatory follicular fluid (PFF) to IVM medium to improve its efficiency (Luis-Calero M., Res in Vet Sci 171:105222,2024). In this study, we aimed to investigate the influence of secretome addition during IVM on the transcriptome of equine embryos produced by intracytoplasmic sperm injection (ICSI) and recovered after transfer to recipient mares. Secretome was isolated as previously described and added to IVM medium at 0 (CTR), 20 µg/ml (S20) or 40 µg/ml (S40), and mature oocytes were then fertilised by ICSI. A cohort of day-9 blastocysts from the CTR (n=5), S20 (n=4), and S40 (n=5) groups, respectively, were produced by ICSI and cryopreserved using the Kitazato protocols. These embryos were warmed, transferred to synchronized recipient mares and recovered 48 hours later via uterine flushing as previously reported (Munoz-García C., Reprod Dom Anim 59(3):e14622,2024). Total RNA was extracted using an RNAeasy mini-kit and RNA sequencing was conducted using Illumina NovaSeq X Plus (PE150; an average of 40 million reads per sample). Raw reads underwent quality control and adapter trimming. Clean reads were then mapped to the reference genome, followed by gene expression quantification. Low-count genes were filtered out using DESeq2 (FDR < .05 log2FC >1) prior differential expression analysis. Functional enrichment analysis of significant genes was performed using Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. In both S20 and S40 embryos, several terms related to cellular component (nucleus, cytoplasm, mitochondria, etc.) were downregulated compared to CTR. S20 embryos showed downregulation of genes involved in protein metabolism and processing, including intracellular protein transport and protein kinase binding. KEGG pathways analysis of these genes showed routes such as protein processing in endoplasmic reticulum and ubiquitin-mediated proteolysis. A cross-comparison between GO terms and KEGG pathways identified a subset of overlapping genes involved in protein regulation, suggesting potential targets for further regulatory gene studies. In contrast, S40 embryos exhibited upregulation of terms associated with lipid-related processes, such as cholesterol metabolic process and ergosterol biosynthetic process, along with KEGG pathways like steroid biosynthesis. In this case, cross-comparison revealed some overlapping genes involved in lipid regulation. In conclusion, the addition of PFF secretome to IVM medium induces changes on the transcriptome of the embryos produced *in vitro* and recovered after uterine transfer. Besides, the dose of secretome used seems to differently affect the embryo transcriptome, and thus, further research is warranted to better understand how PFF secretome during oocyte IVM influence the transcriptome of equine embryos.

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Keywords: equine, preovulatory follicular fluid secretome, embryo transcriptome

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Effect of preovulatory follicular fluid concentrate addition during *in vitro* maturation on the quality and development of ICSI equine embryos

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Equine *in vitro* embryo production remains inefficient due to suboptimal oocyte maturation *in vitro*. Several studies have attempted to develop an effective *in vitro* maturation (IVM) medium by adding pure follicular fluid (Hinrichs, K.; Biology of Reproduction; 67; 256-262; 2002) or extracellular vesicles (Gabrys, J.; Theriogenology; 188; 116-124; 2022) yielding still suboptimal results. In the present work we aimed to assess if the addition of equine preovulatory follicular fluid (PFF) concentrate during IVM influences the quality of *in vitro* produced embryos. To obtain PFF, mares were scanned and when a preovulatory follicle was detected (> 35 mm), 3000 UI hCG were administered intravenously (Luis-Calero, M.; Research in Veterinary Science; 171; 105-222; 2024) and 32 hours later, PFF was obtained by flank aspiration. To obtain PFF concentrate, three ml of PFF were diluted in PBS (1:1; v/v), filtered through a 0.22 µm syringe filter and centrifuged at 4000 g for 1 h at 4°C using 10K Amicon Ultra-15 Centrifugal Filter. Protein concentration was measured using a DC Protein Assay (Bio-Rad Hercules, CA, USA) and the PFF concentrate was stored at – 80°C. Oocytes were obtained in vivo by Ovum Pick-Up (OPU) and sent to the ICSI laboratory (~18 h of transport) in holding medium (40% M199 with Earle's salts, 40% M199 with Hanks' salts, 25 µg/ml gentamycin, and 20% fetal bovine serum). Oocytes were matured, fertilized and cultured as previously described (Sánchez-Calabuig, M.J.; Equine Veterinary Journal; 53; 787-795; 2021). The PFF concentrate was added to IVM medium at 20 µg/ml (C20) or 40 µg/ml (C40), while the control group (CTR) was matured in basal medium. Embryo cleavage was assessed on Day 5 post-ICSI (ranging from 64.5-73%) and embryo development was checked from Day 7 to Day 12 (ranging from 20.4 to 22.2%); results were compared using a chi-square test and no significant differences were observed despite PFF concentrate addition (P>0.05). The speed of development was classified as early (Day 7 or 8 blastocysts) or delayed (Days 9-12). Morphological characteristics of embryos were evaluated following validated criteria (Lewis, N.; Reproduction, Fertility and Development; 35; 338-351; 2023) and graded as follows: A (excellent/good), B (regular), or C (poor). Since morphological grading correlates with foaling only in ICSI produced blastocysts at Day 9 or later, only these were graded. A chi-square test was used to compare the speed of development and grading; significance was set at P<0.05. The results for early vs delayed embryo development were respectively: CTR (60.9% vs 39.1%; n=23), C20 (62.5% vs 37.5%; n=16) and C40 (70.6% vs 29.4%; n=17). Grade classification in delayed embryos (A, B or C) was respectively: CTR (25%, 25% and 50%; n=8), C20 (50%, 16.7% and 33.3%; n=6) and C40 (31.6%, 26.3% and 42.1%; n=5). Although the differences among groups were not statistically significant, PFF concentrate addition showed a trend towards faster embryo development. Notably, C20 treatment seemed to improve morphological features of embryos in Day 9-12. Further research is needed, but PFF concentrate may be a useful additive for equine *in vitro* embryo production.

Keywords: preovulatory follicular fluid, *in vitro* maturation, equine embryo.

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Temporal proteomic profiling of porcine endometrium and extra-embryonic membranes during early gestation

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The immediate post implantation period is critical for establishing a successful pregnancy, as 20-30% of porcine embryos are lost between the third and fourth weeks of gestation, significantly reducing litter size and impacting reproductive efficiency. The mechanism underlying this loss remain unclear, although failures in maternal-embryonic cellular and molecular interactions have been postulated. Therefore, the aim of this study was to analyze dynamic proteomic changes in the porcine endometrium and extra-embryonic membranes during early gestation (days 18 to 24), the period in which most conceptus loss occurs. Sows were inseminated and euthanized on days 18 (d18; n=4 sows) and 24 (d24; n=4 sows) of pregnancy (with the onset of estrus designated as day 0) to collect both extra-embryonic membranes (EEM) samples and endometrial (END) tissue samples from three distinct attachment sites and three implantation areas, respectively. All samples were subjected to proteomic analysis by LC-MS/MS. This analysis identified 306 differentially expressed proteins (DEPs) in the EEM (fold change <-2 or >2; Q<0.05), with 145 upregulated and 161 downregulated in d18 compared to d24 samples. In the END, 13 DEPs were identified (fold change <-2 or >2; P < 0.05; Q < 0.25), with 8 upregulated and 5 down regulated in d18 versus d24. To analyze the functional annotation of DEPs, DAVID (https://david.ncifcrf.gov/; version 2021) was used and pathways and Gene Ontology terms with a P value <0.05 were considered significantly enriched. The functional analysis of the EEM-DEPs revealed 17 pathways and 119 GO terms specifically affected by the gestational day. Pathways with functions in adhesion and angiogenesis, such as cell adhesion, focal adhesion, extracellular matrix-receptor interaction, and complement and coagulation cascades, were enriched, with many associated proteins downregulated on d18 compared to d24. Similarly, proteins significantly downregulated on d18 were enriched in GO terms primarily associated with cell adhesion, cell migration, extracellular matrix organization and blood coagulation and fibrin formation. In the END tissues, protein expression did not differ markedly between d18 and d24 of gestation with only 15 GO terms specifically affected. However, even these minor differences may play a crucial role in sustaining pregnancy. Noteworthy, the proteins downregulated on d18 in the END tissue are involved in immune-related processes. Among these proteins are A0A8D1VZL5, A0A4X1VBN6, A0A8D1IWJ9, and A0A8D1Z2N2, which play key roles in crucial biological processes, including the regulation of interleukin-8 production, the innate immune response, vasoconstriction, and the positive modulation of gene expression. The comprehensive identification of these proteomic changes may provide valuable insights and help elucidate the molecular crosstalk among the endometrium, conceptus, and membranes during this key stage of pregnancy.

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Keywords: Proteome, Implantation, Porcine

Animal Reproduction

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EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Electroporation of porcine zygotes at 25 V does not impair embryo development

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Electroporation is a promising, non-invasive method for introducing CRISPR/Cas9 into oocytes and zygotes. However, its potential effects remain poorly evaluated. In this study, we assessed the impact of electroporation voltage on metaphase II (MII) oocytes and zygotes (ZG) at 2- and 5-hours post-sperm co-incubation. A total of 3,485 *in vitro* MII porcine oocytes were used in this study. Electroporation was performed in OPTIMEM medium without gene editors using either 25 V or 30 V. A subset of MII oocytes was electroporated at the end of the maturation period. Half of them were subjected to *in vitro* fertilization (IVF; MII-25 [n=423] and MII-30 [n=419] groups), while the other half was incubated in IVF medium without sperm to assess parthenogenesis (MIIPG-25 [N=382] and MIIPG-30 [N=329] groups). The remaining MII oocytes were fertilized, and resulting presumptive ZGs were electroporated either 2 h (ZG2-25 [n=383] and ZG2-30 [n=330] groups) or 5 h (ZG5-25 [n=453 and ZG5-30 [n=337] groups) after the onset of sperm co-incubation. Controls were MII oocytes subjected to IVF without electroporation (n=429). Data were analysed with one-way ANOVA or the Kruskal-Wallis test, as appropriated, and are presented as means ± standard deviation. On Day 2 of culture, ZG groups exhibited cleavage rates (range: 56.57 ± 9.7% to 67.4 ± 8.4%) comparable to those of the control group (69.4 ± 8.6%). In contrast, the proportion of cleaved oocytes in the MII-25 (43.9±12.1%) and MII-30 (29.8±7.8%) groups were lower (P<0.01). Plasma membrane disruption in cleaved embryos was comparable between ZG5-25, ZG5-30, and control groups (range: 0.9 ± 1.7% to 5.1 ± 4.4%). Degeneration rates were similar among MII-25, ZG2-25, and ZG2-30 (range: 14.3 ± 14.0% to 34.1 ± 16.6%) but higher than in controls (P<0.05). The MII-30 group exhibited the highest (P<0.05) proportion of degenerated oocytes (52.4±5.2%). A similar trend was observed in blastocyst formation (BF) rates, with the MII-30 group showing the lowest rate (14.0±5.2%; p<0.01). BF rates in ZG2-25, ZG5-25 and ZG5-30 groups were comparable to controls (40.9±8.9%), ranging from 36.3±10.2% to 42.5±9.0%), while the remaining groups showed intermediate values (14.0±5.2% to 28.1±5.9%). Embryo development in MIIPG-25 and MIIPG-30 groups was similar to that observed in their fertilized counterparts. All groups produced blastocysts with similar total cell numbers (range: 38.8 ± 14.0 to 57.0 ± 26.7) and apoptosis indexes (range: 4,6 ± 6.8 to 16.2 ± 19.4%). In conclusion, electroporation of zygotes at 5 h (either with 25 or 30 V) and at 2 h (using 25 V) post-sperm co-incubation supports embryo development comparable to that of controls. In contrast, electroporation of MII oocytes impairs developmental potential and induces parthenogenetic activation, resulting in developmental outcomes similar to those of fertilized oocytes.

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Keywords: Electroporation, zygote, embryo development

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Exogenous IL-6 exposure during *in vitro* maturation of pig oocytes modulates blastocyst transcriptome profiles, upregulating developmental signaling pathways

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Interleukin-6 (IL-6) has, for its critical role in supporting oocyte maturation, embryonic development, and apoptosis regulation, recently emerged as a prominent supplement (at 100 ng/mL) for *in vitro* maturation (IVM), enhancing porcine blastocyst development and total cell numbers and reducing apoptosis following *in vitro* fertilization (IVF) and culture (Garcia-Canovas et al., Theriogenology 244, 117483, 2025). The present study evaluated whether IL-6 supplementation of IVM medium affected gene expression patterns on *in vitro* produced day-7 blastocysts. Oocytes collected from prepubertal gilts were matured in IVM medium (TCM-199 containing glucose, sodium pyruvate, penicillin, streptomycin, PVA, cysteine, epidermal growth factor, eCG and hCG) either supplemented with 100 ng/mL IL-6 (R&D Systems, Minneapolis, USA; IVM+ group) or without supplementation (control group), and subsequently subjected to IVF. The resulting presumptive zygotes were cultured *in vitro* for seven days. At the end of the culture period, six pools of seven viable blastocysts (n=42 blastocysts per group) were placed in sterile Eppendorf tubes, flash-frozen in liquid nitrogen and stored at -80ºC until microarray analysis. These blastocysts were the same as those obtained in our previous study (Garcia-Canovas et al., Theriogenology 244, 117483, 2025), where oocytes from the IVM+ group exhibited higher blastocyst efficiency relative to controls. The embryos were analyzed using the GeneChip® Porcine Genome Array (Affymetrix). To identify differentially expressed genes (DEGs) between groups, an ANOVA with an unadjusted p-value threshold of 0.01 and fold change of <-1.5 or >1.5 was performed. Compared to the control, the IVM+ group exhibited 138 DEGs (91 upregulated and 47 downregulated). The IL-6 treatment enriched a total of 15 pathways. KEGG pathways analysis in the up-regulated list showed enrichment of pathways including genes as ACSL3, ALDH1B1, HADHB, EPT1, PAFAH2, FOS, MAPK4, MAPK14, TLR4, PAK6, LYN, ARHGEF12, SMAD2, WNT2B, PPP2CA, and SPTLC1. Many of these genes are involved in key processes related to lipid metabolism, embryo development, cell proliferation and the immune system. Only two pathways, focal adhesion and protein digestion and absorption, were enriched in the downregulated gene list, comprising genes such as ACTG1, COL9A1, RAPGEF1, and SLC9A3, which have not been reported as essential for embryo development. In conclusion, blastocysts derived from oocytes exposed to 100 ng/mL of exogenous IL-6 during IVM, exhibited altered activity in several immune-related and lipid metabolism pathways. This was associated with the upregulation of specific genes involved in cell proliferation, embryo development, and apoptosis reduction. These findings provide valuable insights into the role of IL-6 during porcine IVM and its influence on oocyte developmental competence.

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Keywords: *in vitro* embryos, IL-6, gene expression

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

New insights on the influence of cumulus cells on the *in vitro* developmental competence of porcine oocytes

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The successful implementation of *in vitro* production (IVP) of porcine embryos would enable genetic exchange, preserve valuable lines, and mitigate the use of live animals for experimental purposes. However, porcine IVP efficiency remains low when compared to that of other species. Cumulus cells (CCs) are specialized somatic cells that closely surround the oocyte and support metabolism, gene regulation, and environmental interactions. However, little is known regarding the impact of CCs on *in vitro* oocyte competence and subsequent embryo development. We aimed to evaluate *in vitro* maturation (IVM), embryo development, and blastocyst quality when selecting cumulus-oocyte complexes (COCs) with different number of CC layers. Immature COCs (n=2397; 5 replicates) collected from ovaries of prepubertal gilts were classified into three groups: Grade 1 (>5 layers), Grade 2 (3–5 layers), and Grade 3 (1–2 layers). A subset from each group was stained with Brilliant Cresyl Blue (BCB; Sigma, St. Louis, USA) to assess glucose-6-phosphate dehydrogenase activity, an indicator of oocyte growth. COCs were incubated for 90 minutes in IVM medium (TCM-199) supplemented with 26 µM BCB and classified as BCB+ (blue cytoplasm) or BCB− (no coloration). The remaining COCs were cultured in 500 µL drops of IVM medium for 44 h. Mature COCs were then denuded and either stained with lacmoid (Santa Cruz Biotechnology, Dallas, USA) to asses IVM rates or inseminated with thawed sperm (1500 spermatozoa/oocyte) in 100 µL drops of *in vitro* fertilization medium (TBMm) for 5 h. Presumed zygotes were cultured in 500 µL drops of *in vitro* culture (IVC) medium (NCSU-23) supplemented with pyruvate and lactate for 2 days and with glucose for an additional 5 days. All incubation steps were performed at 38.5ºC, 5% CO_2, and 99% humidity. Embryos were evaluated after 48, 120 and 144h of IVC to assess embryo cleavage (Day 2), developmental stage (Day 6 and 7), and hatching rates (Day 7). Total cell number per blastocyst was quantified on Day 7 of IVC with Hoechst (Sigma, St. Louis, USA) staining. Data are presented as means ± SEM. Shapiro-Wilk test was used to evaluate data distribution. One-way ANOVA was used for parametric data and Kruskal-Wallis for non-parametric data. On Day 6 and 7 of IVC, blastocyst formation rates differed significantly ($P<0.05$) between Grade 2 (36.32% ±5.5/ 39.94% ±5.0, n=361) and Grade 3 (18,67% ±2.29/ 23.03% ±3.0, n=409) groups, and depicted a significant tendency ($P<0.08$) when comparing Grade 1 (30.52% ±2.79/ 32.60% ±2.6, n=446) and Grade 3 groups. In addition, total cell number was significantly higher ($P<0.05$) in Grade 1 (59.19 ±3.2, n=79) vs Grade 3 (50.27 ±2.8, n=84) groups. We demonstrated that COCs with 1-2 layers of CCs are capable of sustaining nuclear maturation. However, the number of CCs surrounding the oocyte during IVM appears to influence subsequent embryo developmental potential and quality, suggesting that selecting COCs with more than 2 layers of CCs enhances the efficiency of the IVP system.

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Keywords: porcine, embryo, cumulus cells

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Murine oocyte mitochondrial activity following Tirzepatide exposure and washout

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Glucagon-like peptide-1 (GLP-1) receptor agonists, especially dual agonists like Tirzepatide, are promising for obesity treatment due to their metabolic and weight loss benefits. However, their use is discouraged in women around conception, with guidelines advising discontinuation at least one month before planned conception. However, Tirzepatide's effects during the preconception period on oocyte quality remain undescribed. Previous research in diet induced obese (DIO) mouse models have shown that diet normalization as a preconception care intervention (PCCi) only partly restores oocyte quality, leaving persistent oocyte mitochondrial abnormalities. Whether Tirzepatide supplementation can enhance this recovery is unknown. Therefore, this study investigates both the direct effects of Tirzepatide on oocyte mitochondrial activity as well as the effects following a washout period in a DIO mouse model. Outbred Swiss mice were fed a high-fat, high-sugar (HFHS) or control diet for 7 weeks to induce obesity, followed by a 4-week PCCi, during which mice were assigned to 4 treatment groups: (1) C_C, maintained on a control diet; (2) H_H, maintained on a HFHS diet; (3) H_C, switched from HFHS to control diet; and (4) H_T, switched from HFHS to control diet with Tirzepatide injections every 48h during the first two weeks, followed by a two-week washout. After 2 (T1) and 4 weeks (T2) mice were superovulated and sacrificed to collect mature oocytes for JC-1 and CellROX Deep Red staining to assess mitochondrial inner membrane potential (MMP) and reactive oxygen species (ROS) levels, respectively. Six replicates were performed per timepoint, with 5 mice per treatment, analyzing 6-8 oocytes of one mouse per treatment. Confocal microscopy images were analyzed using ImageJ, and statistical analysis was conducted in SPSS using one-way ANOVA for ROS data and Kruskal-Wallis tests for MMP, with significance set at $P<0.05$. HFHS feeding significantly increased MMP ($P=0.025$) and ROS ($P=0.043$) at T1 and tended to increase MMP ($P=0.055$) and ROS at T2 ($P=0.099$) compared to controls. At T1, diet normalization (H_C) restored MMP to control levels and numerically reduced ROS ($P=0.158$). Unexpectedly, supplementation of Tirzepatide resulted in similarly elevated MMP and ROS as in the H_H group. By T2, however, Tirzepatide's effect on MMP appeared to fade, significantly reducing MMP to control levels. In contrast, MMP in the H_C group rose at T2, similar to H_H levels. This study shows for the first time that Tirzepatide may result in acute stress in the oocyte, but, after a washout period, may have beneficial effects on oocyte mitochondrial activity compared to diet normalization alone. The increased mitochondrial activity in the H_T group at T1 is likely linked to the Tirzepatide-induced rapid weight loss, which may have led to elevated free fatty acid concentrations in the bloodstream and subsequent lipotoxicity. Further investigation into additional markers of oocyte health and mitochondrial function is ongoing to fully understand the effects of Tirzepatide exposure.

Keywords: Tirzepatide, Oocyte, GLP-1-R agonist

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Investigating the link between incretin-based therapy-induced weight change and oocyte lipid content

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Obesity and its associated metabolic effects alter the lipid content and composition of ovarian follicles, leading to an increased oocyte lipid content. This creates a nutritional overload, lipotoxicity, and reduced developmental competence. Incretin-based therapy, such as tirzepatide, are recent breakthrough anti-obesogenic medications, inducing up to 20% weight loss in human within a short timeframe. It is not known whether incretin-based therapy can mitigate the lipotoxic effects of obesity on the oocyte. Outbred Swiss mice were allocated to a control diet or a high-fat high-sugar (HFHS) diet for 7 weeks. HFHS mice then underwent a 4-week preconception care intervention (PCCi), and oocytes were collected from 4 groups: (1) C_C, control diet throughout; (2) H_H, HFHS diet throughout; (3) H_C, HFHS diet then switched to a control diet; and (4) H_T, switched to a control diet + tirzepatide injections (5-10 nmol/kg, every 48h for 2 weeks) followed by a 2-week washout. Weight gain/loss was recorded on a weekly basis during the whole experiment. At T1 (2 weeks) and T2 (4 weeks) of PCCi, oocytes were collected from 30 mice per treatment group (6 replicates), with about 20 oocytes per group (3 replicates of ±6 oocytes each) analyzed by BODIPY staining to measure total lipid volume (confocal microscopy was used to quantify the z-stacks). Data were analyzed using repeated measures ANOVA and Kruskal-Wallis tests in SPSS 29, with significance at $P<0.05$. After one week on a HFHS diet, Swiss mice showed significant weight gain ($P<0.001$) ultimately reaching 36% more body weight after seven weeks compared to C_C. Tirzepatide injections led to immediate and significant weight loss compared to H_H ($P<0.001$) and H_C ($P<0.001$) starting from the 2nd injection, whereas H_C mice required one week to reach significant weight loss vs. H_H ($P=0.001$). After two weeks, H_T mice reached weights comparable to controls (C_C), while H_C mice remained 20% heavier than C_C. At T1, lipid content in H_H oocytes was three times higher than in C_C ($P= 0.021$). Strikingly, H_T oocytes also showed elevated lipid levels ($P=0.028$) compared to H_C, whose lipid content had returned to control levels. Three days after the last tirzepatide injection, H_T mice gained weight and were again significantly heavier than C_C ($P=0.039$) reaching 79% of the obese mice weight by T2, whereas H_C weight remained stable during extended PCCi. Still, by T2, oocyte lipid profiles had shifted; lipid content in H_T oocytes did normalize to control levels and only H_H oocytes remained significantly lipid-rich ($P<0.001$). The elevated lipid levels in H_T oocytes at T1 likely result from rapid weight loss leading to marked lipolysis and fat mobilization, aggravating short-term lipotoxicity. In contrast, gradual weight loss in H_C mice allowed earlier normalization of oocyte lipid levels. As folliculogenesis in mice takes 3 weeks, oocytes collected at T2 developed primarily during the wash-out period, possibly allowing oocyte lipid content to return to control levels due to restored free fatty acid storage in adipose tissue. These results highlight the importance of weight loss pace and recovery time after incretin-based therapy in relation to oocyte health.

Keywords: Oocyte, Incretins, Lipid content

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Unveiling potential sperm selection mediators at the mouse utero-tubal junction

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Sperm interact with epithelial cells and other components of the female reproductive tract (FRT) during postcopulatory migration, with only a selected subpopulation of sperm being able to reach the fertilization site. The subsections of the FRT (uterus, utero-tubal junction (UTJ) and the oviduct) have distinct roles in selection, storage and protection of sperm. The UTJ presents a functional barrier between the uterus and the oviduct, where out of millions of ejaculated sperm only hundreds pass, being selected not only for normal motility, but also for specific surface protein properties. The focus of this study is to identify potential direct interactions between sperm and epithelial cells in the murine UTJ. As a first step, we analyzed the FRT transcriptional response to sperm using RNAseq in three groups of 126SV/S2 estrous female mice; unmated control (CTR: n=5), females 6-8h after copulation with intact C57/Bl6 males (COP: n=4) and females 6-8h after copulation with vasectomized C57/Bl6 males (VAS: n=4) to differentiate sperm specific effects. Animals were euthanized by cervical dislocation. RNAseq data were analyzed using HISAT2, Stringtie, DESeq2 and custom R-scripts, comparing expression between the 3 groups in 3 subsections of the FRT (UTJ, uterus and oviduct) (LFC>1; FDR<0.05). Separation of semen derived transcripts was performed, based on strain-specific SNPs and publicly available murine semen RNAseq data. Initial analysis showed that the UTJ has a specific copulatory response related to extracellular matrix composition (UTJ: $F(2)=7.17$, $P<0.001$; CTR vs. COP: $P<0.001$; Uterus: $F(2)=0.4$, $P=0.7$; Oviduct: $F(2)=2.01$, $P=0.13$). We found 216 genes to be significantly upregulated in COP but not VAS compared to CTR, specifically in the UTJ. After filtering out semen derived transcripts and additional LFC filtering to detect highly UTJ specific genes, we produced a list of 85 UTJ response genes. Most of these fall within a STRING network with sperm surface proteins. Out of the genes forming a network we identified several strong candidate genes based on properties of sperm surface proteins and known receptor roles of FRT epithelial proteins. Of these we chose LGALS3 and ITGA8 for further analyses. Their protein expression and epithelial cell localization in the UTJ was confirmed by immunofluorescence and western blot in COP (n=3) and CTR (n=3) females. This indicates their potential involvement in sperm selection at the UTJ and makes them exciting candidates for testing *in vitro* sperm selection. However, further experiments, including interaction assays with sperm surface molecules, are needed to unravel the specific role of each of these molecules. Studying and leveraging these direct interactions between sperm and FRT epithelial cells could significantly enhance and refine artificial reproduction technologies, which are nowadays based mostly on sperm selection by motility and morphology, not utilizing these important natural molecular interactions.

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Keywords: UTJ, sperm selection, ART

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Maternal–embryo crosstalk via uterine extracellular vesicles at the time of embryo reactivation in roe deer (Capreolus capreolus)

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Roe deer exhibit a unique form of embryonic diapause, characterized by a state of extremely low cellular proliferation, rather than the complete suppression observed in other species. The maternal-embryonic interaction underlying this process remains poorly understood. Thus, the objective of this study was to elucidate maternal-embryo communication via uterine fluid extracellular vesicles (EVs) during embryonic reactivation in roe deer, focusing on their RNA cargo. For this, uterine fluid was collected from pregnant does in Guadalajara (Spain) in early December, when the embryo is still in diapause (identified by the presence of at least one ovoid conceptus in the flushed uterine fluid; n=5 does), and once reactivation of development had taken place in late December-early January (identified by the presence of at least one filamentous conceptus; n=6 does). EVs were isolated by size exclusion chromatography and characterised by flow cytometry (Cytoflex S, BeckmanCoulter), detecting Carboxyfluorescein Succinimidyl Ester (a marker of EV integrity), the cargo marker HSP70, and the tetraspanin EV markers: CD63, CD81 and CD9. After EV RNA extraction and sequencing, samples were mapped to the roe deer draft genome (GCA_951849835.1), followed by Trinity genome-guided de novo transcriptome assembly and annotation with Trinotate. Reads were then pseudo-mapped and quantified using Salmon, followed by gene-level differential expression analysis using DESeq2. GO term and pathway overrepresentation analyses of target gene lists were done using custom scripts. We identified 959 differentially expressed genes (padj < 0.05; LFC >1) between samples collected after reactivation of development or at the time of embryonic diapause (635 upregulated and 330 downregulated). Genes downregulated during reactivation of development are involved in biological processes related to the negative regulation of transcription, and cilia-associated functions such as cilium movement and cilium assembly. Biological processes associated to upregulated genes included positive regulation of transcription, cell migration and cell division, as well as regulation of the JNK, MAPK, and ERK1/2 cascades. Both MAPK/JNK and ERK1/2 signalling are required for activation mTOR, which is especially relevant considering that inhibition of this pathway induces diapause in mice, and that mTORC1 activation has been hypothesised to releases roe deer embryos from the decelerated proliferation that marks diapause in this species. In conclusion, these findings suggest that uterine fluid derived EVs play a role in the reactivation of roe deer embryos and offer a foundation for further exploration of the molecular mechanisms governing diapause in mammals.

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Keywords: Roe deer, diapause, extracelular vesicles.

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY AND DEVELOPMENTAL BIOLOGY

Comparative transcriptomics suggests that mice diverge from other mammals in the molecular regulation of first lineage differentiation

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The molecular regulation that drives the differentiation between inner cell mass (ICM) and trophectoderm (TE) is well studied in mouse embryos, but recent findings have observed that such molecular regulation is not conserved in other mammals. For instance, TEAD4, the major regulator of TE differentiation in mice, is not essential for bovine, rabbit and human TE differentiation (Pérez-Gómez et al., Development 2024). The objective of this work has been to contrast the differentially expressed genes (DEGs) between ICM and TE across six mammalian species (cow, pig, rabbit, mouse, macaque and human) by comparing published databases and own data. Own data was generated by conducting RNAseq dataset in 5 samples of ICM and TE biopsies of D7 *in vitro* produced bovine blastocysts. Published raw data were reanalyzed or reannotated if raw files were not available. Briefly, FASTQ files were quality-filtered with fastp, reads were pseudoaligned to each species´ coding transcriptome and quantified with Kallisto. Count matrixes were subjected to differential expression analysis with DESeq2. Fold changes were shrunken with apeglm and genes with |log2FC|>1.5 and adjusted p-value<0.05 were considered as DEGs. A correlation analysis among datasets was performed using a TPM normalized expression matrix including the most variable genes with one-to-one orthologs across all analyzed species (1066 ICM- and 1296 TE-upregulated genes) to calculate Spearman's ranks. In our bovine dataset, 15938 genes were detected, with 870 DEGs (300 upregulated in ICM and 570 in TE). After comparison with 3 other bovine blastocyst datasets we generated a consensus list of 33 ICM markers and 44 TE markers present as DEGs in at least 3 out of the 4 databases. In pigs, the comparison of the DEGs obtained from 2 datasets yielded 104 and 118 common ICM and TE markers, respectively. Both ungulate consensus datasets were contrasted, showing 7 common ICM markers (PDGFRA, HNF4A, OTX2, NOSTRIN, NANOG, SPIC, KIT) and 7 TE markers (DAB2, ARHGAP18, SLC30A2, KRT18, PTGS2, RAB11FIP4, MCOLN2). Analysis of 3 human datasets identified 308 ICM and 109 TE markers shared by at least 2 studies, while 3 mouse datasets yielded 27 ICM and 62 TE consensus markers. For a comparison across mammals, we contrasted the bovine, pig, mouse and human consensus markers and single rabbit and macaque databases, revealing that only NANOG (ICM) and LRP2 (TE) were common to all species. ICM markers SOX2, OTX2 and PDGFRA, and TE markers TFAP2A/C and GATA3 were generally conserved across species. Correlation between databases was higher within species (>0.8), and showed higher similarity for ICM than for TE markers. Compared to human datasets, the highest Spearman´s rank was obtained for macaque followed by rabbit and bovine datasets, whereas mouse exhibited the lowest correlation with all other species, suggesting that first lineage differentiation diverges between mice and other mammals.

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Keywords: RNA-seq, lineage differentiation, mammal.

Animal Reproduction

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CLONING, TRANSGENESIS AND STEM CELLS

Evaluation of two CRISPR/Cas9 electroporation protocols in pig oocytes using BTX and NEPA21 systems

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Electroporation has emerged as a widely used method for delivering CRISPR/Cas9 ribonucleoprotein (RNP) into oocytes and zygotes in different mammalian species, enabling the efficient generation of genetically modified embryos and animals. Compared to traditional methods such as cloning and microinjection, electroporation is simpler, faster and more scalable. It requires only basic equipment such as a stereomicroscope, an electroporator and an electroporation chamber, and allows the simultaneous treatment of large numbers of oocytes or zygotes within seconds. Previous studies have reported comparable embryo development and mutation rates between electroporated and microinjected oocytes (prior to *in vitro* fertilization-IVF) (Navarro-Serna, Theriogenology. 186: 175-184, 2022). While unipolar current electroporation has proven effective, new devices like the NEPA 21 system support more complex electroporation protocols. In this study, we compare the effects of different electroporation procedures involving different equipment (ECM 830 BTX -BTX- and NEPA21 -NEP) on embryo development and CRISPR/Cas9 editing efficiency in porcine oocytes. These two methods differed in terms of number and polarity of pulses. *In vitro*-matured decumulated porcine oocytes (matured in NCSU37) were electroporated in Opti-MEM with 100 ng/µL of CRISPR/Cas9 RNP targeting Calpain-3 gene using 2 RNA guides prior to IVF. BTX and NEP groups followed optimized protocols from previous publications: 4 unipolar pulses in BTX (Navarro-Serna, Therio. 186: 175-184, 2022) and 5 bipolar pulses in NEP (Park, Re:Gen. 4(1), 9–20, 2024), each with its specific electroporation chamber. After electroporation, oocytes were inseminated in TALP with frozen-thawed boar sperm selected by swim-up (3000 spz/mL). 18 hours after insemination, putative zygotes were cultured in NCSU23 medium for an additional 156 hours to evaluate blastocyst yield and gene deletion by PCR, following zona pellucida digestion to remove bound sperm. The experiment was repeated three times with 50-55 embryos per group. All *in vitro* cultures were performed at 38.5ºC, 7% O2 and 5% CO2. Mutation parameters were analyzed by capillary electrophoresis as described in (Navarro-Serna, Therio. 186: 175-184, 2022). Regarding embryo development, cleavage (67.0 vs. 71.3%) and blastocyst rates (14.7-15.8%) were similar between groups (P>0.05). Mutation parameters were also similar between both groups (P>0.05), including total mutation rate (42.5-50.0%), monoallelic knock-out (KO) embryo rate (25.0-34.0%) and biallelic KO embryo percentage (17.5-16.0%). Notably, BTX achieved these results with fewer pulses (4) compared to NEP (10). Previous work by Nishio et al. Rep. Dom. Anim. 53(2), 313–318, 2018 indicated that bipolar pulses may reduce blastocyst yield compared to unipolar pulses when using identical settings. In conclusion, both protocols, each optimized for its respective equipment, produced similar outcomes in terms of embryo development and gene editing efficiency. These findings confirm that both unipolar and bipolar electroporation approaches can effectively support CRISPR/Cas9-based genome editing in porcine embryos.

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Keywords: Gene editing, Electroporation, Pig, Embryo, CRISPR/Cas9

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CLONING, TRANSGENESIS AND STEM CELLS

Cytosine base editing overcomes mosaicism in porcine GJB2 knock-out models of human hearing loss

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The generation of gene edited pigs is gaining importance as they serve as a suitable model to mimic human diseases due to their physiological and anatomical similarities with humans. The gene GJB2 encodes for the protein Connexin 26 protein, whose deficiency is the underlying cause of more than 50% of the cases of nonsyndromic hearing loss (Kenneson, Genet Med Off J Am Coll Med Genet. 4:258-74, 2002). A murine model that recapitulates human disease has been recently generated (Dominguez-Ruiz, BMC Genomics. 25(1): 359. 2024). We have previously generated pig embryos edited by conventional CRISPR/Cas9 system (Piñeiro-Silva, AIDA, 2025), but most of them were mosaic (79.2-95.7%), i.e., they harbored more than two alleles. As an alternative, cytosine base editors (CBE) can be used to generate a point mutation to introduce a premature stop codon. The objective of this study was to generate knock-out (KO) pig embryos for CJB2 gene with CBE. *In vitro* matured oocytes were microinjected with CBE (Pérez-Gómez, Development. 151(20), 2024) and 3 different sgRNA (sgRNA1-3) at two concentrations (100 ng/µl CBE:50 ng/µl sgRNA -1x- and 200:100, respectively -2x). After, they were *in vitro* fertilized and cultured for 6 days. An untreated group was used as control. Blastocyst rate was evaluated on day 6 post-insemination (pi). Mutation rate was analyzed by PCR and Sanger sequencing. Overall efficiency was calculated (blastocyst with stop codon/total oocytes). 144-195 oocytes were analyzed by group (3 replicates). The statistical analysis was performed with the IBM SPSS program using the Krustal-Wallis test (non-normal distribution; $p<0.05$). Embryo microinjection had a negative effect on embryo development, as blastocyst rate was lower in the injected groups (8-18%) compared to the non-injected control (30%, $p<0.05$), with the exception of the group injected with x1 concentration of sgRNA1 (22%, $P>0.05$). Regarding stop codon generation, the mutation rate with sgRNA2 (1x: 11%, 2x: 9%, $P>0.05$) and 3 (1x: 7%, 2x: 10%, $P>0,05$) was similar for both concentrations, while sgRNA1 showed a higher mutation rate at x1 compared to x2 concentration (47 vs. 17%, $P<0.05$). The overall efficiency of all the combinations of guides and concentrations was similar (1.1-3.7%, $P>0.05$). Finally, two synchronized gilts were transferred with zygotes (24h-pi) derived from treated oocytes (microinjected with CBE and sgRNA2) and with in vivo produced zygotes (non-treated with CBE). The transfer was performed by laparotomy into the oviduct (56 treated and 26 in vivo zygotes/gilt). Pregnancy was established by ultrasonography and spontaneous abortion occurred on day 63. Three fetuses out of nine were identified as CBE-treated and 1 of them (33%) had the target mutation that generated a premature stop codon in the GJB2 gene, detected by Sanger sequencing. In conclusion, we were able to generate CJB2 KO pig embryos using CBE, a technology that avoids mosaicism associated to conventional CRISPR/Cas9. This was confirmed by the generation of a mutant fetus, which opens a new possibility to generate pigs as models for this disease.

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Keywords: base editing, CRISPR, microinjection

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Effect of recombinant insect AFPs on *in vitro* bovine embryo cryopreservation

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Cryopreservation of bovine embryos is a highly developed reproductive biotechnology in cattle farms. However, embryos produced *in vitro* (IVP) from ovum pick (OPU) remain particularly sensitive to damage potentially induced by the formation of ice crystals during this process. This damage is manifested in particular by mechanical stress, but also by oxidative stress, which could affect the viability and development of the embryo. The antifreeze proteins (AFPs) produced by certain insects, due to the presence of numerous disulfide bridges, have a strong capacity to bind to emerging ice crystals, thus inhibiting their formation and growth. As a result, these organisms can survive at very low temperatures. Recombinant AFPs (rAFPs) from Anatolica polita have been successfully tested in chilling ewe embryos (Li et al., Cryobiology, 2020) and Xenopus (Jevtić et al., J. Exp. Biol., 2022), but never in bovine IVP embryos. The aim of this project is to evaluate the effect of rAFPs from Anatolica polita (ApAFP80 and ApAFP106) and Tenebrio molitor (TmAFP119) during the cryopreservation of bovine IVP blastocysts. The three rAFPs were produced in a prokaryotic expression system, purified on a nickel column thanks to the presence of a 6 his-tag fused to the protein, checked on an acrylamide gel, then concentrated and lyophilized. They were used at a final concentration of 10µg/ml (Li et al., Cryobiology, 2020). In a first step, a potential deleterious effect of the rAFPs was evaluated on embryo development from the blastocyst stage until hatching. Thus, IVP blastocysts were placed in medium with or without rAFPs on day 7 post fertilization. They were cultivated at 38.5°C, in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. The hatch rate was assessed for the three rAFPs. Twenty blastocysts per batch were tested in three replicates. Hatch rates of 94.1%, 86.4% and 85.7% were obtained for ApAFP80, ApAFP106 and TmAFP119 respectively. Hatch rates for the parallel control batches were 100%, 90% and 95% respectively. The hatch rates observed in the treated groups were not significantly different from those of the controls (Chi^2 test; $P>0.05$). These results indicate that the three rAFPs produced have no apparent deleterious effect at a concentration of 10µg/ml on fresh IVP bovine blastocysts and do not interfere with their hatching. In a second step, the rAFPs were added in VS with 18% EG, 18% DMSO and 0.5 M trehalose during the vitrification procedure. After thawing, the embryo quality was evaluated: analyses including assessment of intracellular oxidative stress (CellROX™ labelling) and early apoptosis (CellEvent™ Caspase-3/7 Detection Reagent labelling) by confocal microscopy and hatching rate are underway. The results (expected in the summer) will allow us to determine whether the three rAFPs we have produced provide additional protection to blastocysts by reducing the potential damage caused by cryopreservation.

A grant was obtained from the INRAE PHASE department (Innovative Project) to carry out this experiment.

Keywords: Bovine embryo, antifreeze protein, cryopreservation

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Post-warm survival of cattle embryos derived from vitrified oocytes supplemented with astaxanthin

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Cryopreservation offers long-term storage of gametes and embryos from valuable and endangered animal species. However, the post-warm viability of vitrified gametes and subsequent embryonic development is poorer compared to fresh ones. Antioxidants are able to mitigate oxidative stress and improve embryo development. The aim of this study was to examine the development and quality of vitrified/warmed embryos produced from vitrified bovine oocytes supplemented with astaxanthin (AX) during the post-warm recovery period. Oocytes, isolated from ovaries of cows at an abattoir, were *in vitro* matured (M199, 10% fetal bovine serum (FBS), 0.25 mM sodium pyruvate, 50 µg/mL gentamicin, 1 I.U. FSH/LH (Pluset)) for 21 h at 38.5°C and 5% CO2. Then partially denuded oocytes were washed in vitrification medium (M199, 10% FBS, 30% ethylene glycol (EG), 1 M sucrose) for 25 s, placed on electron microscopy (EM) grids and plunged into liquid nitrogen (LN). Following warming, oocytes were cultured for recovery during 3 h in IVM medium without (V-0) or with AX addition (1 µM, 2.5 µM; V-1AX and V-2.5AX). Fresh oocytes served as a control (CTR). Afterwards, oocytes were fertilized with frozen bull semen and cultured until day 6 in IVC medium (IVF Bioscience, UK) at 38.5°C and 5% O2. Then, compact morulae and early blastocysts (Bl) were vitrified (M199, 20% FBS, 16.5% DMSO, 16.5% EG) on EM grids and stored in LN for 3–4 weeks. After warming, embryos were cultured for re-expansion (48 h) at 38.5°C and 5% O2 and then analysed for the progress in development, total cell number (TCN) and apoptotic cells (TUNEL). For statistical analysis, Chi-square and t-test were used at the level of significance at $p < 0.05$. Vitrification of oocytes decreased Bl rate ($p < 0.05$) by almost half (51/227; 22.47%) compared to CTR (140/345; 40.58%). AX (2.5 µM) increased ($p < 0.05$) Bl rate (60/188; 31.91%) compared to V-0 and V-1AX (47/223; 21.08%). Morulas and early Bl were again vitrified and after warming and recovery, 62.86% of these embryos reached stage of expanded Bl and 37.14% of them hatching/hatched Bl stage. Oppositely, in CTR, 75% of Bl reached hatching/hatched stage and 25% were expanded. Both AX doses slightly increased ($P > 0.05$) the proportion of hatched Bl (V-1AX – 62.16% and V-2.5AX – 56.82%, resp.) compared to V-0, while expanded Bl represented 37.84 and 43.18%. The TCN of embryos was lower ($p < 0.05$) in V-0 (127.77±6.92) compared to CTR (169.02±4.83) group. AX at both doses increased TCN (169.46±8.72 and 167.77±8.56, resp.) up to the CTR level. Apoptotic cell index (%) of V-0 group was slightly higher (11.04±1.20) than in CTR (9.39±0.61) group. Higher dose of AX reduced ($P < 0.05$) apoptotic cells (8.49±0.78) compared to V-0 group, while being comparable with CTR and V-1AX (10.51±0.86) groups. In conclusion, AX at both doses increased TCN, while given at higher dose it increased Bl rate and suppressed apoptosis in vitrified/warmed embryos. Our results suggests that AX during short exposure after oocyte warming improved their quality and maintained it after embryo warming.

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Keywords: vitrification, embryo, cattle

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Bovine blastocyst vitrification impairs the development of extra-embryonic membranes in a post-hatching culture system

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Embryo cryopreservation is a critical technique that allows the storage and transport of embryos before transfer to synchronized recipients. Unfortunately, the use of cryopreserved embryos is commonly associated with a reduction in pregnancy rates compared to fresh embryos. The underlying developmental defects associated with cryopreservation have remained unknown due to the inaccessibility of the developing embryo following transfer. Currently, advances in post-hatching embryo culture currently allow the analysis of embryo survival and development of specific lineages beyond the Day 7 (D7) blastocyst stage. The objective of this study was to test the developmental ability of *in vitro* produced embryos following vitrification compared to non-vitrified *in vitro* counterparts. *In vitro* produced bovine blastocyst were generated from oocytes collected from slaughterhouse ovaries, which were matured and fertilized *in vitro*. Presumptive zygotes were cultured in conventional SOF medium up to D7 (n=5 replicates, cleavage and blastocyst rate: 87.8 ±2.0% and 31.9±2.3%, mean±s.e.m, respectively). On D7, blastocysts were randomly allocated into two groups: one was transferred directly into a post-hatching system based on N2B27 medium (control group) and the other was vitrified and thawed prior to culture in the same post-hatching system (vitrified group). Vitrification and thawing were performed following the protocol described in Morató et al. Reprod Fertil Dev 2010. After 5 days of post-hatching culture, D12 embryos were fixed in paraformaldehyde and analyzed by IHC to detect epiblast (SOX2+), hypoblast (SOX17+) and trophectoderm (CDX2+) cells. No significant differences in D7 to D12 survival rate were observed between control and vitrified groups (71.0±4.7 and 60.9±1.6% respectively, mean±s.e.m, t-test p>0.05). The percentage of embryos showing epiblast cells (SOX2+) was similar between groups (30/43 (69.8%) vs. 34/46 (73.9%), for vitrified and control group, respectively, Chi-square P>0.05), and no significant differences on epiblast proliferation were observed (21±4 vs. 27±4 epiblast cells/embryo for vitrified and control group, respectively, mean±s.e.m, t-test, P>0.05). The percentage of embryos showing complete hypoblast migration was similar between groups (15/43 (34.9%) vs. 22/46 (47.8%), for vitrified and control group, respectively, Chi-square, P>0.05). However, the proliferation of extra-embryonic membranes, assessed by conceptus diameter by D12, was significantly reduced in vitrified embryos compared to controls (628±40 vs. 826±50 µm for vitrified and control embryos, respectively, mean±s.e.m, t-test, P<0.05). These results suggest that an impaired development of the extra-embryonic membranes, which are essential to prevent luteolysis and for subsequent placentation, could lead to the reduced pregnancy rates associated with blastocyst vitrification.

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Keywords: Vitrification, embryo transfer, *in vitro* culture.

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Bovine oocytes cryopreservation using a human commercial modified vitrification protocol

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The aim of the study was to assess the effects of a human commercial modified vitrification protocol on bovine oocyte, as an animal model for human oocyte vitrification. Bovine oocyte vitrification was performed using the Rapid-i™ Vitrification System (Vitrolife Sweden AB), which employs DMSO-free vitrification solutions, as DMSO is considered a highly toxic cryoprotectant. To further reduce the oocyte exposure time to cryoprotectants, an experimental protocol known as the Manhattan protocol, was applied. Bovine ovaries were collected at a local slaughterhouse, and the cumulus-oocyte complexes (COCs) were recovered and *in vitro* matured. The maturation medium consisted of TCM199 supplemented with 10 ng/ml epidermal growth factor, 100 ng/ml insulin-like growth factor, 0.1 IU/ml porcine FSH-LH, 1.2 mM L-cystein, 1 mM Na-pyruvate and 10% FBS. Oocytes presenting an extruded polar body under the stereomicroscope, indicative of the metaphase II (MII) stage, were randomly allocated into two groups: (1) closed system, oocytes do not come in direct contact with liquid nitrogen (C); and (2) semi-open system, oocytes come in direct contact with liquid nitrogen (SO). Vitrification was performed using the Rapid-i™ Vitrification System kit which provides three different vitrification media (RapidVit™), four warming media (RapidWarm™), and the Rapid-i™ straw as a cryodevice. The Manhattan protocol consists of a modified application of the original Rapid-i™ Vitrification System, in which oocytes are exposed to RapidVitri 2™ for 1 minute, followed by RapidVitri 3™ for 10 seconds, then loaded onto a Rapid-i™ straw. For warming, the Rapid-i™ straw was immersed in RapidWarm 1™ solution at 37°C. After 1 minute, oocytes were transferred to RapidWarm 4™ for 10 seconds, then incubated in H-SOF (Hepes - Synthetic Oviductal Fluid) medium for two hours at 38.5°C under humidified atmosphere in air. Subsequently, the nuclear state and viability of vitrified/warmed oocytes from both groups were evaluated using Hoechst 33342/Propidium Iodide (PI) staining. Data are expressed as mean ± standard deviation and were analysed using SPSS (IBM SPSS Statistics, IBM, Italy), data distribution was assessed using the Shapiro-Wilk test, followed by a GLM test using IBM SPSS Statistics v29. Significance was assessed for $p< 0.05$. A total of 590 bovine oocytes were recovered and matured *in vitro*. Among these,188 oocytes were vitrified using a closed system (C) and 188 using a semi open system (SO). After warming, no statistically significant differences were in the number of recovered oocytes between C (91.9 ± 9.4) and SO (90.5 ± 11.8). A similar rate of immature oocytes was observed in C (13.4 ± 8.3) and SO (13.5 ±15.1). Survival rates, assessed only metaphase II oocytes, also showed no significant differences between the two groups (SO 75.7 ± 10.3; C 76.9 ± 19.3). In conclusion, this study demonstrates that the Manhattan protocol, applied in both semi-open and closed systems, supports good post-warming viability of bovine oocytes, with fertilization potential currently under evaluation.

Keywords: Bovine, oocyte, vitrification

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Comparison of dimethyl sulfoxide and propylene glycol for vitrifying *in vitro* matured bovine oocytes: effects on embryonic development

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Ethylene glycol (EG) and dimethyl sulfoxide (DMSO) are well established cryoprotectants (CPAs) for vitrification of bovine *in vitro* matured (IVM) oocytes. Due to the known cytotoxicity of DMSO, propylene glycol (PG) has been proposed as an alternative CPA. This study investigated the effects of vitrifying in IVM bovine oocytes using PG + EG or DMSO + EG on subsequent embryo development and quality after warming. Both vitrification protocols were optimized in silico by our group to determine the ideal exposure times. Oocytes were obtained from a local slaughterhouse and randomly distributed into 5 groups: A) Control: Oocytes IVM for 24 h; B) VIT-DMSO: Oocytes IVM for 21 h, partially denuded, exposed to 7.5% DMSO-7.5% EG for 2min 30sec and 15% DMSO-15% EG + 0.5M sucrose for <1min, then vitrified in groups of 5 (García-Martínez et al., Theriogenology 184, 110-23, 2022); C) VIT-PG: Oocytes IVM for 21 h, partially denuded, exposed to 7.5% PG-7.5% EG for 1min35sec and 15% PG-15% EG + 0.5M sucrose for <1min, then vitrified in groups of 5 (Diaz-Muñoz et al., Animal Reprod 20 (2),2023). D/E) CPA-DMSO / CPA-PG: oocytes exposed to vitrification solutions as in B/C but not vitrified, to assess CPA cytotoxicity. After treatments, oocytes resumed IVM for 3 h, were fertilized and cultured for 8 days. Cleavage and blastocyst rates were assessed at 48 h post-insemination (pi) and day 8 (D8) pi. D8 blastocysts were assessed for total cell count (TCC; DAPI), Inner Cell Mass (ICM; SOX2) and apoptosis rate (AR; Caspase 3), as described in García-Martínez et al. 2022. Data are presented as mean ± SEM and were analyzed using GraphPad Prism. Normality and variance homogeneity were assessed (Shapiro-Wilk and Levene's tests), followed by one-way ANOVA or Kruskal-Wallis test ($P<0.05$). Developmental analysis included 3 replicates with ≥115 oocytes per replicate. The highest cleavage rate was observed in the Control group (92.3%, n=163). While no differences were observed between the CPA groups, the VIT-DMSO group showed a significantly lower cleavage rate than the Control group (66.5% (n=116) vs. 92,3% (n=163), respectively) ($P=0.0353$). On D8 pi, the percentage of blastocysts was similar across all groups, with 21.9% (n=137) in Control, 19.6% (n=72) in VIT-PG and 20.9% (n=68) in VIT-DMSO groups. No significant differences were observed in TCC between groups (Control: 90.2±12.3, CPA-PG: 108.7±9.1, CPA-DMSO: 98.2±11.2, VIT-PG: 105.7±12.4, VIT-DMSO: 103.5± 6.2) or in AR (Control: 6.3±1.6, CPA-PG: 3.1±0.7, CPA-DMSO: 3.5±1.3, VIT-PG: 4.4±1.2, VIT-DMSO: 2.3±0.3). The CPA-PG had a significantly lower mean ICM count compared to CPA-DMSO (12.9±1.7 and 23.0±4.6, respectively ($P=0.0371$); however, ICM counts in both vitrification groups (VIT-DMSO 21.8±4.8; VIT-PG 22.7±2.9) remained comparable to those in blastocysts derived from control oocytes (13.6±2.2). These findings suggest that PG supports both early cleavage and subsequent blastocyst development effectively, making it a favorable alternative to DMSO for oocyte cryopreservation protocols.

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Keywords: In silico, total cell count, apoptosis index

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Interaction of antioxidant and vitrification media on *in vitro* development of local Egyptian Goat oocytes

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The *in vitro* embryo production method has greatly improved goat breeding. In Egypt, *in vitro* techniques for goats are still a developing concept. Different vitrification media, which have various combinations of cryoprotectants, and additives, can significantly influence key parts of the cryopreservation process, affecting how well oocytes are preserved. This study looked at local Egyptian goat (Baladi breed) oocytes that were matured with or without ascorbic acid (AA; 50 µg/mL) and then vitrified in 2 different media. As a 1st experiment, goat oocytes (1990 oocytes) were matured *in vitro* with or without AA and then vitrified using two different vitrification (homemade (H) and commercial (C)) media. The experimental groups are G1 (H, without AA), G2 (C, without AA), G3 (H, with AA), and G4 (C, with AA). The study aims to investigate the impact of these factors on oocyte survival and subsequent developmental potential. In the 2nd experiment, the oocytes (2240 oocytes) were grouped after *in vitro* maturation (IVM) based on their treatment during the process: G1: Fresh oocytes without AA; G2: Fresh oocytes with AA; G3: Vitrified oocytes in H without AA; G4: Vitrified oocytes in C without AA; G5: Vitrified oocytes in H with AA; G6: Vitrified oocytes in C with AA. This setup allows for a comparison of the effects of AA, different cryoprotectant types (commercial and homemade), and the vitrification process itself on oocyte quality and development. The vitrification of COCs was carried out following 2 steps. At first, COCs were placed in ES, which included 50% VS for equilibration for 2 min. The next step involved vitrification in VS. This solution consisted of basic medium tissue culture media 199 (TCM-199) with the addition of 20% FBS v/v, plus 30% v/v of CPAs (EG and DMSO, 1:1 ratio) and sucrose 0.5 M, for 45 sec. The other vitrification medium was a commercial media kit (Kitazato®, Japan). Briefly, according to the manufacturer's recommended instructions, oocytes underwent an equilibration step in ES for 15 minutes before being moved to VS for 1.5 minutes. Every 5 COCs in 1-2 µl VS were loaded in OPS, then plunged in liquid nitrogen for one hour. Fertilization, blastocyst rates, and immunofluorescence staining of mitochondrial were observed and assessed according to Abd-Elkhalek et al. (2024a, b). All groups were fertilized by fresh epididymal sperm. The chi-square test was used to analyze data of percentage. In the 1st experiment, the percentages of normal-looking oocytes and recovery rates were higher (P≤0.01) in G4 (98.15 & 93.58%) than in the other groups: G1 (75.66 & 79.52%), G2 (85.98 & 83.92%), and G3 (90.96 & 89.92%). Moreover, in the 2nd experiment, blastocyst rates were enhanced in G1, G2, and G6 (34.38, 44.38, and 30.64%) compared to G3, G4, and G5 (9.66, 16.57, and 20.7%). The results indicated that goat embryos showed an increase (P≤0.01) in mitochondrial fluorescence intensity in G6 compared to the G3, G4, and G5 groups. In summary, supplementation of ascorbic acid (50 µg/mL) to IVM enhanced cryotolerance of goat oocytes following vitrification and warming in Kitazato® medium, which finally supported subsequent embryo development.

Keywords: Goats, ascorbic acid, embryo development, Kitazato® media

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Effect of resveratrol (3,5,4'-trihydroxystilbene) on bovine semen viability after thawing

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Cryopreservation of bovine semen often causes oxidative stress and cellular damage, reducing sperm motility, viability, and fertilizing capacity after thawing. One promising approach to mitigate these effects is the use of antioxidants in semen extenders during freezing. Resveratrol, a natural polyphenolic compound, has been shown to possess strong antioxidant, anti-inflammatory, and anti-apoptotic properties. which can help protect sperm cells from oxidative damage induced by the freeze-thaw cycle. Therefore, the objective of the present study is to evaluate the protective effects of resveratrol in the bovine sperm after its thawing. For such purpose, testes with attached epididymides from 12 adult Holstein Friesian bulls were removed immediately after slaughtering and stored on ice. The cauda epididymis was exposed and flushed with warm buffer solution and the sperm-rich fluid collected was evaluated at 37ºC by phase contrast microscopy. The following properties were evaluated: sperm concentration; mass and individual motility, assessed by linear or forward movement, on a scale of 1 to 5 in which grade of 1 indicates minimal or no forward progress, while 5 represents rapid, steady forward movement, as well as sperm morphology and viability by using eosin nigrosin methodology. For the latter, a thin smear of semen was prepared, stained with a mixture of eosin and nigrosin, and then examined under a microscope. Eosin stains dead sperm, while nigrosin provides background contrast, allowing for clear identification of live and dead sperm, as well as assessment of sperm shape and size. After evaluation, the semen was diluted in freezing medium (BioXcell) to a final concentration of 80x106 spz/ml and divided into six batches with different concentrations of resveratrol: 0; 2; 6; 18; 56 and 162 µM. Then, semen was packaged in 0.25 ml French straws (20x106 sperm cells per straw), left to equilibrate for two hours at 5ºC and then frozen in a programmable freezer (Sylab IceCube 1810). Approximately 72 hours later, straws were thawed, and the same parameters evaluated before freezing were reassessed. An Analysis of Variance (ANOVA) was used to determine if there were statistically significant differences in results between different treatments. On average, after thawing, sperm belonging to the control group presented a viability of 73.4% (± 1.6). For the lowest concentration of resveratrol (2 µM) sperm viability rose to 75.4% (± 2.4), slightly decreasing to 67.8% (± 4.2); 61.9% (± 3.3); 44.5% (± 3.0) and 37.2 (± 1.4), to the concentrations of 6, 18, 56 and 162 µM, respectively. The main effect of resveratrol on sperm quality was observed for the parameter of sperm with progressive linear movement. For the control values were 3.8 (± 0.32) rising to 4.0 (± 0.25) and 3.9 (± 0.30) for the 2 and 6 µM of resveratrol, respectively. For the other concentrations tested, a decrease was observed compared to the control. For sperm morphology, no differences were observed. Resveratrol at a concentration of 2 µM, showed promising effects in improving bovine semen quality. Its application in cryopreservation protocols could represent an effective strategy to improve reproductive efficiency in cattle.

Keywords: Bovine epididymal semen, antioxidant resveratrol, viability, freezing/thawing

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Hydrogel-based preservation of ram semen at room temperature: a novel approach

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Semen preservation is crucial for assisted reproduction. In sheep, frozen semen requires surgical application, while refrigerated semen (15°C) maintains acceptable fertility for only 6–8 h. This study aimed to develop a novel hydrogel-based method to preserve ram semen at room temperature for longer periods, considering previous uses of these systems in assisted reproduction (sperm selection and oocyte maturation). Semen was collected using an artificial vagina, pooled from 3 rams, and diluted in a gelatin + alginate (Ge) hydrogel prepared in TALP-HEPES without BSA and $CaCl_2$ at 120 × 10^6 sperm/mL (n=3). This base without hydrogel served as control (C). Hydrogel polymerization was induced with $CaCl_2$, and both groups reached 100 × 10^6 sperm/mL. After depolymerizing the Ge matrix with Tris-citrate-fructose, sperm were released at 50 × 10^6 sperm/mL (final concentration only in Ge). All samples were stored at 20 °C, and sperm quality was assessed at 3, 6, 12, and 24 h. Motility was evaluated with CASA (SCA), and viability, ROS, and membrane disorder by flow cytometry using IP (0.09 µM), CM-H_2DCFDA (0,2 µM), and M540 (0.054 nM). Data were analyzed using ANOVA with ARTool in R ($P<0.05$). A time × treatment interaction was observed: motility in Ge was 80.4 ± 2.4, 74.7 ± 3.3, 67.1 ± 1.7, and 49.0 ± 7.5% at 3, 6, 12, and 24 h, vs 66.3 ± 5.7, 52.8 ± 5.6, 22.8 ± 2.4, and 19.8 ± 1.9% in C. Groups did not differ at 3 h, but Ge remained significantly superior thereafter. Motility in Ge was similar until 12 h, decreasing significantly at 24 vs 3 and 6 h. In C, it dropped significantly at 12 and 24 h vs 3 h. Viability remained stable within groups but was always significantly higher in Ge (3 h: 57.9 ± 6.8 vs 40.8 ± 3.6%, 6 h: 53.5 ± 5.9 vs 38.9 ± 3.2%, 12 h: 52.5 ± 1.8 vs 40.0 ± 3.6%, and 24 h: 52.3 ± 3.2 vs 38.3 ± 3.2%). Similarly, ROS in live cells were significantly lower in Ge (3 h: 2.4 ± 0.7 vs 4.1 ± 0.6 MFI, 6 h: 2.2 ± 0.7 vs 4.6 ± 0.8 MFI, 12 h: 2.3 ± 0.4 vs 4.9 ± 0.8 MFI, and 24 h: 2.5 ± 0.3 vs 5.3 ± 0.7 MFI), with no time-related variation. Live cells without membrane disorder were significantly higher in Ge (3 h: 29.8 ± 4.2, 6 h: 26.1 ± 2.8, 12 h: 25.1 ± 1.3, and 24 h: 23.7 ± 2.0%), while C showed a progressive decline (3 h: 17.7 ± 2.2, 6 h: 12.3 ± 2.1, 12 h: 12.1 ± 1.3, and 24 h: 7.5 ± 1.5%), with significant differences between 3 and 24 h. Overall, Ge outperformed C in all parameters regardless of time, showing significantly higher motility (62.7 ± 3.6 vs 40.4 ± 3.9%), viability (53.1 ± 1.9 vs 39.5 ± 1.6%), and live cells without membrane disorder (25.2 ± 1.1 vs 16.6 ± 1.5%), with lower ROS per live cell (2.4 ± 0.2 vs 4.1 ± 0.4 MFI). In conclusion, gelatin-based hydrogel preserved ram sperm quality better than the control during 24 h of storage at room temperature, offering a field-adapted alternative to conventional semen conservation, but further studies are needed to test fertilization ability.

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Keywords: sperm, conservation, hydrogel

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPPORTING BIOTECHNOLOGIES: DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY, AND OMICS TECHNOLOGIES

Aneuploidy incidence does not differ between *in vitro* produced (IVP) and in vivo derived (IVD) bovine embryos of excellent quality

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Cattle embryo production has shown consistent growth worldwide, with the IETS reports indicating an overall increase of 62.1% between 2010 and 2023. Despite improvements in the quality of IVP embryos, their developmental potential post-transfer remains lower than IVD embryos. Previous studies have linked this to a higher incidence of aneuploidy in IVP cleavage-stage embryos - a trend also observed in other species, such as pigs and sheep. Reported rates of aneuploidy in IVP cattle blastocysts range up to 24% (Tutt, Theriogenology, 161, 108, 2021). However, systematic assessment of aneuploidy in later developmental stages, such as morulae and blastocysts, remains limited. We hypothesized that aneuploidy incidence and developmental competence would differ between IVP and IVD embryos at later stages (morula and early blastocyst), potentially explaining post-transfer outcomes. In this study, we performed a retrospective analysis of 153 cattle embryos (79 IVP and 74 IVD) to evaluate aneuploidy incidence and its parental origin. A biopsy of 8 to 15 blastomeres (morulae) or trophectoderm cells (blastocysts) was collected before cryopreservation, and a subset (n=31) of these embryos was later transferred. At the moment of biopsy, embryos presented excellent quality (IETS grade 1) and were at a morula (IETS code 4) or early blastocyst (IETS code 5) stage. Pregnancy checks were conducted at 28–42 and 60–72 days post-embryo transfer. Preimplantation Genetic Testing for Aneuploidy was performed using SNP-based algorithms. The copy number variation was investigated using signal intensity metrics (B Allele Frequency and Log R Ratio). If an aneuploidy was found, its origin (i.e., parental or mitotic) was assessed through haplotyping. Considering both embryos at the morula and blastocyst stages, the overall aneuploidy rate was 13.1% (n=20/153). Overall, early blastocysts and morulae exhibited an aneuploidy rate of 5.9% (n = 2/34) and 15.1% (n=18/119), respectively (P>0.05, Chi-squared). Contrary to what was expected, IVP embryos (10.1%, n=8/79) did not show a higher incidence of chromosomal abnormalities than IVD embryos (16.2%, n=12/74) (P>0.05, Chi-squared). A total of 31 whole-chromosome errors were detected in 20 aneuploid embryos, with monosomies being the most frequent (61.3%, n=19/31). Most monosomies had a maternal origin (68.4%, n=13/19). When biopsied frozen-warmed embryos were transferred to recipients, 42.11% of IVP and 16.7% of IVD embryos (n=10/12) led to a pregnancy. Moreover, 35.7% of IVP morulae (n=5/14) and 60% of early blastocysts (n=3/5) achieved pregnancy, whereas 18.2% of IVD morulae (n=2/11) and the only one transferred IVD blastocyst led to a pregnancy. In summary, the incidence of aneuploidy in IVP embryos was not statistically different from that of IVD embryos. These findings provide new insights into the developmental competence and chromosomal stability of embryos at later developmental stages.

Keywords: chromosome abnormalities, cattle

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPPORTING BIOTECHNOLOGIES: DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY, AND OMICS TECHNOLOGIES

Unveiling the landscape of bovine oocyte and zygote membrane proteome

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It is proposed that fertilization-competent oocytes display key molecules on their plasma membranes that enable fertilization and are subsequently eliminated after fertilization to ensure the polyspermy block and support viable embryo development. Based on this premise, a comparative proteomic analysis of membrane fractions from mature and fertilized bovine oocytes was designed to identify the proteomic profile of these two cells. To that aim, 3 replicates of 300 *in vitro* matured (IVM) oocytes and 300 zygotes were produced. Briefly, cumulus-oocyte complexes (COCs) were selected and matured *in vitro*. After IVM, oocytes were separated into two groups, i) COCs were treated with hyaluronidase (300 µg/ml) and vortexed to remove cumulus cells, washed twice in PBS, snap-frozen and stored at -80°C until their use; ii) subjected to *in vitro* fertilization. Semen straws were thawed; sperm was selected using a 45/90% Percoll gradient and oocytes were fertilized at 1x106 sperm/mL. After 17 h of co-culture, cumulus cells were removed as explained before, and then, zygotes were washed twice in PBS snap-frozen and stored at -80°C. For cell membrane purification, cells were suspended in a hypotonic lysis buffer, incubated in an ice bath, disrupted using a Dounce homogenizer with a tight-fitting pestle, centrifuged, and concentrated using Vivaspin filters, obtaining 10 µg of total protein from cell membrane fractions. Finally, samples were digested with the single-pot, solid-phase-enhanced sample preparation (SP3) methodology and analyzed on an Evosep One LC system coupled online to a tims-TOF Flex mass spectrometer with DIA-PASEF data acquisition mode at the Proteomics Platform (IMSMI) of IMIBIC, Córdoba, Spain. LC-MS/MS raw data files were analyzed with Spectronaut software. Three different runs were carried out per sample. Proteins with at least three valid values (VV) for LFQ intensity in each group were selected for statistical comparisons using Student's t-test and the Benjamini-Hochberg correction for multiple testing (P≤0.05 significant, differentially regulated). A total of 4148 proteins were detected in both experimental groups across the 3 replicates, 3082 had at least 3 VV of each group. We identified 684 proteins differentially expressed (P<0.05) in bovine eggs or zygotes revealing a clear proteomic profile for each experimental group. Of these 684 proteins, 345 were overexpressed in oocytes compared to zygotes. Furthermore, 320 proteins were exclusively detected in oocytes, and 459 exclusively in zygotes. Moreover, Gene Ontology (GO) analysis revealed the enrichment of several biological process terms potentially related to fertilization. These include processes associated with RNA/protein processing (GO: 0008380, 0042176) and cell division and organization (GO: 0000278, 0007097). These unique proteomic profiles could help unveil new markers related to oocyte quality, embryonic developmental activation, cell communication, or infertility.

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Keywords: Membrane proteome, cattle, oocyte, zygote

Animal Reproduction

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SUPPORTING BIOTECHNOLOGIES: DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY, AND OMICS TECHNOLOGIES

Proteomic response of oviduct epithelial spheroids to sperm binding in cattle

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After migration through the uterus, spermatozoa enter the first part of the oviduct, the isthmus, where a subpopulation bind to luminal ciliated cells to form a reservoir. Then, around ovulation time, spermatozoa migrate toward the ampulla, the site of fertilization. Previous results in pigs (Georgiou, MCP, 4(11):1785-96, 2005) and cattle (Ribeiro, Res. Vet. Sci., 184:105522, 2024), indicate that the oviduct epithelium reacts to insemination by proteomic alterations. In order to decipher the impact of sperm migration in both parts of the oviduct, we compared the proteomic response of bovine ampullar and isthmic epithelial spheroids to sperm interactions with and without binding. Ampullar and isthmic mucosa fragments from healthy pre-ovulatory cyclic cows were cultured for 3 days. Epithelial spheroids with outward ciliary beating were selected according to their uniform shape and size (100-µm diameter). Groups of 60 spheroids were incubated alone (control) or co-cultured with 106/mL frozen-thawed Percoll-washed spermatozoa from a pool of 3 bulls in the well (contact group) or on top of a 0.4 µm insert (non-contact group) in a Tyrode-based medium for 6 h at 38.5°C in a humidified atmosphere with 5% CO2 in air. Five biological replicates with different pools of spheroids were carried out. Sperm-cell interactions were analyzed using confocal and scanning electronic microscopy. At 6 h, all spheroids were vortexed (to remove bound sperm in the contact group) and analyzed by quantitative dia-PASEF nanoLC-MS/MS. Differentially abundant proteins (DAP; t-tests p-value ≤ 0.05 and fold-change ratio ≥ 1.3) were analyzed using Metascape. In both oviduct parts, more than 95% of bound spermatozoa were acrosome-intact and attached by the head to ciliated cells of spheroids. In the contact group, the bound sperm density was 45% higher on ampullar than isthmic spheroids ($P<0.05$). In total, 6989 proteins were identified and quantified in isthmic and ampullar spheroids. Regarding the effect of sperm binding and proximity (contact vs. control groups), 197 and 151 DAP were evidenced in ampullar and isthmic spheroids, respectively. Furthermore, sperm proximity without binding (non-contact vs. control groups) induced 114 and 135 DAP, respectively. In addition, 220 and 171 DAP were attributed to the effect of sperm binding (contact vs. non-contact groups), respectively. Overall, 59% of DAP were increased in abundance after sperm contact or proximity. DAP after sperm binding in isthmic spheroids were mostly involved in cilium functions (TEKT5, AKAP3) and cell response to stress (GPX4, GSTM3), while those in ampullar spheroids were involved in energy generation (ATP5E, PGAM2) and fertilization (SPAM1, FETUB), among others. In conclusion, a region-dependent alteration of the oviduct epithelial proteome upon sperm binding and proximity was shown, providing new evidence of a sperm-female dialog after insemination. Further studies will investigate the role of altered proteins in fertilization and embryo development, and the response of the oviduct to subfertile semen.

Keywords: oviduct spheroid, spermatozoa, proteomic

Animal Reproduction

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SUPPORTING BIOTECHNOLOGIES: DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY, AND OMICS TECHNOLOGIES

Organoid-derived secretions mimic oviductal fluid composition and support bovine sperm function

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Assisted Reproductive Technologies (ART) have revolutionized reproductive medicine by providing solutions for infertility. However, improving success rates still requires mimicking the natural environment of the reproductive tract. Studies in various mammalian models have shown that reproductive fluids enhance *in vitro* fertilization outcomes (Coy et al., Anim Reprod. 1, e20210132, 2022). Yet, individual variability in fluid composition limits their broader application. Producing fluid components under controlled lab conditions offers a promising alternative. This study aimed to generate bovine oviductal organoids, extract and analyse their secretions under pre- and post-ovulatory conditions and test their effect on bovine spermatozoa. Bovine oviductal organoids were generated from oviduct tissue obtained from slaughterhouse-derived cows. To simulate the follicular and luteal phases, organoids were treated with different hormone combinations, E2 or P4 and cAMP respectively. Hormonal responses were confirmed via RT-qPCR and further analysed by bulk RNA-seq. Organoid derived secretions (ODS) were collected and their proteomic profile compared with that of bovine oviductal fluid (BOF) obtained from slaughtered cows. To assess functional effects, frozen-thawed spermatozoa were incubated for 2 hours at 38.5°C and 5% CO_2 in capacitation media supplemented with 10% (v/v) OS from each experimental group. Controls included 10% PBS (negative) and follicular phase BOF-matched protein concentration (positive). Sperm motility, viability, acrosome integrity, and membrane fluidity were evaluated by CASA and flow cytometry. Gene expression analysis confirmed the successful simulation of the follicular and luteal phases in bovine oviductal organoids, revealing 1,144 differentially expressed genes between E2 and P4 stimulation. GO analysis after different hormone stimulation revealed enrichment in gene categories related to secretion and extracellular space. Proteomic analysis identified 4,071 proteins, with 78.3% shared between ODS and BOF. Most non-shared proteins in BOF originated from cytoplasmic, blood, and ciliary sources. Integrated proteomic and transcriptomic analyses showed phase-specific regulation of key proteins, such as oviductin (upregulated during pre-ovulation) and haptoglobin (upregulated during post-ovulation). Regarding sperm parameters, total motility declined significantly less in the pre-ovulatory ODS group (25.5%) than in the negative control (13.5%), reaching values comparable to the BOF group (29.3%). Membrane fluidity and acrosome reaction were also significantly higher in the pre-ovulatory ODS (56.3 & 43.9%) and BOF (57.2 & 44.2%) groups than negative control (46.9 & 29.1%). Previous studies have shown that BOF modulates sperm capacitation, increasing motility and acrosome reaction (Kumaresan A et al., Theriogenology, 124, 48-56, 2019). Our findings demonstrate that pre-ovulatory bovine ODS replicate these effects, aligning with in vivo exposure before fertilization. In conclusion, we successfully modelled the estrous cycle in bovine oviductal organoids and showed their secretions closely resemble natural BOF.

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Keywords: Organoids, oviductal fluid, oviduct, spermatozoa, sperm, capacitation, bovine, cow, estrous cycle, oviductal ephitelial organoid

Animal Reproduction

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SUPPORTING BIOTECHNOLOGIES: DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY, AND OMICS TECHNOLOGIES

Storage temperature modifies the proteomic profile of porcine-derived oviductal and uterine scaffolds

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Decellularization is a process that allows for the isolation of the extracellular matrix (ECM), yielding biocompatible, low-immunogenic scaffolds that retain key components and 3D structure. Beyond mechanical support, ECM contains proteins and growth factors essential for cellular functions. The preservation of these molecules after decellularization and during storage is critical for biomedical use. In reproductive medicine, ECM scaffolds have shown promise for treating fertility disorders and enhanced assisted reproductive technologies. After confirming through histological analysis that storage temperature did not affect collagen, elastin, or glycosaminoglycan levels, we aimed to evaluate the impact of one-month storage at different temperatures (4°C, -20°C, -80°C) on the proteomic profile of porcine oviductal and uterine scaffolds. For this, oviducts and 5 cm uterine sections from prepubertal pigs (Large White × Landrace) were decellularized using a previously described protocol (Martínez-López et al., Theriogenology 231, 36–51, 2025) and stored at the designated temperatures for a period of one month. The controls included native tissues (decellularization control) and freshly decellularized samples (storage control). Each group consisted of four oviducts and four uterine sections from different animals. Following storage, proteins were extracted using a lysis buffer, quantified by Bradford assay, and verified by SDS-PAGE. The samples were then digested with trypsin, and peptides were analyzed by HPLC-MS/MS using a Q-TOF mass spectrometer (Agilent Technologies, USA). Protein identification was performed using the Sus scrofa UniProt database. Bioinformatic analysis was applied to proteins detected in at least 50% of samples per experimental group. Principal component analysis (PCA) was used to evaluate clustering, and differentially abundant proteins (FDR ≤ 0.05) were subjected to functional analysis through gene ontology (GO) enrichment. Proteomic analysis revealed that scaffolds stored at -20°C and -80°C retained more proteins than those stored at 4°C. The PCA showed evident clustering by group in both organs, with the -80°C group exhibiting the closest proteomic profile to freshly decellularized tissues. GO analysis indicated that the most predominant components before and after storage were related to the ECM. Key ECM proteins, including collagens, desmin, decorin, fibrillin-1, fibulin-1, and laminin, were mostly preserved at -20°C and -80°C. Key biological processes such as ECM organization, cell adhesion, and regeneration were better maintained under freezing conditions. Furthermore, storage at -80°C showed the widest range of enriched biological processes and molecular functions. These findings suggest that freezing conditions (-20°C and -80°C) are more suitable for one-month storage of decellularized scaffolds than refrigeration, due to better preservation of protein content and scaffold bioactivity.

Keywords: Reproductive tissue engineering, Proteomic preservation, Scaffold storage

Animal Reproduction

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SUPPORTING BIOTECHNOLOGIES: DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY AND OMICS TECHNOLOGY

AI use in reproductive medicine and the risks that it brings: a review

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Artificial intelligence (AI) is increasingly becoming a tool in numerous medical fields. An example of that is reproductive medicine. Distinct AI programs were and still are being created for andrology, oocyte evaluation and embryos. It is important to keep up with the evolution of tools developed for this area, especially with the challenges they bring. The objective of this article was to analyze existent information on the AI applications conceived for reproductive medicine. For a better understanding of the development and risks of these technologies in this subject, it was also needed to analyze technical information about general AI, to connect how the normal risks of these technologies can be presented in reproductive medicine applications. Detailed research was done on the topics: AI models for andrology, AI models for oocytes, AI models for embryology, but also technical and ethical AI risks. The search platforms used were PubMed, IBM, ACM Digital Library and Science Direct. The quality of the materials used was evaluated thoroughly, with the quality of the journals and websites used classified between Q1 and Q2 according to the Scimago Journal and Country Rank. AI applications are starting to be used in fertility clinics and hospitals in their clinical practices. Sperm analysis and embryo selection are the most developed topics for AI use, with embryo selection being more startling for embryologists. Risks like black-box models and AI hallucinations show the vulnerability of AI applications and increase preoccupation whereas these methods should be used with total reliability. Ethical risks also rise with the use of AI programs in reproductive medicine. Placing emphasis on dehumanization, bias, responsibility gaps, deskilling, transparency and equitable access. It was possible to conclude that technical and ethical risks ensure that AI outputs cannot be taken as entirely reliable or fully trustworthy. The existing risks presented reassure that AI can be used in reproductive medicine, but as a tool, and not as a dependent method. It is important to acknowledge the necessity of quality training data and transparency in AI models. Technical, ethical, and lawful guidelines should be developed or improved for the better use of AI in reproductive medicine. This field of AI still presents great potential for advancement in the future.

Keywords: AI, reproductive medicine, risks

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PRACTITIONER'S AND CLINICAL REPORTS

Factors affecting pregnancy outcomes in dairy heifers 30 days after embryo transfer using *in vitro* produced embryos

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Introduction: In Latvia, ovum pick-up and *in vitro* fertilisation (OPU/IVF) is a new area of veterinary assisted reproductive technology (VetART) in cows. It is well known that many factors influence pregnancy outcomes after transferring *in vitro*-produced (IVP) embryos. Since the field is new, we cannot select the best donors and recipients; we only used the animals that the owners offered. The study aimed to investigate the factors that could affect the IVP embryo (E) transfer (T) result.

Materials and Methods: This work included data from 10 OPU sessions and ovaries from 10 slaughtered Holstein cows, resulting in a total of 183 oocytes, of which 49 developed into blastocysts (26.8%). Subsequently, 22 ET procedures were performed by newly trained personnel in 2024. The recipients were located at two different farms. Before the ET express tests were performed - levels of β-hydroxybutyrate (BHB), lactate (Lac), and glucose (Glc) were assessed in the recipient's blood. Pregnancy-associated glycoproteins were analysed in the recipient's blood 23 days after the ET procedure. Statistical analyses (mean, standard deviation, ANOVA t-test and the Mann-Whitney test to compare two unrelated groups (pregnant, n=7 (31.8%) and non-pregnant, n=15)) were performed using IBM SPSS Statistics 21. Factors included were: method of egg retrieval (OPU n=55 or slaughterhouse n=128), the type (sex-sorted (n=10, pregnant=2) or conventional (n=12, pregnant=5)) of two bull sperm used in IVF, E age (6 days (n=8), 7 days (n=13) or 8 days n=1)), E developmental stage (early blastocyst, blastocyst, expanded blastocyst), E quality before freezing (only grades 1 and 2 were frozen) and ease of ET procedure performing. A statistically significant difference was considered if the P-value was less than 0.05.

Results: No statistically significant effect (P>0.05) on pregnancy outcomes was observed for the method of oocyte retrieval, embryo age, developmental stage, pre-freeze embryo quality, BHB and lactate levels in recipient blood, the complexity of ET procedure, and the recipient farm. Significant effects (P<0.05) were observed for the type of semen used (better results with conventional sperm), the total number of embryos obtained using a given bull's sperm, and the recipient's glucose level (higher Glc was associated with improved pregnancy outcomes: 3.3±4.43 vs 2.6±0.37 mmol/L).

Conclusions: In our laboratory, it is crucial to identify bull sperm that are more suitable for *in vitro* use. Protocols involving sex-sorted sperm require further optimisation. Monitoring recipient glucose levels and considering farm-specific factors may help improve embryo transfer outcomes.

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Keywords: *in vitro*, cow embryo, transfer

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PRACTITIONER'S AND CLINICAL REPORTS

Comparative results of *in vitro* production of bovine embryos using two FSH superstimulation protocols prior to OPU

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The objective of this study was to compare the effects of superstimulation (SS) with FSH (PLUSET, Calier) prior to OPU using two protocols FSHx5 and FSHx1 to simplify the application in large number of donors. SS induces follicular growth and oocyte maturation, synchronizing cytoplasmic maturity with nuclear arrest before OPU. Subsequently, 22 h of IVM completes oocyte capacitation, increases the blastocyst rate and prevents epigenetic malformations. MOET and OPU-IVP services were carried out in 13 farms, indicating OPU-IVP to Bos taurus donors that did not produce embryos with MOET, using both systems adjusted to every donor´s clinical history, and the availability of recipients to transfer fresh IVP embryos. Two SS protocols were used, in both the follicular wave was synchronized with an injection of 20 µg (IM) of GnRH analog Buserelina (Pluserelin, Calier), and 36 h later FSH - PLUSET was injected (IM) in saline solution, 250 IU in heifers and 325 IU in cows. The FSHx5 protocol was given in five injections every 12 h, at a constant dose and 24-30 h coasting until the OPU. The FSXx1 protocol was given the total dose in one injection and 48-54 h coasting until the OPU. OPUs were performed using WTA equipment and in-house made culture media. Oocytes were handled in mobile labs and transported in IVM media for 22 h to the central lab. IVF on day 0 was performed with semen centrifuged in PureSperm, with 1 MM/mL of motile sperm, for 18-21 h. Zygotes were stripped by pipetting and cultured in SOF1. On day 3, cleavage and the first feeding were assessed in SOF2 with Glucose. On day 5, the second feeding was performed in SOF3 with Glucose and Forskolin. On days 6 and 7 blastocysts were frozen or transferred fresh. In 2024, L-Carnitine was added to the IVM medium and SOF along with Forskolin as a moderator of fatty acid metabolism. From 2021 to 2023, 377 OPUs were performed with FSHx5, and in 2024, 230 OPUs were performed with FSHx1. There were no differences between SS protocols with 81% of follicle size of 6 to 14 mm, 55% recovery rate of oocytes/aspirated follicles, and oocyte quality (83% G1 and G2). Slightly smaller follicle size was observed with FSHx1 because the effect of FSH lasts 48 h, instead of 72 h with FSHx5 until the OPU. A total of 1699 and 1003 embryos were respectively produced, with 4.5 vs 4.4 per OPU, 48% vs 50% blastocysts/zygotes, and 41% vs 48% fresh ET pregnancy rate confirmed at 90 days of gestation. Simultaneously, ET with MOET achieved 62% and 55% pregnancy rates with fresh and frozen embryos respectively. SS with FSH did not modify the follicular population and did not improve cryosurvival using to the conventional DT procedures. Furthermore, SS with either protocol of FSH improved oocyte quality and doubled embryo production in comparison with IVP without FSH treatment, reducing OPUs without embryo to 14%, with an abortion rate of 8% as in MOET, normalizing birth weight with less than 10% dystocia and birth of 48% female calves. Moreover, the single injection of FSHx1 simplified time working enabling the SS of IVP donors in large scale on farm, improving animal welfare, and the well-being of the personnel responsible for caring births.

Keywords: embryos, *in vitro*, FSH

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PRACTITIONER'S AND CLINICAL REPORTS

Bovine *in vitro* embryo production using fresh semen from young bulls in a commercial cattle breeding program

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In cattle breeding programs, genetic progress can be achieved by shortening the generation interval. Besides genomic selection, other factors can also help to reduce it. A strategy is the use of fresh semen from young bulls as early as possible in commercial IVP programs.This allows for avoiding the quarantine time required by the European legislation for frozen semen before use. The objectives of this case report are firstly to compare the bull mean age at the time of IVF, for two bull semen use strategies : group 1 (G1), bulls that are used for IVF exclusively with frozen semen; and a second group (G2) of bulls used for IVF, initially with fresh semen (G2F) during the quarantine period, and then, with frozen semen (G2C). Secondly, CASA results, as well as cleavage and embryo development rates, are presented according to the two strategies and the type of semen. The acceptance threshold for sperm abnormalities in an ejaculate to allow its use fresh on IVF is <60%, and frozen <45%. All ejaculates are processed and extended in Optidyl® (IMV Technologies, L'Aigle, France), packed in straws, and conserved at 4°C until use for IVF (24 hours later), or subjected to cryopreservation following the SYNETICS'standard protocol. In this case report, fresh and frozen semen from 138 SYNETICS' bulls were used in 2704 OPU sessions (G1: 86 bulls,1348 OPU, and 406 donors; G2F: 52 bulls, 647 OPU, and 279 donors; and G2C: the same 52 bulls from G2F, 709 OPU, and 299 donors). All data were recorded since June 2022 at the SYNETICS' donor station in France. Holstein heifers between 7 and 12 months of age were subjected to dominant follicle removal followed by a standard ovarian stimulation protocol before OPU; on average 12 oocytes were used per session. IVM, IVF and IVC were performed with the Boviteq IVP culture media system. After density gradient sperm purification, CASA analysis was performed. The mean bull age at the time of semen collection were: G1 – 12.4 and G2 - 12.2 months and at the time of use for IVF were : G1 - 16.0 and G2 – 13.8 months. The values for motility (%), progressive motility (%), and VCL (Curvilinear velocity), for fresh (G2F) and frozen (G2C and G1) semen were on average : 89% vs 81%, 62% vs 57%, and 193 vs 169, respectively. The cleavage (3-days after IVF) and development rates (7-days after IVF) for fresh (G2F) and frozen (G2C) semen were, 79% vs 71%, and 35% vs 30%, respectively; and for only frozen semen (G1) were 73% and 28%. Although it is known that semen from young bulls are of lower quality because they have not yet reached puberty, the tendency for higher CASA results, cleavage and development rates when using fresh samples compared to frozen ones could be explained by the impact of the freezing-thawing process. The results obtained, confirm that it is possible to save time by using fresh semen from young bulls in a commercial IVP program, reducing the generation interval and accelerating genetic progress, all while maintaining stable results on embryo production.

Keywords: young bull, fresh semen, *in vitro* fertilization

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PRACTITIONER'S AND CLINICAL REPORTS

Use of embryo transfer technology under Indian field conditions for production of elite animals

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India remains the top milk producer globally by producing 239 MT milk and contributing 25% of global milk production. India bestowed with largest livestock population in the world with different high milk producing breeds of cattle and buffalo. Average livestock herd size in India and productivity per animals is very less. Livestock rearing is a primary source of income for many rural households, particularly small and marginal farmers in India. Livestock contribute significantly to the income of small farm households. Recently In Vitro Feritlization - Embryo Transfer is being used to accelerate genetic improvement and upgrading the genetic makeup of cattle in the Indian subcontinent. In Vitro Fertilization and Embryo Transfer technologies can be used to produce elite animals from the combination of high milk producing cows and good quality breeding bulls for the sustainable dairy industry. These two technologies also help in the development of herds of genetically valuable females or males. BAIF Development Research Foundation is a Non-Government Organization (NGO) working from the inception for livestock-based activities as a prime instruments used for uplift the livelihoods of rural poor. As a part of this, BAIF initiated field ET activity at farmers' door step from January 2023 as an assisted reproductive tool for production of elite animals at farmers' door step and ultimately to increase their income and food security. Along with production of elite animals, popularizing and adoption of these technologies by marginal Indian farmers was another objective. Basic protocols like recipient selection, their synchronization, heat detection was done by field Artificial Insemination technicians' team. Healthy and regular breed Holstein crossbred heifers and cows (up to third lactation) were selected as recipients at farmers' herd for embryo transfer. Based on their reproductive soundness and ovarian structure oestrus synchronization were planned for embryo transfer like CIDR, 2 injections of PGF2α or single injection of PGF2α. In this study a data set of 227 (Holstein- 189 & Jersey-38) IVF fresh sexed embryo transfer details was studied in farmers'recipients for the period January 2023 to December 2023. For Holstein breed embryos the pregnancy rate was 26.98% & for Jersey breed embryos it was 21.05%. Out of 227 ET done, 140 were done in heifers and 87 ET were done in cows. In heifers the pregnancy rate was observed 27.86% and in cows it was 22.99% respectively with over all pregnancy rate of 25.99%.Statistical analysis using logistic regression method shows that the probability for ET pregnancy ratio in cows and heifers is PP(AA) = 0.5 and PP(AA) = 0.55 respectively. Odds ratio is 1.0 for cows and 1.21 for heifers for ET pregnancies meaning there is no statistically significant effect of parity on the ET pregnancies results in this study. As the technology was newly introduced at farmers' herd so initially the adoption of technology by animal owners and availability of recipients was a bigger challenge. The success rates and propagation of ET technology can be improved more under Indian context once the technology gets popularised and a greater number of quality recipients are available.

Keywords: Embryo, recipient, oestrus synchronization, semen

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PRACTITIONER'S AND CLINICAL REPORTS

Impact of embryo transfer strategy on calving ease and birth weight in Holstein calves

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It is known that *in vitro* produced (IVP) embryos are associated with a higher incidence of prolonged gestation, abortion, stillbirth, dystocia, and reduced offspring viability—partly due to increased birth weights compared to artificial insemination (AI) and in vivo derived (IVD) embryos (reviewed by Nava-Trujillo, H. & Rivera, RM., Animal, 2023). To support CRV's objective of improving calf health and survival, we collected embryo transfer and calving data from 2020 to 2024 across six Dutch Holstein dairy farms. Key variables included recipient category (cow vs. heifer), calving ease (easy vs. difficult), and calf birth weight (kg). A retrospective analysis was performed with the objective to report data records from actual farms and keep track of calving difficulties caused by IVP embryos. Calves born from IVP embryos continue to have a significant ($P<0.05$) higher weight than calves born from AI or IVD (43.3 ± 6.5 IVPa; 40.3 ± 4.8 IVDb; 40.1 ± 5.9c), especially females calves born to heifers, following the same pattern observed in 2023 (Mullaart, E. et al., 39th Annual Meeting A.E.T.E., 2023). During the study period, 630 IVD and IVP embryos were transferred to cows and 202 to heifers. Following a recommendation implemented in 2020 to prioritize transfers into cows, embryo use in heifers was kept to a minimum (less than 40%), especially for IVP embryos (less than 20% of total transfers). Even though, when evaluating birth scoring records, we observed a higher incidence of difficult births when IVP embryos were transferred to heifers compared to cows (13.3% vs. 4.07%, respectively). Such discrepancy was not observed with transferred IVD embryos (3.82% heifers vs. 1.84% cows) or AI (6.31% heifers vs. 5.92% cows). Among IVP embryo recipients, calving data also shown higher birth weights in difficult calving compared to easy, both on embryos transfers to cows (57.2 ± 7.73 vs. 42.9 ± 5.95 kg, respectively) and heifers (46.8 ± 2.48 vs. 39.6 ± 5.34 kg, respectively). Heavy calves were defined as those exceeding 55 kg (95th percentile of the birth weight distribution for male and female calves). A low proportion of heavy calves was observed among cow recipients (2.7%), with a small (<1%) increase in 2023 and 2024 (3.5%), while none were reported among heifers—likely due to the reduced use of IVP embryos in this group. Furthermore, the overall rate of difficult calvings decreased from 2020 (25%) to 2022 (8.6%), but showed a 7% increase in subsequent years. In conclusion, these findings highlight the importance of continuous monitoring of calving outcomes following IVP embryo transfers to identify potential bottlenecks and inform management strategies. In addition, CRV's strategy to reduce IVP embryo transfers to heifers has contributed to a reduction in difficult calvings, supporting improved calf outcomes and farm management practices across Dutch dairy farms.

Keywords: Heavy calves, IVP, embryo transfer

Animal Reproduction

THEMATIC SECTION: 41ST ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PRACTITIONER'S AND CLINICAL REPORTS

First successful application of maternal spindle transfer in equine oocytes to overcome embryo developmental arrest

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Maternal spindle transfer (MST) is a mitochondrial replacement therapy that has been proposed to prevent the transmission of mitochondrial diseases, which are inherited exclusively through the maternal line. It has been shown that MST could also be successfully used to overcome embryo development arrest caused by poor oocyte quality, both in the mouse (Costa-Borges N, Elife, 2020 Apr 29;9:e48591) and human species (Costa-Borges N, Fertil Steril. 2023 Jun;119(6):964-973). This case report describes the first known successful application of MST in the equine species to overcome poor oocyte quality and low embryonic developmental potential in a mare with a long history of failed OPU-ICSI attempts. A 13-year-old mare with six OPU-ICSI cycles had previously yielded a total of 63 oocytes with a maturation rate (MR) of 80.9% (n=51/63). Despite a cleavage rate (CR) of 72.5% (n=37/51), only one blastocyst was obtained (2%, n=1/51). In this case, 18 COCs were retrieved from the patient mare, 13 of which successfully matured *in vitro* (72.2% MR). In parallel, an oocyte donor mare with a previous history of successful blastocyst development provided 11 oocytes, with 72.7% MR (n=8/11). Immature oocytes from both mares were shipped for 24 hours in Emcare®-holding-medium (ICPbio Reproduction) and matured in a humidified incubator at 38°C under controlled conditions (7%CO_2, 7%O_2) using an in-house-made maturation medium (TCM-199, FBS, FSH and EGF) for 29 hours. Micromanipulation procedures were performed on a heated stage under an inverted microscope equipped with a birefringence imaging system to locate the meiotic spindle non-invasively and confirm its removal. MST was performed by removing the meiotic spindle from the patient's oocytes and transferring it into previously enucleated oocytes from the donor mare. Karyoplast-cytoplast fusion was induced by exposure of the karyoplasts to an inactivated protein extract (HVJ-E), prior to reconstruction. Following reconstruction (33hours post-IVM), oocytes underwent Piezo-ICSI (Prime Tech, Japan) using frozen-thawed sperm from a fertile stallion (followed by washing and swim-up procedure). Afterwards, injected oocytes were cultured in a in-house-made culture medium (TCM-199, DMEM/F12 and FBS) in a humidified incubator at 38°C under controlled conditions (7%CO_2, 7%O_2). Cleavage was evaluated on Day-5 and media was renewed; embryo development was evaluated on Days 7-11. MST was successfully performed in 7 out of 8 oocytes, resulting in a 100% CR(n=7/7) and four blastocysts were obtained (57.1%) and vitrified. ICSI was also performed on the six remaining patient mare's oocytes as a control without MST, which resulted in an 83.3% CR(n=5/6), but no blastocyst formation. One Day-7 blastocyst was transferred into a recipient mare, resulting in an ongoing pregnancy with delivery expected in June 2025. Genetic analyses, including parental testing and mitochondrial carryover levels are currently underway and will be performed after the delivery of the foal. To our knowledge, this is the first case in the world in which MST has been applied in the equine species to overcome poor oocyte quality and low embryonic development, demonstrating the feasibility of this technique in horses.

Keywords: spindle transfer, mitochondrial, equine reproduction